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PROBIÓTICOS ENCAPSULADOS APLICADOS EM PRODUTO CÁRNEO

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2016

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RESUMO

PROBIÓTICOS ENCAPSULADOS APLICADOS EM PRODUTO CÁRNEO

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Este trabalho teve por objetivo encapsular microrganismos probióticos, avaliar sua resistência frente à condições adversas de processamento e estudar a aplicação em embutido cárneo fermentado. Para isso o *L. plantarum* ATCC 8041 e *E. faecium* ATCC 700221 foram encapsulados através da técnica de extrusão usando alginato de sódio juntamente com inulina, leite em pó e trealose como materiais de cápsula e submetidos à condições adversas de processamento como altas temperaturas, altas concentrações de cloreto de sódio e nitrito de sódio e armazenamento sob refrigeração. Posteriormente, o *L. plantarum* foi adicionado na forma livre e encapsulado através das técnicas de extrusão, emulsão simples e emulsão dupla em embutido fermentado e avaliado durante seu processamento e armazenamento. Observou-se que a encapsulação foi eficaz na proteção dos probióticos às condições adversas, especialmente quando submetidos à temperatura de 70 ºC e altas concentrações de cloreto e nitrito de sódio. Os probióticos encapsulados se mantiveram em níveis adequados durante os 70 dias de armazenamento sob refrigeração. Ainda, a adição de *L. plantarum* encapsulado por diferentes técnicas não alterou as características físico-químicas do embutido fermentado durante seu processamento e armazenamento. No entanto, a encapsulação através da técnica de extrusão se mostrou mais viável em manter as contagens de *L. plantarum* em níveis mais elevados no produto durante o processamento e armazenamento. A adição de *L. plantarum* encapsulado em emulsão simples e emulsão dupla aumentou a oxidação lipídica dos embutidos fermentados. Ainda, na análise sensorial, os produtos contendo *L. plantarum* encapsulado em emulsão simples apresentaram notas inferiores ao controle nos quesitos sabor, dureza, textura e aceitação geral.

Palavras-chave: probióticos, encapsulação, embutido fermentado, *Lactobacillus plantarum*, emulsões duplas.

ABSTRACT

Encapsulated probiotics applied in meat product

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The aim of this study was to encapsulate probiotic microorganisms, evaluating their resistance against harsh environments and the use in dry fermented sausages. *L. plantarum* ATCC 8041 and *E. faecium* ATCC 700221 were encapsulate in alginate beads in association with inulin, milk powder and trehalose through extrusion technique and submitted to stressful environments such as high temperatures, high concentrations of sodium chloride and sodium nitrite and refrigerated storage period. Subsequently, *L. plantarum* was added to dry fermented sausages as free cells or encapsulated using extrusion, emulsion and double emulsion techniques. Encapsulation was effective to protect probiotics against stress treatments, mainly 70 ℃ temperature and high concentrations of sodium chloride and sodium nitrite. Furthermore, encapsulated probiotics kept their viability throughout 70 days of refrigerated storage. In addition, the use of encapsulated *L. plantarum* did not change physico-chemical properties of dry fermented sausage during processing and storage. However, the extrusion technique seems to be more effective to keep higher counts of *L. plantarum* in dry fermented sausages during processing and storage. The addition of *L. plantarum* encapsulated in emulsion and double emulsion increased lipid oxidation in dry fermented sausages. Furtermore, in sensory analysis, dry fermented sausages containing *L. plantarum* encapsulated in emulsion had lower scores than control treatment in flavor, hardness, texture and overall acceptance attributes.

Key words: probiotics, encapsulation, dry fermented sausage, *Lactobacillus plantarum*, double emulsions.

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APRESENTAÇÃO

Essa tese segue as normas estabelecidas na Estrutura e Apresentação de Monografias, Dissertações e Teses – MDT da UFSM (UFSM, 2015). Os resultados estão apresentados na forma de cinco artigos científicos, sendo que dois deles compõem a **Revisão Bibliográfica** no ítem **DESENVOLVIMENTO**. Os três artigos restantes se encontram no ítem **Parte Experimental** do **DESENVOLVIMENTO**. As seções Materiais e Métodos, Resultados e Discussão encontram-se nos artigos científicos e representam a íntegra desse trabalho. Ao final dessa tese, encontram-se os itens **DISCUSSÃO** e **CONCLUSÕES**, apresentando uma compilação de intepretações e comentários a respeito dos resultados demonstrados nos artigos científicos contidos nesse trabalho. As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** dessa tese.

1   INTRODUÇÃO

A carne é um alimento extremamente nutritivo, rico em proteínas, aminoácidos essenciais, vitaminas e minerais (DECKER & PARK, 2010). Os produtos cárneos geralmente são fabricados com retalhos cárneos em associação com outros ingredientes como sal (NaCl), toucinho, nitritos e conservantes. Nos últimos anos têm se buscado a reformulação dos produtos cárneos, com o objetivos de torná-los mais saudáveis e atrativos para consumidores mais exigentes. Este tipo de reformulação pode-se basear na redução ou substituição do sal, redução de gordura ou sua substituição por gorduras vegetais com maior concentração de ácidos graxos insaturados e a adição de fibras, vitaminas, minerais, antioxidantes e probióticos (OLMEDILLA-ALONSO et al., 2013).

Hoje em dia os consumidores buscam cada vez mais por alimentos que sejam seguros e nutritivos, além de saudáveis e o mais natural possíveis (JIMÉNEZ-COLMENERO et al., 2006). Assim, o mercado dos alimentos funcionais cresceu rapidamente nas últimas décadas, principalmente devido à essas mudanças comportamentais dos consumidores (MENRAD, 2003). Entre os alimentos funcionais, incluem-se os alimentos probióticos e os benefícios que estes promovem estão baseados na presença de cepas selecionadas de bactérias ácido lácticas (BAL) que necessitam estar em quantidades adequadas para exercer efeito positivo na saúde de quem os consome (FAO/WHO, 2002). Conforme Agrawal (2005), os produtos probióticos são uma parcela importante de alimentos funcionais, uma vez que representam 65% do mercado mundial deste tipo de produto.

Os principais microrganismos com características probióticas pertencem aos gêneros *Lactobacillus* e *Bifidobacterium*. Porém, atualmente diversos outros gêneros como *Pediococcus, Enterococcus* e *Saccharomyces* estão sendo utilizados por apresentarem benefícios comprovados à saúde do consumidor (WEINBRECK et al., 2010).. No entanto, estes benefícios são espécie-específicos (KEKKONEN et al., 2007) e para que ocorram é indicado que a dose diária de probióticos seja entre 10^8-10^9 UFC/g (CHAMPAGNE et al., 2005), sendo que a mínima concentração de probióticos no alimento deve ser entre 10^6-10^7 UFC/g no momento do consumo (NULKAEKUL et al., 2012).

Embora produtos lácteos sejam os alimentos mais comuns como veículo para microrganismos probióticos, diversos estudos vêm sendo realizados com adição de bactérias probióticas em produtos cárneos, especialmente os fermentados, com o intuito de aumentar o valor nutricional e caracterizá-los como alimentos funcionais (CAVALHEIRO et al., 2015;

DE VUYST et al., 2008; MACEDO et al., 2008; ROUHI et al., 2013; RUBIO et al., 2013; RUBIO et al., 2014a; RUBIO et al., 2014b; RUBIO et al., 2014c; RUIZ-MOYANO et al., 2011a; RUIZ-MOYANO et al., 2011b; TRZASKOWSKA et al., 2014). Algumas bactérias ácido láticas (BAL) isoladas de produtos cárneos e com características probióticas já vem sendo utilizadas como culturas *starters* em outros produtos cárneos. A principal vantagem é que estes microrganismos apresentam maior resistência às características adversas deste tipo de produto. As BAL mais frequentemente isoladas de produtos cárneos fermentados são *L. plantarum*, *L. fermentum*, *L. sake*, *L. curvatus*, *L. brevis*, *P. pentosaceus* e *P. acidilactici* (BENITO et al., 2007; DE VUYST et al., 2008; REBUCCI et al., 2007; SCHILLINGER et al., 1996). Já em produtos cárneos cozidos, as BAL mais comumente isoladas são *Aerococcus viridans*, *E. faecium*, *L. plantarum* e *P. pentosaceus* (RAMÍREZ-CHAVARIN et al., 2010).

Estudos relatam que a viabilidade dos microrganismos probióticos é baixa quando estes são adicionados como células livres em alimentos (DE VOS et al., 2010). Por isso, o maior desafio na incorporação de probióticos em alimentos está em manter a viabilidade das bactérias durante o processamento e armazenamento até o consumo do produto (GRANATO et al., 2010). Em se tratando de produtos cárneos, o microrganismo necessita resistir ao alto teor de sal e sais de cura, baixo pH e baixa atividade de água dos produtos fermentados e ainda, ao tratamento térmico dos produtos cozidos. Por fim, o microrganismo deve resistir às barreiras naturais do hospedeiro, como as condições adversas do ambiente gástrico para que consiga exercer suas funções diretamente no intestino (KIM et al., 2008).

Nesse contexto, a técnica de encapsulação é uma tecnologia promissora e que já vem sendo utilizada para oferecer proteção aos microrganismos probióticos e aumentar sua sobrevivência em vários tipos de produtos (HEIDEBACH et al., 2012; HOMAYOUNI et al., 2008; OZER et al., 2009). Encapsulação é definida como a tecnologia de empacotamento de materiais sólidos, líquidos ou gasosos em cápsulas que são capazes de liberar seu conteúdo em determinado momento sob influência de condições específicas (ANAL & STEVENS, 2005; ANAL et al., 2006; ANAL & SINGH, 2007; KAILASAPATHY & MASONDOLE, 2005). Também, pode ser definida como um processo físico-químico ou mecânico com o objetivo de desenvolver partículas com diâmetros desde pequenos nanômeros até pequenos milímetros (CHEN & CHEN, 2007), produzindo uma barreira física contra condições de *stress* (CHÁVARRI et al., 2010; NAZZARO et al., 2009). Diversas técnicas distintas podem ser utilizadas para encapsular probióticos e compostos bioativos, sendo a extrusão,

emulsificação, *spray drying* e *freeze drying* as mais comuns. A encapsulação de compostos já vem sendo amplamente utilizada nas áreas têxtil, agroquímica, alimentícia e farmacêutica.

Alguns estudos já vem demonstrando a eficácia da técnica de encapsulação para proteger microrganismos probióticos à características adversas de processamento e ao trato gastrointestinal (REFERÊNCIAS). Este trabalho, traz o uso de alginato de sódio, inulina, leite em pó e trealose como materiais de cápsulas na encapsulação de *Lactobacillus plantarum* e *Enterococcus faecium* com o intuito de proteger-los do alto teor de cloreto de sódio (NaCl), nitrito de sódio (NaNO$_2$), altas temperaturas e armazenamento sob refrigeração. Ainda, este trabalho é inovador no ponto de vista do preparo de emulsões simples (W/O) e dupla (W/O/W) contendo *Lactobacillus plantarum* encapsulado e sua aplicação em um produto cárneo fermentado espanhol (Chorizo).

1.1   Objetivos

1.1.1   Objetivo geral

O objetivo geral deste trabalho foi desenvolver cápsulas contendo os microrganismos probióticos *L. plantarum* e *E. faecium* e avaliar a viabilidade dos probióticos encapsulados sob condições adversas de processamento e armazenamento de alimentos *in vitro*; aplicar *L. plantarum* livre e encapsulado à produção de embutido fermentado espanhol (*Chorizo*) e avaliar a viabilidade dos probióticos adicionados e seus efeitos sobre as características de qualidade do produto durante o processamento e armazenamento.

1.1.2   Objetivos específicos

- Desenvolver cápsulas contendo os microrganismos probióticos *L. plantarum* e *E. faecium* através da técnica de extrusão, utilizando como materiais de cápsula o alginato de sódio, leite em pó, inulina e trealose;

- Avaliar a viabilidade dos microrganismos probióticos *L. plantarum* e *E. faecium* livres e encapsulados à altas temperaturas de processamento (70 e 80 ºC) por diferentes períodos de tempo (5, 10, 20 e 30 min);

- Avaliar a viabilidade dos microrganismos probióticos *L. plantarum* e *E. faecium* livres e encapsulados à diferentes concentrações de cloreto de sódio (NaCl) (0,5; 1,0; 2,5 e 5,0%);

- Avaliar a viabilidade dos microrganismos probióticos *L. plantarum* e *E. faecium* livres e encapsulados à diferentes concentrações de nitrito de sódio ($NaNO_2$) (0,5 e 1,0%);

- Avaliar a viabilidade dos microrganismos probióticos *L. plantarum* e *E. faecium* encapsulados durante o armazenamento por 70 dias a 4 ºC;

- Desenvolver cápsulas contendo o microrganismo probióticos *L. plantarum* através das técnicas de extrusão, emulsão simples (W/O) e emulsão dupla (W/O/W);

- Elaborar embutidos fermentados (*Chorizos*) contendo *L. plantarum* livre e encapsulado pelos métodos de extrusão, emulsão simples e emulsão dupla;

- Analisar as características físico-químicas, composição centesimal, cor e características microbiológicas durante o processamento de *Chorizos* elaborados com *L. plantarum* livre e encapsulado pelos métodos de extrusão, emulsão simples e emulsão dupla;

- Analisar as características físico-químicas, cor, características microbiológicas, oxidação de lipídios, textura e características sensoriais durante o armazenamento de *Chorizos* elaborados com *L. plantarum* livre e encapsulado pelos métodos de extrusão, emulsão simples e emulsão dupla.

2 DESENVOLVIMENTO

2.1 Revisão Bibliográfica

2.1.1 Manuscrito 1

Encapsulação: alternativa para a aplicação de microrganismos probióticos em alimentos termicamente processados

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CIÊNCIAᵉNATURA 35 anos

Encapsulação: alternativa para a aplicação de microrganismos probióticos em alimentos termicamente processados

Encapsulation: an alternative for application of probiotic microorganisms in thermally processed foods

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Resumo

O consumo de alimentos probióticos tem aumentado consideravelmente nos últimos anos e diversos alimentos probióticos já estão disponíveis no mercado. Entre os microrganismos probióticos se destacam os dos gêneros Lactobacillus e Bifidobacterium. No entanto, a aplicação deste tipo de microrganismo é limitada a produtos que não passem por condições extremas de temperatura durante seu processamento. Assim, técnicas de encapsulação vêm surgindo como alternativas para adicionar microrganismos probióticos em produtos processados termicamente. Entre as principais técnicas de encapsulação estão extrusão, spray-drying e emulsificação, sendo que todas apresentam vantagens e desvantagens. Atualmente diversos materiais podem ser utilizados para encapsular bactérias, porém, o alginato é o mais comumente utilizado. Para se obter cápsulas com maior viabilidade mesmo após tratamentos térmicos é necessário associar cepas probióticas termoresistentes e adequados materiais e técnicas de encapsulação.

Palavras-chave: Probióticos. Encapsulação. Termoresistência. Extrusão. Lactobacilli.

Abstract

The consumption of probiotics has increased considerably in recent years and many probiotic foods are already available on the market. Among probiotic microorganisms, Lactobacillus and Bifidobacterium are the most common. However, the application of this kind of microorganism is still limited to foods that not pass through extreme conditions of temperature during processing. Thus, encapsulation techniques are emerging as an alternative to use probiotic microorganisms in thermally processed foods. The main encapsulation techniques are extrusion, spray-drying and emulsification, which each one has its advantages and disadvantages. Currently, various materials can be used for encapsulating bacteria; however, alginate is the most commonly used. To obtain probiotic capsules with high viability even after heat treatments is necessary to associate heat tolerant probiotic strains and appropriated materials and encapsulation techniques.

Keywords: Probiotics. Encapsulation. Thermoresistance. Extrusion. Lactobacilli.

1 Introdução

Atualmente, consumidores buscam cada vez mais por alimentos que sejam seguros, nutritivos e o mais natural possíveis (Jiménez-Colmenero et al., 2006). Por isso, o mercado dos alimentos funcionais cresceu rapidamente nas últimas décadas, devido a essas mudanças comportamentais dos consumidores. Entre os alimentos funcionais, 65% do mercado deste tipo de alimento é representado pelos produtos probióticos (Agrawal, 2005).

O conceito de probióticos surgiu no começo do século 19 devido observações do imunologista russo Elie Metchnikoff que hipotetizou que camponeses búlgaros viviam mais e de forma mais saudável devido ao consumo de leites fermentados contendo *Lactobacillus* benéficos que influenciariam na saúde intestinal (Dixon, 2002). Hoje em dia, probióticos podem ser definidos como "microrganismos vivos que, quando administrados em quantidades adequadas, conferem efeitos benéficos a quem os consome" (FAO/WHO, 2002).

Probióticos já vem sendo incluído em diversos produtos, tanto alimentícios (Anal & Singh, 2007) quanto para alimentação animal (Gaggià et al., 2010). Os principais microrganismos com características probióticos pertencem aos gêneros *Lactobacillus* e *Bifidobacterium* (Weinbreck et al., 2010), porém outras espécies como *Enterococcus*, *Pediococcus* e *Saccharomyces* também podem ser utilizadas (Tabela 1). Entre os benefícios do consumo de microrganismos probióticos, pode-se citar o fortalecimento da imunidade, redução nos sintomas de alergias alimentares, controle da síndrome do intestino irritado/inflamado, aumento na tolerência à lactose e redução nos fatores de riscos para alguns tipos de câncer (Parves et al., 2006). No entanto, para que exerçam os efeitos benéficos à saúde, é indicado que a dose diária de probióticos seja entre 10^{8}-10^{9} UFC/g (Champagne et al., 2005), sendo que a mínima concentração de probióticos no alimento deve ser entre 10^{6}-10^{7} UFC/g no momento do consumo (Nulkaekul et al., 2012).

Por isso, o maior desafio na incorporação de probióticos em alimentos está em manter a viabilidade das bactérias durante o processamento e período de armazenamento até o consumo do produto (Granato et al., 2010). Dependendo do tipo de alimento, os probióticos estarão sujeitos a uma diversa gama de etapas de produção que não são favoráveis a sua viabilidade, além das características próprias do alimento, de seu armazenamento e condições de consumo. Estudos já relataram a baixa sobrevivência de microrganismos probióticos quando são adicionados como células livres aos alimentos (Burgain et al., 2011; De Vos et al., 2010).

Tabela 1- Principais microrganismos utilizados como probióticos (Vuyst et al., 2004)

Espécies de *Lactobacillus*	*L. acidophilus*
	L. casei
	L. gasseri
	L. jhonsonii
	L. paracasei
	L. plantarum
	L. reuteri
	L. rhamnosus
	L. salivarius
Espécies de *Bifidobacterium*	*B. adolescentis*
	B. animalis
	B. bifidum
	B. breve
	B. infantis
	B. lactis
	B. longum
Outras Bactérias Ácido-Láticas	*Enterococcus faecalis*[1]
	Enterococcus faecium[1]
	Lactococcus lactis
	Pediococcus acidilactici
Outros microrganismos	*Escherichia coli* (Nissle 1917)[2]
	Saccharomyces cerevisae

[1] Uso principalmente em animais
[2] Uso principalmente em formulações farmacêuticas

Na indústria de alimentos há uma ampla gama de produtos onde o uso de altas temperaturas no seu processo de fabricação é utilizado. Entre estes, estão a pasteurização ou esterilização de leite e sucos, cozimento de produtos cárneos e pelletização de rações. O processo de fabricação destes produtos inviabiliza a utilização de probióticos uma vez que estes não são capazes de resistir a temperaturas extremas. Nesse contexto a

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encapsulação surge como uma tecnologia promissora e que já vem sendo utilizada para oferecer proteção aos microrganismos probióticos e aumentar a sua sobrevivência em vários tipos de alimentos (Homayouni et al., 2008; Ozer et al., 2009; Heidebach et al., 2012). Diversos estudos estão sendo realizados objetivando um aumento na resistência térmica de probióticos através da sua encapsulação como uma alternativa para sua utilização em alimentos termicamente processados (Cavalheiro et al., 2015; Chen et al., 2007; Chitprasert et al., 2012; Ding & Shah, 2009; Mandal et al., 2006; Sabikhi et al., 2010; Shaharuddin & Muhamed, 2015; Wang et al., 2015; Zhang et al., 2015; Zhang et al., 2016).

Por isso, o objetivo deste trabalho é fazer uma revisão sobre os principais métodos de encapsulação e suas aplicabilidades na tentativa de aumentar a resistência térmica de probióticos.

2 Encapsulação

Encapsulação pode ser definida como uma tecnologia de empacotamento de materiais sólidos, líquidos ou gasosos em cápsulas que são capazes de liberar seu conteúdo em determinado momento sob influência de condições específicas (Anal & Stevens, 2005; Kailasapathy & Masondole, 2005; Anal et al., 2006; Anal & Singh, 2007). A técnica de encapsulação já é amplamente utilizada nas áreas alimentícia, têxtil, agroquímica e farmacêutica.

Em relação à estrutura das cápsulas, o material externo é chamado de agente encapsulante, de cobertura ou parede, enquanto o material encapsulado (probióticos) é chamado de recheio ou núcleo (Gibbs et al., 1999; Champagne & Fustier, 2007; Fávaro-Trindade et al., 2008; Fritzen-Freire et al., 2013). Ainda, as cápsulas podem ser classificadas de acordo com seu tamanho em macrocápsulas, que são aquelas maiores que 5000 μm e, microcápsulas, onde o tamanho varia entre 0,2 e 5000 μm. Os fatores determinantes para o tamanho das cápsulas são basicamente o material de parede e a técnica utilizada para a encapsulação.

Atualmente existem diversas técnicas que podem ser utilizadas para encapsular probióticos, sendo que as mais comumente utilizadas são extrusão, atomização (spray-drying) e emulsificação.

2.1 Técnicas de encapsulação

2.1.1 Extrusão

A extrusão é uma técnica física de encapsulação que utiliza hidrocolóides como materiais encapsulantes. O material a ser encapsulado é completamente homogeneizado com o material encapsulante. A encapsulação ocorre através do gotejamento dessa solução em outra solução solidificante (cloreto de cálcio) através de um bico. Geralmente, este processo produz cápsulas com tamanho variando entre 2 e 5 mm (Figura 1) (Krasaekoopt et al., 2003). Porém, é possível o uso de um sistema com pressão, o que reduz consideravelmente o tamanho final das cápsulas. Por fim, as cápsulas são separadas da solução solidificante e podem ser secas ou usadas *in natura*. A extrusão é um método simples e barato e como não envolve o uso de condições extremas, produz uma grande viabilidade probiótica (Krasaekoopt et al., 2003; Burgain et al., 2011). Geralmente, a eficiência de encapsulação (EE) ao utilizar essa técnica é próxima de 100% (Krasaekoopt et al., 2004; Kushal et al., 2006; Urbanska et al., 2007).
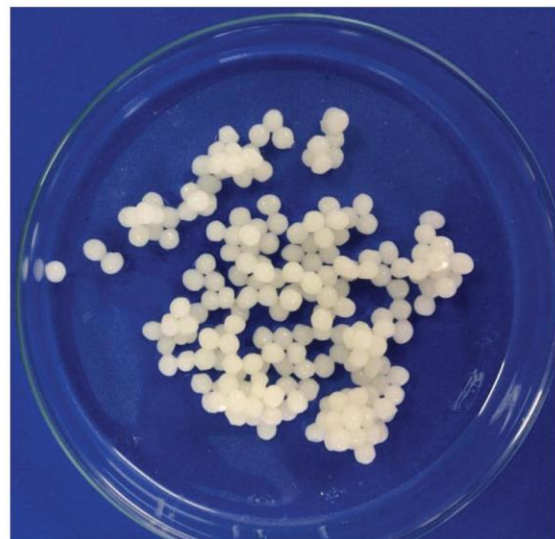
Figura 1 - Cápsulas contendo *Lactobacillus plantarum* obtidas através da técnica de extrusão, utilizando como materiais de cápsula alginato de sódio, trehalose, leite em pó e inulina
Fonte: Os autores

O material mais comumente utilizado para encapsular probióticos através da técnica de extrusão é o alginato, que é um polissacarídeo extraído de diversas espécies de algas e

composto pelos ácidos ß-D-manurônico e α-L-gulurônico. No entanto, as cápsulas obtidas com o uso de alginato são porosas, o que é uma desvantagem quando se deseja proteger os microrganismos de ambientes adversos (Gouin, 2004). Porém, este defeito pode ser compensado pela mistura de alginato com outros polímeros, cobrindo as cápsulas com outros compostos ou aplicando mudanças estruturais no alginato pela adição de diferentes aditivos (Krasaekoopt et al., 2003). Estudos já relataram a mistura de alginato com gelatina, amido, leite entre outras para encapsular probióticos pela técnica de extrusão (Ross et al., 2008; Li et al., 2009; Babu et al., 2009).

2.1.2 Spray-drying

Encapsulação através de spray-drying já é utilizada desde a década de 1950 em uma variedade de aplicações como vitaminas, minerais, óleos de pescado e probióticos (Gouin, 2004; Desai & Park, 2005). Nesta técnica, a solução contendo os probióticos e o material de cápsula é atomizado em uma câmara e a água é evaporada através do contato com o ar quente fazendo com que ocorra a secagem e formação da cápsula. Entre as vantagens desta técnica estão a facilidade de implantação em escala industrial, baixo custo operacional e facilidade de operação contínua. No entanto, devido o uso de altas temperaturas, seu uso é limitado a materiais que apresentem determinada resistência térmica. Apesar de esta ser uma das formas mais tradicionais utilizadas para encapsulas probióticos, alguns estudos não indicam seu uso devido à perda de viabilidade durante o processo (Gibbs et al., 1999; Kailasapathy, 2002; Madene et al., 2006; Mortazavian et al., 2007).

No entanto, a perda de viabilidade probiótica pode ser amenizada através de ajustes nas temperaturas de inlet e outlet para minimizar o choque térmico (Champagne & Fustier, 2007). A perda de viabilidade também vai depender do tipo de material utilizado como material de cápsula. Por exemplo, a adição de amidos aumenta a viabilidade probiótica durante o processo de secagem e armazenamento (Burgain et al., 2011).

2.1.3 Emulsificação

A técnica de emulsificação também é amplamente utilizada para encapsular probióticos. Esta técnica consiste na dispersão da solução contento os microrganismos probióticos e o material encapsulante (fase dispersa) em um óleo (fase contínua). Esta mistura quando submetida à agitação forma uma emulsão água-em-óleo (W/O), que pode ser tanto permanente quanto temporária. O cloreto de cálcio também é utilizado nesta técnica, e quando adicionado à emulsão faz com que ocorra a separação de fases (Krasaekoopt et al., 2003) liberando as cápsulas. No entanto, pode-se utilizar a emulsão permanente contendo os microrganismos encapsulados como ingrediente na preparação de produtos alimentícios.

O alginato também é amplamente utilizado como material de cápsula ao utilizar a técnica de emulsificação (fase dispersa). No entanto, outros materiais também podem ser utilizados como carragenas (Audet et al., 1988), quitosana (Groboillot et al., 1993), gelatina (Hyndman et al., 1993) e celulose acetato phthalate (Rao et al., 1989). Já como fase contínua, para aplicações em alimentos, óleos vegetais como canola, soja, oliva e arroz são normalmente usados (Lamba et al., 2015).

As principais vantagens desta técnica são a facilidade de utilizar-la em grande escala e a obtenção de uma alta eficiência de encapsulação (Chen & Chen, 2007). Ainda, a técnica de emulsão é capaz de formar cápsulas com tamanho muito menor quando comparada à técnica de extrusão, o que pode ser considerado como uma característica desejável dependendo do tipo de alimento onde elas serão incorporadas (Burgain et al., 2011). No entanto, a técnica de emulsificação pode formar cápsulas com formato irregular (Muthukumarasamy & Holley, 2006), e é menos econômica devido à necessidade do uso de óleos vegetais e estabilizantes de emulsão (Gbassi & Vandamme, 2012).

Mais recentemente surgiu o conceito de emulsões duplas ou múltiplas que são sistemas multicompartimentados onde uma emulsão W/O e uma óleo-em-água (O/W) coexistem (Garti, 1997; Marquez & Wagner, 2010; Jimenez-Colmenero, 2013). Assim, surgem as emulsões água-em-óleo-em-água (W/O/W) ou óleo-em-água-em-óleo (W/O/W). Microrganismos como

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L. acidophilus, L. plantarum (Figura 2)*, L. rhamnosus* e *L. salivarius* já foram encapsulados utilizando esta técnica (Shima et al., 2009; Pimentel-González et al., 2009; Rodríguez-Huezo et al., 2014; Zhang et al., 2015).
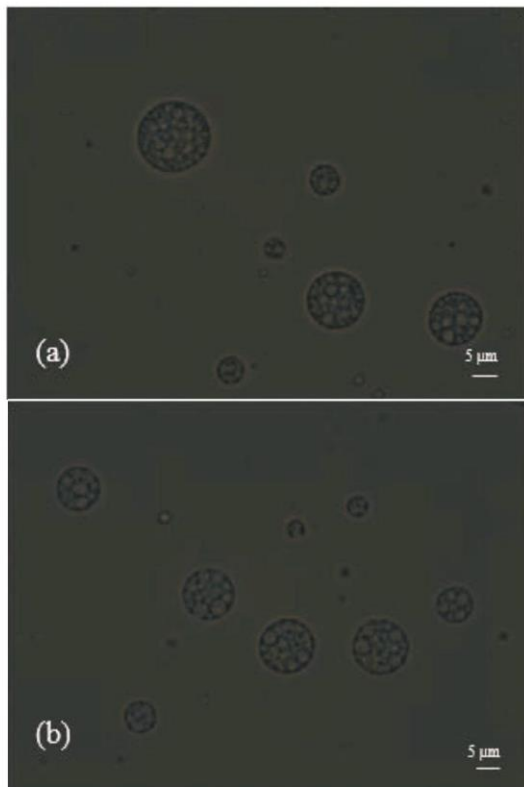
Figura 2 - Cápsulas contendo *Lactobacillus plantarum* obtidas através da técnica de emulsão dupla (W/O/W), utilizando "Aguamiel" (a) e soro de leite (b) como materiais de cápsula. Aumento de 100x
Fonte: Rodríguez-Huezo et al. (2014)

3 Encapsulação de probióticos e resistência térmica

Para que um microrganismo seja incorporado a um alimento, este deve resistir às diversas etapas de processamento, armazenamento e consumo. Sabe-se que, por cada etapa destas ocorrem quedas na viabilidade probiótica, o que acaba por muitas vezes limitar o uso deste tipo de prática na indústria de alimentos. Entre os principais responsáveis na queda da viabilidade probiótica está o uso de altas temperaturas durante o processamento (Ang et al., 1991; Kim et al., 2001). Assim, a encapsulação de probióticos surge como uma alternativa para a incorporação deste tipo de microrganismo em alimentos que são processados termicamente. No

entanto, estudos envolvendo a produção de cápsulas probióticas resistentes á altas temperaturas e sua aplicabilidade em alimentos é escassa. Na tabela 2 estão resumidos os principais estudos realizados com este objetivo.

Conforme Kim et al. (2001), a temperatura subletal e letal de *L. acidophilus* é 53 e 60 ºC, respectivamente. Ao expor este microrganismo tanto na forma livre, quanto encapsulado em alginato de sódio (4%) e amido a temperaturas de 75, 85 e 90 ºC por 30s, Sabikhi et al. (2010) observou reduções entre 8 e 9 log UFC/g na viabilidade de *L. acidophilus* na forma livre. No entanto, quando encapsulados, a redução na viabilidade foi significativamente menor, entre 1 e 4 log UFC/g. A maior proteção aos probióticos encapsulados também está relacionada à concentração de alginato utilizada, uma vez que maiores concentrações oferecem maior proteção aos probióticos durante o tratamento térmico (Hannoun & Stephanopoulos, 1986; Mandal et al., 2006; Sabikhi et al., 2010). Porém, cápsulas de alginato normalmente apresentam poros, o que permite a difusão da temperatura para o interior da cápsula (Mandal et al., 2006; Ding & Shah, 2009; Gouin, 2004). Outros materiais que podem ser utilizados na encapsulação de probióticos, como o carboximetilcelulose também apresentam o problema de gerar cápsulas porosas (Butun et al., 2011). Como alternativa, outros materiais de cápsulas podem ser incluídos como quitosana, trehalose, inulina, leite em pó, maltodextrina, farelo de arroz, entre outros para aumentar a proteção aos probióticos (Cavalheiro et al., 2015; Silva et al., 2015; Chitprasert et al., 2012).

Conforme Cavalheiro et al. (2015), ao encapsular *L. plantarum* em alginato, leite em pó, inulina e trehalose foi possível manter a viabilidade probiótica por um maior período de tempo ao expor estas cápsulas a temperaturas de 70 ºC. No entanto, sempre haverá perda de viabilidade probiótica ao expor as cápsulas aos tratamentos térmicos. Isso se deve a inativação de microrganismos que estão localizados na superfície externa da cápsula e desta forma não possuem a mesma proteção quando comparados aos microrganismos que se localizam na parte interna da cápsula. Simulando o tempo e temperatura utilizados para pelletizar rações de frango (85 ºC por 25 seg), Chitprasert et al. (2012)

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Tabela 2 - Microrganismos probióticos encapsulados submetidos a provas de resistência a altas temperaturas

Microrganismo	Técnica	Material	Temp./Tempo	Referência
L. salivarius	Emulsão S/O/W[1]	Pectina de beterraba	63 ºC / 30 min	Zhang et al. (2016)
L. rhamnosus	Imobilização e extrusão	Alginato e bagaço de cana de açúcar	90 ºC / 40 seg	Shaharuddin & Mohamad (2015)
L. kefiranofaciens	Liofilização	Alginato, goma gelana e leite em pó desnatado	De 25 a 75 ºC, mantendo por 1 min	Wang et al. (2015)
L. salivarius	Emulsão multi-camadas	Isolado proteico de soro, caseinato de sódio e pectina	63 ºC / 30 min	Zhang et al. (2015)
L. plantarum	Extrusão	Alginato, inulina, trehalose e leite em pó	70 ºC / 5, 10, 20 e 30 min	Cavalheiro et al. (2015)
L. plantarum	Emulsão W/O/W[2]	"Aguamiel" e soro de leite	73 ºC / 3 min	Rodríguez-Huezo et al. (2014)
L. reuteri	Emulsão	Carboximetil celulose e farelo de arroz	85º C/ 25 seg	Chitprasert et al. (2012)
L. acidophilus	Emulsão	Alginato e amido	75, 85 e 95 ºC / 30 seg	Sabikhi et al. (2010)
B. bifidum	Extrusão	Alginato e goma gelana	75 ºC / 1 min	Chen et al. (2007)

[1] Emulsão sólido-em-óleo-em-água

[2] Emulsão água-em-óleo-em-água

também relatam que a associação de carboximetilcelulose de alumínio juntamente com farelo de arroz é capaz de promover maior proteção ao *L. reuteri*. Este microrganismo, apesar de apresentar características termotolerantes (Nitisinprasert et al., 2000) não foi capaz de manter uma alta viabilidade após o tratamento térmico empregado (85 ºC/25 seg). Estes resultados evidenciam uma vez mais a necessidade de associar probióticos com características termotolerantes a adequados materiais de cápsulas e técnicas de encapsulação para obter resultados satisfatórios e passíveis de aplicação pela indústria. Ao usar *L. salivarius* que possui uma fraca tolerância térmica (Gardiner et al., 2000), em uma emulsão sólido-em-água-em-óleo (S/W/O), Zhang et al. (2016) não observaram uma proteção eficaz frente aos tratamentos térmicos (63 ºC por 30 minutos). Após o tratamento térmico, foram observados valores de *L. salivarius* na forma livre abaixo dos níveis de detecção (< 1 log UFC/g) enquanto que na forma encapsulada ficaram entre 1,48 e 1,78 UFC/g.

Valores baixos de probióticos não permitem a aplicação das cápsulas em alimentos, uma vez que para ser considerado como alimento probióticos, este deve estar em número superior a 6 log UFC/g no momento do consumo (Nulkaekul et al., 2012).

Utilizar probióticos encapsulados por spray-drying em alimentos processados termicamente não parece ser vantajoso. Devido à utilização de altas temperaturas, o processo de encapsulação pode produzir injúrias nas células probióticas, diminuindo assim sua resistência ao passar por um novo tratamento térmico durante o processamento do alimento.

Outro fator importante é o estudo da aplicabilidade de cápsulas probióticas *in vivo*, ou seja, diretamente no alimento que sofrerá o processo térmico. Nesse contexto, Rodríguez-Huezo et al. (2014) estudaram a viabilidade de *L. plantarum* encapsulados em emulsões duplas contendo "Aguamiel" e soro de leite adicionadas em queijo Oaxaca. Este tipo de queijo é muito popular no México e seu consumo envolve o seu

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derretimento, uma vez que consumido juntamente com "tortillas". Após o tratamento térmico (73 ºC por 3 min), foi observada uma redução de 2,18 log UFC/g para o tratamento sem emulsões duplas enquanto nos tratamentos com emulsões duplas foi de 1,42 e 1,94 log UFC/g, possuindo contagens finais superiores a 6 log UFC/g, que é a mínima concentração para que o alimento seja considerado como probióticos (Nulkaekul et al., 2012). De acordo com os autores, a maior proteção térmica nos tratamentos com *L. plantarum* se deve aos materiais utilizados na encapsulação, uma vez que o "Aguamiel" possui uma grnade concentração de frutoligossacarídeos que agem como termoprotetores (Rodríguez-Huezo et al., 2009) enquanto o soro de leite possui capacidade tamponante contra condições de acidez (Pimentel-González et al., 2009).

Outros estudos também evidenciam a possibilidade de imobilizar bactérias probióticas em pedaços de frutas e assim aumentar a resistência às temperaturas utilizadas na produção de vinho e queijos (Kourkotas et al., 2006a; Kourkotas et al., 2006b).

Porém, apesar do potencial uso da probióticos encapsulados em alimentos termicamente processados, novos estudos são necessários objetivando verificar as possíveis alterações que a inclusão deste tipo de ingrediente pode ocasionar nas características sensoriais dos produtos.

7 Conclusões

Apesar de ser um campo de estudo recente, a encapsulação de probióticos com a finalidade de uso em alimentos termicamente processados é promissora. A escolha de microrganismos que já apresentem maior resistência térmica, assim como adequados materiais e técnicas de encapsulação é fundamental para o sucesso e possibilitar sua aplicação pela indústria. No entanto, mais estudos ainda são necessários para verificar os microrganismos mais adaptados para este fim, assim como materiais e técnicas de encapsulação. Ainda, estudos para verificar as possíveis alterações sensoriais provocadas pelo uso destas cápsulas também estão em falta.

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2.1.2 Manuscrito 2

Application of probiotic delivery systems in meat products

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Review

Application of probiotic delivery systems in meat products

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ABSTRACT

Background: In recent years, probiotic foods have received special attention. The most commonly used probiotic microorganisms are Lactobacillus and Bifidobacterium, and to a lesser degree, Enterococcus and Pediococcus due to their importance for consumer health. Probiotics have also been used as food bioprotectors.
Scopes and approach: This review addresses the potential use of different probiotic delivery strategies for use in meat products to guarantee the viability of the microorganisms throughout the different stages of processing, conservation and preparation, the aim being to obtain probiotic meat products (in some cases even combined with prebiotics) with a positive impact on consumer health.
Key findings and conclusions: In the case of meat products, these studies have mostly focused on fermented meats and, to a lesser degree, on cooked frankfurter-type products or fresh products because the processing to which they are subjected does not guarantee full viability of the microorganisms. Traditionally, starters as free cells have been used to incorporate these microorganisms into meat products. More recently, new microorganism immobilization techniques such as encapsulation have been tested. These new strategies ensure enhanced viability even in meat products subject to thermal treatment during processing or cooking.

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1. Introduction

During the last several decades, a strong link has been established between nutrition and human health (Jiménez-Colmenero, Reig, & Toldrà, 2006). Today, consumer preferences are changing mainly driven by new concerns about wellbeing and a healthier lifestyle (Chen, 2011). This has led to many researches including those designed to enhance the benefits derived from the foods we consume both from a nutritional point of view and in terms of new ingredients incorporated into them with specific functions. In this regard, there has been a major increase in the number of studies conducted on probiotics in recent years. Probiotic foods mainly include fermented dairy products, vegetables, juices and meat products (Burgain, Gaiani, Linder, & Scher, 2011; Khan et al., 2011; Martins et al., 2013; Nulkaekul, Lenton, Cook, Khutoryanskiy, & Charalampopoulos, 2012; Rouhi, Sohrabvandi, & Mortazavian, 2013).

The concept of probiotics emerged from observations early in the 19th century by Russian immunologist Elie Metchnikoff who hypothesized that the long and healthy lives of Bulgarian peasants were rooted in their consumption of fermented milks containing beneficial Lactobacillus and its positive influence on colonic health (Dixon, 2002). Until then, microbes were known only for their negative effects commonly associated with morbidity and mortality (Douglas & Sanders, 2008). Nowadays probiotics can be defined as "live microorganisms which, when administered in adequate amounts, confer health benefits on the consumer" (FAO/WHO, 2002).

The most common probiotics used in food are from the

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Lactobacillus and _Bifidobacterium_ genera, although _Enterococcus_ and _Pediococcus_ have also been used (Weinbreck, Bodnár, & Marco, 2010). Some of these probiotic microorganisms have also been traditionally used for bioprotectors purposes in meat products of various kinds (Bomdespacho, Cavallini, Zavarizi, Pinto, & Rossi, 2014; Chaillou et al., 2014; Sparo, Confalonieri, Urbizu, Ceci, & Bruni, 2013). As probiotic propose a wide range of these pro-biotics have proven clinically effective against many diseases in humans and animals. Probiotics in general can prevent gastroin-testinal infections, enhance host immunity and relieve diarrhea (Douglas & Sanders, 2008). The safety assessment of probiotics for human use is discussed in a review by Sanders et al. (2010). Clinical studies have shown that some probiotics can be safely adminis-tered to immunocompromised patients without side-effects (Bernardeau, Vernoux, Henri-Dubernet & Gueguen, 2008; Sanders et al., 2010). Moreover, the literature reports few correla-tions between adverse events and probiotic consumption (Boyle, Robins-Browne, & Tang, 2006), and also that the risk of lactoba-cillemia is rare (Borriello et al., 2003). However, there is some controversy over the use of enterococci strains as probiotics or bioprotective cultures since they have been described as opportu-nistic pathogens (Morrison, Woodford, & Cockson, 1997). Some enterococci strains are associated with nosocomial infections and cause human diseases such as bacteremia, endocarditis or urinary tract infections, and therefore their use is not indicated in people with immunodeficiency. The main problem of enterococci use is the presence of antibiotic resistance and virulence factors, which are clearly explained in the review by Franz, Huch, Abriouel, Holzapfel, and Gálvez (2011). On the other hand, enterococci strains can bring health benefits to the host, such as immune regulation, lowering of serum cholesterol, and treatment of diarrhea, antibiotic-associated diarrhea and irritable bowel syndrome. According to Allen, Okoko, Martinez, Gregorio, and Dans (2004), _Enterococcus faecium_ SF68 is one of the best documented and effective enterococci strains for use as a probiotic.

Studies have reported the ability of some probiotic strains such as _Bifidobacterium longum_ B6, _B. longum_ ATCC 15708 and _Bifido-bacterium animalis_ DN-173010 to reduce lactose intolerance by increasing production of the enzyme ß-galactosidase (He et al., 2008; Jiang, Mustapha, & Savaiano, 1996). A reduction in the symptoms of inflammatory bowel disease (Chron's disease and Ulcerative colitis) is related to consumption of fermented milk containing _Bifidobacterium bifidum_ Yakult, _Bifidobacterium breve_ Yakult, and _Lactobacillus acidophilus_ YIT 0168 (Ishikawa et al., 2003; Kato et al., 2004). Probiotics can also be helpful in preventing and treating some types of cancer. Studies have shown that the risk of colon cancer is reduced by consuming yoghurt containing _B. longum_ 913 and _Lb. acidophilus_ 145 (Oberreuther-Moschner, Jahreis, Rechkemmer, & Pool-Zobel, 2004) and anti-tumor activity was observed in _Escherichia coli_ Nissle 1917 strains (Stritzker et al., 2007). Probiotic mechanisms of action are likely due to many fac-tors and related to activity against pathogenic microorganisms including the secretion of antimicrobial substances, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system (Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-Llorente, & Gil, 2012).

The health benefits of probiotics depend on many factors asso-ciated to the microorganism (genus, species, strain etc.) and the product (processing, temperature, pH, a_w etc.). However, the beneficial effects of probiotics not only depend on the right strain of microorganism but also on a minimum level of viability. The level of probiotic necessary to bring about positive effects is still unknown and varies in different studies and standards (Rouhi et al., 2013). The higher the level of probiotic microorganisms in food the better,

however, there is a degree of consensus that the minimum pro-biotic concentration in food should be between 10^6 and 10^7 CFU/g or CFU/ml at the moment of consumption (Nulkaekul et al., 2012). However, consumption of high viable counts of probiotics does not guarantee the same survival rate after the arrival of the cells in the intestine (Iravani, Korbekandi, & Mirmohammadi, 2015). The pro-biotic must also survive the upper gastrointestinal tract (GIT) (gastric acid and bile) so that it can adhere to intestinal mucosa and competitively eliminate pathogens and maintain viability (Sharma & Devi, 2014).

Over the last ten years, meat products have generally been related to health problems such as coronary diseases, hypertension, cancer, etc. (Jiménez-Colmenero et al., 2006). However, meat and meat products are an important group of highly nutritious foods forming part of the diet of many consumers around the world. Meat products are an important source of a wide range of nutrients and contribute a considerable proportion of the dietary intake of various nutrients that are essential for optimal growth and devel-opment. In recent years, major efforts have been made to develop meat-based functional foods using strategies related to increasing the presence of beneficial compounds and limiting those with negative health implications. These are based on approaches that basically affect animal production practices (genetic and nutri-tional) and meat processing systems (reformulation processes) (Olmedilla-Alonso, Jiménez-Colmenero, & Sánchez-Muniz, 2013). A number of approaches can be used to remove, reduce, increase, add and/or replace different functional ingredients as, for example, probiotic microorganisms.

A variety of probiotic meat products have been prepared with most of the focus on fermented meat products as these provide a more appropriate environment (no heat treatment) for the viability of microorganisms (Table 1). Studies using probiotic microorgan-isms in other types of meat products such as cooked meat sausages and fresh ground or raw meat are still scarce mainly because of the processing required for these products such as heating, the use of additives (sodium chloride, sodium nitrite, etc.) and consume conditions. Some of these probiotic microorganisms have also been used in these meat products as bioprotectors against pathogenic strains (Table 1). The meat industry is now focusing on producing meat products with potential health benefits by using probiotic microorganisms. To that end, they are searching for delivery stra-tegies to incorporate microorganisms that are able to protect them from harsh environments and storage conditions, limiting the ef-fect of factors that tend to decrease or eliminate the viability of microorganisms in food (Anal & Stevens, 2005; Burgain et al., 2011). Overcoming these difficulties has opened up new challenges for meat research and industry. Although there are various reviews of probiotics in meat products (De Vuyst, Falony, & Leroy, 2008; Khan et al., 2011; Kolozyn-Krajewska & Dolatowski, 2012; Rouhi et al., 2013; Työppönen, Petäjä, & Mattilaa-Sandholm, 2003), they focus on fermented products and/or without giving special consideration to incorporation strategies.

The aim of this review is to give an overview of the different probiotic delivery strategies and their incorporation and behavior of probiotics in different meat matrices. These strategies, based mainly on encapsulation and/or entrapping in gelled dispersions, are designed to enhance viability during manufacture, storage, cooking and gastrointestinal stages with the aim of obtaining healthy meat products. We also review the control of probiotic microorganisms in meat products and the current state of probiotic meat products.

2. Delivery systems for probiotics

Traditionally, probiotics have been added to food as free cells

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Table 1
Free-cell microorganisms used in meat products as bioprotectors and/or probiotic cultures.

Meat products		Microorganisms[a]	Function[b]	Reference
Fermented	Sturgeon sausage	*Lb. plantarum* Y9, *Lb. pentosus* 31-1, *Lb. acidophilus* N4, *Lb. rhamnosus* LGG, *Lb. paraplantarum* S4, L-ZS9 and P	B/P	Wang, Sun et al. (2015)
	Fuet	*Lb. rhamnosus* CTC1679, *Lb. casei/paracasei* CTC1678	P	Rubio, Jofré, Aymerich et al. (2014)
	Fuet	*Lb. rhamnosus* CTC1679	P	Rubio, Martín et al. (2014)
	Fermented sausage	*Lb. fermentum* CTC1693, *Lb. casei/paracasei* CTC1677, CTC1678, *Lb. rhamnosus* CETC1679, *Lb. gasseri* CTC1700, CTC1704	B/P	Rubio, Jofré, Martín, Aymerich, and Garriga (2014)
	Italian salami	*Lb. acidophilus, B. lactis*	P	Ruiz, Villanueva, Favaro-Trindade, and Contreras-Castillo (2014)
	Dry-cured pork loins	*Lb. casei* LOCK 0900	O	Stadnik and Dolatowski (2014)
	Dry-aged pork loins	*Lb. casei* LOCK 0900	O	Stadnik, Stasiak, and Dolatowski (2014)
	Raw-fermented sausage	*Lb. casei* LOCK 0900	B/P	Trzaskowska et al. (2014)
	Mutton fermented sausage	*Lb. acidophilus* CCDM 476, *B. animalis* 241a	P	Holko et al. (2013)
	Italian (*Ciauscolo* and Larded) and Swiss salami	*Lb. rhamnosus* IMC501, *Lb. paracasei* IMC502	P	Coman et al. (2012)
	"Longaniza de Pascua"	*Lb. casei* CECT 475	B/P	Sayas-Barberá et al. (2012)
	Dry-fermented sausage	*Lb. casei* LOCK 0900	P	Wójciak et al. (2012)
	Iberian dry-fermented sausage "Salchichón"	*Lb. reuteri* PL519	P	Ruiz-Moyano, Martín, Benito, Aranda et al. (2011)
	Iberian dry-fermented sausage	*Lb. fermentum* HL57, *P. acidilactici* SP979	P	Ruiz-Moyano, Martín, Benito, Casquete et al. (2011)
	Fermented sausage	*Lb. casei* LC01, *Lb. paracasei* ATCC 1046, *Lb. rhamnosus* ATCC 7469	P	Macedo et al. (2008)
	Dry-fermented sausage	*En. faecalis* CECT7121	B	Sparo et al. (2008)
	Scandinavian type fermented sausage	*Lb. plantarum* MF1291 and MF1298, *Lb. pentosus* MF1300	P	Klingberg, Axelsson, Naterstad, Elsser, and Budde (2005)
	Dry sausage	*Lb. rhamnosus* GG, E-97800 and LC-05	P	Erkillä et al. (2001)
	Fermented sausage	*Lb. gasseri* JCM1131	O	Arihara et al. (1998)
	Fermented sausages	*Lb. plantarum, P. pentosaceus*	O	Kearney et al. (1990)
	Hungarian Salami	*P. pentosaceus, Lb. paracasei, Lb. casei imunitas, Lb. reuteri, Lb. acidophilus, B. longum, B. lactis*	B/P	Pidcock, Heard, and Henriksson (2002)
Cooked	Fat and salt reduced cooked meat sausages	*P. acidilacti* UAM15c, *Lb. plantarum* UAM10a, *P. pentosaceus* UAM17, *Lb. curvatus* UAM18	O	Pérez-Chabela, Díaz-Vela et al. (2013)
	Vienna sausage	*Lb. curvatus, Lb. plantarum, P. acidilactici, P. pentosaceus*	B	Pérez-Chabela et al. (2008)
	Vienna sausage	*L. lactis* ATCC 1154, *Lb. piscícola* MXVK76, *Lb. alimentarius* ATCC 29643, *Enterococcus* sp. MXVK29	B	Victória-León et al. (2006)
Raw	Chicken hamburger	*Lb. acidophilus* CRL1014	B	Bomdespacho et al. (2014)
	Ground beef meat	*Lb. sakei*	B	Chaillou et al. (2014)
	Ground beef meat	*En. faecalis* CECT7121	B	Sparo et al. (2013)

[a] B = *Bifidobacterium*; L. = *Lactococcus*, Lb = *Lactobacillus* and P = *Pediococcus*.
[b] Added as B = Bioprotector, P = Probiotic, O = Other functions, mainly technological properties.

performing a dual function (probiotic and bioprotector). However, interest in including these microorganisms in new products subject to processing and other adverse conditions (preparation, storage, distribution and consumption processes, cooking and conditions present in the GIT), means protecting their viability during these different stages to which all foods, especially meat, are subject. This need for protection has led to the study and development of different probiotic delivery systems enabling microorganisms to remain viable long enough to guarantee beneficial effects (Table 2). This requires naturally or artificially situating microorganisms in an area or space where they are able to maintain their activity and function.

2.1. Encapsulation approaches

Encapsulation is the most widely known microorganism delivery system used to provide a protective environment for microorganisms in adverse conditions (Burgain et al., 2011; Heidebach, Först, & Kulozik, 2012; Mortazavian et al., 2008). Encapsulation has been broadly applied in different fields such as pharmaceuticals, agricultural and chemistry to protect enzymes and medicines (Gibbs, Kermasha, Alli, & Mulligan, 1999). More recently, this technique has been applied to food to control flavor, stability, shelf-life and to protect probiotics (Burgain et al., 2011). Probiotic encapsulation technology has emerged and developed rapidly in recent years due to poor probiotic survival in products containing free probiotic cells (Champagne & Fustier, 2007; Kailasapathy, 2002, 2009).

Encapsulation is the method whereby solids, liquid or gaseous materials are placed in an inert shell (capsule) and are released at a controlled rate under specific conditions (Anal & Stevens, 2005; Champagne & Fustier, 2007). The aim of physicochemical or mechanical encapsulation processes is to entrap substances in a material in order to produce particles with different diameters (Chen & Chen, 2007). In encapsulation there are two well-defined parts, the encapsulated substance which is the core material and the matrix which is the coating or shell (wall material) acting as a barrier to protect the encapsulated substance. Attention must also be paid to the solution (water, saline, lactic etc.) in which the bioactive component is dispersed. This solution must ensure the viability of the cells which need to remain metabolically active for long periods of time.

Table 2
Probiotic microorganisms, encapsulation techniques and wall materials.

Microorganism[a]	Technique	Wall material	Reference
Lb. acidophilus 5, Lb. casei 01	Extrusion	Sodium alginate, Inulin, Galactooligosaccharides, Chitosan	Krasaekoopt and Watcharapoka (2014)
Lb. acidophilus La-5, B. lactis Bb-12	Extrusion	Calcium alginate	Mortazavian et al. (2008)
Lb. acidophilus La-5, B. bifidum BB-12	Extrusion/Emulsion (W/O)	Sodium alginate, Carrageenan	Özer et al. (2009)
B. adolescentis ATCC 15703	Emulsion (W/O)	Chickpea protein, Sodium alginate, Genipin, K-carrageenan	Wang, Korber, Low, and Nickerson (2014)
Lb. plantarum DPC206, P. acidilactici DPC209, Lb. reuteri DPC16, B. lactis HN019	Emulsion (W/O)	Sodium alginate, Chitosan, Sucrose, Lecithin	Chen et al. (2012)
Lb. fermentum CECT5716	Emulsion (W/O)	Sodium alginate, Unmodified starch	Martin et al. (2013)
Lb. salivarius NRRLB-30514	Multiple-layered emulsion	Milk fat, Sodium caseinate, pectin	Zhang et al. (2015)
Lb. acidophilus JCM 1132	Double emulsion	MRS broth, decaglycerol monolaurate	Shima et al. (2009)
Lb. rhamnosus LC705	Double emulsion	Sweet whey	Pimentel-González et al. (2009)
Lb. acidophilus La-5, B. animalis BB-12, Propionibacterium jensenii 702	Spray drying	Goat's milk	Ranadheera, Evans, Adams, and Baines (2015)
Lb. acidophilus 2401, L. lactis ssp. cremoris R-704	Spray drying	Vegetable oil, Sodium caseinate, Fructooligasaccharides, Glucose	Dianawati, Mishra, and Shah (2013)
Lb. kefiranofaciens M1	Freeze drying	Skim milk, Sodium alginate, Gellan Gum	Wang, Ho et al. (2015)
Lb. paracasei subsp. paracasei F19, B. lactis Bb12	Freeze drying	Casein	Heidebach et al. (2010)

[a] B = Bifidobacterium, L = Lactococcus, Lb = Lactobacillus and P. = Pediococcus.

The capsule wall material is the protective matrix designed to enhance the stability of probiotics during the different stages of food processing. A variety of materials such as carbohydrates, polysaccharides, milk proteins, carrageenan, gums etc. has been used for this purpose in encapsulation (Table 2). However, sodium alginate is the most widely used substance for microorganism encapsulation. Alginate is a polysaccharide composed of β-D-mannuronic and α-L-guluronic acids and is nontoxic, biocompatible and inexpensive. The disadvantage of this material is the porosity of the capsules (Gouin, 2004) and its sensitivity to acid conditions (Mortazavian et al., 2008). On many occasions this problem has been solved by combining alginate with other ingredients such as starch, chitosan, etc., which are more resistant to adverse conditions (Krasaekoopt, Bhandari, & Deeth, 2003; Krasaekoopt & Warcharapoka, 2014). The use of milk proteins ensures the biocompatibility of the cells, as they are the natural vehicle of many probiotic bacteria. Cavalheiro et al. (2015) also observed that beads of alginate in association with milk powder, trehalose and inulin can increase the viability of Lactobacillus plantarum to heat stress. Gums, mainly gellan and xanthan are used to encapsulate probiotics because they are very resistant to stomach acids. Carrageenans and gelatins have also been tested for use as encapsulation material, mostly forming gels, which, in the case of gelatin, are thermo reversible (Burgain et al., 2011). In general, selection of one or another of these materials is also based on the encapsulation technique used and other factors such as cost, ease of handing and the type of food or meat products to which they are to be incorporated (Burgain et al., 2011).

Probiotic encapsulation is commonly achieved through techniques such as extrusion, emulsification, spray drying and freeze drying with capsules ranging from a few micrometers to a few millimeters (Champagne & Fustier, 2007; Gibbs et al., 1999; Gouin, 2004; Rathore, Desai, Liew, Chan, & Heng, 2013).

The extrusion technique is the oldest and most common for converting hydrocolloids into capsules due to handling ease, low cost and efficiency (Krasaekoopt et al., 2003). The technique consists of a core material completely mixed with the wall material which is projected into a nozzle in a hardening solution (Desai & Park, 2005). The main ingredients/materials used for this technique are alginate, carrageenan and pectin, which are able to form gels in presence of minerals such as, calcium and potassium and have been used successfully to entrap probiotic microorganisms inside the capsule (Tables 2 and 3). Gel is formed by the bonding of multiple free carboxylic radicals by gelling ions, all done under controlled-environment conditions (Champagne & Fustier, 2007). We can speak of direct and inverse extrusion depending on whether the alginate solution is added to the calcium solution or vice versa. The main advantage of this technique is that it is simple and gentle and does not harm probiotic cells generally resulting in high probiotic viability. Moreover, it is cheap, all conditions can be controlled and it works well in aerobic or anaerobic conditions (Krasaekoopt et al., 2003). Also, the beads with probiotic microorganisms made by means of the extrusion technique can be mixed at the same time with different ingredients such as starch, chitosan and milk protein which enhance the stability of the capsules throughout the process and better protect the probiotic in the gastrointestinal tract (Krasaekoopt et al., 2003; Krasaekoopt & Warcharapoka, 2014). While their use in large-scale production to date is limited due to slow formation of beads, technological advances are overcoming this disadvantage (Burgain et al., 2011). These extrusion capsules have been tested in different foods, mainly dairy products, especially cheese, yogurt drink and fermented milks (Krasaekoopt & Watcharapaka, 2014; Mortazavian et al., 2008; Özer, Kirmachi, Senel, Atamer, & Hayaloglu, 2009). This type of technique has also been tested in fermented meat products (Muthukumarasamy & Holley, 2006, 2007) (Table 3).

The emulsion method is also commonly used for encapsulation of microbial cells. This technique involves dispersion of the cells mixed in a polymer solution (dispersed phase) in an oil (continuous phase). The mixture forms a water-in-oil (W/O) emulsion with the aid of surfactant and stirring followed by the step in which the phases are separated and the dispersed phase encapsulates the probiotic microorganism as core material (Heidebach et al., 2012; Krasaekoopt et al., 2003). Ingredients such as milk proteins or caseinate as well as alginate have mostly been used in the emulsion method (Tables 2 and 3). The main advantage of this technique is large particle size ranging from 0.2 to 5000 μm (Burgain et al., 2011) achieved by controlling the stirring speed and water/oil ratio (Kailasapathy, 2009). Encapsulation using the emulsion technique enhances protection of Lb. plantarum DPC206, Lactobacillus reuteri

Table 3
Application of microorganism delivery system in meat products.

Meat product	Microorganism[a]	Wall material	Delivery technique	Function[b]	Reference
Fermented sausage	*Lb. plantarum, P. pentosaceus*	Calcium alginate	Extrusion − lyophilization	O	Kearney et al. (1990)
Dry-fermented sausage	*Lb. reuteri* ATCC 55730	Sodium alginate	Extrusion − Emulsion	P	Muthukumarasamy and Holley (2006)
Dry-fermented sausage	*Lb. reuteri* ATCC 55730, *B. longum* ATCC 15708	Sodium alginate	Extrusion	B	Muthukumarasamy and Holley (2007)
Dry-fermented sausage	*Lb. casei* ATCC 393	Wheat grains	Immobilization-matrix	B/P	Sidira, Karapetsas et al. (2014)
Dry-fermented sausage	*Lb. casei* ATCC 393	Wheat grains	Immobilization-matrix	P	Sidira, Galanis et al. (2014)
Salami	*Lb. curvatus* MBSa2	Calcium alginate	Extrusion	B	Barbosa, Todorov, Jurkiewicz, and Franco (2015)
Dry-fermented sausage	*Lb. casei* ATCC 393	Wheat grains	Immobilization-matrix	O	Sidira et al. (2015)
Cooked meat batters	*A. viridans* UAM21, *En. faecium* UAM10c, *Lb. plantarum* UAM10a, *P. pentosaceus* UAM17	Arabic gum	Spray drying	B	Pérez-Chabela, Lara-Labastida et al. (2013)

[a] A = *Aerococcus*; B = *Bifidobacterium*; En = *Enterococcus*; Lb = *Lactobacillus* and P = *Pediococcus*.
[b] Added as B = Bioprotector, P = Probiotic, O = Other functions, manly technological properties.

DPC16, *Pediococcus acidilactici* DPC209 and *Bifidobacterium lactis* HN019 from GIT conditions and during storage (Chen et al., 2012). More recently, tests have been conducted on encapsulation in double or multiple emulsions which are multi-compartmentalized systems in which oil-in-water (O/W) and water-in-oil (W/O) coexist and where dispersed phase globules themselves contain even smaller dispersed droplets (Garti, 1997; Márquez & Wagner, 2010). The most common forms of multiple emulsions are water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O). The double emulsion may serve as a suitable wrapper to encapsulate and protect probiotic bacteria during food processing, storage and passage through the human GIT (Jiménez-Colmenero, 2013; Serdaroğlu, Öztürk, & Kara, 2015). In this context, protection of *Lb. acidophilus* JCM 1132 against cytotoxic gastric juice (Shima, Morita, Yamashita, & Adachi, 2006) and bile acids (Shima, Matsuo, Yamashita, & Adachi, 2009) has been reported by incorporating the bacteria into the inner-aqueous phase of a W/O/W emulsion. Also, the entrapment of *Lactobacillus rhamnosus* LC705 in a double emulsion using sweet whey as a hydrophilic emulsifier was tested under simulated gastrointestinal tract conditions (Pimentel-González, Campos-Montiel, Lobato-Calleros, & Pedroza-Islas, 2009). Recently, tests were conducted on another mode of emulsion based on a multi-layered system where emulsion droplets with multiple lipid-protein-pectin layers were used to prepare primary solid/oil/water (S/O/W) for the encapsulation of *Lactobacillus salivarius* NRRLB-30514 (Zhang, Lin, & Zhong, 2015).

Spray drying is commonly used for probiotic encapsulation since it is fast, reproducible, has a low operational cost and adapts well to industrial equipment (Burgain et al., 2011; Desai & Park, 2005; Gouin, 2004). This technique involves the atomization of probiotic cells in a polymeric solution into hot drying air to rapidly evaporate water (Corcoran, Ross, Fitzgerald, & Stanton, 2004). A wide range of wall materials can be used in the spray drying process (Tables 2 and 3) but gum arabic and starches tend to form more spherical microparticles (Chen & Chen, 2007; Kailasapathy, 2009). Several strains of probiotic bacteria such as *Lb. acidophilus*, *Lb. plantarum*, *Bifidobacterium*, etc. have successfully been encapsulated using spray drying technique (Table 2). The main disadvantage of the spray drying technique is that it always has a negative effect on probiotic viability, especially due to the heat generated during the drying process (Burgain et al., 2011). Efforts have been made to reduce viability loss such as optimizing inlet and outlet temperature, using a combination of fluidized bed drying to minimize heat shock, pre-adaptation to heat prior to spray drying (Champagne & Fustier, 2007) and the use of protectants such as granular starch, soluble fibre and trehalose to improve probiotic viability (Burgain et al., 2011).

Freeze-drying technology or lyophilization is based upon sublimation of frozen cells followed by sublimation under high vacuum conditions (Santivarangkna, Kulozik, & Foerst, 2007). These processing conditions are milder than spray drying resulting in comparatively higher probiotic viability (Wang, Yu, & Chou, 2004) but this is an expensive technology compared with other encapsulation methods (Santivarangkna et al., 2007). *Lactobacillus* and *Bifidobacterium* cells had better post drying survival and storage viability rates when first encapsulated into enzymatically gelled sodium caseinate following freeze-drying (Heidebach, Först, & Kulozik, 2010). Recently, encapsulation by freeze-drying using skim milk, sodium alginate and gellan gum proved successful in protecting *Lactobacillus kefiranofaciens* M1 against temperature change (from 25 °C to 75 °C) and adverse intestinal conditions (Wang, Ho, Chen, & Chen, 2015). The use of protectants such as trehalose, glucose, maltodextrin, skim milk, and whey protein is an alternative to protect probiotic microorganisms before freeze drying (Martín, Lara-Villoslada, Ruiz, & Morales, 2015).

2.2. Entrapping probiotics in gelled dispersions approaches

Recently, tests have been conducted on other microorganism immobilization methods in different matrices that trap microorganisms without encapsulation per se (Table 2). Microorganism immobilization may occur naturally by adherence to surfaces or to other microorganisms owing to cellular structures or substances they themselves secrete. The most common of available immobilization techniques are adsorption or attachment of cells to an inert substrate, self-aggregation by flocculation or use of cross-linking agents and entrapment using polymers (Jen, Wake, & Mikos, 1996). Many studies have focused on immobilization or entrapment of probiotic bacteria in materials such as dietary fiber, proteins, minerals and vitamins, starch wheat grains etc. (Mattila-Sandholm et al., 2002; Sidira, Galanis, Nikolau, Kanellaki, & Kourkoutas, 2014; Sidira, Kandylis, Kanellaki, & Kourkoutas, 2015; Sidira, Karapetsas, Galanis, Kanellaki, & Kourkoutas, 2014), some also exhibiting prebiotic activity due to non-digestible carbohydrate content. In contrast with other forms of encapsulation, the cells are not perfectly localized inside the capsules but rather are trapped in a network of different materials (starch, fibre or polysaccharide gel). These immobilized probiotic cell techniques offer the advantage of easy cell separation from the products, greater productivity due to high cell concentration and protection of cells against harsh environments (Rathore et al., 2013). These types of polysaccharide-based gel systems have been used to immobilize or trap oil and to produce food ingredients (oil bulking agent) used in meat reformulation processes with healthy lipid profiles (Herrero,

Carmona, Jiménez-Colmenero, & Ruiz-Capillas, 2014; Herrero, Ruiz-Capillas, Jiménez-Colmenero, & Carmona, 2014).

The successful evaluation of these delivery systems (encapsulation or entrapping) is very important to control the process and the final probiotic viability. Since these processes should maintain high viability of probiotic for application in food, it is important that the encapsulation method not produce alterations. This could be controlled via the encapsulation yield (EY), which is a way to compare the probiotic viability before and after the encapsulation process and is generally calculated by comparing the probiotics (colony forming units) achieved in probiotic solution and in capsules (Heidebach et al., 2012). The encapsulation yield depends on the delivery system used and the conditions produced by the encapsulation process itself (Heidebach et al., 2012). An EY of 100% has been reported using the extrusion method (Krasaekoopt, Bhandari, & Deeth, 2004; Kushal, Anand, & Chander, 2006; Urbanska, Bhathena, & Prakash, 2007). In the case of other systems such as emulsions, double emulsions and freeze-drying EY values close to 100% can also be achieved (Martin, Lara-Villoslada, Ruiz & Morales, 2013; Pimentel-González et al., 2009; Wang, Ho et al., 2015). Spray-drying, on the other hand, produces a lower EY as it is performed at high temperature, which is not compatible with the survival of bacteria (Burgain et al., 2011). In the case of entrapping gelled systems, the yield (entrapping yield) needs to be evaluated in the same way as for encapsulation.

Selection of one or another of these strategies will depend on the industrial management strategy used for the type of meat product in question and processing conditions (heating, freezing, etc.), storage times and conditions, consumer preparation conditions and the GIT conditions. Besides, they have been according to the sensorial and technological characteristic of products where will be incorporated.

3. Application of probiotic delivery systems in the development of healthy meat products

The most common probiotics used in meat products are mainly from the *Lactobacillus* and *Bifidobacterium* genus although others such as *Enterococcus* and *Pediococcus* have also been used (Table 1). Probiotics in meat products have been used as bioprotective cultures against harmful and pathogenic bacteria. The bioprotective effect of probiotics in meat products is due to the bacteriocin production, mainly by lactobacilli and enterococci strains (Dobson, Cotter, Ross, & Hill, 2012).

Due to variability in the processing of meats, the factors affecting the survival of probiotic microorganisms vary greatly depending on the type of product. Therefore, to optimize effectiveness in the use of probiotics in meat products, it is important to have a thorough understanding of the conditions associated with the products in which their use is intended.

3.1. Probiotic fermented meat products

Fermented meat products are the ideal host for probiotics due to the absence of heat in processing and because the sausage matrix protects probiotics in their journey through the GIT allowing them to provide their full health benefits (Ammor & Mayo, 2007; Klingberg & Budde, 2006). The fermentation of meat products involves a large ecosystem in which bacteria, yeasts and molds coexist (Cocolin, Dolci, & Rantsiou, 2011). Different groups of microorganisms play different roles in meat fermentation and are essential in achieving the traditional sensory profile and ensuring that the final product is safe for consumption. The most important groups for meat fermentation are lactic acid bacteria (*Lactobacillus*) and coagulase negative cocci (*Staphylococcus*) (Ammor & Mayo,

2007). Meat fermentation can be spontaneous when caused by microorganisms from the meat itself or from the environment or by means of microorganisms intentionally added as starter cultures. Meat starter cultures are defined as a preparation that contains active or dormant microorganisms that engage in the desired metabolic activity in the meat. Traditionally, meat companies use a commercial starter culture with specific microorganisms aiming to improve the quality (flavor) and safety of their products and standardize production process throughout the ripening period (Lücke, 2000).

Replacement of traditional meat starter cultures with others containing probiotic microorganisms is an interesting alternative for meat companies seeking to produce products that improve consumer health. Studies involving the use of probiotic microorganisms in meat fermentation first emerged in the 90's using strains such as *Lb. plantarum*, *Propionibacterium pentosaceus* and *Lactobacillus gasseri* JCM1131 showing great potential for meat fermentation (Arihara et al., 1998; Kearney, Upton, & McLoughlin, 1990) (Table 1). Subsequent studies showed the ability of *Lb. gasseri* JCM1131 to prosper under high sodium chloride and sodium nitrite levels (Arihara & Itoh, 2000). As a result, a number of studies have been conducted in recent years aiming to verify the adaptation, viability and quality of probiotic microorganisms such as *Lb. acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lb. plantarum*, *Lb. reuteri*, *Lb. rhamnosus*, *B. animalis*, *B. lactis* and *P. pentosaceus* when incorporated into different fermented meat products such as *fuet* (low-acid sausage), Italian salami, Swiss salami, mutton fermented sausage, sturgeon fermented sausage, Norwegian salami, Hungarian salami, *longaniza de Pascua*, *salchichón* (Iberian dry-fermented sausage) and dry cured pork loins (Table 1). Probiotic cultures adapted well to the fermented sausage environment in all cases, *i.e.* high amounts of sodium chloride, sodium nitrate and nitrite (curing salts) with antimicrobial properties, in addition to low pH and a_w during fermentation and ripening (De Vuyst et al., 2008; Khan et al., 2011).

An important aspect to consider when preparing probiotic fermented sausages is their impact on the technological and sensorial properties of the final product as the presence of probiotics could bring changes in physicochemical properties such as pH and a_w, when compared to traditional sausages. Physicochemical alterations in meat products could result in sensory changes, especially taste and texture, resulting in rejection by consumers. These changes depend on probiotic strain and the amount of probiotic and/or traditional meat starter cultures added. Macedo, Pflanzer, Terra, and Freitas (2008) observed low pH values (4.4) in Brazilian dry-fermented sausages following the addition of 10 log CFU/g levels of *Lb. rhamnosus* ATCC 7469, *Lb. paracasei* ATCC 10746 and *Lb. casei* LC01 compared to traditional meat starter cultures (*P. pentosaceus* and *Staphylococcus xylosus*). It is important for fermented sausages to have pH values close to 5.0 to protect products against Gram negative microorganisms and higher acid can have a negative effect on the sensory parameters of the final product such as taste which was also observed in this study (Työppönen et al., 2003). The presence of *P. pentosaceus* associated with probiotic lactobacilli produces higher levels of lactic acid than control treatments causing higher acidification and lower scores in taste parameters. Other studies have also correlated changes in pH, a_w and weight losses with the use of *Lb. casei* LOCK 0900 when compared with traditional meat starter cultures (Trzaskowska, Kolozyn-Krajewska, Wójciak, & Dolatowski, 2014; Wójciak, Dolatowski, Kolozyn-Krajewska, & Trzaskowska, 2012). In any case, physicochemical alterations have proven to be strain-dependent as the same behavior was not observed in other studies with a wide range of *Bifidobacterium*, *Lactobacillus* and *Pediococcus* strains (Erkillä, Suihko, Eerola, Petäjä, & Mattila-Sandholm, 2001; Holko,

Hrabe, Salaková, & Rada, 2013; Rubio, Jofré, Aymerich, Guàrdia, & Garriga, 2014).

From a sensorial point of view, the addition of probiotic microorganisms to fermented meat products should produce only minimum changes in taste, texture and odor in order to prevent rejection of the new probiotic meat product. It has likewise been observed that probiotic microorganisms can produce positive sensory alterations in fermented meat products when fermentation takes place at higher temperatures. According to Trzaskowska et al. (2014), probiotic *Lb. casei* LOCK 0900 can stimulate more intense perception of a pleasant aroma and smoked meat flavor in pork loins fermented between 18 and 20 °C for 20 days. Also, a commercial probiotic mixture Laktoflora® (MILCOM a. s., Prague, Czech Republic) containing *Lb. acidophilus* CCDM 476 and *B. animalis* 241a was able to reduce the typical mutton smell present in mutton fermented sausages (Holko et al., 2013). Meat fermentation at relatively higher temperatures probably stimulates probiotic microorganisms and enzyme reactions against flavor precursors.

Another important factor to consider when using probiotic microorganisms in fermented meat products is high-level viability throughout processing and storage. Generally, probiotic fermented meat products reach lactic acid bacteria (LAB) levels of between 7 and 8 log CFU/g at the end of processing but the addition of probiotic microorganisms to fermented sausages is not a guarantee that the product will have higher LAB levels than a traditional sausage. In this regard, different probiotic delivery systems have been tested in fermented products (Table 3). The first immobilization technique developed to protect microorganisms in meat products was proposed by Kearney et al. (1990) for fermented meat products. In that work *Lb. plantarum* and *P. pentosaceus* were immobilized in lyophilized calcium alginate beads and the time required for meat fermentation (to obtain a pH of 5.0) was reduced by 29% compared to free cells and microorganism viability was also enhanced. This was due to the fact that the capsule protected microorganisms from harsh fermentation conditions such as additives, salts and other antimicrobial agents while the wall material promoted rapid cell activation (Kearney et al., 1990). In contrast, the direct addition of lyophilized free cells to meat is affected by these conditions reducing the viability of microorganisms and increasing the time needed for fermentation (Benkerroum et al., 2005). The reduction in meat fermentation time represents an economic benefit to industry as it reduces the time needed to process meat products. *Lb. reuteri* ATCC 55730 encapsulated in sodium alginate by means of extrusion or emulsion techniques for use in fermented meat products showed that capsules produced by emulsion were irregular and smaller (about 40 μm diameter) than those produced by extrusion (2–3 mm diameter). The technological characteristics were not affected by the encapsulation technique and pH and a_w values were similar in sausages with encapsulated probiotics and control sausages (free cells). However, when incorporated as free cells, the probiotic microorganism count decreased substantially by 2.6 log CFU/g units showing levels under 6 log CFU/g during sausage fermentation and drying whereas alginate-encapsulated *Lb. reuteri* declined by less than 0.5 log CFU/g units.

The use of probiotic microorganisms as a bioprotective agent has also been tested in fermented products. Encapsulated *Lb. reuteri* ATCC 55730 has been tested for inhibition of *E. coli* O157:H7 in dry-fermented sausage (Muthukumarasamy & Holley, 2007). A combination of free and encapsulated cells offers the best results when inactivating *E. coli* O157:H7. The free cell technique exhibited immediate effectiveness at the beginning of the process but viability was less than for the encapsulated technique which proved to be more effective towards the end of the process due to environment conditions.

These promising results show that it is possible to reduce

enterobacteria and *Staphylococcus aureus* in craft dry-fermented sausages produced in Argentina by adding *Enterococcus faecalis* CECT7121 (6 log CFU/g) (Sparo et al., 2008). The same probiotic strain also proved effective as a bioprotective culture against *E. coli* O157, *Clostridium perfringens* and *Listeria monocytogenes* when added to ground beef (4 log CFU/g) (Sparo et al., 2013).

Freshly immobilized probiotics in wheat have been added to dry-fermented sausages (Sidira, Galanis et al., 2014; Sidira, Karapetsas et al., 2014) as a bioprotective agent (Table 3). *Lb. casei* ATCC 393 immobilized in wheat increased the shelf-life of probiotic dry-fermented sausages even with reduced levels of or no added preservatives (sodium chloride, nitrite and nitrate). Resistance to spoilage (white spots on the surface of the sausage due to yeast and mould) was more noticeable in products with immobilized *Lb. casei*. Moreover, there was a drastic reduction in enterobacteria, pseudomonads and staphylococci bacteria in sausages containing immobilized *Lb. casei* (Sidira, Galanis et al., 2014; Sidira, Karapetsas et al., 2014). More recently it was discovered that the addition of immobilized *Lb. casei* in wheat grain also affected the composition of volatiles generated during ripening of probiotic dry-fermented sausages and improved the profile of aroma-related compounds in sausages with high amounts of immobilized cells (Sidira et al., 2015).

Experiments have also been conducted to check the effect that combining the addition of probiotic microorganisms with other healthy strategies have on fermented products to produce potentially functional meat-based foods. These strategies include reducing fat and salt content and adding dietary fiber. In this regard, tests have been conducted combining probiotic lactobacilli with a reduction in salt and fat in *fuet* (Rubio, Jofré, Aymerich et al., 2014). Also, dietary fiber in meat products can act as a prebiotic. These kinds of ingredients can alter the microorganism population in the gut by producing organic acids and improving local and systemic health (Gibson et al., 2010). The addition of citrus fiber (1%) and *Lb. casei* CECT 475 in *Longaniza de Pascua*, a low acid fermented sausage (pH 5.3), provided products with higher sensory scores than those achieved by adding probiotic only or citrus fiber only. Furthermore, the addition of citrus fiber improved the growth of probiotic strains resulting in a faster curing process (Sayas-Barberá, Viuda-Martos, Fernández-López, Pérez-Álvarez, & Sendra, 2012). This is an example of combining pro- and prebiotics, the symbiosis of which is an important option in the search for healthier products.

Extruded alginate beads containing probiotic microorganisms have also been used in dry-fermented sausages (Table 3). Panelists saw these beads as fat particles due to their size and color (Muthukumarasamy & Holley 2006). However, in our lab we observed that alginate capsules used in fermented products such as chorizo (Spanish fermented sausage) had the appearance of small visible balls and offered a degree of resistance when chewed, unlike the smoother consistency of fat. Further studies are required to verify texture and taste alterations produced in meat products by the addition of probiotic alginate beads.

3.2. Cooked meat products

In contrast with fermented products, other meat products are subjected to heat treatment which, while giving these products their typical characteristics (texture, flavor, color, etc.), also destroys microbiota and increasing shelf life. Hence, heat treatment eliminates or reduces the viability of added probiotic strains. Unlike dairy products where starters or probiotic cultures are always added to milk after pasteurization, in cooked meat products meat is heated to a core temperature of 70–72 °C and free cells of probiotic LAB are not able to survive this process. This has limited the use of

probiotics in these types of products (Table 1).

Attempted solutions to this problem include isolating thermo-tolerant LAB from the cooked meat products themselves taking advantage of their ability to adapt to meat product environments and additives (Pérez-Chabela, Totosaus, & Guerrero, 2008; Victoria-León, Totosaus, Guerrero, & Pérez-Chabela, 2006) and immobilizing microorganisms to protect them from heat treatment (Cavalheiro et al., 2015; Pérez-Chabela, Lara-Labastida, Rodriguez-Huezo, & Totosaus, 2013; Wang, Ho et al., 2015). Ramírez-Chavarin, Wacher-Rodarte, and Pérez-Chabela (2010) showed that some isolated strains could survive heat at 65–72 °C for 30–60 min. Possible probiotic strains such as *Aerococcus viridans* UAM21, *En. faecium* UAM10c, *Lb. plantarum* UAM10a, *P. acidilactici* UAM15c and *P. pentosaceus* UAM17 have been isolated from cooked meat sausages and intentionally added as bioprotective cultures to cooked meat products (Pérez-Chabela, Díaz-Vela, Reyes-Menéndez, & Totosaus, 2013; Pérez-Chabela, Lara-Labastida et al., 2013; Pérez-Chabela et al., 2008; Ramirez-Chavarin, Wacher, Eslava-Campos, & Pérez-Chabela, 2013; Victória-León et al., 2006). Thanks to their ability to tolerate heat, these strains can be added during manufacturing and survive heat processing. These specific LAB strains may also enhance nutritional and technological properties of cooked meat (Pérez-Chabela, Lara-Labastida et al., 2013).

Pérez-Chabela et al. (2008) added 8 log CFU/g of *Lactobacillus curvatus*, *Lb. plantarum*, *P. acidilactici* and *P. pentosaceus* to Vienna sausage and observed a bioprotective effect against enterobacteria after 12 days of cold storage by only *Lb. curvatus* and *P. pentosaceus* added (Table 1). Sausages with *Lb. curvatus* added were softer due to higher lactic acid production and lower pH levels and also exhibited lower acceptance values when compared to control treatments, probably due to higher lactic acid levels. Acid flavor was also more pronounced in cooked meat products to which *Lactobacillus alimentarius* ATCC 29643 and *Enterococcus* sp. MXVK29 were added (Victória-León et al., 2006). Changes in sensory attributes have proven to be strain-dependent as cooked meat sausages with *Lactococcus lactis* ATCC 1154 and *Lactobacillus piscicola* MXVK76 exhibited improved flavor (Victória-León et al., 2006).

However, the use of heat tolerant probiotics is not enough to maintain microorganism viability under high-temperature conditions. Therefore, probiotic delivery systems have recently been used to enhance the viability of these microorganisms in cooked meat products but the use of encapsulated probiotics in these products remains scarce (Table 3). Several in vitro studies have shown that encapsulated probiotics are more resistant to heat treatments than free cells. Mandal, Puniya, and Singh (2006) reported that *Lb. casei* NCDC-298 encapsulated in alginate particles was more resistant to heating at 55–65 °C. Recently, Wang, Ho et al. (2015) related the ability of *Lb. kefiranofaciens* M1 encapsulated with alginate, gellam gum and skim milk powder by freeze drying to tolerate heat increase from 25 °C to 75 °C. The results showed a decrease of 1 log CFU/g in encapsulated probiotics after heat treatment compared to no viable free cells after heating. However these were not tested in meat products. Heat tolerant probiotic microorganisms have been micro-encapsulated in Arabic gum by spray-drying and tested in cooked meat batters (Pérez-Chabela, Lara-Labastida et al., 2013). Spray-drying technology has proven to be an effective way to protect heat tolerant LAB and ensure their survival before, during and after processing of cooked meat batters (Table 3). Products cooked with these probiotic capsules exhibited higher LAB counts than cooked meat batters treated with free cells. Furthermore, LAB counts increased during storage time and after 8 days values over 6 log CFU/g were achieved. These capsules also exhibited a bioprotective effect against enterobacteria in cooked meat batters (Pérez-Chabela, Lara-Labastida et al., 2013).

The use of probiotic strains as bioprotective cultures in cooked meat products could be a viable alternative for the food industry, although certain technological challenges still need to be resolved to guarantee the viability of microorganisms in these types of products and control their effect on sensorial characteristics and the quality of products during storage. Immobilization strategies appear to be the trend of the future making it possible to add probiotics to food matrices where high processing temperatures are used (Pérez-Chabela, Lara-Labastida et al., 2013; Wang, Ho et al., 2015). Moreover, we must not overlook the heat tolerance capacity of LAB strains isolated from cooked meat products enabling their inoculation in heat processed foods which will become dominant during vacuum-packed cold storage (Ramírez-Chavarín et al., 2013). This is an interesting point given that their probiotic characteristics are useful in developing functional cooked meat products.

3.3. Raw meat products

Probiotic microorganisms have been added to raw meat products exclusively as bioprotective agents because the subsequent cooking process of these types of products generally inactivates the cells (Table 1). For instance, *En. faecalis* CECT7121 has successfully been tested against pathogenic bacteria such as *E. coli* O157, *C. perfringens* and *L. monocytogenes* in ground beef (Sparo et al., 2013). Recent studies have also shown that the addition of *Lactobacillus sakei* at levels of between 8 and 9 log CFU/g has a bioprotective effect against *Salmonella typhimurium* and *E. coli* in ground beef (Chaillou et al., 2014) and *Lb. acidophilus* CRL1014 added to chicken hamburger (Bomdespacho et al., 2014). In vitro studies have shown that a mixture of *Lb. animalis* SB310 and *Lb. paracasei* subsp. *paracasei* SB137 is a promising antimicrobial alternative against pathogenic strains for future use in ground meat (Tirloni et al., 2014). However, recent developments in probiotic delivery systems being applied in cooked products and mentioned above could be successfully applied to this type of product opening up new possibilities. Some of the probiotic microorganisms used as bioprotectors in these products could also be used with probiotics proposes.

4. Probiotic meat products and human intervention

Irregardless of the type of meat product to which probiotic microorganisms are added or the methodological technique employed to ensure their viability at the time of ingestion, as mentioned above it is also important to keep these bacteria alive as they pass through the gastrointestinal tract. There are few studies involving probiotic meat product intake in humans. The release of entrapped probiotic cells must occur after passage through the stomach, so as to release viable cells into the intestine (Picot & Lacroix, 2004). Generally, the release mechanism depends on the technology and materials used. In most cases, it is based on pH changes, chelating agents and enzymatic action (Martín et al., 2015). To date, studies have shown that the consumption of probiotic bacteria via meat products can increase their number in fecal samples and affect host immunity. Rubio, Martín, Aymerich, and Garriga (2014) observed that levels of probiotic bacteria in fecal samples of healthy volunteers increased from less than 2 to 6-7 log CFU/g after 21 days of consumption of dry-fermented sausage with *Lb. rhamnosus* CTC1679. However, continuous consumption of dry-fermented sausage containing probiotics is needed to maintain higher concentrations of this strain in the GIT, coinciding with previous in vivo studies of other lactobacilli (Saxelin et al., 2010). In a similar study, Holko et al. (2013) also observed an increase in lactobacilli levels in faeces samples following consumption of mutton sausage fermented with *Lb. acidophilus* CCDM 476 and

B. animalis 241a for 14-days. However this phenomenon was not observed for bifidobacteria. In a study in which human volunteers were administered probiotic fermented meat sausage with *Lb. paracasei*, Jahreis et al. (2002), observed an increase in antibodies against oxidized low density lipoproteins with no effects on the serum concentration of different cholesterol fractions and triglycerides. Levels of probiotic bacteria also increased in stool samples.

5. Culture media for probiotic bacteria assessment in meat products

The use of probiotic bacteria in meat products requires appropriate methodologies with which to assess whether the presence of cells is sufficient for the desired probiotic effect. This can be checked using specific and selective culture media able to quantitatively and qualitatively (viability) identify probiotic bacteria throughout the different processing phases, storage, cooking and passage through the GIT.

Proper quantification and identification of probiotic microorganisms added to products is especially important in fermented meat products as meat fermentation is complex and involves a wide range of microorganisms also considered as LAB and *Enterococcus*, genera in which probiotics are included. The presence of intentionally added probiotic strains should be ensured at high concentrations. The most widely used media to assay LAB are Rogosa agar and De Man-Rogosa Sharpe Agar (MRS). However, as these media are not selective, the use of a medium for specific probiotic bacteria is normally encouraged. On the other hand, an overview of culture media that could be used for differential and selective counting for a wide range of *Lactobacillus* strains, *Streptococcus thermophilus* and *Bifidobacterium* spp. in yoghurt is available (Ashraf & Shah, 2011). In the case of *Lactobacillus* strains, MRS agar is typically used as a base medium but with modifications in ingredients and incubation conditions for the purpose of selecting the desired probiotic microorganism. One of these modifications is the use of selective antibiotics for microorganisms and specific sugars for fermentation. In this regard Bujalance, Jiménez-Valera, Moreno, and Ruiz-Bravo (2006) developed a selective differential medium for *Lb. plantarum* (LPSM) based on its resistance to ciprofloxacin and ability to produce acids from sorbitol.

In the case of *Bifidobacterium* strains, counts are performed using MRS agar supplemented with maltose (MRSM) or MRS agar with nalidixic acid, paromomycin, neomycin sulphate and L-cysteine (MRS-NPLP) under anaerobic incubation at 37 °C for 72 h (Ashraf & Shah, 2011). However, it was discovered that transgalactosylated oligosaccharide-mupirocin lithium salt (TOS-MUP) agar is the most effective for selectively counting *Bifidobacterium* spp. even in food products containing mixed populations of other LAB species, such as fermented milks (Süle, Körösi, Hucker, & Varga, 2014).

Specific culture media have also been described for *Enterococcus* spp. counts (Domig, Mayer, & Kneifel, 2003). Generally, Slanetz & Barley (SB) and Kanamycin Aesculin Azide (KAA) agars are used to estimate enterococcal counts in food samples. However, these are only useful if there is no other kind of microorganism in the samples and they are not able to distinguish between different strains of enterococcus. In samples containing multiple microbial components, Bile aesculin azide (BAA) agar has proven more effective and after 24 h of incubation, enterococci colonies are surrounded by a black halo (Domig et al., 2003). However, Cephalexin aztreonam arabinose (CAA) agar is the most suitable in differentiating the two most common enterococci species, *En. faecalis* and *En. faecium* (Ford, Perry, & Gould, 1994). The commercial HiCrome *En. faecium* agar (Fluka) is partly based on CAA agar and is also effective in

isolating *En. faecium* in heavily contaminated samples (Domig et al., 2003).

Alternative probiotic enumeration methods have recently emerged based on the presence of nucleic acid and cellular integrity and are more effective than phenotype methods for swift and sure identification of microorganisms (Davis, 2014). Studies have already successfully identified probiotic microorganisms added to meat products by PCR (Holko et al., 2013; Rubio, Jofré, Aymerich et al., 2014; Ruiz-Moyano, Martín, Benito, Aranda et al., 2011; Ruiz-Moyano, Martín, Benito, Casquete, & Córdoba, 2011; Sidira, Galanis et al., 2014; Sidira, Karapetsas et al., 2014). However, these media are expensive and difficult to use which is why specific media are better for routine identification in laboratory analyses and industry.

Preliminary studies performed in our laboratory consisting of adding probiotic *Lb. plantarum* and *En. faecium* strains to commercial dry-fermented sausage (chorizo) showed that combined culture media is needed to ensure the proper evaluation of the probiotic in meat products containing multiple microorganisms. We use traditional MRS agar in addition to LPSM agar proposed by Bujalance et al. (2006), and HiCrome *En. faecium* agar (Fluka) to distinguish between the two different probiotic strains when added either alone or together in meat products (chorizo) with a naturally wide range of microorganisms. *Lb. plantarum* is not able to grow in HiCrome EF agar while *En. faecium* is able to grow in LPSM agar but the two can be distinguished thanks to the color and size of colonies. As expected, both probiotic microorganisms grow very well in MRS agar. In addition, we observed that the time required for adequate probiotic (from chorizo) growth is different for each culture media, the best conditions being incubation at 37 °C for 24 h for HiCrome EF agar, 48 h for MRS agar and 72 h for LPSM agar.

6. Conclusions

Probiotics have exhibited potential effects against the incidence of many diseases in humans and animals. The bioprotective effect of probiotics in meat is also an important additional consideration in probiotic selection. The main probiotic microorganisms used in meat products are *Lactobacillus* and *Bifidobacterium* genus which have mainly been tested on fermented products. Their use in cooked products presents important problems in terms of maintaining the viability of effective numbers of microorganisms throughout processing and storage periods up to the time of consumption to provide health benefits to consumers. In this regard, the trend is to either isolate heat resistant probiotic microorganisms from meat products themselves or to use different microorganism immobilization strategies.

Microorganism delivery system strategies such as encapsulation by extrusion, emulsion, spray dryer, freezing dryer, and entrapment of gelled dispersions using different matrices are a useful way to protect probiotic microorganisms against the harsh conditions characterizing the processing, storage and cooking of meat products and during passage through the GIT. Encapsulated probiotics exhibit better stability and viability than free cells and encapsulation is an alternative for use in products where the application of probiotic free cells is unfeasible as is the case of cooked meat products.

The key points in immobilization techniques include the encapsulated microorganism, the wall material and encapsulation strategies. The selection of one or another of the encapsulation techniques depends on the management strategy used by the industry for the type of meat product in question and the processing conditions (heating, freezing, etc.), storage times and consumer preparation conditions. The sensorial and technological characteristic of the products in which probiotics will be incorporated have

also been maintained.

Moreover, many of the ingredients used to mobilize prebiotics have additional beneficial effects (e.g. fibre in the case of some prebiotics) such that the development of these products can contribute to the more comprehensive development of healthy products. Probiotics could be a promising market for the meat industry opening up new fields of consumer choice.

Acknowledgments

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2.2   Parte Experimental

2.2.1   Manuscrito 3

Alginate beads to improve *L. plantarum* and *E. faecium* viability during stress treatments.

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Artigo em fase final de revisão.

1 **Alginate beads to improve *L. plantarum* and *E. faecium* viability during stress**
2 **treatments.**

3

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24 **Abstract**

25   This work aimed to investigate the encapsulation of *Lactobacillus plantarum* ATCC

26 8041 and *Enterococcus faecium* ATCC 700221 in alginate beads in association with milk

27 powder, inulin and trehalose and to study the protective effects of such matrix during stress

28 treatments such as high processing temperature, high sodium chloride and sodium nitrite

29 levels, and storage period (4 ºC). Free *L. plantarum* was more resistant to 70 ºC than free *E.*

30 *faecium*. At 80 ºC, encapsulated *L. plantarum* was resistant to 10 minutes with values higher

31 than 6 log CFU/g, while encapsulated *E. faecium* was resistant to 5 min. NaCl even at low

32 concentrations was able to reduce viability of both probiotics when used as free cells.

33 However, higher concentrations of NaCl were able to reduce just the viability of *E. faecium* as

34 free cells. In relation to sodium nitrite, concentrations of 1.0% reduced viability of both

35 probiotics either as free cells or as encapsulated forms. In addition, both encapsulated *L.*

36 *plantarum* and *E. faecium* had counts higher than 6 log CFU/g during the whole storage

37 period (70 days). Then, the encapsulation of probiotics in alginate beads containing inulin,

38 milk powder and trehalose was effective to protect them against harsh environments.

39 **Keywords:** encapsulation, alginate beads, probiotics, trehalose, stress treatments.

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1. Introduction

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer health benefits on the host" (FAO/WHO, 2002). Among the health benefits that probiotics consumption could bring to host are immune enhancement, diarrhea reduction, prevention of some types of cancer, cholesterol reduction, and prevention of gastrointestinal infections (Douglas & Sanders, 2008). It is well known many strains of *Lactobacillus plantarum* are marketed as probiotics since they are one of the most widely used lactic acid bacteria (LAB), have a homofermentative metabolism, moderate acid tolerance and are considered as a GRAS (Generally Regarded as Safe) organism. However, other species are also largely used as probiotic such as *Bifidobacterium, Pediococcus, Enterococcus* and *Saccharomyces* (Weinbreck, Bodnár, & Marco, 2010). Nowadays, a special attention has been given for the use of *Enterococcus* as probiotics, especially for its ability to improve the intestinal microbial balance or can be used in the treatment of gastroenteritis in humans and animals (Giraffa, 2003; Foulquie-Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006).

In relation to the levels of adequate doses of probiotics, it can vary in different standards and regulations. According to Nulkaekul, Lenton, Cook, Khutoryanskiy, & Charalampopoulos (2012), the minimum probiotic concentration in food should be between 10^6 and 10^7 CFU/g or CFU/ml at the moment of consumption, but the recommended daily probiotics intake is between 10^8-10^9 CFU/g or CFU/ml (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011). Nevertheless, with advances in understanding the benefits that consumption of probiotic microorganisms can promote to the host, their stability remains a major concern. Adverse conditions of gastrointestinal tract (GIT), characterized by pH change and bile salt secretions, have been a limiting factor for probiotics application (Su *et al.*, 2011). In addition, the susceptibility of bacteria to drastic temperature changes during storage and

71 processing, and food physico-chemical characteristics are other limiting factors of probiotics

72 application in food (Albertini *et al.*, 2010). Up to now, dairy products such as yoghurt and

73 fermented milks, juices and vegetables are the most common probiotic foods (Burgain,

74 Gaiani, Linder, & Scher, 2011). In meat products, probiotics inoculation is still limited to

75 those are fermented (Cavalheiro *et al.*, 2015).

76   Many reports indicated there is poor survival of probiotics bacteria in products

77 containing free probiotic cells (De Vos, Faas, Spasojevic, & Sikkema, 2010). Therefore, the

78 incorporation of probiotic microorganisms in food generally requires encapsulation of

79 bacterial cells to maintain viability under harsh conditions (Burgain *et al.*, 2011). In general,

80 encapsulation is the method whereby solids, liquids or gaseous materials are placed in an

81 insert shell (capsule) and are released at a controlled rate under specific conditions

82 (Champagne & Fustier, 2007). Even more, encapsulation technology offers good protection to

83 probiotics against harsh environments by isolating them from outside (Anal & Singh, 2007).

84   Among of many techniques and materials that can be used to encapsulate probiotic

85 cells, extrusion technique and sodium alginate as wall material are largely used due to their

86 low cost and easy handling, which causes less damage to probiotic cells and gives high

87 probiotic viability (Krasaekoopt, Bhandari, & Deeth, 2003). Sodium alginate forms a highly

88 versatile, biocompatible and non-toxic matrix offering protection to probiotic microorganisms

89 and cells sensitive to heat, pH, and dissolved oxygen (Pasin, Azón, & Garriga, 2012).

90 However, in general, alginate beads are porous which a drawback is when aiming to protect

91 cells from stressful environments (Gouin, 2004). Due to that limitation, coating beads with

92 biopolymers and the use of other substances along with sodium alginate such as milk proteins

93 and prebiotics has been encouraged aiming to improve probiotic viability (Krasaekoopt *et al.*,

94 2003; Burgain *et al.*, 2011; Fu & Chen, 2011; Nazzaro, Orlando, Fratianni, & Coppola, 2012).

95 In this regard, milk powder supports a strong gel formation due to carbohydrate and protein

96   contents (Ghandi, Powell, Chen, & Adhikari, 2012; Schutyser, Perdana, & Boom, 2012).

97   Prebiotics like inulin have been added along with probiotics because its properties as not

98   hydrolyzed by digestive enzymes and it is fermented in colon by the microbiota (Nazzaro *et*

99   *al.*, 2012). On the other hand, trehalose is also reported as a thermo protectant especially

100   during spray drying of probiotics (Santivarangkna, Higl, & Foerst, 2008).

101   There are some studies in literature addressing the viability of encapsulated

102   probiotics against harsh environments such as TGI characteristics and high temperatures.

103   However, studies evaluating the ability of encapsulated probiotics to survive against different

104   concentrations of sodium chloride and sodium nitrite are still scarce. For example, Studies

105   already shown the probiotic viability of alginate-encapsulated bacteria, such as *L. rhamnosus,*

106   *L. salivarius, L. acidophilus, L. paracasei, L. casei, B. longum* and *B. lactis* was improved

107   when compared to free cells when exposed to temperatures ranging from 40 to 65 ℃

108   (Mandal, Puniya, & Singh, 2006; Ding & Shah, 2007). Recently, the efficacy of using

109   alginate associated with chitosan, xanthan gum or sugarcane bagasse was shown to protect *L.*

110   *plantarum* and *L. rhamnosus* to different heat treatments (Abbaszadeh, Gandomi, Misaghi,

111   Bokaei, & Noori, 2014; Shahrulzaman & Muhamad, 2015). However, encapsulation

112   techniques are generally not efficient to protect probiotic strains that are extremely sensitive

113   to heat treatments. In this case, an interesting alternative is the use of thermoresistant

114   probiotic strains in association with encapsulation. Studies already isolated probiotic strains

115   from cooked meat sausages, which were able to survive to heat treatments between 65 and 70

116   ℃ for 30-60 minutes (Ramirez-Chavarin, Wacher-Rodarte, & Pérez-Chabela, 2010). Possible

117   probiotic strains such as *Aerococcus viridans*, *Enterococcus faecium*, *L. plantarum*,

118   *Pediococcus acidilactici* and *Pediococcus pentosaceus* have been isolated from cooked meat

119   sausages and intentionally added as bioprotective cultures into cooked meat products (Pérez-

120 Chabela, Totosaus, & Guerrero, 2008; Pérez-Chabela, Lara-Labastida, Rodriguez-Huezo, &

121 Totosaus, 2013; Ramírez-Chavarin, Wacher, Eslava-Campos, & Pérez-Chabela, 2013).

122   The present study aim to verify the resistance of *L. plantarum* and *E. faecium* as free

123 cells or encapsulated in alginate with inulin, powder milk and trehalose to stress treatments,

124 such as high processing temperatures, high sodium chloride and sodium nitrite levels. In

125 addition, we analyzed the viability of alginate beads containing probiotic microorganisms

126 when subjected to storage period under refrigeration at 4 ºC.

127

128   **2. Materials and methods**

129   *2.1. Materials*

130   Probiotic strains *L. plantarum* ATCC 8041 and *E. faecium* ATCC 700221 were

131 provided from *Instituto Nacional de Controle de Qualidade em Saúde* (INCQS) of *Fundação*

132 *Oswaldo Cruz* (FIOCRUZ, Brazil). Sodium alginate, calcium chloride, sodium phosphate,

133 NaCl and $NaNO_2$ were provided from Sigma-Aldrich Ltd. (Steinheim, Germany). Inulin was

134 purchased from Sensus (FrutaFit CLR, Roosendal, The Netherlands). Trehalose was provided

135 from Hayashibara (Okayama, Japan). Powder milk was provided from Nestlè (Araçatuba,

136 Brazil). Brain Heart Infusion (BHI) broth, de Man, Rosa and Sharpe (MRS) broth and MRS

137 agar were obtained from Himedia (Mumbai, India). Anaerobiosis kit was provided from

138 Probac (Anaerobac, São Paulo, Brazil). All the glasses were sterilized before used.

139

140   *2.2. Preparation of probiotic inoculum*

141   Probiotic strains were rehydrated using 1% (w/v) of inoculum in MRS broth for *L.*

142 *plantarum* and in BHI broth for *E. faecium*, and incubated to 37 ºC for 24 hours. Cells were

143   harvested by centrifugation at 4000g for 20 min at 4 ℃ (Hitachi CR22GIII, Tokyo, Japan)

144   and, subsequently, washed twice before resuspending them in a NaCl solution (0.9 w/v). The

145   cell suspension obtained had approximately 10^{10}-10^{11} CFU/ml of each probiotic

146   microorganisms, which was determined by plating in MRS agar. In addition, cell suspension

147   was divided into two parts: one part was used for encapsulation and the other was used as free

148   cells for control.

149

150   *2.3. Encapsulation procedure*

151   The extrusion technique of encapsulation followed Krasaekoopt, Bhandari, & Deeth

152   (2004). The slurry was prepared by mixing 1 g of sodium alginate, 1 g of inulin, 1 g of

153   powder milk, 5 g of trehalose and 10 mL of each probiotic in 82 ml of distilled water. The

154   mixture was dropped into 0.1 mol/L calcium chloride from a 10 cm distance using a nozzle

155   attached to a peristaltic pump. The beads were left for 30 min, washed with sterile distilled

156   water and stored at 4 ℃ for the following tests. The treatments were identified as follows:

157   FLP for *L. plantarum* as free cells; ALP for *L. plantarum* in alginate beads; FEF for *E.*

158   *faecium* as free cells; and, AEF for *E. faecium* in alginate beads.

159

160   *2.4. Solubilization of beads and probiotic enumeration*

161   Solubilization of beads was done by adding 1 g in 9 mL of phosphate buffer (0.1

162   mol/L, pH 7.5) as suggested by Sheu and Marshall (1993). Then, the mixture was place in a

163   shaker (model TE-421, Tecnal, Piracicaba, Brazil) under agitation (100 rpm) at room

164   temperature for 20 min. The mixture was vortexed (model K40-1010, Kasvi, Curitiba, Brazil)

165   at high speed for breaking the polymer formed and releasing completely the encapsulated

culture into phosphate buffer. Free or released cells from beads were plated on MRS agar

using ten-fold dilutions and incubation at 37 ºC for 48 h in anaerobic conditions.

2.5. Bead size and encapsulation yield

Size of obtained beads is in the millimeters. For this reason, particle size of beads was assessed using a caliper. Data was collected from 500 beads of alginate beads of each probiotic microorganism. The encapsulation yield (EY) was calculated as: EY (%) = $(N_{beads}/N_{suspension})$ x 100, where N_{beads} were total viable counts recovered from beads and $N_{suspension}$ were total viable counts recovered in cell suspension (free cells) (Doherty *et al.*, 2010).

2.6. Viability of free and encapsulated probiotic to heat stress

One gram of the beads containing *L. plantarum* or *E. faecium* or 1 mL of the culture was transferred to 9 mL of sterile distilled water in glass tubes. The contents were subjected to two different temperatures (70 and 80 ºC) for four different times (5, 10, 20 and 30 min) and immediately cooled by immersing in chilled water. The beads were then subjected to solubilization, serially diluted, and pour plated onto MRS agar. Cells were counted after 48 h of anaerobic incubation at 37 ºC. Duplicate tests were done at each temperature and time.

2.7. Viability of free and encapsulated probiotic to NaCl and NaNO$_2$

One gram of beads was added to 9 mL saline solution containing varying concentrations of NaCl (0.5, 1.0, 2.5 and 5.0%) and NaNO$_2$ (0.5 and 1.0%) for 1 h. The beads

188   were washed, subjected to solubilization, serially diluted, and pour plated onto MRS agar. For

189   the free cells, serially dilutions were pour plated onto MRS agar supplemented with the same

190   concentrations of NaCl or $NaNO_2$ according proposed by Gardiner *et al*. (2000). Cells were

191   counted after 48 h of anaerobic incubation at 37 ℃. Duplicate tests were done at each NaCl

192   and $NaNO_2$ concentrations.

193

194   *2.8.Viability of encapsulated probiotic under refrigerated storage*

195   The beads were stored in sterile falcon tubes at 4º C. The analysis was assessed at 0,

196   10, 20, 30, 40, 50, 60 and 70 days when the beads were subjected to solubilization and serially

197   diluted onto MRS agar. Cells were counted after 48 h of anaerobic incubation at 37 ℃.

198   Duplicate tests were done at each storage period.

199

200   *2.9. Statistical analysis*

201   All experiments were repeated twice. The data analysis was carried out using SPSS

202   17.0 software (SPSS Inc., USA). Analysis of variance (ANOVA) and Tukey's test were used

203   to determine significant differences ($P < 0.05$).

204

205   **3.   Results and discussion**

206   *3.1. Preparation of beads containing probiotic microorganisms, bead size and*

207   *encapsulation yield*

208   Alginate beads in association with inulin, milk powder and trehalose containing *L.*

209   *plantarum* and *E. faecium* were prepared by extrusion technique in this study. Obtained beads

210   were whitish, quite spherical and with smooth surface (Figure 1). Size of beads was 3.79 and

211   3.44 mm (Table 1) for alginate beads containing *L. plantarum* and *E. faecium,* respectively.

212   Previous studies using extrusion technique related bead sizes ranging from 1.3 to 2.5 mm

213   (Krasaekoopt *et al.*, 2004; Krasaekoopt, Bhandari, & Deeth, 2006; Abbaszadeh *et al.*, 2014),

214   depending on the distance between the nozzle and $CaCl_2$ solution and on the nozzle diameter.

215   It was found the protective effect against extreme environmental factors is enhanced with

216   increasing bead diameter (Truelstrup-Hansen, Allan-Wojtas, Jin, & Paulson, 2002), but,

217   oversized beads may cause inappropriate mouthfeel (Dimantov, Greenberg, Kesselman, &

218   Shimoni, 2004). In addition, larger bead sizes could be a drawback for application on food

219   due to possible changes in texture and visual appearance of beads inducing rejection by

220   consumers. However, Muthukumurasamy & Holley (2006) stated alginate beads (2-3 mm)

221   containing *L. reuteri* encapsulated by extrusion technique does not affect the sensory aspects

222   of dry fermented sausages, since the alginate beads resembled, in terms of both size and color,

223   discrete fat particles.

224   Cell viability after the encapsulation process was analyzed and calculated as EY.

225   According to Corbo *et al.* (2016), the evaluation of EY is a complex process and relies upon

226   many different parameters, such as the ability of beads to entrap cells, cell viability

227   throughout bead storage and cell release after immobilization. Obtaining high levels of EY it

228   is important in order to maintain high probiotic viability that allow the use of capsules in food

229   matrices. In our study, EY values were from 80.29 to 93.99% (Table 1) to alginate beads

230   containing *E. facium* and *L. plantarum*, respectively. The high EY values found demonstrate

231   the natural resistance of probiotic microorganisms used and the conditions of encapsulation,

232   which was done at room temperature and without any chemical solvent. In addition, *L.*

233   *plantarum* (ALP) seems to be more resistant for encapsulation procedure than *E. faecium.*

234   Corbo, Bevilacqua, & Sinigaglia (2011) and Corbo, Bevilacqua, Gallo, Speranza & Sinigaglia

235 (2013) related similar results of EY for *L. rhamnosus* (83.33%) and *L. plantarum* (93%) using

236 ionotropic gelation and alginate. Nevertheless, Chávarri *et al.* (2010) found lower EY, varying

237 from 19.5 to 40.2% for encapsulation of *L. gasseri* and *B. bifidum* using ionotropic gelation

238 and alginate and chitosan coating. Lower EY is generally related to the sensitivity of

239 microorganisms to encapsulation procedures.

240 Generally, high EY (close to 100%) when using extrusion technique is related

241 (Krasaekoopt et al., 2004; Krasaekoopt et al., 2006; Urbanska, Bhathena, & Prakash, 2007).

242 However, EY reports in literature vary due to the wide range of microorganisms, the

243 encapsulation technique, and the different wall materials used.

244

245 *3.2.Viability of free and encapsulated probiotic to heat stress*

246 Studies have been shown encapsulation techniques are able to improve survival of

247 probiotics during heat treatments as compared to free cells (Mandal *et al.*, 2006; Ding &

248 Shah, 2007; Abbaszadeh *et al.*, 2014; Shaharuddin & Muhamad, 2015). It is well known loss

249 of viable probiotic cells occurs during the heat treatment. Low survival rate of probiotics upon

250 heat treatment has been a challenge for incorporating viable probiotic organisms in thermally

251 processed food products. The counts of free and encapsulated *L. plantarum* and *E. faecium*

252 after heat treatments are shown on Table 2. An intense decrease ($P < 0.05$) on viability of

253 probiotics was observed after heat treatments. The results showed that free *L. plantarum* were

254 more sensitive to shorter heat treatments than free *E. faecium*. In counterpart, FLP was more

255 thermostable than FEF for larger heat exposures at 70 ºC. FLP and ALP showed levels of

256 probiotic higher than 6 log CFU/g even with 30 min of heat exposure at 70 ºC, meanwhile

257 FEF and AEF showed same levels just after 10 and 20 min of heat exposure, respectively.

258 However, at 80 ºC both FLP and FEF showed an intense viability decrease at 5 min, but ALP

259   and AEF maintained counts higher than 6 log CFU/g for 10 and 5 min, respectively. The

260   lower viability of encapsulated probiotics at 80 ℃ also can be related to that at this

261   temperature capsule can collapse releasing the cells with their consequent death (Xing *et al.*,

262   2014). Other studies already suggested encapsulation using alginate might enhance the

263   thermal resistance of probiotics (Mandal *et al.*, 2006; Ding & Shah, 2007; Abbaszadeh *et al.*,

264   2014).

265   During heat treatments the death of cells is attributed to the destroyed higher-order

266   structures of protein and nucleic acids in cells and the collapsed linkage between monomeric

267   units (Fritzen-Freire, Prudêncio, Pinto, Muñoz, & Amboni, 2013). In this way, encapsulation

268   can provide a physical barrier against harsh environments (Kailasapathy, 2002) which may be

269   the case of the present study (Table 2). Even more, higher concentration of biopolymers

270   (alginate) can improve the viability of probiotics during heat treatments due to lower water

271   diffusion rate, reducing the permeability of heat (Mandal *et al.*, 2006; Shaharuddin &

272   Mohammad, 2015). Mandal *et al.* (2006) stated that slow diffusion of hot water led to higher

273   survival of probiotics after heat exposure.

274   Therefore, our results showed that even using 1% of alginate, the protection of

275   probiotics viability after heat treatments is achieved with association of inulin, milk powder

276   and trehalose. Also mentioned before, milk powder and trehalose are good thermo protectants

277   (Santivarangkna *et al.*, 2008; Ghandi *et al.*, 2012; Schutyser *et al.*, 2012). The better

278   protection of alginate beads is related to the heat intensity transferred to beads compared to

279   free cells. Heat was disseminated to all area of bead and reduced the heat transfer to

280   encapsulated probiotic. The same trend was observed to Shaharuddin & Muhamad (2015)

281   when improved thermotolerance of *L. rhamnosus* immobilized in alginate and sugarcane

282   bagasse. Even more, flat surface of beads enhances the heat transfer on surface and reduce the

283   heat intensity exposed to the probiotics. In this regard, larger beads could improve probiotic

284   viability once the time required for heat transfer would be longer. Capsules made of sodium

285   alginate or with sodium alginate associated with other materials produce larger diameter size

286   which difficult the fast radial mass transfer. In addition, protection against heat can be

287   provided using as low 1% of sodium alginate (Shaharuddin & Muhamad, 2015) and the

288   inclusion of sugarcane bagasse, endorsing the results found in this study with sodium alginate

289   associated to inulin, milk powder and trehalose as wall materials.

290   However, just the use of encapsulation techniques does not appear to be effective in

291   protecting probiotics that are extremely heat sensitive. Thus, use of strains that already are

292   thermoresistant is necessary and so, encapsulation technique serving as a supplement in the

293   resistance to heat exposure. Pérez-Chabela *et al.* (2008) and Ramírez-Chavarin *et al.* (2010)

294   already isolated thermotolerant *L. plantarum* and *E. faecium* from cooked meat sausages. In

295   this perspective, isolation of probiotic from thermally processed foods with subsequently

296   encapsulation could be an alternative for application in other kind of heat-treated foods.

297

298   *3.3.Viability of free and encapsulated probiotic to sodium chloride and sodium nitrite*

299   A wide range of foods, especially meat products, contains salt as a taste enhancer. In

300   this context, NaCl content on foods can affect the probiotic viability. The effect of NaCl

301   concentrations on the survival of *L. plantarum* and *E. faecium* encapsulated in alginate beads

302   with inulin, milk powder and trehalose is shown on Table 3. Low concentrations of NaCl

303   (0.5%) are already able to reduce ($P < 0.05$) the viability of both *L. plantarum* and *E. faecium*

304   as free cells. However, increasing concentrations up to 2.5%, no differences were found, but

305   higher levels of 5% of NaCl significantly reduced probiotic viability as free cells.

306   Furthermore, free cells of *E. faecium* was presented as more sensitively ($P < 0.05$) towards

307   concentrations of 5% of NaCl (Table 3). Nevertheless, NaCl content did not affected probiotic

308   viability of encapsulated *L. plantarum* (ALP). It is well known the osmotic resistance of *L.*

309 *plantarum* and, due to this attribute, its ability to ferment food with high salt concentrations

310 (0.5-10 %) (Ferrando, Quiberoni, Reinhemer, & Suárez, 2015). In relation to encapsulated *E.*

311 *faecium* (AEF), concentrations higher than 2.5% of NaCl are able to reduce viability of this

312 probiotic. Our results are in agreement to those found by Sabikhi, Babu, Thompkinson, &

313 Kapila (2010) who found higher decrease in viability of free cells than encapsulated *L.*

314 *acidophilus* LA1 during exposure to increasing NaCl concentrations (from 1.0 to 2.0%).

315   Sodium nitrite ($NaNO_2$) is commonly used in meat products due to their efficacy

316 against *Clostridium botulinum* and their action on traditionally cured-meat color of products.

317 Therefore, the addition of $NaNO_2$ should not produce large losses on probiotic viability, but

318 studies aiming to verify probiotic viability under different $NaNO_2$ concentrations are scarce.

319 The effect of $NaNO_2$ on the viability of *L. plantarum* and *E. faecium* encapsulated in alginate

320 beads with inulin, milk powder and trehalose is shown on Table 4. It was possible to note that

321 increasing $NaNO_2$ concentrations significantly affected probiotic viability of both *L.*

322 *plantarum* and *E. faecium* as free cells and encapsulated. In ALP and AEF treatments, the

323 higher the concentration of $NaNO_2$, the lower the probiotic viability ($P<0.05$) was observed.

324 However, in FLP and FEF treatments, no significant differences on probiotic viability

325 between addition of 0.5 or 1.0% of $NaNO_2$ were found. Studies related that some probiotic

326 strains, including *Lactobacillus*, isolated from fermented food and human intestine are able to

327 deplete $NaNO_2$ *in vitro* (Ren *et al.*, 2014) and in fermented sausages (Wang, Ren, Wang, &

328 Xie, 2015).

329

330   *3.4. Viability of encapsulated probiotic under refrigerated storage*

331   One of the most considerable prerequisite for the use of probiotics is that they should

332 survive during production process and storage of food products. In this point of view, is

333 important to assess the viability of encapsulated probiotic under refrigerated storage and the

334   values are shown on Table 5. Probiotic viability of alginate beads obtained was at day 0 (P <

335   0.05) of storage for both probiotic microorganisms with counts of ~ 9 log CFU/g of beads. As

336   expected, a reduction of viability was observed throughout the storage. AEF treatment had

337   higher losses than ALP during first 10 days of refrigerated storage (Table 5). However, ALP

338   treatment seems to be more sensitive to longer periods of storage showing higher viability

339   loss during storage period. At the end of 70 days of storage, alginate beads lost 1.74 log

340   CFU/g and 1.22 log CFU/g for ALP and AEF, respectively. Nevertheless, at the end of 70

341   days of storage, values remained higher than 7 log CFU/g for both *L. plantarum* and *E.*

342   *faecium* strains. This way, it is possible to state alginate beads kept their potential probiotic up

343   to the end of storage period, once the minimum probiotic concentration in food should be

344   between 10^6 and 10^7 CFU/g or CFU/ml (Nulkaekul *et al.*, 2012).

345   Several studies already showed the survival of encapsulated bacteria during the storage

346   period was improved in alginate beads/microcapsules as compared to free bacteria

347   (Truelstrup-Hansen *et al.*, 2002; Anekella & Orsat, 2013; Silva *et al.*, 2015; Etchepare *et al.*,

348   2016). In addition, special treatments such as coating beads (Krasaekoopt *et al.*, 2003) and the

349   use of prebiotics in association of alginate improve probiotic viability throughout storage

350   period (Krasaekoopt *et al.*, 2003; Burgain *et al.*, 2011; Fu & Chen, 2011; Nazzaro *et al.*,

351   2012; Okuro, Thomazini, Balieiro, Liberal, & Fávaro-Trindade, 2013; Avila-Reyes, Garcia-

352   Suarez, Jiménez, Martín-Gonzalez, & Bello-Perez, 2014). According to Trabelsi *et al.* (2013),

353   the viability of *L. plantarum* TN8 cells encapsulated on alginate and coated with chitosan

354   showed lower decrease rates during storage at 4 ºC when compared to alginate beads and free

355   cells. Higher storage stability of alginate beads coated with chitosan is due to thicker

356   membranes formed than when only alginate is used (Ribeiro, Neufeld, Arnaud, & Chaumeil,

357   1999). However, the temperature of storage seems to affect probiotic viability. According to

358   Etchepare *et al.* (2016), *L. acidophilus* LA-14 encapsulated with sodium alginate, and sodium

alginate associated with Hi-maize (prebiotic) and chitosan is able to survive at levels higher than 6 log CFU/g up to 135 days of storage at 25 ºC. However, when stored at freezing (-18 ºC) and chilling (7 ºC) temperatures, encapsulated *L. acidophilus* LA-14 were not able to survive at high levels for the same period of time. In another study, Brinques & Ayub (2011) related little loss of *L. plantarum* BL011 when encapsulated in sodium alginate and coated with a solution of chitosan after 38 days of refrigerated storage. Recently, Sathyabama, Kumar, Devi, Vijayabharathi, & Priyadharisini (2014) stated the use of sugarbeet and chicory as prebiotic sources could improve the viability of alginate beads containing *E. faecium* or *Staphylococcus succinus* during 35 days of storage at 4 ºC.

Several factors such as temperature (Strasser, Neureiter, Geppl, Braun, & Danner, 2009) and a_w (Ying *et al.*, 2010) are related to bacterial death during storage period. It has been shown elevated temperatures, even in the range of 4 to 37 ºC correlate with a higher decline in cell viability as dried free cells (Strasser *et al.*, 2009), probably due to a higher rate of lipid and protein oxidation and denaturation (Fu & Chen, 2011). Regarding the effect of a_w values greater than 0.25 can stimulate bacterial metabolism, which is associated with a high molecular mobility in the matrix (Fu & Chen, 2011). Furthermore, in encapsulated probiotic the same factors are also responsible for reduction in probiotic viability (Ying *et al.*, 2010; Albadran, Chatzifragkou, Khutoryanskiy, & Charalampopoulos, 2015). However, according to Albadran *et al.* (2015), it is hard to compare studies involving storage of encapsulated probiotic due to differences on the exact storage conditions, encapsulation and coating materials and microorganisms used.

4. Conclusions

383

384 The encapsulation into alginate beads containing inulin, milk poweder and trehalose

385 was effective to protect the survival of *L. plantarum* and *E. faecium* higher than 6 log CFU/g

386 at 70 ℃ for 30 and 20 minutes, respectively. However, at 80 ℃ the encapsulation was

387 effective to protect probiotics for only 10 and 5 minutes, respectively. In addition, the

388 encapsulation in this context was great to protect probiotics against sodium chloride, sodium

389 nitrite and 70 days of storage period at 4 ℃. Then, encapsulation is an effective form to

390 protective probiotics from stress environments allowing their application in food matrix.

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612 Table 1 – Bead size and encapsulation yield of alginate beads containing *L. plantarum* and *E.*
613 *faecium.*

	ALP	AEF
Size (mm)	3.79 ± 0.50^a	3.44 ± 0.84^a
Encapsulation yield (%)	93.99 ± 1.22^a	80.29 ± 3.04^b

614 Means ± standard deviation.
615 [a] Different letters in the same row indicate significant differences (P < 0.05).
616 ALP – *L. plantarum* in alginate beads; AEF – *E. faecium* in alginate beads.
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Table 2 – Viability of free and encapsulated *L. plantarum* and *E. faecium* in alginate beads to heat treatments (in log CFU/ml or g).

Temperature (ºC)	Time (min)	FLP	ALP	FEF	AEF
Before heating		10.23 ± 0.04^{Ab}	9.61 ± 0.04^{Abc}	11.45 ± 0.44^{Aa}	9.18 ± 0.00^{Ac}
70	5	6.30 ± 0.03^{Cc}	8.40 ± 0.35^{Bab}	9.42 ± 0.36^{Ba}	8.15 ± 0.00^{Bb}
	10	6.20 ± 0.08^{Cb}	8.37 ± 0.10^{Ba}	8.71 ± 0.36^{Ba}	8.37 ± 0.01^{Ba}
	20	6.72 ± 0.02^{Ba}	6.85 ± 0.05^{Ca}	4.60 ± 0.18^{Db}	6.50 ± 0.39^{Ca}
	30	6.04 ± 0.02^{Ca}	6.95 ± 0.60^{Ca}	3.68 ± 0.14^{Db}	4.34 ± 0.26^{Db}
80	5	5.66 ± 0.10^{Db}	8.23 ± 0.08^{Ba}	5.98 ± 0.24^{Cb}	8.00 ± 0.19^{Ba}
	10	2.43 ± 0.23^{Ec}	7.40 ± 0.19^{BCa}	4.28 ± 0.16^{Db}	4.17 ± 0.07^{Db}
	20	2.58 ± 0.05^{Eb}	2.05 ± 0.14^{Db}	4.60 ± 0.34^{Da}	2.65 ± 0.08^{Eb}
	30	$< 1^{Fb}$	1.85 ± 0.11^{Da}	$< 1^{Eb}$	$< 1^{Fb}$

Means ± standard deviation.
FLP – *L. plantarum* as free cells; ALP – Alginate beads containing *L. plantarum*; FEF – E. *faecium* as free cells; AEF – Alginate beads containing *E. faecium*.
[A] Different upper letters in the same column indicate significant differences (P < 0.05).
[a] Different lower letters in the same row indicate significant differences (P < 0.05).

Table 3 – Viability of free and encapsulated *L. plantarum* and *E. faecium* in alginate beads to sodium chloride (in log CFU/ml or g).

	Sodium chloride (%)				
	0	0.5	1.0	2.5	5.0
FLP	10.23 ± 0.04^{Ba}	9.62 ± 0.16^{Ab}	9.67 ± 0.29^{Ab}	9.77 ± 0.09^{Ab}	8.26 ± 0.02^{Ac}
ALP	9.61 ± 0.09^{BCa}	9.54 ± 0.48^{Aa}	9.41 ± 0.02^{ABa}	9.26 ± 0.13^{ABa}	9.20 ± 0.17^{Aa}
FEF	11.45 ± 0.44^{Aa}	9.28 ± 0.14^{Ab}	9.33 ± 0.09^{ABb}	9.24 ± 0.14^{Bb}	5.58 ± 0.45^{Bc}
AEF	9.18 ± 0.00^{Ca}	9.02 ± 0.04^{Aab}	8.86 ± 0.19^{Bab}	8.70 ± 0.09^{Cb}	8.76 ± 0.02^{Ab}

Means ± standard deviation.
FLP – *L. plantarum* as free cells; ALP – Alginate beads containing *L. plantarum*; FEF – *E. faecium* as free cells; AEF – Alginate beads containing *E. faecium*.
[A] Different upper letters in the same column indicate significant differences ($P < 0.05$).
[a] Different lower letters in the same row indicate significant differences ($P < 0.05$).

698   Table 4 – Viability of free and encapsulated *L. plantarum* and *E. faecium* in alginate beads to

699   sodium nitrite (in log CFU/ml or g).

	Sodium nitrite (%)		
	0	**0.5**	**1.0**
FLP	10.23 ± 0.04[Ba]	9.61 ± 0.05[Ab]	9.69 ± 0.07[Ab]
ALP	9.61 ± 0.04[BCa]	9.08 ± 0.08[ABb]	8.69 ± 0.08[Bc]
FEF	11.45 ± 0.44[Aa]	8.54 ± 0.33[Bb]	7.91 ± 0.12[Cb]
AEF	9.18 ± 0.00[Ba]	8.71 ± 0.03[Bb]	8.36 ± 0.13[BCc]

700   Means ± standard deviation.
701   FLP – *L. plantarum* as free cells; ALP – Alginate beads containing *L. plantarum*; FEF – *E.*
702   *faecium* as free cells; AEF – Alginate beads containing *E. faecium*.
703   [A] Different upper letters in the same column indicate significant differences (P < 0.05).
704   [a] Different lower letters in the same row indicate significant differences (P < 0.05).
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727   Table 5 – Viability of *L. plantarum* or *E. faecium* encapsulated in alginate beads during

728   refrigerated storage (in log CFU/g).

Day of storage	ALP	AEF
0	9.62 ± 0.09^{A}	9.19 ± 0.01^{A}
10	9.23 ± 0.16^{B}	8.63 ± 0.16^{B}
20	8.87 ± 0.02^{BC}	8.51 ± 0.08^{B}
30	8.39 ± 0.16^{CD}	8.35 ± 0.09^{B}
40	7.83 ± 0.16^{E}	7.57 ± 0.02^{D}
50	7.93 ± 0.03^{DE}	7.38 ± 0.00^{D}
60	7.70 ± 0.08^{E}	7.43 ± 0.15^{D}
70	7.88 ± 0.03^{E}	7.97 ± 0.02^{C}

729   Means ± standard deviation.
730   ALP – Alginate beads containing *L. plantarum*; AEF – Alginate beads containing *E. faecium*.
731   [A] Different upper letters in the same column indicate significant differences ($P < 0.05$).

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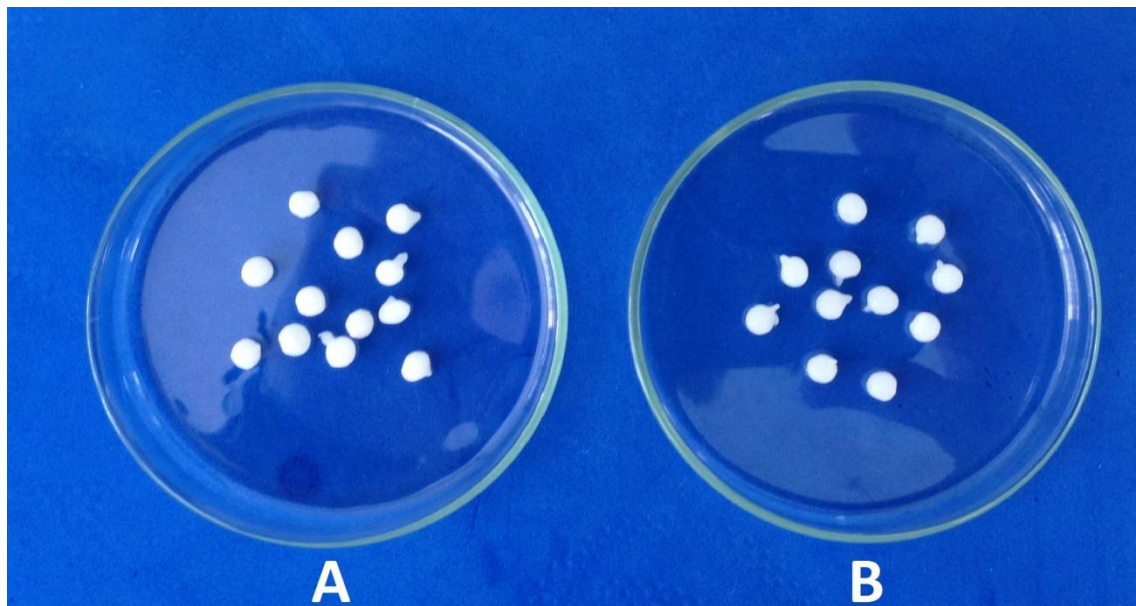
Figure 1 – Alginate beads with inulin, powder milk and trehalose containing *L. plantarum* (A) and *E. faecium* (B).

2.2.2 Manuscrito 4

Processing of dry fermented sausage with free and encapsulated *Lactobacillus plantarum*

Carlos Pasqualin Cavalheiro, Claudia Ruiz-Capillas, Ana M. Herrero, Tatiana Pintado, Francisco Jiménez-Colmenero, Cristiano Ragagnin de Menezes, Leadir Lucy Martins Fries

Artigo em fase final de revisão.

Processing of dry fermented sausage with free and encapsulated *Lactobacillus plantarum*.

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22 **Abstract**

23   The aim of this work was to add *L. plantarum* as free cells or encapsulated in alginate

24 beads, emulsion (W/O) and double emulsion (W/O/W) in a traditional Spanish dry fermented

25 sausage (*chorizo*). Five treatments were produced: a control treatment (Cc) (with no *L.*

26 *plantarum* addition), a treatment with *L. plantarum* added as free cells (Cfc), a treatment with

27 *L. plantarum* encapsulated in alginate beads (Calg), a treatment with *L. plantarum*

28 encapsulated in a water-in-oil emulsion (Cwo), and a treatment with *L. plantarum*

29 encapsulated in a water-in-oil-in-water emulsion (Cwow). Proximate composition was

30 evaluated at the end of processing of dry fermented sausages and weight loss, pH, a_w, color

31 and microbiological parameters were evaluated during processing. The addition of *L.*

32 *plantarum* as free cells or encapsulated affected the composition and physico-chemical

33 characteristics of products. However, all treatments had traditional red color of dry fermented

34 products. In relation to microbiological properties, the adition of *L. plantarum* as free cells or

35 encapsulated in alginate beads had higher levels of probiotics at the end of processing. In this

36 context, encapsulated *L. plantarum* is viable for use in dry fermented sausages. However, the

37 adition of probiotic in alginate beads seems to be more effective due to the higher probiotic

38 counts found at the end of processing.

39 **Key words:** encapsulation, chorizo, probiotic, extrusion, double emulsion.

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45   **1. Introduction**

46   Dry fermented sausages are ready-to-eat products where product is essentially gained

47   by decrease in pH (between 4.5 and 5.0) and water activity (a_w) under the growth of most

48   pathogens (Barbuti & Parolari, 2002). This kind of meat product is of great importance to the

49   meat industry once are largely consumed worldwide. In Spain, the most popular fermented

50   sausage is *Chorizo*, a dry fermented sausage that is resulted of fermentation (first 48h of

51   processing) and ripening steps where microbiological and chemical modifications such as

52   color development, lipolysis and proteolysis occur. Traditionally, dry fermented sausages are

53   produced either by their natural flora or by adding a starter culture mainly composed by lactic

54   acid bacteria (LAB) and staphylococci or micrococci (Hammes & Knauf, 1994). It is well

55   known LAB has positive effect inhibiting the growth of pathogenic micro flora, and on the

56   development of color and flavor (Cenci-Goga, Rossitto, Sechi, Parmegiani, Cambiotti, &

57   Cullor, 2008). Generally, counts greater than 10^7 CFU/g of LAB are found in this type of

58   meat product at the end of process (Benito *et al.,* 2007; Macedo *et al.*, 2008; Zhao *et al.*,

59   2011; Dalla Santa *et al.*, 2014; Rubio, Jofré, Aymerich, Guàrdia, & Garriga, 2014a).

60   Lactobacilli species are of utmost importance and are often used as starter culture

61   (Ammor & Mayo, 2007). Amongst the lactobacilli genus, *L. plantarum* is one of the most

62   widespread and is being widely used in food-related technologies (Sauvageau *et al.*, 2012). In

63   addition, *L. plantarum* can act as a probiotic, which concept is well defined by FAO/WHO

64   (2002) as "live microorganisms which, when administered in adequate amounts, confer health

65   benefits to the consumer". In foods is better to have a high level of probiotics, however, there

66   is a consensus that the minimum concentration of probiotics in food should be between 10^6

67   and 10^7 CFU/g at the moment of consumption (Nulkaekul, Lenton, Cook, Khutoryanskiy, &

68   Charalampopoulos, 2012).

69   Probiotics are being applied in various types of foods, especially dairy. However,

70   some studies have been done using probiotic bacteria in fermented sausages aiming to

71   improve their nutritional value (DeVuyst, Falony, & Leroy, 2008; Macedo *et al.*, 2008; Sayas-

72   Barberá, Viuda-Martos, Fernández-López, Pérez-Alvarez, & Sendra, 2012; Wójciak,

73   Dolatowski, Kolozyn-Krajewska, & Trzaskowska, 2012; Holko, Hrabe, Salaková, & Rada,

74   2013; Rubio *et al.*, 2013: Rubio *et al.*, 2014a; Rubio, Martín, Aymerich, & Garriga, 2014b;

75   Rubio, Jofré, Martín, Aymerich, & Garriga, 2014c; Bagdatli & Kundakci, 2015; Cavalheiro *et*

76   *al.*, 2015). According to Klingberg & Budde (2006), dry fermented sausages are favorable to

77   carry probiotics on, once sausage matrix seems to protect them in their journey through the

78   gastrointestinal tract (GIT). On the other hand, the loss of probiotic viability in dry fermented

79   sausages is unavoidable due to factors such as large amounts of salt and nitrites and low pH

80   and a_w during fermentation and ripening (DeVuyst *et al.*, 2008; Khan *et al.*, 2011).

81   Against this background, encapsulation, entrapment, or immobilization of probiotic

82   bacteria arises as a protection form of cells in the GIT and in food. This is a methodology

83   whereby solids, liquids or gaseous materials are placed in an inert shell (capsule) and are

84   released at a controlled rate under specific conditions (Champagne & Fustier, 2007). Amongst

85   the encapsulation techniques, the most common are extrusion, emulsification and spray

86   drying with capsules ranging from a few micrometers to a few millimeters (Champagne &

87   Fustier, 2007).

88   Extrusion technique is the oldest and most common encapsulation method due to ease,

89   low cost and efficiency, mainly using sodium alginate as wall material (Krasaekoopt,

90   Bhandari, & Deeth, 2003). Alginate recovers the probiotic cells and forms a semi-permeable

91   spherical barrier, which nutrients and metabolites readily cross (Kailasapathy, 2002; Anal &

92   Singh, 2007). Emulsification technique is also largely used for probiotic encapsulation and

93   involves dispersion of the cells mixed in a polymer solution in oil. The mixture forms oil-in-

water (W/O) emulsion. More recently, tests have been conducted on encapsulation in double or multiple emulsions which are multi-compartmentalized systems in which W/O and oil-in-water (O/W) coexist and where dispersed phase globules themselves contain even smaller dispersed droplets (Garti, 1997; Jiménez-Colmenero, 2013).

Probiotics encapsulated by either extrusion or emulsification techniques already have been applied in dry fermented sausages (Muthukumarasamy & Holley, 2006; Muthukumarasamy & Holley, 2007; Barbosa, Todorov, Jurkiewicz, & Franco, 2015). The use of encapsulated probiotic in water-in-oil-in-water (W/O/W) emulsion have been reported in Oaxaca cheese (Rodríguez-Huezo *et al.*, 2014). In meat products, double emulsion (W/O/W) was already used to improve frankfurter lipid content (Freire, Cofrades, Solas, & Jiménez-Colmenero, 2015).

As far as the authors are aware, there are no references in the literature to similar research into delivery probiotic bacteria in fermented sausages as reported in this paper. Thus, the aim of the present work is to add free and encapsulated *L. plantarum* in dry fermented sausage (*chorizo*) and to analyze its physicochemical and microbiological properties during processing.

2. Materials and methods

2.1. Strain and culture conditions

Lactobacillus plantarum was provided by Christian Hansen (SM 199, Hørsholm, Denmark). Stock cultures were produced using MRS broth (Panreac Química S.A., Barcelona, Spain) enriched with 20% of glycerol (Panreac, Darmstadt, Germany). One mL fractions of this cell suspension were stored in eppendorfs at -80 ºC.

117   Before use, the cell suspension was thawed at room temperature for 15 min, and

118   subcultured in MRS broth in a proportion 1/100 (*v/v*) at 37 ℃ for 24 h. Microorganisms were

119   then harvested by centrifugation at 6000 *g* for 20 min at 4 ℃ and suspended in 5 mL of 0.9%

120   NaCl solution. The cell suspensions were referred as free cells (fc) and were used either

121   directly in *chorizos* or subjected to encapsulation in alginate beads, emulsion or double

122   emulsion techniques.

123

124   *2.2. Encapsulation procedures*

125   Extrusion technique was used for the production of alginate beads containing

126   *Lactobacillus plantarum* according to Krasaekoopt, Bhandari, & Deeth (2004) with slight

127   modifications. Briefly, the wall material were prepared containing: 1% sodium alginate

128   (Texturalia, Barcelona, Spain), 1% of milk powder (La Lechera, Nestlè), 1% of dextrin

129   (molecular weight average approximate between 10 and 20 glucose molecule per polymer)

130   (Cargill S. L. U., Barcelona, Spain) and 5% of trehalose (Treha 16400, Cargill, Krefeld,

131   Germany). Then, the solution was mixed with 16.66% (*v/v*) of a 10^9 CFU/ml of free

132   *Lactobacillus plantarum*. The mixture was then dropped into a hardening solution of 0.1

133   mol/L of calcium chloride (Sigma Aldrich, Darmstatd, Germany) through a 200 µm nozzle.

134   Finally, alginate beads were allowed to stand for 30 min for complete hardening, recovered

135   with a strainer and stored at 4 ℃ until their use.

136   The emulsions were prepared according proposed by Cofrades, Antoniou, Solas,

137   Herrero, & Jiménez-Colmenero (2013) with slightly modifications. For W/O emulsion, a one-

138   step emulsification process was used. Briefly, an oil phase was prepared by dispersing 6 g/100

139   g of the lipophilic surfactant PGPR (polyglycerol ester of polyricinoleic acid – SUGIN

140   476/M, Cargill, S.L.U., Barcelona, Spain) in extra virgin olive oil (Hojiblanca, Madrid, Spain)

141   under agitation for 10 min in a TM-3 Thermomix food processor (Vorwerk, Germany) at

142   3250 rpm. The inner aqueous phase was free *L. plantarum* in a 0.6 g/100 g NaCl (Panreac

143   Química S.A., Barcelona, Spain) which was drop-wise added to the oil phase in a blender

144   (Thermomix, Vorwerk, Germany) at 3250 rpm for 5 min. The resulting emulsion (W/O) was

145   passed twice through a two-stage high pressure homogenizer (GEA Niro Soavi model Panda

146   Plus 2000, Parma, Italy) at 4351/435 psi (first-stage pressure/second-stage phase). The

147   emulsion (W/O) was then allowed to cool at refrigeration temperature. Second-step, W/O/W

148   emulsion were prepared by gradually adding a W/O emulsion (40 g/100 g) to an outer

149   aqueous phase (60 g/100 g) with 0.6 g/100 g NaCl and 0.5 g/100 g sodium caseinate (DMV

150   Excellion EM 7, DMV Campina B.V., Veghel, The Netherlands) followed by mixing in a

151   Thermomix blender for 5 min at 700 rpm at room temperature. Resulting W/O/W emulsions

152   were passed twice through a two-stage high pressure homogenizer at 2175/435 psi.

153

154   *2.3. Dry fermented sausage elaboration*

155   Fresh postrigor pork meat and pork backfat were obtained from a local market. Five

156   different formulations of dry fermented sausage (*chorizo*) were produced, according

157   formulation previously reported by Ruiz-Capillas, Triki, Herrero, Rodriguez-Sala, & Jiménez-

158   Colmenero (2012). A control treatment (Cc) without addition of *Lactobacillus plantarum*

159   were prepared as follow: pork meat (75%), pork backfat (18%), choravi (5.3%), NaCl (1.5%)

160   and curavi (0.3%). A treatment with addition of *L. plantarum* as free cells (0.63%) (Cfc), a

161   treatment with *L. plantarum* added as alginate beads (3.75%) (Calg), a treatment with *L.*

162   *plantarum* added in a W/O emulsion (2.30%) (Cwo) and, a treatment with *L. plantarum* added

163   in a W/O/W emulsion (5.70%) (Cwow) were produced. Meat and pork backfat were

164   separately minced at 4.5 mm (Vam.Dall. Srl. Modelo FTSIII, Treviglio, Italy) and then were

165   homogenized (MAINCA, Granollers, Barcelona, Spain) for 2 min. At that time, half of

166   additives (choravi, curavi, and NaCl) were added and the mixture was homogenized for 1

167   min. Then, the remaining additives and the solution containing free cells of *L. plantarum,*

168   alginate beads, W/O or W/O/W emulsions were added and mixture was homogenized for

169   more 2 min. In all cases, the final temperature of meat matrix was less than 11 ℃. Prepared

170   sausage matrix was immediately stuffed into collagen casing (Fibran, S. A. Sant Joan de les

171   Abadesses, Gerona, Spain) using a 4-cm diameter stuffer (MAINCA, Granollers, Barcelona,

172   Spain). Sausages were placed in a ripening cabinet (BINDER model KBF 240 Tuttlingen,

173   Germany) programmed to operate under following conditions: 23 ℃ and 90% relative

174   humidity (RH) for 48 h and 13 ℃ and 75% RH, until the end of ripening (20 days). To

175   monitor processing, samples from each formulation were taken periodically for analysis.

176

177   *2.4. Proximate composition*

178   Proximate composition was done after the end of dry fermented sausage processing

179   (day 20). Moisture and ash were determined (AOAC, 2005) in triplicate in all treatments.

180   Protein content was measured in quadruplicate with a LECO FP-200 Nitrogen Determinator

181   (Leco Corp., St Joseph, MI). Fat content was evaluated in triplicate according to Bligh &

182   Dyer (1959) method.

183

184   2.5. *Weight losses, pH and water activity*

185   Weight losses were evaluated as percentage of initial sample weight. Three sausages

186   for each formulation were used for these determinations. The pH was determined using a pH

187   meter (model 827, pH Lab Methrom, Herisau, Switzerland) on 10 g homogenate samples in

188   100 ml of distilled water. Six measurements were performed for each treatment. Water

189   activity (a_w) was measured at 25 ℃ in a LabMaster-a_w (Novasina, Lachen, Switzerland).

190 Analyses were performed at days 0, 2, 7 and 20 of processing and three determinations were

191 carried out for each treatment.

192

193 *2.6. Color analysis*

194 Color (CIE-LAB tristimulus values, lightness, L^*; redness, a^* and yellowness, b^*) was

195 evaluated on a Chroma Meter CR-400 (Konica Minolta Business Technologies, Inc., Tokyo,

196 Japan). Analyses were performed at days 0, 2, 7 and 20 of processing and ten determinations

197 from each treatment were carried out on cross-sections of the sausage.

198

199 *2.7. Microbiological Analysis*

200 Microbiological analysis of sausages was carried out as follows: 10 g of each

201 treatment was aseptically taken and placed in a sterile plastic bag with 90 mL of buffered

202 peptone water (Panreac, Darmstadt, Germany). After 15 min in a stomacher blender

203 (Stomacher Colworth 400, Seward, London, UK), appropriate decimal dilutions were plated

204 or spread on the media. Plate Count Agar (PCA) (Panreac, Darmstadt, Germany) was used for

205 total viable counts (TVC) (37 ºC for 48 h), De Man, Rogosa, Sharp Agar (MRS) (Merck,

206 Darmstadt, Germany) was used for lactic acid bacteria (LAB) (37 ºC for 48 h). For selective

207 enumeration of *L. plantarum*, LPSM (*Lactobacillus plantarum* selective medium) agar (37 ºC

208 for 72 h) was used according to Bujalance, Jiménez-Valera, Moreno, & Ruiz-Bravo (2006).

209 LPSM medium is composed of (in g/L): D-sorbitol (20), agar (15), bacterial peptone (10),

210 beef extract (10), yeast extract (5), sodium acetate (5), potassium phosphate (2), ammonium

211 citrate (2), magnesium sulfate (0.1), manganese sulfate (0.05), bromocresol purple (0.02) and

212 ciprofloxacin (0.0004). Finally, Violet Red Bile Glucose Agar (VRBG) (Panreac, Darmstadt,

213 Germany) with a double layer was used for *Enterobacteriaceae* (37 ºC for 24 h) enumeration.

214 Homogenization time of samples was according the time need for complete solubilization of

215 alginate beads. All microbial counts were converted to logarithms of colony-forming units per

216 gram (log CFU/g). Analyses were performed at days 0, 2, 7 and 20 of processing and two

217 determinations were carried out for each treatment.

218

219   *2.8. Statistical Analysis*

220   A two-way analysis of variance (ANOVA) was carried out depending on the variables:

221 type of dry fermented sausage (Cc, Cfc, Calg, Cwo, Cwow) and processing time (0, 2, 7 and

222 20). One-way ANOVA was also carried out to evaluate the statistical significance (P<0.05)

223 on the weight losses of the different fermented sausages. Tukey's HSD test was used to

224 identify significant (P < 0.05) differences between types of fermented sausage and time.

225 Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

226

227 **3. Results and Discussion**

228   *3.1. Proximate composition*

229   Proximate composition of dry fermented sausages was measured at the end of

230 processing (day 20). Moisture content of dry fermented sausages ranged from 25.34 to

231 29.83% (Table 1) and showed differences (P < 0.05) according to the formulation. The

232 highest levels of moisture were found in the Cc and Cfc treatments. In the other hand, lowest

233 moisture level was observed in treatments with *L. plantarum* encapsulated in W/O emulsion.

234 Protein content of dry fermented sausages ranged from 27.04 to 29.90% (Table 1) and no

235 differences between treatments were found. Fat levels of dry fermented sausages ranged from

236 30.06 to 35.69% (Table 1) and as expected, two different levels of fat were found. The highest

237 (P < 0.05) fat content were observed in Cwo and Cwow treatments, and this is probably due

238   to the emulsified olive oil added in those formulations. In addition, fat levels were inversely

239   proportional to moisture content. Ash content ranged from 4.85 to 5.61% with no effect of the

240   formulation in qualitative terms (Table 1).

241

242   *3.2. Weight loss, pH and A_w*

243   Weight loss in all treatments increased (P < 0.05) during processing time because of

244   drying process (Figure 1). The most intense weight loss was observed between the days 2 and

245   7 in all treatments, which is related to the decrease in pH values (Table 2) associated to lower

246   RH values on the ripening chamber. At the final of dry fermented sausages processing (day

247   20), the higher weight loss was observed in Calg, Cwow and Cfc treatments (41.74, 40.28,

248   and 38.56%, respectively). However, weight losses values found in all treatments are in the

249   range of 30 to 40%, which is considered ideal for dry fermented sausages (Rust, 1994).

250   During fermentation and ripening steps of dry fermented sausages, three groups of

251   substances are able to influence the pH values: organic acids from fermentation of sugars,

252   substances resulted from proteolysis by microorganisms and organic acids from fat

253   metabolism (Bloukas, Paneras, & Fournitzis, 1997). Processing time affected (P < 0.05) pH

254   values of dry fermented sausages during both fermentation and ripening steps. As shown in

255   Table 2, initial pH values of meat batters (day 0) were between 5.80 and 5.91. The decrease in

256   pH was observed in all treatments regardless of whether or not they were inoculated with *L.*

257   *plantarum*. The decrease in pH was more intense in Cfc and Calg treatments (P < 0.05) at day

258   2, probably due to a more intense metabolism of *L. plantarum* when added as free cells or as

259   in alginate beads. Some studies have shown the entrapment of LAB in alginate improves

260   lactic acid production (Idris & Suzana, 2006; Rao, Prakasham, Rao, & Yadav, 2008). In

261   addition, the entrapment in alginate matrix forms a semi-permeable barrier (Kailasapathy,

262   2002; Anal & Singh, 2007) and the lactic acid produced by probiotics can flow to the meat

263   matrix decreasing pH values. This rapid drop in pH is important for the microbiological

264   quality of the sausages because it contributes to the inhibition of pathogens and spoilage

265   microorganisms, accelerates the reduction of nitrite to nitric oxide, affects the flavor of the

266   product and facilitates meat binding capacity, improving firmness and sliceability (Garriga *et*

267   *al.*, 1996; Castano, Fontan, Fresno, Tornadijo, & Carballo, 2002; Hughes *et al.*, 2002).

268   Even with slower pH decrease in Cc, Cwo and Cwow treatments, at the end of

269   processing (day 20), pH values were less than 5.0 for all treatments. Values of pH lower than

270   5.0 are important for ensure microbiological safety of dry fermented sausages. Value sof pH

271   found in this study are consistent with the results obtained by Erkillä, Suihko, Eerola, Petäjä,

272   & Mattila-Sandholm (2001), Cavalheiro *et al.* (2010), Campagnol, Santos, Wagner, Terra, &

273   Pollonio (2011), Campagnol, Santos, Terra, Pollonio (2012), Rubio *et al.* (2013), Holko *et al.*

274   (2013) in different dry fermented sausages with or without probiotic addition as free cells.

275   Even more, Muthukumarasamy & Holley (2006) related similar values of pH in dry

276   fermented sausages containing *L. reuteri* encapsulated both extrusion and emulsion methods.

277   Water activity of dry fermented sausages decreased ($P < 0.05$) from initial values of

278   0.955-0.962 to 0.879-0.915 (Table 2) at the end of processing (day 20). In the first 48h of

279   processing an increase in a_w values was observed in all treatments, which could be related to

280   the high RH (90%) used in ripening chamber at this step. After the day 2, a_w decreases

281   considerably up to day 20, when reached the lower ($P < 0.05$) a_w values found. Reduction in

282   a_w values is a consequence of weight losses and a decrease in pH values during processing of

283   dry fermented sausages. Cfc treatment showed lower a_w values at the end of processing

284   followed by Calg (0.858 and 0.879, respectively). The lower a_w in treatments Cfc and Calg

285   probably is consequence of their lower pH values (Table 2). According to Mauriello,

286   Casaburi, Blaiotta, & Villani (2004), when pH approaches the isoelectric point of proteins a

287   decrease in water retention occurs, facilitating dehydration and reducing a_w in sausages. As

288   expected, Cc treatment showed higher a_w in the end of processing. Similar results were

289   observed by Ruiz, Villanueva, Favaro-Trindade, & Contreras-Castillo (2014) in Italian-type

290   salami with *L. acidophilus* and *B. lactis* addition.

291

292   *3.3. Color*

293   Color is one of the main organoleptic characteristics used to establish the quality and

294   acceptability of meat products (Ansorena, Peña, Astiasarán, & Bello, 1997). Color values

295   during processing of chorizo were presented in Table 3. All treatments developed typical

296   color characteristics of dry fermented sausages during processing, as well evidenced in Figure

297   2. At the day 2, no differences in color parameters of dry fermented sausages were found

298   amongst treatments, while, by the 7th day, differences in color parameters were observed. The

299   lightness (*L**) values decreased (P < 0.05) during processing in all treatments, due to the

300   concentration of solids during dehydration (Pérez-Alvarez, Sayas-Barberá, Fernández-López,

301   & Aranda-Catalá, 1999). At the end of processing, lowest *L** values were reported in Cwo

302   while highest *L** values were found in Cwow and, this fact could be related to the color of

303   W/O/W emulsion which are more intense in white than W/O emulsion. Even more, the

304   addition of *L. plantarum* as free cells or in alginate beads did not confer changes in *L**

305   parameters. A decrease in *L** values during processing of probiotic fermented sausages

306   already was reported by Sayas-Barberá *et al.* (2012), Wójciak *et al.* (2012) and Bagdatli &

307   Kundakci (2015).

308   Redness values (*a**) of dry fermented sausages were shown on Table 3. No differences

309   between *a** values on days 2 and 7 were found for treatments Cc, Cfc, Calg and Cwow.

310   Nevertheless, a decrease (P < 0.05) in *a** values were observed in treatment Cwo. Higher *a**

311   values are been related to nitrosomyoglobin formation and moisture loss, which promote an

312   increase in heme pigment concentration (Aléson-Carbonell, Fernández-López, Sayas-Barberá,

313　Sendra, & Pérez-Alvarez, 2003). However, a decrease in $a*$ values were found in all

314　treatments which is probably due to lipid oxidation and nitrosopigments reactions (Pérez-

315　Alvarez & Fernández-López, 2006) and it is not related to addition of *L. plantarum* as free

316　cells or as in encapsulated forms. Decrease in $a*$ values during processing of fermented meat

317　products already have been described by Sayas-Barberá *et al.* (2012) in "*Longaniza de*

318　*Pascua*" with addition of *L. casei* CECT 475 and citrus fiber.

319　　　　Yellowness ($b*$) decreased in treatments during processing (Table 3). The $b*$ values

320　ranged from 25.12 and 27.86 at day 2 to 17.26 and 21.18 at the end of processing (day 20).

321　Decrease in yellowness is due to decrease in oxygen consumption by microorganisms from

322　endogenous flora or intentionally added (Pérez-Alvárez *et al.*, 1999; Ruiz *et al.*, 2014).

323

324　*3.4. Microbiological characteristics*

325　　　　Microbiological parameters of dry fermented sausages were shown in Table 4. Cfc

326　treatment showed higher ($P < 0.05$) TVC values at day 0 which could be due to the directly

327　addition of *L. plantarum* as free cells. However, the same behavior was not observed for LAB

328　and specific *L. plantarum* values. Values of TVC in all treatments increased between 2 and 4

329　log CFU/g after fermentation process (day 2) as expected due to LAB growth. Even more, the

330　addition of *L. plantarum* as free cells or encapsulated did not affect the final values of TVC.

331　　　　In respect of LAB values, an intense increase in the beginning of processing was

332　observed for all treatnemts, especially Cc, Cwo and Cwow which ranged from less than 2 and

333　3 log CFU/g at day 0 to more than 7 log CFU/g at day 2 (Table 4). The rapid growth of LAB

334　is desirable in fermented meat products to control pathogenic bacteria and increase the

335　efficiency in manufacture sausage (Raccach, 1992). This growth of LAB were observed only

336　during the fermentation process (day 2) and LAB values remained stable in treatments (except

337   for Cc) up to the end of processing. Even without the addition of probiotic strains or starter

338   cultures, LAB values for Cc treatment were similar at the end of processing to those where *L.*

339   *plantarum* was intentionally added. This is due to endogenous microbiota presented on meat

340   that is able to perform meat fermentation process. It is also known LAB are the dominant

341   microorganisms in fermented sausages even if prepared without starter culture addition,

342   which generally exceeds 8 log CFU/g of LAB counts (Benito *et al.,* 2007; Macedo *et al.*,

343   2008; Zhao *et al.*, 2011; Dalla Santa *et al.*, 2014; Rubio *et al.*, 2014a).

344   Fermentation time, temperature, RH, ingredient and nature of the starter cultures are

345   some factors that can influence on survival of LAB in dry fermented sausages (Barbosa *et al.*,

346   2015). The reduction of LAB during processing and storage of dry fermented sausages

347   already has been related when probiotic microorganisms were added as free cells (Erkilla *et*

348   *al.*, 2001; Ruiz-Moyano *et al.*, 2011a). However, in our study, this reduction was not observed

349   and LAB values of Cfc treatment were 8.81 log CFU/g, being higher (P < 0.05) than values

350   found in treatments with *L. plantarum* added in emulsion (Cwo) and double emulsion (Cwow)

351   forms. At the final of processing, LAB values of Cwo and Cwow treatments were the same as

352   Cc treatment, with no addition of *L. plantarum.*

353   Nevertheless, the LAB group associated with food includes 11 genera:

354   *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc,*

355   *Oenococcus, Pediococcus, Streptococcus, Vagococcus* and *Weissela* (Vries, Vaughan,

356   Kleerebezem, & Vos, 2006). For this reason, the differention of probiotic microorganism

357   intentionally added from LAB that might be present in sausage matrix and are detected by

358   MRS agar is extremely important. Molecular methods already have been used to confirm the

359   presence of specific lactobacilli in meat products (Ruiz-Moyano *et al.*, 2011a; Ruiz-Moyano

360   *et al.*, 2011b; Rubio *et al.*, 2014a; Rubio *et al.*, 2014b), but their use is limited especially due

361   to abundance of false positives and false negatives results (Lauri & Mariani, 2009). In

362   addition, the selective medium for *L. plantarum* developed by Bujalance *et al.* (2006) is an

363   effective form to differentiate this type of microorganism from other kind of LAB normally

364   found on fermented sausages microbiota and well detected by MRS agar. According to Table

365   4, *L. plantarum* as free cells or encapsulated in alginate beads (Calg) and added to dry

366   fermented sausage survived well up to the end of processing. In counterpart, the counts of

367   probiotic microorganism were lower than 2 log CFU/g in Cwo and Cwow treatments at day 0.

368   These values increased after fermentation process (day 2) and decreased (Cwo and Cwow)

369   until the end of processing. As expected, Cc treatment showed lower ($P < 0.05$) *L. plantarum*

370   values that represent the natural microbiota presented on sausage matrix.

371   It is well known high acidification rates as observed in this study are usually followed

372   by fast LAB growth rates. The presented study shows that free and encapsulated *L. plantarum*

373   was able to ferment meat batter. As *L. plantarum* is an excellent acid lactic (Fu & Matthews,

374   1999) and bacteriocin producer (Silva Sabo, Vitolo, González, & Oliveira, 2014), it is easier

375   to compete with other kind of microorganisms present in meat batter. With respect to

376   *Enterobacteriaceae* counts, at day 0 the counts were between 3 and 4 log CFU/g, which is

377   considered hygienically acceptable for raw meat batters. After fermentation (day 2), the levels

378   of *Enterobacteriaceae* increased in all treatments ($P < 0.05$) followed by a decrease up to end

379   of processing (Table 4). At day 20, *Enterobacteriaceae* values were about 3 log CFU/g for

380   Cc, Cwo and Cwow treatments. However, for Cfc and Calg treatments the values were below

381   the detection limit of 1 log CFU/g. The results showed the inhibitory effect of inoculated *L.*

382   *plantarum* against *Enterobacteriaceae,* which is crucial to obtain high quality hygienic

383   sausages (Benito *et al.*, 2007; Rubio *et al.*, 2013). Instead of lactic acid production, the

384   bioprotective effect of *L. plantarum* is due to bacteriocin production and some strains of meat

385   origin already have been characterized as bacteriocin producers (Müller, Carrasco, Tonarelli,

386   Simonetta, 2009; Todorov, Ho, Vaz-Velho, & Dicks, 2010; Barbosa *et al.*, 2016).

387   However, the addition of *L. plantarum* as free cells or encapsulated in alginate beads

388   seems to be more effective against *Enterobacteriaceae*. According to Gouin (2004), alginate

389   beads are porous and this allows the release of a portion of *L. plantarum* cells to meat matrix

390   assisting in meat fermentation and pathogens control. Garriga *et al.* (2005) and Rubio *et al.*

391   (2013) observed an increase of *Enterobacteriaceae* values during the first 7 days of ripening

392   in fuet and chorizo without starter cultures, whereas no growth was found in treatments with

393   starter culture.

394

395   **4. Conclusions**

396   Proximate composition, physico-chemical (weight loss, pH and a_w) and color

397   properties of dry fermented sausages with addition of free and encapsulated *L. plantarum*

398   were considered normal for this kind of product. In relation of microbiological characteristics,

399   total viable counts and lactic acid bacteria counts were similar on treatments with and without

400   addition of *L. plantarum.* However, treatments with addition of free and encapsulated *L.*

401   *plantarum* had higher counts on *Lactobacillus plantarum* selective medium. Nevertheless,

402   treatments with *L. plantarum* as free cells and encapsulated in alginate beads showed counts

403   of *L. plantarum* higher than 6 log CFU/g during the whole processing.

404

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Table 1 – Proximate composition of dry fermented sausage with addition of free and encapsulated *L. plantarum* (in %).

	Moisture	Protein	Fat	Ash
Cc	29.83 ± 0.31^A	29.49 ± 0.30^A	30.06 ± 0.40^B	5.20 ± 0.03^C
Cfc	28.44 ± 0.17^{AB}	29.90 ± 2.16^A	30.62 ± 0.55^B	5.36 ± 0.05^B
Calg	27.24 ± 0.21^B	29.86 ± 0.91^A	30.53 ± 0.45^B	5.61 ± 0.06^A
Cwo	25.34 ± 0.13^C	28.21 ± 0.97^A	35.02 ± 0.10^A	5.03 ± 0.03^D
Cwow	27.30 ± 1.23^B	27.04 ± 0.64^A	35.69 ± 0.32^A	4.85 ± 0.02^E

Means ± standard deviation.

[A] Different letters in the same column indicate significant differences ($P < 0.05$).

Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W emulsion.

653 Table 2 – pH and a_w of dry fermented sausages with addition of free and encapsulated *L.*
654 *plantarum*.

		Day of processing			
		0	2	7	20
pH	Cc	5.93 ± 0.01^{a2}	5.59 ± 0.02^{b1}	5.14 ± 0.01^{c1}	4.92 ± 0.04^{d1}
	Cfc	5.94 ± 0.00^{a1}	4.79 ± 0.00^{b3}	4.54 ± 0.01^{c4}	4.43 ± 0.01^{d3}
	Calg	5.80 ± 0.01^{a4}	4.80 ± 0.06^{b3}	4.52 ± 0.01^{c5}	4.41 ± 0.01^{d3}
	Cwo	5.94 ± 0.01^{a1}	5.55 ± 0.01^{b1}	5.13 ± 0.01^{c2}	4.93 ± 0.02^{d1}
	Cwow	5.91 ± 0.01^{a3}	5.33 ± 0.01^{b2}	5.03 ± 0.01^{c3}	4.84 ± 0.01^{d2}
a_w	Cc	0.956 ± 0.001^{b3}	0.963 ± 0.001^{a3}	0.941 ± 0.001^{c1}	0.915 ± 0.004^{d1}
	Cfc	0.955 ± 0.000^{b3}	0.964 ± 0.001^{a3}	0.938 ± 0.001^{c2}	0.858 ± 0.005^{d4}
	Calg	0.960 ± 0.001^{b12}	0.970 ± 0.001^{a12}	0.937 ± 0.001^{c2}	0.879 ± 0.001^{d3}
	Cwo	0.958 ± 0.002^{b23}	0.966 ± 0.002^{a23}	0.939 ± 0.002^{c12}	0.888 ± 0.001^{d2}
	Cwow	0.962 ± 0.001^{b1}	0.970 ± 0.002^{a1}	0.941 ± 0.001^{c1}	0.896 ± 0.003^{d2}

655 Means ± standard deviation.

656 Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in
657 alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W
658 emulsion.

659 [a] Different letters in the same row indicate significant differences (P < 0.05).

660 [1] Different numbers in the same column for same parameter indicate significant differences (P
661 < 0.05).

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675 Table 3 – Color parameters (lightness, L*; redness, a*; yellowness, b*) of dry fermented
676 sausage with addition of free and encapsulated *L. plantarum.*

		Day of processing		
		2	7	20
*L**	Cc	37.75 ± 2.18^{a1}	35.55 ± 1.44^{b2}	33.23 ± 1.14^{c23}
	Cfc	38.95 ± 1.53^{a1}	40.30 ± 0.75^{a1}	34.64 ± 1.99^{b12}
	Calg	39.69 ± 3.30^{a1}	41.44 ± 1.78^{a1}	34.64 ± 1.22^{b12}
	Cwo	39.14 ± 3.96^{a1}	36.89 ± 1.99^{a2}	32.26 ± 1.18^{b3}
	Cwow	41.46 ± 2.33^{a1}	37.53 ± 2.03^{b2}	35.66 ± 1.97^{b1}
*a**	Cc	27.46 ± 1.31^{a1}	27.27 ± 1.56^{a2}	23.81 ± 2.21^{b1}
	Cfc	28.22 ± 1.61^{a1}	29.37 ± 0.98^{a1}	24.71 ± 1.75^{b1}
	Calg	29.27 ± 1.09^{a1}	28.76 ± 1.51^{a12}	26.21 ± 1.11^{b1}
	Cwo	29.05 ± 1.61^{a1}	26.92 ± 1.96^{b2}	24.67 ± 1.52^{c1}
	Cwow	28.70 ± 2.72^{a1}	29.77 ± 1.12^{a1}	24.05 ± 2.08^{b1}
*b**	Cc	25.59 ± 2.33^{a1}	22.73 ± 3.02^{a2}	17.26 ± 2.44^{b2}
	Cfc	25.12 ± 2.25^{a1}	26.69 ± 1.66^{a1}	18.50 ± 2.31^{b12}
	Calg	26.21 ± 1.85^{a1}	26.43 ± 2.28^{a1}	21.18 ± 1.85^{b1}
	Cwo	27.02 ± 2.46^{a1}	23.52 ± 1.31^{b2}	18.71 ± 2.01^{c12}
	Cwow	27.86 ± 2.77^{a1}	26.64 ± 1.14^{a1}	20.46 ± 2.21^{b1}

677 Means ± standard deviation.

678 [a] Different letters in the same row indicate significant differences ($P < 0.05$).

679 [1] Different numbers in the same column for same parameter indicate significant differences (P
680 < 0.05).

681 Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in
682 alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W
683 emulsion.

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694   Table 4 – Microbiological parameters of dry fermented sausages with addition of free and

695   encapsulated *L. plantarum* (in log CFU/g).

		Day of processing			
		0	2	7	20
TVC	Cc	5.96 ± 0.18^{c2}	7.76 ± 0.46^{b3}	8.41 ± 0.22^{a2}	8.51 ± 0.14^{a1}
	Cfc	6.93 ± 0.15^{c1}	8.92 ± 0.01^{a12}	8.94 ± 0.14^{a1}	8.16 ± 0.05^{b1}
	Calg	5.94 ± 0.04^{b2}	9.03 ± 0.00^{a1}	9.22 ± 0.20^{a1}	8.98 ± 0.76^{a1}
	Cwo	5.95 ± 0.08^{c2}	7.90 ± 0.36^{b23}	8.46 ± 0.06^{a2}	8.52 ± 0.08^{a1}
	Cwow	5.91 ± 0.03^{c2}	8.38 ± 0.10^{b123}	8.52 ± 0.05^{a2}	8.71 ± 0.04^{a1}
LAB	Cc	$<2^{c3}$	7.83 ± 0.01^{b2}	8.26 ± 0.32^{ab2}	8.46 ± 0.16^{a23}
	Cfc	7.39 ± 0.26^{b2}	8.89 ± 0.05^{a1}	8.81 ± 0.08^{a1}	8.81 ± 0.04^{a2}
	Calg	8.35 ± 0.23^{b1}	8.99 ± 0.13^{a1}	9.36 ± 0.38^{a1}	9.29 ± 0.17^{a1}
	Cwo	$<3^{b3}$	7.89 ± 0.01^{a2}	8.16 ± 0.20^{a2}	8.12 ± 0.32^{a3}
	Cwow	$<3^{b3}$	7.85 ± 0.01^{a2}	7.91 ± 0.13^{a2}	8.06 ± 0.16^{a3}
L. plantarum	Cc	$<1^{b2}$	4.28 ± 0.53^{a3}	4.48 ± 0.19^{a3}	4.35 ± 0.31^{a3}
	Cfc	6.41 ± 0.16^{c1}	8.11 ± 0.33^{a1}	8.57 ± 0.34^{a1}	7.06 ± 0.08^{b1}
	Calg	6.23 ± 0.08^{d1}	8.04 ± 0.38^{b1}	8.99 ± 0.16^{a1}	7.28 ± 0.18^{c1}
	Cwo	$<2^{c2}$	6.35 ± 0.22^{b2}	7.15 ± 0.16^{a2}	6.28 ± 0.09^{b2}
	Cwow	$<2^{c2}$	7.01 ± 0.08^{a2}	7.07 ± 0.45^{a2}	5.95 ± 0.06^{b2}
Enterobacteriaceae	Cc	3.44 ± 0.16^{c1}	4.53 ± 0.20^{a2}	3.97 ± 0.04^{b2}	3.52 ± 0.09^{c1}
	Cfc	3.73 ± 0.01^{b1}	4.67 ± 0.04^{a12}	3.20 ± 0.12^{c4}	$<1^{d2}$
	Calg	3.39 ± 0.36^{b1}	4.35 ± 0.21^{a2}	3.73 ± 0.04^{ab3}	$<1^{c2}$
	Cwo	3.70 ± 0.02^{b1}	4.85 ± 0.00^{a12}	4.54 ± 0.01^{a1}	3.44 ± 0.15^{b1}
	Cwow	3.43 ± 0.05^{d1}	5.10 ± 0.10^{a1}	4.52 ± 0.03^{b1}	3.69 ± 0.03^{c1}

696   Means ± standard deviation.

697   [a] Different letter in the same row indicate significant differences (P < 0.05).

698   [1] Different number in the same column in each group of microorganisms indicate significant
699   differences (P < 0.05).

700   TVC = Total viable count, LAB = lactic acid bacteria.

701   Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in
702   alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W
703   emulsion.

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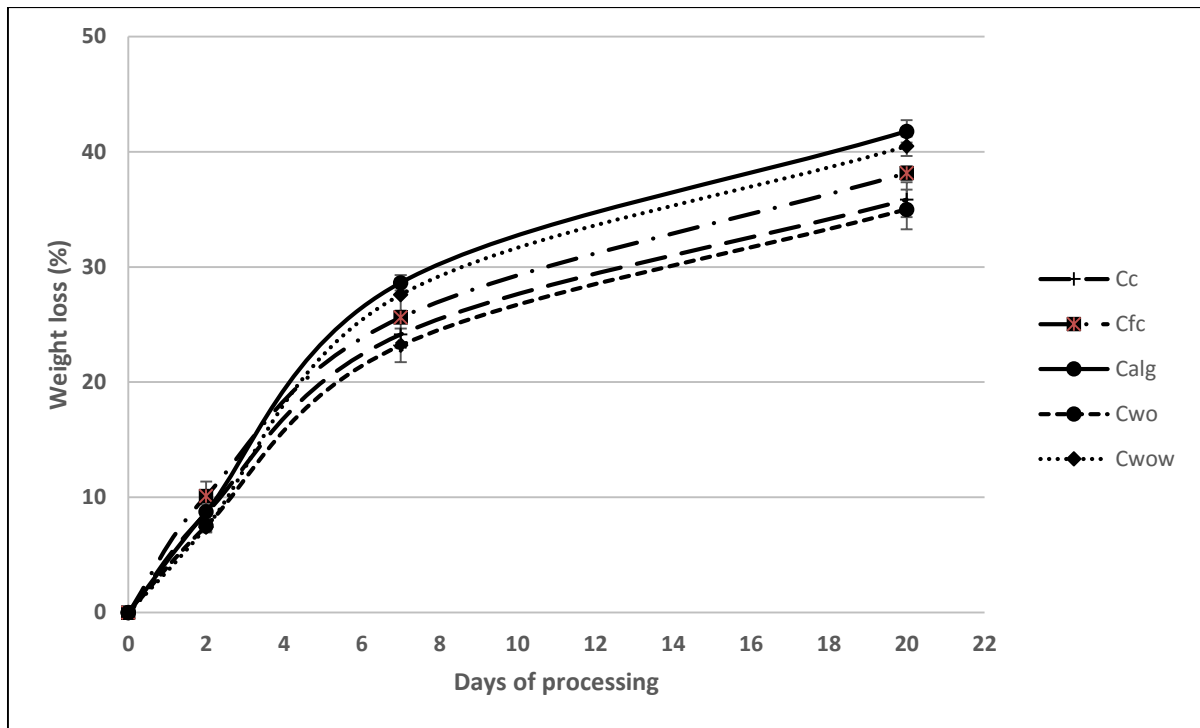
Figure 1 – Weight loss (%) during processing of dry fermented sausages with addition of free and encapsulated *L. plantarum*.

Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W emulsion.

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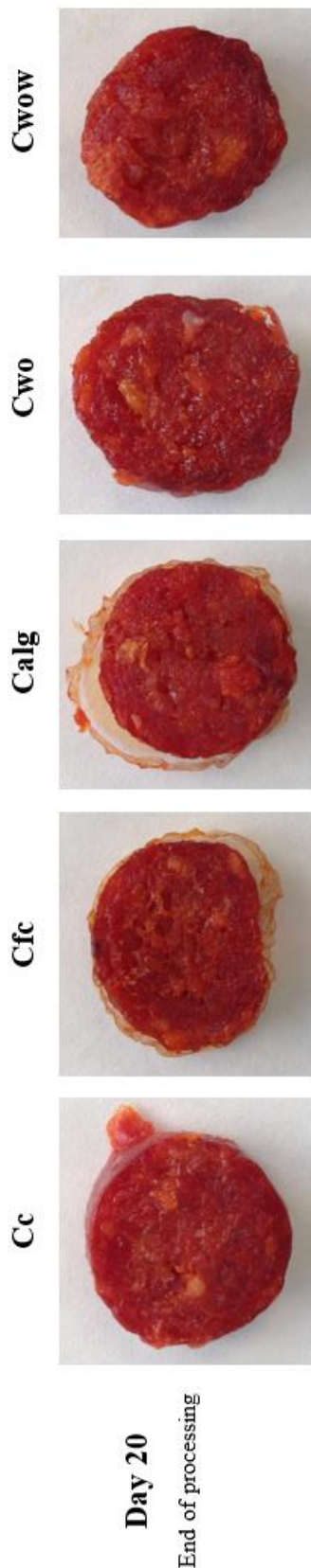
Figure 2 – General aspect of dry fermented sausage with addition of free and encapsulated *L. plantarum* at the end of processing (day 20).

Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W emulsion.

2.2.3 Manuscrito 5

Chilled storage of dry fermented sausage with free and encapsulated *Lactobacillus plantarum*

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Artigo em fase final de revisão

1 **Chilled storage of dry fermented sausage with free and encapsulated *Lactobacillus***

2 ***plantarum***

3

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Abstract

The aim of this work was to analyse physico-chemical, microbiological and sensory characteristics of a traditional Spanish dry fermented sausages (*chorizo*) with addition of *L. plantarum* as free cells or encapsulated in alginate beads, emulsion (W/O) and double emulsion (W/O/W). Five treatments were produced: a control treatment (Cc) (with no *L. plantarum* addition), a treatment with *L. plantarum* added as free cells (Cfc), a treatment with *L. plantarum* encapsulated in alginate beads (Calg), a treatment with *L. plantarum* encapsulated in a water-in-oil emulsion (Cwo), and a treatment with *L. plantarum* encapsulated in a water-in-oil-in-water emulsion (Cwow). Physico-chemical (pH and a_w), color, microbiological characteristics and lipid oxidation were measured during chilled storage of products (60 days). In addition, sensory evaluation was done at the end of processing. Cfc and Calg treatments showed lower pH and a_w values. All treatments kept the typical color of the product, but, in all treatments *a** values decreased during storage. In relation to microbiological characteristics, Calg showed *L. plantarum* counts higher than 6 log CFU/g during the whole storage period. Cwo and Cwow had higher TBARS values at the end of storage, and in relation to sensory evaluation, Cwow showed lower scores, especially flavor and overall acceptability. For this reason, Calg treatment seems to be more indicated for use in dry fermented sausages.

Keywords: encapsulation, probiotic, extrusion, chorizo, double emulsion.

1. Introduction

In Spain, the most popular meat product is *Chorizo*, a dry fermented sausage that is resulted of microbiological and chemical modifications such as dehydration, fermentation, color development, lipolysis and proteolysis. Traditionally, dry fermented sausages are produced either by their natural flora or by adding a starter culture mainly composed by lactic acid bacteria (LAB) and staphylococci or micrococci (Hammes & Knauf, 1994). Amongst the LAB group, *L. plantarum* is one of the most importance due to widespread use in food fermentation and its probiotic activity (Sauvageau *et al*., 2012). According to FAO/WHO (2002), probiotic is defined as "live microorganisms which, when administered in adequate amounts, confer health benefits to the consumer". The higher the level of probiotic in food the better, however, there is a consensus that minimum probiotic concentration in food should be between 10^6 and 10^7 CFU/g or CFU/g at the moment of consumption (Nulkaekul *et al*., 2012).

In last years, application of probiotic microorganisms in meat products has been started. Despite the fact that dairy products are still the most probiotic products, studies already have been reporting the possibility of using probiotics in meat products (DeVuyst *et al*., 2008; Macedo *et al*., 2008; Holko *et al*., 2013; Rubio *et al*., 2013: Rubio *et al*., 2014a; Rubio *et al*., 2014b; Rubio *et al*., 2014c; Cavalheiro *et al*., 2015). According to Klingberg & Budde (2006), fermented meat products are favorable to carry probiotics on, once sausage matrix seems to protect them in their journey through the gastrointestinal tract (GIT). On the other hand, the loss of probiotic viability in fermented meat products is unavoidable due to factors such as large amounts of salt and nitrites and low pH and a_w during fermentation and ripening (DeVuyst *et al*., 2008; Khan et al., 2011). Even more, studies report poor survival of probiotics in food when added as free cells (De Vos *et al*., 2010).

76   In this context, probiotics encapsulation has been emerged as an alternative to protect

77   them from harsh conditions of GIT and food. The encapsulation matrix can provide a physical

78   barrier against stress conditions (Chávarri *et al*., 2010; Nazzaro *et al*., 2012). In addition,

79   capsules should be able to maintain their integrity during passage through the GIT until they

80   reach their target destination (colon), where they should break down and release the probiotics

81   (Ding & Shah, 2007). Amongst the encapsulation techniques, the most common are extrusion,

82   emulsification and spray drying with capsules ranging from a few micrometers to a few

83   millimeters (Champagne & Fustier, 2007).

84   Extrusion technique is the oldest and most common encapsulation method due to ease,

85   low cost and efficiency, mainly using alginate as wall material (Krasaekoopt *et al.*, 2003).

86   Alginate recovers probiotic cells and forms a semi-permeable spherical barrier, which

87   nutrients and metabolites readily cross (Kailasapathy, 2002; Anal & Singh, 2007).

88   Emulsification technique is also largely used for probiotic encapsulation and involves

89   dispersion of the cells mixed in a polymer solution in oil. More recently, tests have been

90   conducted on encapsulation in double or multiple emulsions which are multi-

91   compartmentalized systems in which oil-in-water (O/W) and W/O coexist and where

92   dispersed phase globules themselves contain even smaller dispersed droplets (Garti, 1997;

93   Jiménez-Colmenero, 2013).

94   However, it is important to ensure that the probiotic viability will be maintained in

95   adequate levels during the storage of product. Furthermore, it is important to detect any

96   possible changes in quality and sensorial properties of the sausages that the addition of

97   encapsulated probiotics can promote. Thus, the aim of this study is to assess physico-chemical

98   characteristics, color, microbiological parameters, lipid oxidation, and sensory properties

99   during chilled storage (60 days) of dry fermented sausages (*chorizo*) elaborated with free and

100   encapsulated *L. plantarum*.

2. Materials and methods

2.1. Strain and culture conditions

Lactobacillus plantarum was provided by Christian Hansen (SM 199, Hørsholm, Denmark). Stock cultures were obtained using MRS broth (Panreac Química S.A., Barcelona, Spain) enriched with 20% of glycerol (Panreac, Darmstadt, Germany). One mL fractions of this cell suspension were stored in eppendorfs at -80 ºC.

Before use, cell suspension was thawed at room temperature for 15 min, and subcultured in MRS broth in a proportion 1/100 (*v/v*) at 37 ºC for 24 h. Microorganisms were then harvested by centrifugation at 6000 *g* for 20 min at 4 ºC and suspended in 5 mL of 0.9% NaCl solution. Cell suspensions were referred as free cells (fc) and were used either directly in dry fermented sausage or subjected to encapsulation in alginate beads, emulsion or double emulsion techniques.

2.2. Encapsulation procedures

Extrusion technique was used for the production of alginate beads containing *Lactobacillus plantarum* according to Krasaekoopt, Bhandari, & Deeth (2004) with slight modifications. Wall material were prepared containing: 1% sodium alginate (Texturalia, Barcelona, Spain), 1% of milk powder (La Lechera, Nestlè), 1% of dextrin (molecular weight average approximate between 10 and 20 glucose molecule per polymer) (Cargill S. L. U., Barcelona, Spain) and 5% of trehalose (Treha 16400, Cargill, Krefeld, Germany). Then, the solution was mixed with 16.66% (*v/v*) of a 10^9 CFU/ml of free *Lactobacillus plantarum*. Mixture was then dropped into a hardening solution of 0.1 mol/L of calcium chloride (Sigma Aldrich, Darmstatd, Germany) through a 200 µm nozzle. Alginate beads were allowed to

124 stand for 30 min for complete hardening, recovered with a strainer and stored at 4 ºC until

125 their use.

126   Emulsions were prepared according proposed by Cofrades, Antoniou, Solas, Herrero,

127 & Jiménez-Colmenero (2013) with slightly modifications. For W/O emulsion, a one-step

128 emulsification process was used. Briefly, an oil phase was prepared by dispersing 6 g/100 g of

129 the lipophilic surfactant PGPR (polyglycerol ester of polyricinoleic acid – SUGIN 476/M,

130 Cargill, S.L.U., Barcelona, Spain) in extra virgin olive oil (Hojiblanca, Madrid, Spain) under

131 agitation for 10 min in a TM-3 Thermomix food processor (Vorwerk, Germany) at 3250 rpm.

132 The inner aqueous phase was free *L. plantarum* in a 0.6 g/100 g NaCl (Panreac Química S.A.,

133 Barcelona, Spain) which was drop-wise added to the oil phase in a blender (Thermomix,

134 Vorwerk, Germany) at 3250 rpm for 5 min. The resulting emulsion (W/O) was passed twice

135 through a two-stage high pressure homogenizer (GEA Niro Soavi model Panda Plus 2000,

136 Parma, Italy) at 4351/435 psi (first-stage pressure/second-stage phase). Emulsions were then

137 allowed to cool at refrigeration temperature. In the second-step, W/O/W emulsion were

138 prepared by gradually adding a W/O emulsion (40 g/100 g) to an outer aqueous phase (60

139 g/100 g) with 0.6 g/100 g NaCl and 0.5 g/100 g sodium caseinate (DMV Excellion EM 7,

140 DMV Campina B.V., Veghel, The Netherlands) followed by mixing in a Thermomix blender

141 for 5 min at 700 rpm at room temperature. The resulting W/O/W emulsions were passed twice

142 through a two-stage high pressure homogenizer at 2175/435 psi.

143

144   *2.3. Dry fermented sausage elaboration*

145   Fresh postrigor pork meat and pork backfat were obtained from a local market. Five

146 different formulations of dry fermented sausage (*chorizo*) were produced, according

147 formulation previously reported by Ruiz-Capillas, Triki, Herrero, Rodriguez-Sala, & Jiménez-

148   Colmenero (2012). A control treatment (Cc) without addition of *L. plantarum* were prepared

149   as follow: pork meat (75%), pork backfat (18%), choravi (5.3%), NaCl (1.5%) and curavi

150   (0.3%). A treatment with addition of *L. plantarum* as free cells (0.63%) (Cfc), a treatment

151   with *L. plantarum* added as alginate beads (3.75%) (Calg), a treatment with *L. plantarum*

152   added in a W/O emulsion (2.30%) (Cwo) and, a treatment with *L. plantarum* added in a

153   W/O/W emulsion (5.70%) (Cwow) were produced. Meat and pork backfat were separately

154   minced at 4.5 mm (Vam.Dall. Srl. Modelo FTSIII, Treviglio, Italy) and then homogenized

155   (MAINCA, Granollers, Barcelona, Spain) for 2 min. Subsequently, half of additives (choravi,

156   curavi, and NaCl) were added and the mixture was homogenized for 1 min. Then remaining

157   additives and the solution containing free cells of *L. plantarum*, alginate beads, W/O or

158   W/O/W emulsions were added and mixture was homogenized for more 2 min. In all cases,

159   final temperature of meat matrix was less than 11 ℃. Prepared sausage matrix was

160   immediately stuffed into collagen casing (Fibran, S. A. Sant Joan de les Abadesses, Gerona,

161   Spain) using a 4-cm diameter stuffer (MAINCA, Granollers, Barcelona, Spain). Sausages

162   were placed in a ripening cabinet (BINDER model KBF 240 Tuttlingen, Germany)

163   programmed to operate under following conditions: 23 ℃ and 90% relative humidity (RH) for

164   48 h and 13 ℃ and 75% RH, until the end of ripening (20 days). After the end of processing,

165   dry fermented sausages were immediately stored at 4 ℃ for 60 days.

166

167   *2.4. Physico-chemical and color characteristics*

168   Samples of dry fermented sausages were taken at days 0 (end of processing), 20, 35

169   and 60 of chilled storage. The pH was determined using a pHmeter (model 827pH Lab

170   Methrom, Herisau, Switzerland) on 10 g homogenate samples in 100 ml of distilled water. Six

171   measurements were performed for each sample. Water activity (a_w) was measured at 25 ℃ in

172   a LabMaster-a$_w$ (Novasina, Lachen, Switzerland). Three determinations were carried out for

173   each sample. Color (CIE-LAB tristimulus values, lightness, *L**; redness, *a** and yellowness,

174   *b**) was evaluated on a Chroma Meter CR-400 (Konica Minolta Business Technologies, Inc.,

175   Tokyo, Japan). Ten determinations from each sample were carried out on cross-sections of the

176   sausage. Analyses were performed at days 0, 20, 35 and 60 of storage at 4 ºC.

177

178   *2.5. Microbiological properties*

179   Microbiological analysis of sausages was carried out as follows: 10 g of each sample

180   was aseptically taken and placed in a sterile plastic bag with 90 mL of buffered peptone water

181   (Panreac, Darmstadt, Germany). After homogenizing in a stomacher blender (Stomacher

182   Colworth 400, Seward, London, UK) for 15 min, appropriate decimal dilutions were plated or

183   spread on the media. Plate Count Agar (PCA) (Panreac, Darmstadt, Germany) was used for

184   total viable counts (TVC) (37 ºC for 48 h), De Man, Rogosa, Sharp Agar (MRS) (Merck,

185   Darmstadt, Germany) was used for lactic acid bacteria (LAB) (37 ºC for 48 h). For selective

186   enumeration of *L. plantarum*, LPSM (*Lactobacillus plantarum* selective medium) agar (37 ºC

187   for 72 h) was used, according to Bujalance, Jiménez-Valera, Moreno, & Ruiz-Bravo (2006).

188   LPSM media is composed of (in g/L): D-sorbitol (20), agar (15), bacterial peptone (10), beef

189   extract (10), yeast extract (5), sodium acetate (5), potassium phosphate (2), ammonium citrate

190   (2), magnesium sulfate (0.1), manganese sulfate (0.05), bromocresol purple (0.02) and

191   ciprofloxacin (0.0004). Finally, Violet Red Bile Glucose Agar (VRBG) (Panreac, Darmstadt,

192   Germany) with a double layer was used for *Enterobacteriaceae* (37 ºC for 24 h) enumeration.

193   Homogenization time of samples was according the time need for complete solubilization of

194   alginate beads. All microbial counts were converted to logarithms of colony-forming units per

195   gram (Log cfu/g). Analyses were performed at days 0, 20, 35 and 60 of storage at 4 ºC.

196   *2.6. Lipid oxidation*

197   Lipid oxidation was evaluated by changes in thiobarbituric acid-reactive substances

198   (TBARS). TBARS measurement procedure was based on methods used by López-López,

199   Cofrades, Yakan, Solas & Jiménez-Colmenero (2010). Briefly, the procedure was as follows:

200   5 g of each sample was homogenized in 35 ml of 7.5% trichloroacetic acid for 30 s in a high

201   speed Ultraturrax blender (Ika-Werke, GmbH & Co, Staufen, Germany). The sample was

202   centrifuged (3000 g for 5 min) and 5 ml of the supernatant was mixed with 5 ml of 20 mM of

203   thiobarbituric acid; finally, the solution was mixed and kept in the dark for 20 h at 20 ± 1.5

204   ºC. The pink color formed was determined spectrophotometrically (Lambda 15UV/VIS

205   spectrophotometer, Perkin-Elmer, USA) at 532 nm. A calibration curve was plotted with

206   1,1,3,3-tetraethoxypropane (Sigma Chemical Co., St. Louis, USA) to obtain the

207   malonaldehyde (MDA) concentration as results were expressed as mg MDA/kg of sample.

208   Analyses were performed at days 0, 20, 35 and 60 of storage at 4 ºC and TBARS

209   determinations were performed in triplicate.

210

211   *2.7. Sensory analysis*

212   A 17-member panel carried out sensory analysis of dry fermented sausages. Sausages

213   were sliced into 2 mm slices and a code has been assigned to each sample. Subsequently, all

214   samples were presented to panelists in a random order. A hedonic scale rating test was carried

215   out where panelists evaluated appearance, flavor, hardness, texture, color, odor and overall

216   acceptability. The evaluation was made on 10 cm unstructured line scale with the terms 'I

217   completely dislike' and 'I like very much' at either extreme. Vertical lines drawn by the

218   panelists through the horizontal scale lines were converted into scores. The score sheet also

219   included a comments section. Water and crackers were provided to panelist to cleanse their

220   palates between samples. Sensory analysis was performed one week after preparation of dry

221   fermented sausages.

222

223   *2.8. Statistical analysis*

224   A two-way analysis of variance (ANOVA) was carried out depending on the variables:

225   type of dry fermented sausage (Cc, Cfc, Calg, Cwo, Cwow) and chilled storage time (0, 20,

226   35 and 60 days). One-way ANOVA was also carried out to evaluate the statistical significance

227   ($P < 0.05$) on the sensory analysis of dry fermented sausages. Tukey's HSD test was used to

228   identify significant ($P < 0.05$) differences between samples of fermented sausage and chilled

229   storage time. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL,

230   USA).

231

232   **3. Results and Discussion**

233   *3.1. Physico-chemical and color properties*

234   Values of physico-chemical properties of dry fermented sausages with encapsulated *L.*

235   *plantarum* during chilled storage are shown on Table 1. According to Työpponen et al.

236   (2003), pH values closer to 5 are important to ensure microbiological security of products and

237   to improve changes in color and flavor formation. According to Table 1, pH values of dry

238   fermented sausages during storage ranged from 4.41 to 5.20 ($P < 0.05$), which is beneficial for

239   microbiological stability of meat products (Castano et al., 2002; Garriga et al., 1996; Hughes

240   et al., 2002). Despite the pH values of dry fermented sausages found in this study can be

241   considered normal for this category of meat product (Olivares et al., 2009), some differences

242   were observed between control treatment (Cc) and dry fermented sausages with encapsulated

243   *L. plantarum*. The Cc treatment showed higher pH values during storage, and values slightly

244   increased throughout the storage period ($P < 0.05$). According to Bozkurt and Erkmen (2002),

245   an increase in pH values of fermented sausages during storage is expected due to

246   decarboxylation and amino acids deamination. Treatments with encapsulated *L. plantarum*

247   showed different behaviors in pH values during chilled storage period. Cfc, Calg, and Cwow

248   treatments showed an increase ($P < 0.05$) in pH values followed by a decrease ($P < 0.05$) until

249   the end of storage. A decrease in pH values of fermented sausages close to the final of storage

250   period could be related to lipolysis and consequent fatty acids release. The Cfc and Calg

251   treatments had the lowest pH values during chilled storage. The lactic acid production by *L.*

252   *plantarum* when added as free cells (Cfc) have no physical barrier which is provided by

253   encapsulation techniques, and easily reach the meat matrix. According to Gouin (2004),

254   alginate beads are porous so the lactic acid are also released to meat matrix, assisting in meat

255   fermentation. In counterpart, Cc had the highest ($P < 0.05$) pH values (Table 1) during

256   completely chilled storage period, which is consequence of no *L. plantarum* addition. Some

257   studies have shown the entrapment of LAB in alginate may improve lactic acid production

258   (Idris & Suzana, 2006; Rao, Prakasham, Rao, & Yadav, 2008). According to Table 1, pH

259   values of Calg were similar to those of Cfc during the storage period (except day 35).

260   However, it is not clear if the encapsulation of *L. plantarum* in emulsion (Cwo) and

261   double emulsion (Cwow) reduce the lactic acid production or if the lactic acid produced is

262   placed inside the capsule, not being released into meat matrix. The pH values of dry

263   fermented sausages with encapsulated *L. plantarum* are in agreement with those reported by

264   Campagnol et al. (2012a), Campagnol et al. (2012b), Campagnol et al. (2011), Gelabert et al.

265   (2003), thus, those pH values can be considered for this kind of meat product.

266   Water activity values of dry fermented sausages with encapsulated *L. plantarum*

267   addition during chilled storage are shown on Table 1. It was possible to observe that Cfc

268   treatment showed lower (P < 0.05) a_w at the beginning of storage period (day 0). In addition,

269   in that treatment, even with variations (P < 0.05) in a_w values, no differences were found

270   between the beginning and the end of storage period. In the Cc, Calg, Cwo and Cwow

271   treatments, a decrease (P < 0.05) in a_w values during chilled storage period was observed. At

272   day 0 of storage, Cc treatment had higher (P < 0.05) a_w values (0.915). Nevertheless, at the

273   end of storage period (day 60) even with statistical differences amongst the treatments (Table

274   1), a_w values were quite similar and unable to promote technological and sensorial alterations.

275   CIE *L*a*b** values of dry fermented sausages with encapsulated *L. plantarum* are

276   shown on Table 2. Color is one of the main organoleptic characteristics used to establish the

277   quality and acceptability of meat products (Ansorena, Peña, Astiasarán, & Bello, 1997). All

278   treatments kept the typical color of this kind of product during whole storage period as well

279   evidenced in Figure 1. Values of *L** ranged from 32.26 to 35.66 (P < 0.05) at day 0. In Cc,

280   Cfc, Calg and Cwo treatments was possible to observe an increase (P < 0.05) in *L** values up

281   to 35th day of storage followed by a decrease (P < 0.05) at the 60th day, ranging from 30.54 to

282   33.68.

283   Red intensity (*a**) is the most sensitive parameter for measuring color, red color

284   characterization, and color stability (García-Esteban et al., 2003). The values for *a**

285   parameters of dry fermented sausages with encapsulated *L. plantarum* are shown on Table 2.

286   At the beginning of storage period, no differences were observed amongst all treatments.

287   Starting from day 35 of storage a decrease (P < 0.05) in *a** values in all treatments were

288   found. At the end of storage period, *a** values were lower than at day 0. This means that dry

289   fermented sausages lost the red intensity during storage. According to Pérez-Alvarez et al.

290   (1999), a decrease in *a** values is related to the partial denaturation of nitrosomyoglobin

291   pigment due to lactic acid production or even lipid oxidation in meat products (Fernández-

292   Lopez et al., 2003; Yu et al., 2002). Other studies already related a decrease in *a** parameters

293   during processing and storage period of fermented sausages (Kayaardi & Gök, 2003;

294   Campagnol et al., 2007; Cavalheiro et al., 2013).

295   According to Table 2, values of yellowness parameter ($b*$) of dry fermented sausages

296   also decreased ($P < 0.05$) during storage period. In the beginning of storage period, values of

297    $b*$ parameter ranged from 16.73 to 21.18. However, at the end of storage period (day 60),

298   values ranged from 13.49 to 15.99, being Cc and Cwow treatments with lower $b*$ values than

299   the others. A decrease in yellowness are probably due to the oxygen consumption by

300   microorganisms during their exponential growth and due to metabolites produced that induce

301   the oxidation of meat and fat (Demeyer et al., 1986; Sarasibar et al., 1989).

302   Even with the statistical differences observed amongst the treatments in the $L*a*b*$

303   parameters (Table 2), the effects of addition of encapsulated *L. plantarum* in dry fermented

304   sausages were not evident, which may have been due to the inhomogeneous color typical of

305   fermented sausages (Campagnol et al., 2012a) as can be observed on Figure 1.

306

307   *3.2. Microbiological characteristics*

308   Table 3 shows the microbiological characteristics of dry fermented sausages with

309   encapsulated *L. plantarum* during chilled storage. No differences amongst treatments were

310   observed at day 0 for total viable counts (TVC). Except for the Cfc and Cwo treatments, the

311   others remained stable for TVC counts during whole chilled storage period. In relation to

312   LAB counts, values were higher ($P < 0.05$) in Calg treatment at day 0. During the storage

313   period, in all treatments, LAB values decreased ($P < 0.05$) at day 20, but increased ($P < 0.05$)

314   at day 35. At the end of storage period (day 60), only for the Cwow treatment a decrease ($P <$

315   0.05) in LAB values were observed when comparing to day 35.

316   Nevertheless, the LAB group associated with food includes 11 genera:

317   *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc,*

318   *Oenococcus, Pediococcus, Streptococcus, Vagococcus* and *Weissela* (Vries, Vaughan,

319   Kleerebezem, & Vos, 2006). For this reason, it is important to differentiate the probiotic

320   microorganism that was added from the others that might be present in sausage matrix and

321   that are detected by MRS agar. Molecular methods already have been used to confirm the

322   presence of specific lactobacilli in meat products (Ruiz-Moyano *et al.*, 2011a; Ruiz-Moyano

323   *et al.*, 2011b; Rubio *et al.*, 2014a; Rubio *et al.*, 2014b), but their use is limited especially due

324   to abundance of false positives and false negatives results (Lauri & Mariani, 2009).

325   In addition, the selective medium for *L. plantarum* (LPSM) developed by Bujalance *et*

326   *al.* (2006) is an effective form to differentiate this type of microorganism from other kind of

327   LAB normally found on fermented sausages microbiota and well detected by MRS agar. In

328   this context, LPSM was a good alternative for differentiation of *L. plantarum* from other

329   LAB. According to Table 3, Calg showed higher (P < 0.05) *L. plantarum* values during the

330   whole period of storage. Studies reported that for a food to be considered as a probiotic, the

331   minimum concentration of probiotic microorganism should be between $10^6 - 10^7$ CFU/g at the

332   moment of consumption (Nulkaekul *et al*., 2012). On this basis, Calg treatment could be

333   considered as a probiotic food throughout the whole storage period (Table 3). In addition, it is

334   possible to observe a decrease (P < 0.05) in *L. plantarum* counts in Cfc, Cwo and Cwow

335   treatments to levels below 10^6 CFU/g at day 35 followed by an increase (P < 0.05) at the end

336   of storage. Furthermore, even with no addition of *L. plantarum*, Cc treatment showed *L.*

337   *plantarum* counts between 3 and 4 log CFU/g due to the meat normal flora. However, as

338   expected, those values are lower (P < 0.05) than those observed in treatments where *L.*

339   *plantarum* was added as free cells or encapsulated.

340   In relation to *Enterobacteriaceae* counts (Table 3), at day 0 of storage Cfc and Calg

341   treatments already showed lower than 1 log CFU/g counts, while Cc, Cwo and Cwow

342   treatments showed about 3 log CFU/g counts. However, starting from day 20 up to the end of

343   storage period, all treatments showed counts lower than 1 log CFU/g. Those results indicate

344   the hygienic quality of obtained meat products. Even more, it shows the efficiency of using

345   LAB against pathogenic bacteria mainly due to bacteriocin production. Studies have shown

346   the effectiveness of *L. curvatus* as free cells (Benkerroum *et al.*, 2005) and encapsulated *L.*

347   *plantarum* (Barbosa, Todorov, Jurkiewicz, & Franco, 2015) against *Listeria monocytogenes*

348   in fermented meat sausages.

349

350   *3.3. Lipid oxidation*

351   The TBARS test is the most usual method to follow the evolution of lipid oxidation in

352   meat and meat products (Raharjo & Sofos, 1993). Peroxidation has been proven to produce

353   some harmful substances (Fujioka & Shibamoto, 2004), and the reduction of malonaldehyde

354   (MDA) content of fermented meat products is very important. The changes in TBARS values

355   of dry fermented sausages with encapsulated *L. plantarum* were followed during the 60 days

356   of the storage (Table 4). No differences in TBARS values were found amongst treatments at

357   days 0 and 20 of storage. At 35th day of storage, Cwo treatment had higher ($P < 0.05$) TBARS

358   values than Cc, Cfc and Calg treatment but with no differences with Cwow treatment. This

359   indicates a tendency to greater lipid oxidation in treatments containing emulsions, which has

360   been confirmed at 60th day of storage, where Cwow treatment showed higher ($P < 0.05$)

361   values followed by Cwo treatment.

362   Higher TBARS values of dry fermented sausages with encapsulated *L. plantarum* in

363   emulsion systems are probably due to two reasons: those products had higher levels of fat

364 (data not shown) and the olive oil added in emulsions probably are more susceptible to

365 oxidation due to higher unsaturated fatty acid content. Studies have shown TBARS values of

366 frankfurters containing perilla oil in double emulsion were higher than treatments made with

367 pork backfat (Freire et al., 2016). Delgado-Pando et al. (2011) reported that lipid oxidation

368 can vary according to the type of protein system (caseinate, whey protein isolate, or both

369 combined with transglutaminase) used as a hydrophilic emulsifier to stabilize O/W emulsions.

370   Dry fermented sausages are products that generally take time to ferment and ripe, and

371 in addition, have long storage periods, so is expected to obtain high levels of TBARS values.

372 In addition, the ability to produce hydrogen peroxide is widespread among LAB group (Song

373 et al., 1999), which can be beneficial in terms of food preservation but can increase lipid

374 oxidation (Harel and Kanner, 1985). To control lipid oxidation, natural or synthetic

375 antioxidants are commonly used. In addition, the use of starter cultures also has action to

376 control lipid oxidation in meat products. Nevertheless, in our study no antioxidants were adde

377 to dry fermente sausages. Studies already have shown the *in vitro* and *in vivo* antioxidant

378 activity of some strains of *L. plantarum* (Li et al., 2012) and *L. sake* (Amanatidou, Smid,

379 Bennik & Gorris, 2001; Katikou, Ambrosiadis, Georgantelis, Koidis, & Georgakis, 2005). In

380 the study made by Gao, Li & Liu (2014), fermented sausages with addition of *L. sake* C2

381 showed lower TBARS values when compared to control treatment.

382   Ahmad & Srivastave (2007) stated that sensory alterations related to lipid oxidation

383 are not possible in treatnents with TBARS values lower than 1 mg MDA/kg. Using this value

384 as a threshold of sensory changes detection, only Cwo and Cwow treatments had values

385 higher than 1 mg MDA/kg at day 60 of storage (Table 4).

386

387

388   *3.4.Sensory evaluation*

389   The average scores of appearance, flavor, hardness, texture color, odor and overall

390   acceptability of dry fermented sausages with free and encapsulated *L. plantarum* addition are

391   shown on Table 5. No differences were found amongst the treatments for appearance and odor

392   attributes, which corroborates with Figure 1 showing that all treatments were similar to each

393   other during the whole storage period. Regarding flavor, texture attributes and overall

394   acceptability, only Cwo treatment had lower (P < 0.05) scores compared to Cc treatment. In

395   relation to color, all treatments showed high scores (between 6.74 and 8.11). Cc treatment

396   showed higher (P < 0.05) scores for color and overall acceptability and the results are in

397   agreement with visual aspect of dry fermented sausages (Figure 1) and with *L* a* b** values

398   (Table 2). Finally, for overall acceptability, Cwo treatment showed lowest values but

399   differences between treatments with addition of free or encapsulated *L. plantarum.* These

400   results suggest the potential use of encapsulated *L. plantarum* in fermented sausages. Similar

401   results were reported by Bagdatli & Kudakci (2015) who found the mean scores of surface

402   appeareance, surface color, taste, aroma, texture and overall acceptability of probiotic

403   fermented sausages were above 7 (9-scale) at the end of the fermentation (14 days). Even

404   more, Muthukumurasamy & Holley (2006) related that the addition of encapsulated probiotic

405   in fermented sausages are not able to produce any sensory alterations compared to control

406   treatments.

407

408   **4. Conclusions**

409   The results in this study indicated that dry fermented sausages with addition of free

410   and encapsulated *L. plantarum* in alginate beads showed lower pH and a$_w$ values. All the

411   treatments kept the typical color of fermented meat products, despite the fact that in all

412 treatments, *a** values decreased during the storage period. In relation to microbiological

413 parameters, total viable counts and lactic acid bacteria counts were considered normal for this

414 type of product during the whole period of storage. Even more, low counts of

415 *Enterobacteriaceae* indicate the hygienic quality of products. At the end of storage, excluding

416 Cc, all treatments showed *L. plantarum* counts higher than 6 log CFU/g, but, only treatment

417 Calg kept this level during the whole period of storage. *L. plantarum* encapsulated in

418 emulsion (W/O) and double emulsion (W/O/W) produced higher lipid oxidation in dry

419 fermented sausages, especially at the end of storage period. In genetal, treatments showed

420 good scores for all attributes examined in sensorial evaluation. However, Cwo treatment

421 showed low scores for flavor, texturer and overall acceptability. In this context, Calg

422 treatment seems to be more indicate for dry fermented sausages during chilled storage for 60

423 days.

424

425 **Aknowledgements**

431

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433

434

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647 Table 1 – Physico-chemical characteristics (pH and a_w) during chilled storage of dry
648 fermented sausages with free and encapsulated *L. plantarum* addition.

		Day of storage			
		0	20	35	60
pH	Cc	4.92 ± 0.04^{Ac}	5.00 ± 0.02^{Ab}	5.02 ± 0.03^{Ab}	5.20 ± 0.04^{Aa}
	Cfc	4.43 ± 0.01^{Cc}	4.47 ± 0.01^{Db}	4.57 ± 0.04^{Ca}	4.46 ± 0.00^{Dbc}
	Calg	4.41 ± 0.01^{Cb}	4.49 ± 0.01^{Da}	4.48 ± 0.01^{Da}	4.47 ± 0.01^{Da}
	Cwo	4.93 ± 0.02^{Aa}	4.85 ± 0.02^{Cb}	4.91 ± 0.03^{Ba}	4.83 ± 0.03^{Cb}
	Cwow	4.84 ± 0.01^{Bc}	4.97 ± 0.01^{Ba}	4.92 ± 0.01^{Bb}	4.91 ± 0.01^{Bb}
a_w	Cc	0.915 ± 0.004^{Aa}	0.882 ± 0.001^{Ab}	0.863 ± 0.004^{Ac}	0.853 ± 0.001^{Cd}
	Cfc	0.858 ± 0.005^{Db}	0.876 ± 0.002^{Ba}	0.861 ± 0.001^{Ab}	0.856 ± 0.001^{Ab}
	Calg	0.879 ± 0.001^{Ca}	0.872 ± 0.001^{Cb}	0.854 ± 0.000^{ABc}	0.847 ± 0.001^{Dd}
	Cwo	0.888 ± 0.001^{Ba}	0.875 ± 0.001^{BCb}	0.859 ± 0.005^{Ac}	0.853 ± 0.002^{ABc}
	Cwow	0.896 ± 0.003^{Ba}	0.859 ± 0.001^{Db}	0.847 ± 0.006^{Bc}	0.850 ± 0.001^{CDc}

649 Means ± standard deviation.

650 Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in
651 alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W
652 emulsion.

653 [A] Different upper case letters in the same column for same parameter indicate significant
654 differences (P < 0.05).

655 [a] Different lower case letters in the same row for same parameter indicate significant
656 differences (P < 0.05).

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666 Table 2 – Color parameters (lightness, $L*$; redness, $a*$; yellowness, $b*$) during chilled storage
667 of dry fermented sausage with free and encapsulated *L. plantarum* addition.

		Day of storage			
		0	20	35	60
$L*$	Cc	33.23 ± 1.41^{Cb}	32.75 ± 1.32^{Bb}	36.77 ± 1.61^{Ba}	30.54 ± 1.53^{Cc}
	Cfc	34.64 ± 1.99^{ABbc}	35.77 ± 1.85^{Aab}	37.34 ± 0.94^{Ba}	33.68 ± 1.34^{Ac}
	Calg	34.64 ± 1.22^{ABc}	37.72 ± 1.41^{Ab}	39.79 ± 1.70^{Aa}	33.42 ± 0.92^{Ac}
	Cwo	32.26 ± 1.18^{Cb}	37.71 ± 2.17^{Aa}	37.60 ± 2.31^{Aba}	32.28 ± 1.43^{ABb}
	Cwow	35.66 ± 1.97^{Aa}	33.34 ± 1.21^{Bb}	36.59 ± 1.26^{Ba}	31.11 ± 0.97^{BCc}
$a*$	Cc	23.81 ± 2.21^{Aa}	22.43 ± 2.11^{Ca}	19.97 ± 1.79^{Bb}	19.59 ± 1.49^{Bb}
	Cfc	24.71 ± 1.75^{Ab}	26.57 ± 1.02^{ABa}	23.28 ± 0.73^{Ab}	23.75 ± 0.99^{Ab}
	Calg	26.21 ± 1.11^{Aa}	26.75 ± 1.26^{ABa}	21.27 ± 1.29^{Bc}	23.80 ± 1.14^{Ab}
	Cwo	24.67 ± 1.52^{Ab}	28.12 ± 1.07^{Aa}	20.33 ± 1.32^{Bd}	22.60 ± 1.35^{Ac}
	Cwow	24.05 ± 2.08^{Aa}	25.59 ± 1.63^{Ba}	17.48 ± 0.71^{Cc}	20.12 ± 1.29^{Bb}
$b*$	Cc	16.73 ± 1.97^{Ca}	15.37 ± 1.86^{Cab}	10.47 ± 1.38^{Bc}	13.49 ± 1.87^{Bb}
	Cfc	19.03 ± 1.80^{ABCa}	19.45 ± 1.93^{Ba}	13.63 ± 1.38^{Ab}	15.58 ± 0.80^{Ab}
	Calg	21.18 ± 1.85^{Aa}	21.66 ± 1.55^{Ba}	12.80 ± 1.47^{Ac}	15.80 ± 1.10^{Ab}
	Cwo	18.35 ± 1.82^{BCb}	24.55 ± 1.75^{Aa}	10.47 ± 2.32^{Bc}	15.99 ± 1.69^{Ab}
	Cwow	19.93 ± 1.62^{ABa}	19.40 ± 1.52^{Ba}	16.87 ± 0.61^{Cb}	14.82 ± 1.42^{Bc}

668 Means ± standard deviation.

669 Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in
670 alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W
671 emulsion.

672 [A] Different upper case letters in the same column for same parameter indicate significant
673 differences ($P < 0.05$).

674 [a] Different lower case letters in the same row for same parameter indicate significant
675 differences ($P < 0.05$).

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682 Table 3 – Microbiological parameters during chilled storage of dry fermented sausages with
683 addition of free and encapsulated *L. plantarum* (in log CFU/g).

		Day of storage			
		0	20	35	60
TVC	Cc	8.51 ± 0.14^{Aa}	8.57 ± 0.07^{Ba}	8.55 ± 0.09^{Ba}	8.62 ± 0.02^{BCa}
	Cfc	8.16 ± 0.05^{Ac}	8.90 ± 0.06^{Aab}	8.96 ± 0.16^{Aba}	8.57 ± 0.12^{Cb}
	Calg	8.98 ± 0.76^{Aa}	9.08 ± 0.03^{Aa}	9.48 ± 0.46^{Aa}	8.91 ± 0.23^{Ba}
	Cwo	8.52 ± 0.08^{Ab}	8.33 ± 0.12^{Cb}	8.71 ± 0.43^{Bb}	10.05 ± 0.05^{Aa}
	Cwow	8.71 ± 0.04^{Aa}	8.48 ± 0.12^{Ca}	8.59 ± 0.08^{Ba}	8.62 ± 0.04^{BCa}
LAB	Cc	8.46 ± 0.16^{BCa}	7.30 ± 0.16^{Bc}	7.87 ± 0.20^{Bb}	8.06 ± 0.18^{Db}
	Cfc	8.81 ± 0.04^{Bb}	7.82 ± 0.01^{Ac}	8.97 ± 0.22^{Aab}	9.35 ± 0.04^{Ba}
	Calg	9.29 ± 0.17^{Aa}	8.12 ± 0.16^{Ab}	9.35 ± 0.32^{Aa}	8.86 ± 0.27^{Ca}
	Cwo	8.12 ± 0.32^{Cb}	6.87 ± 0.04^{Cc}	8.79 ± 0.08^{Ab}	9.85 ± 0.01^{Aa}
	Cwow	8.06 ± 0.16^{Cb}	6.93 ± 0.02^{Cc}	8.95 ± 0.08^{Aa}	8.02 ± 0.10^{Db}
L. plantarum	Cc	4.35 ± 0.31^{Cab}	4.14 ± 0.20^{Bab}	4.64 ± 0.04^{Da}	3.72 ± 0.30^{Db}
	Cfc	7.06 ± 0.08^{Ab}	6.87 ± 0.01^{Ab}	5.58 ± 0.20^{Bc}	7.77 ± 0.21^{ABa}
	Calg	7.28 ± 0.18^{Ab}	7.15 ± 0.16^{Ab}	6.69 ± 0.14^{Ac}	8.34 ± 0.24^{Aa}
	Cwo	6.28 ± 0.09^{Bc}	6.92 ± 0.02^{Ab}	5.58 ± 0.09^{Bd}	7.13 ± 0.04^{BCa}
	Cwow	5.95 ± 0.06^{Bb}	6.72 ± 0.08^{Aa}	5.11 ± 0.23^{Cc}	7.05 ± 0.04^{Ca}
Enterobacteriaceae	Cc	3.52 ± 0.09^{ABa}	$<1^{Ab}$	$<1^{Ab}$	$<1^{Ab}$
	Cfc	$<1^{Ca}$	$<1^{Aa}$	$<1^{Aa}$	$<1^{Aa}$
	Calg	$<1^{Ca}$	$<1^{Aa}$	$<1^{Aa}$	$<1^{Aa}$
	Cwo	3.44 ± 0.15^{Ba}	$<1^{Ab}$	$<1^{Ab}$	$<1^{Ab}$
	Cwow	3.69 ± 0.03^{Aa}	$<1^{Ab}$	$<1^{Ab}$	$<1^{Ab}$

684 Means ± standard deviation.

685 Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in
686 alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W
687 emulsion.

688 TVC = Total viable count, LAB = lactic acid bacteria.

689 [A] Different upper case letters in the same column for same parameter indicate significant
690 differences (P < 0.05).

691 [a] Different lower case letters in the same row for same parameter indicate significant
692 differences (P < 0.05).

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697 Table 4 – Lipid oxidation (TBARS) during chilled storage of dry fermented sausages with
698 free and encapsulated *L. plantarum*.

	Day of storage			
	0	20	35	60
Cc	0.236 ± 0.029^{Ac}	0.457 ± 0.069^{Ab}	0.529 ± 0.011^{Ab}	0.971 ± 0.063^{Ba}
Cfc	0.247 ± 0.048^{Ac}	0.537 ± 0.187^{Aab}	0.336 ± 0.093^{Abc}	0.626 ± 0.021^{Ca}
Calg	0.231 ± 0.029^{Ac}	0.360 ± 0.110^{Abc}	0.477 ± 0.127^{Aab}	0.602 ± 0.041^{Ca}
Cwo	0.303 ± 0.049^{Ac}	0.428 ± 0.067^{Ac}	0.808 ± 0.145^{Bb}	1.201 ± 0.184^{Ba}
Cwow	0.291 ± 0.033^{Ac}	0.484 ± 0.089^{Ab}	0.579 ± 0.065^{ABb}	1.949 ± 0.054^{Aa}

699 Means ± standard deviation. Values expressed as mg MDA/kg.

700 Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in
701 alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W
702 emulsion.

703 [A] Different upper case letters in the same column for same parameter indicate significant
704 differences (P < 0.05).

705 [a] Different lower case letters in the same row for same parameter indicate significant
706 differences (P < 0.05).

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717   Table 5 – Sensory evaluation of dry fermented sausages with addition of free and
718   encapsulated *L. plantarum*.

Parameter	Cc	Cfc	Calg	Cwo	Cwow
Appearance	7.21 ± 2.47^A	5.96 ± 1.97^A	6.34 ± 2.00^A	6.76 ± 1.90^A	5.94 ± 2.72^A
Flavor	7.33 ± 2.06^A	5.70 ± 2.49^{AB}	6.36 ± 1.73^{AB}	4.72 ± 2.57^B	6.25 ± 2.19^{AB}
Hardness	4.32 ± 2.40^{AB}	5.96 ± 2.09^A	5.48 ± 2.01^A	3.04 ± 1.50^B	4.14 ± 1.82^{AB}
Texture	7.35 ± 2.05^A	5.50 ± 1.78^{AB}	5.86 ± 1.87^{AB}	5.31 ± 2.40^B	6.42 ± 1.59^{AB}
Color	8.11 ± 1.06^A	6.74 ± 1.51^B	7.24 ± 1.29^{AB}	7.55 ± 1.40^{AB}	7.54 ± 1.38^{AB}
Odor	7.50 ± 1.93^A	6.68 ± 1.82^A	7.29 ± 1.64^A	6.49 ± 1.87^A	7.11 ± 1.94^A
Overall acceptability	7.75 ± 1.74^A	5.92 ± 1.92^{AB}	6.51 ± 1.84^{AB}	5.75 ± 2.30^B	7.03 ± 1.89^{AB}

719   Means ± standard deviation.

720   Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in
721   alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W
722   emulsion.

723   ^{A} Different upper case letters in the same column for same parameter indicate significant
724   differences ($P < 0.05$).

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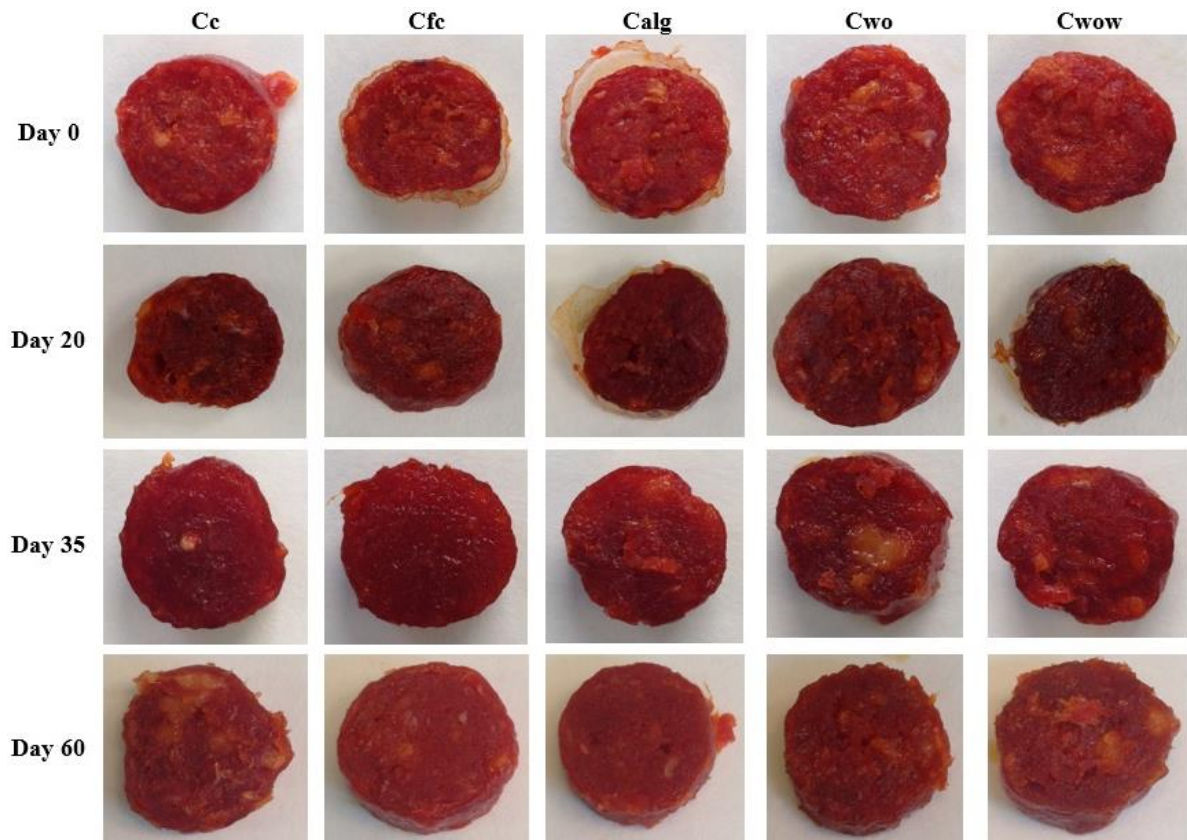
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Figure 1 – General aspect of dry fermented sausage with addition of free and encapsulated *L. plantarum* during chilled storage.

Cc = Without *L. plantarum* addition; Cfc = *L. plantarum* as free cells; Calg = *L. plantarum* in alginate beads; Cwo = *L. plantarum* in W/O emulsion; Cwow = *L. plantarum* in W/O/W emulsion.

3   DISCUSSÃO

Neste trabalho foram testados os microrganismos probióticos *Lactobacillus plantarum* e *Enterococcus faecium* encapsulados em alginato juntamente com inulina, leite em pó e trealose e submetidos à altas temperaturas, diferentes concentrações de cloreto de sódio (NaCl), nitrito de sódio (NaNO$_2$) e armazenamento por 70 dias sob refrigeração. Posteriormente, o *Lactobacillus plantarum* foi encapsulado em cápsulas de alginato, emulsão simples (água-em-óleo, A/O) e emulsão dupla (água-em-óleo-em-água, A/O/A) aplicados em um embutido fermentado espanhol (chorizo) e analisado durante seu processamento e armazenamento.

Em relação à encapsulação de *L. plantarum* e *E. faecium* em cápsulas de alginato contendo inulina, leite em pó e trealose foi possível obter cápsulas esbranquiçadas com aspecto arredondado medindo 3,79 e 3,44 mm, respectivamente. A técnica de encapsulação por extrusão pode promover cápsulas com tamanho variado, desde micrômetros até milímetros (BURGAIN et al., 2011), dependendo da distância entre o bico e a solução de CaCl$_2$ e o diâmetro do bico. Outro fator importante na encapsulação é a determinação da sua eficiência. Os valores de eficiência de encapsulação foram superiores a 80%, o que vai de acordo com outros estudos que relatam uma alta eficiência de encapsulação ao usar a técnica de extrusão (CORBO et al., 2011; CORBO et al., 2013; KRASAEKOOPT et al., 2003; KRASAEKOOPT et al., 2004; URBANSKA et al., 2007).

A encapsulação tem sido usada para aumentar a sobrevivência de microrganismos probióticos frente à tratamentos térmicos (ABBASZADEH et al., 2014; MANDAL et al., 2006; SHAHARUDDIN & MUHAMAD, 2015). No presente estudo, o tratamento térmico reduziu a viabilidade dos probióticos tanto na forma livre como encapsulados. O *L. plantarum* na forma livre se mostrou mais sensível ao ser exposto a 70 ºC por 5 minutos, apresentando uma redução de aproximadamente 4 log UFC/g neste período. No mesmo período, o *E. faecium* na forma livre reduziu sua viabilidade em aproximadamente 2 log UFC/g, mostrando-se mais resistente ao tratamento térmico (70 ºC) por curtos períodos de tempo. No entanto, ao expor ambos microrganismos na forma livre a 80 ºC, a perda na viabilidade (5 log UFC/g) foi semelhante aos 5 minutos e ambos não diferiram estatisticamente. No entanto, na forma livre, o *L. plantarum* se mostrou mais resistente a longas exposições à temperatura quando comparado com o *E. faecium*. Para que um alimento seja considerado probiótico, é necessário que este mantenha contagens superior a 6 log UFC/g (NULKAEKUL et al., 2012). Nesse

contexto, as cápsulas contendo os microrganismos probióticos se mantiveram em níveis superiores a 6 log UFC/g mesmo permanecendo por 30 minutos a 70 ℃. No entanto, na forma livre, o *E. faecium* apresentou contagens inferiores a 6 log UFC/g após 10 minutos a 70 ℃ e o *L. plantarum* após 20 minutos a 70 ℃.

A redução na viabilidade dos probióticos foi mais acentuada quando expostos a 80 ℃. Ambos microrganismos, na forma livre, apresentaram contagens inferiores a 6 log UFC/g quando expostos por apenas 5 minutos a 80 ℃. Já os microrganismos encapsulados também apresentaram contagens superiores a 6 log UFC/g apenas por 5 (*E. faecium*) e 10 (*L. plantarum*) minutos. A baixa viabilidade probiótica das cápsulas a 80 ℃ se deve ao fato destas entrarem em colapso a esta temperature, liberando as células no meio e a consequente morte devido à alta temperatura (XING et al., 2014). Vários fatores podem ser atribuídos no aumento da resistência de probióticos encapsulados, como a concentração dos polímeros usados como material de cápsula (MANDAL et al., 2006), o uso de materiais considerados termoprotetores, como o leite em pó e a trealose (GHANDI et al., 2012; SANTIVARANGKNA et al., 2008; SCHUTYSER et al., 2012) e uma termoresistência natural do microrganismo. Alguns estudos já sugerem que microrganismos isolados de produtos cárneos cozidos apresentam uma maior resistência à altas temperaturas, tanto na forma livre como encapsulados (PÉREZ-CHABELA et al., 2008; PÉREZ-CHABELA et al., 2013; RAMÍREZ-CHAVARIN et al., 2010;).

Baixas concentrações de NaCl foram suficientes para reduzir a viabilidade dos microrganismos probióticos testados na forma livre. Ainda, *E. faecium* na forma livre se mostrou mais sensível quando exposto à altas concentrações de NaCl (5,0%). A encapsulação do *L. plantarum* foi eficaz na sua proteção contra o NaCl. Na forma livre, houve uma perda de aproximadamente 2 log UFC/g após a exposição a 5,0 % de NaCl, porém, não houve redução significativa na viabilidade de *L. plantarum* encapsulado quando exposto ao NaCl. Em relação ao nitrito de sódio ($NaNO_2$), foi possível observar que concentrações de 1,0 % reduziram a viabilidade dos microrganismos probióticos testados tanto na forma livre como encapsulados em alginato, inulina, leite em pó e trealose.

Outro fator importante é a manutenção da viabilidade dos probióticos encapsulados durante o seu armazenamento. É importante manter a viabilidade pelo maior período de tempo possível, aumentando assim as possibilidades de aplicação pela indústria. O período de armazenamento de 70 dias a 4 ℃ reduziu a viabilidade de ambos microrganismos probióticos

encapsulados, porém, as contagens foram superiores a 7 log UFC/g. Diversos estudos mostram que probióticos encapsulados apresentam uma maior viabilidade ao armazenamento quando comparados com probióticos na forma livre (ANEKELLA & ORSAT, 2013; ETCHEPARE et al., 2016; SILVA et al., 2015; TRUELSTRUP-HANSEN et al., 2002).

O microrganismo *L. plantarum* foi utilizado na produção do embutido fermentado tradicional espanhol, o *chorizo*. Nesse estudo, o microrganismo foi adicionado tanto na forma livre como encapsulado através de distintas técnicas de encapsulação: a extrusão, emulsão simples e emulsão dupla e o produto foi avaliado durante o seu processamento (20 dias) e armazenamento sob refrigeração (60 dias).

Os valores de composição centesimal dos produtos foram analisados ao final do período de processamento. Os valores de umidade encontrados foram entre 25,34 e 29,83% e variaram de acordo com o tratamento, sendo que os valores mais altos de umidade foram encontrados nos tratamentos Cc e Cfc e os menores no tratamento Cwo. Os valores de proteína não variaram entre os tratamentos analisados. O teor de gordura foi maior nos tratamentos Cwo e Cwow, provavelmente devido à adição de azeite de oliva utilizado no preparo das emulsões.

Todos os tratamentos perderam peso durante o processamento como resultado do processo de secagem e devido ao decréscimo nos valores de pH durante a fermentação que faz com que os valores se aproximem do ponto isoelétrico das proteínas que tem como consequência uma maior perda de água pelo produto (MAURIELLO et al., 2004). Ao final do processamento os valores de perda de peso ficaram entre 30 e 40%, que é considerado normal para este tipo de produto (RUST, 1994).

O tempo de processamento afetou o pH dos embutidos fermentados. Os valores de pH no dia 0 foram entre 5,80 e 5,91, e diminuíram gradativamente em todos os tratamentos, incluindo o Cc, onde não foi inoculado *L. plantarum*. Este fato deve-se principalmente a presença de outros tipos de bactérias ácido lácticas que fazem parte da flora da carne e que também são capazes de fermentá-la. A rápida queda no pH em produtos cárneos fermentados é importante porque contribui com a segurança microbiológica e melhora o sabor, aroma, firmeza e fatiabilidade (CASTANO et al., 2002; GARRIGA et al., 1996; HUGHES et al., 2002). Durante o período de armazenamento, foi possível obserar um aumento nos valores de pH nos produtos devido às reações de descarboxilação e desaminação dos aminoácidos (BOZKURT & ERKMEN, 2002). Os tratamentos Cfc e Calg apresentaram os menores

valores de pH e isso deve-se ao fato de quando o *L. plantarum* é adicionado aos produtos na forma livre, o ácido lático produzido facilmente atinge a matriz cárnea. Ainda, de acordo com GOUIN (2004), cápsulas de alginato são porosas, o que também faz com que o ácido lático produzido seja facilmente liberado na matriz cárnea. No entanto, não está claro se a encapsulação em emulsão (Cwo e Cwow) diminui a produção de ácido lático pelas BAL ou se o ácido lático produzido permanece no interior do cápsula. Ao final do processamento e armazenamento, todos os tratamentos apresentaram valores de pH inferiores a 5.0, o que é consistente com outros resultados encontrados por Campagnol et al. (2011), Campagnol et al. (2012), Cavalheiro et al. (2010), Erkillä et al. (2001), Holko et al. (2013) e Rubio et al. (2013) em diferentes productos cárneos fermentados com ou sem adição de probiótico. Ainda, Muthukumarasamy & Holley (2006) relataram valores de pH similares em embutidos fermentados contendo *L. reuteri* encapsulados pelos métodos de extrusão e emulsão.

Os valores de atividade de água (Aa) reduziram em todos os tratamentos de acordo com o tempo de processamento. A redução nos valores de Aa é consequência da perda de água e redução no pH dos produtos fermentados. Os menores valores de Aa foram encontrados nos tratamentos Cfc e Calg, que também foram os tratamentos com menores valores de pH durante o período de armazenamento. Os valores de Aa durante o armazenamento variaram entre os tratamentos, porém, todos os valores encontrados são considerados normais para este tipo de produto e não são capazes de promover alterações sensoriais ou tecnológicas.

Todos os tratamentos desenvolveram a cor vermelha típica de produtos cárneos fermentados durante o processamento. Os valores de luminosidade ($L*$) diminuíram durante o processamento devido à concentração de sólidos durante a secagem dos produtos (PÉREZ-ALVAREZ et al., 1999). No final do processamento, os menores valores de $L*$ foram encontrados no tratamento Cwo, enquanto os maiores no tratamento Cwow devido, provavelmente, as próprias diferenças nas colorações das emulsões. Durante o armazenamento foi possível observar um aumento nos valores de $L*$ nos tratamentos Cc, Cfc, Calg e Cwo até o 35º dia, seguido de uma redução até o final do armazenamento (dia 60).

Em relação ao teor de vermelho ($a*$), foram observados valores que não diferiram entre os dias 2 e 7 de processamento para os tratamentos Cc, Cfc, Calg e Cwow. Valores altos de $a*$ são relacionados a formação de nitrosomioglobina e redução da umidade (ALÉSON-CARBONELL et al., 2003). Foi possível observar uma redução nos valores de vermelho em

todos os tratamentos durante o processamento e o armazenamento. Isso ocorre devido a oxidação de lipídios e reações dos nitrosopigmentos (PÉREZ-ALVAREZ & FERNÁNDEZ-LÓPEZ, 2006) e não está relacionada com a adição de *L. plantarum* seja como células livres como na forma encapsulado. Sayas-Barberá et al. (2012) observou a redução nos valores de *a** durante o processamento de *"Longaniza de Pascua"* com adição de *L. casei* CECT 475. Os valores do teor de amarelo (*b**) diminuiram durante o processamento e armazenamento e variaram de entre 25,12 – 27,86 no início do processamento para 17,26 – 21,18 ao final do processamento e para 13,49 – 15,99 ao final do período de armazenamento. A redução nos valores de amarelo ocorrem devido ao consumo do oxigênio por microrganismos que realizam a fermentação (PÉREZ-ALVAREZ et al., 1999; RUIZ et al., 2014).

Em relação a contagem de microrganismos aeróbios mesófilos, foi possível observar que no início do processamento, o tratamento Cfc apresentou maiores contagens em relação aos demais tratamentos. Isto, se deve provavelmente à adição direta de *L. plantarum* como células livres. No entanto, o mesmo não foi observado nas contagens de BAL e de *L. plantarum*. Foi possível observar um aumento nas contagens de BAL no início do processamento dos embutidos fermentados. Conforme Raccach (1992), este rápido crescimento de BAL é importante para controlar o possível crescimento de bactérias patogênicas indesejadas. Mesmo sem adição direta de *L. plantarum* no tratamento Cc, as contagens de BAL foram similares aos demais tratamentos devido a microbiota presente na carne que é composta de outros tipos de BAL que também são capazes de fermentá-la. É esperado para produtos cárneos fermentados que as contagens de BAL sejam superiores a 8 log UFC/g (BENITO et al., 2007; DALLA SANTA et al., 2014; MACEDO et al., 2008; RUBIO et al., 2014a; ZHAO et al., 2011). Ao final do processamento, o tratamento Calg apresentou valores de BAL superiores aos outros tratamentos. Porém, ao final do armazenamento, os maiores valores de BAL foram encontrados no tratamento Cwo.

No entanto, o grupo de BAL associado aos alimentos é composto por 11 gêneros: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Vagococcus* e *Weissela* (VRIES et al., 2006). Por isso é importante realizar a correta diferenciação entre o microrganismos adicionado ao produto com outros que podem estar presentes naturalmente na matriz cárnea. Métodos moleculares já tem sido utilizados para detectar a presença de lactobacilos em produtos cárneos (RUBIO et al., 2014a; RUBIO et al., 2014b; RUIZ-MOYANO et al., 2011a, RUIZ-MOYANO et al., 2011b) porém seu uso ainda é limitado.

O meio de cultura seletivo para *L. plantarum* (LPSM) desenvolvido por Bujalance et al. (2006) mostrou-se eficaz na correta diferenciação do *L. plantarum* de outras BAL presentes nos embutidos fermentados. Foi possível observar que o *L. plantarum* quando adicionado tanto na forma livre como encapsulado nas cápsulas de alginato sobreviveram bem durante o processamento dos embutidos fermentados. No entanto, as contagens de *L. plantarum* nos tratamentos Cwo e Cwow foram inferiores a 2 log UFC/g no início do processamento. Os valores de *L. plantarum* oscilaram durante o processamento e armazenamento, e após 60 dias de armazenamento todos os tratamentos, com exceção do Cc, apresentaram contagens de *L. plantarum* superiores a 7 log UFC/g. Estes resultados, reiteram a importância da enumeração seletiva dos probióticos no alimento, uma vez que no tratamento Cc as contagens de BAL foram superiores a 6 log UFC/g porém a contagem de *L. plantarum* foi inferior ao limite necessário para um alimento ser considerado probiótico. Em relação às contagens de *Enterobacteriaceae*, os valores encontrados ao final do processamento e do armazenamento são considerados adequados para este tipo de produto cárneo.

A oxidação lipídica dos embutidos fermentados foi analisada apenas durante o período de armazenamento dos produtos. Foi possível constatar que a oxidação lipídica aumentou durante o período de armazenamento em todos os tratamentos. A partir do 35º dia de armazenamento, altos teores de oxidação lipídica foram encontrados nos tratamentos Cwo e Cwow, indicando que a adição de emulsão nos produtos aumenta a oxidação lipídica. Estes valores também foram observados ao final do armazenamento e são devido principalmente a dois fatores: a maior quantidade de gordura encontrada nestes produtos e a presença do azeite de oliva que é mais susceptível à oxidação lipídica. Produtos cárneos com valores superiores a 1mg de malonaldeído por kg de amostra sugerem o início da percepção pelos consumidores das alterações sensoriais provocadas pela oxidação lipídica (AHMAD & SRIVASTAVE, 2007). Nesse contexto, os tratamentos Cwo e Cwow apresentaram valores superiores a este limiar aos 60 dias de armazenamento.

Em relação à avaliação sensorial, não foram encontradas diferenças entre os tratamentos para os atributos aparência e odor. Para os quesitos sabor, dureza, textura e aceitação geral, apenas o tratamento Cwo apresentou valores inferiores ao tratamento Cc. Para o atributo cor, todos os tratamentos apresentaram altas notas (entre 6,74 e 8,11), o que reitera a questão que todos os tratamentos apresentaram a cor tradicional de embutidos fermentados. Resultados similares já foram descritos por Bagdatli & Kudakci (2015), que encontraram médias de aparência, cor, sabor, aroma, textura e aceitação geral maiores que 7 (em uma

escala de 9 pontos) em embutidos fermentados probióticos ao final de 14 dias de fermentação. Ainda, Muthukumurasamy & Holley (2006) relata que a adição de probióticos encapsulados tanto pelas técnicas de extrusão como emulsão não provocam alterações sensoriais em embutidos fermentados.

4   CONCLUSÕES

O presente estudo mostrou que a encapsulação de *L. plantarum* e *E. faecium* através da técnica de extrusão produziu cápsulas esféricas, de coloração branca, com tamanho de aproximadamente 3 mm. A eficiência de encapsulação foi superior a 80%. Ainda, a encapsulação dos probióticos se mostrou eficaz contra condições adversas de processamento.

A encapsulação foi eficaz para proteger e manter as contagens de *L. plantarum* e *E. faecium* superiores a 6 log UFC/g após 30 e 20 minutos a 70 ºC, respectivamente, enquanto que a 80 ºC, a encapsulação manteve as contagens superiores a 6 log UFC/g após 10 e 5 minutos, respectivamente. Ainda, a encapsulação protegeu os probióticos à altos níveis de cloreto de sódio, nitrito de sódio e pelo período de armazenamento de 70 dias a 4 ºC.

A encapsulação de *L. plantarum* através de técnicas como extrusão, emulsão simples e emulsão dupla se mostrou uma alternativa para a incorporação de probióticos à um produto cárneo fermentado. Apesar das diferenças entre os tratamentos, as características físico-químicas (perda de peso, pH e Aa) e cor dos embutidos foram consideradas normais para este tipo de produto. Na avaliação sensorial, todos os produtos apresentaram boas notas, com exceção para o tratamento Cwo que apresentou notas inferiores ao controle nos quesitos sabor, dureza, textura e aceitação geral.

Em relação às características microbiológicas, os valores de microrganismos aeróbios mesófilos e bactérias ácido láticas foram semelhantes nos tratamentos sem e com a adição do probiótico. No entanto, a contagem seletiva de *L. plantarum* (LPSM) mostrou a necessidade de diferenciação entre as bactérias acido láticas presentes no produto das bactérias adicionadas intencionalmente. Nesse contexto, as contagens de *L. plantarum* nos tratamentos com adição de *L. plantarum* livre ou encapsulado foram aproximadamente 4 log UFC/g superiores ao tratamento controle. Ao final do período de armazenamento (60 dias), todos os tratamentos com adição de *L. plantarum* apresentaram contagens seletivas do probiótico superiores a 6 log UFC/g. Porém, apenas o tratamento Calg apresentou estes níveis durante todo o processamento e armazenamento. A adição de *L. plantarum* encapsulado através das técnicas de emulsão simples e emulsão dupla fez com que os produtos cárneos apresentassem maiores níveis de oxidação lipídica durante o armazenamento. Nesse contexto, a encapsulação de *L. plantarum* em cápsulas de alginato apresenta melhores resultados quando adicionados em embutido cárneo fermentado.

Os resultados encontrados neste trabalho são promissores do ponto de vista de encapsulação dos probióticos e sua adição em produtos cárneos. Novos estudos serão realizados do ponto de vista de avaliar o uso de diversos óleos no preparo de emulsões para encapsular diversos microrganismos probióticos e aplicá-los em produtos cárneos.

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ANEXOS

ANEXO A - Carta de avaliação dos orientadores do doutorado sanduíche no exterior.

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Madrid, 1 de Diciembre de 2015

A quien corresponda:

Por el presente escrito se certifica que **Don Carlos Pasqualin Cavalheiro** de la Universidad Federal de Santa María (Brasil) ha realizado una estancia de 12 meses, en el Grupo de Carne y Productos Cárnicos del Departamento de Productos del Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC) en Madrid, España, bajo la tutela del **Dr. Francisco Jiménez-Colmenero** y de la **Dra. Claudia Ruiz-Capillas Pérez**. Durante su estancia, ha participado en distintos estudios en relación con la aplicación de microorganismos probióticos encapsulados en productos cárnicos. Los estudios llevados a cabo, que formaran parte de su tesis doctoral, y se enmarcan dentro de las actividades de los distintos proyectos de investigación del grupo.

Estos trabajos han dado lugar a la publicación de 2 artículos científicos

1.- Cavalheiro, C. P., Ruiz-Capillas, C., Herrero, A. M., Jiménez-Colmenero, F., Menezes, C. R., Fries, L. L. M. (2015). Application of probiotic delivery systems in meat produts. Trends in Food Science and Technology, 46 (1), 120-131.

2.- Cavalheiro, C. P., Menezes, C. R., Fries, L. L.M., Herrero, A. M., Jiménez-Colmenero, F., Ruiz-Capillas, C. Alginate beads to improve viability of *Lactobacillus plantarum* to heat stress. Journal of Food Processing & Technology, 6, 126.

Este último trabajo, presentado en las Jornadas de "Food Quality and Safety Control" de la Universidad Complutense de Madrid, ha sido considerado como 2º Mejor Abstract del evento. Jornadas pertenecientes al Congreso Internacional EuroFood 2015.

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Adicionalmente, parte del esfuerzo experimental realizado durante este período, está siendo objeto de análisis y preparación para su publicación.

Durante su estancia, **Don Carlos Pasqualin Cavalheiro** ha realizado satisfactoriamente las distintas actividades y tareas planificadas para llevar a cabo los trabajos de investigación propuestos, poniendo de manifiesto que posee gran capacidad de trabajo, interés y nivel de integración adecuados.

Y para que así conste firmo el presente en Madrid, a 1 de Diciembre de 2015

Dr. Francisco Jiménez-Colmenero

(Profesor de Investigación del CSIC)

Dra. Claudia Ruiz-Capillas

(Investigador Científico del CSIC)

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