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ESTUDO DA ESTABILIDADE EM LONGO PRAZO E APLICAÇÃO DE NANOLIPOSSOMAS CONTENDO NISINA E EXTRATO DE ALHO.

Porto Alegre, 2020

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ESTUDO DA ESTABILIDADE EM LONGO PRAZO E APLICAÇÃO DE LIPOSSOMAS CONTENDO NISINA E EXTRATO DE ALHO

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RESUMO

A necessidade por antimicrobianos naturais cresce continuamente à medida que as pessoas procuram alimentos mais saudáveis, criando um desafio para a ciência de alimentos devido a que as poucas alternativas existentes no mercado para empresas de alimentos, têm alto custo e baixa efetividade. Neste contexto, a nano-encapsulação de antimicrobianos naturais em lipossomas, surge como alternativa tecnológica para desenvolvimento de novos antimicrobianos para alimentos. Portanto, neste trabalho foram explorados o desenvolvimento, propriedades e aplicações de antimicrobianos naturais nanoencapsulados. Este trabalho esta dividido em quatro partes. Na primeira parte do trabalho, foram estudados diferentes materiais estabilizantes da membrana lipídica, avaliando sua influencia nas propriedades oxidativas, características físico-químicas e térmicas de lipossomas contendo a mistura de nisina-extrato de alho. Além disso, foi analisada a estabilidade das preparações lipossomais em estado líquido e liofilizados na presença e na ausência de lioprotector, durante cinco meses em refrigeração; foi concluído que o acido oleico (AO) é um estabilizante promissor para os lipossomas, já que oferece maiores propriedades antioxidantes e de retenção da atividade antimicrobiana. A segunda parte descreve a aplicação AO-lipossomas contendo extrato de alho em pães de trigo. Estes lipossomas foram caracterizados em quanto a seu tamanho, carga, polidispersão, propriedades térmicas e atividade antifúngica *in vitro*, posteriormente foram adicionados a preparações de pão de trigo antes da etapa de forneado para avaliar mudanças da vida de prateleira. Como resultado foi observado que os lipossomas desenvolvidos apresentaram estabilidade térmica melhorada e quando empregados na formulação de pães de trigo, conseguiram retardar o aparecimento de bolores por até cinco dias. A terceira parte descreve o estudo da interação de lipossomas de diferentes composições e características físico-químicas com proteína miofibrilar de peixe (Surimi). Neste estudo foram avaliadas mudanças estruturais da proteína, mediante analise de FTIR, fluorescência, grupos sulfidrila livres e microscopia eletrônica de transmissão. Como resultado, foi observado que lipossomas com grupos carregados na superfície produzem um alto grau de desnaturação da proteína, e lipossomas de composição de 100% fosfatidilcolina produziram desnaturação parcial e um possível efeito de fibrilação. Além disso, foram observadas diferentes dinâmicas de formação de corona proteica, nos lipossomas, dependendo da sua composição. Na quarta e última parte do trabalho se realizou um estudo comparativo da resposta metabólica de células de *Listeria monocytogenes* ATCC 7644, submetidas a concentrações subletais de nisina livre

e encapsulada em lipossomas. Através da extração e identificação de proteínas, foi possível identificar que a nisina livre e encapsulada induze um aumento na atividade metabólica, transcrição de proteínas de transporte e proteínas relacionadas à resposta ao estresse. Não entanto, somente a nisina livre induziu a transcrição das proteínas secD, lmo1539, YfhO e lmo0955, relacionadas com resposta ao estresse e mecanismos de resistência microbiana de *L. monocytogenes*; indicando que a encapsulação em lipossomas modifica o "reconhecimento" inicial da bacteriocina, por parte da bactéria, reduzido assim, fatores de estresse e resistência microbiana.

Palavras chave: Lipossomas, nisina, alho, Antimicrobianos para alimentos, *Listeria monocytogenes.*

ABSTRACT

The need for natural antimicrobials continually grows as people seek healthier foods, creating a challenge for food science because the few alternatives on the market for food companies are costly and low effective. In this context, the nano-encapsulation of natural antimicrobials in liposomes, appears as a technological alternative for the development of new antimicrobials for food. Therefore, in this work were explored the development, properties and applications of nano-encapsulated natural antimicrobials. This work is divided in four parts. In the first part of the work, different stabilizing materials of the lipid membrane were studied, evaluating its influence on the oxidative properties, physical-chemical and thermal characteristics of liposomes containing the mixture of nisin-garlic extract. In addition, the stability of liposomal preparations in liquid and lyophilized form was analyzed in the presence and absence of lyoprotectant, for five months in refrigeration; it was concluded that oleic acid (OA) is a promising stabilizer for liposomes, as it offers greater antioxidant properties and retention of antimicrobial activity. The second part describes the application OA-liposomes containing garlic extract in wheat breads. These liposomes were characterized in terms of their size, charge, polydispersity, thermal properties, and antifungal activity *in vitro*, were later added to wheat bread preparations before the baking step to evaluate changes in shelf life. As a result, it was observed that the developed liposomes showed improved thermal stability and when used in the formulation of wheat breads, they managed to delay the appearance of molds for up to five days. The third part describes the study of the interaction of liposomes of different compositions and physicochemical characteristics with fish myofibrillar protein (Surimi). In this study, structural changes of the protein were evaluated, through analysis of FTIR, fluorescence, free sulfhydryl groups and transmission electron microscopy. As a result, it was observed that liposomes with charger groups on the surface produce a high degree of protein denaturation, and liposomes of 100% phosphatidylcholine composition produced partial denaturation and a possible fibrillation effect. In addition, different dynamics of protein corona formation were observed in liposomes, depending on their composition. In the fourth and last part of the work, was carried out a comparative study of the metabolic response of Listeria monocytogenes cells ATCC 7644, submitted to sublethal concentrations of free and encapsulated nisin in liposomes. Through protein extraction and identification, it was

possible to identify that free and encapsulated nisin induces an increase in metabolic activity, transcription of transport proteins and proteins related to the stress response. However, only free nisin induced the transcription of secD, lmo1539, YfhO and lmo0955 proteins, related to the stress response and microbial resistance mechanisms of *L. monocytogenes*; indicating that encapsulation in liposomes modifies the initial bacteriocin "recognition" by the bacteria, thus reducing stress factors and microbial resistance.

Keywords: Liposomes, nisin, garlic, Antimicrobials for food, *Listeria monocytogenes*

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SUMÁRIO

1. Introdução

O uso de bioconservantes em alimentos vem tornando-se um campo de pesquisa cada vez mais importante. Esta tendência é parcialmente impulsionada pelo surgimento de um número crescente de relatos relacionando produtos químicos em alimentos ao desenvolvimento de doenças crônicas (SEOW et al., 2014). Na atualidade, compostos naturais, como peptídeos e extratos de plantas, apresentam grande potencial de uso em substituição aos conservantes químicos, tendo na nanotecnologia uma ferramenta de desenvolvimento tecnológico para sua aplicação em alimentos, a fim de superar as desvantagens da sua aplicação direta.

A nanotecnologia pode ser utilizada para melhorar a estabilidade antimicrobiana em alimentos, geralmente mostrando vantagens em comparação com os compostos bioativos livres. Nanoestruturas utilizadas como veículos para proteger compostos de interesse, como antimicrobianos, têm sido amplamente estudadas na última década, já que com a encapsulação podem sobreviver à exposição de diferentes estresses ambientais e ajudar no controle de bactérias patogênicas, melhorando assim a sua estabilidade e eficácia (BRANDELLI, 2012).

Dentre as nanopartículas mais empregadas, os lipossomas têm recebido atenção especial. A crescente aplicação destes em alimentos se deve às vantagens que os lipossomas podem fornecer ao serem usados como sistemas encapsulantes de substâncias bioativas, podendo ser utilizados para liberação controlada de componentes funcionais, tais como: proteínas, enzimas, vitaminas, entre outros componentes que podem ser utilizados com o intuito de alterar o sabor ou o aroma dos alimentos (MCCLEMENTS, 2015).

Lipossomas são vesículas esféricas, formados por membranas anfipáticas de bicamadas fosfolipídicas com núcleo aquoso. Têm sido amplamente investigados nos últimos anos, como carreadores coloidais, já que possuem múltiplas vantagens, como a biodegradabilidade, biocompatibilidade e capacidade de liberação controlada de compostos (NEETHIRAJAN & JAYS, 2011). Os lipossomas podem ser utilizados numa vasta gama de aplicações, devido às suas características físicas e químicas, e sua capacidade de incorporar compostos lipofílicos, anfifílicos e/ou hidrofílicos (MCCLEMENTS, 2015). Apresentam ainda diferentes desvantagens como baixa estabilidade e tendência à aglomeração, que podem ser resolvidos facilmente com a liofilização e a adição de estabilizantes (ADITYA et al., 2015).

O alho (*Allium sativum* L.) é uma planta amplamente distribuída em todas as partes do mundo, utilizada não somente como condimento, mas também é considerada uma fonte rica de outros fitonutrientes não voláteis, com importantes propriedades medicinais e terapêuticas. Tais características são atribuídas ao seu conteúdo de flavonoides, saponinas e sapogeninas, compostos fenólicos, óxidos de azoto, amidas e proteínas (LANZOTTI et al., 2014). Com o uso popular do alho, reconhecido como fito terapêutico pela Agência Nacional de Vigilância Sanitária (ANVISA) na RDC n° 26 de 13 de maio 2014 (BRASIL, 2014), e a grande procura pela descoberta de novos produtos naturais com atividade antimicrobiana, surge necessidade de realização de estudos que forneçam parâmetros mais precisos quanto ao seu real potencial antimicrobiano. Neste contexto a alicina, principal componente responsável pela atividade antimicrobiana, é um potencial conservante para ser utilizado na indústria de alimentos. No entanto, a alicina é volátil, e sua estabilidade é baixa. Além disso, pode facilmente causar irritação da mucosa gástrica humana e é susceptível à oxidação por luz e calor (BOSE et a., 2014).

A nisina é uma bacteriocina de amplo espectro, com atividade contra bactérias Gram-positivas e alguns microrganismos associados ao deterioro de alimentos (GHARSALLAOUI et al., 2016). Seu uso como um bioconservante de alimentos é limitado pela falta de efeito contra as bactérias Gram-negativas; Deve também notar-se que a nisina não tem atividade inibidora contra células de levedura, fungos filamentosos e vírus. Em circunstâncias normais, as bactérias Gram-negativas são geralmente resistentes à nisina, principalmente devido às suas membranas externas impermeáveis (GÄNZLE et al., 2003). A combinação de bacteriocinas com outros mecanismos de conservação tem sido relatada como alternativa para reduzir a resistência a bacteriocinas em bactérias e também como meio para ampliar sua atividade inibitória para espécies Gram-negativas (BELFIORE et al., 2007, PINILLA & BRANDELLI, 2016).

Neste contexto, o presente estudo foi focado no desenvolvimento e caracterização de sistemas nano estruturados de fosfolipídios, para incorporação de extrato de alho e nisina com propriedades antibacterianas de amplo espectro, estudando as suas propriedades durante longos períodos de estocagem, sua interação com o alimento, sua aplicação *in situ* e finalmente analisando a interação lipossoma-bactéria. Os resultados deste trabalho proporcionam informações de interesse para novos estudos sobre formulações com lipossomas que contenham misturas complexas de compostos bioativos, empregando como modelo a nisina e os compostos do extrato do alho, explorando seu potencial antimicrobiano contra diversas espécies bacterianas e fúngicas em alimentos, assim como avaliando

aplicações inovadoras em alimentos, que façam possível uma real aplicação desta tecnologia na indústria de alimentos.

2. Objetivos

2.1. Objetivo geral

Desenvolver lipossomas contendo nisina e extrato de alho, avaliando sua estabilidade, propriedades e potenciais aplicações.

2.2 Objetivos específicos

- Desenvolver nano-lipossomas através da metodologia de hidratação de filme lipídico com adição de estabilizantes (colesterol, ácido oleico e octadecilamina) incorporando a mistura de extrato de alho/nisina, avaliando seus características e seu *shelf life* mediante o seguimento de parâmetros de estabilidade e atividade ao longo do tempo.
- Desenvolver lipossomas de alta estabilidade contendo extrato de alho para aplicação em panificação, avaliar suas características físico-químicas e estudando sua aplicação *in situ* em pães de trigo.
- Estudar a interação lipossoma-proteína miofibrilar, mediante a observação de mudanças estruturais da proteína frente a lipossomas com diferentes características de composição, carga e tamanho.
- Investigar os efeitos de concentrações subletais de nisina livre e nano-encapsulada no perfil proteômico de *L. monocytogenes* ATCC 7466, para determinar diferenças na resposta de estresse celular e produção de fatores de virulência nesses tratamentos.

3. Revisão Bibliográfica

3.1. Nanotecnologia na indústria de alimentos

Nanociência e nanotecnologia apresentam um grande potencial em várias áreas do conhecimento, incluindo química, física, ciências da vida, medicina e engenharia. Representa, portanto, uma convergência real entre os diversos campos do conhecimento (ROSSI et al, 2014; KHAN et al., 2015). Diversos projetos de pesquisa e desenvolvimento extensivos estão em andamento com o objetivo de ganhar vantagem competitiva e participação de mercado. Para uma indústria onde a concorrência é intensa e inovação é vital, as nanotecnologias têm surgido como um auxílio potencial para avanços na produção de alimentos de melhor qualidade e com propriedades funcionais (CUSHEN et al., 2012).

As oportunidades e vantagens da nanotecnologia estão se expandindo rapidamente em todos os campos e muitos países estão investindo grandes quantidades de recursos em pesquisa como o governo dos EUA, quem com o objetivo de ter uma grande participação nestas tecnologias, forneceu em 2016 mais de US$ 1,5 bilhões para a National Nanotechnology Initiative (NNI), um investimento contínuo em apoio das prioridades do governo e sua estratégia de inovação. Os grandes investimentos dos países como EUA, estão ligados ao impacto da nanociência em campos ligados à saúde e bem-estar que são hoje muito fortes. Espera-se que as aplicações de nanotecnologias tragam grandes benefícios para o setor de alimentos e nutrição. Os fenômenos que ocorrem em escala nanométrica oferecem muitas oportunidades de inovação que tem o potencial de impactar substancialmente a indústria de alimentos em todo o mundo. As nanotecnologias podem ser aplicadas a toda a cadeia alimentar, desde a produção até o processamento, incluindo sistemas de liberação controlada de compostos, embalagens e produtos para segurança dos alimentos (BRANDELLI & TAYLOR, 2015; CUSHEM et al., 2012, SILVESTRE et al., 2011). A nanotecnologia pode fornecer novas formas e ferramentas para controlar as propriedades e estruturas dos alimentos, introduzindo novos recursos, que podem acrescentar valor a estes produtos.

Os nano materiais são caracterizados por terem pelo menos, uma dimensão com comprimento entre 1 nm e 100 nm, embora o limite superior de 100 nm seja utilizado por consenso geral sem qualquer evidência científica, para apoiar um desaparecimento de nano-propriedades acima deste valor. Alguns autores sugeriram considerar produtos derivados da nanotecnologia como materiais exibindo propriedades ou fenômenos (incluindo efeitos

biológicos) que são atribuíveis às suas dimensões, mesmo que esta esteja fora da faixa de nano escala dos 100 nm (TINKLE et al., 2014).

Um grande número de nutracêuticos e suplementos nutricionais que contêm nano-ingredientes e aditivos (por exemplo, vitaminas, antimicrobianos, antioxidantes) estão disponíveis no mercado. Estes produtos tipicamente oferecem maior absorção e biodisponibilidade dos ingredientes no corpo. Mesmo assim, as funcionalidades de tais nano materiais, como o tamanho de partículas, distribuição do tamanho, índice de polidispersividade e carga elétrica da superfície, podem ser afetadas pela matriz biológica onde eles são colocados (POWERS et al., 2006), como por exemplo a composição de um alimento.

Embora existam muitos benefícios dessas tecnologias há também preocupação com potenciais efeitos negativos. No caso das partículas de tamanho nanométrico que entram em contato com o corpo humano, por exemplo, a redução no tamanho de partícula associada com a nanotecnologia tem potencial de reduzir a eficácia das barreiras à penetração de matérias estranhas no corpo humano permitindo seu livre movimento dentro do corpo (CUSHEN et al., 2012), resultando no acúmulo de contaminantes tóxicos e, por conseguinte, afetando de forma adversa a saúde humana (CHAU et al., 2007).

3.2. Lipossomas: Estrutura e propriedades

Nanopartículas a base de lipídeos (composta por fosfolipídios, triacilglicerídeos, ácidos graxos, entre outros) são amplamente estudadas, uma vez que podem ser produzidas a partir de ingredientes naturais, podendo encapsular compostos com diferentes solubilidades e ser aplicados em nível industrial (PETERS et al., 2011).

Lipossomas são vesículas de bicamada concêntricas, onde o volume aquoso está inteiramente fechado através de uma bicamada lipídica composta principalmente de fosfolipídios, sendo este o nome dado a lipídeos que possuem um resíduo de ácido fosfórico em sua estrutura. Tal molécula possui duas caudas hidrofóbicas ou apolares, compostas de hidrocarbonetos, e um grupo hidrofílico chamado de cabeça polar (RAWAT et al., 2006). A Figura 1 mostra uma molécula de fosfatidilcolina, um tipo de fosfolipídio cujo grupo polar é a colina.

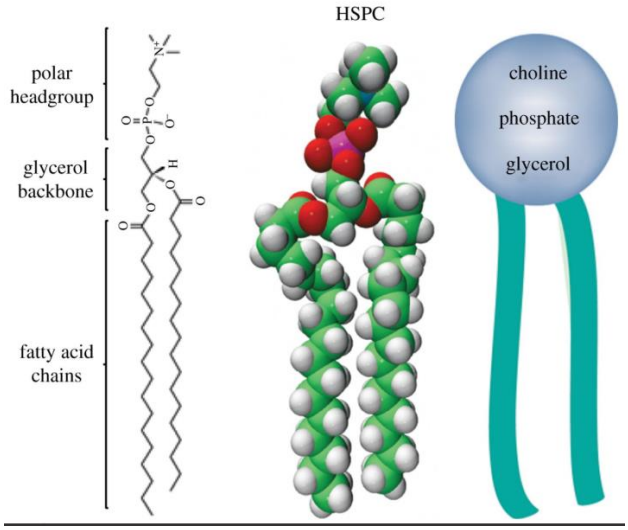

Figura. 1: Estrutura geral da fosfatidilcolina. (MONTEIRO et al 2014)

As moléculas de fosfolipídios são insolúveis em água, porém, quando em ambientes aquosos, dependendo da concentração e da temperatura, formam dispersões e se ordenam em agregados, onde a parte hidrofílica fica em contato com a água, enquanto a parte hidrofóbica se localiza no interior da estrutura, formando as bicamadas lipídicas devido às interações hidrofílicas entre grupos de cabeças polares e interações de van der Waals entre cadeias de hidrocarbonetos com a água (FRÉZARD et al 2005; MCCLEMENTS, 2015). Em solução aquosa, acima de uma determinada concentração, dependendo da temperatura, tais bicamadas lipídicas curvam-se sobre si mesmas dando origem aos lipossomas, onde os fosfolipídios encapsulam parte do meio aquoso onde estão inseridos. A caracterização dos lipossomas é realizada pelo tamanho, carga da superfície e número de bicamadas (RAWAT et al., 2006). Com base no número de bicamadas e vesículas, os lipossomas são classificados como vesículas univesiculares (ULVs, 25 nm a 1 µm), ou vesículas multilamelares (MLVs, 0,1-15 µm) ou vesículas multivesticulares (MVVs, 1,6-10,5 µm), representadas na Figura 2.

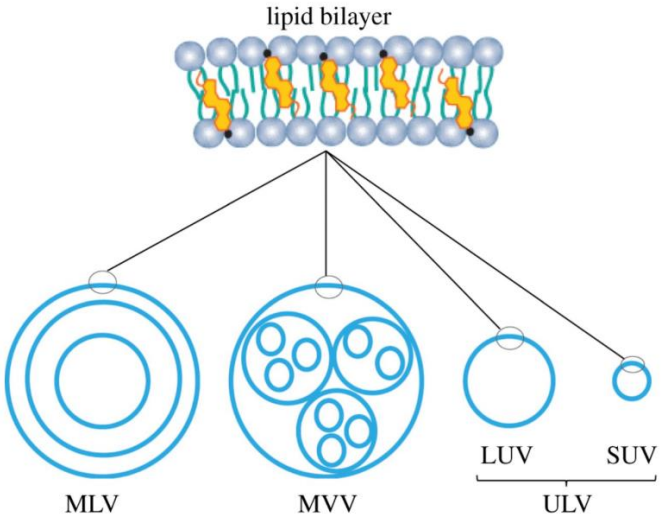

Figura. 2: Estrutura geral dos lipossomas. (MONTEIRO et al 2014). Vesículas univesiculares (ULV), vesículas multilamelares (MLV), vesículas multivesticulares (MVV).

Nanolipossomas são definidos como vesículas de bicamada lipídica (vesículas <30 ou 30-100 nm), que possuem e mantém o tamanho nanométrico durante a aplicação e armazenamento. A característica comum de moléculas formadoras de bicamadas está na sua anfifilicidade, isto é, possuem regiões polares e não polares definidas que podem reter, entregar e controlar a liberação de materiais solúveis em água e lipídeos (BOUWMEESTER et al., 2009).

A aparência das suspensões de lipossomas depende da sua estrutura, determinando o seu comportamento de dispersão de luz. Suspensões que possuem lipossomas pequenos se apresentam opticamente transparentes, devido ao fato de não dispersarem a luz fortemente, enquanto que as suspensões que contêm lipossomas maiores podem apresentar turbidez (KHLEBTSOV, 2001). As características elétricas dos lipossomas dependem do tipo e da concentração de fosfolipídios dentro da formulação, apresentando para a fosfatidilcolina grupos catiônicos e aniônicos ligados na sua cabeça hidrofílica. O grau de ionização dos grupos de cabeça varia de acordo com o pH, onde a carga da fosfatidilcolina pode ser negativa em valores altos de pH e positiva em pH baixo (MCCLEMENTS, 2015; SINGH et al., 2012). As interações eletrostáticas podem promover a agregação dos lipossomas, afetando a sua estabilidade. Para isso, alternativas tem sido utilizadas, como o revestimento da superfície das vesículas com certos polímeros, tais como polietileno glicol (PEG) e quitosana, ou através da utilização de ingredientes catiônicos/aniônicos na estrutura dos lipossomas. Assim, se torna desejável maximizar as forças de repulsão entre as vesículas

para evitar a agregação e sedimentação das mesmas durante o armazenamento, aumentando sua estabilidade. Em geral, as partículas com valores de potencial zeta maiores que +30 mV ou menores que -30 mV possuem maior duração de estabilidade eletrostática (MOZAFARI et al., 2008).

3.3. Aplicação de lipossomas em alimentos

A crescente aplicação de lipossomas na área alimentícia é devida às vantagens que os lipossomas podem fornecer ao serem usados como sistemas encapsulantes de substâncias bioativas, como a proteção de tais substâncias contra alterações químicas e enzimáticas, bem como da temperatura e a variação da força iônica (MOZAFARI et al., 2008).

Numerosos tipos de bioativos hidrofílicos podem ser potencialmente incorporados na fase interna aquosa de lipossomas, incluindo pequenas moléculas e íons (tais como sais, açúcares, peptídeos), biopolímeros (proteínas e polissacarídeos), e partículas (gotículas lipídicas, nanopartículas, e probióticos) (Figura 3). Uma variedade de moléculas bioativas hidrofóbicas (como, vitaminas A, D, E, carotenoides, e coenzima Q10) e hidrofílicos (por exemplo, vitamina C, ferro, cálcio) foram anteriormente incorporados em sistemas de liberação controlada baseados em lipossomas (SINGH et al., 2012).

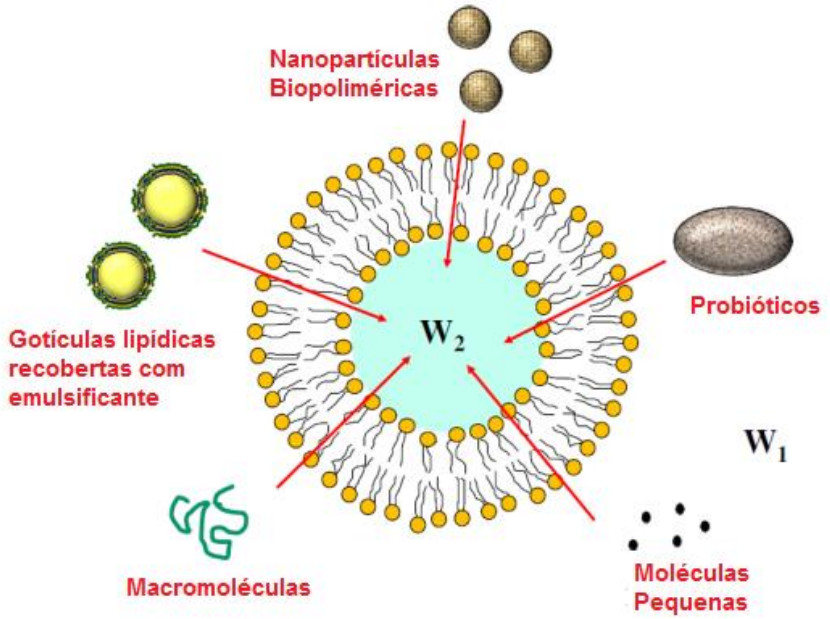

Figura 3. Vários tipos de compostos bioativos hidrofílicos que podem ser incorporados no interior hidrofílico (W2) de lipossomas. Adaptado de MCCLEMENTS (2014).

Um dos primeiros relatos da aplicação de lipossomas em alimentos foi na fabricação de queijo por LAW e KING (1985), com o objetivo de diminuir o tempo e custo de maturação. Para isso, foram adicionadas proteinases encapsuladas em lipossomas na mistura do queijo, demonstrando que a atividade e a estabilidade das enzimas foram melhores com a encapsulação, além de melhorar o sabor do queijo e diminuir os custos de produção.

TONIAZZO et al. (2014) estudaram a encapsulação de β-caroteno em lipossomas para a aplicação em iogurte. Os lipossomas foram capazes de proteger o β-caroteno da degradação por um período de até 95 dias e, quando aplicados nos iogurtes, mostraram que a textura não foi afetada, sugerindo que uma parte dos corantes artificiais poderiam ser substituídos pelos lipossomas encapsulando β-caroteno. Lipossomas foram estudados como transportadores de vitamina C e E, com o objetivo de serem incorporados em suco de laranja. A combinação de formulações de lipossomas e vitaminas não alterou as características organolépticas do suco de laranja, além disso, mostrou estabilidade microbiológica após a pasteurização e armazenamento a 4°C, por 37 dias (MARSANASCO et al., 2011).

Peptídeos antimicrobianos têm sido extensivamente estudados como potenciais bioconservadores, porém, sua atividade antimicrobiana pode ter seu efeito diminuído devido à degradação proteolítica e a interação do peptídeo antimicrobiano com os componentes alimentares. A encapsulação de bacteriocinas em lipossomas pode ser uma alternativa para ultrapassar esse problema (MAHERANI et al., 2011). Neste contexto, MALHEIROS, DAROIT e BRANDELLI (2012) investigaram a eficácia da nisina livre e encapsulada em nanolipossomas para o controle de *Listeria monocytogenes* em queijo minas frescal. Para isso a nisina comercial foi encapsulada em lipossomas de lecitina de soja parcialmente purificada. Os resultados mostraram efeito bactericida com 0,25 mg/mL de nisina livre; efeito bacteriostático para nisina encapsulada em lipossomas e com 0,1 mg/mL de nisina livre. Adicionalmente nosso grupo de pesquisa tem desenvolvido diversos trabalhos de encapsulação de nisina em lipossomas recobertos com polissacarídeos (LOPES et al., 2017), contendo extrato de alho, (PINILLA et al., 2017) e com a mistura nisina-extrato de alho, (PINILLA et al., 2016), demostrando assim as diversas aplicações de este sistema para encapsulamento de antimicrobianos naturais.

É importante notar, no entanto, que o uso potencial de lipossomas como portadores de ingredientes ativos alimentares pode às vezes ser limitado por suas instabilidades físicas e químicas em dispersões aquosas, especialmente para armazenamento de longo prazo (CHEN et al., 2010), devido a existirem diversos desafios que atualmente limitam a sua aplicação

generalizada na indústria de alimentos. Primeiro, são de difícil fabricação em larga escala de forma viável e o processo tem um alto custo. Em segundo lugar, muitas vezes eles têm uma fraca estabilidade física nas condições de processamento e armazenamento de muitos produtos alimentares. Terceiro, eles tipicamente têm uma baixa eficiência de encapsulação para bioativos hidrofílicos porque uma fração apreciável permanece fora dos lipossomas durante o processo de encapsulação (MCCLEMENTS, 2015). No entanto, os avanços na tecnologia de ingredientes, operações de processamento e mecanismos de estabilização, podem levar à utilização mais generalizada de lipossomas na indústria de alimentos.

Neste contexto a liofilização, um processo muito comumente usado na indústria alimentícia, também pode ser empregada como método para estabilizar e preservar lipossomas e estender seu prazo de validade. No entanto, a liofilização de lipossomas pode ter uma grande desvantagem, principalmente o enfraquecimento da membrana das vesículas e perda de integridade. Este problema pode ser gerenciado pelo acoplamento de formulações específicas e parâmetros operacionais apropriados durante a liofilização (CHEN et al., 2010).

3.4. Estabilidade e liofilização de lipossomas

Um desafio no uso de lipossomas em alimentos é sua instabilidade em dispersões aquosas. Eles podem sofrer degradação química e física, eventualmente resultando em eficácia reduzida devido à diminuição da qualidade da formulação e, em alguns casos, até mesmo a geração de produtos de degradação. Os principais mecanismos de degradação responsáveis pela limitada estabilidade química dos fosfolipídios utilizados em formulações lipossômicas são a oxidação e hidrólise (GRID & CROMMELIN, 1993). Embora todos os tipos de ácidos graxos sejam suscetíveis à oxidação por meio do mecanismo em cadeia dos radicais livres, os ácidos graxos insaturados são em geral mais propensos à oxidação do que ácidos gordos saturados devido à presença de ligações duplas nas caudas lipídicas (MOHAMMED et al., 2006). Até certo nível, o processo de oxidação pode ser desacelerado com a adição de antioxidantes adequados ou por armazenamento em atmosferas modificadas. A hidrólise de ligações éster, resultando na geração de ácidos graxos livres, lisofosfolipídios e fosfoglicerol compostos, também pode ser problemático em relação à estabilidade em longo prazo dos lipossomas (ZUIDAM et al., 1995).

A desestabilização física dos lipossomas inclui a fusão de bicamadas de membrana, agregação, diminuição da retenção de materiais encapsulados e conversão em, por exemplo, estruturas micelares, e pode ser mais pronunciada após alterações químicas (INGVARSSON et al., 2011). Todos esses fatores afetam a qualidade da formulação final, e é importante prever soluções para esse problema. Como esses processos ocorrem principalmente em um ambiente aquoso, uma opção é liofilizar as formulações de lipossomas. A estabilização é conseguida reduzindo o teor de água empregando a liofilização. Esta estabilidade melhorada elimina a necessidade da "cadeia de frio" durante a distribuição do produto, que é de grande importância para manter os custos baixos e assegurar produtos estáveis, em especial para a distribuição nos países em desenvolvimento (INGVARSSON et al., 2011).Um processo típico de liofilização consiste em três fases, ou seja, congelamento, secagem primária e secagem secundária. A fase de congelação é um passo de resfriamento onde a maior parte do solvente (por exemplo, água) é separada dos lipossomas e aditivos, resultando na formação de gelo.

Diversos estudos demonstraram a viabilidade do processo de liofilização de lipossomas como uma boa alternativa para superar problemas com a instabilidade físico-química de dispersões aquosas de lipossomas e sua alta suscetibilidade à contaminação microbiológica. Não entanto, muitos fatores que afetam este processo, como a seleção de lioprotetores, a composição da bicamada lipossômica e os protocolos de liofilização, que precisam ser melhorados para obter uma alta retenção dos compostos após a liofilização. (CHEN et al., 2010). A relativa novidade desta tecnologia exige maiores esforços para entender melhor os processos de liofilização de lipossomas e encontrar aplicações apropriadas para esta tecnologia muito interessante e promissora, especialmente para bioativos hidrofóbicos, como carotenoides, óleos essenciais, curcuminoides, flavonoides hidrofóbicos, peptídeos hidrofóbicos, bem como lipídios funcionais (TONIAZZO & PINHO, 2016). A exploração de tecnologias adequadas para a produção de lipossomas é muito importante, pois a liofilização já é um processo amplamente conhecido na indústria alimentícia e, portanto, não seria um limitante no desenvolvimento de um sistema de produção de fosfolipídios liofilizados. Além disso, a caracterização dos pós obtidos é necessária não apenas para elucidar os mecanismos físico-químicos da lioproteção, mas também para otimizar processos de secagem por congelação e para obter lipossomas liofilizados com características únicas, tais como prazo de validade prolongado,

compatibilidade com os requisitos de embalagem de ingredientes alimentares e armazenamento (TANIAZZO & PINHO, 2016).

3.5. Alho (*Allium sativum* L.)

Historicamente o alho tem sido ressaltado como parte de uma dieta saudável. Em textos médicos antigos do Egito, Grécia, Roma, China e Índia o alho é prescrito para uma série de aplicações, incluindo a melhoria do desempenho físico, reduzindo infecções, e protegendo contra toxinas (RIVLIN, 2001). Estas propriedades medicinais, juntamente com suas características de sabor, fizeram do alho um verdadeiro ícone cultural em muitas partes do mundo. Assim, o alho é utilizado tradicionalmente como um intensificador de sabor e tem sido reconhecido não somente como um condimento alimentar, mas também um potente agente terapêutico (YUN et al., 2014).

O alho é originário de zonas temperadas da Ásia Central onde é conhecido popularmente como alho comum. É uma planta herbácea da família *Alliaceae*, de porte baixo, que atinge 0,40-0,70 m de altura. Tem folhas lanceoladas, as quais formam o pseudocaule implantando-se em um caule pequeno e achatado e as gemas do caule formam os bulbilhos (dentes), que em conjunto formam o bulbo (Figura 4). Os bulbilhos têm morfologia ovoide-arqueada e às vezes levemente periforme, que encontram-se envolvidos por uma ou mais folhas protetoras de coloração branca arroxeada. O bulbo é arredondado, periforme e constituído por aproximadamente 5 a 20 bulbilhos (HARVEY, 1995).

Figura 4. Imagem do alho (*Allium sativum* L.), fonte : EMBRAPA, Hortaliças Folders 2013.

O alho apresenta grande importância socioeconômica no Brasil, com uma produção de 132.1 mil toneladas em 2017 segundo o Instituto Brasileiro de Geografia e Estatística (IBGE). Também é importante ressaltar que o Brasil é o segundo consumidor de alho no mundo, alcançando 1,50 Kg de alho/habitante/ano, perdendo somente para a China. Mesmo assim a produção nacional abastece somente 44% do consumo da população e os outros 56% são importados, principalmente da China (40%) e da Argentina (20%), segundo a Associação Nacional de Produtores de Alho (ANAPA 2018).

Do ponto de vista tecnológico, o uso de alho fresco e seus derivados tem sido estudado por muitos autores devido à sua atividade antioxidante e antimicrobiana (antibacteriana, antifúngica e antiparasitária), (ALORAINY et al., 2011; PÂRVU et al., 2011). A ruptura dos bulbos de alho leva à formação de tiossulfinatos, nos quais o composto precursor alliina é transformado em alicina (tiossulfinato de dialil) por ação enzimática; A alicina é o composto bioativo mais importante no alho devido à sua ampla atividade funcional e altas concentrações (BOSE et a., 2014). No entanto, a alinase, que catalisa a conversão de alliina em alicina, é termo lábil, assim como a alicina; o último se decompõe em poucas horas para formar compostos de enxofre mais estáveis, com atividade reduzida.

3.6 Compostos bioativos do alho

O alho possui uma variedade de compostos bioativos, incluindo compostos organossulfurados, saponinas, compostos fenólicos e polissacarídeos (SZYCHOWSKI et al., 2018). Os principais componentes ativos do alho (Figura 5) são seus compostos organossulfurados, como dialil tiossulfonato (alicina), dialil sulfeto (DAS), dialil dissulfeto (DADS), dialil trissulfeto (DATS), E / Z-ajoeno, S-alil -cisteína (SAC) e sulfóxido de S-alil-cisteína (alina) (YOO et al., 2014; KODERA et al., 2017). Além disso, o alho contem mais de 20 compostos fenólicos, com conteúdo mais alto do que muitos vegetais comuns (LIU et al., 2018). O principal composto fenólico descrito é o ácido β-resorcílico, seguido pelo pirogalol, ácido gálico, rutina, ácido protocatecúico e quercetina (NAGELLA et al., 2014). Além disso, foi relatado que os polissacarídeos do alho contêm 85% de frutose, 14% de glicose e 1% de galactose (HANG, 2005).

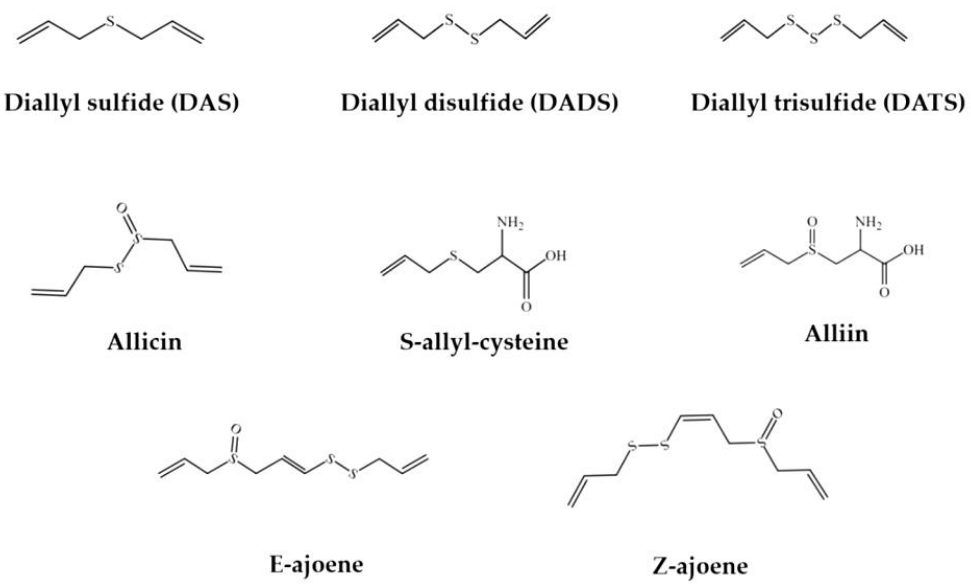

Figura 5. Estruturas químicas dos principais compostos organossulfurados do alho. (SHANG et al., 2019).

3.7 Atividade antioxidante e antimicrobiana do alho

Atividade antioxidante

Diversos estudos tem demostrado que o alho tem fortes propriedades antioxidantes. Em um recente estudo (LOCATELLI, et al., 2017), foram avaliada as capacidades antioxidantes do alho cru e cozido, pelo ensaio de eliminação do radical 1,1-difenil-2-picrilhidrazila (DPPH), eliminação de radicais 2,2'-Azino-bis ácido 3-etil-benzotiazolina-6-sulfônico (ABTS) e ensaio de poder antioxidante redutor de íons férricos (FRAP), onde foi constatado que o alho cru exibia atividade antioxidante mais forte. Em outro estudo, os resultados dos ensaios de DPPH e capacidade de absorção de radicais de oxigênio (ORAC) mostraram que o extrato etanólico de brotos de alho exibia atividades antioxidantes mais fortes que o extrato etanólico de alho cru (ZAKAROVA et al., 2014). Por tanto o alho e seus ingredientes ativos (como fenóis e saponinas) têm efeitos antioxidantes demostrados, mas os diferentes métodos de processamento afetam a atividade antioxidante do alho.

Atividade antimicrobiana

O alho possui um amplo espectro de propriedades antibacterianas e antifúngicas (LIU et al., 2017). Os compostos organossulfurados presentes do alho são responsáveis por sua

atividade antimicrobiana, sondo a alicina o principal composto responsável pela sua atividade antimicrobiana. De entre os mecanismos propostos para a atividade antimicrobiana da alicina, incluem: 1) capacidade de permear a membrana e capacidade de destruir a estrutura celular, (LI et al., 2016; PRAGER-KHOUTORSKY et al., 2007); 2) capacidade de alterar a expressão gênica dos microrganismos, (LI et al., 2016); 3) reatividade com enzimas que contem grupos tiol, induzindo assim o estresse oxidativo (RABINKOV et al., 1998). De entre as diversas formas de obter os compostos bioativos do alho, o óleo essencial de alho tem sido um dos produtos mais amplamente estudados, demostrando atividades antibacterianas e bacteriostáticas contra *Staphylococcus aureus*, *Escherichia coli* e *Bacillus subtilis* (GUO, 2014). Em outro trabalho, verificou-se que o óleo essencial de alho inibia o fungo *Penicillium funiculosum*, provavelmente penetrando nas células e organelas, destruindo a estrutura celular e induzindo o vazamento de macromoléculas do citoplasma (LI et al., 2014). O alho cru e extratos de alho tem sido objeto de estudo. Em um ensaio clínico, o tratamento com alho cru inibiu o *Helicobacter pylori* no estômago de pacientes com infecção por esta mesma bactéria (ZARDAST et al., 2016) e mais recentemente, foi demostrado o efeito anti-biofilme de nano partículas carregadas de extrato de alho contra (MRSA) *Staphylococcus aureus* (GIRISH et al., 2019). Adicionalmente, no nosso grupo de pesquisa demostramos o potencial do extrato de alho livre e encapsulado em lipossomas no controle de *Listeria monocytogenes* (PINILLA et al., 2017).

3.8 Nisina

O antimicrobiano nisina é uma bacteriocina produzida por *Lactococus lactis*, pertencente à família de lantibióticos, na qual contém grupos de lantionina e metil lantionina. A nisina é um peptídeo antimicrobiano catiônico e anfifílico, que possui ponto isoelétrico acima de 8,5 e tem sido utilizado como conservante em alimentos, por ser eficiente em baixo pH e a altas temperaturas (SALMIERI et al., 2014; MEIRA et al., 2015).

A nisina é um polipeptídeo pequeno, com 34 aminoácidos, que se apresenta com as variantes A, Z, M, Q, que são produzidos por *Lactococcus lactis*, enquanto os tipos U e U2 são obtidos a partir de *Streptococcus uberis*, e nisina P é produzida por *Streptococcus suis* e *Streptococcus gallolyticus* subsp. *pasteurianus* (QI et al., 2012; WU et al., 2014). As variantes A e Z se diferem por um único aminoácido na posição 27, sendo histidina em nisina A, e asparagina em nisina Z. Dentre esses tipos, a nisina A é considerado o mais ativo contra patógenos (PROMBUTARA et al., 2012). Quando aplicada em combinação com o

calor e baixo pH, a nisina aumenta sinergicamente a sua atividade contra esporos. AOUADHI et al. (2016) concluíram que os esporos de *Bacillus sporothermodurans* LTS27 submetidos a meio ácido sofrem alterações morfológicas. Estas alterações podem torná-los mais sensíveis à inativação por tratamento térmico em combinação com nisina, o que torna este processo muito eficaz em produtos alimentares de baixa acidez e com tratamento térmico.

A viabilidade do uso da nisina está baseada em que bactérias lácticas (BAL) e seus metabólitos têm sido processados e consumidos em todo o mundo, sem efeitos adversos. Alimentos contendo bacteriocinas, compostos purificados e extrato de cultura de BAL têm sido avaliados (YANG et al., 2012; SIROLI et al., 2016). A nisina é amplamente utilizada em produtos alimentares, incluindo queijo, saladas, sopas enlatadas, gelo para armazenamento de peixe, alimentos para bebês, milk-shakes e produtos de panificação (SAMELIS et al., 2005; DISCHINGER et al., 2014), tendo características ideais para um aditivo alimentício, na medida em que não apresenta efeitos sobre a microbiota normal do intestino, é atóxica, não afeta a cor ou sabor dos alimentos e apresenta estabilidade térmica.

Atualmente a Comissão da FAO/WHO permite uma ingestão diária de até 33.000 unidades de nisina por 70 kg de peso corporal. O limite máximo diário de ingestão de nisina varia de país para país. Na Austrália, Grã-Bretanha e França a nisina é permitida sem limite máximo, enquanto que nos EUA, o limite máximo é de 10.000 UI/g; na Rússia, o limite máximo é de 8000 UI/g. No Brasil, a nisina tem o seu uso permitido pela Legislação Brasileira (DETEN/MS nº 29, de 22 de janeiro de 1996) com a função de conservador para queijos pasteurizados no limite máximo de 12,5 mg/kg (500 UI/g). Não entanto seu uso é limitado devido a que a nisina não tem atividade contra bactérias Gram-negativas, fungos filamentosos, células de levedura e vírus, inibindo principalmente os principais géneros bacterianos *Micrococcus, Lactococcus, Leuconostoc, Lactobacillus, Pediococcus, Listeria* e *Staphylococcus* (GHARSALLAOUI et al., 2016).

De acordo com diferentes estudos, a adsorção de moléculas de nisina produz uma desregulação da superfície da membrana bacteriana, sendo o principal mecanismo da atividade antimicrobiana da nisina (BAHRAMIA et al., 2019). A ligação das moléculas de nisina ao lipídeo II é uma maneira que facilita a infiltração de nisina através da membrana bacteriana (Figura 6). O lipídio II é um material precursor necessário para a biossíntese de parede bacteriana. A molécula de nisina se liga ao lipídeo II e impede o crescimento da rede

de peptidoglicano. Além disso, a parte N-terminal da nisina se conecta à parte carboidrato-pirofosfato do lipídeo II, o que permite que a seção C-terminal da nisina se infiltre na membrana bacteriana. Em seguida, os complexos nisina-lipídio II criam um poro na membrana (PUNYAUPPA-PATH et al., 2015).

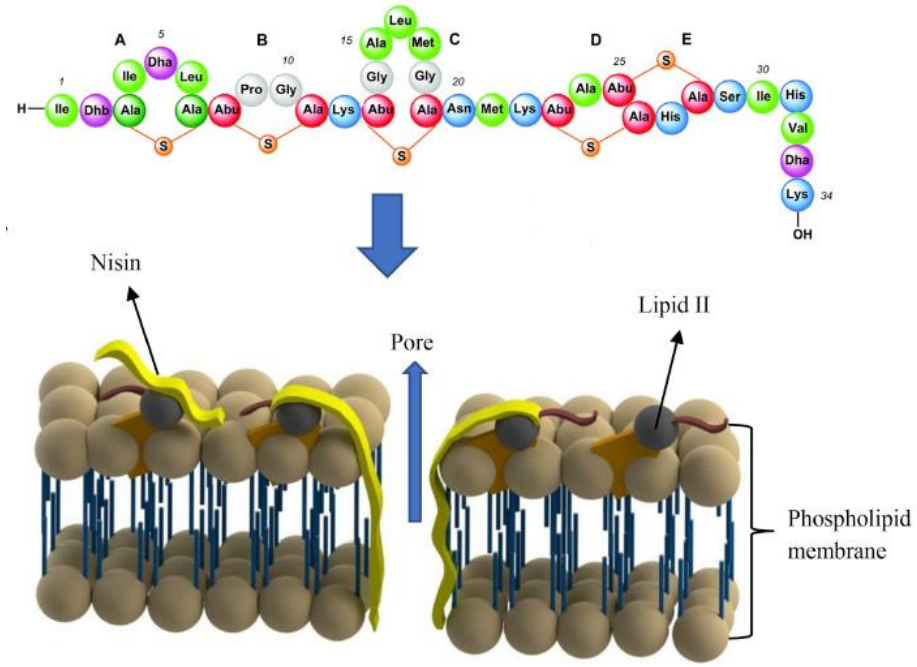

Figura 6. Nisina e seu mecanismo de atividade contra bactérias. (BAHRAMI, et al. 2019).

A capacidade antimicrobiana da nisina contra uma ampla gama de microrganismos e sua rápida atividade levou à extensão de sua aplicação como um agente antimicrobiano popular e natural nos produtos lácteos, sucos, carnes e vegetais (BAPTHO et al., 2017). No entanto, existem alguns desafios que restringem o uso de nisina, incluindo um baixo desempenho antibacteriano durante o armazenamento de alimentos, sensibilidade ao estresse ambiental, suscetibilidade à proteólise e interações indesejáveis com componentes alimentares (BISWARO et al., 2018). Para superar esses desafios, é importante ter uma liberação sustentada de nisina durante o prazo de validade dos alimentos, projetando um sistema de entrega eficiente por meio de técnicas de encapsulamento (BAHRAMI, et al. 2019). Nesse processo, a nisina, como material do núcleo, é revestida com os materiais da parede. Portanto, os materiais das paredes agem como uma barreira contra as tensões ambientais, tanto para proteger a nisina quanto para controlar sua liberação.

Recentemente, novas tecnologias de encapsulamento (incluindo a nanotecnologia) foram usadas para melhorar as propriedades funcionais dos antimicrobianos para alimentos (ARPAGAUS et al., 2018; JAFARI & MCCLEMENTS, 2017). Os sistemas de nano-encapsulação, fornecendo uma área superficial mais alta, podem apresentar várias vantagens, incluindo liberação controlada e alta biodisponibilidade de compostos no alvo (FARIDI et al., 2018). Em alguns casos, o encapsulamento de nisina usando outros materiais GRAS, incluindo quitosana, pectina, alginato / pectina, entre outros, demonstrou potencial para estender a atividade antimicrobiana da nisina quando testada in vitro (MCCLEMENTS, 2018). No entanto, os desenvolvimentos precisam ser avaliados em uma matriz alimentar para demonstrar que o sistema de encapsulamento pode realmente aumentar a atividade antimicrobiana da nisina.

4. Artigo No.1

Effect of oleic acid, cholesterol, and octadecylamine on membrane stability of freeze-dried liposomes encapsulating natural antimicrobials.

Resumo:

Os lipossomas têm sido amplamente estudados como sistemas de transporte de compostos bioativos, embora sua estabilidade relativamente baixa permaneça como limitação para aplicação comercial. Neste estudo, lipossomas de fosfatidilcolina (PC) foram preparados xontendo uma mistura de extrato de alho (GE) e nisina (Nis) usando colesterol (CHO), ácido oleico (OA) ou octadecilamina (ODA) como estabilizadores de membrana, para avaliar suas propriedades físicas, químicas, bioativas e de estabilidade, em estado totalmente hidratado e após liofilização. GE / Nis lipossomas apresentaram diâmetro hidrodinâmico abaixo de 200 nm e índice de polidispersividade abaixo de 0.30, típico para pequenas vesículas unilamelares produzidas pelo método de hidratação de filme. Sob oxidação induzida, os lipossomas PC-OA-GE / Nis apresentaram 91% menos de peroxidação lipídica em comparação com os lipossomas de PC não carregados. A análise por espectroscopia no infravermelho por transformada de Fourier (FTIR) revelou um alto nível de ligações de hidrogênio no grupo da cabeça polar do PC após a adição de GE / Nis em todas as formulações de lipossomas, em concordância com os altos valores de atividade da água e higroscopicidade encontrados nas amostras após liofilização. Durante 5 meses armazenamento a 4 ° C, lipossomas totalmente hidratados e liofilizados mostraram um incremento em seu tamanho médio e índice de polidispersividade, mas esses valores foram reduzidos pela adição de trealose como lioprotetor. Todas as preparações de lipossomas mantiveram 100% de atividade contra *Listeria monocytogenes*; no entanto, foi observada uma redução gradual da atividade contra *Salmonella enterica* serovar Enteritidis, sugerindo uma perda parcial de compostos ativos de GE. Apesar de algumas modificações físicas, lipossomas liofilizados contendo OA como estabilizador apresentaram melhores propriedades antimicrobianas e alta resistência à oxidação lipídica, constituindo uma abordagem promissora estabilizar GE / Nis para armazenamento em longo prazo.

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Effect of oleic acid, cholesterol and octadecylamine on membrane stability of freeze-dried liposomes encapsulating natural antimicrobials

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Abstract

Liposomes have been broadly studied as delivery systems for bioactive compounds, although its relatively low stability remains a limitation for commercial application. In this study, phosphatidylcholine (PC) liposomes were prepared entrapping a mixture of garlic extract (GE) and nisin (Nis) using cholesterol (CHO), oleic acid (OA) or octadecylamine (ODA) as membrane stabilizers to evaluate their physical, chemical, bioative and stability properties, in fully-hydrated state and after freeze-drying. GE/Nis-loaded liposomes presented hydrodynamic diameter below 200 nm and polydispersity index below 0.30, typical for small unilamellar vesicles produced by thin film method. Under induced oxidation, the PC-OA-GE/Nis liposomes presented 91% less lipid peroxidation compared with the unloaded PC liposomes. The Fourier Transform Infrared Spectroscopy (FTIR) analysis revealed a high level of hydrogen bonds in the polar head group of PC after addition of GE/Nis in all liposome formulations, in agreement to the high values of water activity and hygroscopicity found in the samples after freeze-drying. During 5 months storage at 4°C, fully-hydrated and lyophilized liposomes showed an increment in their average size and polydispersity index, but these values were reduced by the trehalose addition as lyoprotector. All liposome preparations maintained 100% activity against *Listeria monocytogenes*, nevertheless, a gradual reduction of activity against *Salmonella Enteritidis* was observed, suggesting a partial loss of GE active compounds. Despite some physical modifications, freeze-dried liposomes containing OA as stabilizer showed best antimicrobial properties and high lipid oxidation resistance, constituting a promising approach to stabilize GE/Nis for long-term storage.

Keywords: Soy phosphatidylcholine liposomes; long-term stability; natural antimicrobials; nanotechnology

Introduction

Liposomes are versatile phospholipid carriers for both hydrophilic and hydrophobic bioactive molecules, with some advantages including biodegradable, non-toxic and sustained releasing properties (Singh et al. 2012). These lipid vesicles are used for many applications, and during the past few decades, they have been intensively studied as delivery vehicles of many bioactive compounds, ranging from small molecules and peptides to proteins or complex mixtures (Lopes and Brandelli 2018). Food applications of these colloidal structures have been quite increased lately, due to the encapsulation techniques which be used to overcome diverse limitations and for the development of value-added products (Celli et al. 2015). Several examples of encapsulated substances used in food fortification and antimicrobial protection can be found in the literature such as carotenoids (Moraes et al. 2013), functional peptides (Mohan et al. 2018), clove essential oil (Sebaaly et al., 2015) and natural antimicrobial agents (Pinilla and Brandelli 2016; García-Toledo et al. 2019).

However, in respect to the potential use of liposomes as carriers for food additives, some remaining challenges need to be overcome, such as their physical and chemical instabilities in aqueous dispersions, especially for long-term storage (Chen et al. 2010). The colloidal nature of liposomes increase their susceptibility to microbiological contamination and implicate some stability problems related to handling, storage, and transport, which restrict their widespread use for food processing applications (Toniazzo et al. 2016). In a water suspension, unsaturated acyl chains oxidation and ester bonds hydrolysis are the main causes of liposome chemical degradation, that compromises its structural integrity, leading to membrane leakage (Gibbs et al. 1999). Fusion and structural transformations of the liposomes and increased levels of reactive oxygen species (ROS) are known to cause

damage and might influence their performance (van Winden 2003). Other factors present in the food production chain, such as fluctuations in pH, temperature, light exposure, and ingredient interactions, can also affect the liposomes stability. Therefore, it is an important goal in liposomes production that bioactive loaded colloidal dispersion remains physically and chemically stable when exposed to environmental stress (McClements 2015).

In this context, many methods for liposomes stabilization have been described, like freeze-drying, freezing, spray-drying and spray-chilling (Nedovic et al. 2011). Due to their chemical instability for long-term storage, lyophilization, a process commonly used in the food industry, is the main approach used to preserve and extend their shelf life. However, liposomes freeze-drying have a major drawback: weakening phospholipid membrane integrity, a problem that could be managed by treating the formulations with appropriate operational parameters (Chen et al. 2010).

Besides the process variables, formulation parameters, such as the specific type of lyoprotectant and the lipid composition of bilayer might be even more critical for the liposomal product stabilization in drying process, which determines bilayer order degree and the liposome behavior during storage (Ingvarssson et al. 2011). In addition, the presence of some compounds within the liposomes, like ascorbic acid, anthocyanins, and catechins as antioxidants, not only avoids the degradation of entrapped bioactives, but also inhibits lipids oxidation that are used in liposome production (Viljanen et al. 2004). Therefore, if encapsulated compounds can also help to reduce the lipid oxidation, improving liposome stability, would be an interesting approach.

The reduction of nisin tolerance in target strains and extension of activity spectrum to Gram-negative bacteria may be achieved by combination with other antimicrobials, but reports describing the co-encapsulation of nisin and other agents are limited (Xiao et al. 2011; Lopes et al. 2019). The present work aimed to evaluate cholesterol (CHO), oleic acid

(OA) or octadecylamine (ODA) as membrane stabilizers for long term storage of PC liposomes co-encapsulating a mixture of garlic extract and nisin (GE/Nis). For this, PC-liposomes containing the antimicrobial mixture GE/Nis were prepared in the absence and presence of stabilizers, and the stability of freeze-dried and fully hydrated liposomes was evaluated, in terms of physical characteristics and antimicrobial activity over five months of storage.

Materials and Methods

Nisin and garlic extract preparation

Garlic bulbs were obtained from a local market in Porto Alegre, Brazil, and commercial nisin (Nisaplin®) from Danisco (Vargem Grande, Brazil). Garlic extract (50% w/v) was prepared using ultrapure water by the methodology described previously (Pinilla et al. 2017). Nisin solution was prepared with 0.1 M citric acid to obtain a 2.5 mg mL^{-1} stock solution and filtered through 0.22 μm sterile membranes (Sartorius, Götingen, Germany) prior to use.

Nisin-GE loaded liposomes preparation

Encapsulation of nisin and GE in phosphatidylcholine (Phospholipon 90G® Lipoid Ludwigshafen, Germany) liposomes was carried out by the thin-film hydration method, described in our previous work (Pinilla and Brandelli 2016). Briefly, individual formulations of phosphatidylcholine (PC)-Cholesterol (CHO) molar ratio 7:2, PC-oleic acid (OA) molar ratio 7:2, and PC-octadecylamine (ODA) molar ratio 7:1, were prepared using to the same amount of PC in each sample (106.4 mg). These mixtures were dissolved in chloroform and the organic solvent was removed in a rotary evaporator at 40°C. The resulting dried lipid film was dispersed by adding 3 mL GE and 3 mL of nisin solution (1.25 mg mL^{-1}). Then,

such mixtures were vortexed at 55°C during three minutes before sonication for five minutes in a probe-type sonicator (frequency 20 kHz, power 250 W, Unique OF S500, São Paulo, Brazil) and finally filter-sterilized by 0.22 μm membranes. PC liposomes with and without stabilizers were prepared as control for each formulation using 6 mL of 10 mM phosphate buffer (pH 7.0). Part of this fresh prepared liposomal dispersions (PC, PC-CHO, PC-OA and PC-ODA) was used for particle size, polydispersity index (PDI), zeta potential, entrapment efficiency, lipid peroxidation and antioxidant activity analyses. The remaining was divided in three parts: one was freeze-dried adding trehalose in the liposome suspension (1 mg mg^{-1} of PC) as lyoprotector, the second was freeze-dried without lyoprotector and the third was maintained as liquid suspension. All the liposomes were stored at 4°C for the stability analysis.

Characterization of the liposome formulations

Particle size, PDI and zeta potential (ζ) analyses were carried out after formulations dilution in ultrapure water using a Zetasizernano-ZS ZEN 3600 equipment (Malvern Instruments, Herrenberg, Germany).

The encapsulation efficiency (EE) of freshly prepared liposomes was performed by liquid chromatography analysis. Vesicles were separated from the solution by ultrafiltration with a 10 K membrane (Ultracel®, Millipore, Cork, Ireland). Nisin and allicin filtrate concentrations were determined using the HPLC methodology described previously (Pinilla and Brandelli 2016). HPLC analyses were performed in a Shimadzu chromatograph (Kyoto, Japan), equipped with quaternary pumps (LC-20AD) and a diode array detector (DAD) (SPD-M20A).

$ABTS^{\bullet+}$ radical capture

For the antioxidant capacity analysis, an ABTS-ethanol stock solution was produced by mixing the ABTS radical (7 mM) with 2.45 mM potassium persulfate (final concentration) for 16 h before use in the dark at room temperature. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for the measurements. Then, 10 µL aliquots of each hydrated liposome suspension was added to 1.0 mL of radical $ABTS^{\bullet+}$ solution and mixed, after 6 min reaction in the dark, samples were read at 734 nm in a spectrophotometer. The analyses were carried out in triplicate and the antioxidant activity was expressed, based on the construction of a Trolox (Sigma, St. Louis, MO, USA) standard curve, as µmol of Trolox equivalents per mL of liposome suspension.

Lipid peroxidation assay (TBARS)

Liposomes peroxidation was induced by hydroxyl radical ($^{\bullet}OH$). This radical was produced as described by Nogueira et al. (2018), with some modifications. A 2 mL solution containing 2.8 mM H_2O_2, 0.04 mM $FeCl_3$, 0.1 mM ascorbate in 10 mM KH_2PO_4 (pH 7.4) and 100 µL of liposome dispersion were incubated for 120 min at 37°C and then centrifuged for 2 min at 3000 *g*. The supernatant was recovered and the lipid peroxidation level was obtained by thiobarbituric acid reactive substances (TBARS) method (Fagali and Catalá, 2012). Two control experiments were performed in parallel by adding (i) reactants except liposomes and (ii) reactants with the free mixture GE/Nis. TBARS amount was estimated by a spectrophotometer at 535 nm using an extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$. Results were expressed as mg TBARS per mL of liposome suspension.

Fourier transform infrared spectroscopy (FTIR)

The interaction between PC liposomes with different additives (CHO, OA, and ODA) and the GE/Nis mixture was investigated through FTIR spectroscopy, performed in a

spectrometer Perkin Elmer Spectrum 400 (Waltham, USA). Freeze dried liposomes were analyzed by the KBr compression method. The spectra of each empty and loaded formulation were recorded in the range of 4000-400 cm^{-1}.

Water activity (a_w), hygroscopicity and moisture of freeze-dried liposomes

Water activity (Aw) was directly measured by an electronic meter (AquaLab 3TE, Decagon, Pullman, USA) at 24°C. The hygroscopicity was determined according to Tonon et al. (2008), where samples were placed in an airtight glass container with saturated NaCl (75% relative humidity), stored in an incubator chamber (411/FDP Ethik Technology, Brazil) at 25°C for 7 days, and after that weighed every 24 h until a constant weight was achieved. Hygroscopicity was expressed in grams percentage of adsorbed moisture per 100 g of dry solids. Moisture content was calculated from samples weight loss after heating at 105°C, using thermogravimetric analyzer model TGA Pyris 1 (Perkin Elmer, Shelton, USA).

Thermal properties of freeze-dried liposomes

Differential scanning calorimetry (DSC) studies were developed in a DSC Q 8500 apparatus (Perkin Elmer). Approximately 10 mg of freeze-dried samples were placed in aluminum pans and then heated from -30 to 150°C (heating rate of 10°C min^{-1}) with continues nitrogen atmosphere at a flow rate of 40 mL min^{-1}. A clean and empty pan sealed was used as a reference sample.

Liposome stability evaluation

For the liposome stability test, 1 mL of each formulation, made with different additives (CHO, OA and ODA), was placed in individual containers. Three groups were evaluated: full hydrated liposomes, freeze-dried liposomes without lyoprotectant and freeze-dried

liposomes with trehalose as lyoprotectant. Samples were stored at 4°C during 5 months. Stability test was carried out by monitoring changes in some parameters, including particle diameter, polydispersity index (PDI), zeta potential (ζ) and antimicrobial activity, every month until the end of the study. Freeze-dried samples rehydration was performed by adding 1 mL of ultra-purified water to each individual liposomal paste before manual shaking for 1 min.

Antimicrobial activity assay

The antimicrobial activity was tested every month and determined by the Tetrazolium/formazan test (TTC) (Moussa et al. 2013). Briefly, 500 μL of fully hydrated liposomes was poured in 10 mL BHI medium containing 20 μL of indicator microorganisms (*S. Enteritidis* ATCC 13076 or *L. monocytogenes* ATCC 7644) in 10^8 cell mL^{-1} concentration. All flasks were incubated at 37°C with shaking for 3 h. Then, 1 mL from each sample containing treated and control cultures was added to tubes containing 100 μL of TTC sterile solution (5 mg mL^{-1}), and incubated again at 37°C until the red formazan was observed in control samples. As control, the bacterial strains were incubated without liposomes. The resulted formazan was centrifuged at 4000 *g* for 5 min, and then pellets obtained were suspended in ethanol 50% and centrifuged again using identical conditions. The red formazan solution was measured by spectrophotometer at 480 nm and the antimicrobial activity was calculated using absorbance (Abs) values, according to the following equation:

$$\% \text{ Activity} = (\text{Abs control} - \text{Abs sample}) / (\text{Abs control}) \times 100$$

Statistical Analysis

The results were analyzed by one-way ANOVA and Tukey means comparison test at 5% significance level, using the STATISTICA® program (StatSoft. Inc., Tulsa, USA) version 10.

Results and Discussion

Liposome characterization

Empty and GE/Nis loaded liposomes with different wall materials were produced and characterized. The hydrodynamic diameters of each liposome formulation are shown in Table 1. Formulations of PC, PC-CHO and PC-OA presented negative zeta potential with sizes of less than 100 nm for empty and load GE/Nis vesicles. As expected, the addition of oleic and cholesterol increase the zeta potential and liposome size. The mean diameter variations are related to changes in the membrane packing of vesicles (Lee et al. 2017) and the zeta potential increment are due to the presence of carboxylic groups from OA. Formulations with ODA presented positive zeta potential and the higher size of 155.5 nm and 177.6 nm for empty and loaded liposomes, respectively. These results are in agreement with the fact that ODA generally increases the size of nanovesicles (Mattheolabakis et al. 2013). No statistical difference was observed between the size or PDI values of load and empty liposomes with the same wall composition, indicating that the added antimicrobials had no influence on these parameters. Only the PC-ODA-GE/Nis showed a significant reduction of zeta potential value possibly by anionic compounds in the GE. In addition, formulations with CHO and ODA presented highest EE values for nisin, 90.7% and 94.7%, respectively. For allicin, samples showed EE values superior to 80%, with no significant differences among formulations. Considering the obtained results, parameters for liposomes produced with PC alone are in agreement with our previous report on liposomes

encapsulating GE/Nis (Pinilla and Brandelli 2016) and reports of liposome encapsulation of garlic juice (Rodríguez-Jimenes et al. 2014) and pure nisin (Lopes et al. 2019).

Induced lipid oxidation by TBARS assay

In Figure 1A are displayed the results of induced lipid peroxidation by hydroxyl radical on the liposome formulations. Cyclic peroxides (or endoperoxides) were determined spectrophotometrically at 512 nm, analyzing a pink chromophore resultant of the reaction between breakdown products (malondialdehyde) at elevated temperatures with thiobarbituric acid (TBA). As result, thiobarbituric acid reactive substances (TBARS) values were significantly higher in the liposomes prepared with only phosphate buffer, and no statistical difference was found between PC, PC-CHO and PC-ODA vesicles, indicating similar sensibility to oxidative radicals. However, the PC-OA liposomes showed about 36% less lipid oxidation as compared with the PC vesicles used as control. Similar antioxidant effect of OA was reported previously (Lee et al. 1998), which showed that OA addition to PC-liposomes act as $^{\bullet}$OH scavenger, delaying the production of lipid peroxides.

In contrast, when the antimicrobial mixture (GE/Nis) was incorporated into PC-liposomes, it was observed a reduction in the TBARS after induced peroxidation by $^{\bullet}$OH radical (Fig 1A), which is attributed to a GE compounds with free radical scavenging activity. Garlic radical scavenging properties are related to the presence of phenolic compounds and flavonoids as myricetin and apigenin (Aditya and Ko 2015). These compounds, that probably are located on the liposome surface, could capture radicals from the external aqueous phase and thus avoiding the liposome oxidation. Prevention of the initial radical attacks from the aqueous phase against the phospholipid membrane is essential to obtain its antioxidant protection; as free radicals can be constantly generated in the aqueous phase (Cotelle 2001). The best result was for the PC-OA loading GE/Nis that

reduced in 91% the lipid oxidation as compared with pure PC-liposomes, demonstrating high protection against oxidative radicals that can affect the lipid structure integrity, in agreement with the unloaded PC-OA liposomes. In addition, the PC-CHO-GE/NIS showed low TBARS values and this result could be explained due to the presence of cholesterol, increasing bilayer order and packing that makes the liposome membrane more resistant to attack of radicals from an external aqueous phase (Bhattacharya and Haldar 2000; Mosca et al. 2011).

The effect on ABTS$^{\bullet+}$ radical

The ABTS assay is a good tool for determining antioxidant activity of hydrogen-donating compounds and chain-breaking antioxidants (Nalinanon et al. 2011). Antioxidant activity of different liposome formulations loaded with GE/Nis was evaluated by ABTS assay and compared with the same formulations without antimicrobials (Fig. 1B). No significant difference (p>0.05) in radical scavenging activity was registered between the liposomal encapsulated GE/Nis and the free mixture (GE/Nis), indicating retention of the antioxidant properties after nano-encapsulation. The quantity of phenolic compounds and flavonoids in garlic extracts has a positive correlation with ABTS radical scavenging (Jang et al. 2017), due to electron donation from active hydroxyl groups present. Our findings indicate that garlic bioactive compounds with ABTS$^{\bullet+}$ scavenging properties were not affected by the encapsulation process and are in agreement with encapsulation efficiency results (Table 1), since thiosulfinates such as allicin also have scavenging activity and the antioxidant activity of garlic is not solely due to its phenolic compounds (Ghasemi et al. 2015). In contrast, some works reported a reduction of antioxidant activity of natural extracts due to degradation of phenolic compounds during the liposome production (Lopes de Azambuja et al. 2015; Marín et al. 2018a). However, this could be explained by the negative effect of some factors such

as pH, fabrication method and the presence of surfactants or cholesterol in the membrane composition, on the liposome radical scavenging activity (Tai et al. 2017).

Fourier transform infrared spectroscopy (FTIR)

Variations of several vibrational modes were evaluated to determine changes in the liposomal membrane composition after stabilizers and antimicrobials incorporation (Fig. 2). Groups located in specific lipid regions were observed, as follows: phosphate antisymmetric stretching vibration frequency (from 1260 to 1220 cm^{-1}); choline antisymmetric stretching vibrations around 970 cm^{-1}; carbonyl stretching mode frequency (from 1725 to 1740 cm^{-1}) of lipid interfacial region and the symmetric and antisymmetric stretching vibrations of the acyl chain methylene around 2850 and 2920 cm^{-1}, respectively. These vibrations are main groups related to phosphatidylcholine (Manrique-Moreno et al. 2009).

FTIR spectra of control PC-liposomes (Fig. 2A) showed characteristic bands of phosphatidylcholine groups. Remarkable changes can be seen in the liposome infrared spectra after incorporation of antimicrobials. In the PC-GE/Nis nanovesicles (Fig. 2A), it was observed a reduction in the *v* C=O ester bond stretch peak at 1735 cm^{-1} and changes in the v_s CH_2 v_{as} CH_2 at 2920 and 2850 cm^{-1} respectively. The v_{as} PO_{-2} band at 1238 cm^{-1} was shifted to 1228 cm^{-1}; and was also shifted the v_{as} CN^+C band from 977 to 936 cm^{-1}. In addition, the presence of –OH groups at the PC-GE/Nis liposomes surface was confirmed by the presence of a broad band from 3550 to 3300 cm^{-1}. Similar behavior of the C=O stretch reduction, shifted bands and presence of –OH groups was observed in the liposomes formulated with CHO and OA as wall materials after antimicrobials incorporation, as showed in Fig. 2B and 2C, respectively. Also, modifications were observed on shape and frequency values of the choline CN^+C band at 977 cm^{-1} in the PC, PC-CHO and PC-OA spectra (Fig. 2A, 2B and 2C).

The shifts of the v_{as} PO_{-2} and v C=O wavenumbers is indicative of a higher hydration degree of the phospholipid head group due to the increase of weak hydrogen bonds in the phosphate groups by water or other compounds (Sousa et al. 2013; Nogueira et al. 2018). Also, variations on the acyl chain methylene (2925 and 2852 cm^{-1}) reflect changes in the lipid group mobility and order parameters from hydrophobic region (Herec et al. 2007) and the alterations in the wavenumber in the v_{as} $CN^{+}C$ reflect the presence of water molecules that separate the choline from the neighboring lipid phosphate (Severcan et al. 2005).

Thus, our results confirmed that the antimicrobials influenced the polar, interfacial and hydrophobic groups of the PC affecting its conformation and mobility. Shifted values in the v_{as} P=O stretch and C=O groups have been reported as result of interactions between hydrogen bonds among the –OH group of disaccharides and the phosphate group of liposomes (Cacela and Hincha 2006; Chen et al. 2010). Similar modifications of the on FTIR patterns were presented by liposomes containing essential oil obtained from Brazilian cherry (*Eugenia uniflora L.*) using trehalose as lyoprotector (Yoshida et al. 2010).

On the other hand, PC-ODA liposomes showed absence of v C=O peak around 1735 cm^{-1} (Fig 2D) and low intensity in the 2920 and 2850 cm^{-1} bands, indicating low mobility of the acyl chain methylene and high the hydration degree of phospholipid head group. However, after the addition of antimicrobials, FTIR spectra presented similar behavior to the other formulations, but without shifted bands, indicating a different level of membrane hydration caused by the ODA and mixture GE/Nis characterized by low membrane mobility.

Physical and thermal properties of the freeze-dried liposomes

Physicochemical properties of freeze-dried liposomes depend on several factors such as the properties of encapsulated compound, lipid composition and the type of lyoprotectant used (Ingvarsson et al. 2011). The effect of CHO, OA, ODA, antimicrobials and lyoprotectant on

the physical and thermal properties of PC liposomes were investigated and are shown in Table 2. The water activity (a_w) is related to interactions between powder components and water, being commonly used as parameter to measure the stability of dried products during storage (Sun-Waterhouse et al., 2015). All freeze dried samples presented a_w values between 0.2 and 0.3, close to the recommended limit to ensure powder stability (<0.30). Water activity values between 0.129 and 0.496 with low powder recovery were obtained in spray-dried garlic juice, prepared with Arabic gum and DE-10 maltodextrins as wall material (Rodríguez-Jimenes et al. 2014). According to the authors the low output air temperatures was the principal factor that affected the garlic powder a_w.

Residual water content is a good indicator of the primary quality control for freeze-dried cakes (Chen et al., 2010). Samples moisture content ranged from 1.34 to 10.49%. In the PC, PC-CHO and PC-ODA formulations, a significant moisture reduction was observed when the antimicrobials mixture (GE/NIS) was added, and especially when the lyoprotector (TREA) was present, indicating the facility of bonding water molecules were highly depend on the composition. Hence, it can be assumed that the competition between water and sugar molecules to interact with the phospholipid polar head groups determines the freeze-dried liposomes stability (Chen et al. 2010). Similar results were observed by Luzardo et al. (2000), where the amount of tightly bound water decreased significantly with the lyoprotectant addition, indicating that water molecules had been partially replaced by the applied excipient mediated by hydrogen bond formation with the PC (Crowe et al. 1996), reducing the liposomes moisture after freeze drying process. A different behavior was observed in the formulations with OA, where the moisture values increased with the antimicrobials and lyoprotector addition, as well as the hygroscopicity. These results indicate a saturation of OA between PC molecules reducing the number of weak H-bonds available for the TREA and the garlic compounds, so these compounds were possibly

located on the liposome surface providing an abundance of hydroxyl groups available for hydrogen bond formation.

The hygroscopicity values ranged from 9.22 and 22.81 g water/100 g dry matter (Table 2), indicating a hygroscopic behavior in all formulations. Empty PC and PC-ODA liposomes had the lower hygroscopicity values, 9.39 and 9.22 g water/100 g dry matter, respectively. This is related to their high moisture values before the test (Table 2), indicating bilayer saturation by water molecules weakly linked to the PC. However, high hygroscopicity values were observed in the liposome powders, probably due to presence of hydrophilic compounds and available hydrogen bonds in the polar lipid head. Hygroscopicity reduction was reported for PC liposomes loaded with essential oil of *Eugenia uniflora* L. by the addition of TREA as lyoprotectant (Yoshida et al. 2010). In contrast, our results showed no significant difference was found in the hygroscopicity values between the GE/NIS loaded liposomes in presence or absence of TREA, indicating that the GE/Nis are available to form weak H-bonds in the bilayer but not replace water upon dehydration.

Differential scanning calorimetry (DSC) is commonly applied to study the effects of stabilizers on dehydrated liposomes and permit a determination of the phase transition temperature (*T*m), which reflects the transition of the lipid bilayer molecules from a hindered, all-*trans* hydrocarbon chain conformation to a phase where there is increase in the rotational freedom of the lipid (Ingvarsson et al. 2011). The liposome powders *T*m values ranged from 32.59°C to 79.73°C (Table 2). The PC liposomes *T*m was located at 62.15 ± 1.14°C and this value was reduced to 53.32 ± 1.34°C and 41.75 ± 0.92°C (Fig.4A) by the presence of CHO and OA, respectively. These *T*m values were reduced even more when present the antimicrobial mixture and the lyoprotector, indicating that the single mixture GE/Nis affect the membrane mobility as well as the addition of CHO, OA and TREA. These

*T*m reductions are possibly due to these compounds which increase the spacing between the hydrocarbon chains by intercalation, resulting in decreased van der Waals interactions between the hydrocarbon chains (Ingvarsson et al. 2011). The thermal properties of GE on PC/OA liposomes was discussed in a previous work (Pinilla et al. 2019), where DSC patters of full hydrated PC-OA-GE vesicles showed a reduction of *T*m value with an increase of enthalpy indicating increase in membrane rigidity which follow a similar mechanism of the sugars used commonly as lyoprotectors. Regardless to this effect, is also well known that the addition of disaccharides such as trehalose depresses the liposomes *T*m (Doxastakis et al. 2005). This depression of *T*m exerted by stabilizers is an important factor, especially for liposomes composed of lipids with low *T*m, changing the degree of membrane fluidity, which is directly related to the liposome retention capacity (Chen et al. 2010). The ODA showed a different behavior, increasing the *T*m value to 72.17 ± 1.02°C (Fig.4D), this could indicate that ODA incorporation in the lipid bilayer result in a ordering of lipids in the bilayer, favoring the solid state (Pamunuwa et al. 2016).

Long term stability of liposomes containing GE/NIS

Liposome stability during dehydration and in the dry state is dependent not only on the stabilizer used, but also on the production parameters, the lipid composition, the storage conditions and the properties of the incorporated compound (Ingvarsson et al. 2011). Storage stability of liposome formulations was monitored during 5 months at 4°C, in order to verify the influence of the wall composition in their physical and bioactive properties. Three conditions were studied, namely liposomes in liquid suspension, freeze-dried without TREA and freeze-dried with TREA. Changes in particle size and polydispersity index (PDI) are presented in Fig. 3. It could be observed that, in liquid suspension (Fig. 3A), a significant change of liposome size occurred after 5 months with increments of 2-, 5- and 3-fold for

CHO, OA and ODA formulations respectively, as compared with the initial size. The PDI values remained below 0.35 in all formulations. The liposome storage at low temperature delays the oxidative degradation of unsaturated fatty acids in the lipid bilayer, causing low permeability of coating layers, as well as inhibition of aggregation by low molecular mobility (Gibis et al. 2014). The zeta potential values showed no significant changes during storage period and the antimicrobial activity against *L. monocytogenes* remained at 100% (data not shown). However, activity against *S. Enteritidis* decreased gradually until last month in all liposome formulations, which indicate a loss by oxidation of garlic reactive compounds such as allicin, responsible by the synergetic effect of nisin-garlic extract activity against Gram-negative bacteria (Pinilla and Brandelli 2016). Liposomes in full hydrated state are subjected to high concentrations of reactive oxygen species that can affect their physical and chemical stability (McClements 2015). The peptide fraction from cooked shrimp muscle with antioxidant and ACE-inhibitory activities was encapsulated into PC liposomes and the use of ε-polylysine as stabilizer provided increased stability and antimicrobial activity against food pathogens like *L. monocytogenes*, *S. aureus*, *E. coli* and *Yersinia enterocolitica* (Alemán et al. 2016).

The characterization of freeze-dried samples without lyoprotectant is depicted in Figure 3B. It was observed an increment in size and PDI values for all formulations after re-hydration. Liposome size and PDI significantly increased after freeze-drying, in accordance with literature (Sebaaly et al. 2016). Highest increment was found in the PC-OA-GE/NIS liposomes, which in five months had a size increase from 201.1 to 3253.1 nm and PDI from 0.20 to 0.57. The liposomes with CHO and ODA increase 5- and 8-fold after 5 months. The zeta potential presented low changes and the antimicrobial activity followed same trend of the liposomes without lyophilization, showing 100% of activity against *L. monocytogenes* and a gradual reduction of the activity against *S. Enteritidis* during the 5 months.

Nevertheless, the PC-OA-GE/NIS liposomes retained 22.5% of its initial activity against *Salmonella*. Important changes in size diameter are well known in freeze-dried liposomes due to the fusion or aggregation produced by the membrane changes during drying process that can affect liposome morphology after hydration (Chen et al. 2010). Liposomes containing OA showed important size changes after freeze-drying, and this could be related to lamellar phase destabilization. In a recent work, using small-angle X-ray scattering, Godoy et al. (2015) observed changes in the microstructure of soybean lecithin-OA membranes due to cubic structures formation, which can result in a swelling potential increment.

The addition of TREA to freeze-dried liposomes reduced the fusion effect comparing with the freeze-dried samples without TREA in PC-OA-GE/Nis and PC-ODA-GE/Nis (Fig. 3C). The parameters PDI, zeta potential and antimicrobial activity have undergone a few changes as compared to freeze-dried samples without lyoprotectant in PC-CHO-GE/Nis liposomes. Cholesterol in liposome bilayers exerts an ordering effect forming a liquid-ordered phase that reduces the freeze damage (van Winden, 2003). PC-ODA-GE/Nis liposome size changed from 177.5 nm to 256.1 nm, indicating a high ordered and hydrated bilayer that permit a better TREA-water replacement, increasing the lyoprotector effect in freeze-dried state.

As demonstrated above by DSC analysis, TREA reduced the membrane mobility, decreasing the head group spacing between lipids during dehydration and fusion, in agreement with the water displacement theory. Regarding the antimicrobial activity, freeze-dried liposomes with TREA also lost their effect against *S. Enteritidis* during the time with no significant differences with the other two treatments (data not shown). However, it is noteworthy that the antimicrobial activity decreases as the liposome fusion and aggregation increase.

There are few reports on encapsulation of complex mixtures of bioactive compounds into liposomes, their properties and stability after freeze-drying process. An increase in the average diameter of PC liposomes containing different food waste hydrolysates and fruit extract after freeze drying has been reported (Marin et al. 2018b). Opposing to our results, a decrease in diameter and zeta potential parameters of freeze-dried liposomes loaded with food waste materials was observed after 7 months storage (Marín et al. 2018a). This could be due to the use of partially purified PC rich in tocopherols and the properties of encapsulated compounds, which improve their stability.

Conclusions

The GE/Nis incorporation in PC-CHO, PC-OA and PC-ODA liposomes was achieved and the stabilizers increase the liposomes physical stability in terms of mean particle size and zeta potential. The formulations containing OA or CHO plus the antimicrobials presented a synergetic effect in the reduction of induced lipid oxidation and also improved thermal properties of PC liposomes, increasing membrane order and rigidity. Considering storage stability, the freeze-drying process increased fusion and aggregation in all liposome formulations, but this effect was reduced using trehalose as lyoprotector. However, only the liposomes prepared with OA presented improved antimicrobial activity as compared with full hydrated liposomes. Therefore, the upgraded resistance to lipid oxidation by OA added to retention of bioactive properties after freeze drying during storage, suggest that OA have great potential as stabilizer of liposomes containing complex mixtures of bioactive compounds.

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Conflicts of interest

Authors declare no conflicts of interest regarding this manuscript.

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Figure Legends

Fig. 1. Antioxidant activity of empty liposomes and liposomes encapsulating nisin/garlic extract (GE/NIS) measured by TBARS (A) and ABTS radical scavenging assay (B). PC, phosphatidylcholine liposomes; PC-CHO, formulations with cholesterol; PC-OA, formulations with oleic acid; and PC-ODA, formulations with octadecylamine. Bars are the means ± standard deviation of three independent experiments. Different letters represents significant differences ($p<0.05$).

Fig. 2. FTIR spectra of control and GE/Nis-loaded liposomes. (A) PC and PC-GE/NIS liposomes, (B) PC-CHO and PC-CHO-GE/NIS liposomes, (C) PC-OA and PC-OA-GE/NIS liposomes, and (D) PC-ODA and PC-ODA-GE/NIS liposomes.

Fig. 3. Particle size (columns) and PDI (lines) of liposomes during storage at 4°C. (A) Liquid liposomes suspension, (B) lyophilized liposomes, (C) lyophilized liposomes with trehalose as lyoprotectant. Formulations: PC-CHO-GE/Nis = black column and square symbol; PC-OA-GE/Nis = grey column and triangle symbol; PC-ODA-GE/Nis = light grey column and circle symbol. Values are the means ± standard deviation of three independent experiments.

Fig. 4. DSC analysis of freeze dried liposomes. (A) Line 1: PC; Line 2: PC-GE/NIS; Line 3: PC-TREA-GE/NIS. (B) Line 1: PC-CHO; Line 2: PC-CHO-GE/NIS; Line 3: PC-CHO-TREA-GE/NIS. (C) Line 1: PC-OA; Line 2: PC-OA-GE/NIS; Line 3: PC-OA-TREA-GE/NIS. (D) Line 1: PC-ODA; Line 2: PC-ODA-GE/NIS; Line 3: PC-ODA-TREA-GE/NIS.

Table 1. Characterization of PC liposomes load with the mixture of nisin and garlic extract produced with cholesterol (CHO), oleic acid (OA) or octadecylamine (ODA) as wall stabilizers.

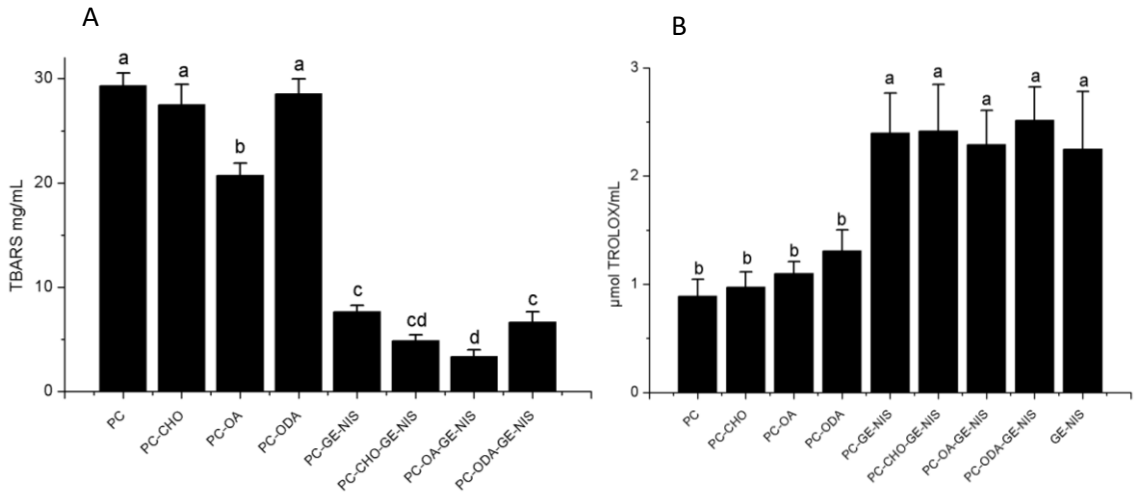
Sample	Size (nm)	PDI	ζ potential (mV)	EE Allicin (%)	EE Nisin (%)
PC	86.6 ± 5.2^{b}	0.22 ±0.01^{a}	-11.6 ± 5.8^{d}	-	-
PC-CHO	93.4 ± 3.6^{b}	0.17 ±0.03^{b}	-20.5 ± 6.8^{d}	-	-
PC-OA	65.6 ± 2.1^{c}	0.16 ±0.01^{b}	-34.8 ± 3.4^{c}	-	-
PC-ODA	155.5 ± 9.6^{a}	0.21 ±0.02^{a}	+29.6 ± 5.9^{a}	-	-
PC-GE/NIS	95.1 ± 4.7^{b}	0.23 ±0.02^{a}	-15.59 ± 5.9^{d}	85.1 ± 3.3^{a}	80.7 ± 2.4^{c}
PC-CHO-GE/NIS	94.0 ± 3.2^{b}	0.24 ±0.04^{a}	-24.2 ± 4.0^{d}	83.9 ± 2.7^{a}	90.7 ± 1.2^{b}
PC-OA-GE/NIS	69.1 ± 7.3^{c}	0.16 ±0.02^{b}	-28.2 ± 6.1^{c}	85.2 ± 3.1^{a}	80.7 ± 3.0^{c}
PC-ODA-GE/NIS	177.6 ± 12.4^{a}	0.19 ±0.01^{a}	+19.7 ± 3.8^{b}	82.1 ± 1.5^{a}	94.7 ± 1.6^{a}

Values are the means ± standard deviation of three independent experiments. Different superscript letters within each column represents significant differences (p<0.05).

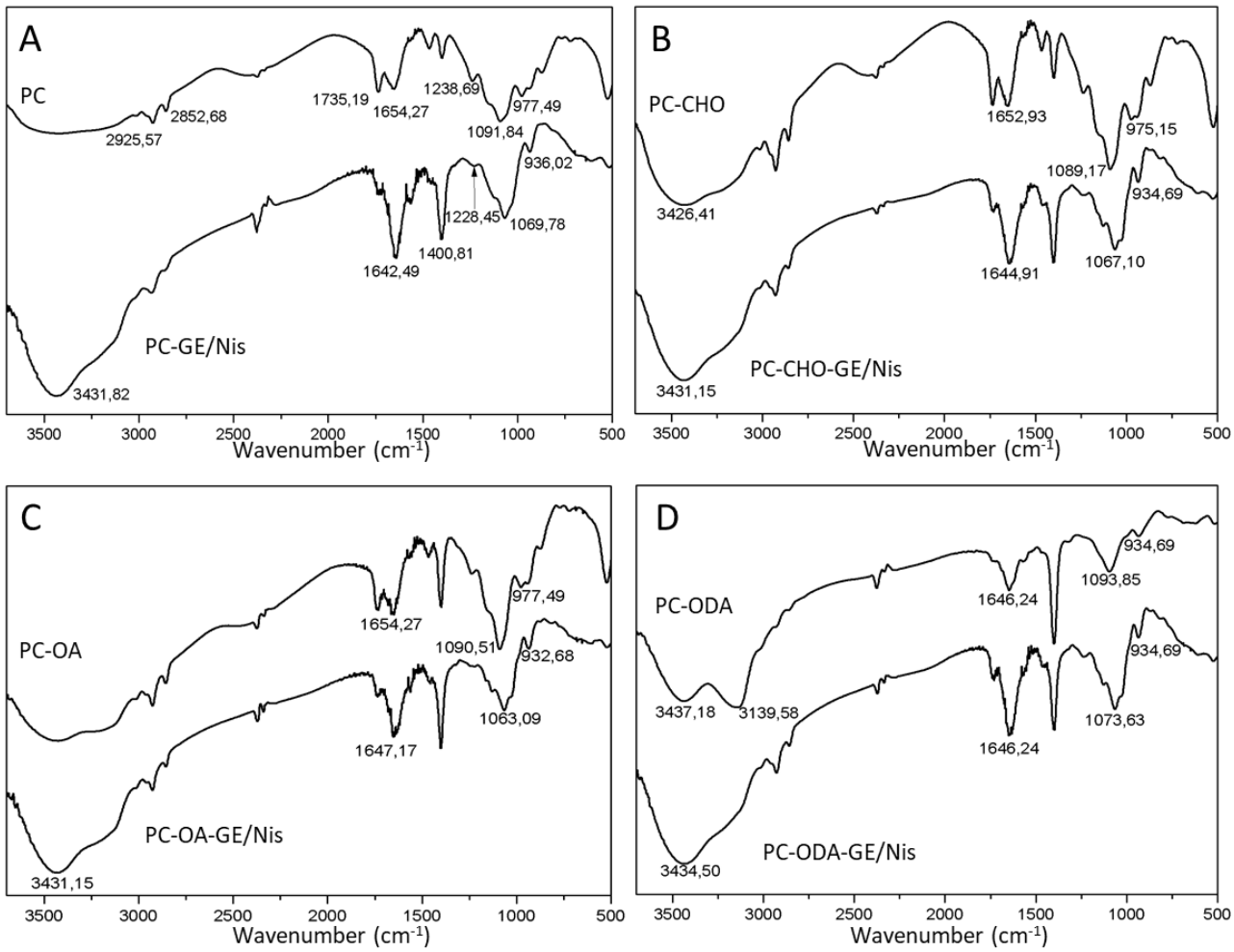
Table 2. Physical and thermal properties of different freeze-dried liposomes formulations.

Liposome formulation	a_w	Hygroscopicity (%)	Moisture (%)	T_m (°C)
PC	0.304 ± 0.005^{a}	9.4 ± 0.3^{ef}	7.3 ± 0.4^{b}	62.2 ± 1.1^{bc}
PC-GE/NIS	0.201 ± 0.003^{f}	22.8 ± 0.2^{a}	4.7 ± 0.3^{c}	37.9 ± 0.7^{d}
PC-TREA-GE/NIS	0.248 ± 0.006^{d}	22.4 ± 0.1^{a}	1.6 ± 0.1^{e}	32.6 ± 2.2^{e}
PC-CHO	0.279 ± 0.001^{b}	11.4 ± 0.6^{e}	2.4 ± 0.1^{d}	53.3 ± 1.3^{c}
PC-CHO-GE/NIS	0.238 ± 0.002^{e}	18.1 ± 0.6^{b}	1.9 ± 0.1^{de}	48.2 ± 1.1^{c}
PC-CHO-TREA-GE/NIS	0.269 ± 0.002^{c}	16.5 ± 0.3^{c}	1.3 ± 0.1^{e}	47.0 ± 0.3^{c}
PC-OA	0.221 ± 0.001^{e}	14.7 ± 0.3^{d}	1.5 ± 0.1^{e}	41.7 ± 0.9^{cd}
PC-OA-GE/NIS	0.225 ± 0.004^{e}	16.1 ± 0.3^{c}	2.0 ± 0.1^{de}	35.3 ± 0.7^{d}
PC-OA-TREA-GE/NIS	0.268 ± 0.003^{c}	19.5 ± 0.2^{ab}	3.6 ± 0.0^{d}	34.6 ± 0.8^{de}
PC-ODA	0.288 ± 0.003^{b}	9.2 ± 0.4^{ef}	10.5 ± 0.7^{a}	72.2 ± 1.0^{ab}
PC-ODA-GE/NIS	0.220 ± 0.001^{e}	18.4 ± 0.1^{b}	5.5 ± 0.3^{c}	79.7 ± 1.9^{a}
PC-ODA-TREA-GE/NIS	0.272 ± 0.002^{b}	18.7 ± 0.3^{b}	3.4 ± 0.1^{d}	71.8 ± 0.9^{b}

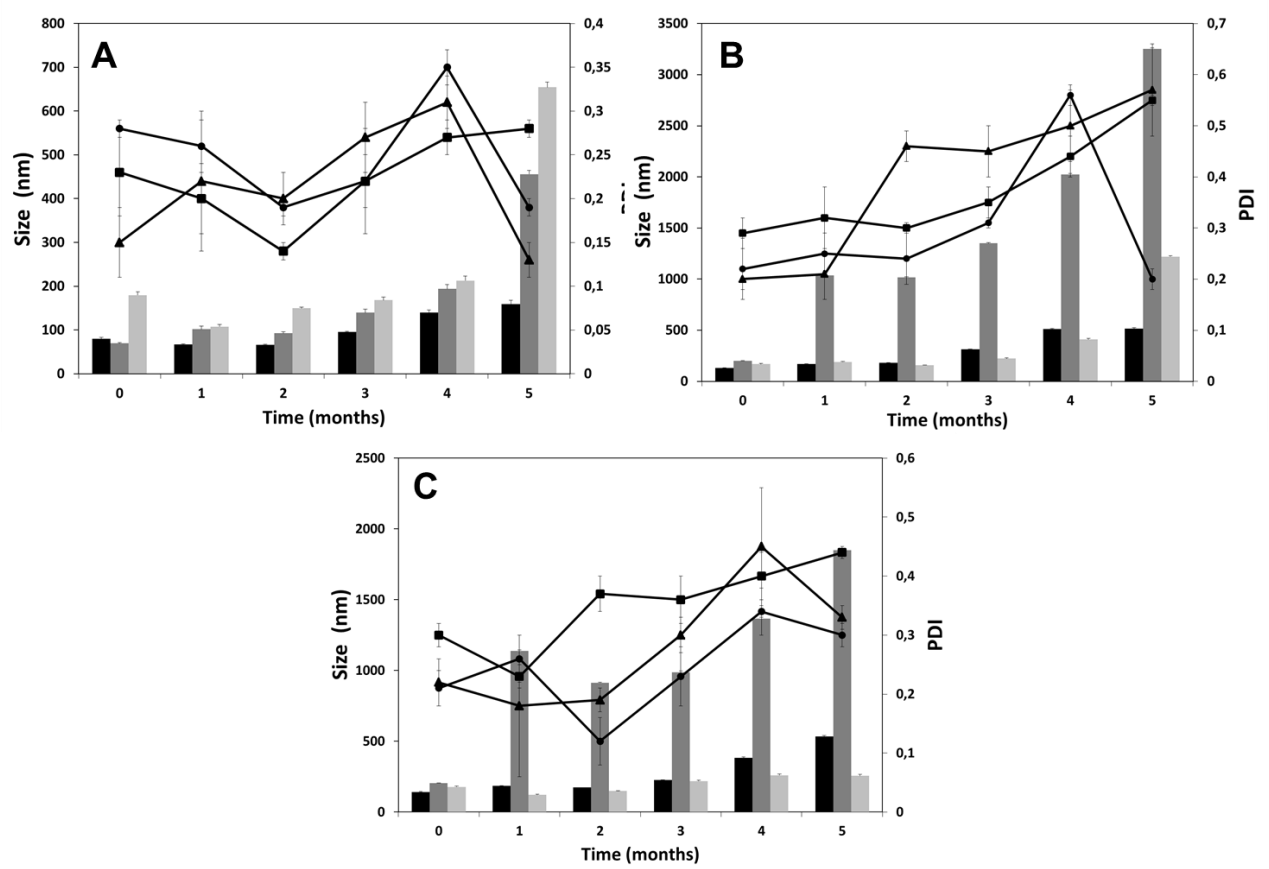
Values are the means ± standard deviation of three independent experiments. Different superscript letters within each column represents significant differences ($p<0.05$).



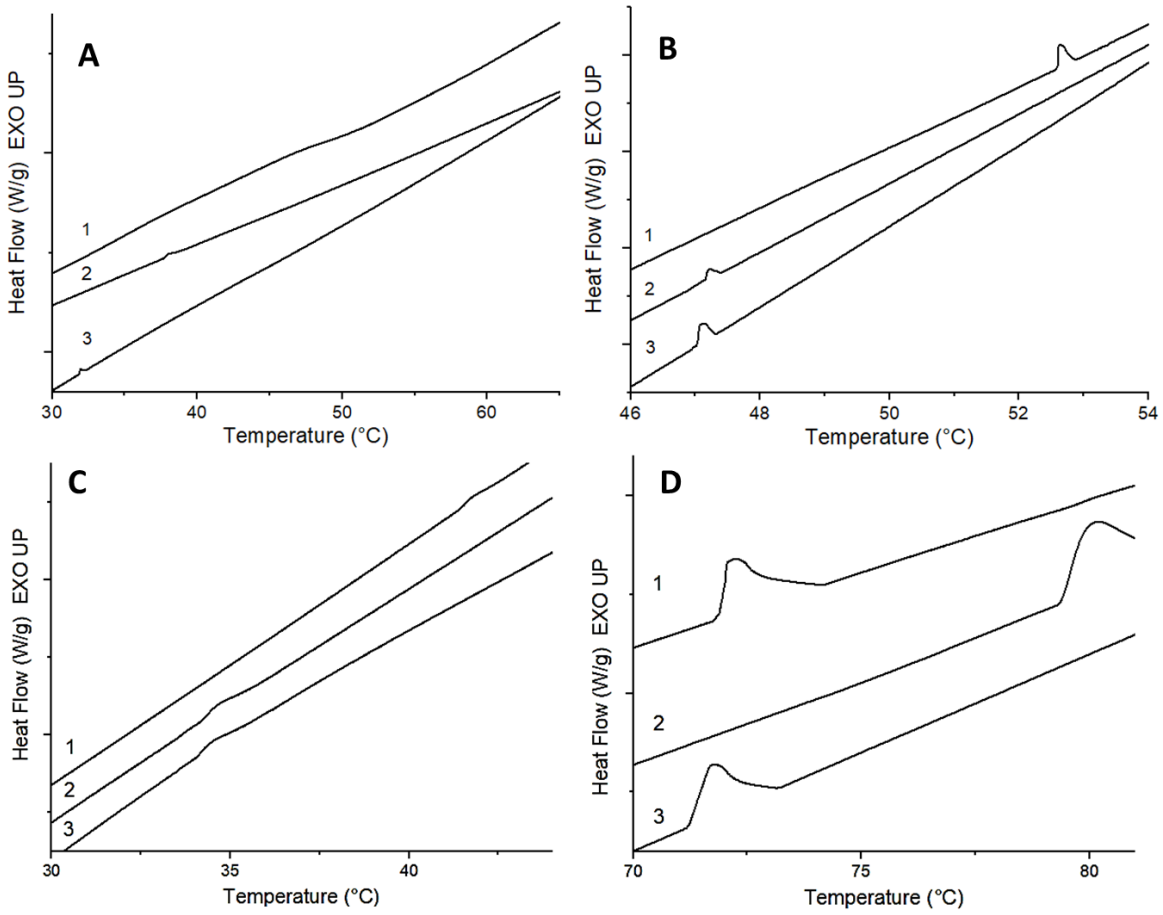
Pinilla et al., Fig. 1



Pinilla et al., Fig. 2



Pinilla et al., Fig. 3



Pinilla et al., Fig. 4

5. Artigo No.2

Antifungal properties of phosphatidylcholine-oleic acid liposomes encapsulating garlic extract against environmental fungal in wheat bread

Resumo:

Foram desenvolvidos lipossomas de extrato de alho (GE) encapsulado em fosfatidilcolina (PC) e ácido oleico (OA). Vesículas esféricas com distribuição de tamanho estreita, eficiência de aprisionamento de 79,7% e potencial zeta de -27,9 mV foram obtidas. O teste antifúngico in vitro mostrou atividades inibitórias perceptíveis para GE livre e encapsulado contra cepas de fungos selecionadas. A análise termogravimétrica revelou que a presença de OA e GE na formulação melhorou a estabilidade térmica dos lipossomas, em comparação com os lipossomas de PC puro. A análise DSC mostrou alterações na temperatura de transição principal e na entalpia para lipossomos PC-OA-GE, devido a um forte efeito rigidificante induzido pelo calor. Os testes de vida útil mostraram o potencial biopreservador dos lipossomas PC-OA-GE no pão de trigo. As formulações com GE livre e encapsulado a 1% (V/W de farinha de trigo) foram microbiologicamente estáveis por mais tempo em comparação com o controle, mesmo após o cozimento a 220 ° C. Os lipossomas formulados com OA e GE têm potencial como agente antifúngico em produtos de panificação.

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Antifungal properties of phosphatidylcholine-oleic acid liposomes encapsulating garlic against environmental fungal in wheat bread

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Abstract

Liposomes have gained great interest in the food and pharmaceutical industry as colloidal carriers of bioactive compounds. In this work, liposomes of phosphatidylcholine (PC) and oleic acid (OA) encapsulating garlic extract (GE) were developed to determine its aptitude as antifungal agent in wheat bread. The influence of GE on the properties of liposomes were followed by determination of size, Zeta potential, Fourier transform infrared patterns (FTIR), morphology, differential scanning calorimetry (DSC) and thermogravimetric (TGA) techniques. The produced PC-OA-GE liposomes showed spherical morphology with narrow size distribution, entrapment efficiency of 79.7% and zeta potential of -27.9 mV. *In vitro* antifungal test showed noticeable inhibitory activities for free and encapsulated GE against selected fungal strains. TGA analysis revealed that the presence of OA and GE in the formulation retards the liposomal thermal decomposition, as compared with the pure PC liposomes and the DSC enthalpy and main transition temperature variation in PC-OA-GE liposomes, suggested a strong heat-induced rigidifying effect that could be attributed to the presence of garlic polysaccharides in the liposome surface, observed by FTIR. In the *in situ* test, the breads formulations with free or encapsulated GE (0.65 mL/100g of dough) were microbiologically more stable as compared with the controls, showing mold inhibition for five days. Therefore, the liposomes formulated with OA and GE showed potential as natural antifungal agent in bakery products.

Key words: antimicrobial; biopreservative; natural compounds; nanobiotechnology

1. Introduction

Bread is an important food constituent of the diet in many countries and is consumed daily almost over the world. Bread spoilage by microorganism is a serious concern for consumers and the resulting waste is also a problem that causes high economic losses in bakery industry and consumers (Melikoglu and Webb, 2013). Bacteria, yeast and moulds can cause spoilage of bread. However, contamination originates mainly post baking by fungal spores being deposited from the bakery environment (Spicher, 1980). The principal factors to controlling the growth of spoilage fungi on food stuffs are oxygen, temperature, pH, and water activity (a_w); generally, breads have relatively high moisture content and a_w between 0.94-0.97 at a pH of about 6 on sliced, prepacked and wrapped breads being very susceptible bakery products for fungal spoilage (Hager et al., 2012). Apart from the unpleasant sight of visible mould growth, fungi can also produce of mycotoxins and off-flavors (Magan et al., 2003). Thus, spoiled breads represent a hazard and can cause health problems to the consumer.

The use of biopreservatives in food has become an increasingly important field of research. This trend is partially driven by the emergence of an increasing number of reports linking chemicals in foods with the development of chronic diseases (Tajkarimiet et al., 2010; Seow et al., 2014). Additionally, this term can also be used in bio-active plant ingredients or plant extracts as alternative to the weak organic acids such as propionic and sorbic acids, which are commonly added as chemical preservatives to prevent the growth of unwanted microorganisms and improve the shelf life of bakery products (da Cruz-Cabral et al., 2013). Nevertheless, the disadvantages associated with the use of chemical preservatives, has motivated the research of alternative natural agents to control spoilage fungi in food products.

Garlic (*Allium sativum*) has been known for centuries as important food flavoring agent with recognized medicinal properties (Martins et al., 2016). Several studies revealed

that garlic has antimicrobial activity against bacteria and fungi (Borlinghaus et al., 2014; Fratianni et al., 2016; Pinilla et al., 2017). The principal constituents of garlic associated to its bio-active properties are the allicin, ajoene, thiosulfinates and others organosulphurate compounds (Ledezma and Apitz-Castro, 2006). It is related that allicin and thiosulfinates, the compunds responsibles for the strong flavor of garlic, can decompose under different temperature and pH conditions to form additional sulfur constituents that include diallyl, methyl allyl, and diethyl polysulfides, the vinyldithiins and also the antimicrobial compounds (E)- and (Z)-ajoene (Rose et al., 2005). The sensitive character and especially the strong odor of garlic compounds, restricts its application in food products and only a few technological approaches have been reported to overcome this limitation (Wang et al., 2012).

In this context, the use of nanocarrier systems like liposomes may stabilize different bioactive compounds against environmental and chemical changes as well as increase their bioavailability and stability, improving its shelf-life (Mozafari et al., 2008). Liposomes are well known as spherical bilayer with an aqueous core inside. This vesicles are made by phospholipid membranes with amphiphilic characteristics and present multiple advantages such as biodegradability, biocompatibility and controlled release behavior of water-soluble, lipid-soluble, and amphiphilic compounds (Neethirajan and Jayas, 2011; Brandelli et al., 2017). The antimicrobial activity of liposomes containing garlic extract (GE) was related in our previous work (Pinilla et al., 2017) and it was possible to conclude that liposomes constitute a suitable system for encapsulation and controlled release of garlic active compounds with inhibitory effect against *Listeria* spp.

The present study aimed to produce phosphatidylcholine-oleic acid liposomes as carrier for GE and investigate its potential as inhibitor of spoilage fungi in wheat bran.

Therefore, the physicochemical and morphological characteristics of PC-OA-GE liposomes were investigated as well the antifungal proprieties through both *in-vitro* and *in-situ* assays.

2. Materials and methods

2.1. Garlic extracts production

Preparation of garlic extract (GE) was carried out according to our previous work (Pinilla and Brandelli, 2016). Briefly, selected bulbs of garlic acquired from a local market at Porto Alegre (Brazil) without injuries or contamination were washed with purified water; then were cut in small pieces and mixed in a ratio 1:1 (w/v) with distillated water. The garlic juice (50%, w/v) was sonicated at 40 kHz for 15 min in a bath-type ultrasound (Unique USC 700, São Paulo, Brazil) at room temperature and centrifuged 10 min at 10,000 *g* under refrigeration at 4^{o}C. Thereafter, the supernatant obtained was filter sterilized with 0.22 μm membrane (Sartorius, Göttingen, Germany) and stored at 4°C for further use.

2.2. Preparation of liposomes

The GE liposomes were produced according to Pinilla and Brandelli, (2016) by the thin-film hydration method with some modifications. Briefly, purified phosphatidylcholine (PC) Phospholipon 90G® (Lipoid, Ludwigshafen, Germany) and oleic acid (Labsinth, São Paulo, Brazil) molar ratio (3:1) were dissolved in mixture chloroform/methanol (1:1 v/v) in a round-bottom flask, the solvent was recover by rotary evaporator at 40°C, until obtaining a thin film on the flask wall and finally dried in desiccator for 24 h. The obtained lipid film was recovery by the addition of 4 ml of GE and 2 ml of 10 mM phosphate buffer (pH 7), then vortexed at 50°C in three times for 30 s, sonicated in an ultrasonic cell disrupter (Model DES500, Unique Group, São Paulo, Brazil) and finally sterilized by filtration through 0.22 μm membranes (Sartorius, Göttingen, Germany). In addition, empty liposomes with and

without oleic acid (OA) were prepared with 10 mM phosphate buffer pH 7.0 and used as control.

2.3. Size, polydispersity, zeta potential and morphological characterization

The size of and polydispersity index (PDI) of nanovesicles was determined by dynamic light scattering essentially as described elsewhere (Teixeira et al., 2008; Lopes et al, 2017). The measurements of size and PDI were made using a Brookhaven system (He-Ne laser of 35 mV) in a fixed scattering angle of 90°. The zeta potential analyses of liposomes were carried out after dilution (1:100) on purified water, using a Zetasizernano-ZS ZEN 3600 (Malvern Instruments, Worcestershire, UK), the mean value was determined by repeating the reading by 10 times. The vesicles morphology was observed by transmission electron microscopy (TEM). Each liposome sample was stained in 25 g/L uranyl acetate on a copper grid with formvar-coated of 300 mesh. The stained and dried samples were visualized on an electron microscope (JEM1200, Jeol Ltd., Tokyo, Japan).

2.4. Entrapment efficiency

The encapsulation efficiency (EE) of PC-OA-GE liposomes was determined considering the allicin as main component of the GE. The HPLC method for allicin described by Liang et al. (2013) was employed, using ethylparaben as secondary reference standard for allicin. Freshly prepared samples of PC-OA-GE liposomes were separated from the liquid media by membrane ultrafiltration (Ultracel YM-10; Millipore, MA, USA) and the filtrate without liposomes was used for determination of total allicin concentration by HPLC. The EE values were calculated by the equation:

$$\%EE = \frac{[\text{Allicin used in preparation}] - [\text{Allincin in the filtrate}]}{[\text{Allicin used in preparation}]} \times 100$$

The chromatographic analyses were performed in a HPLC (Shimadzu HPLC, Kyoto, Japan) equipped with quaternary pumps (LC-20AD) and diode array detector (DAD) (SPD-M20A), using an XBridge® C18 (150 mm 4.6 mm, 5 mm) column, with a isocratic flow (0.7 mL/min) of methanol:water (65:35 v/v) acidified with formic acid (0.04%, v/v) for 15 min. The column temperature was maintained at 20°C and the volume injected in the equipment was 10 µl for sample. Mobile phases were filtered using 0.22 µm nylon filters and degassed previous to use.

2.5. Fungal inhibition assays

For determination of antifungal activity, the selected fungal strains *Penicillium expansum*, *Aspergillus niger, Penicillium herquei, Fusarum graminearum* and *Aspergillus. flavus* were previously cultured on PDA plates for 5 days at 30°C. A sterile solution of Tween 80 at 0.05% (v/v) was transferred on each plate and the spores harvested with a Drigalski loop. This suspension was collected and the concentration of spores was determined and adjusted with sterile distilled water until reaches 1×10^6 spores/mL using a Neubauer chamber. For antifungal activity tests, a solution of sterile PDA with 1% of this spore suspension was homogenized at 45°C and transferred to a sterile plate. After solidification of the media, sterile paper disks were placed on the agar plates and then 20 µL of each sample was transferred to the disks. The diameters of inhibition halos were measured after incubation for three days at 30 ± 2°C.

2.6. FTIR Analysis

In order to investigate the possible alterations occurred in phospholipid membranes, the FTIR spectra of the liposomes and its components were acquired, using a Bruker Alpha FTIR spectrometer (Bruker, Billerica, MA, USA) in the range of 4000 to 400 cm^{-1}. The

spectral resolution of the spectrometer is 4 cm^{-1}. Samples of PC, PC-oleic acid and PC-OA-GE liposomes, were freeze dried before the analysis, mixed with KBr and then pressed into a tablet.

2.7. TGA and DSC analyses

The thermal stability evaluation was carried out in a thermogravimetric analyzer model TGA Pyris 1 (Perkin Elmer, Shelton, USA). The lyophilized samples of liposomes were heated from 25°C to 600°C at the rate of 20°C min^{-1} under nitrogen atmosphere (flow rate 40 ml/min). Differential scanning calorimetry (DSC) studies were developing in a DSC Q 8500 apparatus (Perkin Elmer, Shelton, USA). Liquid samples were pre- concentrated in a factor of 1:3 using a Ultracel YM-50 Membrane (Millipore), then approximately 10 mg were placed in aluminum pans and heated from -30 to 100 °C with a heating rate of 10 °C/min under nitrogen atmosphere. A clean and empty pan sealed with it cover was used as a reference sample.

2.8. Bread preparation and in situ antifungal activity

Bread was manufactured at Cereal Laboratory of Food Science and Technology Institute (Porto Alegre, Brazil) and all the bread ingredients were obtained from local suppliers of bakery products. The loaf bread recipe included 500g wheat flour (Orquidea, Caxias do Sul, Brazil), 15g vegetal oil (Klemm & Cia, Santa Cruz do Sul, Brazil), 10g baker yeast (Fleischmann, Petrópolis, Brazil), 10g NaCl, 25 g sucrose, 0.045g ascorbic acid (Granolab, Araucaria, Brazil), 300 mL tap water and 5 mL of free or liposome encapsulated GE to achieve a concentration of 0.65 mL/100g of dough. The ingredients were kneaded for 5 min, then the bread dough was maintain resting for 10 min and cut in pieces of 165g before fermentation at 30°C for 90 min. Baking was performed at 220°C for 20 min in a deck oven

(Tadesco ITT150E, Caxias do Sul, Brazil). The loaves were kept for 60 min on cooling racks at room temperature. Each bread was cut in 3 slices with approximately 10 cm high by 3 cm wide. The slices were exposed to the laboratory environment for 5 min and after that, packet in plastic bags, which were closed and stored at room temperature during 15 days. Every day until 15 day of analysis, the bread slices were examined to determine the visible fungal growth and to estimate if shelf life was improve or not. The breads were baked in triplicate, and the slides of the each assay were analyzed twice. The shelf life of breads was monitored using the mold environmental challenge method, based on the analysis of the fungal outgrowth as a percentage of the total area of each bread slice (Axel et al., 2015).

2.9. Statistical analysis

Results are presented as average ± SD. Statistical analysis were carried out by Tukey test, using SSPS software package 16.0 (IBM SPSS Statistics, Inc., Chicago, IL, USA). and difference between groups were considered significant at $P < 0.05$

3. Results and discussion

3.1. Liposome characterization

Different liposome formulations prepared by the thin film method were characterized. Garlic extract loaded PC-OA liposomes differ ($P<0.05$) with the liposomes without GE, in the parameter of particle size, polydispersity index (PDI) and zeta potential (Table 1). The empty PC-liposomes presented higher size and lower zeta potential than the PC-OA and PC-OA-GE liposomes that showed the narrow size distribution (110–140 nm), similar to previous reports of PC-OA liposomes (Srisuk et al., 2012). The polidispersivity index reflects the particle size distribution and is often considered satisfactory when it is below 0.3 that indicate a narrow distribution of the liposomes. In this work, PDI values oscillated

between 0.32 for empty liposomes and 0.16 for PC-OA-GE liposomes. In the Figure 1A, are presented the size distribution histograms of PC-OA-GE liposomes and the controls PC and PC-OA liposomes. The formulations of control liposomes showed a broader monomodal size distribution profile, as result of variability in the size distribution profile in the samples. On the other hand, the PC-OA-GE liposomes showed a narrow monomodal curve, and 90% of the nanovesicles had diameters smaller than 150 nm (Fig. 1A). The PC-OA-GE liposome formulations formed stable translucent suspensions as compared with the controls without GE (Fig. 1B).

High value of electronegative zeta potential is indicative of a good stability of the liposomal suspensions due to the increase of electrostatic repulsion among liposome (Müller et al., 2001). The suspension of PC-OA-GE-liposomes had a zeta potential value estimated as -24.89 mV (Table 1) with no statistical difference with the PC-AO. The addition of fatty acid resulted in a decrease in size and an increase in zeta potential of liposomes due to the changes in the membrane packing of vesicles (Lee et al., 2017), and this characteristics of size and charge make it possible that the particles with the same charge can repel each other providing stability and resistance to aggregation (Sou, 2011). The presence of oleic acid improved the size and PDI parameters and increased the surface charge, providing better stability as compared with the PC-GE liposomes developed in our previous work. Those liposomes encapsulating GE presented an average particle size of 174.6 nm with a PDI of 0.26, thus showing a larger mean diameter, broader size distribution, and zeta potential value of -16.2 mV (Pinilla et al., 2017).

Allicin concentration in the GE was 1.82 mg/mL. The developed liposomes containing GE and oleic acid showed an encapsulation efficiency (EE) of 79.7% for allicin. This value was higher as compared with those obtained in other studies that have been conducted for liposome encapsulation of allicin into matrices like lecithin-cholesterol at

3.77:1 ratio, reaching EE values about 75.2% (Lu et al., 2014) and using pure PC with maximum EE of 47.5% (Pinilla et al., 2017). The allicin was the unique component analyzed for EE determination, but the garlic extract has other water-soluble components that may be present in the liposomes. These include flavonoids, phenols and other radical scavenging compounds that potentially increase its bioactive properties and applications.

The prepared liposomes were analyzed by TEM. The structure of liposomes containing OA as wall material and loading GE can be observed in Fig. 1C. The electronic microscopy revealed spherical structures for both PC-OA-GE and PC liposomes, showing typical spherical morphologies found in PC liposomes containing peptides (Malheiros et al., 2011; Yan et al., 2014) (Fig. 1D). In our previous work (Pinilla et al., 2017), was observed that the GE liposomes prepared only with PC presented amorphous structures with a tendency to form aggregates. Thus, the addition of OA to the preparation, improved its morphology and stability.

3.2. Antifungal activity

Garlic compounds has been widely used in antibacterial, antifungal, antioxidant activities. The formulated liposomes with GE were initially tested against environment and pathogenic fungi *P. expansum*, *A. niger*, *P. herquei*, *F. graminearum* and *A. flavus*. The control PC-OA liposomes caused no fungal growth inhibition, while GE and the liposomes containing GE showed inhibition against all tested fungi (Table 2). The inhibitory zones were significantly higher against *P. herquei*, *F. graminearum* and *A. flavus* as compared with those observed for *P. expansum* and *A. niger*. No significant difference ($P<0.05$) was found between the inhibitory zones caused by GE and PC-OA-GE liposomes. Using the same antifungal assay, a 16.3 mm inhibition halo was observed for 20 µg pure ketoconazole against the aflatoxin producer fungus *A. flavus* (Veras et al., 2016). In addition, inhibition

halos ranging from 5 to 11.33 mm were reported in the antifungal activity of some GE prepared with two different varieties of garlic from the Campania Region, Southern Italy, against *P. expansum*, a mycotoxin-producer strain (Fratianni et al., 2016). According with the results obtained in this work (Table 2), it can be concluded that nanoencapsulation did not affect the antifungal activity of the GE.

3.3. Fourier Transform Infrared Spectroscopy

The intermolecular interaction between the load compounds and the wall materials of liposomes commonly leads to changes in the FTIR patterns. Infrared spectroscopy can be used to evaluate structural and conformational changes produced by entrapment of different compounds at different parts of the phospholipid bilayer (Toyran and Severcan, 2003). The FTIR spectrum of the PC-OA-GE liposomes (Fig. 2D) showed phosphatidylcholine and oleic acid characteristic peaks (Fig. 2A and 2B) corresponding to the asymmetrical and symmetrical -CH_2 stretching at 2924 and 2853 cm^{-1} and the C=O stretching at 1738 cm^{-1}. For the GE (Fig. 2C), it was observed the C=C stretching frequency of diallyl disulfide at 1630 cm^{-1}, vinyl group that appeared at 1026 cm^{-1} and the –OH broad stretching in 3358 cm^{-1} (Pinilla et al., 2017). The presence of these characteristic peaks in the FTIR spectrum of PC-OA-GE liposomes (Fig. 2F), indicated the presence and effective incorporation of GE into the nanovesicles. The infrared spectrum of PC-OA-GE liposomes showed no other specific peaks and no variations were found in wavenumbers at 2924 cm^{-1} and 2853 cm^{-1}, revealing no changes in the bilayer acyl chains. Recently, Ezner and collaborators (2017) reported that alliin increases membrane dynamics DMPC liposomes and induces compositional and structural changes with significant increase due to the increased hydration of these polar groups. However, in the present work, no differences were observed in the absorption band at 1738 cm^{-1} (C=O stretching), evidencing the absence of structural changes

associated with the hydration state of carbonyl groups on the membrane interfacial parts (Toyran & Severcan, 2003). For PC-OA-GE liposomes (Fig. 2F) in the spectral range of 1200-900 cm^{-1}, was possible observe a prevalence of bands attributed to C-C, C-O stretching and C-O-H, C-O-C bending, characteristic of polysaccharides as inulin, which could be present in the GE. The band at 1646 cm^{-1} observed in the PC and PC-OA empty liposomes (Fig.2 D and E) used as controls, could be attributed to the bending vibration of water molecules in a weak phospholipid interlayer space (Marcos and Rodriguez, 2016). The FTIR pattern in the PC-OA-GE liposomes showed that no new chemical bonds were formed, indicating that the compounds of GE, PC and OA were combined through physical interaction.

3.4. Thermal properties

Thermogravimetric analysis (TGA) was used to determinate the physical and chemical changes of the freeze dried nanoliposomes when subjected to high temperatures. From TGA analysis, PC liposomes moisture content was 10.1%, for PC-OA liposomes 7.9% and for PC-OA-GE liposomes 6.9%. The TGA analysis (Figure 3A) showed the degradation curve of the liposome samples. The first decay in the curves is related to the loss of moisture and loss of volatile compounds from de samples. PC control liposomes showed a significate weight loss (about 25 wt%) at 120°C, Compared with PC-OA and PC-OA-GE liposomes that presented a slight weight loss (about 9 wt%) at 190°C, corresponding to the evaporation of adsorbed water. From the liposomes containing GE curve, the highest rate of degradation started in 190 °C and when reached 600 °C the residue was 28%, while the liposomes without GE presented a final residue of 48%. The better thermal stability under 200°C of liposomes containing OA compared to pure PC liposomes could be due to the intercalated OA molecules in the bilayer that cause interruption of tight packing of lipids promoting

chain disorder and reducing the lipid mobility by the formation of a liquid/crystalline solution at room temperature (Cacela and Hincha, 2006; Ricker et al., 2003).

DSC results of fresh liposomal suspensions are presented in Fig. 3B. Fully hydrated PC-OA liposomes incorporating GE showed thermograms consisting of broad melting transitions, high enthalpy, abolition of the pre-transition and without visible liquid–crystalline phase transition in the range of temperatures tested (-30 to 90°C). In DSC analysis, small molecules presented a very sharp peak, while for larger molecules, such as polymers or lipid bilayers, the melting transition is broad (Demetzos, 2008). Liposome suspensions were characterized by a highly cooperative endothermic and broad transition at temperatures between 3°C to 9°C. The PC-OA empty liposomes used as control, showed a minor endothermic event at 3.5°C (ΔH = 128.36 J/g). The empty PC liposomes present the higher melting temperature (9.1°C) and an enthalpy value of 263.20 J/g. In contrast, the PC-OA-GE liposomes showed a temperature transition of 5.41°C and the higher endothermic event of 293.79 J/g. These findings indicated structural changes in the bilayer membrane of the liposomes containing OA and GE, probably owing to interactions with the polar head groups and their intercalation in the bilayer membrane must be lowered. Thus, the addition of GE into the liposomes resulting in domains with heterogeneous distribution of PC and OA, that increased the ΔH by induction of interdigitated gel phase and gives rise to more rigid bilayers. In agreement with our results, Marin et al. (2018) reported a subzero melting transition of phosphatidylcholine liposomes prepared with different food waste compounds. The ΔH was lower for empty PC liposomes (120.6 J/g) and higher (166.8 J/g) for PC liposomes containing shrimp lipid extract, concluding that liposomes loaded with lipophilic compounds may suffer a strong heat-induced rigidifying effect.

3.5. Shelf life of wheat bread

The presence of visible molds on the surface of bread slices is considered a critical point of spoilage evaluation for the consumers. The Figure 4 presents the results of the shelf life assay and the characteristic behavior of mould growth in breads with the addition of PC-OA-GE liposomes and also for the control samples, in the 15 days of test. A significate difference was observed between the results of mould growth in the breads treated with GE and the control ones without antifungal agent. With the addition of GE and PC-OA-GE liposomes the mold-free shelf life was prolonged for at least 5 days compared to the non-treated control and the PC-OA control liposome, where the moulds were visible and cover large portions of the slides after 2 days. This early presence of moulds is explained because wrapping can prevent moisture loss from the bread slices allowing suitable growth conditions for fungi in the humid atmosphere. At the end of the 11th day of storage, the non-threated bread slices were completely covered for moulds. In contrast, the treatments with free GE and PC-OA-GE liposomes showed respectively only 4 and 2 slides totally mouldy at the day 15, respectively.

Breads were baked at 220°C and according with Bosmans et al, (2013) at this condition the crumb temperature is around 98°C. Therefore, bread preservatives should be heat stable and resistant to losses due to evaporation or decomposition during the baking process. Heat stability of liposomes content GE was showed in the Figure 3. The results indicated good liposomal stability in temperatures around 100°C and a rapid decomposition over 225°C. The GE encapsulation into liposomes could prevent the loss of antifungal compounds by the high temperatures used in the bakery process, improving its retention in the matrix. In addition, the antifungal compound ajoene, formed when garlic juice is exposing at high temperatures could be formed during baking. Yoo, Lee, Kim, & Shin (2014) studied the optimal conditions for ajoene production from garlic juice, reporting that the temperature of reaction, possibly will be the key factor in the ajoene formation, due to

the increases of temperature resulted in higher ajoene content in oil-macerated garlic and the optimal conditions for *E*- and *Z*-ajoene formation was 98.80°C in 6.87 h and 42.24°C in 9.71 h. Thus, the presence of native antifungal compounds of garlic and the possible formation of others as ajoene could be responsible for the positive results of free and encapsulated GE in mould control. However, additional studies must be conducted to determine the changes in the volatile compounds profile of garlic extract into liposomes at high temperatures.

In our knowledge, this is the first report on free and nanoencapsulated GE used to prevent of mould spoilage for wheat bread. A complete review about the recent strategies for bread shelf life extension was published by Axel et al. (2016), where they highlighted the use of specific compounds produced by lactic acid bacteria that extend the shelf life of bread as the main area of study, followed by the use of antifungal peptides, ethanol and plant extracts as antifungal preservatives. These authors found only a few studies dealing with the direct addition of plant extracts in bakery products. In a more recent work, a challenge and shelf life tests were performed to investigate the mould inhibition potential of thyme oil in par-baked wheat and sourdough bread, finding inicially *in-vitro* antifungal activity, but when performed an *in situ* test, using different concentrations of thyme oil (0.08, 0.15 and 0.20 ml/100g dough), no clear shelf-life extension was observed in the samples of par-baked bread. According to the authors, the activity was affected by food matrix and for a not homogeneous distribution on the thyme oil in the bread (Debonne, et al., 2018). In another study, Rizzello and collaborators using legume flour hydrolysates as ingredient for making bread under pilot plant conditions and they observed that the legume flour hydrolysates extended the bread shelf-life, without affecting the rheological and sensory properties. According with the authors, the antifungal activity were attributed to specific peptides produced during the legume flour hydrolyze.

4. Conclusions

The addition of OA improved multiple proprieties of liposomes as size distribution, PDI and the entrapment efficiency of GE. Oleic acid-liposomes entrapping bioactive compounds could serve as carrier of potential ingredients that improve the microbiological stability of baked food products, due to its thermal properties that make possible their use at high temperatures maintaining the properties of sensitive bioactive products encapsulated as the antifungal volatile compounds of garlic extract, which resulted in an extension of the shelf life baked wheat bread.

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Conflicts of interest

Authors declare no conflicts of interest regarding this study.

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Figure legends

Fig. 1. (A) Histograms of size distribution obtained by laser dynamic light scattering of PC liposomes (full line), PC-OA liposomes (dotted line) and GE-PC-OA liposomes (dash line). (B) Image of the liposome suspensions, from left to right: GE-PC-OA, PC-OA and PC liposomes. (C,D) Transmission electron microscopy images of PC-OA-GE and control PC liposomes containing phosphate buffer, respectively. Bar =100 nm.

Fig. 2. FTIR spectra of (A) pure PC, (B) pure OA, (C) GE, (D) PC-liposomes, (E) PC-OA liposomes, (F) PC-OA-GE liposomes.

Fig. 3. Thermal analysis of liposomes. Samples of control PC liposomes (full lines), PC-OA liposomes (dotted lines) and GE-PC-OA liposomes (dash lines) were subjected to TGA (A) and DSC (B) analysis.

Fig. 4. Shelf life wheat bread slices with liposomes after challenging against environmental moulds during a 15-day storage period. Bread spoilage is designated as percentage of the total surface area of each slice where fungal outgrowth was observed: Mould free slices (white area), 10% mouldy (black vertical striped area), 10-24% (grey area), 25-49% (black horizontally striped area) and 50% mouldy (black area). (A) Control without treatment, (B) PC liposomes, (C) Free GE and (D) PC-OA-GE liposomes. Values are means of duplicate samples originated for three independent fermentations each; error bars indicate standard deviations.

Table 1. Characterization of PC-OA liposomes containing garlic extract.

	PC	PC-OA	PC-OA-GE
Particle size (nm)	172.2 ± 5.5^{a}	143.1 ± 21.7^{b}	113.3 ± 3.3^{c}
Polydispersity	0.33 ± 0.56^{a}	0.32 ± 0.41^{a}	0.16 ± 0.31^{b}
Zeta potential (mV)	-13.9 ± 4.5^{a}	-36.7 ± 7.8^{b}	-27.9 ± 6.5^{b}
EE (%)	-	-	79.7 ± 2.2

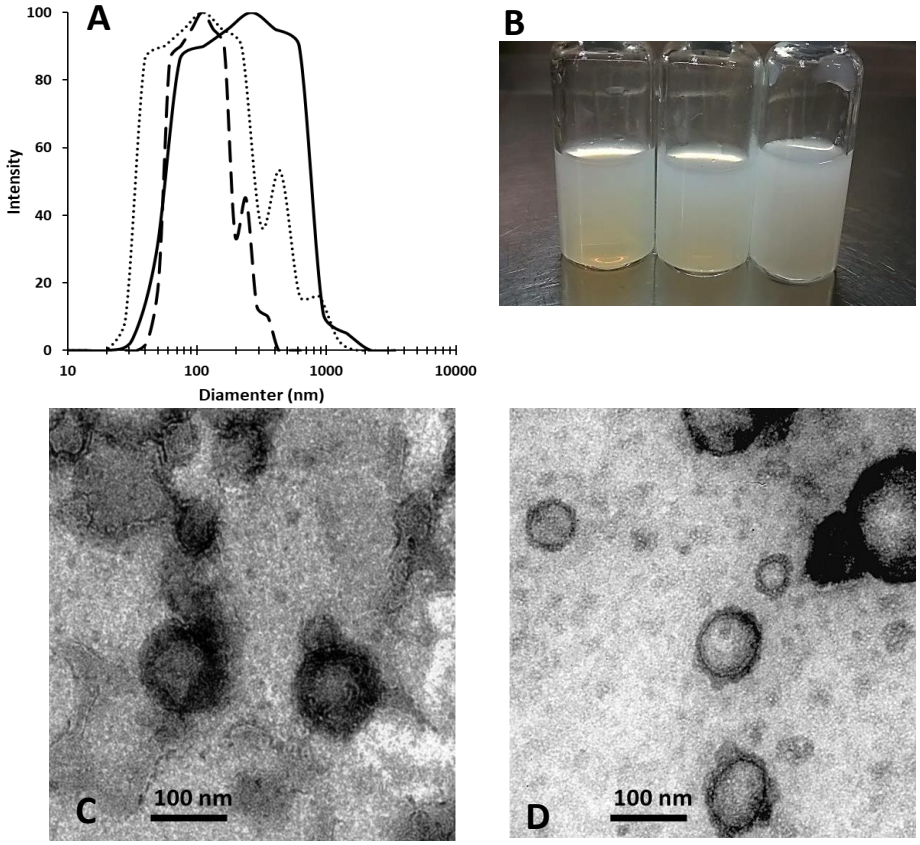
Different letters (a, b, c) indicate significant differences ($P < 0.05$)

Values are the means ± standard deviation of three independent experiments.

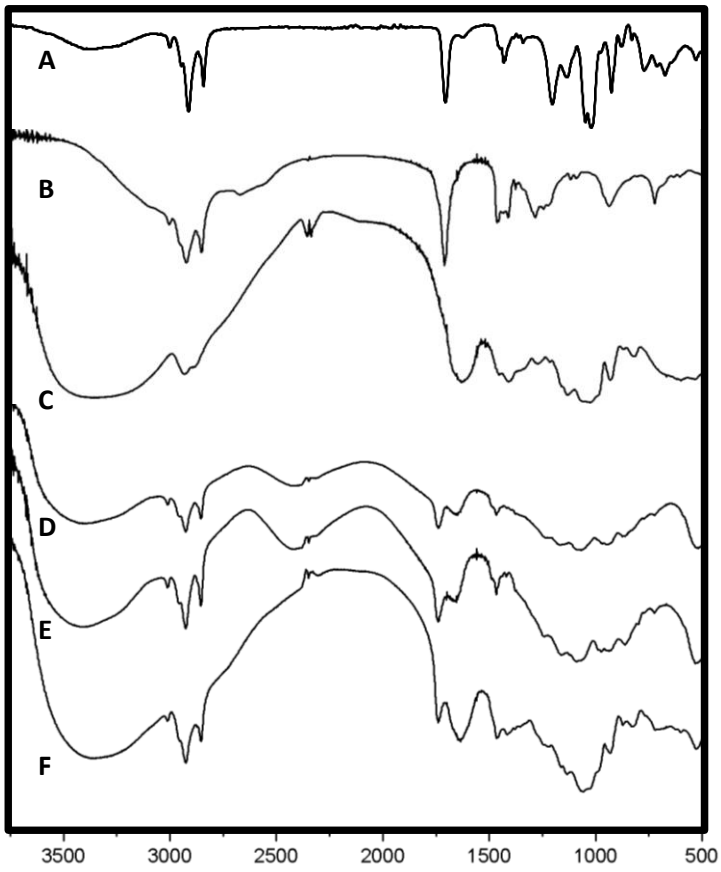
Table 2. Inhibition of fungal growth by PC-OA liposomes containing garlic extract.

Fungi	GE	PC-OA-GE	PC-OA
	Inhibition halo (mm)		
Penicillium expansum	6.2 ± 0.8	6.5 ± 0.7	0.0
Penicillium herquei	34.4 ± 0.2	35.3 ± 0.5	0.0
Fusarium graminearum	33.6 ± 0.7	34.1 ± 0.9	0.0
Aspergillus flavus	34.1 ± 0.6	35.3 ± 0.5	0.0
Aspergillus niger	6.3 ± 0.4	6.1 ± 0.6	0.0

Results are the means ± standard deviations of three independent experiments. Garlic extract and PC-OA were used as controls.



Pinilla et al., Fig. 1



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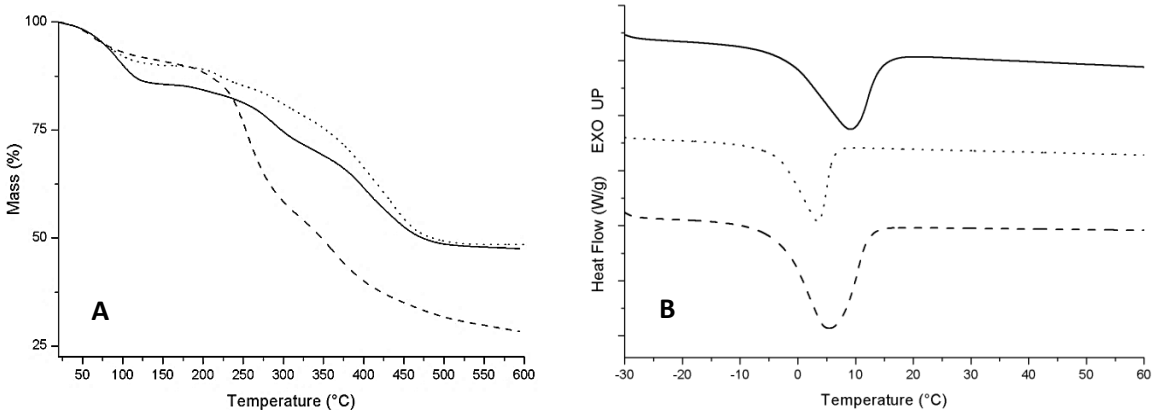

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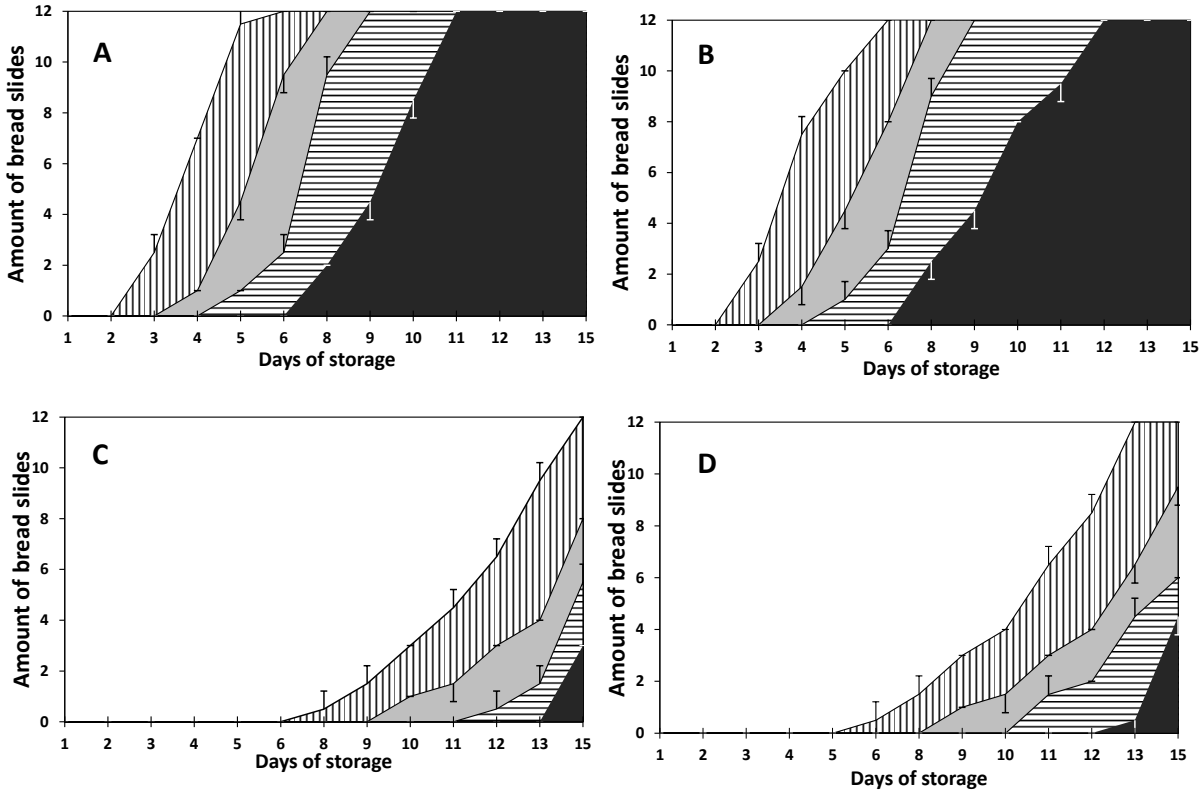

Pinilla et al., Fig. 4

6. Artigo No.3

Influence of liposome composition on interaction and structural features of surimi protein.

Resumo:

O objetivo do presente estudo é investigar as interações entre lipossomos e proteína muscular de peixe (surimi, SURP), avaliando o papel da composição e concentração lipídica lipossômica nas propriedades dessa proteína. Lipossomas de fosfatidilcolina ultrapura (UPCL) ou fosfatidilcolina de soja parcialmente purificada (PPCL) foram preparados e dispersos em diferentes proporções de peso em (SURP); as mudanças na estabilidade e estrutura da proteína foram determinadas usando espectroscopia no infravermelho por transformada de Fourier (FTIR), fluorescência intrínseca e grupos sulfidrila livre. Além disso, as alterações nas características físico-químicas e na morfologia dos lipossomas carregados e descarregados de nisina foram avaiadas por Espalhamento Dinâmico de Luz e Microscopia Eletrônica de Transmissão (TEM). Como resultado, observou-se que o PPCL promoveu desnaturação e agregação de SURP desdobrada, refletida em perda de estrutura secundária, exposição de resíduos de tirosina e ligeiro incremento de grupos sulfidrila livres. A UPCL produziu desdobramento parcial e alterações na estrutura secundária da proteína SURP de α-helicoidal para fita β, essas estruturas foram observadas pelo TEM ao redor da UPCL, que podem resultar de interações hidrofóbicas entre UPCL-SURP e interações proteína-proteína. Além disso, a estabilidade do lipossoma foi afetada pelo SURP, UPCL e PPCL descarregados aumentaram seu tamanho em 40% e também foi mudada sua carga superficial, indicando uma formação de proteína corona, relacionada aos locais de ligação disponíveis nos lipossomas, resultando em morfologia e composição diversa da proteína corona. Portanto, a carga superficial e a composição dos lipossomas são os principais fatores envolvidos na redução da estabilidade da SURP e podem exercer diferentes efeitos na rede de proteínas miofibrilares, o que é importante para determinar as propriedades tecnológicas dos lipossomas em produtos surimi.

Influence of liposome composition on interaction and structural features of surimi protein

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Abstract

The aim of the present study is to investigate the interactions between liposomes and surimi ground salted muscle (SURP), evaluating the role of liposomal lipid composition and concentration in the properties of such protein. Liposomes of ultrapure egg phosphatidylcholine (UPCL) or partial purified soybean phosphatidylcholine (PPCL) were prepared and dispersed in different weight ratio on (SURP); changes in protein stability and structure were determined using Fourier Transform Infrared Spectroscopy (FTIR), intrinsic fluorescence and free sulfhydryl groups. Also, changes in physicochemical characteristics and morphology of nisin loaded and unloaded liposomes were attempted by Dynamic Light Scattering and Transmission Electronic Microscopy (TEM). As a result, it was observed that PPCL promoted denaturation and aggregation of unfolded SURP, reflected in secondary structure loss, exposure of tyrosine residues and slight increment of free sulfhydryl groups. The UPCL produced partial unfolding and changes in the secondary protein structure of SURP from α-helical to β-strand, these structures were observed by TEM surrounding the UPCL and could be resulting from hydrophobic interactions between UPCL-SURP and protein-protein interactions. In addition, the liposome stability was affected by the SURP, unloaded UPCL and PPCL increasing its size in 40% and modifies its surface charge indicating a protein corona formation, related to available binding sites in the liposomes, resulting in diverse protein corona morphology and composition. Therefore, the surface charge and composition of liposomes are the main factors involved in reduction of SURP stability and could exert different effects in the myofibrillar protein network, what is important to determine the technological properties of liposomes in surimi products.

1. Introduction

Nanotechnology is widely used in the food industry to improve the color, taste, and texture, as well as to preserve food products. Among different types of nanocarriers, lipid-based devices have advantages such as higher encapsulation efficiency, compatibility, low toxicity, and continuous production possibility (Katouzian, Esfanjani, Jafari, & Akhavan, 2017). However, concerns remains regarding the potential undesirable effects of nanoparticles into foods, such as those used as delivery systems for colors, flavors, preservatives, nutrients, and nutraceuticals or food packaging (McClements & Xiao, 2017). It is expected that interaction of nano structured materials with food ingredients will influence their behavior (Yusoff et al., 2018).

In the food science and technology field, it has been reported some adverse effects attributed to interactions between liposomes and proteins in food matrixes. Recently (Xiangzhou and collaborators (2019) related that ferrous sulfate liposomes affect whey protein emulsifying properties by modification of its secondary structure. In the case of muscle protein, it has been reported that soy phosphatidylcholine liposomes slightly modified the viscoelastic behavior and interfered with the thermal aggregation of hake salt-ground muscle proteins (Marín et al., 2018a) and freeze-dried soy phosphatidylcholine liposomes loaded with various bioactive compounds were found to reduce the gel strength of squid surimi (Marín et al., 2018b). In addition, liposomes can also be affected by the food matrix, reducing its stability and control release (Chen et al. 2010). The addition of carbohydrate polymers, peptides and oils were studied as a way of enhancing the liposome delivery efficacy and the resistance to chemical and physical degradation in complex food systems (Lopes, Pinilla, & Brandelli 2019; Marín et al 2018a; Ghorbanzade et al., 2017) using different encapsulating

materials, as commercial ultra-purified phosphatidylcholine (PC) and partial purified PC derived from non-synthetic lecithins, such as soy lecithin. However, still there is little information in the scientific literature about how food-interest proteins behave in response to liposomes incorporation as additive and questions about how the liposome composition and charge affect the protein-protein interactions continues unclear.

Interactions between nanostructured delivery systems and human plasma proteins have been extensively studied in pharmaceutical research. In this context, it has been stablished the concept of a 'protein corona', recognized as proteins bounded to liposome surface (del Pino et al., 2014 Foteini et al., 2019). Several liposome characteristic could have considerable influence on proteins adsorption, which include its size (Wolfram et al., 2014), hydrophobicity (Rahman et al. 2013), lipid composition (Caracciolo et al., 2015a) the surface charge (Rahman et al. 2013; Aggarwal et al. 2009), and also topography, curvature, protein affinity, and protein binding sites (Caracciolo, 2015b). In a recent work in the field of food science, Oehlke et al., (2019) studied the adsorption of β-lactoglobulin on solid lipid nanoparticles (SLN) loaded with ferulic acid or tocopherol; they concluded that the formation of protein layer was dependent of the SLN composition, where the presence of tocopherol, increase the amount of bounded β-lactoglobulin. All these studies have been carried out working preferentially with globular proteins, but scarce information is available regarding these effects on myofibrillar proteins, as is the case of fish surimi. Additionally, the physicochemical properties of liposomes can change after adsorption of proteins on its surface e.g. liposomes become larger because of protein binding and changes in surface charge can also be observed, as the case of adsorption of serum proteins, usually negatively charged, that decrease the zeta-potential of cationic liposomes (Capriotti et al. 2019).

The objective of this research were to study the physicochemical interactions between liposomes of different composition and surimi myofibrillar proteins. Liposomal formulations composed of high purified PC and partial purified PC were prepared, with and without nisin loaded. This research examines how the liposomes affect the surimi ground salted muscle SURP, as well as changes in nisin-loaded and unloaded liposomes, after mixture with SURP. This work could enhance our understanding of the interaction between myofibrillar proteins and liposomes when incorporated in food formula, and provide insights for effective application of liposomes as nanocarriers of bioactive compounds in protein-structured foods such as surimi gels.

2. Materials and methods

2.1 Preparation of surimi protein

Grade KA Alaska Pollack surimi (Theragra chalcogramma), kindly supplied by Angulas Aguinaga S.A. (Guipuzcoa, Spain) in 20 kg frozen blocks was used in the present study. To solubilize the surimi protein, 1% of sodium chloride (Panreac Quimica, S.A.; Barcelona, Spain) was added and the moisture was adjusted to 80%. After total solubilization in Thermomix ® (Wuppertal, Germany), the resultant surimi ground salted muscle (SURP) was freeze dried and then stored at -4°C for the further analyses.

2.2 Liposome preparation

Partially purified phosphatidylcholine (PPC) was made as described by Taladrid et al (2017). Summarizing, commercial soybean lecithin (Manuel Riesgo, S.A., Madrid, Spain) was dissolved in ethyl acetate (1:5, w/v) and washed five times with acetone (1:2, w/v), and dried in desiccator for 48h. The chemical composition of the partial purified PPC powder used in the present study, were also described in the same work (Taladrid et al., 2017).

Ultra-purified phosphatidylcholine (UPC), Phospholipon 90G®, was provided by Lipoid (Ludwigshafen, Germany). Both phosphatidylcholines were stored at –20 °C until use. Nisin solution was prepared according with Pinilla and Brandelli (2016). Empty and nisin loaded liposomes were prepared by the heating method reported by Marín et al, (2018a) with some modifications. Briefly, the phospholipid (UPC or PPC) was suspended in 0.1M phosphate buffer (pH 7) or a solution of 62.5µg/mL of nisin in phosphate buffer (pH 7). UPC or PPC at (20mg/mL) was mixed with each solution and kept in a water bath at 55 °C for 40 min and 100 rpm. Then, each solution was vortexed at 60 °C for 1 min, cooled and sonicated in ice bath using an ultrasonic cell disrupter (Unique OF S500, frequency 20 kHz, power 250 W) at 90% amplitude, for 5 min with a 60 s stop every min. Finally the produced liposomes were filter sterilized with 0.22 µm membranes (Sartorius, Göt, Germany) and stored at 4°C until use (UPCL, PPCL, or UPCLN and PPCLN for nisin loaded liposomes).

2.4 Measurement of zeta potential and particle size

The particle sizes and zeta potentials were measured by electrophoretic light scattering using a Zetasizer Nano-ZS 90 instrument (Malvern Instruments, Worcestershire, UK) at 25 °C. Determinations were carried out after dilution ratio 1:100 in ultrapure water. Samples were equilibrated for 180 s inside the instrument, and data were collected at least 12 sequential readings. All determinations were performed in triplicate. Liposome suspension after preparation and mixtures UPCL-SURP and PPCL-SURP, with increasing weigh ratio (1:0, 1:2, 1:4, 1:6, 1:8 and 1:10 w/w) of SURP, were analyzed. For this, 1mL of liposome suspension was mixtured with freeze dried SURP and starring in vortex by 2 min follow for 15 min of rest and finally, centrifuged at 3000g for 5 min before dilution and lecture in the instrument.

2.5 Fourier transform infrared (FTIR) spectroscopy analysis

Infrared spectra between 4,000 and 650 cm−1 were recorded using a FTIR Nicolet 6700 Spectrometer (Thermo Scientific., MA, USA) equipped with Attenuated Total Reflection (ATR). The spectral resolution was 4 cm−1. Measurements were performed at room temperature using freeze-dried samples. Spectra were collected in 64 scans. All determinations were performed in triplicate. A second-derivative spectrum was determined to providing more accurate information about the changes in the protein secondary structure. To that end, the amide I region (1700–1600 cm^{-1}) of the myofibrillar proteins spectrum was selected because the Amide I band components correlated closely with each secondary structural element of the proteins (Jackson & Mantsch, 1995). For this analysis, first, were prepared suspensions of SUR (20 mg/mL) in 0.1M phosphate buffer (pH 7), by homogenization in vortex for 2 min. Then, were added liposomes to attempt the ratio 1:1, 1:5 and 1:10 w/w and the mixtures were freeze dried and stored at -20°C.

2.6 Measurement of intrinsic fluorescence spectra

The intrinsic fluorescence spectra of mixtures SURP-UPCL, SURP-PPCL and free SURP were obtained using a RF-5301 PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) with a quartz cuvette (1 cm pathlength) at 25 °C. The SURP protein was diluted to 0.1 mg/mL with hydrogen phosphate buffer solution (0.01 mol/L, pH 6.8) and then was added the UPCL or PPCL in different mass ratio from 1:0.2 to 1:1 w/w. Each sample was homogenized in vortex and the lecture of the fluorescence spectra was carried out after 15 min of rest. The emission spectra were recorded in triplicate, using the wavelength range of 300–450 nm (emission slit width = 5 nm) after excitation at 296 nm (excitation slit width = 5 nm) and 200 nm/min of scan rate.

2.8 Quantification of free sulfhydryl (SH) groups.

Free sulfhydryl groups determination was conducted according to the method related by Ellman (1959) using the reactive buffer (Tris-HCl 50 mM, NaCl 0,6 M, EDTA 6 mM, Urea 8 M, SDS 2 % pH 8) and DTNB solution (5,5'-dithiobis-2-nitrobensoic acid 0.01 M in buffer Tris-HCL 50 mM). Briefly, solutions of SURP (1mg/mL) in Tris-HCL 50 mM with different concentrations of UPCL, PPCL, from 1:0.2 to 1:1 w/w SURP-liposomes ratio were prepared. Then, 500 µL of the reactive buffer and 200 µL of DTNB solution was mixed with 500 µL of each SURP-liposome solution and homogenized in vortex. The mixed solutions were stored in dark at 25 °C for 30 min; the absorbance was measured at 412 nm (UV-VIS Spectrophotometer, SHIMADZU CORP). To determine the sulfhydryl values was used the molar extinction coefficient (EM= 13,600 M/cm). The analyse was carried out at least in duplicate and the results were expressed in terms of micromoles of sulfhydryl per gram of protein.

2.7 Transmission electron microscopy

The changes on liposomes morphology after incubation with SURP were observed by transmission electron microscope (TEM). The samples were obtained by the dilution of UPCL and PPCL suspensions to 1mg/mL. In addition, mixtures SURP-Liposomes (1:1 w/w) were prepared in 0.1M phosphate buffer (pH 7). These mixtures were homogenized and incubated for 15 min at 25 °C and 100 rpm. Then were dropped onto a Formvar-coated 300 mesh copper grids and stained in 2.5 % uranyl acetate for 30 seconds. The dried samples were visualized by Transmission Electron Microscope (JEM-1200, JEOL Ltd., Japan) operated at 100 KV.

3. Results and Discussion

3.1 *Fourier Transform Infrared Spectroscopy*

The signature of a conformational change in the spectrum can then be used to identify transient conformational states of proteins. The absorption bands in amide I (~1655 cm^{-1}) and amide II (~1545 cm^{-1}) are hardly affected by the nature of the side chain and offer important information of hydrogen-bonding patterns, dipole–dipole interaction, and geometry of the protein polypeptide backbone (Yang et al., 2015; Krimm and Bandekar 1986). Therefore, this vibration is used commonly for secondary structure analysis.

The SURP amide I band and the mixtures with liposomes are presented in Fig 1. In the samples SURP-PPCL (Fig. 1A), was observed a gradual reduction of intensity of the amide I band with the increase of concentration of PPCL, reducing in 95% the band area at the higher concentration of PPCL compared with the SURP control (solid line). Additionally, the amide I band was shifted to a higher value, from 1641.9 cm^{-1} to 1654.4 cm^{-1} in the SURP-PPCL ratio 1:10 w/w (dash-dot line). The peak at 1738 cm^{-1}, which appears in all samples with liposomes, represents the carbonyl stretching of the phosphatidylcholine. The FTIR spectra of SURP-UPCL samples (Fig 1B) showed a reduction of 70% in the amide I band area at the concentration 1:10 w/w of UPCL (dash-dot line), which also was shifted gradually from 1641.9 cm^{-1} to around of 1652 cm^{-1} with the increase of UPCL ratio. The intensity of the amide I band is usually assigned to C=O stretching vibrations originated from α-helical structures and water vibration in the myofibrillar proteins and its reduction is related to protein aggregation and denaturation (Jackson & Mantsch., 1995; Boubellouta et al., 2010). Characteristic of unordered structure, unfolded protein shows a broad, featureless amide I band centered near 1650 cm^{-1}, due to changes in the α -helical structure (Barth, 2007). According with the FTIR results, increase the SURP-PPCL ratio produce pronounced alterations in the α-helical structure resulting in high degree of protein denaturation and

aggregation (Fig. 1A). The fact that the amide band I maintains some of its intensity, even at high concentrations of UPCL, suggests that UPCL produce a different effect in the SURP secondary structure, as compared with the PPCL. This different trend could indicate that the UPCL affect preferentially weak protein-protein interactions, as hydrogen, ionic and other bonds, which result in protein intermediates with α-structures (Fig. 1B). This results are in agreement with recent studies about of liposomes-proteins interactions in models of peptides and enzymes, in some of these works were highlight the electrostatic interactions between the peptide/enzyme peripheral surface and the polar head group of phospholipids as the main factor that affect the protein structure and function (Colletier et al., 2002; Inda et al., 2014; Güler et al., 2016). The amide II band intensity, presenting almost total reduction in the presence of PPCL and partial decrease with the UPCL, follows the same pattern to the amide I results.

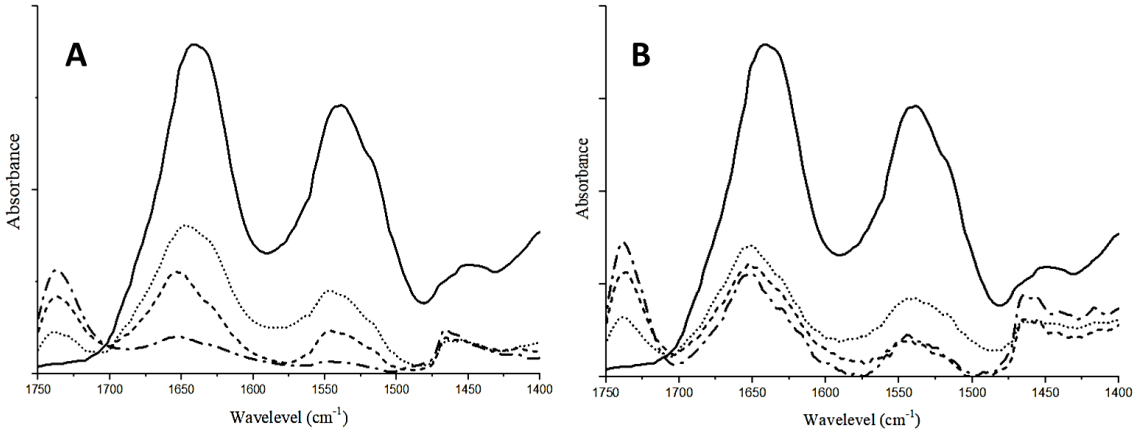

Fig 1. Normalized FTIR-ATR spectra of SURP after addition of PPCL (A) or UPCL (B) in different weight ratio: Solid line (1:0); dotted line (1:1); dashed line (1:5) and dash-dot line (1:10).

To analyze the amide I band component, Fourier second derivative spectra (FSD) was used to separate some overlapped absorption peaks in order to enhance the spectral resolution and gain insight to the secondary structure of the myofibrillar proteins (Kong & Yu, 2007). Bands near 1650–1660 cm−1, 1600–1640 cm−1 and 1640–1650 cm−1 represent α-helical

structures, β-sheet structures, and random coil structures, respectively (Barth, 2007). However these structures are probably partially denatured because the proteins have been solubilized.

The FSD of SURP mixed with PPCL and UPCL are shown in Fig. 2A and 2B, respectively, reveal significant spectral alterations in the amide I region by the liposome addition with more marked changes at higher concentration of liposomes. The SURP band at 1,652 cm^{-1} corresponds to α-helical structures; the bands at 1,618, 1,630 cm^{-1} to β-sheet fractions; and the bands at 1,682 and 1,667 cm^{-1} to β-turns; random structures are located at 1,658 and 1,646 cm^{-1}. The addition of PPCL produce a down-shift of 4 cm^{-1}in the ratio 1:1 w/w in all bands, while it was reduced to 2 cm^{-1} in 1:10 w/w. The FSD of SURP plus UPCL (Fig. 2B) presented a down-shift of 1 cm^{-1} in ratio 1:1 w/w (dotted line) and, with the liposome increment, the band change to up-shift of 3 cm^{-1} in the 1:10 w/w ratio (dash-dot line). Changes between the low and the high frequency in the β-components have been described as characteristic for protein aggregation, indicating intermolecular β-sheet structure with strong hydrogen bonds (Shivu et al., 2013). In addition, all the amide I bands almost disappear in the relation 1:10 w/w of SURP and PPCL respectively (Fig. 2A), indicating gradual protein denaturation. For the sample with UPCL, all bands presented significant reduction; nevertheless, α-helical, random coils and β-sheet structures maintained some intensity in the 1:10 ratio of SURP-UPCL, indicating partial denaturation and changes in SURP secondary structure. Destabilization of the α-helix structure is caused by changes in intramolecular hydrogen bonds between the carbonyl oxygen and amino hydrogen of the peptide chains (Cao & Xiong, 2015). Liposomes form hydrogen bonds and polar interactions between the water molecules of the aqueous environment and their polar heads to promote stabilization (Bozzuto & Molinari, 2015). Thus, PPCL that contains tocopherols and others charged molecules in its surface, produce changes in electrostatic forces around

the protein charged groups affecting the intramolecular hydrogen bonds of SURP, disturbing the secondary structure and causing denaturation and aggregation. On the other hand, the UPCL, probably due to its low surface charge and less polar groups as compared with PPCL, produce reduced damage in SURP secondary structure. This is important for the technological properties of surimi due to gel-forming ability is related to the α-helix transforms into other types of secondary structures, such as β-sheet and random coil, and β-sheet contributes more to increase gel strength (Wei et al., 2018).

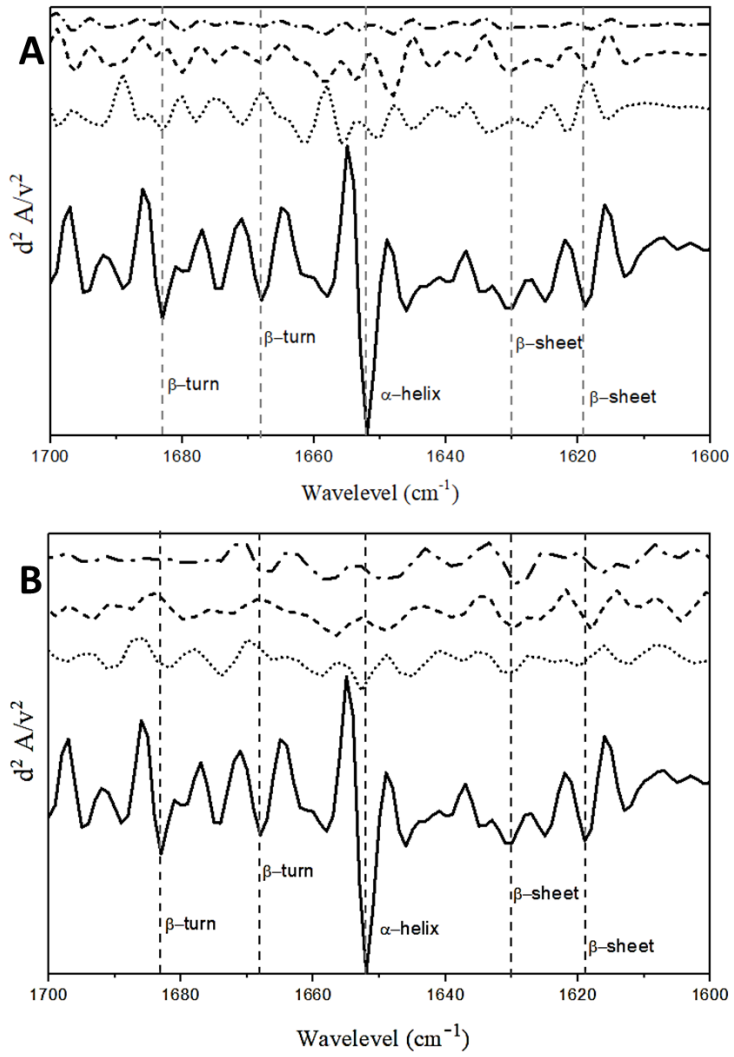

Fig. 2. Second derivate FTIR spectra of surimi protein (SURP) after addition of PPCL (A) or UPCL (B) in different weight ratio: Solid line (1:0); dotted line (1:1); dashed line (1:5) and dash-dot line (1:10).

3.2. *Fluorescence spectra*

Intrinsic fluorescence spectroscopy provides a sensitive means of conformational and structural changes in proteins according to the polarity of the microenvironment of aromatic amino acids, especially for tryptophan residues. Changes in the emission spectra of tryptophan often occur in response to conformational transitions, subunit association, substrate binding, or denaturation (Nguyen et al., 2017). Changes in fluorescence spectra upon incorporation of UPCL or PPCL into SURP were monitored. As result, the

fluorescence intensity of the surimi ground salted muscle gradually decreased with increasing weight ratio of PPCL and increase with the UPCL (Figure 3A and 3B). Studies in fish myosin protein during storage, pointed out that the decrease in intrinsic fluorescence could be an indication of denaturation and exposure of indole side chain of Trp to the polar environment (Lina et al., 2013; Wang et al., 2019; Xu et al., 2019); the exposition of buried Trp residues due to unfolding of native myosin may play an important role in the protein-protein interactions accompanying detrimental changes as the formation of aggregates (Xue et al., 2017). It could be deduced that the PPCL increase de polarity in the protein core, exposing Trp residues and, creating a hydrophobic interactions that favored a more aggregated state of the protein, as observed in the FTIR analyze.

In contrast, the increment of fluorescence intensity was observed in the SURP-UPCL (Fig, 3B). Han et al, (2017) reported the increase of emission intensity of myosin after treatment with of ultrahigh pressure in combination of temperature, according with the authors this effect was result of alteration of protein structure from folding to stretching. Thus, our results indicated an interaction SURP-UPLC that maintain the tryptophan residue located within the core of the protein (a hydrophobic environment), increasing the fluorescence intensity, that result in protein unfolding and partial denaturation.

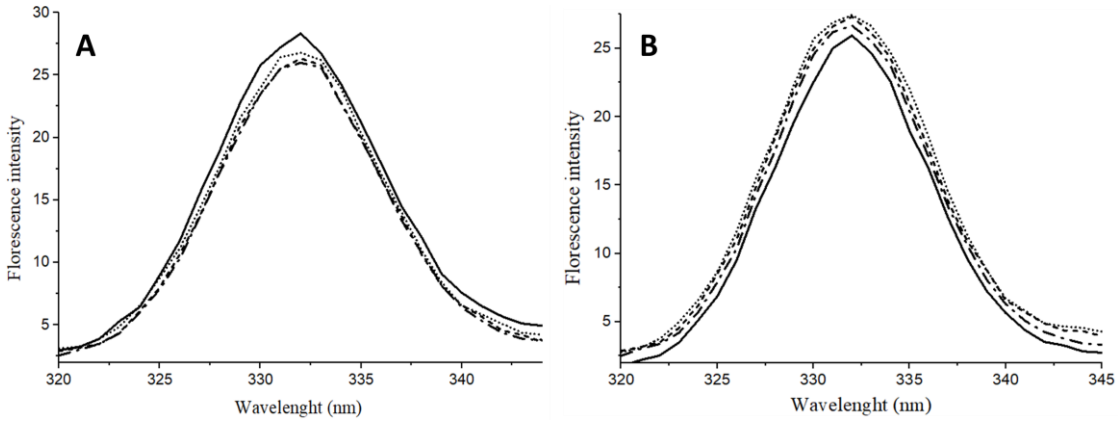

Fig 3. Changes in the intrinsic fluorescence intensity of SURP proteins (black line) and the same protein after addition of PPCL (A) or UPCL (B) in different weight ratio respectively: Solid line (1:0); dotted line (1:1); dashed line (1:5) and dash-dot line (1:10).

3.3 *Effects of the interaction between SURP and liposomes on the free sulfhydryl content*.

Sulfhydryl groups (SH) contribute significantly to the functional properties of proteins (Cao et al. 2012). Changes in total sulfhydryl content demonstrates the formation and/or breaking of protein molecular disulfide bonds. The quantification of sulfhydryl groups indirectly indicates the amount of aggregation, since the sulfhydryl groups are representative of native proteins and contribute to detect changes in the functional properties of proteins (Poowakanjana and Park 2013, Cao et al. 2012).

As shown in Fig. 4, the total sulfhydryl content of SURP increased slightly as the protein-PPCL ratio increased from 1:0.6 to 1:1 min. This indicated that the presence of liposomes from partially purified phosphatidylcholine (PPCL) does not change the amount of sulfhydryl groups present until the ratio is 1:6, from which they cause exposure of thiol groups buried in the interior of myofibrillar protein, leading to the increase (4,5%) in total sulfhydryl content (Tadpitchayangkoon et al., 2010). Therefore, transfer of thiol to the surface suggests disruption of the myosin α-helices by presence of PPCL, as confirmed by FTIR spectroscopy. This is in agreement with the results above, indicating that the mixture SURP-PPCL renders proteins more susceptible to formation of aggregates affecting its functional properties. This result is consistent with Marín et al (2018b), who reported that the addition of aqueous dispersions of liposomes, prepared with partial purified PC from soy lecithin, favored protein unfolding and reduction in gel strength when incorporated in salt-ground hake (*M. merluccius*) muscle. On the other hand, as the UPCL ratio increase, the free sulfhydryl content of SURP decreased around 20% (Fig. 4). Usually, the decrease of

total sulfhydryl content from fish muscle proteins is related to simultaneous refolding and formation of aggregates, during longer heat treatments or frozen storage (Xu et al., 2019; Wang et al., 2013). In this work, the gradual reduction could be explained by the increasing hydrophobic interactions and H-bonds between UPCL and SURP, which favors the formation of disulfide linkages protein-protein and also interactions phosphatidylcholine-thiol groups, reducing consequently the free thiol groups available; this result in partially folded structures, as observed in the FTIR of SURP-UPCL (Fig, 1B). The phosphatidylcholine property of interact hydrophobically with thiols groups has been recently explored in formulations of redox-responsive liposomes (Kwon & Kim, 2017, Guo & Kim, 2016), in this studies the strong hydrophobic interaction between liposome membrane and free thiol groups is used as a initiator of liposome content release in response to a reducing agent.

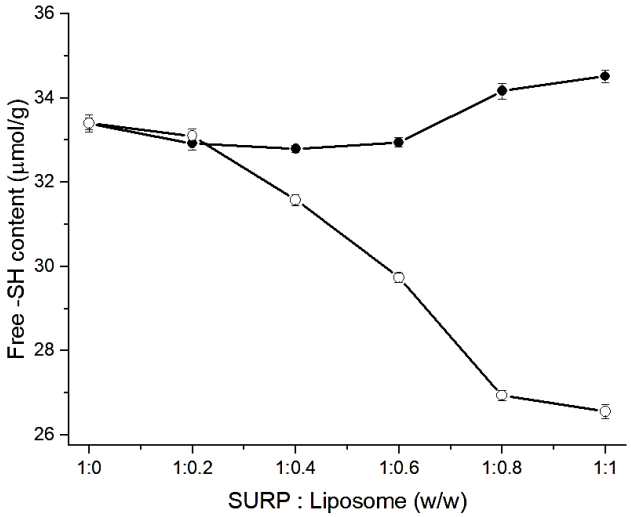

Fig 4. Effects of SURP-Liposome ratio on free –SH content. PPCL (black circle) and UPCL (white circle) in different weight ratio.

3.4 *Transmission electron microscopy of liposomes plus SURP*

To observe changes in the liposomes-SURP morphology, electronic microscopy was carried out. The single PPCL (Fig. 5A) and UPCL (Fig. 5C) presented spherical morphology with

sizes around 100 and 150 nm, respectively. After interaction with SURP protein ratio 1:1 w/w, some changes in PPCL (Fig. 5B) shape and surface were observed, but not in size, indicating that some positive charged protein residues were attached. This could be due to partial protein denaturation (Fig. 2A and Fig 3A) and the production of protein residues with reactive amino acids that participates as a hydrogen donor in hydrogen-bonding interactions with the polar groups of PPCL.

Interestingly, in the mixture SURP-UPCL (Fig. 5D) an elongated structure reworked with what appear to be "free protein fibers" was observed. In the same figure it is possible to see loose "fibers" of protein and also a minor circular structure alone and with a liposome core. Based on the results of the previous analyzes on the SURP-UPCL interaction, we have hypothesized that the UPCL effectively favors the formation of a protein secondary structure fold in β-strand, to the detriment of α-helices, in which the backbone of the peptides is stretched with disulfide and hydrogen bonds and is almost totally unfolded (Banerjee et al., 2018). Nanoparticles can introduce thermodynamic instability to the surrounding proteins that can induce structural changes in proteins that lead to chemical denaturation and fibril formation (Saptarshi, Duschl & Lopata, 2013), where, in the case of promotion of protein fibrillation, the nanoparticles serving as a template for protein self-assembling to result in microstructures with different features determined by the outer surface layer directly involved in the interaction (Shemetov et al., 2012). In this context, Miriani et al (2014) reported that polystyrene nanoparticles (NPs) enhance the formation and stability of a specific refolding-prone conformation of the small proteins Rubredoxins; according with the authors, the structural changes ensuing from protein interactions with the hydrophobic surface of polystyrene NP produce a "selective" denaturation of protein regions that lead to specific protein folding events. In another study, fibrillation of proteins caused by gold

nanoparticles was associated to binding stoichiometry of peptide-nanoparticle and the protein chemical properties (Wagner et al., 2010).

Thus, the UPCL could initially, favor the unfolding creating a hydrophilic environment as observed in the fluorescence analysis (Fig 3B) acting at the same time as donor of hydrogen bonds with the hydrophilic groups of the unfolded protein to form and maintain the β-strand structure. After this initial process, others fibers in β-strand structure may co-assembly by electrostatic force to oppositely charged peptide groups forming a facial amphiphilic design. The final protein rearrangement could be result of its interaction with the nonpolar surface of UPCL. However, the mechanism that naturally orientates the protein on the hydrophobic surface of UPCL remains unknown.

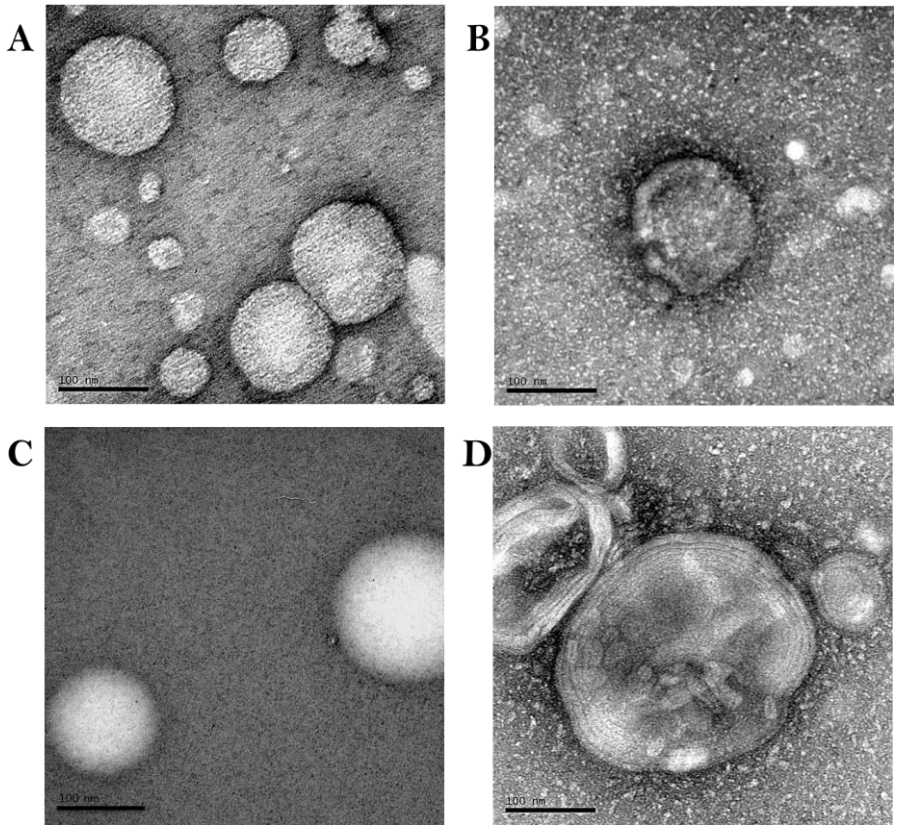

Fig 5. Representative transmission electron microscopy images of liposomes and the mixture of liposomes-Surimi protein (1:1 w/w). The images correspond to PPCL (A), SURP-PPCL (B), UPCL (C), and SURP-UPCL (D).

3.5 *Effect of SURP in liposomes formulated with nisin.*

Liposomes of UPCL and PPCL containing nisin (UPCL-N or PPCL-N), were made and mixed with SURP in different weigh ratio monitoring size and zeta-potential, to determine changes in the liposomes by the interaction with SURP and also to determine if the addition of the peptide nisin in the liposome membrane can affect its interaction with the surimi ground salted muscle (SURP). Initially, the liposomes were found to be small-size vesicles with mean diameter between 90 and 140 nm (Fig 6A). After mixing with SURP, the mean diameter of formulations without nisin presented the higher increments for PPCL (Fig. 6A). The increase after incubation with SURP 1:10 w/w ratio, were of 25% and 41% for nisin load and unload PPCL respectively. However the addition of UPCL SURP was around 7% for UPCL-N and 42% in the UPCL. Liposomes usually become larger because of protein binding which form a protein corona (Papageorgiou et al., 2019), however, the presence of nisin produce a stabilizing effect reducing the membrane mobility (Taylor et al., 2005), which could reduce bind sites available in the liposome surface. In relation to surface charge the PPCL presented characteristic negatively zeta-potential of -53.9 mV and -46.5 mV for PPCL-N due to the presence of anionic phospholipids as phosphatidylinositol (Taladrid et al., 2017). The formulations UPCL and UPCL-N exhibited zeta-potentials value of -4.5 and -10.5 mV, respectively. After incubation with SURP, the zeta-potential values present an increment until almost -25 mV in UPCL and UPCL-N preparations. On the contrary, the charge of both PPCL and PPCL-N formulations after incubation with SURP were reduced to around -41 mV (Fig. 6B). The mayor changes in zeta-potential were for the unloaded liposomes. Myofibrillar protein exhibits negative zeta potential in of around -12mV in low ionic strength medium (Zhang et al., 2015) and its reduction is related with destabilization processes as flocculation. The increase in zeta-potential values of UPCL and UPCL-N, indicated the presence of negative charged groups on the UPCL surface, coming

from peptide residues of the denatured SURP. On the other hand, the zeta-potential of PPCL suffered a small change to less negative charge, probably by binding positive protein groups, since charged lipids provide liposomes with a repulsive electrostatic barrier against the negatively charged proteins. Caracciolo et al., (2015a) reported that cholesterol increase the zeta-potential of liposomes and promote the formation of domain structure, which may induce differences in the protein adsorption profile of human plasma proteins. Thus, the differences in zeta-potential and mean diameter increment found in the present work indicated that different protein domains were bound in each liposome depending on lipid formulation.

Comparing the initial size distributions of liposomes with those of liposome–SURP mixtures, we conclude that a protein corona was formed and affected in different ways the zeta-potential in UPCL and PPCL. Most of the studies of liposome-protein corona formation using simulated biological fluids, as human plasma (Caracciolo, 2015b; Digiacomo et al., 2017) and animal serum (Guan et al., 2018), demonstrated that several factors could affect the protein binding on liposomes as its surface chemistry, topography, charge, protein affinity, and protein binding sites. Based in our findings, the liposome membrane packing could be an important factor to shaping protein adhesion, observed before as size increment and changes of zeta potential in the UPCL and PPCL (Fig. 5). The nisin tightens the lipid packing affecting the liposome surface charge intensity and also reducing the protein binding sites increasing the membrane thickness and reducing the structural defects, which has a crucial role in protein interactions with lipid bilayers (Róg et al. 2009; Briuglia et al. 2015). These packing defects expose hydrophobic domains on the surface of the bilayer that increase the contact between proteins and the phospholipid (Róg et al. 2009).

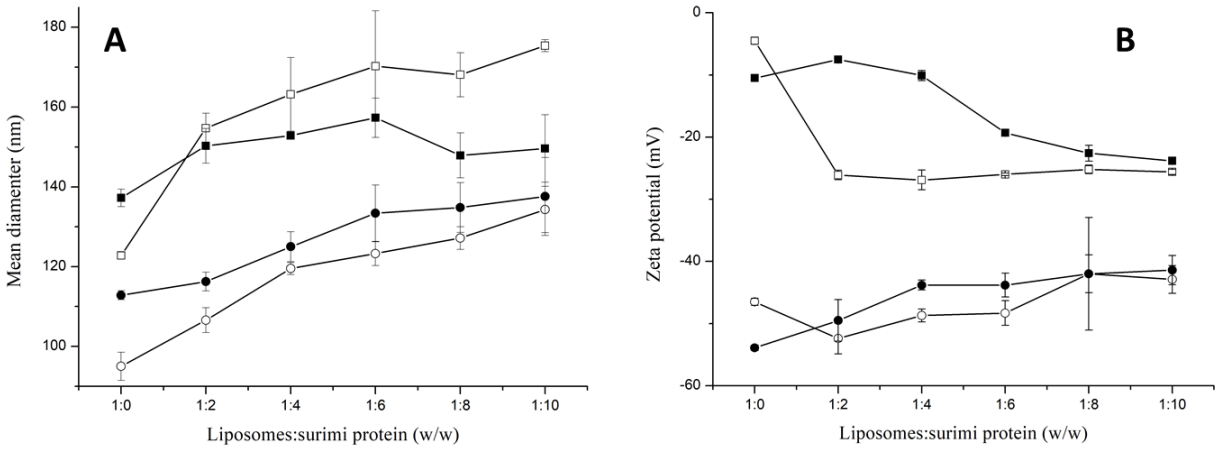

Fig 6. Effect of the Liposome-surimi protein on the particle sizes (A) and zeta-potentials (B) of nisin load PPL (black circle), unload PPL (white circle), nisin load UPL (black square) and unload UPL (white square).

Conclusion

This study showed that the ability of liposomes to bind myofibrillar protein was related with structural changes, which were found to be strongly affected by the lipid composition and surface charge. With the increase of SURP-PPCL weight ratio, partial denaturation and aggregation of protein was observed, attributed to the high polar surface of the PPCL that increased structure unfolding of SURP by the exposure of sulfhydryl groups and hydrophobic bonding sites. The hydrophobic surface and low zeta potential of UPCL generate less level of protein denaturation and favors the formation of β-strands structures, which result in an unfolded structure with liposome affinity, due to the electrostatic interactions lipid-protein. The formation of protein corona was observed and protein binding to liposomes was dependent of liposome composition and zeta potential. The present study describes an interesting relationship between liposome composition and its effect in the stability of surimi ground salted muscle, which would be an important point for development of fish gel products using liposomes as carriers of bioactive compounds.

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7. Artigo No.4

Comparative proteomic analysis of Listeria monocytogenes ATCC 7644 submitted to subletal concentrations of free and liposome encapsulated nisin.

O artigo a seguir analisa os dados obtidos por analise proteômica de células de *Listeria monocytogenes* ATCC 7644, submetidas a concentrações subletais de nisina. O antimicrobiano foi colocado em estado libre e encapsulado em lipossomas de fosfatidilcolina, com o objetivo de avaliar as respostas em expressão proteica da bactéria frente a este estresse, e verificar possíveis efeitos em redução de resistência, resposta ao estresse e virulência.

Artigo a ser submetido para publicação na revista *Food microbiology*, na versão em inglês.

Comparative proteomic analysis of *Listeria monocytogenes* ATCC 7644 submitted to subletal concentrations of free and liposome encapsulated nisin

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Abstract

Growth of *Listeria monocytogenes* on refrigerated ready-to-eat food has a significant concern in food safety. Natural antimicrobials, such as nisin, can be used to control this pathogen, but the increasing reports of nisin tolerance and resistance make necessary novel approaches for effective delivery, such as nanoencapulation. The goal of this study was to investigate, using a proteomics strategy, how *L. monocytogenes* ATCC7466 regulates and shapes its proteome in response to sublethal doses of nisin and nisin-loaded phosphatidylcholine liposomes (lipo-nisin), comparing to untreated cells growing under optimal conditions. Total proteins were extracted from *L. monocytogenes* cells treated for 1 h with free and liposome loaded nisin. As results, 805 proteins were initially identified and from those, 61 proteins were differentially upregulated for the treatments with nisin and lipo-nisin. The upregulation of 5 exclusive proteins was also observed in the treatment with nisin and 1 protein in the lipo-nisin. Adaptation and shaping of *Listeria* proteome in response to both treatments containing nisin were mainly concerned to ATP-binding cassette (ABC) transporter system, transmembrane proteins and RNA-binding proteins. Some of the proteins uniquely detected in free nisin-treatment were the membrane proteins secD, lmo1539 and the YfhO enzyme, which are related to translocation of *L. monocytogenes* virulence factors, activation of the SigB-mediated stress defense and glycosylation of wall tectonic acid, respectively. These results suggest that nisin-loaded liposomes reduce some stress response of *L. monocytogenes* membrane proteins as compared with the free nisin, turning the nisin, less recognizable by the innate immunity sensors and therefore reducing response and nisin-resistance factors of *L. monocytogenes*. Therefore, encapsulation of nisin into liposomes could be a strategy to reduce the risk of nisin resistance.

Key words: *Listeria*; nisin; lantibiotics; liposomes; proteomic

1. Introduction

Listeria monocytogenes is a facultative intracellular foodborne pathogen that can cause listeriosis, a serious disease with high deadly rate (approximately 16%) and has been considered a major concern to public health and the food industry (Scallan et al 2011; Lee, 2020). Infections with *L. monocytogenes* are more common among the elderly, pregnant women, infants, or immunocompromised individuals and are primarily associated with ingestion of contaminated food products (Farber and Peterkin, 1991). Thus, much care is taken to ensure the safety of meat and ready-to-eat products to ensure its safety, since this pathogen is capable of multiplying to high levels during refrigerated storage (Pradhan et al., 2009).

The increasing consumers demand for green, natural, and minimally processed foods is a big challenge for food biopreservation. Bacteriocins such as nisin, produced by food grade microorganisms, can be used to control pathogenic and food spoilage microorganisms with little changes of flavor or texture. Bacteriocins are a promising option to be used for biopreservation in food industry (Garsa et al. 2014). However, some studies have revealed that foodborne microorganisms can develop resistance against cationic antimicrobial peptides, including class I bacteriocins such as nisin (Lather et al. 2015). After the exposure to sublethal concentrations of bacteriocins, susceptible strains may acquire resistance with different levels of frequency and stability (Gravesen et al. 2002), which raises concerns about the possible emergence of bacteriocin-resistant strains. Particularly, strains of *L. monocytogenes* in foods present more nisin resistance than other Gram-positive pathogens (Field et al., 2010), probably because the foods produce additional environmental stresses to this pathogen, such as osmotic stress, organic acid stress, and cold stress, that increase nisin resistance (Bergholz et al., 2013).

In this scenario, different strategies, such as the use of nanostructured materials, can be developed to overcome bacterial resistance. Nanostructured materials can be used to convey antimicrobials, to assist in the delivery of antimicrobials and may circumvent drug resistance mechanisms in bacteria (Baptista et al., 2018). Among various nanoparticle platforms, liposomes are widely used vehicles for drug delivery, owing to their proven biocompatibility, biodegradability, and ability to encapsulate both hydrophilic and hydrophobic compounds (Kube et al., 2017). The liposomal lipid bilayer interacts directly with the lipids comprising the cell/bacteria membrane, delivering the cargo directly to the cell membrane without having to rely on active or passive uptake of the nanostructure by target bacterial cells (Wang et al., 2016). This represents a promising approach for improved antimicrobial compounds delivery.

The emergence of tolerance and resistance to bacteriocins among strains of *L. monocytogenes* requires the understood about how the bacteria detect and respond on the presence of these antimicrobial agents (Jiang et al., 2019). To attempt this goal, the proteomic approach can be used to obtain information on potential biological functions and molecular mechanisms of *L. monocytogenes* adaptation to bacteriocins (Guevara et al., 2015). Considering this scenario, the aim of this study was to investigate the effects of subletal concentrations of free and liposome loaded nisin on proteomic profile of *L. monocytogenes* ATCC 7466, searching for possible different responses of cell stress and virulence factors to these treatments.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. monocytogenes ATCC 7644 was maintained in BH broth containing 20% (v/v) glycerol for long-term preservation. To acclimatize the strain to the experimental conditions, 100 µL

of the culture was inoculated into 9.9 mL of Brain Heart Infusion broth (BHI) (Kasvi, São José dos Pinhais, Paraná, BR) and incubated for 24 h at 37°C and 125 rpm. Subsequently, the cells were propagated at 37°C for 24 h in BHI broth using a 1% (v/v) inoculum.

2.2. Preparation of liposomes

The nisin liposomes were produced according to (Pinilla & Brandelli, 2016) by the thin-film hydration method. Briefly, purified phosphatidylcholine (PC) (Phospholipon 90G®, Lipoid, Germany) was dissolved in chloroform on a round-bottom flask, the solvent was recovered by rotary evaporator at 40°C, until obtaining a thin film on the flask wall and finally dried in desiccator for 24 h. The obtained lipid film was recovery by the addition of a nisin solution (Nisaplin®, Danisco DuPont, Copenhagen, Denmark) at 3 µg/mL, prepared as described by Lopez et al. (2019) in 10 mM phosphate buffer (pH 7.0). The suspension was vortexed at 50°C in three times for 30 s, sonicated in an ultrasonic cell disrupter (Model DES500, Unique Group, S.P. Brazil) and finally sterilized by filtration through 0.22 mm membranes (Sartorius, Göt, Germany). This method provides nisin loaded liposomes with more than 90% of entrapment efficiency, cationic charge and size around 150 nm, as reported previously (Malheiros et al., 2010). In addition, bare liposomes were prepared with 10 mM phosphate buffer pH 7.0 instead nisin and used as control.

2.3. Antimicrobial treatments

Initially, *L. monocytogenes* ATCC7644 cells grown in BHI broth (Kasvi, São José dos Pinhais, Paraná, BR) for 24 h at 37°C were inoculated (0.1% v/v) in fresh BHI broth to attempt the concentration of 2 log CFU/mL. Cells were then cultivated at the optimum growth temperature (37°C and 125 rpm) until reaching the mid-exponential growth phase (OD_{600} nm about 0.4). At this point, it was added in separated treatments nisin at 3 µg/mL

final concentration, nisin-loaded liposomes (3 µg/mL nisin), and the control equivalent of bare liposomes. This concentration of nisin was determined as subletal by verification of growth curve in the concentration range from 0.1 to 10 µg/mL (data not shown). After incubating at 37°C for 1 h, the control and treated cells were harvested by centrifugation at 5000 *g* at 4°C for 10 min, and the pellet was washed three times with 2 mL of PBS pH 7.4 and reserved for protein extractions. Each treatment was performed in triplicate (biological replicates).

2.4. Preparation of protein extracts

Bacterial cells from each treatment were recovered during the mid-log growth phase, suspended in 1 mL of 50 mM tris-HCl buffer pH 7.5, containing 10 µL of Halt™ protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Cell suspensions were sonicated in an ice bath for 5 cycles in ultrasonic cell disrupter (Model DES500, Unique Group, São Paulo, Brazil) of 30s with a 60s of interval between each cycle. Then, the samples were centrifuged at 13,000 *g* for 20 min and the supernatants were stored at -80°C and lyophilized. Protein concentration was determined by the Lowry method, using bovine serum albumin as a standard.

2.5. Protein preparation for mass spectrometry

Protein digestion was carried out by the protocol of Villén and Gygi (2008), with some modifications. Approximately 100 µg of *L. monocytogene*s protein extracts were first denatured with 8 M urea (1:1 v/v) for 30 min, and then reduced and alkylated in 5 mM dithiothreitol (DTT, Sigma-Aldrich) and 14 mM iodoacetamide (IAA, Sigma-Aldrich), respectively. A further addition of 5 mM DTT for 15 min was performed to eliminate the remaining IAA. Samples were diluted in 50 mM ammonium bicarbonate (1:5 v/v) and 1 mM

$CaCl_2$ was used as a trypsin co-factor. Proteins were digested with 20 μg of trypsin (Sequencing Grade Modified Trypsin V5111, Promega, Fitchburg, WI, USA) by incubation at 37°C during 16 h. The digestion was stopped with formic acid 5% (v/v) and then centrifuged 14,000 *g* for 20 min, then, the supernatant was collected and desalinated in a C18 Sep-Pak column (Waters, Milford, MA, USA). Three biological replicates for each culture (nisin, lipo-nisin, bare liposomes and the control without treatment) were utilized for proteomic experiments. Each sample eluted from the C18 resin was run three times (LC-MS/MS technical replicates).

2.6. Protein analysis

After the preparation procedure, the samples were dried in a vacuum concentrator and reconstituted in 10 μL of 0.1% (v/v) formic acid. An aliquot of 3 μL (0.88 μg of protein) was analyzed on LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) connected to the EASY-nLC system (Proxeon Biosystem, West Palm Beach, FL, USA) through a Proxeon nanoelectrospray ion source. Peptides were separated by a 2-90% (v/v) acetonitrile gradient in 0.1% (v/v) formic acid using an analytical column PicoFrit Column (20 cm x ID75 μm, 5 μm particle size, New Objective) at a flow rate of 300 nL/min over 80 min. The nano electrospray voltage was set to 2.2 kV and the source temperature was 275°C. All instrument methods were set up in the data dependent acquisition mode. The full scan MS spectra (*m/z* 300-1600) were acquired in the Orbitrap analyzer after accumulation to a target value of 1 × 10E6. The resolution in the Orbitrap was set to *r* = 60,000 and the 20 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 5,000 and fragmented in the linear ion trap using low-energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 1,000 counts. Dynamic exclusion was enabled with an exclusion size list of 500,

exclusion duration of 60 s, and a repeat count of 1. An activation q = 0.25 and activation time of 10 ms were used.

2.7. Protein identification

Raw data were processed using MaxQuant v1.3.0.3 software (Cox and Mann, 2008) and MS/MS spectra were searched against *Listeria monocytogenes* UniProt database using the Andromeda search engine (Cox et al., 2011). For the false discovery rate, selected were identified proteins with valid LFQ intensity values present in ≥ 50% of the samples were considered for the statistical analysis. MetaboAnalyst 3.068 was used for statistical analysis. Principal Component Analysis (PCA) was carried out the variation grade between the control and the nisin-treated samples. Partial least squares discriminate analysis (PLS-DA) was performed and important features were selected with a threshold of VIP (Variable Importance in Projection) ≥1.0 and used for the hierarchical clustering heatmap analysis. All the proteins with ≥1.0 VIP score were identified using Uniprot (The UniProt Consortium 2019) and regrouped in down and up regulated proteins as reported on the heatmap. The terms with *p*-value <0.05, count numbers >2 and multiple linkage threshold value 0.5, were considered as significant. Additionally, Protein Analysis Through Evolutionary Relationships (PANTHER) classification system was used to determine the ontology categories. Proteins were classified by their molecular function, cellular localization, and class, using PANTHER 21. Finally, we used DAVID 6.8 (Huang et al., 2009) to find significant functional annotation clustering for the sets of differentially expressed proteins found by Andromeda and MetaboAnalyst.

3. Results

3.1. Effect of nisin and lipo-nisin on L. monocytogenes

Proteins extracted after 60 min treatment corresponds to the proteins that were expressed and accumulated as response of the cells to nisin, lipo-nisin and bare liposomes. This set of proteins represents the overall mechanism, in terms of protein expression and triggered by *Listeria* cells. A total 805 proteins were identified in the four conditions (Supplementary material 1). Compared to the control cells growing under optimal conditions, *L. monocytogenes* ATCC 7644 treated for 1 h showed increases (VIP score > 1.0) in the levels of 141 proteins (Heat map, Supplementary material). The proteins with VIP score under 1.0 were not consider for the analyses. The Venn diagram representing the interactions among the up-regulated proteins with significant VIP score is depicted in Figure 1. In detail, the treatments (nisin, lipo-nisin and lipo), exhibited increased level of 75 proteins compared to the control (without treatment). From these, 61 proteins were over expressed exclusively in the treatments nisin and lipo-nisin, 5 proteins were upregulated exclusively in response to free nisin and 1 protein by the nisin-loaded liposomes. These three protein groups resulted by treatments with nisin and liposomes were taken for ontology and functional analyses.

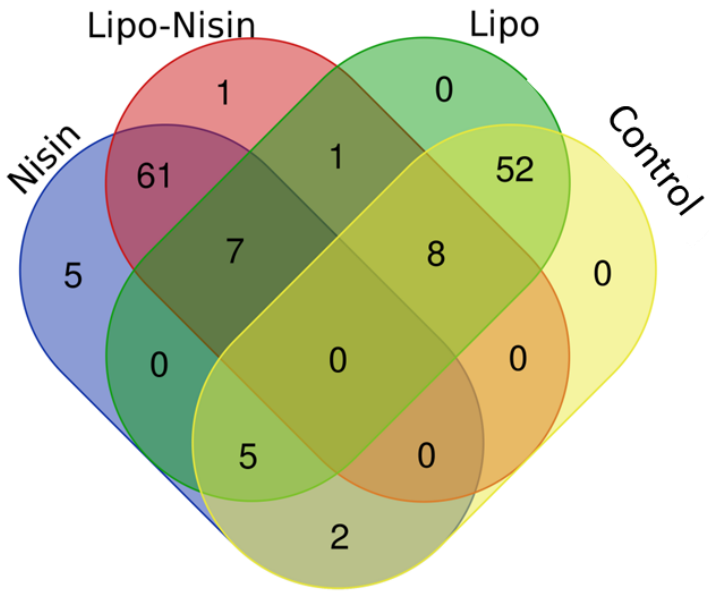

Fig 1. Distribution of *L. monocytogenes* ATCC 7644 proteins obtained under treatment with nisin, nisin loaded liposomes and unloaded liposomes. Venn diagram shows the dispersion of the identified upregulated proteins.

3.2. Gene ontology analysis of differentially expressed proteins by the treatments nisin and lipo-nisin

The results of Gene Ontology (GO) analysis provide a global description and relationship of the proteins uniquely upregulated by *L. monocytogenes* in response to subletal concentrations of free nisin and nisin-loaded liposomes. The 61 proteins common to the groups nisin and lipo-nisin, were categorized according to GO annotation, being assigned to biological processes, molecular function and protein class. According to the results (Fig 2), the main biological processes related to the proteins identified after the treatments were essentially associated with cellular process, metabolic process, localization, and cellular component organization and biogenesis (Fig 2A). Interestingly, proteins associated with locomotion and stimulus response were also differentially expressed. Regarding to the molecular function, proteins related to nucleic acid binding, translation and transport were the main groups identify by GO (Fig 2B). Considering the proteins class, metabolite interconversion enzymes, transport and translational proteins were the main classes identify (Fig 2C).

3.3. Functional annotation clustering of differentially expressed proteins by the treatments nisin and lipo-nisin

In order to determine specific molecular functions, the clustering annotation profile of the same 61 proteins differentially expressed in the treatments with nisin was carried out. The division of a network into groups of nodes with relatively dense connections within the

group provides practical and important information and helps to reveal substructures with common functionality or association (Zhu et al., 2007). Functional classification showed an important diversity of biological roles. A total of 10 clusters were identified (Fig. 3). A number of different classes of proteins were enriched with binding, transport, and secondary metabolites biosynthesis activity, representing the protein categories with more proteins. The RNA binding proteins comprised the most relevant category (enrichment score = 3.03 and 2.87) follow by ABC transport related proteins (enrichment score = 1.04). Other interesting categories were the secondary metabolites biosynthesis and transmembrane proteins. From a biological perspective, most of these categories are closely involved in adaptation and response to environmental stresses.

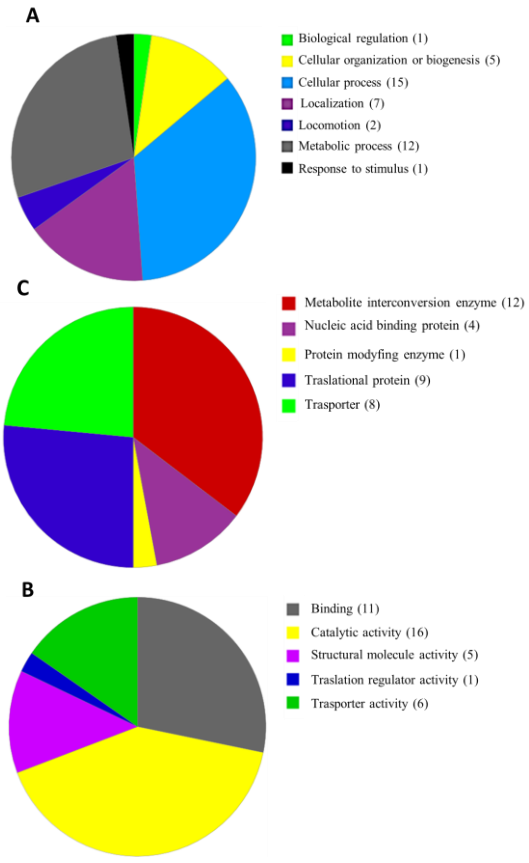

Fig. 2. Gene Ontology annotation of (A) biological process, (B) molecular function and (C) protein class of the differentially expressed proteins obtained from *L. monocytogenes* ATCC 7644 after treatment with free nisin and lipo-nisin.

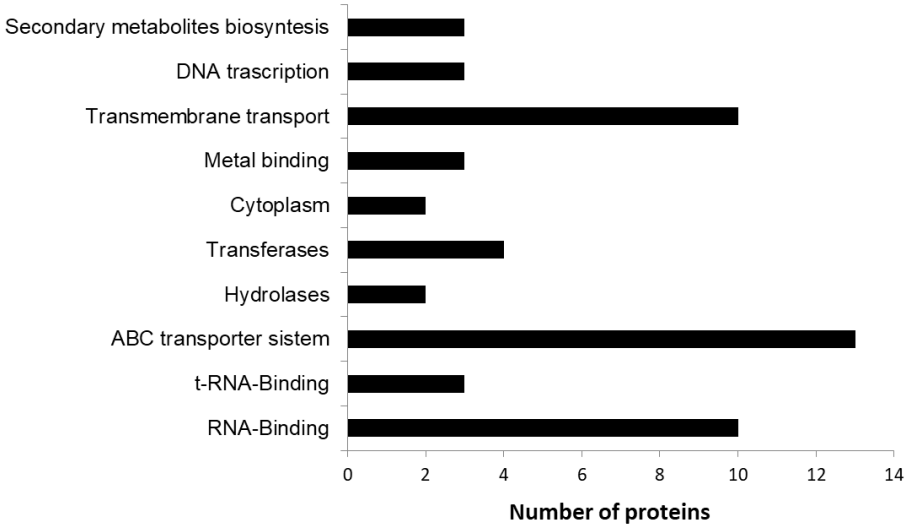

Fig 3. Cluster enrichment profile of differentially expressed proteins between nisin- and lipo –nisin treated cells of *L. monocytogenes* ATCC 7644. The bar charts represent the functional annotations of most relevant molecular groups.

3.4. Specific effects of free nisin or lipo-nisin on L. monocytogenes

To differentiate the effects of free or nisin-loaded liposomes on *L monocytogenes*, the proteins that were upregulated proteins by free nisin and the unique protein that was upregulated by lipo-nisin treatment were analyzed. The proteins upregulated in cells treated with free nisin and lipo-nisin are listed in Table 1. It could be identified the membrane protein lmo1539, involved in glycerol uptake with the higher VIP score (2.95); the SecD protein, that make part to the general secretion (Sec) system; YfhO that is as glycosyltransferase related to maintenance of wall teichoic acid (WTA) and the lmo 0955 that is a hypothetical protein, but present alignment with the PspA/I30 protein family. On

the other hand, only one protein was exclusively upregulated by the lipo-nisin treatment, the cysteine persulfate intermediate lmo1384 that belong to the rhodaneses group of enzymes.

Table 1. Proteins differentially regulated by free nisin or lipo-nisin treated cells of *Listeria monocytogenes* ATCC 7644. Positive VIP score numbers represent upregulated proteins in the treated cells compared with other treatments and the control.

Gene	Accession number	Protein description	Location	VIP score
lmo1539	CAC99617.1	Channel activity	Membrane	2.95
secD	CAC99605.1	Multifunctional fusion protein	Membrane	1.27
lmo0653	CAC98731.1	No information	Membrane	1.53
yfhO	CAC99157.1	Glycosyltransferase (Lmo1079)	Membrane	1.45
lmo0955	CAC99033.1	Hypothetical protein	No information	1.67
lmo1384	CAC99462.1	Cysteine persulfide intermediate	No information	1.97

4. Discussion

Nisin A is a broad-spectrum class I lantibiotic, produced by *Lactococcus lactis*, characterized by five intermolecular lanthionine rings that confer inherent heat and protease stability. It is the most studied bacteriocin and commercially approved as a food additive by regulatory agencies including the World Health Organization (WHO), the Food and Drug Administration (FDA) in the USA and the European Food Safety Authority (ESFA) in Europe (Chikindas et al., 2018). To improve its efficiency in foods, nisin-loaded nanoparticles have been engineered. Improved antimicrobial activity can be achieved since the nanometric range provides high surface area for particles, which increases the capacity to interact with biological substrates. In addition, nanoencapsulation may lead to protection of

antimicrobials against environmental stresses and provides controlled-release (Lopes et al., 2017).

However, some studies about the resistance to nisin have demonstrated that *L. monocytogenes* subjected to additional environmental stresses, such as osmotic stress, organic acid stress, and cold stress can contribute to the development of *Listeria* resistance (Bonnet et al., 2005; van Schaik et al., 1999). The exposure to antibiotics, when not lethal, induces stress responses and resistance development in bacteria as well. Since antibiotics represent stresses, they often elicit protective responses in bacteria that reduce antibiotic activity (Poole, 2012). Conversely, stress can impact antimicrobial susceptibility. As a specific example, stresses trigger gene expression of regulatory RNAs that influence antibiotic resistance by pairing with target mRNAs expressing drug efflux pumps, antibiotic transporters, or enzymes involved in drug catabolism (Felden and Cattoir, 2018).

To obtain a general view of the molecular mechanisms of the *L. monocytogenes* response to free or encapsulated nisin, we explored a broad proteomic approach to identify the differential protein profile of this bacterium. The analysis of the 61 proteins specifically upregulated by nisin and lipo-nisin, the most abundant proteins were related to transport proteins (Fig. 2), some of them with activity in the ATP-binding cassette (ABC) transporter system. ABC transporters are ubiquitous multi-domain integral membrane proteins that couple the energy of ATP hydrolysis to translocate molecules across cellular membranes in the cells of all domains of life (Jones and George, 2004). This system plays essential role in bacteria that recognize a wide range of ligands from metal ions to sugars, amino acids and peptides, including bacterial cell wall-derived murein tripeptide (Maqbool et al., 2015). The ABC transporter is also regulated by the two-component signal transduction systems (TCS) and is believed to participate in bacteriocin resistance by mediating its export from the cells (Blake et al., 2011). He and co-workers (2015) reported that under some stress conditions,

Listeria induces the overexpression of several ABC transporters to maintain cellular homeostasis, which could be crucial for cell survival. It was also reported that TCS/ABC transporter multicomponent system is involved in resistance to nisin and bacitracin in *L. monocytogenes* (Collins et al., 2010). In another work, the overexpression of the ABC transport system was observed in *Salmonella,* due to oxidative stress induced by cold plasma treatment (Ritter et al., 2018).

Other important group of upregulated proteins by the treatments with nisin was related to ribosomal proteins that act in the RNA binding (Fig. 2 and Fig. 3). Two essential ribosomal functions are structurally dominated by rRNA: decoding and peptide bond formation (Ogle et al., 2001). This group of proteins is very sensitive to oxidative stress, and it's over regulation is related to ribosomal reparation (Mirzaei and Regnier, 2007). Ribosome repair by r-protein exchange might therefore help bacteria to withstand environmental stresses during various growth conditions. Indeed, ribosome repair makes it possible to avoid the full degradation/synthesis metabolic cycle for each damaged ribosomal particle (Pulk et al., 2010). The adaptation to different antimicrobials leads to modification of the amount of some ribosomal proteins involved in translation, ribosomal structure and biogenesis which involve the 30S and 50S ribosomal proteins (Aleksashin et al., 2019). In a previous work, *L. monocytogenes* cells subjected to triple-stress conditions (low pH, high salinity and low temperature) showed the overexpression of the 30S ribosomal protein S1 (He et al., 2015).

Other stress-response proteins upregulated by nisin and lipo-nisin treatments were the penicillin-binding protein lmo2229 that contributes to spontaneous nisin-resistant of *L. monocytogenes* (Gravesen et al., 2001); the flagellar motor protein MotA (lmo0685) related to cold stress response (Mattila et al., 2011); and DltD protein that acts in the D-alanine esterification of lipoteichoic acid and wall teichoic acid and is related to cationic antimicrobial peptide resistance (Neuhaus and Baddiley, 2003). Analyzing this scenario, it is

possible suggested that the *L. monocytogenes* cells underwent structural damages in membranes and other cell structures, and the increased levels of transport proteins corroborate that free nisin produce diverse stress response, increased regulation and transcription of nisin-resistance factors, as well the transmembrane transport and metabolic processes.

From the set of membrane proteins uniquely overexpressed in the free nisin treatment we highlighted the lmo1539, secD, YfhO and lmo 0955. The presence of the glycerol kinase lmo1539 (*glpK*) was previously associated with changes in *L. monocytogenes* sugar metabolism. When the expression of genes encoding enzymes of the second part of glycolysis are reduced, it was found that an operon encoding glycerol kinase and the glycerol uptake facilitator (lmo1538 to lmo1539) and glycerol-3-phosphate dehydrogenase (lmo1293) are upregulated, indicating that glycerol was being used as additional carbon source for cellular growth in the cytosol of the host cells (Joseph et al., 2006). More recently, the study of Koomen et al., (2018) using two stress resistant *L. monocytogenes* LO28, related the upregulation of specific genes involved in glycerol uptake (*glpF*, *glpK*, *glpD*) as one of the features that can contribute to the activation of the SigB-mediated stress defense. Additionally, the stress response sigma factor SigB has been associated with salt tolerance and nisin resistance (Bergholz et al., 2013) in *L. monocytogenes*.

Typically, Gram-positive bacteria general secretion (Sec) system is composed of the protein conducting SecYEG channel and the auxiliary SecD-SecF complex that is fused into a single protein (SecDF) encoded by a single gene (Schneewind and Missiakas, 2012). Protein secretion is driven by the SecA ATPase, which energizes translocation of secreted and trans-membrane proteins through the membrane-embedded SecYEG pore (Trost et al., 2005).

However, a recent investigation on *L. monocytogenes* SecDF showed that the Sec system plays a role as a chaperone that facilitates the translocation of *L. monocytogenes* virulence factors during infection (Burg-Golani et al., 2013). The authors suggest that *Listeria* could modify the composition and/or the stoichiometry of the translocon apparatuses as a mechanism to cope with different secretion/translocation requirements, such a strategy could enable *L. monocytogenes* to manage the enhanced secretion of virulence and housekeeping proteins during infection. Taken together, the data link the secretion of *L. monocytogenes* virulence factors to the Sec system and evidence the importance of this system in *L. monocytogenes* pathogenesis.

The putative membrane protein designated as YfhO (lmo1079) features similarity to glycosyltransferases and it was reported as responsible for modification of the poly(ribitol-phosphate) in the bacterial wall teichoic acid (Eugster et al., 2015). Teichoic acids are important cell wall polymers produced by Gram-positive bacteria. They are either covalently linked to the peptidoglycan and denoted as wall teichoic acid (WTA) or embedded in the cytoplasm via a glycolipid anchor and called lipoteichoic acid (LTA) (Neuhaus and Baddiley, 2003, Percy and Gründling, 2014). WTA/LTA subunits can be typically substituted with sugars or esterified with D-alanine, as a result of the action of specific glycosyltransferases, forming chemically diverse WTA monomers; the most common are glycerol or ribitol- phosphate. These cell wall polymers are essential for the maintenance of the cell integrity and play an important role in antimicrobial resistance, cation homeostasis, regulation of peptidoglycan autolysins, cell division and virulence for the glycosylation of WTA (Rismondo et al., 2018). Given that D-alanine is positively charged at physiological pH, the addition of this molecule to teichoic acids represents a mechanism used by bacteria to fine-tune their surface charge in response to adverse environmental conditions (Carvalho

et al., 2014). This process is particularly important for the protection against cationic antimicrobial peptides (Carvalho et al., 2015).

Other interesting protein upregulated exclusively by free nisin was the lmo 0955. This protein is reported as hypothetical, but his sequence producing significant alignments with the PspA/I30 family proteins. The PspA from enterobacteria, IM30 (Vipp1) from cyanobacteria and plants, and LiaH from Firmicutes are the best characterized member of the PspA/IM30 family, and presents common features with respect to their oligomerization, membrane binding and putative membrane protecting function (Thurotte et al., 2017). The LiaH and LiaI have been shown to be strongly induced by LiaR in both *L. monocytogenes* and *Bacillus subtilis*. In the work of Bergholz and co-workers (2013), the increase of LiaH and LiaI transcription was reported as result of salt-stress in *L. monocytogenes*, confirming the LiaR regulation was induced. According with the authors, these increase of transcript levels of LiaR also favorite the cross-protection against nisin, due to LiaR acts as mediator of expression of genes involved in nisin resistance.

As mentioned above, only one protein was exclusively upregulated by the lipo-nisin treatment, namely lmo1384. This protein acts as cysteine persulfide intermediate and belongs to the rhodanese group of enzymes. Rhodaneses are ubiquitously present in nearly all living organisms, including archea, bacteria, fungi, plants and animals (Cipollone et al., 2007). Rhodanese and/or rhodanese-like proteins are known to play a critical role in sulfur traffic by delivering sulfur in a “safe” chemical species to biosynthetic pathways, using their labile persulfide group (Aussignargues et al., 2012). Despite numerous studies, the physiological role of rhodaneses as well as their physiological substrates remain unclear and are still widely debated. It has been proposed that they accomplish essential cell functions as they may be involved in the maintenance of redox homeostasis (Remelli et al., 2012) and in

the elimination of toxic cyanide (Cipollone et al., 2007). However, there is a forming notion that rhodanese may have an antioxidant role. Through the above studies, rhodaneses were suggested to have an important role in such an essential and ubiquitous metabolic process, the antioxidant defense. In the bacterium *Azotobacter vinelandii*, rhodanese was found to be regulated by stressor induction (Remelli et al., 2010), indicating possible roles of rhodanese-domain proteins in physiological processes related to xenobiotic-induced oxidative-stress and detoxification. This experimental evidence suggests that rhodanese domains might function as regulatory devices in specific physiological situations, but the redundancy of these proteins in the same organism make it difficult to define their different cellular roles.

4. Conclusion

Environmental stresses similar to those present on foods can lead to cross-protection of *L. monocytogenes* against nisin. In conclusion, our results indicated that encapsulation of nisin into phosphatidylcholine liposomes, exerts a different effect on protein expression by *L. monocytogenes* as compare with the free nisin. These differences include the reduction of multiple mechanisms of stress response, virulence and nisin resistance. Thus, the liposome probably changes the way the cell membrane recognizes the nisin, retarding the initial defense response. The group of proteins differentially upregulated upon both treatments with nisin and nisin-loaded liposomes, indicate that the response mechanism involves diverse biochemical functions as result of the stress imposed to the cells, compromising motility, transport, and cellular defenses. Therefore, the use of liposomes could potentially be used as carrier of nisin reducing potential factors of *Listeria* resistance.

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7.1 Supplementary material (Artigo 4)

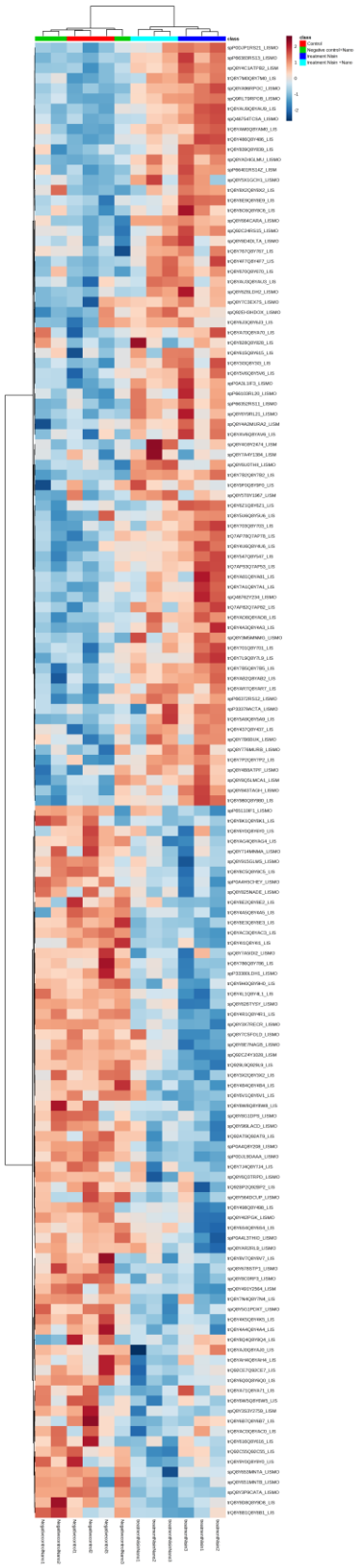

Figure 1. Heat map of the different up and down regulated proteins for all treatments

Table 1. List of proteins upregulated exclusively by nisin and lipo-nisin

UNIPROT_ ACCESSON	Gene Name
P0A3L1	translation initiation factor IF-3(infC)
P0DJP1	30S ribosomal protein S21(rpsU)
P33379	actin-assembly inducing protein precursor(actA)
P66103	50S ribosomal protein L20(rplT)
P66352	30S ribosomal protein S11(rpsK)
P66372	30S ribosomal protein S12(rpsL)
P66383	30S ribosomal protein S13(rpsM)
P66401	30S ribosomal protein S14(rpsN)
Q48754	CD4+ T cell-stimulating antigen, lipoprotein(tcsA)
Q48762	hypothetical protein(lmo0234)
Q7AP53	peptide ABC transporter ATP-binding protein(lmo2193)
Q7AP78	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
Q7AP82	flagellar motor protein MotA(lmo0685)
Q8Y3M5	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA(gidA)
Q8Y486	peptide ABC transporter substrate-binding protein(lmo2569)
Q8Y4A3	transcription termination factor Rho(rho)
Q8Y4B8	ATP synthase F0F1 subunit B(atpF)
Q8Y4C1	ATP synthase F0F1 subunit beta(atpD)
Q8Y4F7	hypothetical protein(lmo2487)
Q8Y4G9	hypothetical protein(lmo2474)

Q8Y4U6	PTS fructose transporter subunit IIABC(fruA)
Q8Y547	penicillin-binding protein(lmo2229)
Q8Y5A9	oxidoreductase(lmo2159)
Q8Y5T8	toxic ion resistance protein(lmo1967)
Q8Y5U6	ferrichrome-binding protein(lmo1959)
Q8Y5V6	hypothetical protein(lmo1949)
Q8Y5X1	GTP cyclohydrolase I(folE)
Q8Y664	carbamoyl phosphate synthase small subunit(pyrAa)
Q8Y670	fibronectin-binding proteins(lmo1829)
Q8Y6J3	deoxyuridine triphosphate nucleotidohydrolase(lmo1691)
Q8Y6U0	thiamine biosynthesis protein ThiI(lmo1592)
Q8Y6Y9	50S ribosomal protein L21(rplU)
Q8Y6Z6	L-lactate dehydrogenase(lmo1534)
Q8Y701	hypothetical protein(lmo1529)
Q8Y767	hypothetical protein(lmo1434)
Q8Y7A1	sugar ABC transporter ATP-binding protein(lmo1389)
Q8Y7B2	branched-chain alpha-keto acid dehydrogenase subunit E2
Q8Y7B5	dihydrolipoamide dehydrogenase(lmo1371)
Q8Y7C3	exodeoxyribonuclease VII small subunit(lmo1362)
Q8Y7L9	PTS trehalose transporter subunit IIBC(lmo1255)
Q8Y7M0	alpha,alpha-phosphotrehalase(lmo1254)
Q8Y7P2	DNA polymerase beta(lmo1231)
Q8Y843	teichoic acid ABC transporter ATP-binding protein(lmo1075)
Q8Y8C6	peptidase(lmo0982)

Q8Y8D4	D-alanine--poly(phosphoribitol) ligase subunit 1(dltA)
Q8Y8Q5	calcium-transporting ATPase(lmo0841)
Q8Y9F0	hypothetical protein(lmo0579)
Q8YA81	sugar ABC transporter ATP-binding protein(lmo0278)
Q8YA96	DNA-directed RNA polymerase subunit beta'(rpoC)
Q8YAB2	serine O-acetyltransferase(cysE)
Q8YAD4	bifunctionalN-acetylglucosamine-1-phoshate (glmU)
Q8YAD8	lmo0193(lmo0193)
Q8YAM0	PTS mannose transporter subunit IID(lmo0098)
Q8YAR7	lmo0047(lmo0047)
Q8YAU3	GntR family transcriptional regulator(lmo0020)
Q8YAU9	AA3-600 quinol oxidase subunit I(qoxB)
Q8YAV6	DNA gyrase subunit A(gyrA)
Q92C24	30S ribosomal protein S15(rpsO)
Q92EH3	lmo0484(lmo0484)
Q9RLT9	DNA-directed RNA polymerase subunit beta(rpoB)

Table 2. Cluster enrichment profile of differentially expressed proteins between nisin and lipo-nisin-treated cells of *L. monocytogenes* ATCC 7644

Annotation Cluster 1	**Enrichment Score: 3.03**	**Count**	**P_Value**	**Bonferroni**
	RNA-binding	11	1.0E-6	7.8E-5

	Ribosomal protein	8	1.2E-4	9.2E-3
	Ribonucleoprotein	8	1.4E-4	1.0E-2
	rRNA-binding	7	1.4E-4	1.0E-2
	structural constituent of ribosome	8	5.4E-4	3.4E-2
	rRNA binding	6	1.7E-3	1.0E-1
	translation	8	2.9E-3	1.6E-1
	Ribosome	8	1.4E-2	4.3E-1
	small ribosomal subunit	3	1.6E-2	3.3E-1
	RNA binding	3	4.0E-1	1.0E0
Annotation Cluster 2	**Enrichment Score: 2.87**	**Count**	**P_Value**	**Bonferroni**
	RNA-binding	11	1.0E-6	7.8E-5
	tRNA-binding	3	2.4E-2	8.5E-1
	tRNA binding	3	1.0E-1	1.0E0
Annotation Cluster 3	**Enrichment Score: 1.41**	**Count**	**P_Value**	**Bonferroni**
	Hydrolase	9	1.2E-2	6.2E-1
	Nuclease	3	1.2E-1	1.0E0
Annotation Cluster 4	**Enrichment Score: 0.95**	**Count**	**P_Value**	**Bonferroni**
	Transport	10	7.4E-3	4.4E-1
	Nucleotide-binding	13	8.4E-3	4.8E-1
	ATP-binding	12	8.9E-3	5.0E-1
	AAA+ ATPase domain	6	7.2E-2	1.0E0
	ATP binding	12	1.2E-1	1.0E0
	AAA	6	1.6E-1	9.5E-1
	ABC transporter, conserved site	4	1.6E-1	1.0E0

	Oxidative phosphorylation	3	2.5E-1	1.0E0
	ABC transporter-like	4	2.5E-1	1.0E0
	P-loop containing nucleoside triphosphate hydrolase	7	2.6E-1	1.0E0
	ATPase activity	3	4.7E-1	1.0E0
	ABC transporters	4	5.8E-1	1.0E0
	nucleotide phosphate-binding region:ATP	3	8.5E-1	1.0E0
Annotation Cluster 5	**Enrichment Score: 0.75**	**Count**	**P_Value**	**Bonferroni**
	Acyltransferase	3	4.2E-2	9.6E-1
	Nucleotidyltransferase	3	9.4E-2	1.0E0
	Transferase	8	2.8E-1	1.0E0
	Metabolic pathways	16	8.7E-1	1.0E0
Annotation Cluster 6	**Enrichment Score: 0.61**	**Count**	**P_Value**	**Bonferroni**
	Cytoplasm	10	1.1E-1	1.0E0
	cytoplasm	11	5.5E-1	1.0E0
Annotation Cluster 7	**Enrichment Score: 0.54**	**Count**	**P_Value**	**Bonferroni**
	Metal-binding	8	1.2E-1	1.0E0
	Zinc	3	3.6E-1	1.0E0
	zinc ion binding	3	5.6E-1	1.0E0
Annotation Cluster 8	**Enrichment Score: 0.53**	**Count**	**P_Value**	**Bonferroni**
	Transport	10	7.4E-3	4.4E-1
	Cell membrane	8	4.8E-2	9.8E-1
	Ion transport	3	6.9E-2	1.0E0

	plasma membrane	7	4.6E-1	1.0E0
	Membrane	14	7.6E-1	1.0E0
	phosphoenolpyruvate-dependent sugar phosphotransferase system	3	8.1E-1	1.0E0
	Phosphotransferase system (PTS)	3	8.3E-1	1.0E0
	Transmembrane helix	11	9.3E-1	1.0E0
	Transmembrane	11	9.3E-1	1.0E0
	integral component of membrane	10	1.0E0	1.0E0
Annotation Cluster 9	**Enrichment Score: 0.13**	**Count**	**P_Value**	**Bonferroni**
	Transcription	4	6.0E-1	1.0E0
	DNA binding	6	7.1E-1	1.0E0
	transcription, DNA-templated	3	9.3E-1	1.0E0
Annotation Cluster 10	**Enrichment Score: 0.02**	**Count**	**P_Value**	**Bonferroni**
	Microbial metabolism in diverse environments	4	8.9E-1	1.0E0
	Biosynthesis of antibiotics	4	9.6E-1	1.0E0
	Biosynthesis of secondary metabolites	4	1.0E0	1.0E0

8. Conclusões

- Os estabilizadores aumentaram a estabilidade física dos lipossomas em termos de tamanho médio de partícula e potencial zeta. As formulações contendo ácido oleico e colesterol apresentaram um efeito sinérgico na redução da oxidação lipídica e também melhoraram as propriedades térmicas dos lipossomas, aumentando a ordem e rigidez da membrana. Considerando a estabilidade do armazenamento, o processo de liofilização aumentou a fusão e a agregação em todas as formulações de lipossomas, mas esse efeito foi reduzido usando a trealose como lioprotetor. Entretanto, apenas os lipossomos preparados com AO apresentaram atividade antimicrobiana melhorada. Portanto o AO tem um grande potencial como estabilizador de lipossomos contendo misturas complexas de compostos bioativos.

- A adição de acido oleico na composição dos lipossomas contendo extrato de alho melhorou a distribuição tamanha, eficiência de encapsulação e polidispersividade, mediante a estabilização da estrutura lipossomal, resultado do efeito combinado das moléculas de acido oleico no interior da membrana e polissacarídeos do extrato de alho na superfície. Também, esta estratégia de veiculação do extrato resultou exitosa no controle de bolores em pães de trigo, mantendo ausência de bolores por cinco dias a mais, quando comparado com o controle.

- A capacidade dos lipossomas de se desestabilizar a proteína miofibrilar esta relacionada à composição lipídica e carga superficial. A alta polaridade de superfície dos lipossomas feitos com fosfatidilcolina parcialmente purificada aumentou o desdobramento da estrutura da proteína pela exposição de grupos sulfidrila e sítios de ligação hidrofóbicos. Por sua vez, a superfície hidrofóbica e o baixo potencial zeta dos UPCL geram menor nível de desnaturação da proteína e favorecem a formação de estruturas *β-strand*, com afinidade pela superfície do lipossoma. Além disso, formação da proteína corona foi observada e a ligação da proteína aos lipossomas foi dependente da composição lipossômica e do potencial zeta.
- O encapsulamento de nisina em lipossomas de fosfatidilcolina exerce efeito diferente na expressão de proteínas por *L. monocytogenes,* quando comparado com a nisina

livre; reduzindo mecanismos de resposta ao estresse, resistência e virulência. O lipossoma provavelmente altera a maneira como a membrana celular reconhece a nisina, retardando a resposta de defesa inicial. O grupo de 61 proteínas positivamente reguladas pelos tratamentos com nisina livre e encapsulada, indica que o mecanismo de resposta da *L. monocytogenes*, como resultado do estresse imposto às células, envolve diversas funções bioquímicas, como motilidade, aumento dos sistemas de transporte e síntese de metabólitos secundários.

9. Considerações finais

Na atualidade conceitos como saudabilidade, CLEAN LABEL e PLAN-BASED tem sido o foco da indústria de alimentos nos últimos anos e todos giram em torno de alimentos mais seguros e saudáveis para as pessoas, sendo uma tendência mundial.

O conhecimento adquirido na realização deste trabalho, permitiu ter uma visão mais completa de aplicações, estabilidade química, física e da interação lipossoma-alimento. Com isto conseguimos mudar a ideia dos lipossomas, de uma possibilidade *in vitro* de alto custo, para uma alterativa viável, fonte de mais pesquisas e transferência tecnológica.

Esperamos aprofundar nas pesquisas nesta área, para assim, junto com parcerias da indústria de alimentos, trazer alterativas aos conservantes sintéticos e assim aportar à segurança dos alimentos o e bem-estar das pessoas.

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