HELOÍSA HELENA DE ABREU MARTINS

NANOEMULSIONS CONTAINING ESSENTIAL OILS: CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY AGAINST FOOD CONTAMINATING BACTERIA

LAVRAS-MG

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Prof.ª Dra. Roberta Hilsdorf Piccoli

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NANOEMULSÕES CONTENDO ÓLEOS ESSENCIAIS: CARACTERIZAÇÃO E ATIVIDADE ANTIMICROBIANA CONTRA BACTÉRIAS CONTAMINANTES EM ALIMENTOS

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RESUMO

A indústria de alimentos, impulsionada pela crescente demanda dos consumidores por alimentos mais saudáveis e livre de aditivos sintéticos busca por novas opções de conservantes. Nesse sentido, os óleos essenciais (OEs) tem grande potencial pois já são estudados há algum tempo por possuírem atividade antioxidante e antimicrobiana. Embora os óleos essenciais apresentem atividade biológica contra diferentes microrganismos, o seu uso em alimentos ainda é um desafio. OEs tem baixa solubilidade em água e a sua incorporação nos alimentos é dificultada. Além disso, seu uso pode levar a alteração de gosto e sabor nos produtos alimentícios. Baseado nisso, as nanoemulsões dos óleos essenciais têm sido bastante estudadas. O objetivo desse trabalho foi preparar e caracterizar nanoemulsões contendo óleos essenciais e verificar a sua efetividade contra bactérias contaminantes de alimentos. Primeiramente, nanoemulsões de orégano, tomilho, cravo e capim-limão foram eleboradas com Tween 80. As mesmas foram avaliavadas quanto à influência do pH, temperatura e força iônica sobre a estabilidade física através do tamanho de partícula e potencial zeta. Ainda, as nanoemulsões foram avaliadas em relação à atividade antimicrobiana contra endósporos e células vegetativas de *C. sporogenes*. Os resultados mostraram que a estabilidade física das nanoemulsões é dependente do tipo de óleo essencial e do tamanho inicial da gotícula, bem como, da temperatura e do pH. O uso do sal influenciou apenas na repulsão eletrostática das gotículas. Além disso, foi possível obervar ação esporicida melhorada das nanoemulsões contendo os óleos essenciais quando comparadas aos óleos essenciais livres, sinergismo e redução no crescimento de células vegetativas de *C. sporogenes.* Em um segundo experimento, nanoemulsões contendo óleos essenciais de orégano e capim-limão foram elaboradas com proteína, pectina, e complexo proteína-pectina como surfactantes. As mesmas tiveram seu potencial antimicrobiano avaliado contra *Escherichia coli.* As nanoemulsões elaboradas usando complexo proteína-pectina se apresentaram mais estáveis com o tempo de armazenamento. Nesse estudo foi possível observar que a diferença de tensão interfacial entre os surfactantes utilizados afetou a atividade das nanoemulsões contra *E. coli*. Em ambos os estudos é evidente que a composição do óleo essencial afeta a atividade antimicrobiana da nanomeulsões. Além disso, conclui-se que o uso de diferentes surfactantes impacta no tamanho de gotícula e na estabilidade das nanoemulsões, bem como na atividade contra microrganismos.

Palavras-chave: Aditivo natural; Nanotecnologia; Microfluidização; Complexo químico; Ação antimicrobiana

ABSTRACT

The food industry, driven by growing consumer demand for healthier foods and free of synthetic additives, is looking for new preservative options. In this sense, essential oils (EOs) have great potential because they have been studied for some time due to their antioxidant and antimicrobial activity. Although essential oils have biological activity against different microorganisms, their use in food is still a challenge. EOs have low water solubility and incorporation into food is difficult. Moreover, their use may lead to change in taste and flavor in food products. In this way, nanoemulsions of essential oils have been extensively studied. The objective of this work was to prepare and characterize nanoemulsions containing essential oils and to verify their effectiveness against food contaminating bacteria. First, nanoemulsions of oregano, thyme, clove and lemongrass were elaborated with Tween 80. They were evaluated for the influence of pH, temperature and ionic strength on physical stability through particle size and zeta potential. Nanoemulsions were also evaluated for antimicrobial activity against endospores and vegetative cells of *C. sporogenes*. The results showed that the physical stability of nanoemulsions is dependent on the type of essential oil and initial particle size, as well as temperature and pH. The use of salt influenced only the electrostatic repulsion of the droplets. Moreover, it was possible to observe improved sporicidal action of nanoemulsions when compared to free essential oils, synergism and reduced growth of *C. sporogenes* vegetative cells. In a second experiment, nanoemulsions containing oregano and lemongrass essential oils were made with protein, pectin, and protein-pectin complex as surfactants. They had their antimicrobial potential evaluated against *Escherichia coli*. Nanoemulsions made using protein-pectin complex were more stable with storage time. In this study it was observed that the difference in interfacial tension between the surfactants used affected the activity of nanoemulsions against *E. coli*. In both studies it is evident that the composition of the essential oil affects the antimicrobial activity of nanomeulsions. In addition, it is concluded that the use of different surfactants impacts on droplet size and nanoemulsion stability as well as activity against microorganisms.

Keywords: Natural Additive; Nanotechnology; Microfluidization; Chemical complex; Antimicrobial action

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A grande mudança no perfil alimentar da população, principalmente no que diz respeito à crescente preocupação com a saudabilidade, leva a necessidade da adaptação do segmento alimentar à nova realidade. Os consumidores buscam alimentos naturais e saudáveis, e quando adicionados de aditivos, estão preferindo os que sejam naturais. Apesar dos vários métodos de conservação de alimentos existentes, faz-se necessário o estudo e a utilização de novas tecnologias, ou a combinação de métodos para suprir as necessidades do consumidor.

A utilização de aditivos é imprescindível sob o ponto de vista tecnológico na produção de alimentos. Porém, é necessário estar atento aos possíveis riscos toxicológicos que podem ser acarretados pela ingestão frequente dessas substâncias. Diversas pesquisas têm mostrado reações tóxicas incididas por alguns aditivos sintéticos, quer seja aguda ou crônica, que desencadearam processos alérgicos, alterações neurocomportamentais e, em longo prazo, neoplasias, assim, a indústria busca por novas opções de conservantes, preferencialmente obtidos de fontes naturais.

Neste contexto, os óleos essenciais surgem como alternativa promissora, já que nos últimos anos vem sendo comprovadas cientificamente as propriedades antimicrobianas de substâncias presentes nos óleos essenciais produzidos pelas plantas, como consequência do seu metabolismo secundário. Devido a estas propriedades, alguns destes óleos essenciais já vêm sendo estudados e aplicados na indústria farmacêutica, como alternativa ao uso de antibióticos, e na indústria alimentícia, na substituição parcial ou total de conservantes químicos sintéticos, porém existem ainda certas dificuldades na aplicação desses óleos. Embora os óleos essenciais tenham desejada atividade antimicrobiana sobre vários microrganismos patogênicos e deterioradores, os ensaios realizados *in vitro*, de um modo geral, mostram que concentrações elevadas destes são necessárias para obter o mesmo efeito dos conservantes comumente utilizados em alimentos, e este fato pode conduzir a alteração sensorial do produto, podendo ultrapassar o limite de sabor aceitável pelo consumidor. Além disso, os óleos essenciais são bastante instáveis às condições ambientais e pouco solúveis em água.

A fim de contornar essas limitações e utilizar os óleos essenciais de plantas em sistemas alimentares, é crucial o desenvolvimento de novas metodologias. Assim, tem sido amplamente

proposto utilizar a nanotecnologia, uma vez que os sistemas nanoestruturados podem ser capazes de melhorar a estabilidade, solubilidade e biodisponibilidade dos óleos essenciais, além de poder controlar a liberação do óleo essencial, minimizando assim as alterações sensoriais. As nanoemulsões são sistemas emulsionados que possuem gotas com tamanho médio na faixa de 20 a 200 nm. Alguns autores consideram uma faixa de 20 a 500 nm. Devido ao pequeno tamanho da gota, as nanoemulsões possuem maior estabilidade cinética quando comparadas a emulsões normais, o que as pode tornar estáveis por longo tempo de armazenagem. Em adição, o uso de nanoemulsões aumenta a atividade antimicrobiana dos óleos essenciais.

Diante do exposto, esse trabalho tem como objetivos a produção e caracterização de nanoemulsões de óleos essenciais, bem como a avaliação da atividade antimicrobiana contra microrganismos contaminantes em alimentos.

2 REFERENCIAL TEÓRICO

2.1 Bactérias em alimentos

A deterioração de alimentos por microrganismos causa grandes perdas para as indústrias de alimentos, determinando na maioria dos casos, o final da vida útil dos produtos. Para diminuir estes prejuízos, é importante conhecer as características dos microrganismos envolvidos, bem como conhecer técnicas para inativação dos mesmos. Além dos microrganismos deteriorantes, os alimentos podem ser facilmente contaminados durante manipulação e processamento por microrganismos patogênicos, causadores de doenças transmitidas por alimentos (DTAs) (CHAN, KRIEG & PELCZAR, 1996).

De acordo com a Organização Mundial da Saúde (OMS) as doenças transmitidas por alimentos são aquelas de natureza infecciosa ou tóxica causadas pela ingestão de alimentos ou água contaminados por agentes biológicos, químicos e físicos, representando um sério risco à saúde (BRASIL, 2010). As bactérias causadoras de DTAs podem ser divididas em grupos, de acordo com as manifestações clínicas. As infecciosas mais importantes são *Salmonella* spp, *Campylobacter* e *Escherichia coli*, as principais intoxicantes são *Bacillus cereus*, *Staphylococcus aureus* e *Clostridium botulinum* e no grupo das toxigênicas, a *E. coli* enterotoxigênica, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Clostridium perfringens* e *Bacillus cereus* são exemplos clássicos (BRASIL, 2010).

Nos últimos 17 anos, entre os principais agentes envolvidos em surtos de DTA no Brasil e no mundo está a *Escherichia coli* (BRASIL, 2018). A *E. coli* pertence à família Enterobacteriaceae. São bastonetes gram negativos, não esporulados, móveis ou não e anaeróbios facultativos (PINTO, 1996). Essa bactéria é habitante comensal do trato entérico de humanos e animais sadios (LÓPEZ-BANDA et al., 2013; QADRI et al., 2005; RYU et al., 2012; TANGI et al., 2015). A presença de cepas em alimentos indica contaminação direta ou indireta de origem fecal, sendo considerada indicador de possível presença de outros microrganismos patogênicos. Contagens elevadas de *E. coli* também podem estar relacionadas à falta de higiene e à falha no processamento de alimentos (YUCEL; ULUSOY, 2006). Esse microrganismo é facilmente eliminado por processamento térmico, ou tem seu crescimento inibido em condições adversas como acidez ou refrigeração.

Diferentemente, há microrganismos que são resistentes à condições adversas. São esses os chamados microrganismos esporulados, como *Bacillus* spp e *Clostridium* spp. Endósporos de bactérias são extremamente resistentes à temperatura, pH, radiação UV, ação enzimática, ácidos orgânicos, e podem permanecer dormentes durante longos períodos. A sobrevivência bem sucedida de um microrganismo esporulado depende da habilidade desse sistema dormente (endósporo inativo) ser ativado, germinar, crescer e se multiplicar em condições favoráveis para o seu desenvolvimento (TANAKA et al., 1799). Os endósporos de *Clostridium botulinum*, microrganismo causador do botulismo, estão entre as formas mais resistentes que se têm encontrado entre os agentes bacterianos, podendo sobreviver por mais de 30 anos em meio líquido e, provavelmente, mais tempo ainda em estado seco. Podem tolerar temperaturas de 100°C durante horas. Para reduzir o número de endósporos nos alimentos, os mesmo devem ser aquecidos a 120°C por 30 minutos (KETCHAM, GOMEZ, 2003). Desta forma, bactérias esporuladas constituem um problema especial para a indústria de alimentos.

Tratamentos térmicos e o uso de aditivos químicos estão entre as técnicas mais utilizadas para eliminação/inibição de bactérias e consequente conservação de alimentos. Porém, o uso de calor pode alterar as características sensoriais e nutritivas dos alimentos como alteração da cor, perda de aromas e perda de compostos benéficos à saúde do consumidor (BARBOSA-CÁNOVAS et al, 1998). Em relação aos aditivos, estes são amplamente utilizados na indústria de alimentos, com diversas funções, no entanto, o uso de alguns aditivos sintéticos vem demonstrando algumas controvérsias em relação ao seu consumo, como manifestações clínicas, complicações metabólicas e efeitos carcinogênicos, dentre outros (SOUZA et al., 2009).

2.2 Óleos essenciais como aditivos em alimentos

Durante séculos os aditivos alimentares têm sido utilizados, exercendo funções tecnológicas nos alimentos, estendendo a vida útil, e promovendo a segurança alimentar. De acordo com a Legislação Brasileira (BRASIL, 1997), aditivo alimentar é qualquer ingrediente adicionado intencionalmente aos alimentos, sem propósito de nutrir, com o objetivo de modificar as características físicas, químicas, biológicas ou sensoriais, durante a fabricação, processamento, acondicionamento, transporte ou manipulação de um alimento.

A FAO (*Food and Agriculture Organization*), juntamente com a WHO (*World Health Organization*) agrupa os aditivos alimentares em três categorias amplas, com base em suas funções: (i) agentes aromatizantes, que são adicionados aos alimentos para melhorar o aroma e sabor, (ii) preparações enzimáticas, que podem ou não acabar no produto final. São enzimas que aumentam as reações bioquímicas, usadas principalmente na produção de vinhos, sucos e massas alimentícias, e (iii) outros aditivos, utilizados por várias razões, como conservantes, colorantes e adoçantes (WHO, 2018). Os conservantes são uma importante classe dos aditivos alimentares, usados para aumentar a vida útil e impedir a contaminação dos alimentos por microrganismos patogênicos, que podem levar a surtos de doenças transmitidas por alimentos (BRASIL, 1997).

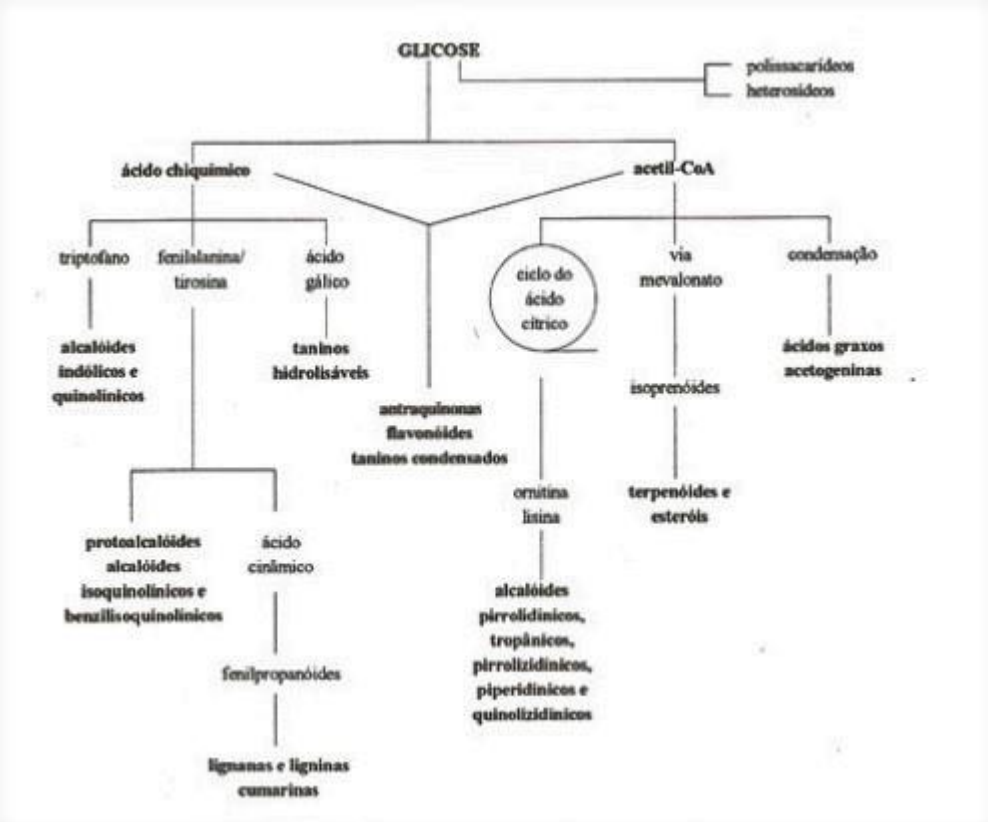
Embora os aditivos de grau alimentício sejam considerados como GRAS (*Generally recognized as safe*), eles podem ser potenciais causadores de alergia. De acordo com Gultekin & Doguc (2012), a sensibilidade ou as reações alérgicas aos aditivos alimentares vem aumentando nos últimos 20 anos, o que se deve ao aumento do consumo de produtos industrializados e à exposição frequente a esses compostos químicos.

Em vista dos relatórios recentes sobre impactos negativos de alguns aditivos sintéticos, e da crescente conscientização do consumidor em relação à saudabilidade, as indústrias de alimentos estão sendo direcionadas a procurar alternativas naturais. Nesse contexto, os óleos essenciais (OEs) de plantas aromáticas possuem forte potencial antimicrobiano e antioxidante, podendo ser utilizados como conservantes naturais para atender à demanda do consumidor por alimentos seguros e saudáveis (PRAKASH & KIRAN, 2016).

Os óleos essenciais são líquidos oleosos aromáticos voláteis que são identificados por cheiro forte e são produzidos por plantas aromáticas como metabólitos secundários. Consistem em misturas de vários componentes ativos, extraídos dos botões florais, flores, galhos, folhas, cascas, madeira, frutos e raízes da planta (TEPE et al., 2004).

Nas células, os metabólitos secundários são sintetizados a partir do acetil-CoA, ácido chiquímico, ácido mevalônico e metileritrol fosfato, que são intermediários da via glicolítica. O ácido chiquímico é sintetizado a partir da combinação de um intermediário da glicólise (fosfoenolpiruvato) com um componente da via das pentose-fosfato (eritrose-4-fosfato). Sua via leva à formação dos aminoácidos aromáticos fenilalanina, tirosina e triptofano, que são precursores dos metabólitos secundários aromáticos, como alcaloides, ácido cinâmico, fenilpropanoides e ligninas. A condensação de três moléculas de acetil-CoA origina o ácido mevalônico, enquanto a combinação do piruvato com o gliceraldeído 3-fosfato (via glicolítica) forma o metileritrol fosfato. A via biossintética do metileritrol fosfato, juntamente com a via do mevalonato, origina os esteróis e os terpenoides (Figura 1) (DEWICK, 2009; SANTOS, 2004).

Figura 1 - Ciclo biossintético dos metabólitos secundários em plantas



Fonte: Simões e Spitzer (2004)

As plantas aromáticas e especiarias são comumente usadas em fitoterapia pois os óleos essenciais apresentam diversas atividades, como antimicrobianas, antioxidantes, antifúngicas, carminativa, hepatoprotetora, antiviral e atividades anticancerígenas. São protetores naturais das plantas e possuem baixa ou nenhuma toxicidade para humanos (BURT, 2004).

Devido suas várias características, pode ser utilizado como alternativa aos conservantes químicos sintéticos. Por ser considerado um produto natural, conhecido como aditivo geralmente seguro GRAS (FDA, 2014), classificado como aromatizante natural pela legislação brasileira (BRASIL, 2007); diminuir deteriorações e oxidações, apresentando eficiência nas funções antioxidantes, e antimicrobianas em alimentos, os óleos essenciais (OUSSALAH, 2006; SACCHETTI et al., 2005) têm ganhado cada vez mais importância.

2.2.1 Mecanismo de ação dos óleos essenciais

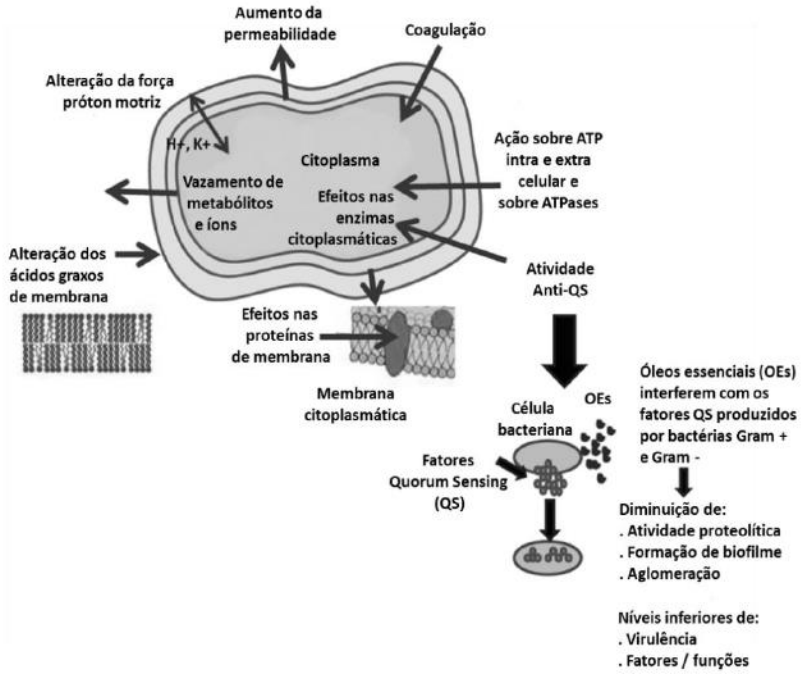
A atividade antimicrobiana ou outras atividades biológicas dos OEs estão diretamente correlacionadas com a presença de seus componentes voláteis bioativos (MAHMOUD, CROTEAU, 2002). Quimicamente, os OEs consistem em compostos de terpeno (mono-, sesqui- e diterpenos), álcoois, ácidos, ésteres, epóxidos, aldeídos, cetonas, aminas e sulfetos (BAKKALI et al., 2008).

Os efeitos antimicrobianos dos óleos essenciais foram testados contra vasta gama de microrganismos ao longo dos anos, entretanto seus mecanismos de ação ainda não são completamente compreendidos.

Vários mecanismos têm sido propostos para explicar as ações dos componentes químicos contidos nos OEs (COX et al., 2000; BURT, 2004;). Os óleos essenciais são constituídos por vários componentes e a sua atividade antimicrobiana não pode ser confirmada com base apenas na ação de um composto (BAJPAI, BAEK, KANG, 2012).

É proposto que a ação antimicrobiana dos OEs pode ser atribuída à sua capacidade de permear as membranas externa e citoplasmática, e uma vez no interior da célula, exibir atividade inibitória sobre as propriedades funcionais da célula e às suas propriedades lipofílicas (SMITH-PALMER, STEWART, FYFE, 1998, FISHER, PHILLIPS, 2009, GUINOISEAU et al., 2010, BAJPAI et al; 2012). Devido ao grande número de diferentes grupos de componentes químicos presente nos óleos essenciais, é provável que sua atividade antibacteriana não seja atribuída a um mecanismo específico, mas sim a vários alvos na célula como demonstrado na Figura 2.

Figura 2 Localidades e mecanismos de ação de componentes dos óleos essenciais em células bacterianas. Danos à membrana citoplasmática e proteínas de membrana; fluxo de constituintes intracelulares; coagulação do citoplasma e depleção da força próton motiva.



Fonte: Nazzaro et al. (2013) com modificações

A natureza fenólica dos OEs desencadeia resposta antimicrobiana sobre bactérias patogênicas transmitidas pelos alimentos (SHAPIRA, MIMRAN, 2007, BAJPAI et al., 2012). Os compostos fenólicos rompem a membrana celular resultando na inibição das propriedades funcionais da célula e, eventualmente, causam vazamento do conteúdo interno da célula (BAJPAI et al., 2012). Os mecanismos de ação podem relacionar-se à capacidade dos compostos fenólicos de alterar a permeabilidade das células microbianas, danificar as membranas citoplasmáticas, interferir com o sistema de geração de energia celular (ATP) e interromper a força motora do próton, levando à lise da célula (BAJPAI et al., 2012; BURT, 2004; FRIEDLY et al., 2009, LI et al., 2011).

Uma característica importante dos óleos essenciais e seus componentes é sua hidrofobicidade, que permite interações com os fosfolipídios da membrana celular bacteriana, tornando-a mais permeável (BURT, 2004; FRIEDLY et al., 2009). A interação de OEs com membranas de células microbianas resulta na inibição do crescimento de algumas bactérias Gram-positivas e Gram-negativas (CALSAMIGLIA et al., 2007).

Bactérias Gram-positivas como *Staphylococcus aureu*s, *Listeria monocytogenes* e *Bacillus cereus* são mais susceptíveis a OEs do que bactérias Gram-negativas como *Escherichia coli* e *Salmonella enteritid*is (CHORIANOPOULOS et al., 2004). Acredita-se que células Gram-negativas são mais resistentes, pois possuem parede celular hidrofílica (KIM et al., 2011), a qual ajuda a prevenir a penetração de componentes hidrofóbicos (CALSAMIGLIA et al., 2007; RAVICHANDRAN et al., 2011).

2.2.2 Óleos essenciais em matrizes alimentares

Fatores presentes em matrizes alimentares complexas, tais como teor de gordura, proteínas, atividade de água, pH e enzimas, podem potencialmente diminuir a eficácia dos OEs contra microrganimos (BURT, 2004). Métodos adicionais para aumentar a atividade de OEs incluem o aumento do teor de sal e a diminuição das temperaturas de armazenamento (BURT, 2004; FRIEDLY et al., 2009).

A produção de sabor desagradável ou forte odor limita o uso de óleos essenciais como conservantes de alimentos (BAJPAI et al., 2012; SOKOVIĆ et al., 2010; SOLORZANO-SANTOS & MIRANDA-NOVALES, 2012, TIWARI et al., 2009). Como resultado, concentração inibitória (concentração mínima na qual não será observado crescimento bacteriano) em vez de concentração bactericida (que irá matar as bactérias) é geralmente aplicada (CHORIANOPOULOS et al., 2006; LI et al., 2011).

Em sua pesquisa, Firouzi et al. (2007) relataram que embora o trabalho in vitro com OEs e seus componentes indicassem que óleos como o de orégano e de noz-moscada possuíam atividade antimicrobiana substancial, quando usados em sistemas alimentares as quantidades requeridas eram aproximadamente 1 a 3% mais altas, suficiente para causar alterações sensoriais.

Muitos óleos essenciais também mostram atividade antimicrobiana sinérgica quando utilizados em combinação. Por exemplo, a concentração mínima bactericida (CMB) de carvacrol, timol e eugenol contra *Listeria innocua* foi de 150, 250 e 450 mg / kg, respectivamente. Já as misturas de 62,5 mg / kg de timol e 75 mg / kg de carvacrol, ou 56,25 mg / kg de timol e 125 mg / kg de eugenol inibiram completamente o crescimento de *L. innocua*, assim como combinação de carvacrol, timol e eugenol de 75, 31,25 e 56,25 mg / kg (GARCÍA-GARCÍA, LÓPEZ-MALO, PALOU, 2011). Portanto, combinações de OEs podem reduzir a quantidade necessária para inibir o crescimento dos microrganismos, diminuindo assim o custo e potenciais impactos no sabor e aroma dos alimentos.

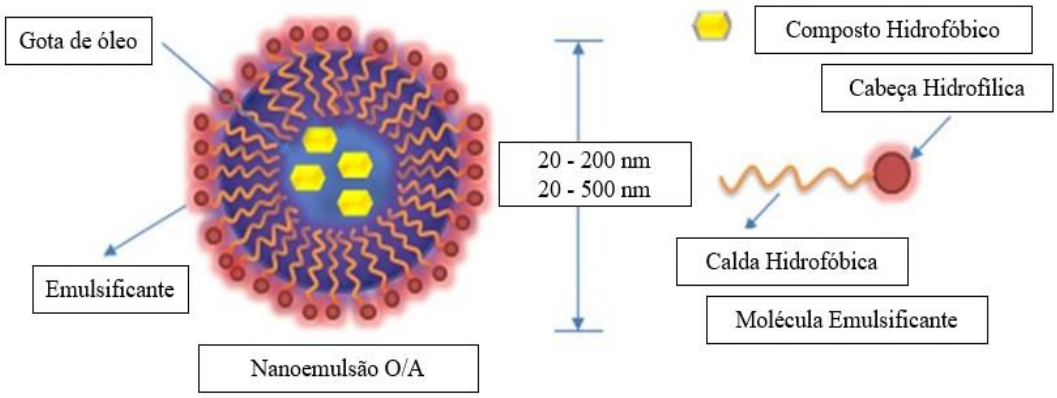
Além da utilização em combinação, para manter a sua atividade biológica e minimizar ao mesmo tempo o impacto nas propriedades organolépticas dos alimentos, os OEs podem ser adicionados em sistemas de distribuição, tais como nanoemulsões, que são compatíveis com aplicações alimentares (BURANASUKSOMBAT et al., 2011). Os sistemas podem ser formulados com ingredientes de uso alimentar e podem ser facilmente dispersos nos alimentos, onde os microrganismos crescem e proliferam (DONSI et al., 2011). A adição de emulsões em escala nanométrica, oferecem vantagens adicionais, tais como a minimização do impacto sobre as propriedades organolépticas dos produtos alimentares, bem como aumento da bioatividade, devido ao tamanho subcelular e melhor difusão (DONSI et al. 2012).

2.3 Nanoemulsões: Aspectos gerais

Nanoemulsões são dispersões coloidais formadas por uma combinação de duas fases imiscíveis estabilizadas por um surfactante, com gotas de óleo de diâmetros na faixa de 20–200 nm (SAGALOWICZ & LESER, 2010, MCCLEMENTES, 2012). O tamanho pequeno da gota torna os sistemas de nanoemulsões cineticamente estáveis, apresentando maior resistência à agregação de partículas do que as emulsões convencionais (MCCLEMENTES, 2012). Outro aspecto importante das nanoemulsões é que elas podem apresentar aspecto translúcido em comparação com emulsões convencionais. Além disso, uma redução no tamanho das gotículas e a aumento associado na área da superfície da gota pode aumentar a funcionalidade de compostos bioativos encapsulado em nanoemulsões (SALVIA-TRUJILLO et al., 2017).

As nanoemulsões são formuladas misturando pelo menos três componentes: óleo, água e um emulsificante. A Figura 3 ilustra uma nanoemulsão óleo em água.

Figura 3 Esquema de nanoemulsão óleo em água.



Fonte: Manickam (2013) com modificações

As características e a concentração dos principais componentes de uma nanoemulsão determinam suas propriedades finais (SALVIA-TRUJILLO et al., 2017). A fase lipídica das nanoemulsões pode consistir inteiramente no lipídio bioativo (como um óleo essencial) ou pode consistir em um lipídio bioativo (como uma vitamina ou nutracêutico) dissolvido em um óleo transportador (como o milho, soja, girassol ou azeite de oliva) (MCCLEMENTS & RAO, 2011). A fase oleosa pode ser formulada usando diferentes compostos não polares, como triglicerídeos, óleos minerais, óleos ou óleos essenciais (MCCLEMENTS, 2011, MCCLEMENTS & RAO, 2011). As características físico-químicas do óleo, como viscosidade, densidade, índice de refração e tensão interfacial, afetam a formação e a estabilidade de emulsões e nanoemulsões (MCCLEMENTS, 2005). A viscosidade pode influenciar no tamanho da gota; os óleos essenciais por exemplo possuem baixa viscosidade e tensão interfacial e podem produzir menores tamanhos de gotículas do que triglicerídeos de cadeia longa, que possuem maior viscosidade. No entanto, nanoemulsões contendo óleos essenciais podem ter menor estabilidade a longo prazo devido a fenômenos de desestabilização, como amadurecimento de Ostwald ou coalescência (MCCLEMENTS & RAO, 2011).

A composição da fase aquosa desempenha um papel importante na determinação das propriedades físico-químicas das nanoemulsões. Uma variedade de constituintes solúveis em água, incluindo minerais, ácidos, bases, aromas, conservantes, vitaminas, açúcares, surfactantes, proteínas e polissacarídeos, pode ser adicionada à fase aquosa para alterar suas propriedades (MCCLEMENTS, 2005). O pH e a força iônica da fase aquosa afetam as interações eletrostáticas entre as gotículas de óleo, o que pode alterar a estabilidade da agregação das gotículas (QIAN & MCCLEMENTS, 2011). As nanoemulsões são sistemas termodinamicamente instáveis e, portanto, são necessários estabilizadores apropriados para facilitar a formação de pequenas gotículas durante a homogeneização e para impedir sua agregação durante e após a homogeneização (TADROS et al., 2004, WOOSTER et al., 2008).

2.4 Emulsificantes

Os emulsificantes são moléculas anfifílicas com superfície ativa que possuem partes hidrofílicas e lipofílicas em sua estrutura molecular. Assim, uma parte possui afinidade com meios não polares (como óleo) e outra parte possui afinidade com meios polares (como água) (HASENHUETTL & HARTEL, 2008). Os emulsificantes adsorvem à interface óleo-água durante a emulsificação, protegendo as gotículas de óleo contra agregação (KRALOVA &

SJOBLOM, 2009, MCCLEMENTS, 2005). A concentração de emulsificante é um fator importante a ser considerado no desenvolvimento de nanoemulsões devido à sua grande área superficial (SALVIA-TRUJILLO et al., 2017). Foi relatado que o tamanho das gotículas de óleo diminui em nanoemulsões com o aumento da concentração de emulfificante nos métodos de homogeneização de alta energia (QIAN & MCCLEMENTS, 2011) e de baixa energia (RAO & MCCLEMENTS, 2011). Além disso, o tipo de emulsificante a ser utilizado deve ser cuidadosamente selecionado de acordo com a fase lipídica.

Os emulsificantes podem ser classificados de acordo com suas características elétricas, como catiônicos (positivos), aniônicos (negativos), não iônicos (neutros) ou zwitteriônicos (positivos e negativos). As propriedades elétricas de um emulsificante têm um grande impacto na formação, estabilidade e propriedades funcionais das nanoemulsões (MCCLEMENTS, 2005). Os surfactantes aniônicos incluem dodecilsulfato de sódio (DSS), lauril sulfato de sódio (LSS), e alguns ésteres (LEONG et al., 2009, QIAN & MCCLEMENTS, 2011, WOOSTER et al., 2008). Os surfactantes não iônicos incluem ésteres de sacarose, ésteres de polioxietileno sorbitano (Tweens) e surfactantes de éter de olioxietileno. Não é esperado que surfactantes não iônicos levem a uma carga elétrica em gotículas de óleo, mas podem causar uma carga devido à adsorção preferencial de íons da fase aquosa ou se contiverem impurezas carregadas (HSU & NACU, 2003). De fato, os surfactantes de pequenas moléculas, como os Tweens são geralmente os mais adequados para a produção de nanoemulsões devido à sua baixa carga superficial, cinética de adsorção rápida e baixa tensão interfacial (MCCLEMENTS, 2011). No entanto, a maioria dos surfactantes de moléculas pequenas são ingredientes sintéticos e, portanto, podem ser inadequados para algumas aplicações comerciais. Os surfactantes catiônicos são raramente utilizados na indústria de alimentos (ZIANI et al., 2011). Finalmente, o último grupo corresponde aos emulsificantes zwitteriônicos, que são moléculas com dois ou mais grupos ionizáveis com cargas opostas na mesma molécula. Lecitinas e proteínas são os dois tipos mais comuns de emulsificantes zwitteriônicos para aplicações alimentares (TROTTA et al., 1996, HOELLER et al., 2009).

As proteínas lácteas têm sido amplamente utilizadas como emulsificantes nos alimentos, pois adsorvem à interface das gotículas de óleo, formando uma película protetora forte e coesa que ajuda a evitar a agregação de gotículas (LEE & MCCLEMENTS, 2010). Elas também são eficazes como emulsificantes nas nanoemulsões. As proteínas do soro de leite (α-lactalbumina, β-lactoglobulina, albumina sérica bovina, lactoferrinas e imunoglobulinas) constituem cerca de 20% da proteína total no leite (~80% caseínas) e têm um alto valor nutricional devido ao seu alto conteúdo de aminoácidos essenciais. Sua natureza anfifílica permite adsorver as superfícies

das gotículas de óleo (TIROK et al., 2001) e estabilizar as recém-criadas gotículas de emulsão contra a desestabilização (VAN DER VEN et al., 2001). A hidrólise enzimática controlada das proteínas do soro produz peptídeos menores, possuem menos estruturas secundárias e terciárias e têm um núcleo hidrofóbico parcialmente exposto (TIROK et al., 2001, GAUTHIER & POULIOT, 2003, CHRISTIANSEN et al., 2006). Essas características são responsáveis pela maior taxa de difusão na interface óleo/água e por sua capacidade de cobrir uma área maior da interface do que a proteína intacta (DAVIS et al., 2005, O'REGAN & MULVIHILL, 2010).

Ainda, polissacarídeos com estruturas anfifílicas tendem a ser usados como emulsificantes, como goma arábica, amido modificado, celulose modificada, alginato modificado e pectinas (DICKINSON, 2003). A atividade superficial desses polissacarídeos tem sido atribuída à presença de grupos químicos não polares ou componentes de proteínas ligado ao esqueleto hidrofílico do polissacarídeo (DICKINSON, 2009). A atividade emulsificante da pectina é atribuída principalmente à sua capacidade de aumentar a viscosidade da fase aquosa (DICKINSON, 2003). Além disso, pode conferir carga elétrica negativa (a pH cerca de 3,5) aos arredores das gotículas de óleo devido à sua natureza aniônica, contribuindo também para a estabilidade eletrostática das emulsões óleo/água (OZTURK & MCCLEMENTS, 2016, ALBA & KONTOGIORGOS, 2017; ARTIGA-ARTIGAS et al., 2018). Recentemente, foi sugerido que a pectina também pode atuar como emulsificante ativo de superfície, ou seja, como um verdadeiro surfactante (ACEVEDO-FANI et al., 2015), embora o mecanismo de adsorção real ainda não esteja claro. A estabilidade das nanoemulsões preparadas pela pectina depende do tipo de pectina, do grau de esterificação e da concentração de pectina na formulação da nanoemulsão.

2.5 Métodos de Fabricação das Nanoemulsões: Microfluidizador

As abordagens usadas para formar nanoemulsões são tipicamente classificadas como métodos de alta energia ou baixa energia (MCCLEMENTS & RAO, 2011, TADROS et al., 2004). Métodos de alta energia consistem de aplicar altas forças disruptivas com dispositivos mecânicos capazes de causar a ruptura de gotículas de óleo dispersando-as na fase aquosa, como homogeneizadores de alta pressão, microfluidizadores e sonicadores. Abordagens de baixa energia dependem da formação de pequenas gotículas de óleo em sistemas mistos óleo-água-surfactante quando a solução ou as condições ambientais são alteradas, como emulsificação espontânea e métodos de temperatura de inversão de fase (ANTON & VANDAMME, 2009, SOLANS et al., 2005). Os métodos de obtenção das nanoemulsões pertencentes ao grupo de

alta energia têm potencial para utilização nas indústrias de alimentos devido tanto à disponibilidade de equipamentos para uso em nível industrial, quanto à capacidade para produzir sistemas nanoemulsificados sem a adição de solventes orgânicos (SALVIA-TRUJILLO et al., 2013).

Existem vários tipos de homogeneizadores de alta pressão, sendo os mais comuns os homogeneizadores e microfluidizadores de válvulas de alta pressão. No caso de homogeneizadores de válvulas de alta pressão, uma emulsão normal é bombeada através de uma válvula estreita no final de uma câmara (MCCLEMENTS & RAO, 2011). As intensas forças disruptivas que o líquido experimenta ao passar através da válvula causam a decomposição de gotículas maiores em pequenas. A geometria do homogeneizador de válvula de alta pressão usado tem um impacto significativo na distribuição do tamanho de gota das nanoemulsões produzidas (DONSI et al., 2012). Além disso, a pressão operacional e o número de passagens pela câmara são fatores importantes que determinam a distribuição do tamanho das gotas produzidas: normalmente, quanto maior a pressão e o número de ciclos, menor o tamanho das gotas (DONSI et al., 2012).

Os microfluidizadores são semelhantes aos homogeneizadores de válvulas de alta pressão, mas o design da câmara onde ocorre a ruptura de gotículas é substancialmente diferente. Nesse caso, uma emulsão grossa também é passada através de uma câmara de interação usando um dispositivo de bombeamento de alta pressão (JAFARI et al., 2007). No entanto, a câmara de interação consiste em dois canais de fluxo, projetados para fazer com que duas correntes da emulsão grossa se colidam em alta velocidade, criando assim uma ação de cisalhamento muito alta que fornece uma emulsão excepcionalmente fina (MAHDI JAFARI et al., 2006). Diversas formas de canal de microfluidização estão disponíveis para criar nanoemulsões, sendo o canal em forma de Y o mais comum. A aplicação bem-sucedida da microfluidização para produzir nanoemulsões foi relatada por vários autores (QIAN & MCCLEMENTS, 2011, SALVIA-TRUJILLO et al., 2013), com o tamanho da gota diminuindo com o aumento da pressão operacional e número de ciclos. No entanto, o superprocessamento durante a microfluidização pode levar a um aumento no tamanho das gotículas de óleo devido à rápida recoalescência das gotículas (JAFARI et al., 2008).

2.6 Propriedades físicas e estabilidade das nanoemulsões

O tamanho de partícula, bem como a distribuição do diâmetro médio das partículas, conhecido como índice de polidispersividade (PDI), são propriedades importantes para a

caracterização de nanoemulsões, relatadas em quase todos os estudos envolvendo a aquisição desses sistemas. A determinação do diâmetro médio é fundamental, principalmente por razões científicas e tecnológicas para caracterizar e confirmar se as dimensões desejadas foram obtidas e, principalmente, se elas são mantidas durante o armazenamento ou processamento subsequente (BUNJES, 2005; MCCLEMENTS, 2013; TAMJIDI et al., 2013). O controle das dimensões das nanopartículas é essencial, pois esse parâmetro influencia as propriedades físico-químicas e funcionais, bem como o potencial de aplicação. A redução do diâmetro médio pode promover aumento da translucidez, viscosidade, estabilidade e biodisponibilidade das nanopartículas lipídicas. Geralmente, essa propriedade está relacionada à composição da matriz lipídica e ao processo utilizado para obter as nanoemulsões (DA SILVA SANTOS, RIBEIRO & SANTANA, 2019).

O PDI é outro fator relacionado à estabilidade física das nanopartículas lipídicas. Segundo Tamjidi et al. (2013), para obter suspensões com estabilidade a longo prazo, os valores do PDI devem estar na faixa de 0,1 a 0,25. Valores acima de 0,5 indicam distribuição de tamanho de partícula muito ampla, caracterizando baixa estabilidade física.

As partículas coloidais geralmente exibem carga superficial, devido à disponibilidade de grupos ionizados ou adsorção de íons do meio de dispersão. Essas cargas superficiais e a força e extensão do campo elétrico ao redor das partículas desempenham um papel muito importante na repulsão mútua das nanoemulsões e, portanto, em sua estabilidade eletrostática. Como o potencial superficial das partículas não pode ser medido diretamente, o zeta potencial (potencial elétrico na superfície de cisalhamento hidrodinâmico ao redor das partículas coloidais) é geralmente determinado como um parâmetro característico da carga de nanopartículas (ROBLES et al., 2008). Os valores de zeta potencial de aproximadamente |30| mV caracterizam sistemas coloidais com boa estabilidade e valores em torno de |60| mV são considerados ótimos. Este sistema é suscetível à desestabilização entre 5 e 30 mV, e a ocorrência de floculação pode ser observada; e em valores menores que |5| mV, o sistema apresenta uma tendência notável para a coagulação de partículas (SANTOs et al., 2012; SHAH et al., 2015; BADEA et al., 2015; MADUREIRA et al., 2015).

Ao estudar as nanoemulsões, o zeta potencial é determinado principalmente para obter informações sobre seu comportamento de dispersão. Também pode representar uma indicação de estabilidade do sistema, permitindo ajustes de formulação e permitindo a determinação do comportamento de interação com diferentes compostos incorporados. Alterações no zeta potencial podem ser observadas ao longo do tempo, sendo indicadores de desestabilização do

sistema, com consequentes expulsões dos compostos bioativos incorporados durante o período de armazenamento (BUNJES, 2005; SHAH et al., 2015).

Os métodos analíticos comumente usados para determinar o diâmetro médio e o PDI são o espalhamento dinâmico de luz (DLS), também conhecido como espectroscopia de correlação de fótons (PCS) e difração a laser (LD). O DLS / PCS mede a flutuação da intensidade da luz dispersa como resultado do movimento browniano das partículas. O LD é baseado no ângulo de difração do laser focalizado, diretamente relacionado ao diâmetro médio das partículas na suspensão. O uso combinado de feixes de luz com diferentes comprimentos de onda permite medições que variam de nanômetros a alguns milímetros, abrangendo um espectro maior de tamanhos médios de partículas. As duas técnicas devem ser usadas para melhor caracterização do sistema e para evitar mal-entendidos, pois esses métodos não medem diretamente o tamanho das partículas, mas a dispersão da luz, usada para calcular o diâmetro médio (ROBLES et al., 2008; WU, ZHANG & WATANABE, 2011; YOON, PARK & YONN, 2013).

O potencial zeta não pode ser medido diretamente. Assim usa-se algum tipo de medida indireta, a partir da qual se calcula o potencial zeta. A técnica mais usada e mais aceita é através da mobilidade eletroforética, introduz-se uma suspensão coloidal diluída em uma cuba com dois eletrodos e aplica-se um potencial elétrico à suspensão. As partículas com carga elétrica líquida irão se mover na direção do eletrodo de carga contrária, tão mais rapidamente quanto maior a sua carga elétrica e maior o campo elétrico aplicado. O quociente da velocidade de deslocamento pelo campo elétrico chama-se mobilidade eletroforética, expressa em $m^2/V.s$. Esse valor entra numa equação (as mais usadas são as aproximações de Smoluchowski ou a de Debye) para calcular o potencial zeta (ROBLES et al., 2008).

2.7 Atividade antimicrobiana de nanoemulsões com óleos essenciais

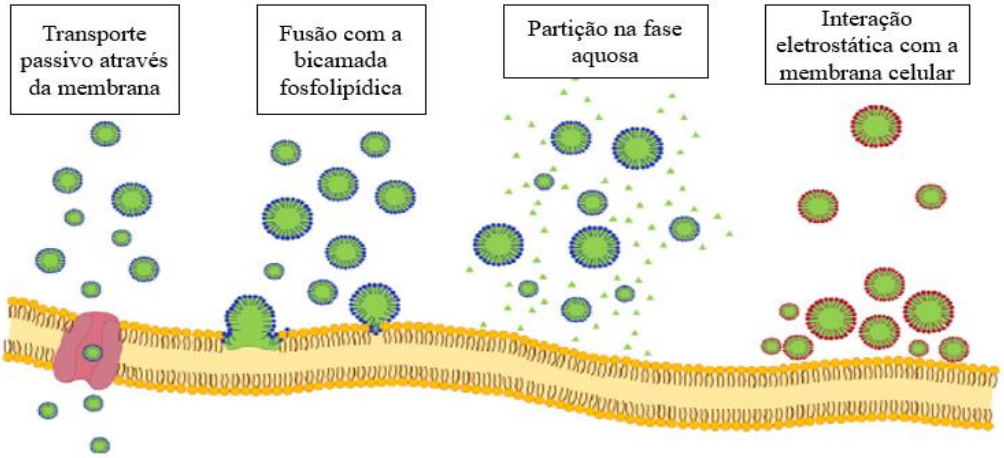
A atividade antimicrobiana dos óleos essenciais tem sido amplamente reconhecida há décadas e intensamente explorada nos últimos anos, impulsionada pela busca de alternativas naturais frente a aditivos alimentares sintéticos (SEOW et al., 2014). As ações antimicrobianas dos óleos essenciais envolvem múltiplos alvos dentro da célula, em vez de depender de um mecanismo específico. OEs, principalmente através de seus compostos fenólicos, que interagem fortemente com os lipídeos da membrana celular, aumentando a permeabilidade da membrana, perturbando as estruturas celulares originais, quebrando a homeostase e causando a fuga de íons e conteúdo citoplasmático (SALVIA- TRUJILLO et al., 2015).

A encapsulação em nanoemulsões, ao mesmo tempo em que aumenta a dispersibilidade em matrizes alimentares e melhora a estabilidade físico-química de OEs, tem necessariamente um efeito significativo na sua interação com as células microbianas, bem como na sua atividade biológica. O tamanho médio das gotas e a carga superficial, de fato, influenciam no transporte de OE para a membrana celular, bem como na interação com os múltiplos sítios moleculares na membrana celular (DONSI & FERRARI, 2016).

As nanoemulsões permitem dispersar os OEs na fase aquosa a uma concentração mais elevada do que a sua solubilidade em água. Portanto, quando a concentração mínima de inibição de OE está acima da sua solubilidade em água, o uso de nanoemulsões aumenta a atividade antimicrobiana resultante (DONSI et al., 2011; LIANG et al., 2012). No entanto, vários autores relataram que as nanoemulsões podem aumentar a atividade antimicrobiana de OEs também quando usadas em concentrações abaixo de suas solubilidades em água.

A interação com as membranas de células microbianas pode ocorrer através de quatro vias principais, mostradas da Figura 4.

Figura 4 Esquema das diferentes rotas promovidas pelas nanoemulsões para a interação do óleo essencial com as membranas celulares microbianas.



Fonte: Donsì, Ferrari (2016)

- O aumento da área superficial e o transporte passivo através da membrana celular melhoram a interação com as membranas citoplasmáticas (DONSI et al., 2012). Pequenas microesferas de nanoemulsão com superfícies hidrofílicas são capazes de passar através da membrana celular através das abundantes proteínas

de porina que servem como canais transmembranares hidrofílicos para bactérias Gram - (NAZZARO et al., 2013). No caso das bactérias Gram + e das células de levedura, as gotículas de nanoemulsão contribuem para colocar as moléculas de OE em contato com os seus locais de ação (MAJEED et al., 2016). As pequenas gotas de nanoemulsão são capazes de trazer os OEs para a superfície da membrana celular, melhorando a acessibilidade às células microbianas e permitindo a ruptura da membrana celular, possivelmente alterando a integridade da bicamada dos fosfolipídios ou interferindo com proteínas de transporte incorporadas na bicamada fosfolipídica (MOGHIMI et al., 2016).

- A fusão das gotículas de emulsionante com a camada fosfolipídica da membrana celular provoca provavelmente a liberação dos OE nos locais desejados. A evidência desta via provém da observação de que a utilização de diferentes tensioativos resulta em atividade antimicrobiana diferenciada apesar do tamanho de gotícula semelhante, tal como no caso de eugenol emulsionado por Tween 80 contra *E. coli* (LI et al., 2015), ou de óleo de tomilho emulsionado por amido modificado ou Tween 80 contra *S. aureus, E. coli* e *L.monocytogenes* (MAJEED et al., 2016).
- A liberação prolongada ao longo do tempo dos OEs a partir das gotas de nanoemulsão, impulsionadas pela separação de OE entre as gotas de óleo e a fase aquosa, prolonga a atividade dos OE. As gotas de nanoemulsão atuam como nano tanques, com moléculas de OEs em equilíbrio dinâmico entre a fase de óleo dispersa e a fase aquosa (DONSI et al., 2012). De acordo com essa hipótese, vários autores observaram uma taxa de inativação inicial menor de OEs em nanoemulsões do que os OEs livres, enquanto que durante períodos prolongados de tempo, os níveis de inativação alcançados pelas nanoemulsões foram maiores (MAJEED et al., 2016).
- A interação eletrostática de gotas de nanoemulsões carregadas positivamente com paredes de células microbianas carregadas negativamente aumenta a concentração de OEs no local de ação (CHANG et al., 2015). No entanto, esta hipótese ainda é controversa.

Pode-se esperar que a utilização de sistemas de liberação adequados para óleos essenciais reduza a concentração de OEs necessária, por dispersar melhor nos alimentos, e alterar a velocidade de liberação, e assim, diminuir o impacto sensorial (DONSI et al., 2012),

reduzir a taxa de evaporação, bem como evitar a interação com outros componentes dos alimentos (SHAH, DAVIDSON & ZHONG, 2012). Além disso, a utilização de diferentes OEs, capazes de desenvolver efeitos antimicrobianos sinérgicos em baixas concentrações, também poderia contribuir para minimizar a alteração das características sensoriais do alimento (GYAWALI & IBRAHIM, 2014).

Diversos estudos antimicrobianos já foram realizados utilizando nanoemulsões com óleos essenciais ou compostos majoritários. Chuesiang et al., 2019 investigaram o impacto da composição da fase oleosa na concentração inibitória mínima (CIM) das nanoemulsões de óleo de canela contra *Escherichia coli*, *Salmonella* enterica sorovar *Typhimurium*, *Staphylococcus aureus* e *Vibrio parahaemolyticus*. Os autores concluíram que a atividade antimicrobiana das nanoemulsões de óleo de canela aumentou à medida que os tamanhos das gotículas diminuíram. Li et al. (2018) avaliaram nanoemulsão de *Citrus medica* (conhecido como cidreira) e concluíram que a nanoemulsão quando comparadas ao óleo livre apresentou melhor atividade antibacteriana contra *E. coli*, *B. subtilis* e *S. aureus*. O mesmo foi percebido por Donsi e colaboradores (DONSI et al. 2011), e Liang e colaboradores (LIANG et al. 2012), que estudaram as propriedades antimicrobianas de nanoemulsões contendo compostos aromáticos. Nos dois estudos, foi relatada uma atividade antimicrobiana mais alta das nanoemulsões em comparação com a mesma concentração de óleos essenciais não emulsionados. Salvia-Trujillo et al., (2014) também relataram uma inativação mais rápida de *Escherichia coli* em contato com nanoemulsões de óleo de capim-limão do que emulsões convencionais. Outro estudo (LI et al., 2017) avaliou a ação de nanoemulsão de tomilho contra *Escherichia coli* O157:H7 e *Salmonella Typhimurium*, concluindo que as mesmas foram eficazes contra os patógenos no estado planctônico e de biofilme. Nanopartículas carregadas com eugenol exibiram forte atividade antibacteriana contra *Staphylococcus aureus*, *E. coli* O157: H7 e *Pseudomonas aeruginosa* (SHAO et al., 2018). Ziani e colaboradores (ZIANI et al., 2011) estudaram o impacto da carga das partículas na interação entre nanogotículas de óleo de tomilho e células microbianas usando diferentes tipos de surfactantes (catiônicos, aniônicos e não iônicos). Eles levantaram a hipótese de que, como as superfícies dos microrganismos são tipicamente carregadas negativamente, as gotas de óleo com uma superfície carregada positivamente são atraídas eletrostaticamente e, portanto, devem exibir uma atividade antimicrobiana aprimorada. No entanto, a conclusão do estudo foi que a partição de moléculas de surfactante catiônico ou aniônico entre as gotículas de óleo e a superfície microbiana reduziram a eficácia das nanoemulsões. Portanto, nesse caso, seria mais adequado usar surfactantes não iônicos para formular sistemas de distribuição de óleos essenciais para inativar microrganismos.

Embora existam na literatura muitos estudos sobre a ação antimicrobiana de nanoemulsões de óleos essenciais, grande parte deles são referentes a microrganismo não formadores de endósporos. Dessa maneira, é preciso que sejam realizados mais estudos, também com endósporos microbianos, já que esses possuem maior resistência do que as células vegetativas. Assim, haverá mais dados para comprovar a efetividade das nanoemulsões de óleos essenciais como potencial conservante natural em alimentos.

3 CONCLUSÕES

Os estudos desenvolvidos na presente tese mostraram que a estabilidade das nanoemulsões contendo óleos essenciais depende da composição química dos óleos essenciais e do tamanho inicial das gotículas. Foram identificadas condições em que as nanoemulsões são mais estáveis ao longo do tempo. A estabilidade da nanoemulsão varia de acordo com a temperatura, pH e força iônica.

A ação antimicrobiana das nanoemulsões de óleos essenciais também depende do óleo essencial utilizado e do tamanho da gotícula. As nanoemulsões se mostraram mais eficientes contra endósporos de *C. sporogenes* do que os óleos essenciais livres. Além disso, houve efeito sinérgico quando as nanoemulsões de orégano e tomilho foram misturadas, diminuindo a concentração mínima esporicida. Houve menor crescimento de células vegetativas de *C. sporogenes* quando nanoemulsões de óleos essenciais foram adicionadas ao caldo de carne, havendo diferenças entre as amostras armazenadas a 7 e a 15°C.

Os resultados também mostraram que é possível desenvolver nanoemulsões contendo óleos essenciais estabilizadas com proteína, pectina e com complexo proteína-pectina. Há evidências de que a composição interfacial não apenas determina as propriedades físico-químicas, mas também a atividade antimicrobiana das nanoemulsões contra *E. coli*.

Os estudos realizados evidenciam que nanoemulsões contendo óleos essenciais são efetivas contra microrganismo e podem ser usadas como sistemas carreadores de antimicrobianos em bebidas e alimentos.

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SEGUNDA PARTE: ARTIGOS

ARTIGO I: Nanoemulsions containing essential oils: Influence of pH, temperature and ionic strength on physical stability, and activity against *C. sporogenes* endospores (reference to *C. botulinum*)

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ABSTRACT

The application of essential oils (EOs) in foods has been widely studied in recent decades, stimulated by the search for natural additives to replace synthetic preservatives. However, these compounds have low water solubility, so nanoemulsions are being developed to improve the incorporation of essential oils in food and increase activity against microorganisms. The aims of this manuscript were (i) to form and characterize nanoemulsions containing essential oils of clove, thyme, oregano and lemongrass, (ii) evaluate the influence of pH, temperature, and ionic strength on the physical stability of nanoemulsions, (iii) evaluate the sporicidal action of isolated essential oils, nanoemulsions and their combinations against *C. sporogenes*, (iiii) to evaluate the inhibitory effect of oregano:thyme nanoemulsions against *C. sporogenes* endospores through growth curve in meat broth. According to the particle size and zeta potential results it was observed that the nanoemulsions were more stable at pH 7.0 and 4°C. Moreover, there was influence of salt addition and heat treatment on physical stability. Regarding to antimicrobial activity, only oregano and thyme nonemulsions showed effective results against *C. sporogenes* endospores, and when combined, it was possible to decrease the minimum sporicidal concentration. Growth curves in meat broth showed that low concentrations of nanoemulsions did not prevent *C. sporogenes* endospore germination, but reduced the growth rate of vegetative cells at 15°C.

Keywords: Natural additives, Antimicrobial activity, Endospote, Oregano, Thyme

1 INTRODUCTION

Essential oils (EOs) contain a complex mixture of non-volatile and volatile compounds produced by aromatic plants as secondary metabolites (Bakkali et al., 2008, Burt, 2004). They are used in medicine, cosmetics and food industry. The use of these oils as flavor and aroma ingredients has increased due to growing consumer demand for natural foods free of synthetic additives. Essential oils are lipophilic and therefore are able to interact with biological membranes of microbial cells, causing cell changes and consequent leakage of cytoplasmic content and cell lysis (Burt, 2004). Thus it has been widely studied as natural antimicrobials in food preservation (Salvia-Trujillo et al., 2015, Guerra-Rosas et al., 2016, Dávila-Rodríguez et al., 2019). Although EOs have potential in the food industry, they are chemically unstable and have low water solubility (Burt, 2004, Sánchez-González et al., 2011). Therefore new technologies such as nanoemulsions have been developed to incorporate these compounds in foods (Donsi & Ferrari, 2016). Moreover, the impact of EOs on the taste of food may impair their use, in this sense, the combined use of essential oils with each other or with other preservation technologies has also been suggested (García-García, López-Malo, & Palou, 2011).

Nanoemulsions are colloidal systems that can encapsulate, protect and release lipophilic bioactive components. They are characterized by the mixture of two immiscible liquids, where at least one of them is dispersed in the form of nano droplets, smaller than 200 nm (McClements, 2012). Other authors consider in the nano range particle sizes smaller than 500 nm (Otoni et al., 2016). Like coarse emulsions, nanoemulsions are thermodynamically unstable and must be formulated to ensure sufficient kinetic stability for industrial applications. Due to the small droplet size, nanoemulsions generally show good physical stability and some transparency (Wooster, Golding, Sanguansri, 2008). On the other hand, the reduced droplet size increases the surface/volume ratio, which can increase the transport of active molecules across biological membranes and lead to improved functionality of the encapsulated active compound (Solans et al., 2005, McClements, 2012, Salvia-Trujillo et al., 2015). Nanoemulsions can be prepared using different methods such as high pressure homogenization, microfluidization, high speed mechanical homogenization, ultrasonography, spontaneous emulsification and others (Qian & McClements, 2011, Walker, Decker, & McClements, 2015, Jin et al., 2016). However, microfluidization nanoemulsions are reported to have improved antimicrobial activity, while ultrasound appears to have a deleterious impact on antimicrobial activity (Salvia-Trujillo et al., 2014).

Studies have shown that nanoemulsified essential oils have greater antimicrobial action than those in emulsion. The effectiveness of cinnamon essential oil nanoemulsions has been shown against *Listeria monocytogenes* and *Salmonella* spp. (Paudel, Bhargavaa & Kotturi 2019), cinnamon, rosemary and oregano oils against *Escherichia coli* and *L. monocytogenes* (Dávila-Rodríguez et al. 2019), lemongrass and other nanoemulsions against *E.coli* (Salvia-Trujillo et al. 2015), *Eugenia brejoensis* oil against *Pseudomonas fluorescens* (Mendes et al. 2018) and other essential oils and major components over a wide range of Gram-negative and Gram-negative bacteria (Li et al., 2017, Li et al., 2018, Shao et al., 2018, Chuesiang et al., 2019). However, there are no reports of the action of nanoemulsions containing essential oils against against endospore germination. Therefore, to confirm the effectiveness of the nanoemulsion action of essential oils against bacteria, further research involving growth curves in a food model as well as other microorganisms is needed.

In this sense, the present work aimed to apply nanoemulsions of different essential oils (clove, oregano, thyme and lemongrass) against endospores of *Clostridium sporogenes* ATCC 3584. *C. sporogenes* is included in the group of reducing sulfite clostridia and is an endospore-forming bacteria. This microorganism is used as a model to *C. botulinum* and its endospores have high heat resistance. It is a putrefying microorganism, decomposing proteins with subsequent production of ammonia and sulfuric acid (Brown, Tran-dinh & Chapman, 2012). Both *C. sporogenes* and *C. botulinum* are associated with contamination of low acid foods, such as cured meat products, which contain synthetic additives such as nitrates and nitrites to prevent the growth of *C. botulinum*. Although widely used, the use of nitrites and nitrates over time is related to carcinogenic effects. This study works on the hypothesis that if nanoemulsions of essential oils are effective against *C. sporogenes* endospores germination, it will be possible to infer that these nanoemulsions could be used in the future to partially replace these synthetic additives in meat products.

2 MATERIAL AND METHODS

2.1 Materials

Oregano (*Origanum compactum*), clove (*Syzygium aromaticum*), thyme (*Thymus vulgaris*) and lemongrass (*Cymbopogon citratus*) essential oils were purchased from Essential'aroms® (Dietetica Intersa, Lleida, Spain). Tween 80 and other reagents used in this

study were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Ultrapure water, obtained from Millipore Milli-Q filtration system (0.22 mm), was used for the formulation and analysis of nanoemulsions. *Clostridium sporogenes* ATCC 3584 (INCQS 00004) was donated by the National Institute for Health Quality Control (INCQS) of the Oswaldo Cruz Foundation (FIOCRUZ-Brazil). Ak N.2 agar and Differential Reinforced Clostridial (Broth and Agar), used for microbiological analysis, were purchased from HiMedia Laboratories (Einhausen, Germany).

2.2 Preparation of nanoemulsions

Nanoemulsions were formulated with EOs (2% w / w) as the lipid phase and ultra pure water as the aqueous phase. Tween 80 (4% w / w) was the surfactant used. Thus, the nanoemulsions contained 2% essential oil, 4% surfactant and 94% ultra pure water. The mixture was homogenized using a laboratory mixer (Ultra-Turrax T25 homogenizer, IKA® Works, Inc. Wilmington, NC, USA) at 7600 rpm and 3 min, which led to the formation of a coarse emulsion. Afterwards, coarse emulsions were subsequently passed through a microfluidizer (MP-110 Microfluidics, MA, USA) at 100 MPa for 3 cycles, and were cooled down at the outlet of the microfluidization unit through an external coil immersed in a water bath with ice, so the temperature was maintained at approximately 10 ° C. The pH of nanoemulsions were adjusted to 7.0 and 3.0 after passing through the microfluidizer. Samples were divided and kept at 4 ° C and 25 ° C under light protection during the experiment time.

2.3 Characterization of the nanoemulsions

The characterization of nanoemulsions was performed by particle size and ζ-potential. The particle size was measured by either light scattering technique using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). For dynamic light scattering measurements sample was diluted in MilliQ water and the particle size was reported as z-average (nm). The ζ-potential (mV) was measured by phase-analysis light scattering (PALS) with a Zetasizer Nano ZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). Samples were prior diluted in ultrapure water at pH 3,0 or 7,0 according to sample pH, using specific dilution factor sample-to-solvent. Clove nanoemulsion was diluted 1:99 (sample-to-solvent), lemongrass nanoemulsion 6:94, and oregano and thyme nanoemulsions 5:95. The differences in dilutions

are due to the quality criteria pointed by the equipment. The same dilution factors were maintained throughout the experiment.

2.4 Stability of the nanoemulsions

Several factors can destabilize a nanoemulsion. In this study, the stability of nanoemulsions was evaluated considering different pH and temperatures (°C), heat treatment and ionic strength, which are factors normally related to food processing. These analyzes were performed in triplicate.

2.4.1 Influence of pH and temperature during storage

After passing through the microfluidizer the nanoemulsions containing essential oils had the pH adjusted to 7.0 and 3.0 using HCl and NaOH. The nanoemulsions were divided into glass tubes and samples were stored at 4 ° C and 25 ° C without light. Stability was evaluated by particle size (nm) and ζ-potential (mV) during storage for 52 days.

2.4.2 Thermal stability

Thermal stability was evaluated at time 0. An amount of each nanoemulsion (10mL) at pH 7.0 was heated sequentially at different temperatures (50°C/10min, 60°C/10min, 70°C/10min and 80°C/10min) simulating a thermal food process. After each heating time and temperature, samples were taken and evaluated for particle size.

Phase contrast microscopy images of nanoemulsions after submitted to temperatures of 50 and 80°C were obtained with an optical microscope (BX41, Olympus, Göttingen, Germany) equipped with UIS2 optical system. All images were processed using the instrument software (Olympus cellSense, Barcelona, Spain).

2.4.3 Ionic strength

Nanoemulsions (pH 7.0) were diluted with equal amounts of NaCl solution at different concentrations finally obtaining nanoemulsions with 150 mM NaCl and 300 mM NaCl. Nanoemulsions mixed with NaCl solutions were stirred for 5 min and stored at 4 ° C for 48 h, and then evaluated for particle size and ζ-potential.

2.5 Antimicrobian activity

Antimicrobial activity was evaluated using *C. sporogenes* ATCC 3584 endospores. First, the Minimum Sporicidal Concentrations (MSC) of essential oils and their nanoemulsionsoils (cloves, oregano, thyme and lemongrass) and combinations were determined. Then a growth curve was made using non-sporicidal concentrations at different temperatures (7 and 15°C) to check whether non-sporicidal concentrations could inhibit endospore germination.

2.5.1 Endospore formation and inoculum standardization

Clostridium sporogenes was activated in Differential Reinforced Clostridial Broth (DRCB) (10g/L peptone protease, 10g/L meat extract, 1,5g/L yeast extract, 1g/L starch, 5g/L sodium acetate, 1g/L glucose; 0.5g/L L-cysteine) at 37°C / 24 h in anaerobiosis using mineral oil. 0.1 mL aliquots were transferred to tubes containing the same culture medium and incubated at 37°C/24 h. After incubation, 0.1 mL aliquots of the culture were transferred to AK n° 2 agar (Himedia®) and incubated at 37ºC/120 h in anaerobiosis using anaerobic generator (Anaerobac, Probac do Brasil®) to obtain endospores. Endospores´s number of was standardized after washing the agar surface with 10 mL saline solution (0.9% w / w). The spore suspension obtained was observed under optical microscopy for visualization of endospores that were stained by the Wirtz-ConKlin technique using Malachite Green stain and Safranin stain. After verifying the presence of endospores, the suspension was subjected to thermal shock with heating (75ºC/15 min) and rapid cooling in an ice bath, aiming to eliminate all vegetative cells. Endospore inoculum was standardized in peptone water (0.1%w/w) and pour plate method using Differential Reinforced Clostridial Agar (DRCA). The plates were incubated in anaerobiosis at 37ºC/ 48 h, and then counted. The suspension was standardized to 10^6 CFU / mL of endospores.

For culture maintenance, endospore culture samples were transferred to microtubes (1mL), which were centrifuged at 2500 xg for 5 min. The supernatant was discarded and 1 mL freezing medium (prepared with 30 mL glycerol, 0.5 g bacteriological peptone, 0.3 g yeast extract, 0.5 g NaCl and 100 mL distilled water) was added. The tubes were kept at -18 °C throughout the experimente.

2.5.2 Determination of Minimum Sporicidal Concentration

Minimum Sporicidal Concentrations (MSC) of essential oils and nanoemulsions were determined using the broth dilution technique (Clinical and Laboratory Standards Institute, 2018) with modifications. DRCB with Tween 80 (0.5% w/ w) was used to evaluate the action of isolated essential oils.

The culture media was placed in tubes containing nanoemulsions (pH7.0) and essential oils at concentrations of 2, 1, 0.5, 0.25% (w / w), for a total of 5mL. Aliquots of endospores (10^5 CFU/mL) were added to the tubes and incubated at 37 °C/24h under anaerobic conditions. After this period, pour plate method was performed using DRCA in double layer. The plates were incubated at 37 °C/48 h, considering as Minimum Sporicidal Concentration (MSC) one that there was no growth of the microorganism in plates.

After determining the MSCs, only the oils that showed sporicidal action were used to do a combination. Thus were selected the oregano and thyme oils as well as their nanoemulsions. The ratios used were 50:50, 30:70, 70:30 oregano:thyme. These combinations were evaluated at concentrations 2, 1, 0.5, 0.25% (w/w) in the same manner as mentioned above for the determination of isolated MSCs. The objective was to verify if the combined oils and nanoemulsions could decrease the sporicidal concentration. This experiment was performed in triplicate.

2.5.3 Growth curve evaluation at different temperatures

Growth curves were performed at non-sporicidal concentrations (0.5 and 0.25% w/w). Aliquots of the standardized inoculum were transferred to 5mL meat broth (10 g meat extract, 10 g meat peptone, 5 g tryptone, and 5 g glucose in 1 L water) containing the nanoemulsions (50:50, 70:30, 30:70 oregano-thyme) at a final concentration of 10^5 UFC/mL, and incubated at 7°C e 15°C for 20 days. Periodically (every 2 days), aliquots were taken for plate counting using pour plate method and DRCA medium in double layer. The plates were incubated at 37°C/48 h in anaerobiosis. The growth curves were performed in duplicate. This analysis aimed to evaluate if non-sporicidal concentrations would be able to inhibit endospore germination and vegetative cell growth.

The growth curves were adjusted by linear regression. The following equations were used to calculate the μ-value (day $^{-1}$), that represents the growth rate of microorganisms.

1. $\mu = \frac{dN}{dt}$
2. $lnN - lnN0 = \mu\,(t - t0)$
3. $logN - logN0 = \frac{\mu}{2{,}303}\,(t - t0)$
4. $\mu = \frac{logN - LogN0}{t - t0}\,.\,2{,}303$

where μ is the specific growth rate, *N* is the final cell count, *N0* is the initial count, *t* is the final time, and *t0* is the initial time. The μ-values were calculated considering the logarithmic part of the curve (after day 4).

2.6 Statistical analysis

Statistical analyzes for the characterization of nanoemulsions and growth curves were performed using Statistica 8.0 software.The results were submitted to analysis of variance (ANOVA) and the comparison between the means was established by Tukey test at a significance level of 5%.

3. RESULTS AND DISCUSSION

3.1 Influence of pH and temperature on particle size of essential oil nanoemulsions

Nanoemulsions are unstable colloidal systems that are subjected to many destabilizing phenomena (aggregation, sedimentation and creaming). Although they exhibit improved kinetic stability during storage compared to emulsions, variations in temperature and pH may affect their physicochemical properties (Fernandez-Avila & Trujillo (2017), so the stability of nanoemulsions under different conditions during storage was evaluated. In general, droplet size was significantly ($p<0{,}05$) affected by EOs type, pH, storage time and temperature. Initially the nanoemulsions had particle sizes of 40.26 ± 1.58 nm, 124.55 ± 3.06 nm, 44.37 ± 2.59 nm and 26.86 ± 0.9 nm for oregano oil, clove, lemongrass and thyme respectively, and these sizes increased during storage time. When visualizing the graphs (**Figure 1**), under neutral pH and refrigeration temperature, the final particle sizes were smaller when compared to acidic pH and room temperature.

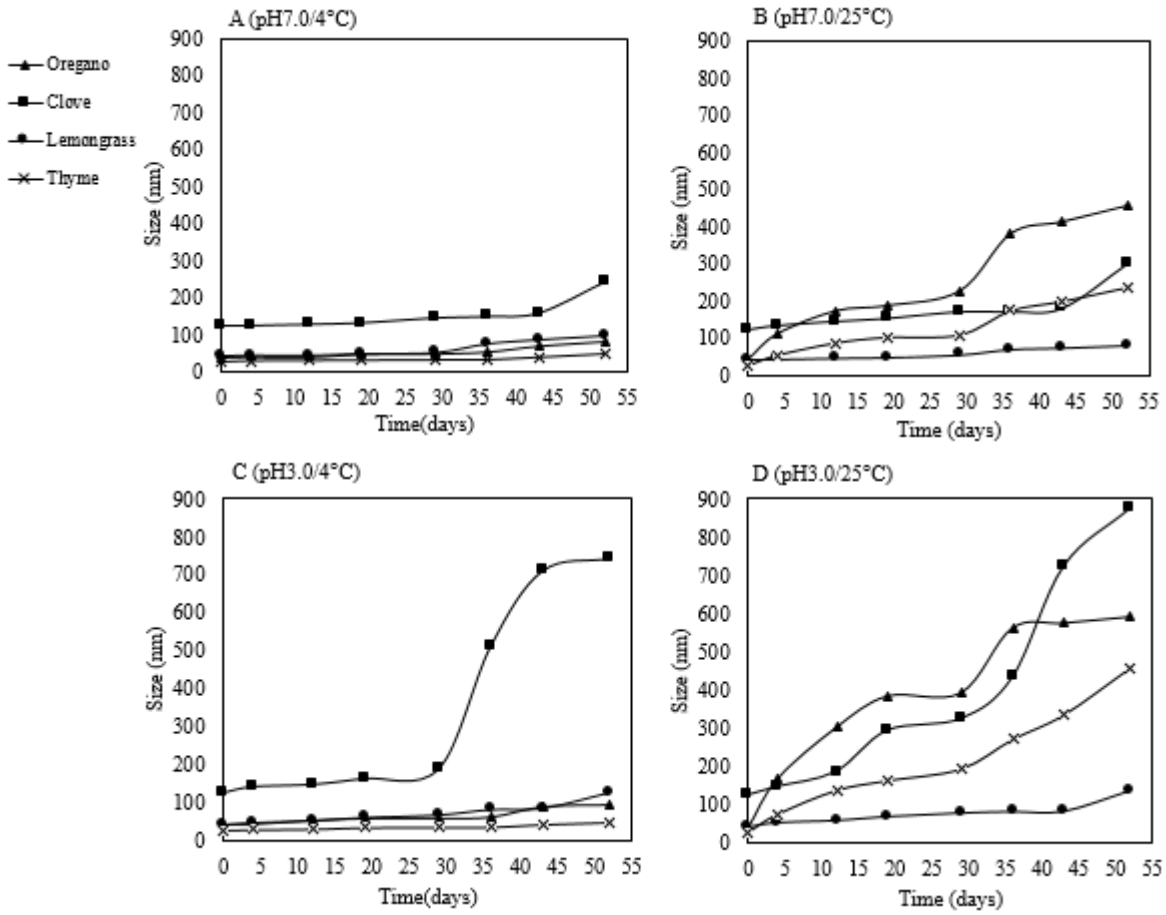

Figure 1. Particle size of nanoemulsions containing different essential oils under different pH and temperature conditions. Storage stability at pH 7.0/4°C (A), pH 7.0/25°C (B), pH 3.0/4°C (C) and pH 3.0/25°C (D).

Clove nanoemulsion showed final particle sizes of 245 ± 27.06 nm (pH 7/4°C), 300.7 ± 46.53 nm (pH 7/25°C), 743.85 ± 3.63 nm (pH 3/4°C), 876 ± 3.46 nm (pH 3/25°C), presenting less stable; remaining at the nanometer scale only at pH 7. As a consequence of the increase in particle size, in **Figure 2** it is possible to observe the phase separation in clove nanoemulsions at pH3 (4 and 25°C) after 52 days of storage, there was accumulation of the essential oil at the bottom of the tube.

Regarding to oregano nanoemulsion, the final particle sizes were 80.85 ± 0.12 nm (pH 7/4°C), 445.95 ± 3.63 nm (pH 7/25°C), 94.75 ± 5.29 nm (pH 3/4°C) and 591.6 ± 11.31 nm (pH 3/25°C). In this case it is possible to observe that the temperature influenced more than the pH, and that only the sample stored at pH3/25°C was outside the nanometer scale, when considering the scale between 10 and 500 nm (Otoni et al., 2016). Although there was an increase in particle size, oregano nanoemulsions did not undergo phase separation (**Figure 2)**, however, the

destabilization at 25°C was evidenced by the increase in nanoemulsion turbidity, and this turbidity is already perceived in the sample at pH3/ 4°C. Increased turbidity at higher temperatures was previously reported by Chang & McClements (2014), who fabricated orange oil nanoemulsions and also associated this increase with increased droplet size.

Lemongrass nanoemulsion showed final particle sizes of 99.50 ± 1.0 nm (pH 7/4°C), 82.03 ± 0.83 nm (pH 7/25°C), 124.62 ± 3.78 nm (pH 3/4°C) and 138.32 ± 4.63 nm (pH 3/25°C). These nanoemulsions showed particle size within the nanometer scale from 20 - 200 nm (McClements, 2012) under all conditions tested, however, looking at the tube figures (**Figure 2)**, it can be seen that all tubes containing lemongrass nanoemulsion showed clarification at the bottom of the tube and consequent creaming formation at the top.

Thyme nanoemulsion showed final particle sizes of 48.32 ± 0.62 nm (pH 7/4°C), 236.7 ± 1.50 nm (pH 7/25°C), 49.59 ± 0.27 nm (pH 3/4°C) and 456.2 ± 5.88 nm (pH3/25°C). For this oil, as well as for oregano, a greater influence of temperature on the stability of nanoemulsion is observed, and visually (**Figure 2)**, the behavior was also similar, being that thyme nanoemulsions also showed increased turbidity at 25°C.

In general, the increase in particle sizes was evident after 30 days for all evaluated nanoemulsions. It can also be said that the destabilization of nanoemulsions was emphasized at acid pH and room temperature. Previous studies have also shown that there is an increase in particle sizes of nanoemulsions containing essential oils with increasing temperature. This is probably because the rate of coalescence of oil drops increases at elevated temperatures (Rao & McClements, 2011, Pongsumpun et al., 2020). According to McClements (2015), nanoemulsions that are stabilized with Tween 80 do not change significantly with pH change because it is a nonionic surfactant. However, this study shows the opposite. Probably, due to the variation of chemical components present in the essential oils, oxi-reduction reactions occur at acid pH changing the particle charges and consequently decreasing the electrical repulsion between the droplets, causing coalescence and droplet aggregation. These results are later evidenced by the ζ-potential data (**Figure 3**).

Overall, nanoemulsion exhibited distinct behaviors regarding to particle size and visual appearance. Thyme nanoemulsion showed smaller particle sizes at the end of storage at 4°C, and lemongrass nanoemulsion at 25 ° C. It can also be observed that there was a smaller increase in particle sizes for EOs nanoemulsions that had smaller initial droplet size. It has been shown in a previous study (Walker, Decker & McClements, 2015) that the droplet growth rate depends on the initial particle size. The authors suggested that the initial structural organization of surfactant and oil molecules has an important role in determining the stability of nanoemulsions.

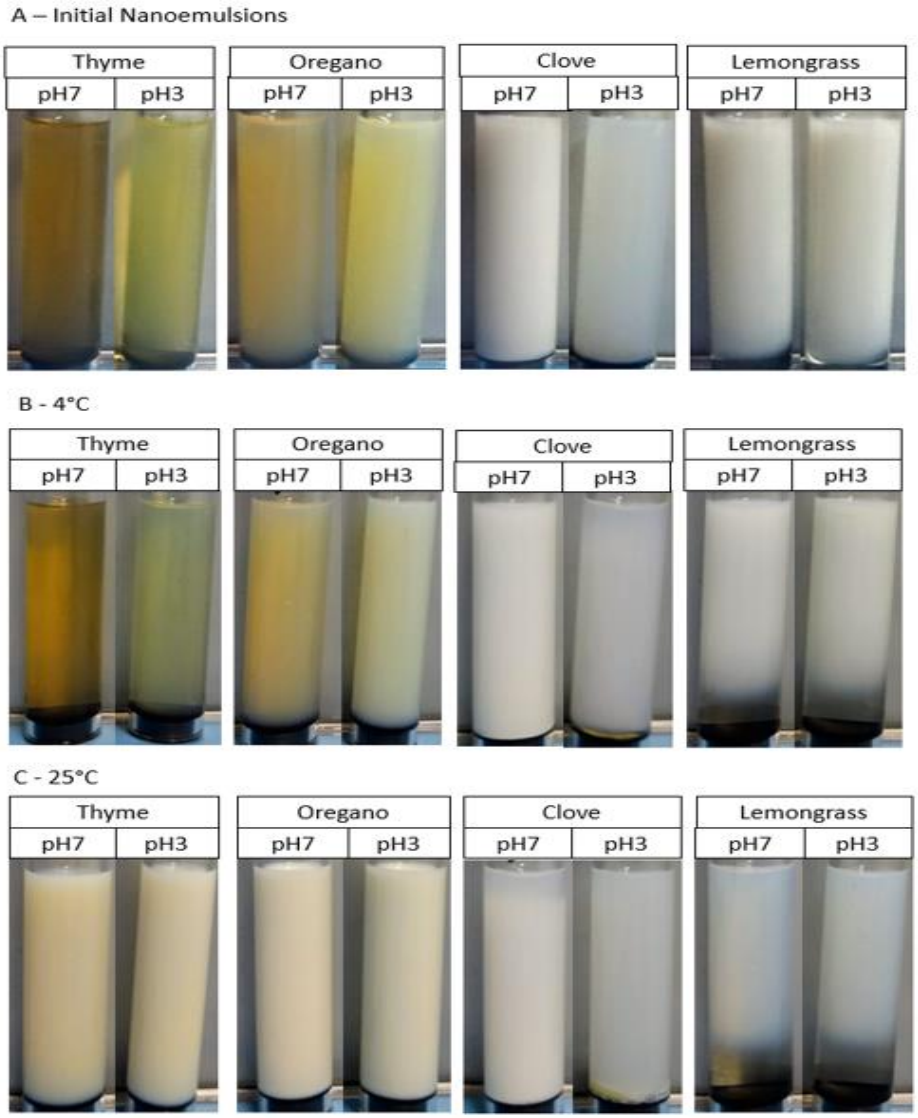

Figure 2. Photos of nanoemulsions stored at rest. Initial nanoemulsions (A) and nanoemulsions after 52 days at 4°C (B) and 25°C (C).

The formula for each nanoemulsion was identical, changing only the type of essential oil. Thus, the difference in stability between nanoemulsions can be attributed to Ostwald ripening and can be explained by differences in chemical composition. Ostwald ripening is a phenomenon observed in solid or liquid solutions that describes the change in an inhomogeneous structure over time. It can be observed in liquid-liquid systems such as oil in water emulsion. In this case, Ostwald ripening is a result of the diffusion of individual molecules or atoms from smaller droplets to larger droplets due to the higher solubility of single monomer molecules in larger monomer droplets resulting from the difference in pressure (Yamashita, Miyahara & Sakamoto 2017). According to Rao & McClements (2011) and Wan

et al. (2019), the change in mean droplet radius (r) with time (t) due to Ostwald ripening can be represented by the following equation:

$$\omega = \frac{dr^3}{dt} = \frac{8}{9}\left(\frac{8C\infty \Upsilon \mathrm{Vm} \mathrm{D}}{9RT}\right)$$

where r is the number average droplet radius, t is the storage time, $C\infty$ is the water solubility of EOs, Υ is the interfacial tension, Vm is the molar volume of the lipid, D is the translational diffusion coefficient of the EOs through the water, R is the gas constant, and T is the absolute temperature. EOs with relatively higher amount of water soluble compounds owns greater C∞, thus tending to promote nanoemulsion droplet growth due to Ostwald ripening (Rao & McClements, 2012).

EOs may vary in chemical composition according to species, season, region and weather; however, some major compounds are often associated with some oils. Oregano essential oil for example usually has carvacrol as main chemical component; thyme oil has thymol as major compound, and clove and lemongrass oils have eugenol and citral, respectively (Velluti et al., 2003, Usai et al., 2011, Bonfati et al., 2012, Verma et al., Dávila-Rodríguez et al., 2019, Wan et al., 2019). In this sense, eugenol, the main constituent of clove oil, has a relatively high solubility (Wan et al., 2019) which explains a larger particle size than other nanoemulsions, as well as the increase in particle size observed during storage time. In other hands, the major compounds of oregano, thyme, and lemongrass essential oils have relatively low solubility in water (Chen, Davidson & Zhong, 2013, Wan et al., 2019) which may have inhibited droplet growth through a compositional ripening effect, especially when observing the data at 7 °C.

3.2 Influence of pH and temperature on ζ-potential of essential oil nanoemulsions

The influence of pH and temperature on ζ-potential are presented in **Figure 3**.

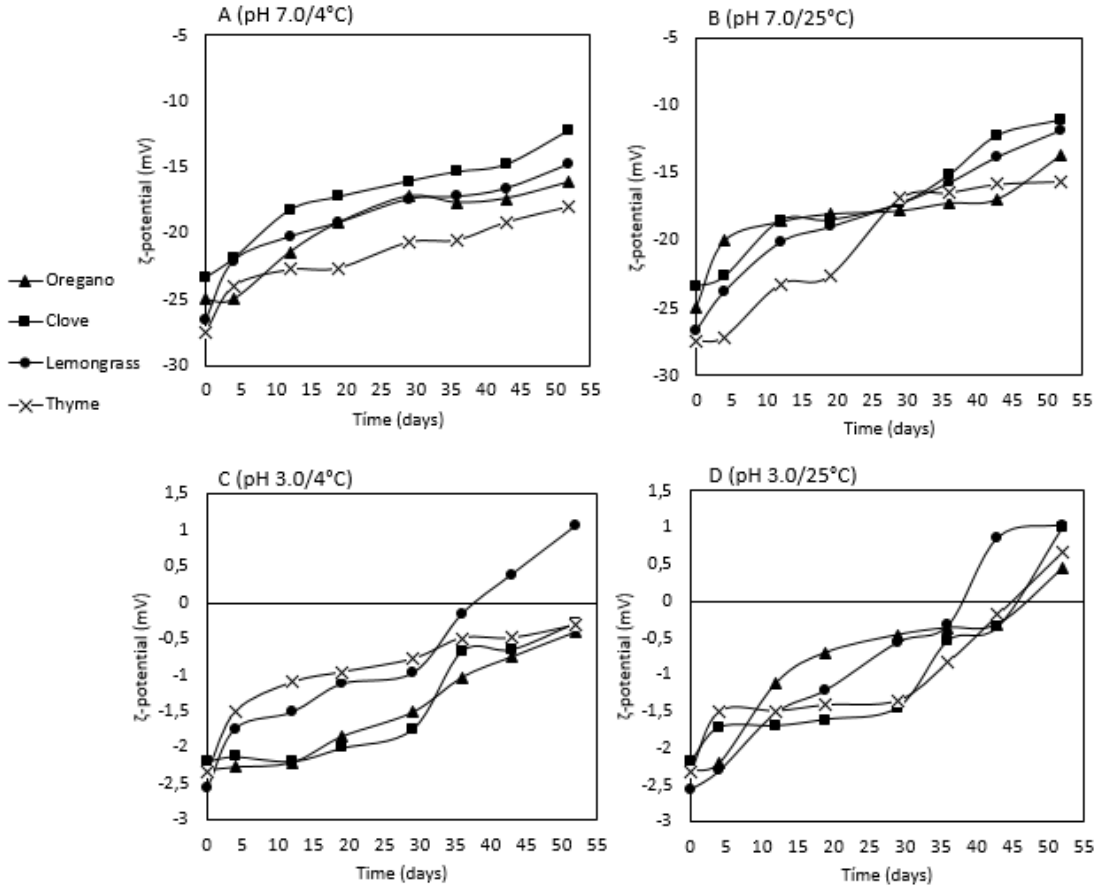

Figure 3. ζ-potential of nanoemulsions containing different essential oils under different pH and temperature conditions. Storage stability at pH 7.0/4°C (A), pH 7.0/25°C (B), pH 3.0/4°C (C) and pH 3.0/25°C (D).

The initial ζ-potentials of nanoemulsions at pH 7.0 were -24.97 ± 0.55 mV, -23.32 ± 2.15 mV, -26.6 ± 1.72 mV and -27.43 ± 2.15 mV for oregano, clove, lemongrass and thyme oils respectively. Although there are significant differences ($p < 0.05$) between them, the values do not vary widely. Differences in ζ-potential between essential oils nanoemulsions may be due to the different adsorption of surfactant molecules at the interface, where the presence of Tween 80 may increase the electrostatic repulsive forces between emulsion droplets. The same is reported by Guerra-Rosas et al (2016), using pectin to formulate nanoemulsions containing different essential oils.

Nanoemulsions at pH 3 showed least negative ζ-potentials values such as -2.31 ± 0.16 mV, -2.18 ± 0.27 mV, -2.33 ± 0.64 mV and -2.31 ± 0.22 mV for oregano, clove, lemongrass and thyme respectively. During storage, all factors (oil type, temperature, pH and time) had a significant impact on ζ-potential. However, in the graph (**Figure 3**) is noted especially the influence of pH. For all conditions evaluated, the ζ-potential became less negative over time.

At pH 7.0/ 4°C final values range from -12.17 ± 0.46 to -18 ± 0.55 mV, while at pH 7.0/25°C final values range from -11.05 ± 0.39 to -15.57 ± 0.26 mV. Clove nanoemulsion showed less negative ζ-potential and thyme nanoemulsion more negative. When evaluating nanoemulsions at pH3.0 after 52 days of storage, those stored at 4°C have zeta potential values close to zero and those stored at 25°C have positive values.

The zeta potential (ζ-potential) is the difference in electrical charge between the dense ion layer that surrounds the particles and the charge of most of the suspended fluid that surrounds this particle (Lu et al., 2005). It is an indirect measure of the surface charge of oil droplets, which provides an indication of stability during storage. In general, all samples studied presented a negative interfacial charge despite the use of a nonionic surfactant. According to Uskokovic (2012), theoretically, if the absolute value of the particle potential is below 30 mV, a nanoemulsion exhibits poor stability whereas for absolute values greater than 30 mV nanoemulsions are considered stable due to electrostatic repulsion. Indeed, although the electrical charge of EOs nanoemulsions is below -30 mV, the small initial droplet size appears to be sufficient to prevent instability phenomena throughout storage.

Essential oils consist mainly of volatile components including terpenoids and phenolic compounds (Cosentino et al., 1999). It is reported that these components may undergo oxidation leading to the formation of hydroperoxides that decompose with increasing acidity. As a consequence of terpenoid degradation, terpenes become water-soluble and pass into the aqueous phase (Mercier et al., 2009), thereby releasing ions of different charges, which explains the significant change in zeta potential of essential oil nanoemulsions at pH3. Moreover, some terpenes are simple unsaturated hydrocarbons, but many have extra functional groups and may be alcohols, ketones or carboxylic acids. The carboxylic acid group for example is negatively charged (COO-) when exposed to high pH (pH≫pKa). On the other hand, these groups are not charged (COOH) at low pH (pH≪pKa) (Chen et al., 2018), the same can be inferred for the hydroxyl group present in phenolic compounds. In this way, the electric charge decreases at acid pH, thus explaining the results found.

3.3 Thermal stability of nanoemulsions

For commercial applications it is of great importance that nanoemulsions remain physicochemical stable, especially under processing conditions. Therefore it is important to evaluate the thermal stability. Nanoemulsions were heat treated using temperature/time binomials of 50°C/10min, 60°C/10min, 70°C/10min, 80°C/10min. After each temperature,

nanoemulsions were evaluated for particle size. The particle sizes obtained are shown in **Table1**. Photos of nanoemulsions were taken after application of 50 and 80°C (**Figure 4**).

Table 1. Influence of heat treatment on particle size (nm) of nanoemulsions containing different essential oils.

	Temperature (°C)									
Nanoemulsion	**25°C**		**50°C**		**60°C**		**70°C**		**80°C**	
Oregano	40,85	±1,58 ^{aA}	54,58	±1,12 ^{aA}	79,01	±0,71 ^{aB}	94,78	±0,53 ^{aC}	106,53	±1,09 ^{aC}
Thyme	26,84	±0,11 ^{aA}	62,06	±0,88 ^{aB}	79,48	±2,23 ^{aB}	126,9	±4,09 ^{bC}	180,6	±0,69 ^{bD}
Lemongrass	44,86	±2,56 ^{aA}	70,09	±8,58 ^{aB}	84,42	±1,96 ^{aB}	183,5	±2,29 ^{cC}	214,77	±0,75 ^{cD}
Clove	125,9	±27,03 ^{bA}	223,4	±30,37 ^{bB}	229,7	±7,38 ^{bB}	298,2	±0,12 ^{dC}	303,87	±11,14 ^{dC}

Means followed by the same lowercase letters under the same column, and uppercase letters under the same line do not differ from each other after the Tukey test (p> 0.05).

There was a significant difference (p <0.05) between nanoemulsions and applied temperatures. In general, at 25, 50 and 60°C, the clove nanoemulsion was the only one that differed from the others with larger particle size. Analyzing the action of temperature, when it increases from 25 to 50°C there is a representative increase in particle sizes, from 50 to 60°C there is no difference between particle sizes, there is only for oregano nanoemulsion. From 60 to 70°C a significant increase is observed for all nanoemulsions, and from 70 to 80°C a representative increase in particle sizes is observed only for thyme and lemongrass nanoemulsions. Overall, can be said that temperatures of 70 and 80°C had more impact on the physical stability of nanoemulsions. It can also be concluded that oregano nanoemulsion showed smaller particle sizes at higher temperatures, followed by thyme, lemongrass and clove nonemulsions, the latter having a larger particle size than the others. The difference between essential oils is probably due to the solubility of the different chemical components and Ostwald ripening. **Figure 4** shows the increase in particle sizes of EOs nanoemulsions observed with optical microscope.

The significant increase in particle size at 70 and 80 ° C can be explained by what Teo et al. (2016) and Chen et al. (2018) reported. Both state that droplet size increased after application of temperatures considered above the surfactant cloud point. In this case, the Tween 80 has cloud point near 65°C. According to McClements (2015), a nonionic emulsifier such as Tween may lose its ability to stabilize an emulsion at temperatures near cloud point, resulting in droplet aggregation and coalescence. Cloud point is defined as the temperature at which the

surfactant solution becomes insoluble and phase separated due to dehydration of the major group of surfactant molecules at higher temperatures (Mahajan, Chawla, & Bakshi, 2004). When a nonionic surfactant reaches the cloud point, its hydrogen bonds are broken and its water solubility decreases (Rahman et al., 2017). Thus, its ability to promote the union of oil/water interfaces undergoes a change, which can lead from a small increase in particle size to phase separation processes.

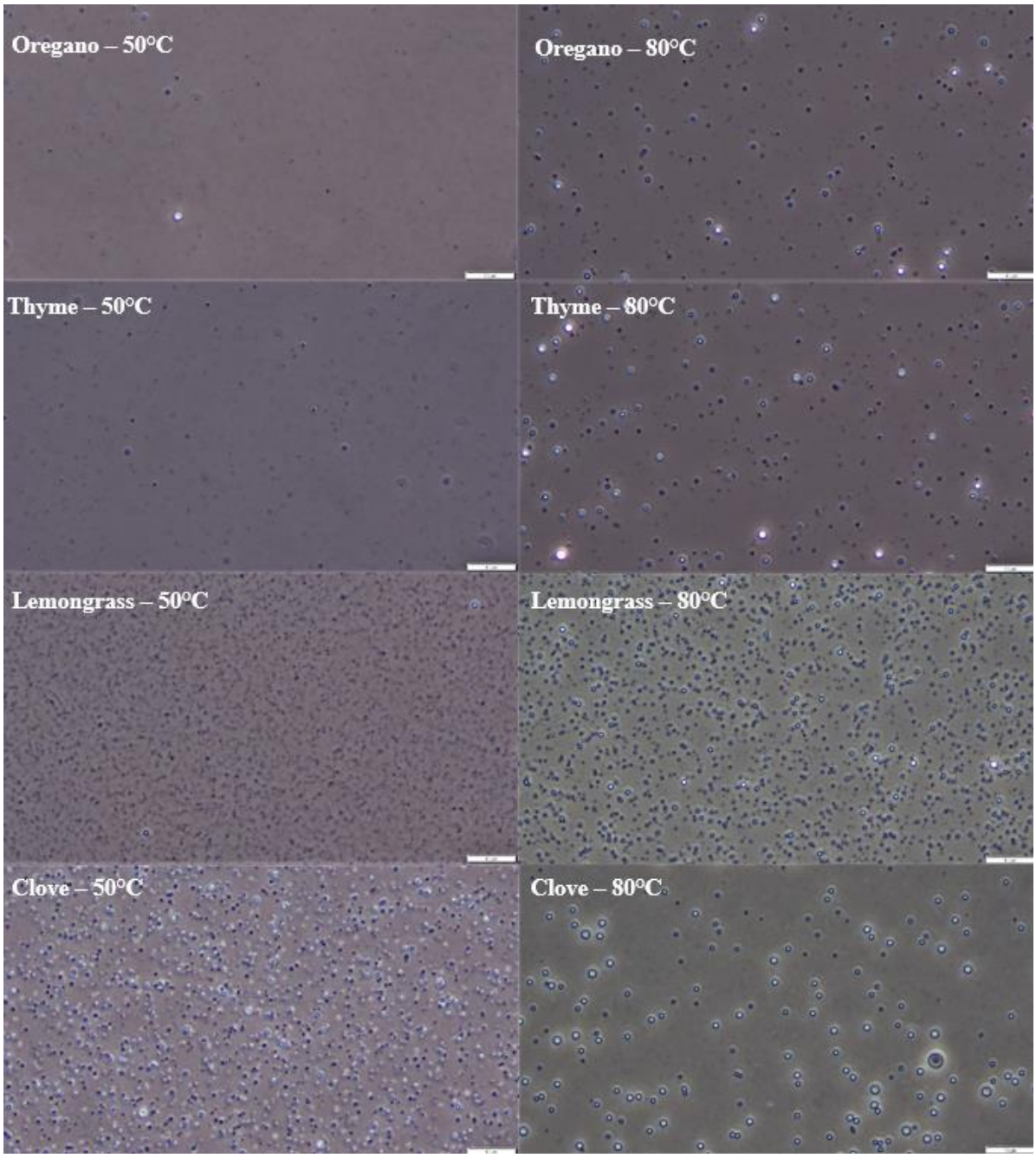

Figure 4. Phase contrast nanoemulsion images using 100x magnification.

3.4 Influence of ionic strength

The ionic strength varies with the food product. Thus it is essential to study the influence of ionic strength on the stability of nanoemulsions. The ionic strength was evaluated by the addition of NaCl (150 and 300 mM). Nanoemulsions mixed with the solutions NaCl were stored at 4°C/48h and further analyzed for particle size and ζ-potential. The data are presented in **Figure 5**.

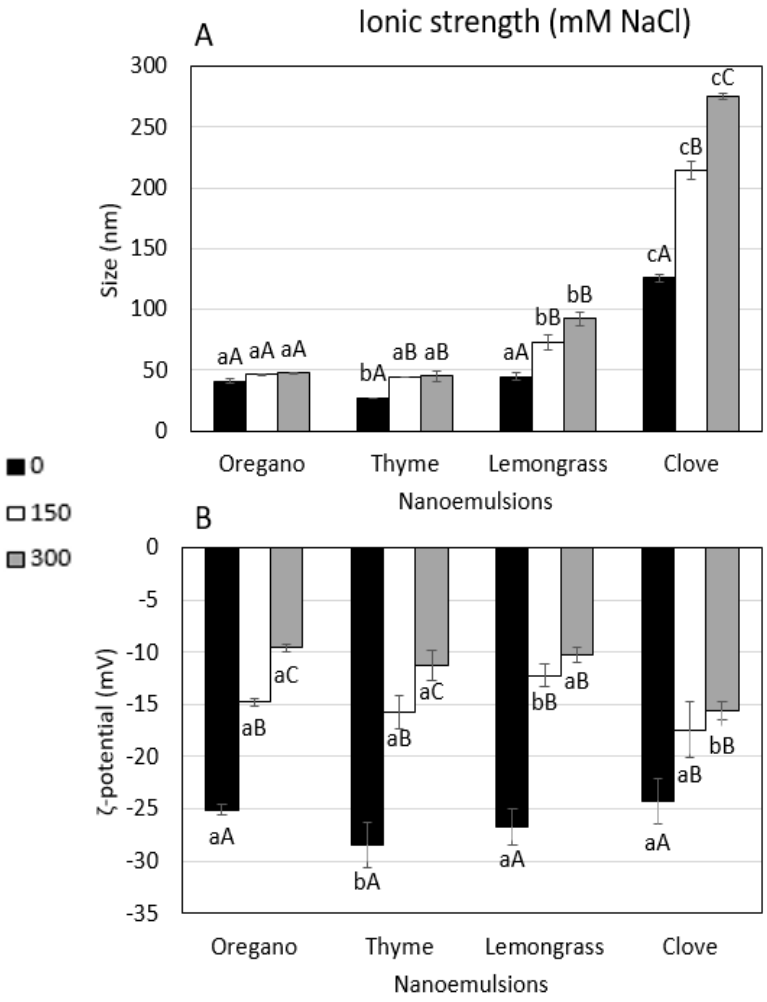

Figure 5. Influence of salt addition on different nanoemulsions (0, 150 and 300 mM NaCl): Particle size (A) and ζ-potential (B).

Lower case letters show significant difference for Tukey test ($p < 0.05$) between different nanoemulsions. Capital letters show differences between salt concentrations.

Regarding to particle sizes and salt concentrations used, only oregano nanoemulsion kept the size stable for all concentrations tested. Observing the thyme and lemongrass nanoemulsions it is noted that the addition of 150 mM NaCl significantly increased particle

size, however, this difference is not observed when comparing the addition of 150 mM NaCl or 300 mM NaCl. For clove nanoemulsion, there was a gradual and significant increase in particle sizes with increasing salt concentration. Overall, oregano and thyme nanoemulsions behaved similarly when subjected to salt concentrations, differing from others and being more stable. Lemongrass and clove nanoemulsions were significantly different from each other. The final particle size at the highest salt concentration followed the order of initial sizes, with thyme nanoemulsion showing smaller particle size, followed by oregano, lemongrass and clove nanoemulsions. Similar results were found by Quian et al (2012), who reported that the addition of salt caused little change in particle diameter. On the other hand, the same authors reported that high levels (> 400 mM NaCl) may result in particle aggregation. Teo et al. (2016) also evaluated the stability of nanoemulsions in the presence of NaCl (0-500 mM) and identified good stability to NaCl with little change in particle size at all concentrations tested.

Regarding the ζ-potential, it is noted that the addition of salt has a significant influence, making the potential load less negative. This is noticeable for all nanoemulsions when 150 mM NaCl is added. However, when comparing the tested salt concentrations, only the oregano and thyme nanoemulsions show significant ζ-potential decrease. Initially, without the addition of salt, the values were -25.07 ± 0.55 mV, -28.40 ± 2.15 mV, -26.70 ± 1.72 mV and -24.23 ± 2.15 mV for the oregano, thyme, lemongrass and clove nanoemulsions respectively; and after the addition of salt (300 mM NaCl) the zeta potentials were -9.59 ± 0.35 mV, -11.3 ± 1.42 mV, -10.19 ± 0.72 mV and -15.57 ± 0.82 mV, following the same previous order. The results suggest that the presence of dissociated Na^+ cations screened the electrical charges and reduced the electrostatic repulsion. Several authors report that a significant decrease of charge in the presence of salts, is to say that the potential becomes less negative (Anarjan et al., 2013, Teo el al., 2016, Bai & McClementes, 2016, Patel el al., 2019). McClementes (2015) states that mineral ions increase the ionic strength of the aqueous phase, leading to a reduction in electrostatic repulsion between particles. Moreover, he reports that at relatively low salt levels, electrostatic repulsion is still strong enough to overcome hydrophobic attraction and van der Waals, but above a critical salt level is not strong enough for attractive forces to dominate, leading to droplet aggregation.

3.5 Minimum sporicidal concentrations (MSC)

The Minimum Sporicidal Concentration (MSC) of each oil and its nanoemulsions were evaluated against *C. sporogenes* endospores. The concentrations tested were 2, 1, 0.5 and

0.25%. Subsequently, the oregano and thyme oils and nonemulsions were chosen to determine the MSCs of the combinations. The combinations were made using oregano:thyme ratios of 50:50, 70:30 and 30:70. All combinations were evaluated at concentrations of 2, 1, 0.5 and 0.25%. Results are presented in **Table 2**.

Table 2. Minimum sporicidal concentrations (MSC) of the essential oils, nanoemulsions and combinations against *C. sporogenes* endospores.

MSC of individual oils and nanoemulsions			
Essential oil	MSC (%)	Nanoemulsion	MSC (%)
Oregano	>2%	Oregano	2%
Clove	>2%	Clove	>2%
Lemongrass	>2%	Lemongrass	>2%
Thyme	>2%	Thyme	2%
MSC of combined oils and nanoemulsions (oregano: thyme)			
Essential oil	MSC (%)	Nanoemulsions	MSC (%)
50:50	>2%	50:50	2%
70:30	>2%	70:30	1%
30:70	>2%	30:70	2%

The individual or combined essential oils showed no sporicidal action at the concentrations tested. However, when the nanoemulsions were evaluated, the oregano and thyme nanoemulsions had a MSC of 2%. Moreover, when nanoemulsions were evaluated in combination, a reduction in MSC to 1% was observed when 70:30 (oregano: thyme) ratio was used.

The antimicrobial activity of essential oils has been heavily exploited in recent years driven by consumer demand for natural alternatives to synthetic additives (Seow et al., 2014). It is reported by Donsi & Ferrari (2016) that nanoemulsions may increase the dispersibility of EOs in foods, protect these compounds from degradation and produce an interactive effect with microbial cells thereby improving biological activity against microorganisms. In fact, this can be observed in the results found in this study, since the essential oils did not exhibit sporicidal action and the oregano and thyme nanoemulsions were effective. The small size of the nanoemulsion droplets, the surface charge and the exposure of the hydrophilic groups of the emulsifying molecules make the essential oils efficiently transport across the membrane and

interact with multiple molecular sites, leading to cell death (Donsi & Ferrari, 2016). Although nanoemulsions of essential oils have antimicrobial potential, it is important to emphasize that this efficacy is dependent on the components present in the essential oils, as well as the concentration, particle size and strain of the microorganism.

In this study, only oregano and thyme nanoemulsions showed sporicidal concentration, even nanoemulsions having the same formulation. In this sense, two distinct points are identified. The first is the chemical composition of essential oils and the second is the difference between particle size. Oregano essential oil is known to contain large amounts of phenolic compounds including carvacrol and thymol (Burt, 2004, Silva et al., 2010, Amorati, Foti, & Valgimigli, 2013). Moreover, it is reported that the main components of thyme oil are thymol and carvacrol (Bakkali et al., 2008, Sienkiewicz et al., 2012). Carvacrol is a thymol position isomer. Thus it can be said that the two essential oils have similar chemical characteristics, which explains the sporicidal action of the oregano and thyme nanoemulsions being equal. Several studies report the antibacterial effects of thymol and carvacrol (Lambert et al., 2001, Palaniappan & Holley, 2010, Memar at al., 2017). These compounds can inhibit the growth of gram-positive and gram-negative bacteria. Some researchers attribute the effect of thymol as a result of disturbance of lipid fraction of cytoplasmic membrane of bacteria resulting in alterations of membrane permeability and loss of intracellular contente (Lambert et al., 2001, De Souza et al., 2010). In addition, Lambert et al (2001) attributed the effect of carvacrol to a disruption in membrane integrity, which further affects pH homeostasis and the balance of inorganic ions. Thus, the antimicrobial action of carvacrol and thymol depends on its ability to permeabilize, depolarize and disrupt the cytoplasmic membrane (Memar at al., 2017). On the other hand, there is the influence of particle size. It is observed in **Figure 1** that the oregano and thyme nanoemulsions have the smallest particle sizes at neutral pH when compared to the other nanoemulsions. According to Donsi & Ferrari (2016) and Salvia-Trujillo et al. (2014), particle size is critical for antimicrobial efficiency. A smaller particle size facilitates the transport of essential oil droplets across the membrane and the action of chemical components on the bacterial cell.

Regarding to the nanomeulsion combination, when there is a combination of essential oils three distinct results can be found: synergistic, additive or antagonistic. Synergism exists when the action obtained by combining two antimicrobial compounds produces antibacterial activity greater than the sum of antibacterial activity of individual componentes (Bush at al., 2011). This was observed when the oregano and thyme nanoemulsions were combined, decreasing the MSC to 1%. The same was reported by Sharma et al. (2018) combining clove

and lemongrass nanoemulsions, and by Zhang et al (2017) with clove and cinnamon nanoemulsions.

Although the action of nanoemulsions has been widely reported, studies are usually done with non-endospore-forming bacteria, and there are few studies reporting the action of essential oils against endospores. Chaibi et al. (1997) studied nine essential oils against endospores and vegetative cells of *C. botulinum* and *B. cereus*. The authors reported the sporicidal action of some oils up to 0.3% against C. botulinum, and attributed the action of essential oils by inhibition of commitment-to-germinate of endospores. Ismaiel et al. (1990) evaluated clove, thyme, black pepper, origanum, garlic, onion, and cinnamon essential oils against *C. botulinum* endospore germination. All essential oils completely prevented 0.2% endospore germination. Aleixo (2014) reported the use of up to 2.5% clove essential oil to inhibit *C.botulinum* endospore germination. Isidoro el al. (2019) analyzed several essential oils against endospore germination of *C. sporogenes* and found positive results at a concentration of 3%. Alanazi et al. (2018) demonstrated the use of isolated major compounds, such as cinnamaldehyde, eugenol and carvacrol, reporting a positive effect against *C. perfringens* Type A endospore germination at low concentrations. Since there are few studies showing the action of nanoemulsions containing essential oils against endospores, this study is important because it shows that the essential oils were not effective against *C. sporogenes* endospore germination, but that the use of oregano and thyme nanoemulsions was effective and that the combination of nanoemulsions of these oils decreased MSC (1%), showing that in the future these nanoemulsions may be used against *C. sporogenes* (or *C. botulinum*) endospore germination in food. Thus more studies are needed following this approach. It is necessary to consider that *Clostridium* spp. endospores resist under very hostile conditions such as lack of nutrients, heat treatments, chemical treatments and others, and that *C. sporogenes* endospore is highly resistant to heat treatment, more than the endospores of other *Clostridium* species (Peck, 2009, Brunt et al., 2014). Thus, the results found in this study are positive because indicate the use of a natural additive to inhibit the endospore germination of *C. sporogenes*.

3.6 Growth Curves

Growth curves were performed with the combined nanoemulsions (oregano: thyme) at 50:50, 70:30 and 30:70 ratios. The objective was to evaluate the action of concentrations lower than 1% on the germination of *C. sporogenes* endospores. Thus, concentrations of 0.5 and 0.25% were used. Moreover, the curves were performed at two different temperatures (7 and

15°C). Endospores (10^5 CFU / mL) were inoculated into broth meat containing nanoemulsions and a control curve was performed using only broth meat. The growth of the microorganism was evaluated for 20 days by plaque counting. The curves are shown in **Figures 6**.

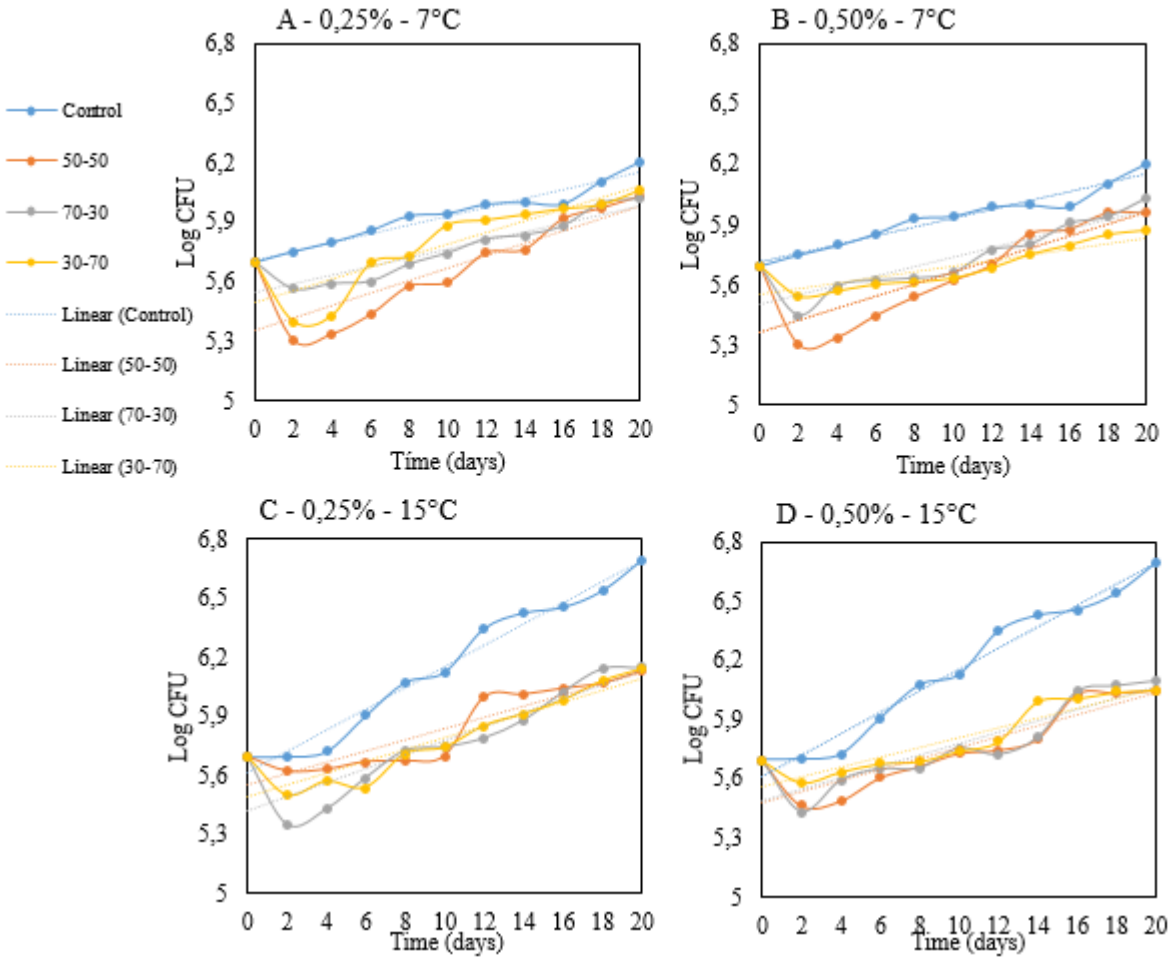

Figure 6. Growth curves of *C. sporogenes* in broth meat containing nanoemulsion combinations (oregano: thyme): 0.25% / 7°C (A), 0.50% / 7°C (B), 0.25% / 15°C (C) and 0.50% / 15°C (D)

Table 3. Slope of the straight, representing the specific growth rate (μ)

7°C- 0,25%		**7°C – 0,50%**	
Sample	**μ**	**Sample**	**μ**
Control	0,058	**Control**	0,058
50-50	0,074	**50-50**	0,089
70-30	0,061	**70-30**	0,063
30-70	0,090	**30-70**	0,043
Sample	**μ**	**Sample**	**μ**
15°C- 0,25%		**15°C – 0,50%**	
Control	0,140	**Control**	0,140
50-50	0,072	**50-50**	0,080
70-30	0,103	**70-30**	0,093
30-70	0,082	**30-70**	0,058

The growth rate was considered using only the logarithmic part of the growth curve. It is shown in **Table 3** that in general, for the temperature of 7 °C there was an increase in the growth rate when in the presence of nanoemulsions, when compared to the control, regardless of the concentration used. These data show that at 7 ° C there was an opposite effect as expected, the presence of nanoemulsion contributed to the growth of the microorganism.

However, when looking at the data at 15 °C, it is noticed that in the samples containing nanoemulsions the growth rate was slower than the control. Considering that the temperature of 15 °C is closer to the optimal growth temperature of the microorganism (37 °C), when there is usually accelerated growth, the data can be considered positive, since it is concluded that there was a decrease in the growth rate. The microorganism grows more slowly in the presence of nanomulsions containing essential oils. Considering all phases of the growth curve, **Table 4** shows that there was a decrease in growth (Log/CFU) in samples containing essential oils nanoemulsions.

Table 4. Growth of *C. sporogenes* during 20 days storage at different temperatures represented by Log CFU.

	0,25%			**0,50%**	
	7°C	**15°C**		**7°C**	**15°C**
Control	0,50 ±0,02 ^{aA}	1,0 ±0,01 ^{aB}	**Control**	0,50 ±0,02 ^{aA}	1,0 ±0,01 ^{aB}
50:50	0,33 ±0,14 ^{bA}	0,44 ±0,05 ^{bB}	**50:50**	0,30 ±0,07 ^{bA}	0,35 ±0,02 ^{bA}
70:30	0,32 ±0,15 ^{bA}	0,45 ±0,04 ^{bB}	**70:30**	0,33 ±0,05 ^{bA}	0,40 ±0,03 ^{bA}
30:70	0,36 ±0,08 ^{bA}	0,45 ±0,04 ^{bB}	**30:70**	0,17 ±0,03 ^{cA}	0,34 ±0,05 ^{bB}

Means followed by the same lowercase letters under the same column, and uppercase letters under the same line do not differ from each other after the Tukey test ($p > 0.05$).

The decrease in growth observed in **Table 4** is mainly due to the drop in Log CFU present on the growth curve (day 2) (**Figure 6**). These results suggest that initially there was an immediate action of essential oils nanoemulsions on endospores. Probably the essential oils in contact with the structure of the endospore causes damage that prevents their germination. However, it is clear that these damages are not fatal, since later, the remaining endospores germinate and there is growth of vegetative cells.

There was no significant difference in using 0.5% or 0.25% of oregano:thyme nanoemulsion. Moreover, there was no difference between the oregano: thyme ratios, but there was difference between incubation temperatures with higher growth of *C. sporogenes* at 15°C.

Overall, there was greater bacterial growth in controls that did not receive nanoemulsions. Although data show that there was a significant difference ($p<0.05$) between controls and nanoemulsion treatments, it is difficult to say that the concentrations used are effective to inhibit growth of *C. sporogenes*. This is because growth was not very evident (> 1 Log CFU) even for controls. Furthermore, the temperatures used (7 and 15°C) certainly contributed to inhibit the growth of the microorganism, thus there was no isolated effect of nanoemulsions. Further, studies involving food matrix are needed because it is known that the presence of fat, protein and other food components can protect the microbial cell from the action of essential oils (Firouzi et al., 2007). Thus, low concentrations of nanoemulsions (0.25 and 0.5%) may not be effective when applied to food.

According to Labbe (1989), a suitable storage condition for *Clostridium* endospores should prevent germination and keep dormant endospores so that viable cell counts decrease or remain constant over time. Thus, this study shows that the use of nanoemulsions and low temperatures did not make *C. sporogenes* endospores unviable, but only inhibited the growth of vegetative cells. Storage temperature may influence the viability of vegetative cells and

endospores. As expected, at higher temperature (15°C) there was more growth. The same was observed by Mah, Kang and Tang (2009), who evaluated different temperatures (refrigeration, freezing and room temperature) on the viability of *C. sporogenes* endospores. The authors report that endospore viability exists even at 4 ° C, meaning that endospore germination occurs at refrigeration temperature; and also reported that the growth rate at 4 ° C is low and increases at temperatures above 25°C. The findings agree with previous studies, which report that temperatures above 4°C do not cause endospore unviability (Odlaug & Pflug 1977; Freeman & Wilcox 2003). In contrast to this study, Alanazi et al (2018) evaluated different major components of essential oils and concluded that they were able to inhibit *C. perfringens* endospores germination in chicken meat, however they found different results for the *Clostridium* strains used, showing that the action of major components against endospores depends on the microbial strain. Furthermore, the authors mentioned that the mechanism of action of the essential oils in the endospores is unknown, but suggest that are the same as those already known for vegetative cells. It is likely that the chemical components present in essential oils act at different points, permeating the endospores and exhibiting inhibitory activity on the functional properties of the cell, that explains the lower growth rate found at 15°C (**Table 3**) when EOs nanoemulsions were evaluated.

The results found suggest that the essential oil nanoemulsions associated with low temperatures decreases growth of *C. sporogenes* vegetative cells but are not able to prevent endospore germination. Further studies about efficacy of essential oils and nanoemulsions against endospore germination, using different *Clostridium* strains and foods, are needed to provide more information regarding this.

4 CONCLUSIONS

The present study showed that the stability of essential oil nanoemulsions is dependent on the chemical composition of the essential oils and the initial droplet size. In general, there is influence of temperature and pH; nanoemulsions were more stable at neutral pH and refrigeration temperature. It was also possible to evaluate the action of heat treatment and ionic strength on nanoemulsions. The results showed that the use of temperatures of 70 and 80 ° C considerably increases the droplet size. The use of salt at low concentrations interferes with electrostatic repulsion of particles but does not cause a significant increase in droplet size. All results contribute to identify suitable conditions for future use of essential oil nanoemulsions in foods.

Furthermore, this study allowed to verify that nanoemulsions were more effective against endospore germination of *C. sporogenes* than the isolated essential oils, since these did not present minimum sporicidal concentration (MSC) in the tested concentrations. Only the oregano and thyme nanoemulsions presented MSC. The antimicrobial action of nanoemulsions was also dependent on the chemical composition of the essential oils and the initial size. The combined use of oregano:thyme nanoemulsions was able to decrease MSC from 2 to 1%, showing that there is a synergistic effect. Concentrations of 0.5 and 0.25% of oregano:thyme nanoemulsions did not prevent the germination of *C. sporogenes* endospores even at low temperatures, but were able to decrease the growth rate of vegetative cells in meat broth at 15°C.

Although there is potential for the use of essential oil nanoemulsions against endospores and vegetative cells of *C. sporogenes* (reference to *C. botulinum*), further studies with this approach are needed, especially with different strains and application of these nanoemulsions in foods.

5 ACKNOWLEDGMENT AND CONFLICT OF INTEREST

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The authors of the article declare that there is no conflict of interest

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ARTIGO II: Interfacial composition of O/W nanoemulsions containing essential oils effects their antibacterial activity

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<Single and complex-stabilized nanoemulsions as antimicrobial delivery systems

ABSTRACT

The aim of this manuscript was to *(i)* form and characterize biopolymer complexes composed of whey protein isolate (WPI) and two pectins being high or low methoxylated (HMP or LMP, respectively) and assess their surface activity; (*ii*) evaluate their capacity in comparison with the single biopolymers to form and stabilize nanoemulsions with different essential oils (EOs) being oregano and lemongrass; and (*iii*) study the impact of the interfacial composition on the antimicrobial killing kinetics of nanoemulsions against *Escherichia coli*. According to ζ-potential results and microscopic images, complexation started at pH below 5. Moreover, electrostatic interactions between LMP and WPI were stronger than those with HMP, leading to higher complexes. On the other hand, although WPI showed the greatest interfacial activity, it was quantitatively shown that pectins were capable of reducing the interfacial tension of an oil droplet regardless its degree of methoxylation, evidencing their surface active properties. Moreover, complexes behaved similarly to the pectins alone thus suggesting that is the pectin that dominates this parameter. Regarding their capacity to form and stabilize EO nanoemulsions, it was observed that ζ-potential and viscosity parameters were mainly affected by the emulsifier, whereas the type of EO had an influence on the particle size and creaming stability. Actually, both LMP and HMP contributed to emulsify nanoemulsions containing lemongrass EO leading to particle sizes <0.5 μm that remained stable during at least 30 days of storage. Lastly, it was determined that the interfacial composition had an effect on the antimicrobial activity of the resultant nanoemulsions. In fact, those HMP: WPI complex-stabilized emulsions prevented *E. coli* survival in a higher extent, while LMP: WPI complexes led to so compact interfaces that slowed down EOs release.

Keywords: Whey protein isolate: citrus pectin complexes; nanoemulsions; *Escherichia coli*; antimicrobial activity; essential oils

1 INTRODUCTION

The crescent interest in the use of essential oils (EOs) as natural antimicrobials and preservatives in the food industry has been driven by the growing consumers' demand for natural products with improved microbial safety. The antimicrobial properties of EOs are mainly due to their volatile components, including terpenoids and phenolic compounds (Cosentino et al., 1999). However, the high reactivity and hydrophobicity of EOs represent a great challenge to their direct incorporation in food and beverage products. In this way emulsion-based delivery systems has been developed (Donsi & Ferrari, 2016). Recently, emulsions with small droplet size, typically less than 200 nm, also called nanoemulsions, are being investigated as lipophilic drug delivery systems in food, cosmetic and pharmaceutical products (Bernardi et al., 2011, He et al., 2011). It has been observed that nanoemulsions in relation to coarse emulsions are more effective against microorganisms (Liang et al., 2012, Salvia-Trujillo,Rojas-Graü, Soliva-Fortuny & Martín-Belloso, 2015). The reduced droplet size increases the transport of active molecules through biological membranes and also increase the surface area/volume ratio. Thus, it is generally assumed that nanoencapsulated lipophilic antimicrobials would be able to penetrate more easily though microbial membranes, thus leading to an improved bactericidal action (Donsì et al., 2011, Salvia-Trujillo,Rojas-Graü, Soliva-Fortuny & Martín-Belloso, 2015).

Despite their potential as natural antimicrobial agents, essential oils are typically hydrophobic substances with a relatively low water solubility, which means they tend to easily destabilize mainly by a mechanism called Ostwald ripening (Ryu, McClements, Corradini & McLandsborough, 2018). In this sense, nanoemulsions require the presence of emulsifiers, which have the ability to adsorb and remain at the oil/water interface, thus forming a stable layer surrounding the oil droplet surface and preventing the breakdown of emulsions structure once it is formed (Dickinson, 2003). A range of surfactants are available, including small molecule emulsifiers, biopolymer surfactants and ionic or non-ionic. Among them, small-molecule surfactants (e.g Tween 80) are those most commonly used to stabilize nanoemulsions, as they adsorb rapidly at the oil/water interface due to the presence of a fatty acid in their molecular structure (Komaiko, McClements, 2016). Although small-molecule surfactants are very effective emulsifiers, they are typically obtained synthetically. In this way, there is a trend to replace them by emulsifiers of natural origin. Proteins from natural origin as well as certain types of biopolymers have shown interfacial properties, thus being able to act as natural emulsifiers.

Proteins predominantly stabilize emulsion droplets through electrostatic repulsion due to the presence of charged groups on the surface and steric effects (McClements, 2015). In the recent years, whey protein isolate (WPI) has been broadly studied as a hydrophilic emulsifier for O/W emulsions for encapsulating hydrophilic bioactive compounds (Pérez-Masiá & López-Nicolás, 2015; Teng et al., 2015; Assadpour et al., 2016). Pectin is a natural biopolymer present mainly in fruits and vegetables that has shown some adsorption capacity at oil-water interfaces and can improve emulsion stability (Alba & Kontogiorgos, 2017; Artiga-Artigas, Guerra-Rosas, Morales-Castro, Salvia- Trujillo & Martin-Belloso, 2018). Pectins are classified according with different degrees of methoxylation (DM) into high methoxyl pectins (HMP with DM > 50%) and low methoxyl pectins (LMP with DM < 50%) (Alund, Smistad & Hiorth, 2013). Low methoxyl pectins have shorter chains and higher negative charge compared to pectins with higher DM. These properties may affect system density and hence the nanoemulsion stability (Nguyen, Alund, Hiorth, Kjoniksen & Smistad, 2011).

Pectin-protein-containing nanoemulsions are being considered more than pure polymer nanoemulsions due to the synergistic combination between functional groups and greater physicochemical stability, thus complexation is a highly promising nanocapsilation technique (Schmitt et al., 1998, Matalanis, Jones & McClements 2011). The interactions between proteins and polysaccharides lead to the formation of complexes with a good emulsifying function, which results from rapid absorption of proteins in the interfacial layer, stearic repulsion and increasing the viscosity due to the presence of polysaccharides (Benichou et al., 2004). Although it is not clear how this interaction interferes with the antimicrobial activity of essential oils, there are indications that the properties of the interfaces directly affect the antimicrobial capacity of emulsions with essential oils. Essential oils act by increasing the permeability of the bacterial membranes thereby leading leaking of cytoplasmic content and and cell death (Bakkali et al., 2008). In this sense, anything that affects the direct contact of the EOs droplets with the cell membrane can affect antimicrobial activity. Indeed, there are studies that claim that the electrical charge on nanoemulsion droplets can be manipulated in many different ways, for example by using one or more emulsifiers with different charge characteristics, thereby changing antimicrobial capacity (Ziani, Chang, McLandsborough, & McClements, 2011); (Chang, McLandsborough, & McClements, 2012).

Therefore, the aim of this work was to (*i*) study the formation of whey protein isolate (WPI) and pectin being high methoxyl pectin (HMP) or low methoxyl pectin (LMP) complexes and characterize them in terms of their interfacial behavior; (*ii*) evaluate their capacity in comparison with the biopolymers alone to form and stabilize O/W nanoemulsions with different

EOs being oregano and lemongrass EOs being analyzed in terms of their particle size during storage, ζ-potential, viscosity and stability against creaming, and (*iii*) assess the impact of the interfacial composition on the antimicrobial killing kinetics.against *Escherichia coli*.

2 MATERIAL AND METHODS

2.1 Materials

Oregano (*Origanum compactum*), and lemongrass (*Cymbopogon citratus*) essential oils (EOs) were purchased from Essential'aroms® (Dietetica Intersa, Lleida, Spain). Whey Protein Isolate (WPI) (mixture of β- lactoalbumin and α-lactoalbumin concentrated by ultrafiltration and spray drying containing a 50% protein) was kindly donated by El Pastoret (Lleida, Spain). Food-grade High Methoxyl Pectin (HMP, Classic CU201 from citrus source) with a degree of esterification (DE°) from 68 to 76% and Low Methoxyl Pectin (LMP, Classic CU701 from citrus source) with DE° from 34 to 38% were kindly provided by Herbstreith & Fox corporate group **(**Neuenbürg- Germany), and the rest of chemicals used in this study were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Citric acid buffer (10 mM - pH 3,5) was prepared with ultrapure water, obtained from Millipore Milli-Q filtration system (0.22 mm), and used for the formulation and analysis of nanoemulsions.

2.2 Protein:pectin complex formulation and characterization

2.2.1 Formation

First, solutions of HMP (1%), LMP (1%) and WPI (1%) were prepared separately using ultrapure water. These solutions were mixed with magnetic stirrer for at least 4 hours (until complete hydration). Then, the solutions were adjusted to different pHs (7, 6, 5, 4, 3.5 and 3) using HCl and NaOH and characterized by measuring the ζ-potential. Secondly, mixtures of LMP: WPI and HMP: WPI were obtained in 1: 1 ratio using the previously prepared solutions. These were adjusted to pH 7.0 and stirred for 30 minutes. Therefore, the pH of the solutions was changed (7, 6, 5, 4, 3.5 and 3) and the ζ-potential measured. The formation of the pectin-protein complex was observed at different pHs visually and using optimal microscopy. Better complex formation occurred at pH 3.5.

2.2.2 ζ-potential

The ζ-potential (mV) was measured by phase-analysis light scattering (PALS) with a Zetasizer Nano ZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). Samples were prior diluted in ultrapure water using a dilution factor of 1:99 sample-to-solvent.

2.2.3 Optical microscopy

Phase contrast microscopy images using darkfield and objective magnification of 40x were taken with an optical microscope (BX41, Olympus, Göttingen, Germany) equipped with UIS2 optical system. All images were processed using the instrument software (Olympus cellSense, Barcelona, Spain). Images were obtained for the HMP: WPI and LMP: WPI mixtures at different pH.

2.2.4 Dynamic interfacial tension

A pendant drop tensiometer (Drop Shape Analyzer – DSA30R, KRÜS - Hamburg, Germany) was used to evaluate the change in interfacial tension caused by HMP, LMP and WPI, as well as pectin-protein complexes formed at pH 3.5. The measuring principle involves evaluating video images of drops at a dosing needle to measure the surface or interfacial tension. The interface size is sinusoidally changed during this process. The interfacial tension (IFT- mN/m) is measured as a function of the surface change and is also sinusoidal in the case of samples containing surfactants.

The following solutions were prepared with citric acid buffer: LMP, HMP, WPI, LMP: WPI, HMP: WPI (1% *w/w* and pH 3.5); then for the interfacial tension analysis, the previously prepared solutions were diluted using 0.1% (*w/w*) of the samples in ultrapure water. MCT oil (Mygliol® 812N) was used to form a droplet at the tip needle, while the emulsifier solutions were located in a cuvette. The pendant drop measurements recording the changes in the interfacial tension were performed for 2 hours and in duplicate.

2.3 Nanomulsion formation and characterization

Nanoemulsions were formulated with EOs (1% *w/w*) as lipid phase and emulsifier solutions as aqueous phase. For each emulsifier solutions prepared with citric acid buffer (HMP, LMP, WPI, HMP: WPI, LMP: WPI - 1% *w/w* - pH 3.5), emulsions were made using oregano and lemongrass EOs. The lipid phase and the aqueous phase were mixed by using a laboratory mixer (Ultra-Turrax T25 homogenizer, IKA® Works, Inc. Wilmington, NC, USA) at 15000 rpm and 2 min, which led to the formation of a coarse emulsion. Afterwards, coarse emulsions were subsequently passed through a microfluidizer (MP-110 Microfluidics, MA, USA) at 100 MPa for 3 cycles, and were cooled down at the outlet of the microfluidization unit through an external coil immersed in a water bath with ice, so the temperature was maintained at approximately 10 ° C. The pH of the emulsions was adjusted to 3.5 before and after passing through the microfluidizer. Nanoemulsions were kept at 4 ° C under light protection during the experiment time. The characterization of nanoemulsions was performed by particle size, ζ-potential, viscosity and creaming stability. In parallel, nanoemulsions containing oregano and lemongrass EOs were evaluated for their antimicrobial activity against *Escherichia coli* 1.107.

2.3.1 Particle size

The particle size was measured by static light scattering technique using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK). For static light scattering measurements, sample was dispersed in a liquid dispersion unit containing citric acid buffer (pH 3.5) and the particle size was reported as volume/surface mean d_{32} (μm).

2.3.2 ζ-potential

The ζ-potential (mV) was measured by phase-analysis light scattering (PALS) with a Zetasizer Nano ZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). Samples were prior diluted in citric acid buffer (pH 3.5) using a dilution factor of 1:99 sample-to-solvent.

2.3.3 Viscosity

A vibro-viscometer (SV-10, A&D Company, Tokyo, Japan) vibrating at 30 Hz was used to measure the viscosity (mPa.s) of 10 mL aliquots of the nanoemulsions. Moreover, the viscosity of water, which was used as dispersant phase, was 0.89 mPa.s. The viscosity of water was considered as the viscosity of the aqueous phase, conducted at 22 ± 2 °C.

2.3.4 Creaming stability

The stability of the prepared EOs nanoemulsions was performed in duplicate through a turbidity study with a Turbiscan ™Classic MA 2000 (Formulaction, Toulouse, France) during 30 days of storage at room temperature. The turbidity measurement allows the detection of the most common destabilization mechanisms of emulsions such as creaming, sedimentation, flocculation, or coalescence by multiple light scattering. Then, the Turbiscan software enables to interpret the obtained data easily. Stability analysis was carried out as a variation of backscattering (BS). The following equation was applied:

$$BS = 1/\sqrt{\lambda}* \qquad \textbf{eq.1}$$

where λ was the photon transport mean free path in the analyzed dispersion. From the physical point of view, the *(˚,d) value in the analyzed dispersion was evaluated by using the following equation:

$$\lambda * (\Phi, d) = \left[\frac{2d}{3\Phi\,(1-g)Qs}\right] \qquad \textbf{eq.2}$$

where Φ is the volume fraction of particles, *d* is the mean diameter of particles and *g(d)* and *QS (d)* are the optical parameters given by the Mie theory.

The obtained BS data were then elaborated as ΔBS profiles by the Turbisoft MA 2000, monitoring the clarification process.

2.4 Antimicrobial activity

The antimicrobial activity of essential oil nanoemulsions was assessed evaluating the in vitro inhibition of *E. coli*, following the method described previously by other authors with some modifications (Ferreira et al., 2010). A strain of *E. coli* 1.107 (culture collection of the Department of Food Technology, University of Lleida, Spain) was cultured in tryptone soy

broth (TSB) and incubated at 37 °C with continuous agitation at 100 rpm for 9 h to obtain cells in stationary growth phase. The final concentration reached in the culture was 10^8 colony forming units/millilitre (CFU/mL).

2.4.1 Survival fraction determination

For the nanoemulsion containing oregano essential oil, 0.5 mL aliquot of bacterial culture was mixed with 0.075 mL of the nanoemulsion and 4.5 mL of sterile ultrapure water. For lemongrass nanoemulsion, 0.3 mL of nanoemulsion was used. These concentrations were defined by previous tests. Aliquots of 0.1 mL were taken after 0, 10, 20, 30, 40, 50 and 60 min and were serially diluted and spread on McConkey agar plates to determine the inactivation kinetics. A blank determination was performed with the same method, replacing the nanoemulsion by sterile Mili-Q water. Colony counts were determined after incubation of agar plates at 37 °C for 24 h.

The survival fraction was determined by the eq.3:

$$S = log\ N/N0 \qquad \textbf{eq.3}$$

where *S* is the survival fraction, *N* is the bacterial population at the momento (10, 20, 30, 40, 50 and 60 min) and *N0* is the bacterial polulation in the initial time.

2.4.2 Kinetic modelling

The inactivation kinetics of EOs nanoemulsions against *E. coli* was adjusted to a Weibull distribution model, by eq.4 (Peleg, 2006):

$$\ln S(t) = -(\frac{t}{\propto})^{\beta} \qquad \textbf{eq.4}$$

where S is the survival fraction (N/N0) of *E. coli*, t is the reaction time (min), β is the shape factor, and α is the scale factor. The scale factor represents the contact time (min) necessary to inactivate the first log-cycle of the microbial population, whereas the shape factor is related to the trend of the inactivation curve. A shape factor below 1 indicates a concavity upwards while a value greater than 1 means a concavity downwards. Estimated parameters were determined using the JMP Pro 14 statistical software (SAS Institute Inc.). The fit of the model was assessed by calculating R^2 and visually analyzing the residue plots. Significant differences between the

estimated parameters of different samples were determined by calculating the confidence intervals (95%).

2.4.3 Fluorescence microscopy

Interactions between nanoemulsion droplets and grown *E.coli* cells were observed using fluorescence microscopy with an optical microscope (BX41, Olympus, Göttingen, Germany) equipped with UIS2 optical system. Cells were stained using a fluorescent agent named propidium iodide 95% (Acros Organics, Fisher Scientific, New Jersey, USA) to distinguish between living and dead cells. Dead cells were stained red. A dye solution was prepared by dissolving 1 mg of the fluorescent agent in 1 mL of ultrapure water. After 9 hours of incubation, 1 mL of the bacterial culture was placed in eppendorf tube along with 15 µL of oregano essential oil nanoemulsion or 50 µL of lemongrass essential oil nanoemulsion and 50 µL of fluorescent agent. After contact times (0, 10, 30 and 60 min), the mixture was centrifuged for 2 min at 6000 rpm, the supernatant was discarded and the precipitated cells mixed with 100 µL of ultrapure water. Then, aliquots of the samples were transferred to microscope slides and observed. All images were processed using the instrument software (Olympus cellSense, Barcelona, Spain).

2.5 Statistical analysis

All experiments were performed in triplicate, using at least two measurements of each determination. An analysis of variance was carried out and the Student's t test was run to determine significant differences at a 5% significance level ($p < 0.05$) with statistical software JMP Pro 14 (SAS Institute Inc.).

3 RESULTS AND DISCUSSION

3.1 Protein:pectin complex formation and characterization

Throughout this study, the changes in ζ-potential of single LMP, HMP and WPI solutions at varying pH were evaluated in order to determine their optimal electrical charge for electrostatic complex formation (Fig. 1A). Subsequently, the formation of LMP: WPI and

HMP: WPI complexes, was assessed determining their ζ-potential (Fig. 1B) and microstructure (Fig. 2).

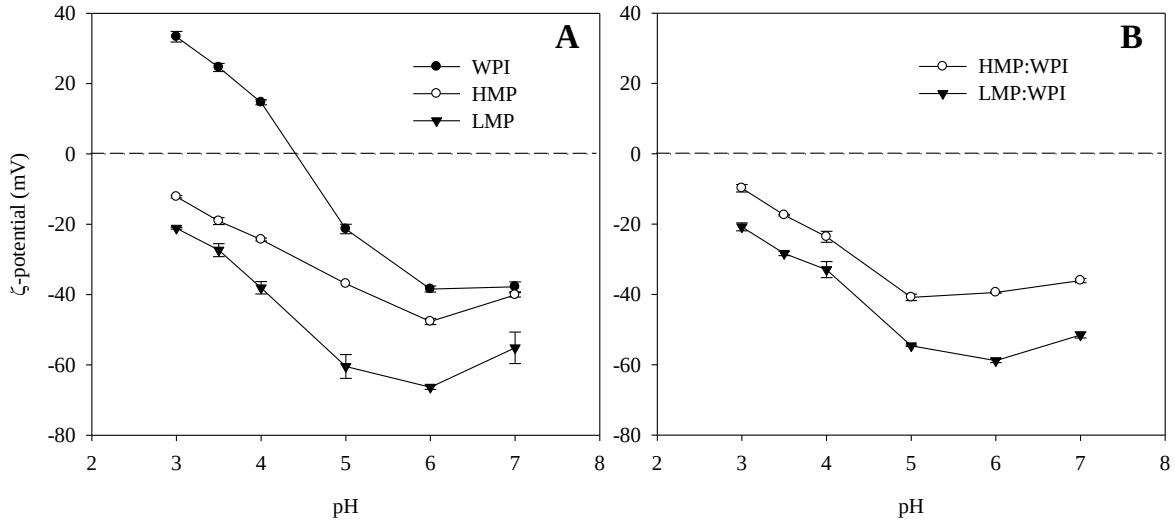

Figure 1. ζ-potential (mV) of single biopolymer solutions (A) or protein:pectin complexes (B) at 1 % (w/w) at varying pH.

WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.

3.1.1 Complex formation

WPI was negatively charged at high pH values (pH 7), and started becoming positively charged below its isoelectric point (WPI pI ≈ 4.6) (Fig. 1A) (Zeeb, Mi-Yeon, Gibis, & Weiss, 2018). That may be probably because at low pH amines from WPI are protonated ($-NH_3^+$). In contrast, LMP and HMP molecules remained negatively charged going from values of respectively, -58 to -20 mV and -40 to -16 mV as reducing the pH from 7 to 3 due to carboxylic groups of pectin may remain deprotonated at a wide range of pH values (Fig. 1A). In this regard, the most favorable pH for the formation of LMP: WPI and HMP: WPI complexes should be the one in which pectin and protein molecules are oppositely charged enough to boost the electrostatic interaction. Thus, pH 3.5 was selected since the ζ-potential of WPI was +22 mV, whereas for LMP and HMP it was -22 mV and -19 mV, respectively. Similar to the single pectin molecules, the ζ-potential of LMP: WPI and HMP: WPI complexes, also endured negatively

charged during the studied range of pH. Indeed, the ζ-potential of LMP: WPI complexes varied from -58 to -20 mV and it was between -39 to -15 mV for HMP: WPI complexes as reducing the pH from 7 to 3. The overall change of ζ-potential towards less negative values as reducing the pH might be because most of the pectin carboxylate groups were protonated ($–CO_2H$) at the lower pH ($pK_a \approx$ pH 3.5) (Surh, Decker, & McClements, 2006; Thakur, Singh, & Handa, 1997). Complexation occurs not only due to the attractive forces between oppositely charged biopolymers but also by hydrogen bonding (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). Based on the same approach, HMP may interact less strongly with WPI molecules than LMP since the former contains the most of its $–CO_2^-$ groups in form of esters ($–CO_2CH_3$) that cannot be protonated. In fact, microscopic images in Fig. 2 showed that in the case of LMP, soluble complexes formation began at pH 5 and these complexes grew as the pH decreased (acidic conditions) probably due to the increase of complexation strength caused by an increase of hydrogen bonds (Chen, Li, Ding, & Suo, 2012; Doublier, Garnier, Renard, & Sanchez, 2000). In this regard, it should be taken into consideration that working at pH bellow 3.5 may cause associative phase separation due to the coexistence of two phases: a rich solvent phase with very small amounts of biopolymers and a rich biopolymers phase thus provoking the complex precipitation (Doublier et al., 2000). In case of microscopic images corresponding to HMP: WPI complexes, however, the observed complexes were smaller compared to those LMP: WPI. It suggested that electrostatic interactions between the HMP and the protein were probably weaker than those of LMP. Therefore, microscopic results are consistent with the measured ζ-potential values and the observed different behavior can be attributed to the dissimilarities of pectin structures.

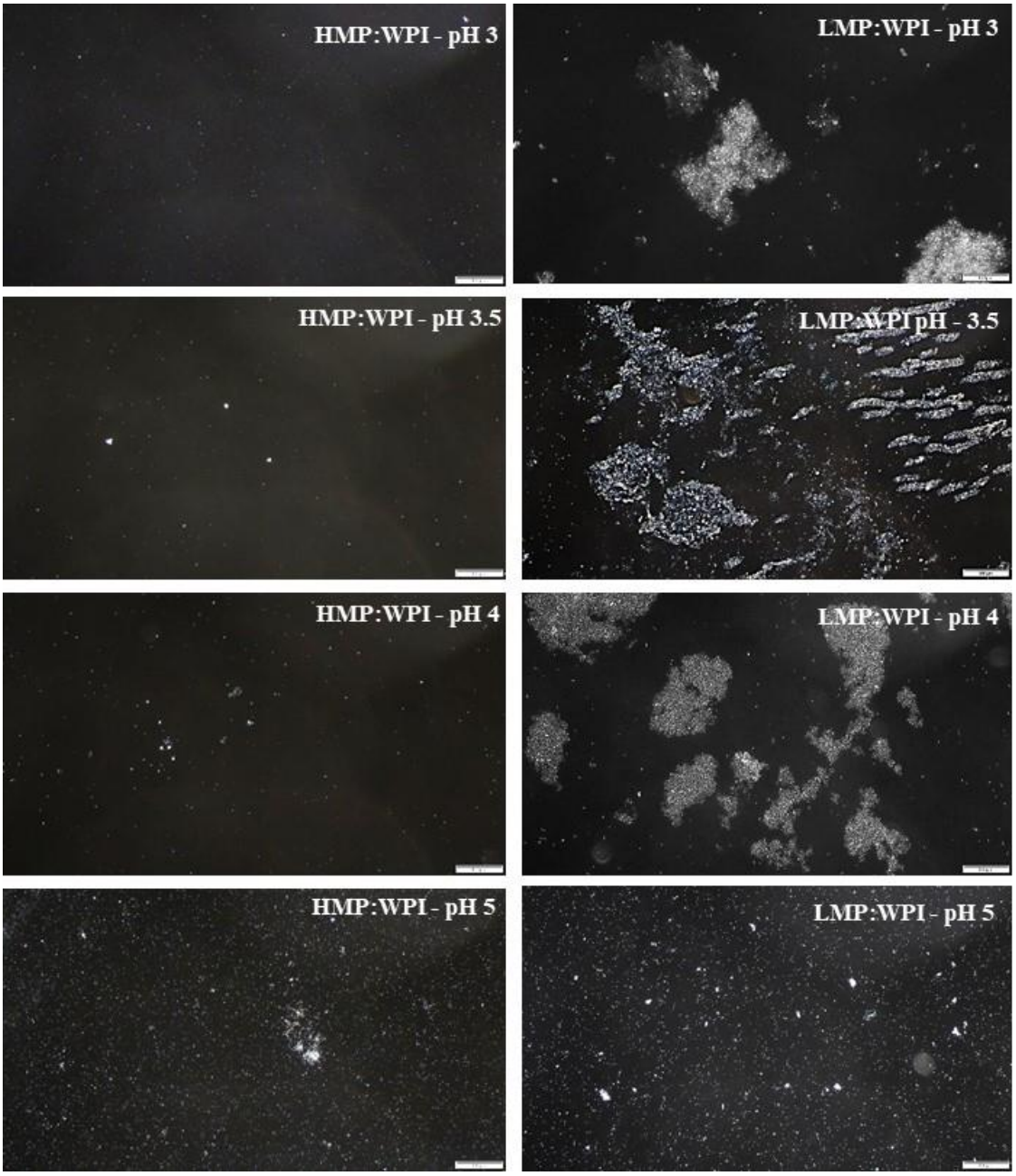

Figure 2. Microscopy images with dark field mode of single biopolymer solutions (A) or protein:pectin complexes (B) at 1 % (w/w) at varying pH.

WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.
Scale bar is 100 μm.

3.1.2 Interfacial behavior

The interfacial activity of HMP, LMP and WPI, as well as pectin-protein complexes formed at pH 3.5 was evaluated. Fig. 3 exhibits the results obtained of the dynamic interfacial

tension of an MCT droplet present in a single-biopolymer emulsifier solution (0.1% w/w LMP, HMP or WPI) or in a biopolymeric complex solution (0.1% w/w LMP: WPI or HMP: WPI) at pH 3.5. As a control, the interfacial tension of the MCT was measured against ultrapure water at pH 3.5.

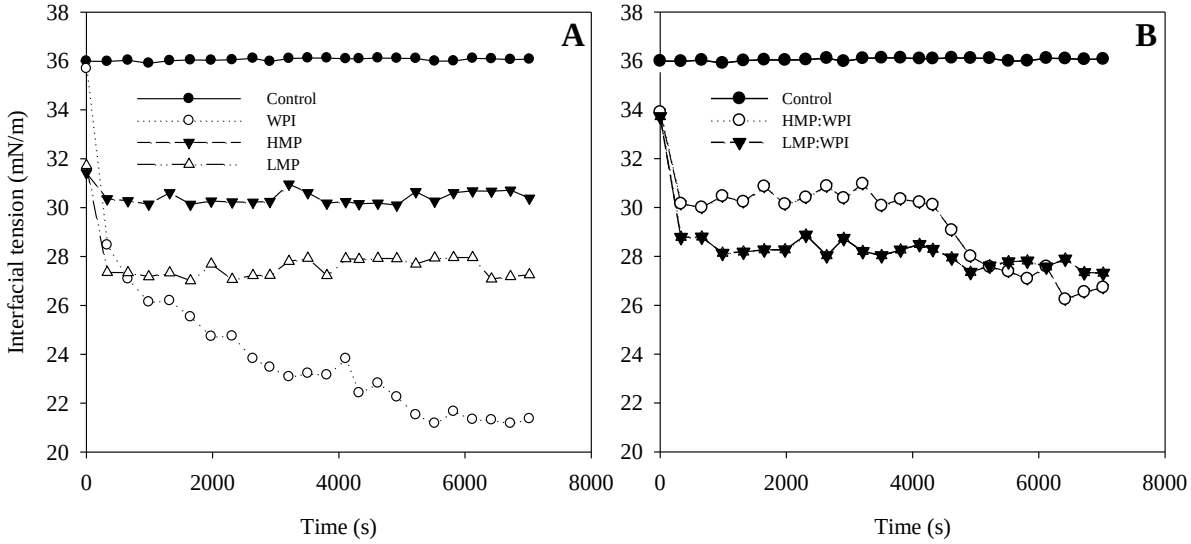

Figure 3. Dynamic interfacial tension (mN/m) of single biopolymer solutions (A) or protein:pectin complexes (B) measured with medium chain tryglyceride oil (MCT) with the inverted pendant drop method.

Control is the interfacial tension of the MCT oil in water. WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.

Regarding the control, it could be observed that the interfacial tension (36 mN/m) did not change along the time since the MCT is free of surface-active components. Overall, all emulsifier solutions were able to decrease the interfacial tension of the MCT droplet (Fig. 3). In the case of single-biopolymer emulsifier solutions, the WPI solution decreased gradually the oil droplet interfacial tension, which diminished from 36 to 22 mN/m, approximately, being the one in reaching the lowest final values (Fig. 3A). It could be attributed to the surfactant nature, electrostatic function and hydrophobic effect of this globular protein, which allow its effective adsorption at droplets interface (Yi, Lam, Yokoyama, Cheng, & Zhong, 2014). Therefore, it can be postulated that WPI effectively adsorbs to the oil-water interface and subsequently decreases the interfacial tension (Verkempinck et al., 2018). Apropos LMP and HMP solutions, an effect of pectin DM on the change in interfacial tension could be observed. Interestingly, the LMP emulsifier solution decreased the interfacial tension of the MCT droplet faster and to a

lower plateau value than the HMP emulsifier solution (27.5 and 30 mN/m, respectively) both at pH 3.5; indicating a higher surface activity. HMP can be assumed to have a more hydrophobic character compared to LMP as it contains more acetyl and methoxyl ester groups. However, Akhtar, Dickinson, Mazoyer, & Langendorff (2002) investigated citrus pectin with DM ranging from 22 to 73% and concluded that the degree of esterification was of minor importance for the emulsifying properties of pectin than other factors such as the protein moieties content and the molecular weight of pectin. Since it is reported that LMP have shorter chains compared to HMP, the rapid adsorption of LMP onto droplets surface observed in Fig. 3A, could be attributed to its lower molecular weight (Nguyen, Alund, Hiorth, Kjoniksen & Smistad, 2011).

In the case of both biopolymeric complex solutions, the rate with which the interfacial tension of the oil droplet decreased was similar than of the HMP emulsifier solution and in any case much lower than the one of WPI (Fig.3B). Consequently, we hypothesized that it is the pectin who conducted the interfacial behavior. In this regard, the charged backbone of pectin preferably oriented to the bulk aqueous phase may generate electrostatic and stearic repulsion forces phase able to slow down the deposition of WPI molecules at MCT droplets interface. (Verkempinck et al., 2018).

3.2 Nanoemulsion formation and stabilization

The capacity of LMP: WPI and HMP: WPI complexes in comparison with the biopolymers alone to form and stabilize nanoemulsions containing oregano or lemongrass EOs as dispersed phase, was assessed. In this regard, EOs-in-water nanoemulsions stabilized by WPI, LMP, HMP or LMP: WPI and HMP: WPI complexes in a ratio 1:1 were analyzed in terms of their particle size during storage expressed as d_{32} (μm) (Fig. 4), ζ-potential (mV) and apparent viscosity (mPa·s) (Fig. 5); and stability against creaming (Fig. 6).

3.2.1 Nanoemulsion particle size

Static light scattering was used to measure changes in the particle sizes over time. According to the observed results, not only the type of emulsifier but its interaction with the two EOs influenced the particle size of the prepared nanoemulsions. As it is shown in Fig. 4A, the particle sizes of all the prepared emulsions containing oregano EO were in the nano-range (<500 nm) (Otoni, Avena-Bustillos, Olsen, Bilbao-Sáinz, & McHugh, 2016), regardless the

emulsifier used. Both pectin complexes used as emulsifiers led to the smallest particle sizes and maintained this parameter stable over time without observing significant differences among them. Indeed, the particle size (d32) increased from 0.289 ± 0.013 to 0.358 ± 0,013 μm in the case of LMP: WPI complexes and from 0.304 ± 0.011 to 0.521 ± 0.013 μm in those nanoemulsions HMP: WPI complex-stabilized. Interestingly, the final particle sizes remained below 1 μm in both cases even though the lipid phase is oregano EO, whose partial solubility in water provokes its easy migration to the aqueous phase leading to nanoemulsions destabilization. According to Rodriguez Patino & Pilosof (2011) protein-polysaccharide complexes have the ability to form thicker adsorbed layers than biopolymers alone being more efficient in stabilizing nanoemulsions containing EOs. Regarding the single-biopolymer stabilized nanoemulsions, those containing WPI as emulsifier maintained steady their particle sizes during at least 12 days before starting to growth. Nonetheless, at day 21, the particle size increased sharply up to 6.16 ± 0.16 μm and remained steady for the rest of the study (Fig. 4A). Oppositely, the particle size of LMP and HMP-stabilized nanoemulsions started growing from the first 24 h after their preparation reaching values of 1.24 ± 0.12 and 2.94 ± 0.05 μm, respectively, after the 30 days of experiment (Fig. 4A). Nanoemulsions containing EOs are prone to suffer Ostwald ripening, which is a phenomenon by which bigger droplets grow at expenses of the smallest ones (Zeeb, Gibis, Fischer, & Weiss, 2012).

Probably, the reason why WPI was able to slow down the Ostwald ripening is because it is a globular protein that provides a high interconnected monolayer around the droplets and greater surface coverage of the interfacial area than pectins (Combrinck, Otto, & du Plessis, 2014; Dickinson, 2009; Ghosh & Bandyopadhyay, 2012). It is well established that at pH 3.5, -CO2H residues from LMP and HMP chains are protonated and they acquire compact conformations in which the hydrophobic groups can go towards the oil interface and adsorb (Alba & Kontogiorgos, 2017). However, the steric repulsion among pectin chains oriented to the bulk aqueous phase might lead to the appearance of interfacial gaps through which the dispersed phase can migrate increasing the particle size.

On the contrary, regarding Fig. 4B where lemongrass EO was used as dispersed phase, nanoemulsions with particle sizes (d32) lower than 0.328 ± 0,021 μm were exclusively obtained in case of incorporating single or complexed pectin as emulsifier. Moreover, nanoemulsions stabilized with LMP, HMP or LMP: WPI complex, respectively experimented a slow and gradual increase of their particle size up to 0.54 ± 0.03, 0.63 ± 0.02 or 0.73 ± 0.05 μm during the 30 days of experiment. Specifically, those HMP: WPI-stabilized nanoemulsions showed a remarkable increase of their d32 from day 20 leading particle sized over 1 μm (Fig.4B).

Actually, it is reported that pectin contributes positively in the emulsification of lemongrass EO mainly dominating the particle size (Artiga-Artigas, Guerra-Rosas, Morales-Castro, Salvia-Trujillo, & Martín-Belloso, 2018). In contrast, WPI could not effectively emulsify dispersions containing lemongrass EO leading to emulsions with particle sizes of 1.12 ± 0.06 µm (Fig. 4B). In addition, the increase in the d32 throughout the study was abrupt reaching values of 4.68 ± 0.48 µm. This suggested that probably WPI adsorbed significantly more slowly on lemongrass EO droplets than on those of oregano EO. Therefore, the observed slow adsorption of WPI at droplets interface together with the reported high susceptibility of lemongrass EO to be volatilized may favor oil migration and in turn, the particle size increase in protein-stabilized nanoemulsions (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015b).

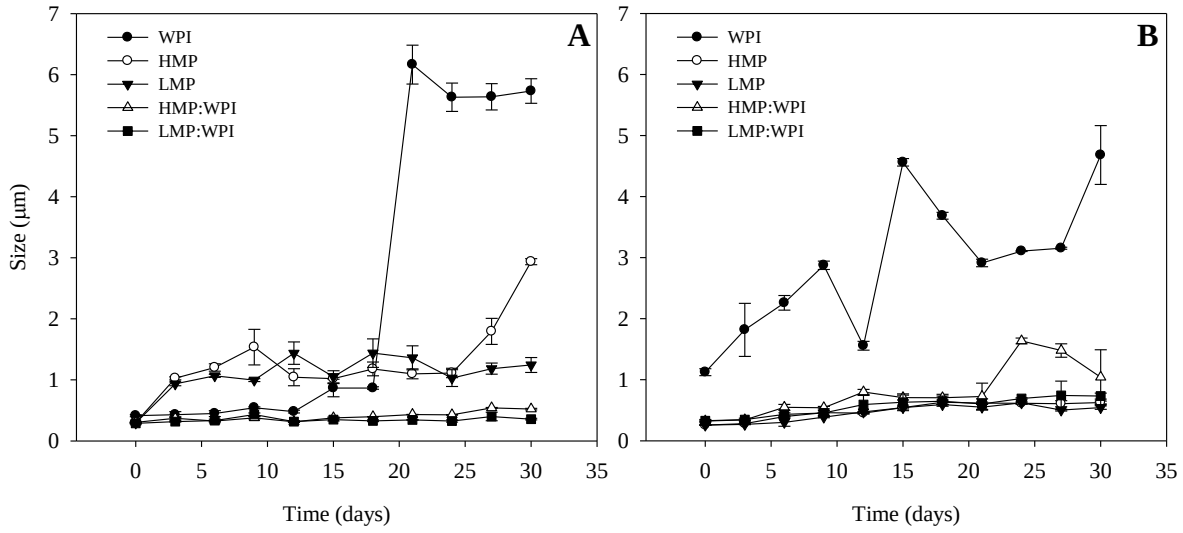

Figure 4. Particle size in d_{32} (µm) *versus* storage time (days) at room temperature of O/W nanoemulsions formulated with oregano (A) or lemongrass essential oils (B) stabilized with single biopolymer solutions or protein:pectin complexes.

WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.

3.2.2 Nanoemulsion ζ-potential and viscosity

The ζ-potential (mV) and the apparent viscosity (mPa·s) of the resultant nanoemulsions stabilized with the different evaluated emulsifiers are shown in Fig. 5.

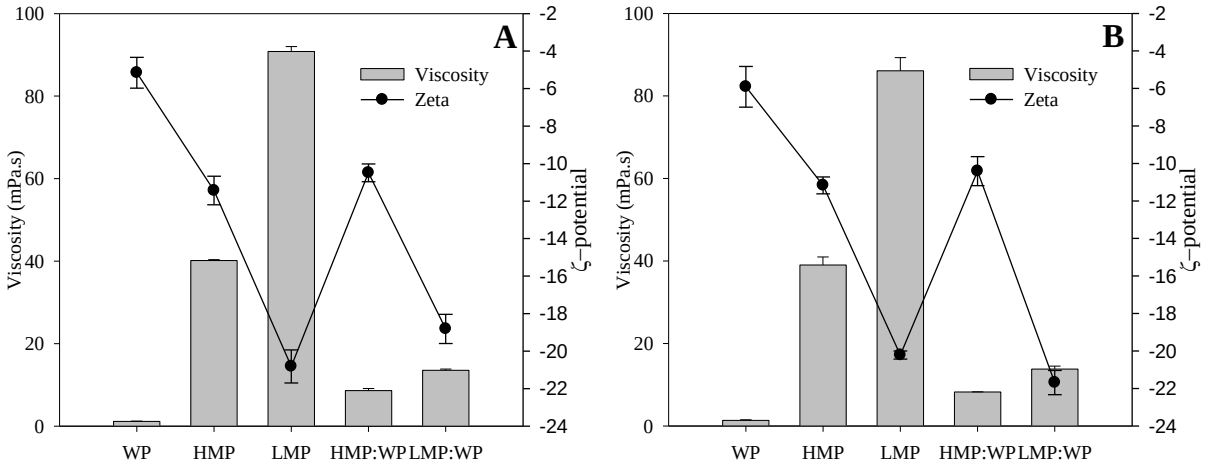

Figure 5. Initial ζ-potential (mV) (black lines) and apparent viscosity values (mPa.s) (grey bars) of O/W nanoemulsions formulated with oregano (A) or lemongrass essential oils (B) stabilized with single biopolymer solutions or protein:pectin complexes.

WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.

The less negative ζ-potential (≈ -5.5 ± 1 mV) was observed in those nanoemulsions containing WPI as emulsifier since the protein was positively charged at pH 3.5 for both oregano (Fig. 5A) and lemongrass (Fig. 5B) EOs. The ζ-potential of the WPI in solution was 24.62 ± 1.16 mV, whereas it was about -6 mV within nanoemulsions (Fig. 1 and 5, respectively). Therefore, the adsorption of hydroxyl ions at the oil-water interface is the most probable mechanism to explain the accumulation of negative charge at droplets interface (Marinova et al., 1996). In the absence of WPI, the net electrical charge of the LMP and HMP-coated droplets was respectively, around -20 and -11 mV, regardless the EO used. This is because the adsorbed pectins were negatively charged at pH 3.5 (Fig. 1). After complexation, LMP: WPI and HMP: WPI-stabilized nanoemulsions presented ζ-potential values of (-20.25 ± 0.71 and -10.45 ± 0.62 mV, respectively) similar to their analogous single-stabilized nanoemulsions. This suggested that protein moieties of the complex may be primarily adsorbed at the o/w interface while pectin moieties of the complex may be potentially located at the outer region of the o/w interface contributing with its charge to a greater extent.

Moreover, the apparent viscosity of the resultant nanoemulsions was also measured in order to assess their rheological behavior. Nanoemulsions containing WPI alone, exhibited the lowest viscosity with values around 2 mPa·s without regard to the type of EO (Fig. 5). WPI

adsorbs at the surface of oil droplets as an interconnected monolayer in which the functional groups are busy forming hydrogen and specific covalent bonds between molecules, as well as, interacting hydrophobic- and electrostatically in the interface (Murray, 2002). Oppositely, pectin-stabilized nanoemulsions and especially those containing LMP, showed the highest viscosities reaching values of 85 mPa·s in case of using LMP and values near 40 mPa·s for nanoemulsions with HMP. These difference can be attributed to a higher amount of long chains in the HMP than in the LMP, which may be more exposed to the bulk aqueous phase due to steric repulsion. At pH 3.5, these chains contain protonated carboxylates oriented to the bulk aqueous phase that may rearrange resulting in compact conformations (Alba & Kontogiorgos, 2017). Therefore, these conformations, which are able to hold high quantity of water in their structures may be responsible for the increase in the nanoemulsions viscosity (Artiga-Artigas et al., 2018; María Inés Guerra-Rosas, Morales-Castro, Ochoa-Martínez, Salvia-Trujillo, & Martín-Belloso, 2016). In spite of complex-stabilized nanoemulsions presented similar ζ-potential values to their analogous single-stabilized, the viscosity of the former was much lower (Fig. 5). Indeed, viscosity of nanoemulsions containing LMP: WPI or HMP: WPI complexes fell until ≈ 9 or 15 mPa·s, respectively. During complexation, some of the pectin chains are occupied in forming the complex. Hence, there might be a smaller amount of pectin chains forming the previously mentioned compact conformations and thus, the viscosity was lower than in case of using the pectins alone.

3.2.3 Nanoemulsion creaming stability

The stability of the prepared EOs nanoemulsions was evaluated during 30 days in terms of backscattering difference (BSD) (Fig. 6) since it is directly related to creaming phenomenon.

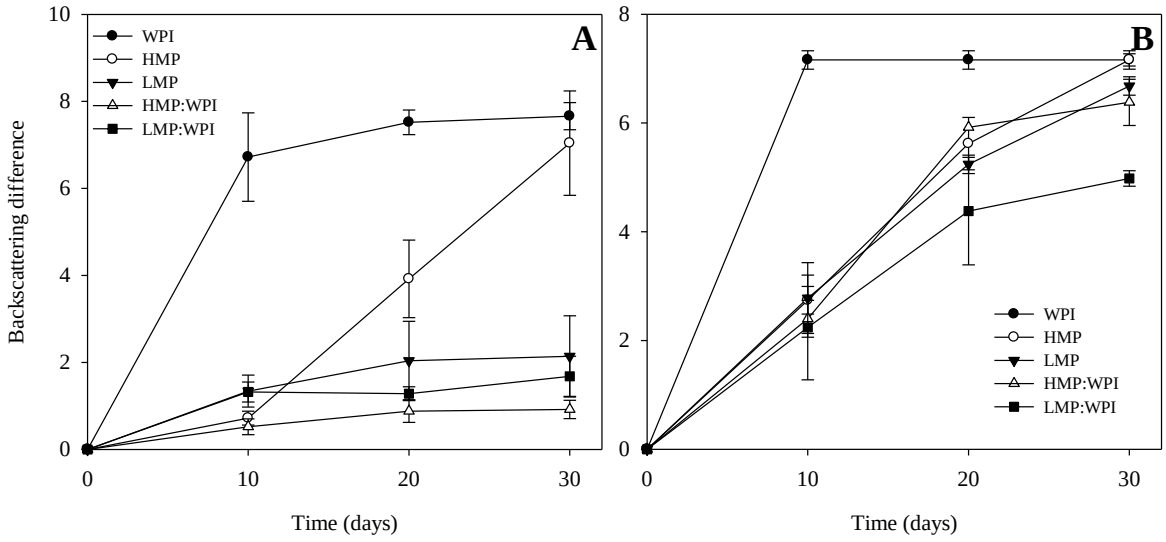

Figure 6. Backscattering difference values during storage time (days) of O/W nanoemulsions formulated with oregano (A) or lemongrass essential oils (B) stabilized with single biopolymer solutions or protein:pectin complexes.

WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.

Rapid creaming was observed in WPI-stabilized emulsions regardless the type of EO. However, nanoemulsions containing lemongrass EO (Fig. 6B) reached a faster plateau than those with oregano EO (Fig. 6A) after 10 days of room temperature storage with a BSD of 7%, approximately. Likewise, the increase of creaming in nanoemulsions containing pectin, either single or complexed, was greater in the case of lemongrass EO nanoemulsions. Certainly, the measured BSD valued of lemongrass EO nanoemulsions containing pectin already exceeded the one of oregano EO nanoemulsions after 2 days of storage. This can be attributed to the high volatility of lemongrass EO, which may promote a partial phase separation due to a biopolymer concentration gradient (Guzey & McClements, 2006; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015a). Therefore, this phase separation may favor flocculation and irreversible creaming formation. Nonetheless, it is worthwhile to note that LMP: WPI complexes were more effective than the rest of emulsifiers on reducing the creaming of lemongrass EO nanoemulsions (BSD ≈ 4.5%) (Fig. 6B). Probably because pectin chains that are not intervening in the formation of the LMP: WPI complex may acquire compact

conformations due to hydrogen bonds formation between protonated -CO_2H groups generating compact interfaces that might prevent oil droplets migration.

Regarding oregano EO nanoemulsions, those stabilized with HMP suffered a huge increase of creaming from day 10 of experiment finally reaching similar BSD values to WPI-stabilized nanoemulsions (Fig. 6A). Nonetheless, nanoemulsions stabilized by LMP showed a gradual and continuous increase of creaming being the final BSD below 2% (Fig. 6A). Specifically, LMP: WPI or HMP: WPI complexes-stabilized nanoemulsions presented BSD values lower than 1% during the 30 days of experiment resulting more efficient in preventing creaming than biopolymers alone. It is in agreement with the approach that protein-polysaccharide complexes may form compact adsorbed layers at droplets interface (Rodriguez Patino & Pilosof, 2011).

3.3 Antimicrobial activity

Additionally, the impact of the interfacial composition of EOs-in-water nanoemulsions on the antimicrobial killing kinetics against *Escherichia coli* was evaluated (Fig. 7 and 8). A Weibull equation was fitted to experimental data of the survival curves with R^2 values higher or equals 0.95 for oregano EO nanoemulsions (Table 1) and greater or equals 0.81 for lemongrass EO nanoemulsions (Table 2).

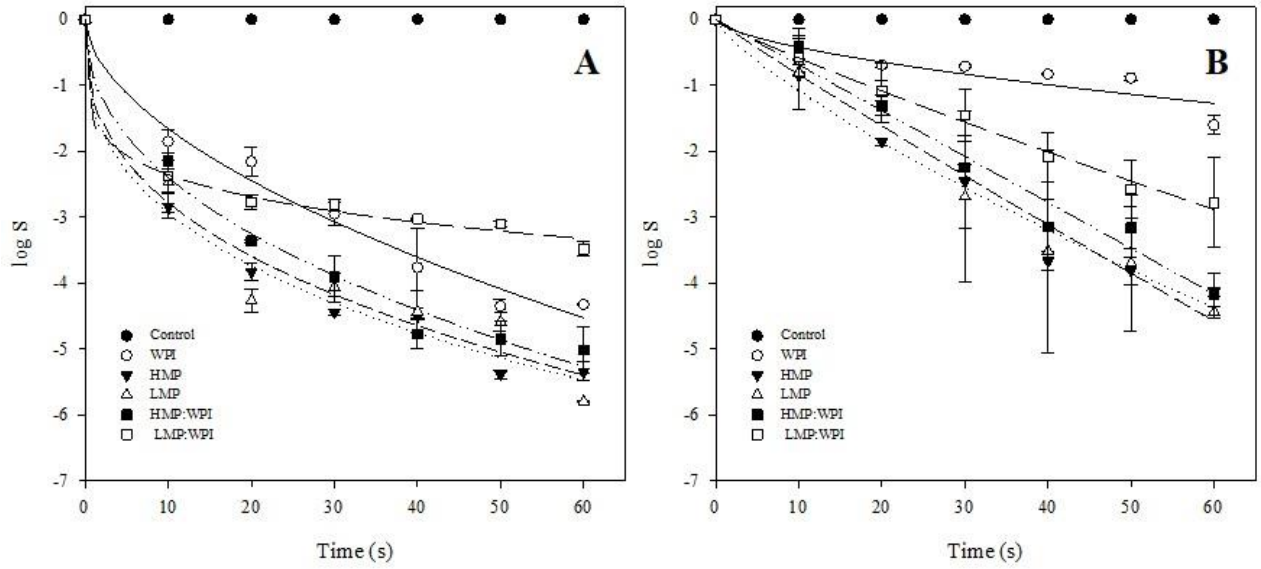

Figure 7. Survival kinetics (log S) during time (s) of *Escherichia coli* cells exposed to O/W nanoemulsions formulated with oregano (A) or lemongrass essential oils (B) stabilized with single biopolymer solutions or protein:pectin complexes.

Control is referred to the bacterial culture in water during time (s). Full symbols represent experimental data and lines represent the predicted curves with a Weibull model. Full line: predicted WPI; dotted line: predicted HMP; dashed: predicted LMP; dotted-dashed: predicted HMP:WPI; semi-solid line: predicted LMP:WPI.
WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.

Regarding lemongrass EO nanoemulsions, the inactivation of *E. coli* followed a linear model regardless the type of emulsifier since their β factor is approximately 1 (Table 2). Therefore, the efficiency of inactivation may be mainly determined by the α factor in this particular case. According to eq.2, when $\beta = 1$, the higher the α factor, the less negative the ln S(t) and thus, the greater the *E. coli* survival. Actually, nanoemulsions stabilized with WPI that presented the highest α factor (40.6 ± 16.0) (Table 2) also exhibited the lowest *E. coli* inactivation with less than 2 log reduction after 60 min of contact time (Fig. 7B).

Table 1. Estimated kinetic parameters of the Weibull model fitted to experimental data of the survival curve (log S) of *Escherichia coli* exposed to nanoemulsions containing oregano essential oil and stabilized with single biopolymer solutions or with protein:pectin complexes.

	α			β			
	Estimated	Lower CI	Upper CI	Estimated	Lower CI	Upper CI	R^2
WPI	4,073[a]	1,417	6,728	0,561[a]	0,405	0,717	0,9821
HMP	0,449[b]	-0,032	0,932	0,347[a]	0,263	0,431	0,9915
LMP	0,634[ab]	-0,799	2,0676	0,370[ab]	0,166	0,574	0,9544
HMP:WPI	1,370[ab]	0,1143	2,6259	0,439[a]	0,320	0,559	0,9863
LMP:WPI	0,117[ab]	-0,109	0,3446	0,192[b]	0,127	0,258	0,9929

α is the scale parameter and represents a characteristic time (min), β is the shape parameter of the curve. $\beta < 1$ represents a concavity upwards, $\beta = 1$ indicates a linearity and $\beta > 1$ indicates a concavity downwards. Different lower case levels indicate statistically significant differences in the same estimated kinetic parameter between the differently stabilized nanoemulsions.

WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.

Table 2. Estimated kinetic parameters of the Weibull model fitted to experimental data of the survival curve (log S) of *Escherichia coli* exposed to nanoemulsions containing lemongrass essential oil and stabilized with single biopolymer solutions or with protein:pectin complexes

	α			β			
	Estimated	Lower CI	Upper CI	Estimated	Lower CI	Upper CI	R^2
WPI	40,601[a]	24,640	56,561	0,615[a]	0,0431	1,188	0,8128
HMP	9,020[c]	4,681	13,359	0,779[a]	0,549	1,010	0,9771
LMP	12,071[bc]	6,372	17,769	0,948[a]	0,623	1,273	0,9680
HMP:WPI	14,471[bc]	9,272	19,670	1,003[a]	0,705	1,302	0,9764
LMP:WPI	18,221[b]	15,521	20,922	0,889[a]	0,754	1,024	0,9934

α is the scale parameter and represents a characteristic time (min), β is the shape parameter of the curve. $\beta < 1$ represents a concavity upwards, $\beta = 1$ indicates a linearity and $\beta > 1$ indicates a concavity downwards. Different lower case levels indicate statistically significant differences in the same estimated kinetic parameter between the differently stabilized nanoemulsions.

WPI: whey protein isolate; HMP. high methoxyl pectin; LMP: low methoxyl pectin; HMP:WPI: high methoxyl pectin:whey protein isolate complexes; LMP:WPI: low methoxyl pectin:whey protein isolate complexes.

As it has been discussed in the previous sections, WPI adsorption is slower than pectins thus, allowing a partial volatilization of lemongrass EO during nanoemulsions formation. Therefore, these nanoemulsions might be loaded with less amount of EO, which may contribute to reduce their antimicrobial efficiency since it directly proportional to its concentration (Kavas & Kavas, 2014). Moreover, the high interconnected network that WPI forms around droplets

may slow down the release of the antimicrobial compound. Additionally, HMP-, LMP- and HMP: WPI complex-stabilized nanoemulsions did not show significant differences in their α factors (9.020, 12.071 and 14.471, respectively) and they were the most effective in preventing *E. coli* survival with ≈4.3 log reductions after the final time of exposure. Pectin affinity for lemongrass EO may prevent lemongrass EO migration in a higher extent preserving the antimicrobial properties of nanoemulsions. In line with our results, Guerra-Rosas, Morales-Castro, Cubero-Márquez, Salvia-Trujillo, & Martín-Belloso (2017) also observed that lemongrass-pectin nanoemulsion, exhibited a strong antimicrobial potential against *E. coli*. Lastly, nanoemulsions stabilized with LMP: WPI complexes exhibited just 2.5 log reductions after being in contact 60 min with the *E. coli* and an α factor of 18.2 ± 2.7 (Table 2). These results evidenced the lower antimicrobial activity of LMP: WPI complex-stabilized nanoemulsions compared to those stabilized by the HMP: WPI complex. This hypothesis is consistent with the approach of that the interaction of LMP and WPI is stronger than those between HPM and the protein. Subsequently, LMP: WPI complexes may led to compact interfaces preventing creaming (Fig. 6B) and particle size increase (Fig. 4B) but also the complete release of the antimicrobial compound.

Overall, the antimicrobial activity of oregano EO nanoemulsions was greater than the one of lemongrass EO nanoemulsions regardless the emulsifier used. The antimicrobial activity of each EO is attributed to their phenolic compounds and it strongly influenced by their concentration and their chemical structure (Pesavento et al., 2015). Indeed, many works have reported the higher bactericidal action of carvacrol, main component of oregano EO, compared to citral, main component of lemongrass EO (Artiga-Artigas, Acevedo-Fani, & Martín-Belloso, 2017; Burt, 2004; Rojas-Graü et al., 2007). Nanoemulsions containing oregano EO exhibited β factors below 1 (Table 1) meaning that the antimicrobial inactivation was fast at the beginning becoming more moderate during the time until reaching a plateau (Fig. 7A). Similar to nanoemulsions with lemongrass EO, the type of emulsifier played a relevant role on the antimicrobial capacity of oregano EO nanoemulsions. In fact, nanoemulsions LMP, HMP and HMP: WPI complex-stabilized, were the most efficient at killing *E. coli* showing respectively, 5.79, 5.34 and 5.0 log reductions after 60 min in contact with the microorganism. In contrast, nanoemulsions stabilized with WPI presented 4.32 log reductions after the same contact time suggesting that these nanoemulsions has less antimicrobial effect than the ones containing pectin. Based on the results, LMP: WPI complex-stabilized nanoemulsions presented higher antimicrobial efficiency during the first 20 min of contact with the microorganism but they mostly lost their effect from that moment (Fig. 7). The β factor of LMP: WPI complex-

stabilized nanoemulsions (0.192 ± 0.065) suggested that the complex forms an interface so compact that it only allows a small amount of EO to be released (Table 1). This is in agreement with fluorescence images of Fig. 8 where it can be observed that HMP: WPI complex-stabilized nanoemulsions presented more quantity of dead cells over time than those WPI-stabilized; and the latter in turn, exhibited more killing activity than nanoemulsions stabilized with LMP: WPI complexes.

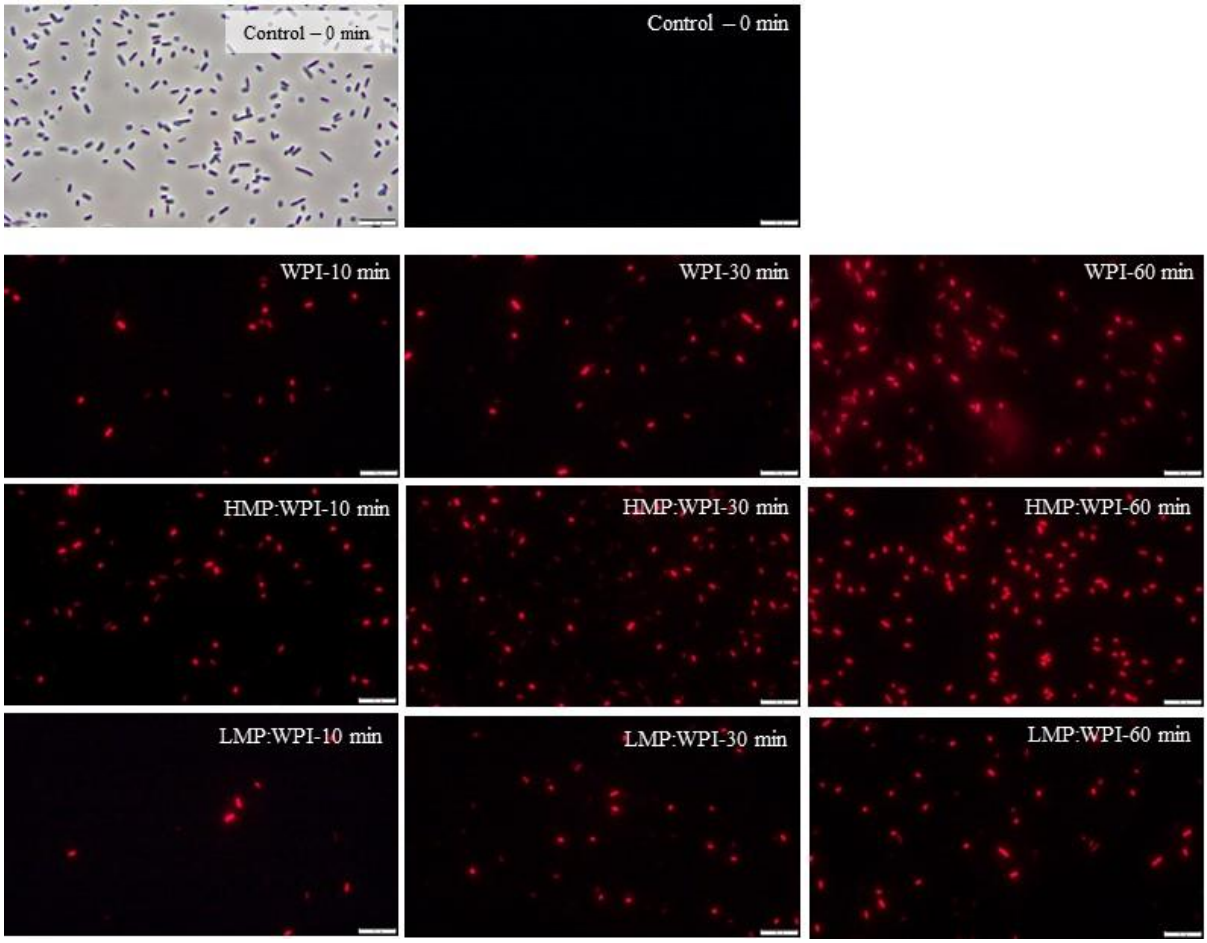

Figure 8. Optical and fluorescence microscopy images of *Escherichia coli* cells in contact with water (control) or after 10, 30 and 60 min in contact with O/W nanoemulsions stabilized with Whey Protein Isolate (WPI), High Methoxyl Pectin:Whey Protein Isolate (HMP:WPI) or Low Methoxyl Pectin:Whey Protein Isolate (LMP:WPI). Red cells are dead cells.

4 CONCLUSIONS

This work evidences that the interfacial composition not only determines the physicochemical properties but the antimicrobial activity of EO-loaded nanoemulsions. The greatest achievement of this study was the formation of stable nanoemulsions whose dispersed

phase was entirely an EO, preserving their antimicrobial efficiency against *E. coli*. The results showed that oregano EO nanoemulsions stabilized by 1% *w/w* HMP: WPI complexes, which maintained a droplet size below 0.5 μm after 30 days of room temperature storage, exhibited the strongest antimicrobial potential against the microorganism. It demonstrates that HMP: WPI complexes may form interfaces sufficiently compact around EO droplets to ensure the nanoemulsions stability, which in turn, allow the release of the antimicrobial compound through them. In fact, although LMP: WPI complexes also guaranteed the stability of EO-loaded nanoemulsions over time, the resultant interfaces were so compact that the antimicrobial compound had more difficulties to go through it, which resulted in higher *E. coli* survival. Also LMP and HMP acted as efficient emulsifiers for lemongrass EO nanoemulsions according to their high stability and their small particle sizes. The most relevant result regarding WPI was their slow adsorption at oil droplets interface, which might favor EO migration impacting on the antimicrobial efficiency of the resultant nanoemulsions. Conclusively, the results obtained in the present study, have firstly demonstrated the potential of pectin: protein complexes to stabilize EO-loaded nanoemulsions during at least 30 days of room temeperature storage and secondly, to preserve their antimicrobial killing kinetics against *E. coli*. Moreover, this manuscript evidences the effectiveness of HMP: WPI complex-stabilized nanoemulsions to be used as antimicrobial delivery systems in beverages and foods.

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