NELSON JUSTINO GOMES NETO

EFEITO DA EXPOSIÇÃO AO ÓLEO ESSENCIAL DE *Rosmarinus officinalis* L. E 1,8-CINEOL NA TOLERÂNCIA DIRETA E CRUZADA EM BACTÉRIAS PATOGÊNICAS CONTAMINANTES DE ALIMENTOS

2015

RECIFE

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Tese apresentada ao Programa de Pós-Graduação em Nutrição do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, para obtenção do título de Doutor em Nutrição.

Orientador: Prof. Dr. Evandro Leite de Souza

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Tese aprovada em 31 de julho de 2015.

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Ao meu bom, inefável e maravilhoso Deus,
Inesgotável fonte de misericórdia, amor e graças em minha vida.

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Razões de minha existência e exemplos personificados de amor, simplicidade, luta e dedicação, que sempre levarei como legado

Dedico.

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RESUMO

A ocorrência de tolerância por parte dos micro-organismos contaminantes de alimentos a agentes e/ou tratamentos antimicrobianos clássicos, impõe a busca de tecnologias alternativas para emprego na conservação de alimentos, a exemplo dos óleos essenciais, em especial aqueles obtidos da espécie *Rosmarinum officinalis* L. (OERO). A ação antimicrobiana do OERO tem sido atribuída ao 1,8-cineol (CIN) frequentemente citado como seu constituinte majoritário. O objetivo deste estudo foi investigar o efeito da exposição a concentrações subinibitórias do OERO e CIN na modificação da tolerância direta e cruzada de algumas bactérias contaminantes de alimentos (*Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* e *Salmonella* Typhimurium). Ainda, foi avaliada a influência dos fatores sigma alternativos σ^S e σ^B na tolerância de *E. coli* MG1655 e *L. monocytogenes* EGD-e, e seus mutantes isogênicos *E. coli* MG1655 Δ*rpoS e L. monocytogenes* EGD-e Δ*sigB*, frente ao OERO e ao óleo essencial de *Origanum vulgare* L. (OEOV) bem como na modificação da tolerância bacteriana direta e cruzada ao campo elétrico pulsado (CEP). Para isso, foram realizados ensaios de determinação da Concentração Inibitória Mínima (CIM) e de modificação da tolerância direta e cruzada após exposição das cepas às concentrações subinibitórias (sub-CIMs) (1/2 CIM e 1/4 CIM) do OERO ou do CIN em caldo base carne (18 h) e em modelo base carne (72 h). Para os ensaios de verificação de injúria subletal e interferência dos fatores sigma na tolerância bacteriana, células de *E. coli* MG1655 e *L. monocytogenes* EGD-e foram expostas a concentrações inibitórias do OERO ou OEOV para, em seguida, serem cultivadas em meios adicionados de agentes seletivos (NaCl ou sais de bile). Os valores de CIM variaram entre 20 e 40 μL/mL e 40 e 80 μL/mL para o OERO e CIN, respectivamente. Os ensaios de modificação de tolerância não revelaram aumento de tolerância direta e cruzada nas cepas bacterianas testadas. A CIM do OERO e OEOV frente às células mutantes foi menor daquele encontrado frente às células parentais. As células mutantes também apresentaram maior diminuição da sua viabilidade quando tratadas com os óleos essenciais (OES) em relação às células parentais, bem como maior proporção de células apresentando lesão subletal. Ainda, as células mutantes mostraram maior sensibilidade ao CEP quando comparadas as parentais. A ausência dos fatores sigma diminuiu a tolerância direta e ao CEP nas células pré-expostas ao OES em sub-CIMs. Os resultados obtidos neste estudo demonstram que: i) a exposição das cepas bacterianas a sub-CIMs do OERO ou CIN não aumentou a tolerância direta e cruzada; ii) os fatores sigma σ^S e σ^B influenciaram a sensibilidade das cepas de *E. coli* MG1655 e *L. monocytogenes* EGD-e, respectivamente, frente ao OERO e OEOV ao CEP; e iii) os fatores sigma não influenciaram no aumento da tolerância bacteriaia direta e cruzada ao CEP. Estes resultados demonstram o potencial do OERO e do CIN para uso como antimicrobiano na conservação de alimentos, quando considerado o seu efeito inibitório sobre bactérias contaminantes de alimentos e a ausência de indução de aumento na tolerância bacteriana direta e cruzada.

Palavras-chave: Óleos essenciais, Alecrim, Tolerância bacteriana, Conservação dos alimentos, Bactérias patogênicas.

ABSTRACT

The occurence of tolerance in food contaminant microorganisms to agents and/or classic antimicrobial treatments has required the search of alternative technologies for use in food preservation, such as essential oils, especially those obtained from the specie *Rosmarinum officinalis* L. (ROEO). The antimicrobial action of ROEO has been attributed to 1.8-cineol (CIN), frequently cited as its main constituent. The aim of this study was to investigate the effect of exposure to subinhibitory concentrations of ROEO and CIN on the modification of direct tolerance and cross-tolerance of some food-contaminant bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella Typhimurium*). Still, the influence of alternative sigma factors σ^S and σ^B on tolerance of *E. coli* MG1655 and *L. monocytogenes* EGD-e, and its isogenic mutant *E. coli* MG1655 and *L. monocytogenes* EGD Δ*rpoS* and Δ*sigB* to the ROEO and the essential oil of *Origanum vulgare* L. (OVEO), as well as direct on the modification of the bacterial direct tolerance and cross tolerance the pulsed electric field (PEF) were investigated. For this, assays to determine the Minimum Inhibitory Concentration (MIC) and the modification of the direct tolerance and the cross-tolerance after exposure of the strains to subinibitory concentrations (sub-MICs) (1/2 MIC and 1/4 MIC) of ROEO or CIN in meat broth (18 h) and model based meat (72 h) were performed. For verification of sublethal injury and interference of sigma factors on bacterial tolerance, *E. coli* MG1655 cells and *L. monocytogenes* EGD-e were exposed to inhibitory concentrations of ROEO or OVEO and further cultivated in media added selective agents (NaCl or bile salts). The MIC values ranged between 20 and 40 μL/mL and 40 to 80 μL/mL for ROEO and CIN, respectively. The tolerance modified assays revealed no increase in either the direct tolerance or the cross-tolerance in the tested bacterial strains. The MIC of ROEO and OVEO against the mutant cells was lower than that found against the parental cells. Mutant cells showed also a greater decrease in viability when treated with essential oils (EOS) compared to the parental cells, as well as a higher proportion of cells with sublethal injury. Still, the mutant cells showed higher sensitivity to PEF when compared to parental cells. The absence of sigma factors decreased the direct tolerance and tolerance to the PEF in cells pre-EOS exposed to sub-MIC. The results of this study show that: i) exposure of the bacterial strains to sub-MICs of ROEO or CIN did not increase the direct or the cross-tolerance; ii) the sigma σ^S and σ^B factors influenced the sensitivity of strains of *E. coli* MG1655 and *L. monocytogenes* EGD-e, respectively, to the OVEO and ROEO and PEF; and i) the sigma factors did not influence the increase in direct bacterial tolerance and cross tolerance to the PEF. These results demonstrate the potential of ROEO and CIN for use as antimicrobials in food preservation, when considering their inhibitory effect on bacterial food-contaminants and the absence of induction to direct or cross bacterial tolerance.

Keywords: Essential oils, Rosemary, Bacterial tolerance, Food preservation, Pathogenic bactéria.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ATCC - American Type Culture Collection

ATM - Pressão Atmosférica

ATP - Adenosina Trifosfato ou Trifosfato de Adenosina

ATSEL - Ágar Tripsona de Soja suplementado com Extrato de Levedura

CEP - Campo Elétrico Pulsado

CIM - Concentração Inibitória Mínima

CIN - 1,8-Cineol

CTSEL - Caldo Tripsona de Soja suplementado com Extrato de Levedura

DAEC - *Escherechi*a *coli* de adesão difusa

DNA -

DVA's - Doenças Veiculadas por Alimentos

EAEC - *Escherechia coli* enteroagressiva

EDTA - Ethylenediamine Tetraacetic Acid

EHEC - *Escherechia coli* enterohemorrágica

EIEC - *Escherechia coli* enteroinvasiva

EPEC - *Escherechia coli* enteropatogênica

FDA - Food and Drug Administration

FIOCRUZ - Fundação Osvaldo Cruz

g - Grama

Gras - Generally Recognized as Safe

h - Hora

Hz - Hertz

KGy - kilogray

kJ/kg - kilojoules per kilogram

kV/cm - kilovolt per centimeter

Log $_{10}$ - Logaritmo na base 10

m - Metro

Min - Minuto

mL - Mililitro

mS/cm - MiliSiemens por centímetro

NaCl - Cloreto de sódio

ng - Nanogramas

º C - Graus Celcius

OE - Óleo essencial

OEOV - Óleo Essencial de *Origanum vulgare*

OERO - Óleo essencial de *Rosmarinus officinalis*

OES - Óleos Essenciais

OMS - Organização Mundial de Saúde

PCR - Polymerase Chain Reaction

pH - Potencial hidrogeniônico

RNA - Ribonucleic Acid

rpm - Rotação por Minuto

s - Segundo

SNC - Sistema Nervoso Central

SUS - Sistema Único de Saúde

UFC - Unidade Formadora de Colônia

WHO - World Health Organization

σ^{B} - Fator Sigma B

σ^{S} - Fator Sigma S

SUMÁRIO

1 APRESENTAÇÃO

Os alimentos são passíveis de contaminação por diferentes agentes microbianos, os quais podem causar deterioração e/ou doenças decorrentes da ingestão de micro-organismos patogênicos ou de suas toxinas pré-formadas presentes nestes alimentos (CÉBRIAN et al., 2010). As modificações da tolerância, particularmente o seu aumento, por parte de micro-organismos frente a diversos compostos ou processos antimicrobianos clássicos utilizados em alimentos tem se apresentado como importante fator motivador da busca por alternativas para emprego em sistemas de conservação de alimentos (OLIVEIRA et al., 2010).

A exposição a condições estressantes (subinhibitórias ou subletais) pode causar danos às células microbianas que, durante o seu processo de reparação, podem adquirir a habilidade de desenvolver-se na presença dos agentes estressores. Esta habilidade tem como repercussão o desenvolvimento de tolerância aumentada, com impactos negativos sobre a segurança alimentar (SILVA-ÂNGULO et al., 2014). Essas respostas microbianas podem resultar em um aumento da tolerância a outros agentes estressores, fenômeno denominado como tolerância cruzada (LUZ et al., 2014a; LUZ et al., 2014b).

A modificação da tolerância microbiana cruzada entre agentes conservantes tem importantes implicações na conservação de alimentos, quando, geralmente, tensões são aplicadas com o intuito de controlar o crescimento e sobrevivência dos micro-organismos (LUZ et al., 2014a). Estudos anteriores já reportaram a capacidade de aumento de tolerância direta e cruzada em micro-organismos contaminantes de alimentos quando expostos a condições estressoras causadas pela acidez, temperaturas elevadas e baixas, sais e radiação (ÁLVAREZ-ORDÓÑEZ et al., 2009; ESPINA et al., 2011; GRUZDEV et al., 2011; SOMOLINOS et al., 2010a).

A indústria alimentícia procura atender regras gerais que objetivam em prolongar o período de armazenamento dos alimentos, fazendo com que permaneçam adequados para o consumo (SOUZA et al., 2013a). As mudanças no processamento e a crescente exigência do consumidor por alimentos mais naturais, com uma vida útil prolongada, mantendo a qualidade nutritiva e sensorial, impulsionam a realização de pesquisas para obtenção de novos ingredientes, que possam atender a novas perspectivas do mercado (MONTE et al., 2014). Ainda, a legislação de alimentos tem progressivamente restringido e/ou limitado o uso de alguns conservantes químicos que são tradicionalmente utilizados em alimentos (WANG et al., 2013).

Neste contexto, torna-se de interesse para a ciência de alimentos a possibilidade da descoberta de novos agentes antimicrobianos e sua possível aplicação prática na conservação de alimentos. Assim, diversos estudos têm sido conduzidos a fim de se aprofundar nesta possibilidade de aplicação de novos compostos antimicrobianos, tomando como base resultados observados em experimentos *in vitro* (BARROS et al., 2012; ESPINA et al., 2014; LUZ et al., 2012a; OLIVEIRA et al., 2015).

O uso de óleos essenciais (OES) como antimicrobianos surge como alternativa tecnológica para emprego em sistemas de conservação de alimentos (MONTE et al., 2014). As principais características relacionadas ao uso de OES em sistemas de conservação de alimentos referem-se a sua origem natural e sua composição complexa em diversidade de constituintes, os quais apresentam diferentes mecanismos de ação antimicrobiana, tornando, desta forma, mais difícil à ocorrência de processos adaptativos por parte dos micro-organismos (BURT 2004; BARBOSA et al., 2015; LUZ et al., 2014b).

Entre os diversos OES que apresentam potencialidade antimicrobiana, aquele obtido da espécie vegetal *Rosmarinus officinalis* Linn. (OERO) tem mostrado destacáveis resultados na inibição de diferentes micro-organismos, e se mostrado como possível alternativa para uso como antimicrobiano em alimentos. Compostos como eugenol, 4-terpineol, α-terpineol, δ-terpineol e tricicleno são alguns dos constituintes encontrados no OERO, sendo que 1,8-cineol (CIN) tem sido frequentemente relatado como o seu componente majoritário (AZEREDO et al., 2011; BARBOSA et al., 2015; MULYANINGSIHA et al., 2010; OLIVEIRA et al., 2015).

O OERO, bem como o CIN, têm se mostrado eficazes na inibição de diversas bactérias contaminantes de alimentos, a citar *Staphylococcus aureus, Listeria monocytogenes Pseudomonas aeruginosa* e *Salmonella* Tiphymurium (AZÊREDO et al.;, 2012; GACHKAR et al., 2007; SOUSA et al., 2012). Embora esta atividade antimicrobiana já tenha sido estudada, não se dispõe de dados na literatura científica que tenham como ênfase a observação de modificações na tolerância direta e cruzada de cepas bacterianas quando expostas a condições estressantes causadas pela exposição a concentrações subinibitórias do OERO e CIN.

2 PERGUNTA CONDUTORA

A exposição a concentrações subinibitórias do óleo essencial de *R. officinalis* e de seu constituinte majoritário 1,8- cineol induz o aumento na tolerância direta e cruzada das bactérias contaminantes de alimentos *L. monocytogenes*, *S. aureus*, *P. aeruginosa* e *S.* Typhimurium?

3 OBJETIVOS

Objetivo geral

Investigar a capacidade de desenvolvimento de tolerância em bactérias contaminantes de alimentos quando expostas a concentrações subinibitórias do OERO e de seu constituinte majoritário CIN.

Objetivos específicos

a) Avaliar a influência da exposição a diferentes concentrações do OERO e CIN sobre a viabilidade de cepas de *L. monocytogenes*, *S. aureus*, *P. aeruginosa* e *S.* Typhimurium;

b) Avaliar a modificação na tolerância direta e cruzada (termotolerância, ácidotolerância e osmotolerância) nas cepas bacterianas teste quando expostas a concentrações subinibitórias do OERO e CIN;

c) Verificar a influência dos fatores sigma alternativos σ^S e σ^B na tolerância ao OERO, e na ocorrência de injúria subletal, em cepas de *E. coli* e *L. monocytogenes*.

4 REVISÃO DA LITERATURA

Implicações do crescimento microbiano em alimentos

Desde muito tempo, reconhece-se a magnitude das implicações da deterioração microbiana em alimentos (SOUZA et al., 2013b). As perdas de alimentos decorrentes da deterioração microbiana no âmbito mundial chegam a bilhões de dólares por ano, afetando principalmente países em desenvolvimento (CARFORA et al., 2015). O crescimento de micro-organismos em alimentos pode causar uma gama de alterações organolépticas e nutricionais, conduzindo a um estado de impropriedade para o consumo humano (JUNEJA; HWANG; FRIEDMAN, 2010). A deterioração microbiana de alimentos pode ocorrer como conseqüência do crescimento de micro-organismos, ou ainda da liberação de enzimas extra ou intracelulares no substrato após lise da célula microbiana (JAY, 2005).

Os micro-organismos quando presentes no alimento crescem por meio da utilização dos nutrientes disponíveis através de via oxidativa ou fermentativa, com a finalidade de obtenção de energia necessária para manutenção da viabilidade da célula microbiana e síntese de componentes celulares (SARKER et al., 2007). A condução das reações químicas envolvidas na utilização dos nutrientes presentes no alimento promove a formação de produtos intermediários e finais, os quais são responsáveis pelas alterações organolépticas e nutricionais (YI et al., 2010).

Além dos problemas decorrentes do crescimento microbiano em relação a deterioração e consequentes perdas dos alimentos, em todo o mundo as doenças provocadas pelo consumo de alimentos contaminados com micro-organismo patogênicos e/ou suas toxinas têm grande impacto sobre a saúde pública (SPANU et al., 2012). Segundo a Organização Mundial da Saúde (OMS), doenças de origem alimentar são definidas como “doenças de natureza tóxica ou infecciosa, causadas pelo consumo de alimentos ou água contaminados” (BAKKALI et al., 2008). A ocorrência de dois ou mais casos de uma mesma doença, como resultado da ingestão de um alimento ou água em comum define surtos ocasionados por Doenças Veiculadas por Alimentos (DVA’s) (SILVA-WEISS, 2013). Dentre os perigos biológicos relacionados com o surgimento das DVA’s, as bactérias são consideradas seus principais agentes etiológicos, representando mais de 2/3 dos surtos registrados (WINK, 2012).

Os surtos provocados pelo consumo de alimentos contaminados por micro-organismos patogênicos podem sofrer variações quanto ao seu período de incubação, intensidade e duração da doença de acordo com a suscetibilidade individual (idade, estado nutricional e

grau de sensibilidade), quantidade do alimento contaminado ingerida e, em alguns casos, com tipo e quantidade de toxina (pré) formada (FRANCO; LANDGRAF, 2005).

De modo geral, as doenças microbianas de origem alimentar apresentam como sintomas mais comuns a dor abdominal, náuseas, vômitos, diarréia, prostração, hipotermia e hipotensão (LAWRYNOWICZ-PACIOREK et al., 2007). Estes sintomas, por se apresentarem leves, terem baixa letalidade e um curso restrito, muitas vezes não são tratados nos serviços de saúde e, portanto, não sofrem notificação pelos órgãos de saúde oficiais, consequentemente (WHO, 2014). As bactérias *Staphylococcus aureus, Salmonella Typhi, S. Paratyphi, Shigela disenteriae, Clostridium botulinum, C. perfringens, Bacillus cereus* e *Escherichia coli* são exemplos de alguns micro-organismos frequentemente envolvidos como agentes etiológicos de surtos alimentares (PIMENTEL-FILHO et al., 2014).

A transmissão de patógenos e/ou suas toxinas para os alimentos pode ocorrer através de fezes, mãos de manipuladores, animais voadores ou rasteiros e pela água contaminada. Neste sentido, todo alimento é potencialmente suscetível à contaminação por ação microbiana, necessitando um sistemático controle de qualidade em todas as etapas da sua cadeia produtiva (STEFANAKIS et al., 2013). Nos países em desenvolvimento são detectados anualmente mais de um bilhão de casos de diarreia aguda em crianças menores de cinco anos, das quais 2 milhões chegam ao óbito, segundo registros da OMS (SILVA et al. 2013).

Estima-se que em países que apresentam sistemas de registro de notificação, até 100 milhões de pessoas sejam acometidas por doenças decorrentes do consumo de alimentos anualmente (HAMADI et al, 2014). No Brasil, de todas as internações realizadas em hospitais do Sistema Único de Saúde (SUS), compreendendo o período de 2010 a 2013, entre 4,5% a 4,8% tiveram diagnóstico de infecção intestinal, representando aproximadamente 70 mil internações com um custo estimado de 110 milhões de reais (ZELENY et al, 2015). Por esta razão, no sistema de produção de alimentos, torna-se fundamental que medidas sejam tomadas para que seja assegurada a inocuidade e estabilidade dos seus produtos finais durante toda a sua vida de prateleira (WANG et al., 2013).

O consumo de alimentos fora dos padrões higiênico-sanitários além de não atender a legislação, pode resultar em aumento das taxas de absenteísmo no trabalho, despesas com tratamento médico e aumento do número de hospitalizações, com repercussões econômicas e sociais significativas (MUTHAIYAN; RICKE; GUSTAFSON et al., 2014). Diversos fatores ambientais, sociais e econômicos, como produção de alimentos em larga escala, intenso comércio de alimentos, urbanização, mudanças no comportamento humano, aumentada mobilidade e aumento no número de indivíduos imunodeprimidos têm contribuído para o

surgimento, e, em alguns casos, para o agravamento da realidade acerca da emergência de micro-organismos patogênicos (MAZZARIN et al., 2015; PESAVENTO et al., 2015).

Staphylococcus aureus

A espécie *S. aureus* faz parte do grupo de estafilococos coagulase positiva, sendo considerada a mais virulenta do gênero (BARROS et al., 2012). A peculiaridade de seu habitat torna estes micro-organismos largamente distribuídos na natureza, sendo transmitidos aos alimentos principalmente pelos manipuladores, na maioria, portadores assintomáticos. Deste modo, pode-se esperar a presença de *S. aureus*, mesmo em pequenas quantidades, em quase todos os alimentos de origem animal ou naqueles diretamente manipulados (DEVERE; PURCHASE, 2007).

S. aureus tem se destacado como um dos mais importantes agentes etiológicos de DVA's notificadas em todo o mundo (ARAGON-ALEGRO et al., 2007; CEBRIAN et al., 2010; ZELENY et al., 2015), sendo responsável por aproximadamente 45% das toxinfecções associadas à ingestão de alimentos *in natura* e processados (AIT-OUAZZOU et al, 2011). Isso se deve a capacidade que algumas cepas de *S. aureus* possuem de produzir enterotoxinas dentro de matrizes alimentares, causando intoxicação alimentar estafilocócica (ARGUDIN et al., 2010), que consiste em uma gastroenterite resultante da ingestão de 100 a 200 ng de toxinas pré-formadas em alimentos. Essa quantidade de toxina pode ser alcançada quando a contagem de *S. aureus* ultrapassa 10^5 e 10^6 UFC/g de alimento (BENNETT, 2005).

Os alimentos frequentemente envolvidos em surtos de intoxicação estafilocócica incluem produtos cárneos, aves, ovos, leite e produtos lácteos (HAMADI et al., 2014), ou seja, alimentos com elevado teor protéico, que requerem manipulação durante o processamento, muitas vezes associada ao aquecimento e/ou armazenamento inadequado destes produtos (ALABOUDI et al., 2012; HUMMERJOHANN et al., 2014; WALLIN-CARLQUIST et al., 2010).

As toxinas estafilocócicas causam diarreia e vômito quando ingeridas, e são responsáveis pelo chamado envenenamento alimentar estafilocócico. As enterotoxinas estafilocócicas caracterizam-se como proteínas imunologicamente distintas, hidrossolúveis e resistentes à ação do calor. As temperaturas requeridas para a sua inativação são muito mais elevadas do que aquelas necessárias para eliminar a bactéria do alimento (EL-BAKRY et al., 2014).

Listeria monocytogenes

L. monocytogenes é um patógeno oportunista, capaz de sobreviver e se multiplicar em meios adversos como substratos nutritivos muito simples, temperaturas de refrigeração, ambientes ácidos e alcalinos, além de elevadas concentrações de NaCl (LUZ et al, 2012a).

L. monocytogenes é conhecida por causar a listeriose, importante e grave doença de origem alimentar, que atinge principalmente indivíduos com o sistema imune comprometido, como grávidas, idosos, recém-nascidos, portadores de neoplasias e da Síndrome da Imunodeficiência Adquirida (LIN et al., 2011). A listeriose é considerada um grave problema de saúde devido à severidade dos sintomas e alta taxa de mortalidade (em torno de 30%), mesmo com adequado tratamento com antibióticos (DALMASSO; JORDAN, 2014). Os relatos de surtos causados por esta bactéria incluem alimentos *in natura* e processados armazenados sob refrigeração por longos períodos, submetidos a aquecimento insuficiente, incluindo vegetais, produtos cárneos, leite e seus derivados (MELO et al., 2015).

A dose infectante para o estabelecimento da doença depende da virulência e patogenicidade da cepa de *L. monocytogenes*, bem como do estado de saúde do hospedeiro, entretanto pelo menos 10^2 UFC (Unidades Formadoras de Colônia) por grama ou mililitro de alimento são necessários para causar a listeriose (JEMMI; STEPHAN, 2006). As manifestações clínicas da listeriose em adultos são decorrentes, principalmente, de infecções no sistema nervoso central (SNC) como meningite e encefalite, além de outras infecções como endocardite, peritonite, pneumonia e ostiomielite (BARANCELLI et al., 2014). Em gestantes, *L. monocytogenes* pode provocar abortamento, nascimento prematuro, morte fetal, meningite ou septicemia neonatal (RAJKOVIC et al., 2011).

Pseudomonas aeruginosa

P. aeruginosa é uma bactéria ubiquitária, que requer necessidades mínimas de nutrientes para seu crescimento (TYAGI; MALIK, 2010). Transmitida por água e alimentos contaminados, esta bactéria é considerada um patógeno oportunista, podendo causar infecções e enterites quando o sistema imunológico do hospedeiro encontra-se deprimido (MASSIER et al., 2012). Por possuir intensa atividade metabólica, sua presença em quantidades elevadas reduz a vida de prateleira dos produtos alimentícios, devido a alterações nas características químicas e sensoriais (YI et al., 2010). *P. aeruginosa* pode também ser monitorada como um indicador de outras contaminações bacterianas, como as de origem fecal (SILVA et al., 2008).

Aproximadamente 10^5 UFC/g de alimento são necessárias para causar a patogenia alimentar (MASSIER et al., 2012), sendo a dose infectante influenciada tanto pela susceptibilidade do hospedeiro quanto pela linhagem envolvida (YI et al., 2010). As infecções causadas por *P. aeruginosa* estão associadas a um alto padrão de mortalidade, e são difíceis de serem erradicadas do sangue ou de tecidos infectados, pois esses micro-organismos têm uma suscetibilidade limitada a antimicrobianos (HASSANI et al., 2007).

Além disso, tem sido descrito que *P. aeruginosa* apresenta tolerância a uma ampla variedade de condições físicas, incluindo capacidade de se multiplicar mesmo sob condições extremas de temperatura e pH, com elevadas concentrações de aditivos e sais, propriedades que contribuem para sua presença em diversos ambientes (VICTORIA et al., 2012). Sua tolerância a diferentes antimicrobianos pode ser intrínseca, devido à baixa permeabilidade de sua membrana e a capacidade de formar biofilmes (LUZ et al., 2014).

Salmonella enterica sorovar Typhimurium

Salmonella enterica subespécie *enterica* sorovar Typhimurium é uma bactéria amplamente disseminada na natureza e de grande importância em saúde pública, por se tratar de um dos principais patógenos envolvidos em surtos de origem alimentar (GUNDUZ et al., 2009). Diversas espécies animais podem servir de reservatório para *S.* Typhimurium, proporcionando sua constante eliminação no meio por animais infectados assintomáticos. Assim, sua disseminação entre espécies de animais destinadas à produção de alimentos representa um grande risco de contaminação da cadeia alimentar (MARTÍN et al., 2014).

Os alimentos de origem animal, em especial, os ovos e carnes de aves, suína e bovina são os principais veículos de transmissão da *S.* Typhimurium, (PHILLIPS et al., 2008). A dose infectante oscila entre 10^2 a 10^6 UFC/g, ocorrendo variação quanto ao alimento e cepa envolvidos (GUNDUZ et al., 2009).

A salmonelose acomete principalmente indivíduos com o sistema imune comprometido, sendo caracterizada por febre, diarreia, vômitos e cólicas abdominais (MARTÍN et al., 2014; BAJPAI; BAEK; KANG, 2012). Estima-se, que apenas nos Estados Unidos ocorram a cada ano, cerca de 1 milhão de episódios envolvendo gastroenterites com 19.000 internações e 400 óbitos devido a infecções causadas por *S.* Typhimurium (LV et al., 2014). Por muitos anos, *S.* Typhimurium foi o sorovar mais envolvido em quadro de diarreia aguda no homem em diversos países, inclusive o Brasil (DUBOIS-BRISSONNET et al., 2011).

4.1.5 *Escherichia coli*

Pertencente ao grupo dos Coliformes Termotolerantes, as células de *E. coli* são importantes como possíveis patógenos veiculados por alimentos (EL-BAKRY et al., 2014). Como micro-organismo indicador, a presença de *E. coli* nos alimentos em quantidades elevadas é utilizada para indicar contaminação fecal (ABDOLLAHZADEH et al., 2014).

De modo geral, a *E. coli* é considerada um comensal inofensivo, no entanto, diversos sorovares da espécie apresentam potencial patogênico, sendo capazes de causar infecções intestinais e extra-intestinais por diferentes mecanismos, assim como infecções no trato urinário e meningites (EL-BAKRY et al., 2014). Estima-se que a dose infectante da *E. coli* para adultos seja de 10^5 a 10^8 UFC/g, todavia, esta dose varia de acordo com o estado imune e/ou a idade do indivíduo exposto (ABDOLLAHZADEH et al., 2014). De modo geral, infecções causadas por *E. coli* caracterizam-se por diarréia aquosa, náusea, vômito, cólicas abdominais e febre baixa ou ausente (MELO et al., 2015).

A transmissão de *E. coli* se dá por meio da ingestão de água ou alimentos contaminados não processados, ou que tiveram algum tipo de contaminação fecal durante a sua produção. Vários surtos têm sido associados ao consumo de carne bovina mal cozida e diversos produtos á base de carne, leite cru, vegetais consumidos crus e molhos preparados para saladas (LV et al., 2014).

Sorotipos de *E. coli* têm sido implicados em doenças diarreicas, se constituindo num grave problema de saúde pública no mundo, com mais de dois milhões de mortes relatadas anualmente (MELO ET AL., 2015). As cepas de *E. coli* são classificadas de acordo com os seus mecanismos de patogenicidade (toxinas, adesinas, invasibilidade), danos a animais de laboratório e padrões de adesão a células eucarióticas em cultura. A classificação, considerando estes aspectos inclui: i) *E. coli* enteropatogênica (EPEC); ii) *E. coli* enteropatogênica atípica (A-EPEC); iii) *E. coli* enterotoxigênica (ETEC); iv) *E. coli* enterohemorrágica (EHEC); v) *E. coli* enteroinvasiva (EIEC); vi) *E. coli* de adesão difusa (DAEC); e vii) *E. coli* enteroagregativa (EAEC) (LIN et al., 2011).

Controle do crescimento microbiano em alimentos

A necessidade de conservar alimentos sempre foi uma preocupação do homem e tem se tornado, uma necessidade cada vez mais presente diante da crescente demanda de consumo de alimentos em todo o mundo. Estes aspectos obrigam os setores produtores a manterem

níveis mínimos de perdas decorrentes da deterioração de alimentos, porém sem esquecer a necessidade de o alimento não representar riscos a saúde do consumidor devido a contaminação com micro-organismos patogênicos (GUTIERREZ; BARRY-RYAN; BOURKE, 2008). Os micro-organismos podem ser inseridos no alimentos em qualquer uma das etapas do processamento, promovendo alterações sensoriais, ou comprometendo a segurança do produto final (HAVELAAR et al., 2010).

O processo de conservação dos alimentos baseia-se em uma busca pela obtenção de um produto final que seja possuidor de uma alta qualidade nutricional, atrelada a uma longa vida útil (BENKEBLIA, 2004). A segurança e a qualidade microbiana dos alimentos podem ser estabelecidas por meio da boa aplicação de métodos que garantam a inativação ou inibição do crescimento e proliferação de bactérias deteriorantes e/ou patogênicas (HADDADIN et al., 2010).

Métodos que incluem aquecimento, congelamento, secagem, liofilização, irradiação, pressão hidrostática elevada, fermentação ou adição de compostos químicos ou antimicrobianos, têm sido tradicionalmente empregados para o controle da contaminação bacteriana em alimentos. O uso de ácidos orgânicos fracos (ácido lático, benzóico, sórbico, cítrico), peróxido de hidrogênio, agentes quelantes (ácido cítrico, sais de cálcio e de sódio, ácido etilenodiaminotetracético-EDTA) e biomoléculas orgânicas, entre outros artifícios químicos (modulação do potencial de óxido-redução, da pressão osmótica) e físicos (temperatura, embalagens) são exemplos de métodos aplicados para conservar diferentes matrizes alimentares (PINCHUK; BESWICK; REYES, 2010). Estes processos atuam causando injúria e/ou dano permanente à célula microbiana, com comprometimento ou perturbação do seu processo homeostático, adaptativo, de crescimento e multiplicação (WU, 2008).

Os procedimentos empregados para o controle microbiano em alimentos promovem uma série de alterações na matriz alimentar, que podem comprometer suas características nutricionais, funcionais e sensoriais (HAVELAAR et al., 2010). Essas alterações têm levado a indústria a reduzir a intensidade das tensões ou obstáculos utilizados. A combinação de técnicas de conservação, aplicadas em níveis mais brandos, de acordo com o conceito da tecnologia de obstáculos ou barreiras (LEISTNER, 2000), tem se mostrado uma alternativa promissora para o alcance do controle microbiano em alimentos. Na tecnologia de barreiras, o entendimento das interações complexas entre temperatura, atividade de água, pH e conservantes químicos, é utilizado para criar uma série de barreiras que garantam a segurança microbiológica do alimento processado.

A crescente tendência dos consumidores na busca de alimentos seguros, porém com baixos níveis ou sem a adição de aditivos sintéticos, bem como o surgimento de cepas bacterianas resistentes aos processos de conservação tradicionalmente aplicados tem direcionado a busca de novas tecnologias que permitam a manutenção dos atributos naturais e a segurança dos alimentos. Dentre essas alternativas inovadoras que vem sendo estudadas atualmente, aplicadas de forma isolada ou em combinação, podem ser citadas as tecnologias não térmicas, como alta pressão hidrostática, campo elétrico pulsado, embalagens ativas e bacteriocinas, culturas protetoras e compostos vegetais (ESPINA et al., 2014; LUZ et al., 2012c).

Tolerância de micro-organismos a procedimentos de conservação de alimentos e influência dos fatores sigma

A falta de critérios na aplicação dos procedimentos físicos e o uso abusivo e desordenado dos conservantes químicos sintéticos para o controle microbiano têm favorecido o surgimento de micro-organismos cada vez mais tolerantes aos métodos e agentes antimicrobianos classicamente utilizados pela indústria de alimentos (SOUZA et al., 2013b). Este fato tem contribuído para o surgimento de cepas bacterianas com perfil de tolerância a múltiplos agentes antibacterianos em todo o mundo (HAMADI et al., 2014).

Vários autores reportam sobre o crescente número de isolamentos de cepas microbianas com elevada tolerância aos procedimentos tradicionais antimicrobianos aplicados pela indústria de alimentos (ÁLVAREZ-ORDÓÑEZ et al., 2009; CHUENCA et al., 2015; ESPINA et al., 2013; DUBOIS-BRISSONNET et al., 2011).

A tolerância é definida como a capacidade temporária ou permanente do micro-organismo e sua descendência de permanecer viável e/ou multiplicar-se sob condições que poderiam ser consideradas inibitórias ou letais. A tolerância aos antimicrobianos pode ser natural (intrínseca), adquirida, ou ainda ser transmitida dentro da mesma ou de espécies diferentes de bactérias (HEMAISWARYA; KRUTHIVENTI; DOBLE, 2008). A tolerância a antimicrobianos é principalmente adquirida através de três mecanismos principais: (1) transferência de genes de tolerância de micro-organismos resistentes a susceptíveis; (2) adaptação genética, por exemplo, mudança no alvo da droga; e (3) adaptação fenotípica, primariamente pelo aumento de expressão de maquinaria celular existente, tais como bombas de efluxo (SHELDON, 2005; KOHANSKI et al., 2007; KOHANSKI et al., 2010).

A exposição de um micro-organismo repetidas vezes a concentrações subletais de um agente antimicrobiano pode resultar no aumento da sua tolerância, o que repercute em aumento da sua capacidade de sobreviver mesmo quando exposto a altas concentrações desse agente. A exposição de bactérias ao estresse subletal pode resultar ainda em um aumento da tolerância a outros tipos de estresses não relacionados, fenômeno denominado como tolerância cruzada (SKANDAMIS et al., 2008). As condições subletais que têm demonstrado induzir respostas ao estresse, resultando em um aumento da tolerância, incluem o choque térmico, a exposição a um pH extremo, o choque oxidativo, tal como a exposição ao peróxido de hidrogênio, entre outros (SOKOVIC et al., 2010).

Uma das principais estratégias microbianas para sobreviver em ambientes desfavoráveis envolve mudanças nas propriedades físico-químicas da membrana Este processo adaptativo se reflete em modificações nas propriedades físicas da membrana celular, tais como a temperatura da fase de transição e microviscosidade, que se relacionam com a permeabilidade e consequentemente viabilidade da célula (ALVAREZ-ORDONEZ et al., 2008; DUBOIS-BRISSONET et al., 2011). As mudanças na membrana em resposta ao estresse, como resposta do micro-organismo pode depender das condições de adaptação específica impostas durante a fase estacionária de crescimento, com subsequente estresse subletal (SOMOLINOS et al., 2010b).

Modificações na fluidez da membrana citoplasmática demonstraram estar envolvidas na adaptação ao estresse ambiental (por exemplo, variações de temperatura, pressão, concentrações de íons, pH, disponibilidade de nutriente e xenobióticos) (MYKYTCZUK et al., 2007). Como exemplo, a adaptação à baixa temperatura de crescimento em *Bacillus subtilis* envolve a mudança na fluidez da membrana celular, com uma rápida desnaturação de cadeias de ácidos graxos presentes nos fosfolipídios por meio da indução de enzimas desnaturases de ácidos graxos (BERANOVÁ et al., 2008). Por outro lado, em *P. aeruginosa*, a composição de ácidos graxos dos fosfolipídios de membrana pode ativar a expressão prematura de alguns genes, suportando a hipótese de que mudanças na estrutura de membrana podem ser decorrentes de modulação gênica relacionada ao estresse (PATRIGNANI et al., 2008).

Di Pasqua et al. (2006) verificaram um mecanismo adaptativo mediante exposição a concentrações subletais de diferentes compostos ativos de OES em células de *E. coli, Salmonella* spp. e *Brochothrix thermosphacta* que resultou em uma maior concentração de alguns ácidos graxos insaturados na membrana citoplasmática. Da mesma forma, nos resultados obtidos por Luz et al. (2014b), foi observado que células de *S.* Typhimurium

ATCC 14028 submetidas a concentrações subletais do óleo essencial de *Origanum vulgare* e do seu fitoconstituinte majoritário carvacrol apresentaram aumento da síntese de ácidos graxos insaturados e isômeros *trans*, embora, o desenvolvimento de tolerância direta ou cruzada (termotolerância, ácidotolerância e osmotolerância) não tenha sido observado. Aparentemente, as alterações na susceptibilidade a OES poderiam ser explicadas por adaptação fenotípica, devido a mudanças reversíveis na composição dos lipídios de membrana e efluxo (PAPADOPOULOS et al., 2008).

Além das mudanças na membrana, os micro-organismos dispõem de diferentes estratégias para se adaptar e sobreviver às constantes mudanças que ocorrem no meio em que estão inseridos e em resposta às tensões ambientais. O uso de fatores sigma alternativos é uma das mais importantes estratégias que a célula bacteriana é capaz de utilizar quando exposta a condições de *stress*. Fatores sigma alternativos são reconhecidos pela enzima RNA-polimerase, e a partir deste reconhecimento os genes que serão expressos dependem de qual fator sigma está ligado à enzima (AIT-OUAZZOU et al., 2011).

Nas bactérias Gram-negativas, o fator σ^S, também conhecido como fator sigma de fase estacionária, é o principal regulador da resposta geral ao *stress* em *E. coli* e em outras bactérias entéricas. O fator σ^S é codificado pelo gene *rpoS* e induzido quando as células são submetidas a diversas situações de adversidade ambiental, como escassez de nutrientes, altas e baixas temperaturas e entrada na fase estacionária de crescimento (MONFORT et al., 2012; WEBER al., 2005).

Assim, o fator σ^S regula a expressão de genes associados com o aumento da tolerância a alguns dos tratamentos de conservação de alimentos (DODD; ALDSWORTH, 2002). O fator σ^S regula direta ou indiretamente a expressão de até 10% dos genes de *E. coli* (WEBER et al., 2005), sendo portanto, considerado como fator crucial neste micro-organismo, não só por seu papel na tolerância ao estresse, mas também por sua grande influência sobre a fisiologia celular em condições de crescimento adversos.

Para as bactérias Gram-positivas, existem menos informações disponíveis sobre os fatores biológicos que fundamentam o fenômeno de sua tolerância. No entanto, sabe-se que, de forma semelhante ao que acontece nas bactérias Gram-negativas, há um importante mecanismo de regulação da transcrição de determinados genes em *L. monocytogenes* frente a mudanças bruscas das condições ambientais. Alguns fatores sigma alternativos ativos em células Gram-positivas, como exemplo, o fator σ^B (considerado homólogo ao fator σ^S em bactérias gram-negativas), permanecem em estado de repouso até que a célula encontra-se em

condições ambientais desfavoráveis, para então requerer a expressão de determinados genes reconhecidos por este fator (ESPINA et al., 2013; KAZMIERCZAK et al., 2005).

O fator sigma alternativo σ^B é codificado pelo gene *sigB*, e tem sido demonstrado sua contribuição na habilidade de *L. monocytogenes* multiplicar-se e sobreviver em condições de stress ambientais (GRAY et al., 2006), como a exposição a elevada osmolaridade, temperaturas extremas, etanol, EDTA, ou início fase estacionária de crescimento (BECKER et al., 1998). O estudo de caracterização do fator σ^B de *L. monocytogenes* durante a fase estacionária de crescimento identificou mais de 170 genes regulados diretamente por este fator (KAZMIERCZAK et al, 2003; RAENGPRADUB et al, 2008). O fator σ^B já foi identificado também em *S. aureus* e *B. subtilis* (KAZMIERCZAK et al., 2005).

Apesar de sua recente descoberta, vários estudos têm demonstrado a influência dos fatores σ^S e σ^B para *E. coli* e *L. monocytogenes,* respectivamente, na tolerância a importantes técnicas utilizadas na conservação de alimentos, tais como tratamento térmico (ESPINA et al., 2010), exposição á condições ácidas (AIT-OUAZZOU et al., 2012), campo elétrico pulsado (ROBEY et al., 2001; WEMEKAMP-KAMPHUIS et al., 2004), pressão hidrostática (SOMOLINOS et al., 2008) e compostos vegetais antimicrobianos (SOMOLINOS et al., 2010a).

No entanto, há ainda muitos aspectos a serem esclarecidos, uma vez que os estudos realizados não abordaram, por exemplo, as condições ideais de tratamento que poderiam influenciar a expressão destes fatores, ou como esta expressão poderia ser potencializada ou suprimida. De forma geral, o conhecimento da influência dos fatores sigma alternativos e seu papel fisiológico sobre a sobrevivência microbiana mediante os processos de conservação dos alimentos é um aspecto de interesse para o desenvolvimento de processos de conservação mais eficazes.

Óleos essenciais como antimicrobianos para uso em alimentos

Considerando que alguns conservantes químicos são suspeitos ou são tóxicos aos consumidores, em soma a capacidade de desenvolvimento de tolerância direta e cruzada decorrente da exposição microbiana a compostos sintéticos e/ou processos antimicrobianos clássicos, têm ocorrido uma pressão sobre a indústria alimentícia para uma progressiva diminuição da sua utilização e conseqüente adoção de alternativas mais naturais para obtenção dos seus propósitos (SOUZA et al., 2010; BARROS et al. 2009). Neste cenário, o

uso de OES como antimicrobianos para uso em alimentos surge como alternativa tecnológica para emprego em sistemas de conservação de alimentos.

Os OES são líquidos aromáticos extraídos de materiais vegetais como flores, folhas, caules, raízes e frutas e desempenham diversas funções necessárias à sobrevivência vegetal, exercendo papel fundamental na autodefesa contra micro-organismos e na atração de polinizadores (SIQUI et al., 2000). Estas substâncias são límpidas e raramente coloridas, lipossolúveis e solúveis em solventes orgânicos com densidade geralmente menor que a da água, e são armazenadas em células secretoras, cavidades, canais, células epidérmicas ou tricomas glandulares (BAKKALI et al., 2008; LUZ, et al 2012c; BARBOSA et al., 2015; FORSYTHE, 2002).

O perfil químico dos componentes dos OES difere na quantidade e tipos estereoquímicos das moléculas presentes em relação ao procedimento utilizado para a sua extração, o qual deve ser escolhido de acordo com a proposta de uso dos OES (AZEREDO et al., 2012). Além disso, a composição do material extraído pode variar em qualidade e quantidade de acordo com o clima, composição do solo, órgão da planta, idade e estágio do ciclo vegetativo (OLIVEIRA et al., 2010). Existem diversos métodos para extração de OES, que podem incluir o uso de dióxido de carbono líquido, micro-ondas, destilação com alta ou baixa pressão com água fervente ou vapor quente. Portanto, com a finalidade de obter OES de composição constante, estas substâncias devem ser extraídas sob as mesmas condições (ANGIONI et al., 2006; ABAD et al., 2012).

Avalia-se que a produção brasileira de OES gera em torno de 85 milhões de dólares, vindo a corresponder a 12% da produção mundial (LV et al., 2014). O maior problema ao desenvolvimento da agroindústria produtora de OES é a concorrência com similares sintéticos. Atualmente, a indústria alimentícia, a qual mais necessita deste tipo de produto, tem substituído os produtos sintéticos por naturais, em função das exigências atuais dos mercados (ABDOLLAHZADEH et al., 2014).

As propriedades bioativas dos OES têm sido exploradas em diversos setores da indústria farmacêutica, agronômica, de produtos cosméticos e de perfumaria (WATSON; PREEDY, 2008; ALIM et al., 2009). Conhecidos por suas propriedades antissépticas (bactericida, virucida e fungicida), além da sua fragrância, os OES são usados na produção de embalagens ativas, como analgésicos, sedativos, antiinflamatórios, espasmolíticos, anestésicos tópicos e como antimicrobianos (BAKKALI et al., 2008).

Os OES constituem-se em misturas complexas de até mais de 100 compostos orgânicos, pertencentes às mais diversas classes químicas (hidrocarbonetos terpênicos, alcoóis

simples, aldeídos, cetonas, fenóis, ésteres e ácidos orgânicos) e são caracterizados por dois ou três constituintes majoritários em concentrações mais elevadas (20-70%) (CASTRO et al., 2004; SOUZA et al., 2013b). Porém, os terpenos e os fenilpropanos são as classes mais comumente encontradas. Os terpenos encontrados com maior frequência são os monoterpenos, que podem constituir até cerca de 90% do óleo. Sesquiterpenos, e diterpenos são geralmente encontrados como constituintes minoritários (BAKKALI et al., 2008; OLIVEIRA et al., 2015).

Em relação a suas propriedades biológicas, pode-se dizer que a atividade antimicrobiana dos OES não depende apenas de sua composição química, mas também de sua configuração estrutural, grupos funcionais e possíveis interações aditivas e sinérgicas entre seus componentes (QUESTED et al., 2010).

Cerca de 60% dos OES têm ação antifúngica e 30% exibem propriedade antibacteriana. Já foi comprovada ação antimicrobiana de OES contra um amplo número de bactérias Gram-negativas e Gram-positivas (SOUSA et al., 2012). A maior ou menor efetividade antimicrobiana depende do OE ensaiado, da sua concentração, do tipo de micro-organismo alvo e da composição do substrato teste (BARROS et al., 2009; MONTE et al., 2014; LUZ et al., 2012a; MUTHAIYAN; RICKE; GUSTAFSON, 2014). Os mecanismos pelos quais os OES inibem o crescimento e/ou sobrevivência dos micro-organismos podem, em parte, estar relacionados a sua hidrofobicidade. Estas substâncias conseguem passar pela parede celular e membrana citoplasmática, destruindo a estrutura dos polissacarídios, lipídios e fosfolipídios, o que provoca um dano à membrana e é responsável pela toxicidade que apresentam na célula alvo (BURT, 2004; PRAKASH et al., 2015).

Uma sequência de eventos envolvendo diferentes modos de ação dos OES pode ocorrer conduzindo ao estabelecimento da inviabilidade ou morte microbiana, dependendo dos compostos majoritários presentes no OE. Entre estes, podem ser citados: sensibilização da dupla camada fosfolipídica com perturbação na função e na composição da membrana plasmática; aumento da permeabilidade e perda de componentes intracelulares vitais; inativação do mecanismo enzimático mitocondrial, inibindo o transporte de elétrons para produção de energia e síntese protéica; extravasamento de material citoplasmático; lise e eventual morte celular (PESAVENTO et al., 2015; SANTIESTEBAN-LÓPEZ et al., 2007).

Alguns autores relatam que os OES apresentem duas principais características como agentes antimicrobianos para uso em sistemas de conservação de alimentos: i) sua origem natural, o que significa mais segurança para os consumidores e para o meio ambiente; e ii) são considerados como possuidores de baixo risco de desenvolvimento de tolerância microbiana.

A segunda característica citada toma como base o fato de que os OES são compostos por uma grande variedade de constituintes, os quais, aparentemente, apresentam diferentes mecanismos de atividade antimicrobiana, tornando, desta forma, mais difícil uma possível adaptação dos micro-organismos frente a sua ação (BURT, 2004; SOUZA et al., 2007).

Nos Estados Unidos e em países da Europa alguns produtos à base de OES e seus componentes são registrados e liberados para uso na conservação de alimentos, de modo que esta realidade de conhecimento e aplicação foi originada de pesquisas acadêmicas através de consórcios envolvendo diferentes países. OES são considerados como tendo baixo risco para a saúde dos consumidores, e tal propriedade se estende aos constituintes carvacrol, carvona, cinamaldeído, citral, eugenol, mentol, timol, p-cimeno, limoneno, entre outros (AIT-OUAZZOU et al., 2013; SILVA-ÂNGULO et al., 2014).

Outro fator importante a ser ressaltado é que a ação combinada de OES e/ou seus constituintes pode potencializar o seu efeito antimicrobiano, como verificado por Gutierrez, Barry-Ryan e Bourke (2008), em estudo com a combinação dos óleos essenciais de *O. vulgare* e *Thymus vulgaris*. Este é um aspecto interessante para o uso de OES, pois para alcançar o efeito antimicrobiano desejado na matriz alimentar, as concentrações requeridas de um OE podem causar alterações sensoriais indesejáveis, particularmente no sabor e odor do alimento (DEVLIEGHERE et al., 2004). Neste sentido, a aplicação combinada de OES ou seus constituintes em concentrações subinibitórias, pode permitir a manutenção da segurança do produto, garantindo sua vida de prateleira, porém minimizando o sabor indesejável e ou alterações sensoriais associadas (AZERÊDO et al., 2011; SOUSA et al., 2012).

Estudos prévios têm relatado que a combinação de alguns OES e/ou seus constituintes pode contribuir mais efetivamente que um óleo essencial/constituinte aplicado de maneira isolada (AZEREDO et al., 2012; BARBOSA et al., 2015; SOUZA et al., 2015; SOLORZANO-SANTOS; MARTÍN et al., 2014). Os efeitos benéficos dessa combinação podem ser aditivos ou sinérgicos, sendo de grande interesse industrial, uma vez que menores concentrações seriam requeridas para atuar na preservação de alimentos. No estudo realizado por Azerêdo et al. (2011) verificou-se efeito sinérgico entre os OES *O. vulgare* e *R. officinalis*, cujos componentes majoritários são, respectivamente, carvacrol e 1,8-cineol, frente à *A. hydrophila, L. monocytogenes* e *Y. enterocolitica*, e efeito aditivo contra *P. fluorescens.*

Em alguns estudos, é sugerido o uso combinado de OES com outros métodos antimicrobianos, visando um efeito sinérgico para a conservação de alimentos, a citar: baixos valores de pH e atividade de água, agentes quelantes, baixa tensão de oxigênio, nisina e

empacotamento anaeróbico no intuito de aumentar a estabilidade e segurança microbiológica. Esta combinação de tratamentos de conservação permite que um nível exigido de proteção seja alcançado, ao mesmo tempo em que se retêm os atributos de qualidade organoléptica do alimento como cor, sabor, odor, textura e conteúdo nutricional (NAZER et al., 2005; NAVEENA et al., 2006; DIMIRTIEVIAE et al., 2007; GUTIERREZ; BARRY-RYAN; BOURKE, 2008).

A atividade antimicrobiana dos OES em matrizes alimentares tem sido avaliada de forma isolada e combinada em diversos estudos e em geral, é reduzida quando comparado aos testes *in vitro* (Burt, 2004). Segundo Gutierrez (2008), os componentes nutricionais presentes nos alimentos poderiam proteger as bactérias contra a ação dessas substâncias, além de possibilitar uma reparação mais rápida das células injuriadas em decorrência da maior disponibilidade de nutrientes neste substrato, quando comparada aos meios laboratoriais. Assim, o uso de meios-base alimento pode auxiliar na obtenção de dados mais realísticos a cerca do real potencial de aplicação de OES como conservantes em alimentos.

Propriedades antimicrobianas do óleo essencial de *Rosmarinus officinalis* L. e 1,8-cineol

Entre os diversos OES que apresentam potencialidade antimicrobiana, aquele obtido da espécie vegetal *Rosmarinus officinalis* L. (alecrim - OERO) tem mostrado destacáveis resultados na inibição de diferentes micro-organismos de interesse em alimentos, embora os resultados tenham sido variados de acordo com os micro-organismos ensaiados, origem do óleo essencial e técnica utilizada (BARBOSA et al., 2015).

A espécie *Rosmarinus officinalis* L., conhecida popularmente como alecrim, é originária da Região Mediterrânea e cultivada em quase todos os países de clima temperado de Portugal à Austrália. No Brasil sua produção se localiza, principalmente, na região sul do país (MONTE et al., 2014). A planta possui porte subarbustivo lenhoso, ereto e pouco ramificado de até 1,5 m de altura (Figura 1). Suas folhas são lineares, coriáceas e muito aromáticas, medindo 1,5 a 4 cm de comprimento por 1 a 3 mm de espessura. As flores são azulado-claras, pequenas e de aromas forte e muito agradável (LORENZI; MATOS, 2006). A planta exala aroma forte e agradável, sendo uma erva usada para fins culinários, medicinais e aromáticos. O OE é aplicado em cosméticos e perfumaria (LORENZI; MATOS, 2006; LIN et al., 2011).

A atividade antimicrobiana do OERO tem sido relacionada a mecanismos comuns aos demais OES, que tem início com a perturbação da membrana, podendo ser seguida de interferência em eventos celulares fundamentais para a viabilidade da célula microbiana (AZERÊDO et al., 2011). Já foi comprovado que muitos dos seus compostos são inibidores de diversos micro-organismos, como *S. aureus, Bacillus subtillis, Klebsiella pneumoniae, P. aerugionosa, E. coli, S.* Enteritidis e *Shiguella sonei* (SANTOYO et al., 2005; BOZIN et al., 2007).

Dentre os constituintes do OERO (Figura 2), eugenol, 4-terpineol, α-terpineol, δ-terpineol e α-pineno podem ser encontrados, porém o 1,8-cineol (CIN) é frequentemente relatado como sendo seu componente majoritário podendo representar até 47% do mesmo (ATTI-SANTOS et al., 2005; GOMES NETO et al., 2012a). Azerêdo et al. (2011) avaliaram a composição química do OERO, e verificaram a presença de 13 compostos distintos, sendo o CIN o composto em maior quantidade representando 32,2% da massa total do óleo.

Figura 1. Espécie vegetal *Rosmarinus officinalis* L. (Alecrim)
Fonte: jornalagricola.wordpress.com

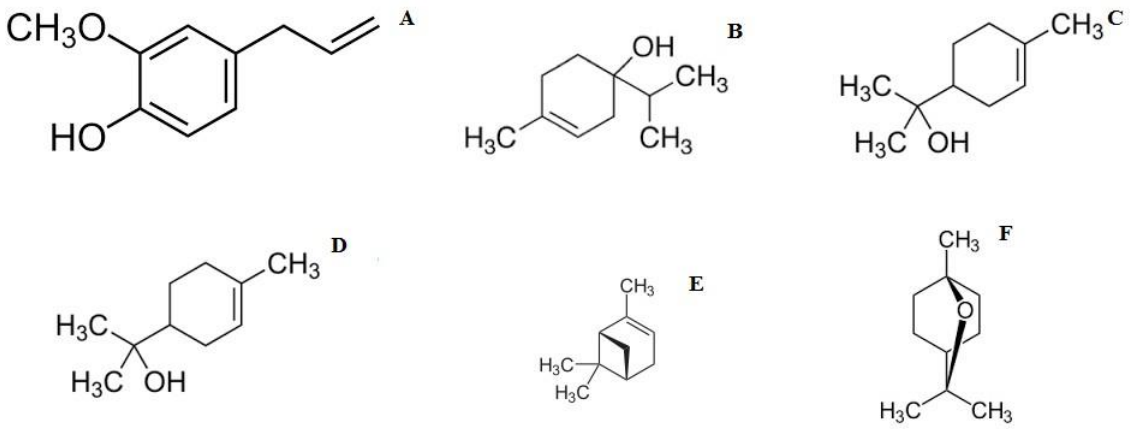

Figura 2. Estrutura química dos principais constituintes do OERO. (a): eugenol; (b): 4-terpineol; (c): α-terpineol; (d): δ-terpineol; (e): α-pineno; (f): 1,8-cineol.
Fonte: www.quimicabrasil.com.br

O CIN ou eucaliptol, como também pode ser denominado, possui fórmula química $C_{10}H_{18}O$ e peso molecular de 154,24 $g.mol^{-1}$. É um composto monoterpênico cíclico reconhecido como seguro (GRAS), aprovado pela FDA para o uso em alimentos (FDA, 2009) e indicado como flavorizante pela *Flavor and Extract Manufacturer's Association* (SOUSA et al., 2012).

Embora o OERO seja constituído por muitos compostos individuais, suas propriedades antimicrobianas geralmente são atribuídas ao seu composto majoritário CIN. Devido a suas propriedades hidrofóbicas, o CIN é capaz de penetrar na célula microbiana onde interfere em mecanismos essenciais para o metabolismo microbiano (ATTI-SANTOS et al., 2005; CELIKTAS et al., 2005). Perturbação da membrana citoplasmática, ruptura do fluxo de elétrons, perturbação do transporte ativo, inibição de atividade de enzimas e coagulação do conteúdo citoplasmático são alguns dos mecanismos envolvidos na promoção do efeito antimicrobiano do CIN (VILJOEN et al., 2013).

O OERO, bem como o seu constituinte CIN, têm demonstrado efetividade na inibição de diversas bactérias contaminantes de alimentos, a citar: *S. aureus, L. monocytogenes P. aeruginosa* e *S.* Tiphymurium (AZÊREDO et al., 2011; GOMES NETO et al., 2012b; SOUSA et al., 2015; OLIVEIRA et al., 2015). Mulyaningsih et al. (2010) obtiveram valores de concentração inibitória mínima do CIN variando entre 8 e 64 mg/mL frente 13 micro-organismos diferentes, incluindo bactérias Gram-negativas (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603 e *Acinetobacter baumanii* ATCC BAA 747), Gram-positivas (*B. subtilis* ATCC 6051, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 14990, *S. saprophyticus* ATCC 15305, *S. pyog*enes ATCC 12344, *S. agalactiae* ATCC

27956, *Enterococcus faecalis* ATCC 29212) e leveduras (*C. albicans* ATCC 90028 e *C. glabrata* ATCC MYA 2950).

Dado o exposto, a capacidade possível que alguns micro-organismos possuem de adaptar-se a condições adversas do ambiente precisa ser avaliada, considerando a potencialidade antimicrobiana de óleos essenciais e seus constituintes com a finalidade de uso como conservantes naturais em alimentos.

Ainda, considerando de forma pragmática as limitações de interpretação de dados decorrentes de sistemas experimentais de estudo que envolvam somente avaliações fenotípicas, bem como a necessidade de avanços na área de estudo em tela, a realização de estudos vindouros se fazem necessários para inferir hipóteses e prospectivas a respeito do possível envolvimento dos fatores sigma alternativos no comportamento de cepas bacterianas, quando expostas à ação de óleos essenciais.

5 MATERIAIS E MÉTODOS

O presente estudo foi conduzido em duas etapas. Na primeira, foram investigadas as mudanças na tolerância direta e cruzada em cepas de bactérias contaminantes de alimentos (*L. monocytogenes, S. aureus, P. aeruginosa* e *S. Typhimurium*) quando expostas a concentrações subinibitórias do OERO e do CIN (experimentos conduzidos no Laboratório de Microbiologia dos Alimentos do Departamento de Nutrição da Universidade Federal da Paraíba, Campus I).

Na segunda etapa, avaliou-se a influência dos fatores sigma alternativos σ^S e σ^B na tolerância de cepas de *E. coli* e *L. monocytogenes*, respectivamente, ao OERO e OEOV, bem como nas modificações da tolerância direta destas cepas quando expostas a concentrações subinibitórias de ambos os OES testados (experimentos conduzidos no *Laboratorio de Microbiología de los Alimentos, del Departamento de Producción Animal y Ciencias de los Alimentos, Facultad de Medicina Veterinaria, Universidad de Zaragoza*, Espanha).

Primeira Etapa

OERO e CIN

O OERO tipo *food-grade*, foi obtido da Aromalândia Ind. Com. Ltda. (Minas Gerais, Brasil), a qual obtém os produtos através de processo de arraste por vapor d'água (hidrodestilação) em escala industrial. O óleo essencial obtido atendeu todas as especificações de controle de qualidade (aparência, cor, impurezas, odor, densidade a 20 °C: 0.90, índice de refração a 20 °C: 1.47) conforme boletim técnico emitido pelo fornecedor. Por sua vez, o constituinte CIN foi adquirido da Empresa Sigma Aldrich Brasil Ltda.

Cepas teste e inóculo

As cepas tipo padrão *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027 e *S.* Typhimurium ATCC 14028 utilizadas como micro-organismos teste nos ensaios antimicrobianos foram obtidas da Coleção de Micro-organismos de Referência, Instituto Nacional de Controle de Qualidade em Saúde, FIOCRUZ, Rio de Janeiro, Brasil.

Para a realização dos ensaios antimicrobianos, as cepas teste foram cultivadas em caldo Infusão de Cérebro e Coração (Himedia, Índia) por 18 h a 35 °C. Feito isso, as células

bacterianas foram separadas por meio de centrifugação (4000 rpm por 12 min a 4 °C), lavadas duas vezes com tampão fosfato-salino (0.1 g/100mL, Oxoid, Brasil) e ressuspendidas em tampão fosfato-salino. As suspensões bacterianas foram ajustadas em espectrofotômetro para uma densidade óptica de 1,5 a 620 nm, correspondente a um inóculo de aproximadamente 8 $\log_{10}$ UFC/mL (CARSON; MEE; RILEY, 2002). Culturas estoque foram mantidas em tubos de ensaio com Ágar Nutriente (Himedia, Índia) inclinado a 7 ºC.

Preparação do caldo base carne

Os ensaios de viabilidade celular e de investigação da modificação de tolerância (direta e cruzada) das cepas bacterianas foram realizados utilizando caldo carne como substrato de cultivo. Para isso, uma porção de carne bovina do "tipo patinho" (500 g) Foi separada da gordura externa e cortada em pedaços de tamanho uniforme (3 x 3 x 3 cm), que foram fervidos em água destilada (1000 mL) durante 30 min. Em seguida, a carne foi retirada, obtendo-se cerca de 500 mL de caldo que foram submetidos à filtração a vácuo utilizando papel filtro Whatman No. 1. O filtrado foi esterilizado em autoclave durante 15 min (121 atm), sendo, posteriormente, armazenado a -20 °C em alíquotas de 50 mL. Quando necessário, as alíquotas foram descongeladas sob refrigeração (7 ± 1 °C) e utilizadas nos ensaios (OLIVEIRA et al., 2010).

Preparação do modelo base carne

Um modelo base carne foi utilizado como substrato de cultivo no ensaio de modificação da tolerância direta em células de *S.* Typhimurium ATCC 14028. Para isto, porções de carne bovina "tipo patinho" moída previamente irradiadas (25 kGy; 2h) para eliminação da microflora contaminante foram assepticamente distribuídas em embalagens plásticas (30g/embalagem), submetidas à vácuo e congeladas a -20 ºC. Quando necessário, as amostras foram descongeladas sob refrigeração (7± 1ºC) e utilizadas nos ensaios (JUNEJA; HWANG; FRIEDMAN, 2010). Amostras aleatórias foram testadas previamente para verificar a eliminação ou inativação de microflora por diluição (1:1) em tampão fosfato-salino (0,1 g/100mL, Oxoid, Brasil), seguida por plaqueamento direto da suspensão (0.1 mL e 1 mL) em superfície de Ágar Nutriente (Himedia, Índia) e incubadas aerobicamente a 35 ºC por 48 h.

Determinação da Concentração Inibitória Mínima (CIM)

A CIM do OERO e CIN foi determinada pela técnica de macrodiluição em caldo (NOSTRO et al., 2001). Inicialmente, 1 mL da suspensão bacteriana foi inoculado em 4 mL de caldo Nutriente (Himedia, Índia). Em seguida, foram adicionados 5 mL da emulsão do OERO ou da solução de CIN em concentrações variando de 2 a 100 μL/mL. A mistura foi agitada durante 30 segundos utilizando aparelho tipo Vortex, e incubada a 35 °C por 24 h. Após o período de incubação, a menor concentração do óleo essencial ou constituinte capaz de inibir o crescimento microbiano visível (turvação) foi considerada como a CIM.

Ensaios de viabilidade bacteriana

Os ensaios de influência do OERO e do CIN (1/4 CIM, 1/2 CIM e CIM) sobre a viabilidade das cepas bacterianas teste foram realizados utilizando o método de contagem de células viáveis (SAGDIÇ, 2003). Inicialmente, 1 mL da suspensão bacteriana foi inoculado em 4 mL de caldo carne adicionado de 5 mL das emulsões do OERO ou do CIN nas diferentes concentrações ensaiadas. O sistema foi incubado a 35 °C, e nos intervalos de 0 (imediatamente após a homogeneização do sistema), 15, 30, 45, 60 e 120 minutos pós-incubação, uma alíquota de 1 mL da suspensão foi diluída seriadamente (1:9 v/v) em tampão fosfato-salino (0.1 g/100mL, Oxoid, Brasil) estéril e inoculada em Ágar Nutriente (Himedia, Índia), seguido por incubação a 35 ºC por 24 – 48 horas. O experimento controle foi constituído por caldo carne inoculado sem adição do OERO ou do CIN. A contagem do número de células viáveis foi expressa em $\log_{10}$ UFC/mL (BARROS et al., 2009).

Avaliação de mudanças na tolerância bacteriana direta

A capacidade das cepas bacterianas de modificar a tolerância direta ao OERO ou CIN foi avaliada por meio da sua exposição continuada em cultivo *overnight* em caldo carne adicionado de concentrações subinibitórias da substância/composto ensaiado. Para isso, 18 mL de cada substrato de crescimento contendo inóculo inicial de 8 $\log_{10}$ UFC/mL (2 mL) foram adicionados do OERO ou de CIN em quantidade suficiente para obtenção de uma concentração final de 1/4 CIM ou 1/2 CIM. Sistemas de cultivo das cepas bacterianas sem adição do OERO ou do CIN foram usados como ensaio controle. Os sistemas foram incubados a 35 ºC durante 18 h (LUZ et al., 2012c) e, posteriormente, uma alíquota de 2 mL

foi utilizada como inóculo para a contagem de células viáveis das cepas adaptadas (tratadas) e não adaptadas (controle) em caldo carne (20 mL) adicionado do OERO e CIN nas concentrações ajustadas à CIM correspondente. A análise de contagem de células viáveis foi realizada nos intervalos de 0, 30, 60, 120, 180 e 240 min por meio da contagem de células viáveis em Ágar Nutriente (Himedia, Índia) de acordo com metodologia descrita por Sagdiç (2003), sendo os resultados expressos em $\log_{10}$ UFC/mL.

Para determinar se a tolerância bacteriana direta foi modificada, as contagens de células viáveis ao longo do tempo de exposição ao OERO ou CIN foram calculadas e comparadas com as obtidas a partir de suspensões bacterianas não expostas.

Avaliação de mudanças na tolerância bacteriana cruzada

As modificações na tolerância cruzada das cepas bacterianas foi avaliada por meio da exposição *overnight* a concentrações subinibitórias do OERO ou CIN em caldo de carne, seguida por exposição a distintas condições ambientais estressantes causadas por temperatura moderada, baixo pH e NaCl, como descrito por Luz et al. (2012c).

Para isso, testes preliminares foram realizados para estabelecer a máxima condição não inibitória para as cepas ensaiadas quando expostas a diferentes temperaturas (40-60 °C), ácido láctico (pH 4,5 – 6,0, a 35 °C) e NaCl (5-15 g/100 mL, a 35 °C) em caldo carne sem adição do OERO ou CIN. Após o estabelecimento das condições de estresse, uma alíquota de 2 mL de cada suspensão bacteriana foi inoculada em 18 mL de caldo carne adicionado do OERO ou CIN (concentrações finais de 1/4 CIM ou 1/2 CIM; tratamento de adaptação), sendo os sistemas incubados *overnight* a 35°C. Em seguida, uma alíquota de 2 mL de cada tratamento foi inoculada em 18mL de caldo carne (incubada estaticamente a 35 °C) acidificado com ácido láctico (VETEC Química Fina Ltda, Brasil) a pH de 5.2 ou em caldo carne contendo NaCl (Qeel, Brasil) a 5g ou 10g/100 mL, para avaliar mudanças na ácidotolerância e osmotolerância, respectivamente. Para analisar mudanças na termotolerância, uma alíquota de 2 mL de cada tratamento foi inoculada em 18 mL de caldo carne, seguido por incubação estática a 45 °C. O número de células viáveis para todos os ensaios foi obtido (0, 30, 60, 120, 180 e 240 min) como descrito anteriormente. Os resultados foram expressos como $\log_{10}$ UFC/mL (SAGDIÇ, 2003).

Para determinar modificações na tolerância bacteriana cruzada, as contagens de células viáveis ao longo do tempo nas suspensões submetidas a exposição ao OERO ou CIN foram

calculadas e comparadas com as obtidas a partir de suspensões bacterianas não expostas a estes compostos.

Avaliação da modificação da tolerância bacteriana direta utilizando ciclos de habituação

A ocorrência de modificações na tolerância direta nas cepas bacterianas teste foi também avaliada por meio de habituação utilizando exposição sucessiva a diferentes e crescentes concentrações do OERO e CIN (1/16 CIM, 1/8 CIM, 1/4 CIM, 1/2 CIM, CIM e 2 CIM) em caldo carne (20 mL) de acordo com o procedimento descrito por To et al. (2002). Inicialmente, as cepas foram expostas em caldo Nutriente à menor concentração do OERO ou CIN ensaiada (1/16 CIM) e incubadas por 24 h a 35°C, sendo, em seguida, observado o crescimento através da inoculação de uma alíquota (100 μL) do meio de cultivo em Ágar Nutriente (Himedia, Índia), seguido por incubação a 35 °C por 24 - 48 h. Em seguida, uma alíquota do sistema (2 mL) foi inoculada ao mesmo meio de cultivo adicionado do OERO ou CIN na concentração crescente subsequente (1/8 CIM), sendo o sistema incubado sob as mesmas condições e realizada a avaliação do crescimento bacteriano como previamente descrito. Este procedimento se repetiu até ser alcançada a última concentração testada dos compostos antimicrobianos que não foi detectado o crescimento bacteriano (2 x CIM). Sistemas de cultivo das cepas bacterianas sem adição do OERO ou do CIN foram usados como ensaios controle.

As modificações na tolerância bacteriana direta por meio dos ciclos de habituação foi avaliada utilizando a comparação da CIM obtida para as células submetidas ao tratamento de habituação ao OERO ou CIN com aquela obtidas para as células não tratadas.

Avaliação de mudanças na tolerância direta em modelo base carne

Os ensaios de investigação de modificação da tolerância direta de *S.* Typhimurium ATCC 14028 utilizando modelo base carne foi realizada por meio da exposição da cepa teste a concentrações subinibitórias do OERO ou CIN (concentrações finais de 1/4 CIM e 1/2 CIM) em amostras de carne irradiada, armazenadas previamente a 7 °C por 18 horas. Para isto, as amostras de carne moída (30g) contendo o OERO ou CIN foram inoculadas com 3 mL da suspensão bacteriana e homogeneizadas em Stomacher (Modelo MA440, Marcone Ltda., Piracicaba, Brasil) por 5 minutos para assegurar uma distribuição uniforme da suspensão nas

amostras. Depois disso, as amostras de carne moída inoculadas foram adicionadas do OERO ou CIN (concentração final ajustada à CIM), e novamente misturadas em Stomacher (Modelo MA440, Marcone Ltda., Piracicaba, Brasil) por 5 min e incubadas a 7 ºC. O número de células viáveis nos sistemas foi determinado ao longo do tempo de armazenamento (60, 120, 180 e 240 min, e 24, 48 e 72 h) utilizando diluição em série (10^{-1} - 10^{-5}) das amostras em tampão fosfato-salino (0,1 g/100mL, Oxoid, Brasil), seguido de plaqueamento em Ágar Nutriente (Himedia, Índia), e incubação por 24 - 48 h a 35 °C. Sistemas não adicionados do óleo essencial ou constituinte foram testados similarmente como controle, e os resultados expressos em $\log_{10}$ CFU/mL (SAGDIÇ, 2003).

Para determinar se a tolerância bacteriana direta foi modificada, as contagens de células viáveis, ao longo do tempo, nos sistemas submetidos ao processo de adaptação ao OERO ou CIN foram calculadas e comparadas com as contagens obtidas no sistema não submetido ao processo de adaptadas.

Análises estatísticas

Os ensaios de investigação de modificações de tolerância nas cepas bacterianas foram realizados em triplicata e em duas repetições, sendo os resultados expressos como médias dos diferentes ensaios. As análises estatísticas foram realizadas utilizando o teste de Tukey para determinação de diferença significativa entre as médias obtidas, considerando $p < 0,05$. As análises estatísticas foram realizadas utilizando o software Sigma Stat. 3.1.

Segunda Etapa

Cepas testes e inóculo

As cepas teste utilizadas no estudo foram *E. coli* MG1655 e sua mutante isogênica *E. coli* MG1655 Δ*rpoS* e *L. monocytogenes* EGD-e e sua mutante isogênica *L. monocytogenes* EGD-e Δ*sigB*. Ambas as cepas já foram utilizadas em diversos ensaios de indução de modificações na tolerância bacteriana (direta e cruzada) quando expostas a vários compostos e procedimentos antimicrobianos (FONTENOT et al., 2013; CHUECA et al., 2015; SOMOLINOS et al., 2010a; AIT-OUAZZOU et al., 2012).

A mutante *rpoS* é uma estirpe derivada da cepa *E. coli* BW25113 (proveniente da coleção Keio e disponibilizada pelo laboratório Böehm do “Institute for Molecular Infection

Biology"- Würzburg, Alemanha), construída a partir da transdução do fago P1. Os transductantes foram confirmados e selecionados pela aquisição de tolerância a canamicina a partir da amplificação de DNA por PCR (Polymerase Chain Reaction). A remoção do cassete de tolerância a canamicina foi realizada utilizando o plasmídeo pCP20 (BABA et al., 2006). Já a mutante *L. monocytogenes* EGD-e Δ*sigB* foi gentilmente cedida pelo Professor Chakraborty (Instituto de Microbiologia Médica, Giessen, Alemanha). Esta mutante foi construída utilizando o plasmídeo suicida pAUL-A resultando em uma deleção cromossômica a partir de aquecimento.

As cepas foram mantidas em criotubos (Scharlau, Barcelona, Espanha) a -80 °C para depois serem cultivadas semanalmente em placas de Ágar Triptona de Soja (Oxoid Ltd. Basingstoke, Hampshire, Inglaterra) suplementado com 0,6 % de Extrato de Levedura (ATSEL) durante todo o período de realização da pesquisa.

Para o preparo das suspensões microbianas realizou-se pré-cultivos a partir da transferência de uma colônia isolada (da placa de ATSEL) para tubos contendo 5 ml de Caldo Tripsona de Soja (Oxoid Ltd. Basingstoke, Hampshire, Inglaterra) suplementado com 0,6 % de Extrato de Levedura (CTSEL) estéril, para em seguida serem incubadas por 18 h a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente. Transcorrida a incubação, alíquotas foram transferidas do pré-cultivo para frascos contendo 50 mL de CTSEL estéril (cultivo) até se alcançar uma população final de 9 $\log_{10}$ UFC/mL. Imediatamente após, os frascos foram incubados sob agitação contínua de 130 rpm (Selecta, modelo Rotabit, Barcelona, Espanha) por 18 h a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente. Estes períodos de incubação são estabelecidos como ideais para as células atingirem a fase estacionária de crescimento, de modo que nesta fase de crescimento se mostram geralmente mais tolerantes a diversas tecnologias empregadas na conservação de alimentos (MAÑAS; PAGÁN, 2005).

Para a realização dos ensaios com os antimicrobianos, as suspensões bacterianas (1 mL) foram separadas por meio de centrifugação (6000 rpm por 6 min), lavadas duas vezes com tampão fosfato-salino (0.1 g/100mL, Oxoid, Espanha) e ressuspendidas em tampão Mcllvaine (10 ml) estéril a pH 7,0. As suspensões bacterianas foram ajustadas em espectrofotômetro para uma densidade óptica de 1,5 a 620 nm, correspondente a um inóculo de aproximadamente 7 $\log_{10}$UFC/mL.

Óleos essenciais

Os óleos essenciais de *R. officinalis* L. e .*O.vulgare* L. utilizados nestes ensaios foram obtidos pela Aromalândia Ind. Com. Ltda. (Minas Gerais, Brasil), conforme descrito no item **5.1.1.**

Determinação da CIM

A CIM do OERO e OEOV foi determinada utilizando a técnica de macrodiluição em caldo (utilizando como caldo de cultivo o CTSEL), conforme descrito no item **5.1.4.** O sistema foi incubado sob agitação de 130 rpm durante 24 h a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente.

Efeito dos óleos essenciais sobre a sobrevivência bacteriana

Inicialmente, as suspensões microbianas em tampão Mcllvaine a pH 7,0 foram acrescidas com o OERO ou OEOV (concentração final de 2 x CIM), seguido de incubação sob agitação (130 rpm) à temperatura apropriada. Nos intervalos de 0 (imediatamente após a homogeneização do sistema), 10, 20, 30, 40, 50, 60, 70 e 80 minutos pós-incubação, uma alíquota de 1 mL da suspensão foi diluída seriadamente (1:9 v/v) em tampão fosfato-salino (0.1 g/100mL, Oxoid, Espanha) estéril e inoculada em ATSEL e incubada durante 24 h a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente. Os resultados foram expressos através da contagem do número de células viáveis em $\log_{10}$ UFC/mL.

Detecção de injúria subletal

Para este ensaio, após as suspensões bacterianas terem sido ressuspendidas em tampão Mcllvaine a pH 7,0, lhes foram acrescidas o OERO ou OEOV (concentração final de 2 x CIM), durante 10 minutos sob agitação (130 rpm) à temperatura apropriada. Decorrido tal período, as células foram então semeadas em ATSEL suplementado com cloreto de sódio (NaCl, 3 g/100 ml para *E. coli* e 6 g/100 mL para *L. monocytogenes*) ou ATSEL suplementado com sais biliares (0,35 g/100 ml para *E. coli*), para, em seguida, serem incubados durante 48 h (meios seletivos) e 24 h (meios não seletivos) a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente.

As concentrações ensaiadas de NaCl e sais biliares foram previamente determinadas como sendo as concentrações máximas não inibitórias para as células na fase estacionária de cada cepa testada. Sistemas que não sofreram exposição ao OERO ou OEOV foram analisados de forma semelhante como o tratamento controle. A extensão da lesão subletal nas membranas citoplasmática e externa, causada por NaCl e sais biliares, respectivamente, foi expressa como a diferença entre as contagens de células viáveis tratadas com o OERO ou OEOV e semeadas nos meios seletivo e não seletivo em comparação com as contagens de células viáveis obtidas nos ensaios do tratamento controle (SOMOLINOS et al, 2010b).

Efeitos sobre a resposta ao agente estressor homólogo

Os ensaios para avaliar possíveis alterações na tolerância bacteriana frente ao OERO ou OEOV deu-se por meio da sua exposição continuada em cultivo *overnight* em CTSEL adicionado de concentrações subinibitórias destes compostos. Para isso, 18 mL de CTSEL contendo inóculo inicial de 7 $\log_{10}$UFC/mL (2 mL) foram adicionados do OERO ou de OEOV em quantidade suficiente para obtenção de uma concentração final de 1/2 CIM.

Os sistemas foram incubados sob agitação (130 rpm) por 24 h a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente. Após o período de incubação, alíquotas de 1 mL foram centrifugadas (6000 rpm por 5 min), lavadas duas vezes com tampão fosfato-salino (0.1 g/100mL, Oxoid, Espanha) e ressuspendidas em tampão Mcllvaine estéril a pH 7,0 para uma população final de aproximadamente 7 $\log_{10}$ UFC/mL, para depois lhes serem acrescidas OERO ou OEOV (concentração final de 2 x CIM) e em seguida incubadas novamente á temperatura apropriada para cada micro-organismo.

A contagem de células viáveis ocorreu ao longo do tempo (0, 10, 20, 30, 40, 50, 60, 70 e 80 min) através de semeadura em ATSEL para 24 - 48 h a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente. Sistemas de cultivo das cepas bacterianas sem adição do OERO ou OEOV foram usados como ensaio controle.

Os resultados foram expressos como a diferença entre a contagem de células viáveis (fração $\log_{10}$ UFC/mL) de cada tempo ensaiado com a da população bacteriana-UFC/mL no tempo zero ($\log_{10}N_0 - \log_{10}N$; onde N_0 foi a contagem inicial no tempo zero, enquanto N correspondeu a contagem após cada tempo de incubação á temperatura apropriada para cada micro-organismo).

Efeitos sobre a resposta ao agente estressor heterólogo

A avaliação de possíveis alterações na tolerância bacteriana frente ao Campo Elétrico Pulsado (CEP) após exposição das cepas teste ás concentrações subinibitórias de OERO ou OEOV em CTSEL foi realizada conforme descrito anteriormente no item anterior e por García et al., 2005. Para isso, 18 mL de CTSEL contendo inóculo inicial de 7 $\log_{10}$UFC/mL (2 mL) foram adicionados do OERO ou de OEOV em quantidade suficiente para obtenção de uma concentração final de 1/2 CIM.

Os sistemas foram incubados sob agitação (130 rpm) por 24 h a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente. Após o período de incubação, alíquotas de 2 mL foram centrifugadas (6000 rpm por 5min), lavadas duas vezes com tampão fosfato-salino (0.1 g/100mL, Oxoid, Espanha), ressuspendidas em CTSEL fresco e por fim transferidas (1 mL) para 10 mL de tampão Mcllvaine estéril a pH 7,0 para uma população final de aproximadamente 7 $\log_{10}$ UFC/mL. A condutividade elétrica do sistema foi ajustada para 2 mS/cm (semelhante aos produtos alimentares).

Alíquotas de 0,5 mL de cada sistema ensaiado foram introduzidas dentro da câmara de tratamento do CEP através de uma seringa estéril. A avaliação do efeito do tratamento de CEP foi realizada a temperatura ambiente (aproximadamente 22 ºC) com intensidade de campo elétrico de 30 kV/cm durante 50 pulsos de 2 μs de duração aplicados a uma freqüência de 1 Hz. A energia específica de cada pulso foi de 4,72 kJ/kg. O equipamento de CEP utilizado no presente ensaio é capaz de aplicar os pulsos elétricos por meio de queda exponencial, conforme descrito por Garcia et al. (2005).

Após o tratamento de CEP, as suspensões bacterianas foram diluídas seriadamente (1:9 v/v) em tampão fosfato-salino (0.1 g/100mL, Oxoid, Espanha) estéril e inoculadas em ATSEL por 24 h a 37 °C ou 30 °C para *E. coli* e *L. monocytogenes*, respectivamente.

Os resultados foram expressos como a diferença entre a contagem de células viáveis (fração $\log_{10}$UFC/mL) com a da população bacteriana-UFC/mL no tempo zero ($\log_{10}N_0 - \log_{10}N$; onde N_0 foi a contagem inicial no tempo zero, enquanto que N correspondeu a contagem após exposição de cada tratamento.

O limite de detecção de células viáveis foi de 2 $\log_{10}$ UFC/mL para todas os experimentos.

Reprodutibilidade e análise estatística

Os ensaios foram realizados em triplicata com três experimentos independentes, sendo os resultados expressos como média de todos. Os dados foram analisados utilizando o software SAS 9.1 e submetidos à comparação entre as médias de significância ($p \leq 0,05$) através do teste *t* de Student para os dados (redução de ciclos em log_{10}UFC/mL) de injúria subletal nas membranas citoplasmática e externa de células pré-expostas e não expostas aos OES testados. Para os ensaios de determinação de CIM, os resultados foram expressos como valores modais, uma vez que foram os mesmos em todas as repetições. E por fim, a Análise de variância (ANOVA) e teste de Tukey para os dados de resposta log_{10} (UFC/mL de fração de sobrevivência) aos agentes estressores homólogos e heterólogos das cepas pré-expostas e não expostas aos OES testados a comparação de médias, com $p < 0,05$ para significância para nos demais ensaios.

6 RESULTADOS E DISCUSSÃO

Os resultados e discussão produzidos durante o desenvolvimento da tese de doutorado estão expostos em formato de artigos científicos publicados (presentes no item Anexos) em revistas científicas indexadas, e formatados de acordo com as normas dos periódicos de publicação.

7 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Os resultados obtidos neste estudo revelam que a exposição de cepas bacterianas contaminantes de alimentos a concentrações subinibitórias do OERO ou CIN não aumentou a tolerância bacteriana direta e cruzada, quando cultivadas em caldo e modelo base carne. Ainda, demonstraram a influência dos fatores sigma σ^S e σ^B na sensibilidade das estirpes individuais de *E. coli* MG1655 e *L. monocytogenes* EGD-e, respectivamente, frente ao OVEO e ROEO, revelando evidenciando que as células mutantes eram ligeiramente mais sensíveis do que as parentais. Estes achados revelam o potencial antimicrobiano do OERO e do CIN em sistemas de conservação de alimentos, quando considerada a sua capacidade de inibição do crescimento das cepas utilizadas no estudo, concomitante ao não aumento da tolerância bacteriana direta e cruzada seguinte a exposição a condições de estresse causadas por concentrações subinibitórias dos OES ensaiados.

Ademais, tomando como base os resultados obtidos nos ensaios com as cepas parentais e mutantes em relação aos fatores sigma abordados no estudo, pode-se inferir que a compreensão das condições ambientais, incluindo os parâmetros relacionados com os fatores intrínsecos e extrínsecos dos alimentos, envolvidos no desenvolvimento de recursos bacterianos que influenciem na tolerância microbiana (como a ativação dos fatores sigma) poderá ser útil na criação de estratégias mais eficazes para o controle de crescimento e sobrevivência de diversas bactérias patogênicas e deteriorantes por meio da aplicação de OES e seus constituintes individuais em associação (sequencial ou combinada) com outros métodos de conservação dos alimentos.

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ANEXOS

Anexo A

Título: *Pseudomonas aeruginosa* cells adapted to *Rosmarinus officinalis L*. essential oil and 1,8-cineole acquire no direct and cross protection in a meat-based broth

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Short communication

Pseudomonas aeruginosa cells adapted to *Rosmarinus officinalis* L. essential oil and 1,8-cineole acquire no direct and cross protection in a meat-based broth

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ABSTRACT

This study assessed the efficacy of *Rosmarinus officinalis* L. essential oil (ROEO) and 1,8-cineole (CIN) in inhibiting the survival of *Pseudomonas aeruginosa* ATCC 9027, and the capacity to induce direct protection and bacterial cross protection against lactic acid, NaCl and high temperature following exposure to different sublethal amounts of the compounds in a meat based broth. Overnight exposure of *P. aeruginosa* to sublethal concentrations of both compounds revealed no induction of direct protection and cross protection. Cells subjected to 24-hour cycles of adaptation in increasing amounts of the antimicrobials showed no increase in direct tolerance, as they were able to survive in growth medium containing up to 1/4 and 1/2 of the minimum inhibitory concentration of the ROEO and CIN, respectively. The results of this study revealed evidence for lack of induction of direct protection or cross protection in *P. aeruginosa* ATCC 9027 when exposed to sublethal amounts of ROEO or CIN in a meat-based broth, as determined by monitoring cell survival and growth behavior.

1. Introduction

Pseudomonas aeruginosa is a ubiquitous environmental bacterium with great adaptability and metabolic versatility. It is an important opportunistic human pathogen associated with the spoilage of a variety of foods (Yi, Zhu, Fu, & Li, 2010). Resistance to antimicrobials and biocides is a long recorded aspect of *Pseudomonas*, and adaptation of *Pseudomonas* species by serial passage in increasing concentrations of these compounds is already well documented (Tattawasart, Maillard, Furr, & Russell, 1999).

The special risk posed by *P. aeruginosa* has provoked studies on development of novel technologies to control this bacterium (Mann, Cox, & Markham, 2000; Yi et al., 2010), and the essential oils of plants and their constituents have been featured as interesting alternative to control this bacterium in foods (Victoria et al., 2012). Previous studies showed that *Rosmarinus officinalis* L. (rosemary) essential oil (ROEO) possesses strong antimicrobial activity against food-related pathogenic bacteria, including species of *Pseudomonas* (Azerêdo, Stamford, Nunes, Gomes-Neto, & Souza, 2011; Tyagi & Malik, 2010). The antimicrobial property of ROEO has been linked to the monoterpene 1,8-cineole (CIN), which is often found to be the major compound therein (Azerêdo et al., 2011; Sousa et al., 2012).

This study assessed the efficacy of the ROEO and CIN in inhibiting the growth and survival of *P. aeruginosa* ATCC 9027 and evaluated the development of direct protection and cross protection when the strain was exposed to sublethal concentrations of these substances in a meat-based broth.

2. Material and methods

2.1. Essential oil and CIN

ROEO (batch ROSTUN04; density at 20 °C: 0.94; refractive index at 20 °C: 1.51) was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil. The compound CIN was purchased from Sigma Aldrich (Sigma, France). Solutions of ROEO and CIN (160–0.075 μL/mL) were prepared in nutrient broth (Himedia, India) using bacteriological agar (0.15 g/100 mL) as a stabilizing agent (Bennis, Chami, Chami, Bouchikhi, & Remmal, 2004).

2.2. Test microorganism

P. aeruginosa ATCC 9027 was obtained from the Collection of Reference Microorganisms at the National Institute of Control Quality in Health (FIOCRUZ, Rio de Janeiro Brazil). Inocula of *P. aeruginosa* ATCC 9027 used in the assays (*c.a.* 7 log of cfu/mL) were obtained as previously described (Sousa et al., 2012).

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2.3. Preparation of meat broth

Time-kill assays and assays to determine the development of direct protection and bacterial cross-protection were performed using a meat-based broth (Oliveira, Stamford, Gomes Neto, & Souza, 2010) as a substrate for bacterial cultivation.

2.4. Determination of the Minimum Inhibitory Concentration (MIC) and time-kill assay

MIC values of ROEO and CIN were determined using macro-dilution in broth. (Nostro et al., 2001). The effect of ROEO and CIN (MIC, 1/2 MIC and 1/4 MIC) on the bacterial viability in meat broth over 120 min was evaluated by the viable cell count procedure (Barros et al., 2009), and the results were expressed in the log of cfu/mL.

2.5. Induction of bacterial direct and cross protection

For assessing the induction of bacterial direct protection, the strain was exposed overnight to sublethal concentrations (1/2 MIC and 1/4 MIC) of ROEO or CIN in meat broth at 35 °C (Luz, Gomes Neto, Tavares, Magnani, & Souza, 2012a; Luz et al., 2012b). The induction of direct protection was determined by comparing the viable cell counts (log cfu/mL) in the treated and untreated (cells not previously exposed to ROEO or CIN) suspensions upon further inoculation into meat broth at 35 °C, to which the same stress agent was added at its MIC values.

The induction of bacterial cross protection was performed by an overnight exposure of the bacterium to sublethal amounts of the ROEO or CIN in meat broth, followed for exposure to other stressing agents (45 °C, pH 5.2, NaCl 10 g/100 mL, conditions that modestly inhibited the growth of the assayed strain) (Luz, Gomes Neto, Tavares, Magnani, & Souza, 2012a, Luz, Gomes Neto, Tavares, Nunes, et al., 2012b). The induction of bacterial cross protection was measured by comparing viable cell counts (log cfu/mL) in the treated and untreated samples following inoculation into growth media either containing stressful additives or exposed to environmental stressful conditions.

The capacity of the tested bacterial strains to develop tolerance to the ROEO and CIN was also assessed through exposure of the bacterium to increasing amounts of these compounds (1/16 MIC–2 × MIC) throughout successive 24 h habituation cycles in meat broth, regarding a more prolonged time of exposure according to a previously described procedure (Luz, Gomes Neto, Tavares, Magnani, & Souza, 2012a, Luz, Gomes Neto, Tavares, Nunes, et al., 2012b).

2.6. Reproducibility and statistics

All assays were performed in triplicate on three separate occasions and the results were expressed as an average of the assays. The significant differences ($P<0.05$) were calculated by ANOVA followed by Tukey tests using the Software Sigma stat 3.1.

3. Results and discussion

The ROEO and CIN displayed MIC value of 40 and 80 μL/mL for *P. aeruginosa* ATCC 9027, respectively. ROEO at MIC strongly inhibited the viability of *P. aeruginosa* with a steady kill-rate throughout 120 min of exposure. The ROEO at 1/2 MIC and 1/4 MIC displayed an initial inhibition of the cell viability up to 15 min of exposure, however from this time onward the viable cell counts revealed a linear and progressive increase, with viable counts between 6–7 log cfu/mL after 120 min of exposure. For all of the concentrations tested (MIC, 1/2 MIC and 1/4 MIC) CIN inhibited the viability of *P. aeruginosa* throughout 120 min of exposure. CIN at 1/2 MIC and 1/4 MIC decreased the viable cell count to approximately 5 log cfu/mL after 120 min of exposure, revealing that these concentrations were inhibitory to the growth of the tested strain, but not lethal.

The overnight exposure of *P. aeruginosa* to sublethal amounts of both ROEO and CIN (1/2 MIC and 1/4 MIC) did not reveal induction of bacterial direct protection (Fig. 1A–B), as demonstrated by the viable cell counts over 240 min of exposure. Interestingly, pre-adapted cells and cells that were not pre-adapted showed similar kill curve shapes over the time intervals that were examined, although with differences ($P>0.05$) in the counts of viable cells over time. *P. aeruginosa* that had been previously challenged with sublethal concentrations of ROEO or CIN displayed a decrease in viable counts ($P<0.05$) when the cells were further cultivated in growth medium to which the same compound (at its MIC) was added. Moreover, there was no difference ($P>0.05$) between the viable counts when compared the cells habituated to both ROEO or CIN at 1/2 MIC and 1/4 MIC following further cultivation in growth medium to which the same compound (at its MIC) was added.

Consistent with the results of the bacterial direct protection assays, *P. aeruginosa* cells that were exposed overnight to sublethal concentrations (1/4 MIC and 1/2 MIC) of ROEO or CIN showed no induction of cross-protection to lactic acid (pH 5.2), salt (NaCl at 10 g/100 mL) or high temperature (45 °C) (Figs. 2A–C and 3A–C), as determined by the viable cells counts throughout the 240 min of exposure. The

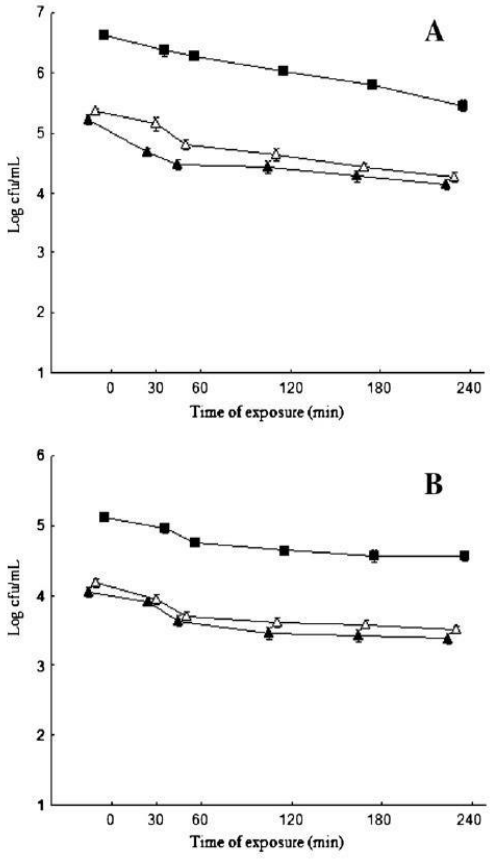

Fig. 1. Viable cell counts of *P. aeruginosa* ATCC 9027 in meat broth to which the essential oil and 1,8-cineole (at its MIC) were added following overnight exposure at 35 °C to sublethal concentrations of *R. officinalis* L. essential oil (A): (■) Control, non-adapted cells; (▲) cells pre-adapted at 1/2 MIC, 20 μL/mL; (Δ) cells pre-adapted at 1/4 MIC, 10 μL/mL and 1,8-cineole (B): (■) Control, non-adapted cells; (▲) cells pre-adapted at 1/2 MIC, 40 μL/mL; and (Δ) cells pre-adapted at 1/4 MIC, 20 μL/mL.

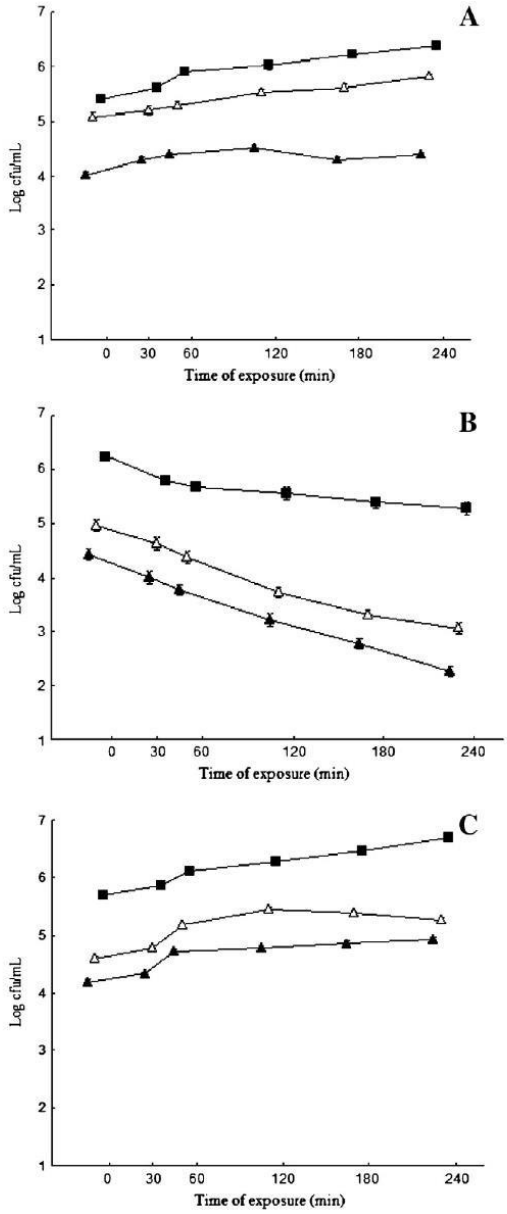

Fig. 2. Viable counts of *P. aeruginosa* ATCC 9027 in meat broth with lactic acid at pH 5.2 (A), with 10 g/100 mL NaCl (B) and at 45 °C (C) after overnight exposure to sublethal concentrations of *R. officinalis* L. essential oil. (■) Control, non-adapted cells; (▲) cells pre-adapted at 1/2 MIC, 20 μL/mL; and (Δ) cells pre-adapted at 1/4 MIC, 10 μL/mL.

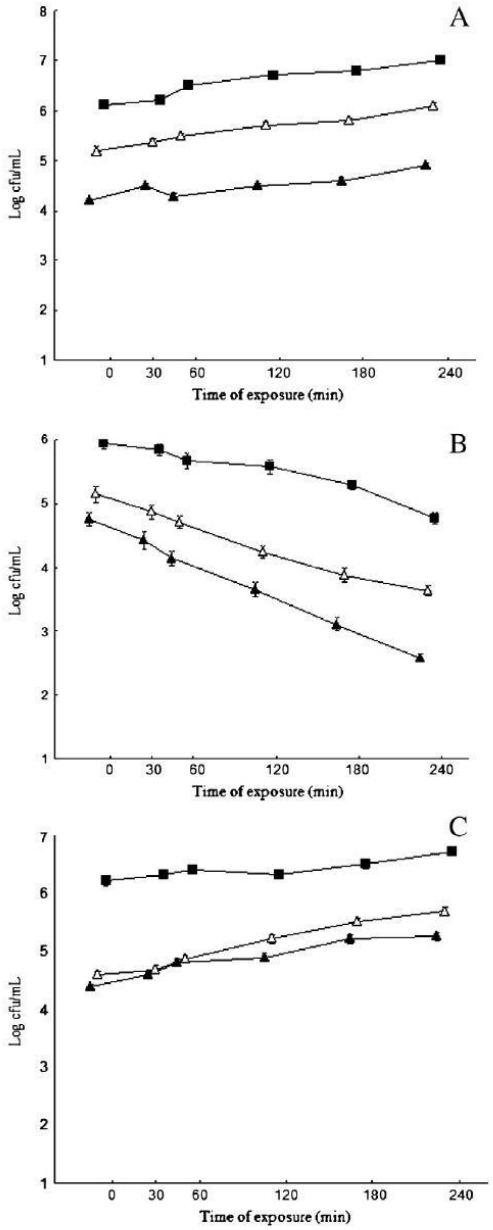

Fig. 3. Viable counts of *P. aeruginosa* ATCC 9027 in meat broth with lactic acid at pH 5.2 (A), with 10 g/100 mL NaCl (B) and at 45 °C (C) after overnight exposure to sublethal concentrations of 1,8-cineole. (■) Control, non-adapted cells; (▲) cells pre-adapted at 1/2 MIC, 40 μL/mL; and (Δ) cells pre-adapted at 1/4 MIC, 20 μL/mL.

sensitivity presented by pre-adapted cells to the heterologous stressing agents was always higher ($P<0.05$) or similar ($P>0.05$) than the sensitivity revealed by the non-adapted cells over the assessed time intervals.

Cells of *P. aeruginosa* exposed (24 h cycles) to increasing amounts of ROEO or CIN were able to survive (as demonstrated by viable cell counts) in broth to which the ROEO and CIN were added in concentrations up to

the 1/4 MIC and 1/2 MIC, respectively, suggesting that the exposure of the cells to increasing amounts of both substances did not stimulate the development of higher tolerance. The inhibition of the cells already present in the broth upon the addition of sublethal amounts of the tested substances could be related to the manifestation of cell injury; when bacterial cells are kept continuously exposed in a stressing but non-lethal environment, as was provided by the sublethal amounts of the ROEO and CIN, the cells may lose viability and the capacity to survive over time (Dodd, Sharman, Bloomfield, Booth, & Stewart, 1997).

The absence of any induction of direct or cross protection in *P. aeruginosa* following an one-step challenge (18 h exposed to sublethal concentrations) and a multi-step challenge (24-hour cycles of exposure to sublethal concentrations) of both ROEO and CIN is particularly interesting in light of the previously documented development of homologous and heterologous resistance of *P. aeruginosa* upon adapted with sublethal amounts of some antibiotics, biocides or antimicrobial procedures (Hassani, Mañas, Pagán, & Condón, 2007; Joynson, Forbes, & Lambert, 2002; Massier et al., 2012).

The results of this study reveal that *P. aeruginosa* ATCC 9027 was not able to acquire direct protection and cross protection (acid-tolerance, osmotolerance and thermotolerance) when exposed to sublethal amounts of the ROEO and its majority compound CIN in a meat based broth. The lack of induction of tolerance in *P. aeruginosa* by ROEO and CIN reinforce the possibility of the use of the substances as anti- *P. aeruginosa* compounds in foods regarding their efficacy to establish an inhibitory effect, besides the low capacity to induce bacterial tolerance.

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Anexo B

Título: *Rosmarinus officinalis L*. Essential Oil and Its Majority Compound 1,8-Cineole at Sublethal Amounts Induce No Direct and Cross Protection in *Staphylococcus aureus* ATCC 6538

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Rosmarinus officinalis L. Essential Oil and Its Majority Compound 1,8-Cineole at Sublethal Amounts Induce No Direct and Cross Protection in *Staphylococcus aureus* ATCC 6538

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Abstract

In this study, the inhibitory efficacy of *Rosmarinus officinalis* essential L. (ROEO) and 1,8-cineole (CIN) in inhibiting the growth and survival of *Staphylococcus aureus* ATCC 6538 and the induction of direct and bacterial cross protection (lactic acid pH 5.2; NaCl 100 g/L; high temperature 45°C) were evaluated following exposure to sublethal and increasing amounts of these treatments in meat broth. All of the concentrations of the ROEO and CIN examined in this study (minimum inhibitory concentration [MIC], 1/2 MIC, and 1/4 MIC) inhibited the viability of *S. aureus* throughout the 120 min of exposure. The overnight exposure of *S. aureus* to sublethal amounts of both ROEO or CIN in meat broth did not result in direct or cross protection. Cells progressively subcultured (24-h cycles) in meat broth with increasing amounts of ROEO or CIN showed no increased direct tolerance. These results reveal the antimicrobial efficacy of ROEO and CIN for use in food conservation systems as anti–*S. aureus* compounds given their efficacy at inhibiting bacterial growth, in addition to their lack of induction for the development of homologous and heterologous resistance.

Introduction

Staphylococcus aureus is among the leading causes of foodborne diseases worldwide (Normanno *et al.*, 2007). *S. aureus* foodborne disease is typically caused by the ingestion of staphylococcal enterotoxins, with symptoms including abdominal cramps, prostration, projectile vomiting, lowered body temperature (never fever), lowered body pressure, and sometimes diarrhea (Lawrynowicz-Paciorek *et al.*, 2007).

Some researchers have found resistance in isolates of *S. aureus* from foods to some antimicrobials used in food conservation and sanitization (Carson *et al.*, 2002; Severin *et al.*, 2004). Exposure of microorganisms to sublethal concentrations of inhibitory compounds may lead to adaptation to the same or cross tolerance to a range of unrelated stressing agents (Hill *et al.*, 2002). The assessment of the response to the sublethal injuries in food-related microorganisms could be helpful for reducing the uncertainty about the establishment of the true microbiological risk related to the lack of knowledge about the physiological behavior of the microbial cells surviving in sublethal stress (Luz *et al.*, 2012).

Concern over the negative consumer perception of chemical preservatives has increased interest in the use of natural compounds to control the growth and survival of bacteria in foods (Barros *et al.*, 2009). Early studies showed that *Rosmarinus officinalis* L. essential oil (ROEO) possesses strong and wide-spectrum antimicrobial activity against spoilage and pathogenic food-related bacteria (Santoyo *et al.*, 2005; Prabuseenivasan *et al.*, 2006). The antimicrobial property of ROEO has been mainly related to the monoterpene 1,8-cineole (CIN), which has been often found as its majority compound (Naveena *et al.*, 2006).

This study assessed the efficacy of ROEO and its main component CIN in the growth inhibition and survival of *S. aureus* ATCC 6538, and evaluated the development of direct and/or cross protection when the strain was exposed to sublethal concentrations of these substances in food-based media.

Materials and Methods

Essential oil and 1,8-cineole

ROEO (batch ROSTUN04; density at 20°C, 0.94; refractive index at 20°C, 1.51) obtained by steam distillation was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil), and its quality parameters were described in an

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accompanying technical report. CIN was purchased from Sigma Aldrich (Sigma, Lyon, France). A previous study (Azerêdo *et al.*, 2011) found CIN (32.2 g/100 g) as the majority compound of the ROEO examined here. Solutions of the antimicrobials were prepared in nutrient broth (Himedia, Mumbai, India) in a range of 160 to 0.075 μL/mL using bacteriological agar (0.015 g/L) as a stabilizing agent (Bennis *et al.*, 2004).

Test microorganism

S. aureus ATCC 6538 was obtained from the Collection of Reference Microorganisms, National Institute of Control Quality in Health (FIOCRUZ, Rio de Janeiro, Brazil). A stock culture was kept on nutrient agar (Himedia, Mumbai, India) under refrigeration (4 ± 1°C). Unless stated otherwise, the inocula [approximately 10 log of colony forming units per milliliter (log CFU/mL)] used in the assays were obtained from stationary phase suspensions of the strain and prepared according to a previously described procedure (Carson *et al.*, 2002). The obtained suspension was serially diluted (10^{-1} to 10^{-3}) in 0.1 M phosphate-buffered solution [PBS (NaCl 9 g/L; pH 7.4)] to provide a viable cell count of approximately 7 log CFU/mL.

Preparation of meat broth

Time-kill and assays to determine the development of direct and cross protection were carried out using cultures grown in a meat-based broth as the substrate for bacterial cultivation. To generate this broth, bovine meat steaks were trimmed of all external fat and cut into uniform-sized pieces (3 × 3 × 3 cm). Meat pieces were then boiled in distilled water for 20 min at 90°C. Approximately 500 mL of meat broth was obtained and vacuum filtered using Whatman no. 1. The filtrate was sterilized by autoclaving for 15 min (1.21 atm) and stored at −20°C in aliquots of 50 mL. For use in the functional assays, one aliquot was thawed at a time under refrigeration (7 ± 1°C) (Oliveira *et al.*, 2010).

Determination of the minimum inhibitory concentration

Minimum inhibitory concentration (MIC) values of ROEO and CIN were determined using macrodilution in broth. Double strength nutrient broth (4 mL) was inoculated with 1 mL of the bacterial suspension, mixed with 5 mL of the antimicrobial solution (160 to 0.075 μL/mL) and vortexed for 30 s. The system was statically incubated for 24 h at 35°C. The MIC was defined as the lowest concentration of ROEO or CIN that was required to prevent visible bacterial growth (Nostro *et al.*, 2001). Control flasks without the antimicrobials were tested similarly.

Time-kill assay

The effect of ROEO and CIN (MIC, 1/2 MIC, and 1/4 MIC) on the viability of *S. aureus* in meat broth over a time span of 120 min was evaluated by a viable cell count procedure. Meat broth (4 mL) was inoculated with 1 mL of the bacterial inoculum, 5 mL of the antimicrobial solution was added to the system, and the culture was gently shaken for 30 s using a vortexer. The system was incubated at 35°C, and at increasing time intervals (0, 15, 30, 45, 60, and 120 min) 1 mL of the suspension was serially diluted (10^{-1} to 10^{-5}) in PBS, spread on sterile nutrient agar, and allowed to grow for 24 h at 35°C (Nostro *et al.*, 2001). Control flasks without ROEO or CIN were tested similarly. The results were expressed in measurements of log CFU/mL.

Evaluation of the occurrence of bacterial direct protection

Bacterial strains were exposed overnight to sublethal amounts of ROEO or CIN in meat broth according to a previously described procedure (Luz *et al.*, 2012). Bacterial suspensions (1 mL) were inoculated in 20 mL of meat broth, the ROEO or CIN was added (final concentrations of 1/2 MIC or 1/4 MIC), and the cultures were shaken for 30 s using a vortexer (adaptation treatment). Control flasks without the ROEO or CIN were assayed similarly (non-adaptation treatment). The flasks were incubated overnight (18 h) at 35°C. Following the incubation period, an aliquot (2 mL) of each treatment was used to inoculate fresh meat broth (18 mL) to which the ROEO or CIN (final concentration of the MIC was determined previously) was added. The cultures were shaken for 30 s and incubated at 35°C. Viable cells were enumerated over time (0, 30, 60, 180, and 240 min) by serial dilution (10^{-1} to 10^{-5}) in PBS, plated on nutrient agar, and grown for 48 h at 35°C. The results were expressed in measurements of log CFU/mL. The induction of bacterial direct protection was determined by comparing the viable cell counts over time in the treated and untreated suspensions upon further inoculation into meat broth, to which the same stress agents were added at their MIC values.

Evaluation of the occurrence of bacterial cross protection

Measurements of cross protection induction were performed according to a previously described procedure (Luz *et al.*, 2012). Bacteria were exposed overnight to sublethal amounts of ROEO or CIN in meat broth, followed by exposure to other stress agents (high temperature, low pH, and NaCl). Preliminary experiments were performed to evaluate the salt tolerance, acid tolerance, and thermotolerance of the bacteria. Untreated cultures were used to inoculate meat broth or meat broth containing NaCl (Qeel Ltda., São Paulo, Brazil; 50–150 g/L, at 35°C), lactic acid (VETEC Química Fina Ltda., Rio de Janeiro, Brazil; pH 4.5–6.0, at 35°C), or meat broth that was incubated at increasing temperature (40–55°C) to determine the NaCl concentration, pH value, and temperature that modestly inhibited cell growth.

After establishing these conditions, an aliquot (1 mL) of a newly obtained bacterial suspension was used to inoculate 20 mL of meat broth, to which the ROEO or CIN was added (final concentrations of 1/2 MIC or 1/4 MIC), and the cultures were shaken for 30 s using a vortexer (adaptation treatment). Control flasks without the ROEO or CIN were assayed similarly (non-adaptation treatment). The flasks were incubated overnight (18 h at 35°C). To evaluate the induction of acid tolerance and osmotolerance, respectively, an aliquot (2 mL) of each sample was used to inoculate fresh meat broth (18 mL) that was acidified with lactic acid to a pH of 5.2 or to which NaCl at 100 g/L was added, and the samples were incubated at 35°C. To measure thermotolerance induction, an aliquot (2 mL) of each treatment was used to inoculate fresh meat broth (18 mL) and incubated at 45°C. Viable cells were

determined with increasing time (0, 30, 60, 180, and 240 min) by serial dilution (10^{-1} to 10^{-5}) in PBS followed by plating on nutrient agar and growth for 24–48 h at 35°C. The results were expressed as log CFU/mL. The induction of bacterial cross protection was measured by comparing viable cell counts with increasing time in the treated and untreated samples following inoculation into growth media either containing stressful additives or exposed to environmental stressful conditions.

Evaluation of the occurrence of the induction of bacterial direct tolerance throughout successive 24-h habituation cycles

The capacity of *S. aureus* to develop tolerance over a more prolonged exposure time was determined according a previously described method (To *et al.*, 2001; Luz *et al.*, 2012). Bacteria were exposed to increasing amounts of the ROEO or CIN (1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC, 2×MIC, and 4×MIC) throughout successive 24-h habituation cycles in meat broth. Specifically, bacterial suspensions (1 mL) were used to inoculate 20 mL of meat broth containing ROEO or CIN (final concentration of 1/16 MIC). The cultures were shaken for 30 s using a vortexer and then incubated for 24 h at 35°C. After the incubation period, an aliquot (1 mL) of each sample was serially diluted (10^{-1} to 10^{-5}) in PBS and plated on sterile nutrient agar for viable cell determination (35°C for 24 h). At the same time, an aliquot (1 mL) of meat broth to which the ROEO or CIN was added at 1/16 MIC (to allow for bacterial growth) was inoculated in a fresh meat broth to which the same compound at the next highest concentration (1/8 MIC) was added, followed by incubation at 35°C and viable cell detection according to the conditions described above. This procedure was repeated with bacteria exposed to increasing concentrations of ROEO or CIN (1/4 MIC – 4×MIC) until a concentration was reached at which no viable cells were detected.

The detection limit of the method used for viable cell counting was 2 log CFU/mL for all of the assays.

Statistical analysis

The assays were performed in triplicate on two separate occasions, and the results were expressed as an average of the assays. Statistical analysis was performed to determine significant differences ($p < 0.05$) by analysis of variance, followed by Tukey tests using the SigmaStat 3.1 computer program.

Results and Discussion

ROEO and CIN displayed a MIC value of 20 and 40 μL/mL, respectively, against *S. aureus* ATCC 6538. For all of the concentrations tested (MIC, 1/2 MIC, and 1/4 MIC), the ROEO and CIN inhibited ($p < 0.05$) the cell viability of *S. aureus* when compared to the control assay. Inhibition of bacterial viability occurred as early as 15 min after exposure, and no subsequent increase in viable counts was observed for the remainder of the exposure. ROEO and CIN at 1/2 and 1/4 MIC decreased the viable count of *S. aureus* to approximately 5 log CFU/mL after 120 min of exposure, revealing that these concentrations were inhibitory to the growth of the tested strain, but not lethal.

The overnight exposure of *S. aureus* ATCC 6538 to sublethal amounts (1/2 MIC and 1/4 MIC) of ROEO or CIN did not reveal induction of direct protection (Fig. 1A and B), as demonstrated by the viable cell counts over 240 min of exposure. The kill-curves of *S. aureus* previously challenged with both tested sublethal amounts of the ROEO presented similar viable counts ($p > 0.05$) when exposed to the same agent at the MIC previously determined (Fig. 1A). Cells that have been previously challenged with sublethal concentrations of CIN displayed a decrease in viable counts ($p < 0.05$) when the cells were further cultivated in growth medium to which the same compound (at its MIC) was added when compared to non-adapted cells (Fig. 1B). However, the cells challenged with sublethal concentrations of CIN displayed a kill-curve shape revealing a decreased relative reduction in the viable cells over 240 min of exposure to the same antimicrobial (at its MIC), while the non-adapted cells displayed a linear and

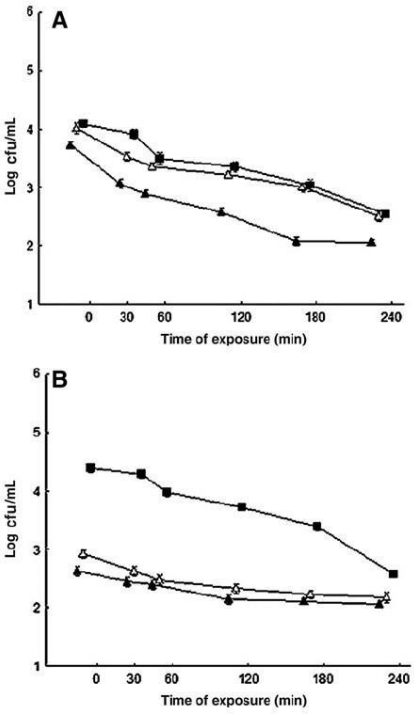

FIG. 1. Viable cell counts of *Staphylococcus aureus* ATCC 6538 in meat broth to which the essential oil and 1,8-cineole (at its minimum inhibitory concentration [MIC]) were added following overnight exposure at 35°C to sublethal concentrations of *R. officinalis* L. essential oil. **(A)** Control, non-adapted cells (■); cells pre-adapted at 1/2 MIC, 10 μL/mL (▲); cells pre-adapted at 1/4 MIC, 5 μL/mL, and 1,8-cineole (Δ). **(B)** Control, non-adapted cells (■); cells pre-adapted at 1/2 MIC, 20 μL/mL (▲); and cells pre-adapted at 1/4 MIC, 10 μL/mL (Δ).

sharp reduction in viable count over the time intervals that were examined.

Exposure of *S. aureus* to sublethal concentration (20 μg/mL) of Epigallocathechin gallate (EGCC), the major phenolic compound of green tea extract, in Muller Hinton broth for 120 min led to adaptation and cross resistance to vancomicyn, ampicillin, and oxacillin with increased MIC values (two- to eightfold increase) (Sing *et al*., 2007). Still, adaptation to EGCG increased the heat resistance in pre-adapted cells. The authors assumed that exposure of *S. aureus* to EGCC might cause an increase in the expression of heat shock proteins (HSPs). The HSP DnaK has been shown to play a significant role in the survival of *S. aureus* under various stress conditions, such as high temperatures, oxidative stress, and in the presence of cell-wall-active antibiotics. HSP is also found in *Escherichia coli*

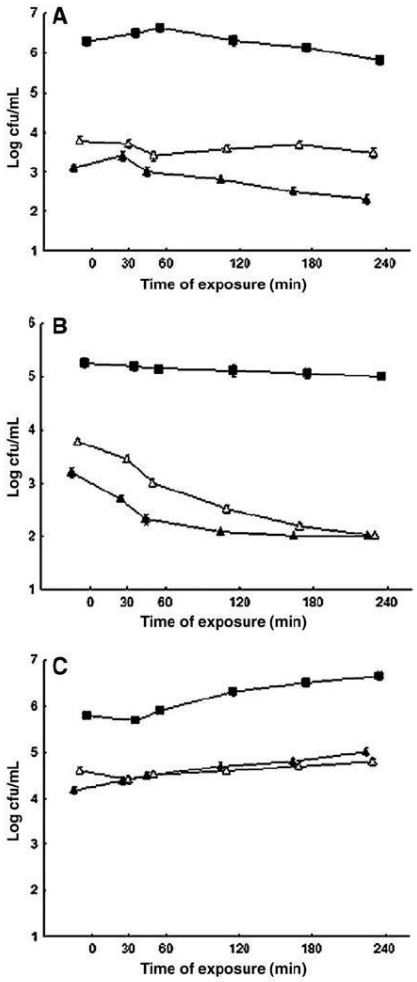

FIG. 2. Viable cell counts of *Staphylococcus aureus* ATCC 6538 grown in meat broth incubated at high temperature 45°C **(A)** or to which lactic acid pH 5.2 **(B)** or NaCl 10 g/100 mL **(C)** was added following overnight exposure at 35°C to sublethal concentrations of *R. officinalis* L. essential oil. Control, non-adapted cells (■); cells pre-adapted at minimum inhibitory concentration (MIC)/2, 10 μL/mL (▲); and cells pre-adapted at MIC/4, 5 μL/mL (Δ).

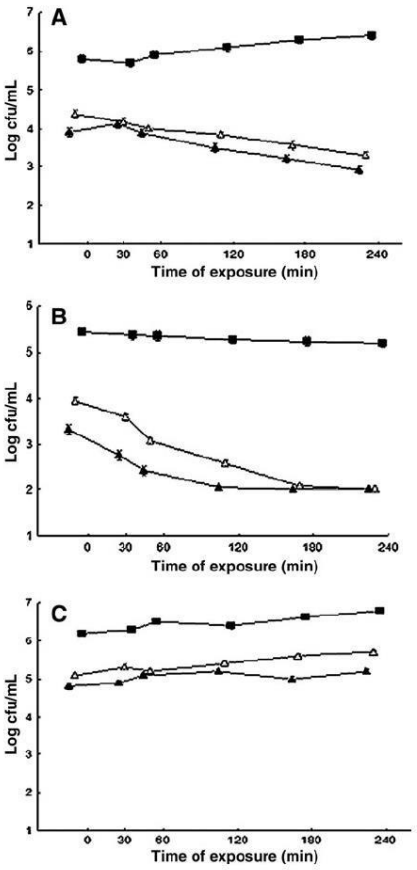

FIG. 3. Viable cell counts of *Staphylococcus aureus* ATCC 6538 grown in meat broth incubated at high temperature 45°C **(A)** or to which lactic acid pH 5.2 **(B)** or NaCl 10 g/100 mL **(C)** was added following overnight exposure at 35°C to sublethal concentrations of 1,8 cineole. Control, non-adapted cells (■); cells pre-adapted at minimum inhibitory concentration (MIC)/2, 20 μL/mL (▲); cells pre-adapted at MIC/4, 10 μL/mL (Δ).

cells challenged with ethanol, high osmotic pressure, high temperature, and presence of the phenolic carvacrol (Burt *et al.*, 2007).

Consistent with the results of the bacterial direct protection assays *S. aureus* ATCC 6538 cells that were exposed overnight to sublethal concentrations (1/4 MIC and 1/2 MIC) of ROEO or CIN showed no cross protection to high temperature (45°C), lactic acid (pH 5.2), and salt (NaCl at 100 g/L) (Figs. 2A–C and 3A–C). For the most systems, kill-curves of pre-adapted and non pre-adapted cells revealed similar shapes over the time intervals that were examined. No difference ($p > 0.05$) was found between the counts of viable cells of *S. aureus* submitted to pre-adaptation with the ROEO or CIN (at 1/2 MIC and 1/4 MIC) upon further exposure to heterologous stressing agents (low pH, NaCl, and high temperature). The cells submitted to pre-adaptation with ROEO or CIN revealed increased sensitivity to lactic acid, high temperature and NaCl when compared to the non-adapted cells, noted for smaller ($p > 0.05$) viable counts over 240 min of exposure.

Cebrián *et al.* (2010) reported the ability of *S. aureus* CECT 44459 to develop direct and cross resistance when exposed (5 min to 2 h) to sublethal conditions of acid (hydrochloric acid pH 5.5) and alkaline pH (sodium hydroxide pH 8.0–10.0), hydrogen peroxide (0.01 and 10 mM), and heat (40°C and 48°C) in tryptone soy broth. These researchers noted that the presence of the antibiotics rifampicin and chloramphenicol in the growth media concomitantly to the other stressing agents completely abolished the increase in homologous resistance to pH and hydrogen peroxide. Other studies have also noted that addition of protein synthesis inhibitors, such as rifampicin and chloramphenicol, at different levels in growth media completely prevent the development of resistance to acid pH in bacteria (e.g., *Enterococcus faecalis*, *Lactococcus lactis*, *Listeria monocytogenes*, *Salmonella typhimurium*), suggesting that protein synthesis is necessary for the development of the resistance responses (Cebrián *et al.*, 2010). Essential oils and their compounds even at levels lower than the MIC have been cited to suppress the synthesis and activity of enzymes in a number of *S. aureus* strains resulting in protein synthesis block (Nostro *et al.*, 2001; Oliveira *et al.*, 2010), and this could be related to the difficulty of *S. aureus* ATCC 6538 to develop direct or cross adaptation in the conditions used in this study. Moreover, terpenes found in ROEO (such as CIN, β-pinene, and α-terpinene) have the ability to disturb and penetrate the lipid structure of the bacterial cell wall leading to denaturing of proteins, destruction of cell membrane, cytoplasm leakage, cell lysis, and eventually death. The decrease of pH due to the cell membrane disruption caused by terpenes in bacteria means that control of cell processes such as DNA transcription, protein synthesis, and enzyme activity is lost (Oussalah *et al.*, 2006).

Barros *et al.* (2009) reported that the cultivation of *S. aureus* ATCC 6538 in nutrient broth supplemented with sublethal amounts of *O. vulgare* essential oil (1/2 MIC, 0.3 μl/mL; and 1/4 MIC, 0.15 μL/mL) for 24 h interfered with the metabolic activity of the strain with reduction in salt (NaCl) tolerance, lipase and coagulase activity, and enterotoxin production. The authors stated that the changes in metabolic activity of the bacterium could be related to a sublethal injury of the cell provided by subinhibitory concentrations of the essential oil, which may alter the ability of the cell to osmoregulate adequately, exclude toxic material, and synthesize some physiological determinants, such as proteins and enzymes.

Regarding possible assay limitations for the two sublethal concentrations of ROEO and CIN and the chosen exposition time (18 h) for the pre-adaptation further experiments were performed to assess the induction of tolerance following exposure to 24-h cycles in meat broth to which increasing amounts of the ROEO or CIN (1/16 MIC – 4 × MIC) were added. The results of these assays showed that *S. aureus* ATCC 6538 was able to survive (as demonstrated by viable cell counts) in the broth to which the ROEO or CIN were added in concentrations of up to the respective 1/2 MIC, suggesting that the exposure of the cells to increasing sublethal amounts of the both substances for a more prolonged time (24 h) could not induce the development of adaptive responses towards these compounds. The inhibition of the cells already in the broth added of 1/2 MIC of the tested antimicrobials could be related to the manifestation of injury because when bacterial exponentially growing cells are exposed and continuously kept in a stressing but non-lethal environmental condition, as that provided by the sublethal amounts of the tested antimicrobials, they could lose their viability and capacity to survive over time (Rees *et al.*, 1995). Sublethally injured bacterial cells with the imposed stress caused by levels of antimicrobials lower than the MIC may develop an imbalance between anabolism and catabolism that is sufficient to disrupt the growth and becoming cells unable to form colonies on agar without affecting the metabolic rate, although it can result in self-destruction of the cells through free-radical attack (burst radical production) mainly because of a failure in cell repair mechanisms (Dodd *et al.*, 1997).

The results obtained in this study highlight the antimicrobial efficacy of ROEO and its majority component CIN for use in food conservation systems regarding their efficacy to inhibit the growth and survival of *S. aureus*, in addition to a lack of development of homologous and heterologous resistance to high temperature, pH, and salt. Further studies are needed to assess the induction of genetic changes, synthesis of stress proteins, and modification of lipid membrane composition in *S. aureus* after exposure to sublethal amounts of ROEO and CIN, and the occurrence of induction of bacterial tolerance by the tested compounds in *S. aureus* when cultivated in food matrices.

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Disclosure Statement

No competing financial interests exist.

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Anexo C

Título: *Rosmarinus officinalis L*. essential oil and the related compound 1,8-cineole do not induce director cross-protection in *Listeria monocytogenes* ATCC 7644 cultivated in meat broth

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Rosmarinus officinalis L. essential oil and the related compound 1,8-cineole do not induce direct or cross-protection in *Listeria monocytogenes* ATCC 7644 cultivated in meat broth

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Abstract: *Listeria monocytogenes* has the capability of adapting to 1 or more antimicrobial compounds or procedures applied by the food industry to control the growth and survival of microorganisms in foods. In this study, the effects of *Rosmarinus officinalis* essential oil (EO) and the related compound 1,8-cineole on the inhibition of the growth and survival of *L. monocytogenes* ATCC 7644 were determined. The ability of the *R. officinalis* EO and 1,8-cineole to induce direct and cross-protection of bacteria against various stresses (lactic acid, pH 5.2; NaCl, 3 g/100 mL; high temperature, 45 °C) was also determined. At all concentrations tested (minimum inhibitory concentration (MIC), ½ MIC, and ¼ MIC), both compounds inhibited the cell viability of *L. monocytogenes* over 120 min of exposure. Overnight exposure of *L. monocytogenes* to sublethal amounts of either the *R. officinalis* EO or 1,8-cineole in meat broth revealed no induction of direct or cross-protection against lactic acid, NaCl, or high temperature. Similarly, cells subjected to 24 h cycles of adaptation with increasing amounts (½ MIC to 2× MIC) of the EO and 1,8-cineole showed no increase in direct tolerance, as they were able to survive in growth medium containing up to ½ MIC of either substance. These results show the antimicrobial efficacy of *R. officinalis* EO and 1,8-cineole for use in systems, particularly as anti-*L. monocytogenes* compounds.

Key words: Rosmarinus officinalis L., 1,8-cineole, *Listeria monocytogenes*, bacterial adaptation.

Résumé : *Listeria monocytogenes* a la capacité de s'adapter à un ou plusieurs composés antimicrobiens, ou à des procédés appliqués par l'industrie alimentaire pour contrôler la croissance et la survie des microorganismes dans les aliments. Les effets de l'huile essentielle (HE) de *Rosmarinus officinalis* et d'un composé qui lui est relié, le 1,8-cinéole, sur l'inhibition de la croissance et de la survie de *L. monocytogenes* ATCC 7644 ont été déterminés dans cette étude. La capacité de l'HE de *R. officinalis* et du 1,8-cinéole d'induire une protection directe et croisée des bactéries contre différents stress (acide lactique, pH 5,2, NaCl 3 g/100 mL, température élevée à 45 °C) a aussi été déterminée. Les deux composés inhibaient la viabilité de *L. monocytogenes* pendant la période d'exposition de 120 min et ce, à toutes les concentrations testées (concentration inhibitrice minimale (CIM), ½ CIM et ¼ CIM). Une exposition de 18 heures de *L. monocytogenes* à des quantités sublétales d'HE de *R. officinalis* ou de 1,8-cinéole dans du milieu de culture à l'extrait de viande n'a révélé aucune induction de protection directe ou croisée contre l'acide lactique, le NaCl ou la température élevée. De la même façon, les cellules soumises à des cycles d'adaptation de 24 h à des concentrations croissantes (½ CIM à 2× CIM) d'HE et de 1,8-cinéole ne montraient aucune augmentation de tolérance directe, étant capable de survivre dans du milieu de culture contenant jusqu'à ½ CIM d'une des deux substances. Ces résultats révèlent l'efficacité antimicrobienne de l'HE de *R. officinalis* et du 1,8-cinéole, particulièrement comme composés anti-*L. monocytogenes*.

Mots-clés : Rosmarinus officinalis L., 1,8-cinéole, *Listeria monocytogenes*, adaptation bactérienne.

[Traduit par la Rédaction]

Introduction

Listeria monocytogenes is a ubiquitous psychrotrophic human pathogen found in a wide variety of raw and processed foods. Milk and dairy products, meats and meat products, cabbage, and seafood and fish products have been associated with *Listeria* contamination and implicated in several outbreaks of human listeriosis (Vitas et al. 2004; Gandhi and Chikindas 2007). Listeriosis is a foodborne disease that occurs primarily in pregnant women, the elderly, and immunosuppressed people and leads to illness, miscarriage, and death (Swaminathan and Gerner-Smidt 2007).

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Previous studies have found that isolates of *L. monocytogenes* are capable of survival and growth when exposed to many procedures applied by the food industry to control microbial growth in foods, such as low temperatures, low pH, and high salt concentrations (Koutsoumanis and Sofos 2004; Lin and Chou 2004; Gandhi and Chikindas 2007). When challenged with sublethal stresses including food processing intervention, *L. monocytogenes* may undergo an adaptive response involving signal transduction systems and the coordinated expression of genes related to cellular defense mechanisms. These responses result in changes in cell physiology and the acquisition of reduced sensitivity to the same (homologous) or other (heterologous) stresses (Shigapova et al. 2005; Patrignani et al. 2008).

In the last few decades, food preservation techniques have become milder in response to consumer demand for higher quality and more convenient foods, which are mildly heated, less heavily preserved (e.g., containing less acid, salt, and sugar), and less reliant on preservatives such as sulfite and nitrite (Abee and Wouters 1999; Koutsoumanis and Sofos 2004; McMahon et al. 2007). Concern over the negative consumer perception of chemical preservatives has also increased interest in the use of natural compounds to control the growth and survival of bacteria in foods (Burt et al. 2007; Patrignani et al. 2008; Skandamis et al. 2008; de Oliveira et al. 2010). In this sense, the essential oils (EOs) and their constituents, possessing a wide spectrum of antimicrobial effects, have been featured as interesting alternatives to synthetic antimicrobials in foods (Skandamis et al. 2008; Bikels-Goshen et al. 2010). Early studies showed that *Rosmarinus officinalis* L. EO possesses antimicrobial activity against spoilage and pathogenic food-related bacteria (Santoyo et al. 2005; Prabuseenivasan et al. 2006). The antimicrobial property of *R. officinalis* EO is mainly due to the monoterpene 1,8-cineole, which is often found as the major component (Randrianarivelo et al. 2009; de Azerêdo et al. 2011). The efficiency of these alternative antimicrobials needs to be assessed, especially regarding the potential of food pathogens to adapt to a variety of stress conditions.

Researchers have stated that the amounts of EOs (or their compounds) required to substantially inhibit bacterial growth in foods are often higher than the levels that are organoleptically acceptable (Naveena et al. 2006; de Souza et al. 2009). In this sense, the use of EOs or their constituents at subinhibitory concentrations in combination with other antimicrobial methods could provide a balance between sensory acceptability and antimicrobial efficacy (de Souza et al. 2005; Gutierrez et al. 2008; de Azerêdo et al. 2011). It is thought that the antimicrobial efficacy of an EO is due to the inability of microorganisms to develop adaptations or tolerance to the compounds making up the EOs, although there are few scientific reports providing sound evidence for this phenomenon (Di Pasqua et al. 2006; Galindo et al. 2010). Still, despite the fact that EOs and their compounds are known as potential antimicrobials for use in foods, there is a lack of reports on the possible development of direct and (or) cross-protection by food-related microorganisms when exposed to sublethal concentrations of these compounds.

This study assessed the ability of *R. officinalis* EO and its related component, 1,8-cineole, to inhibit the growth and survival of *L. monocytogenes* and evaluated the development of direct and cross-protection when the strain was exposed to sublethal challenges with these substances in food-based media.

Material and methods

EO and 1,8-cineole

The commercial EO from *R. officinalis* L. (batch ROSTUN04; density at 20 °C, 0.94; refractive index at 20 °C, 1.51), as obtained by steam distillation, was purchased from Aromalândia Ind. Com. Ltd. (Minas Gerais, Brazil), and the quality parameters were described in an accompanying technical report. Solutions of the EO and 1,8-cineole were prepared in nutrient broth (Himedia, India) in a range of concentrations (160–0.075 μL/mL) using bacteriological agar ((0.15 g/100 mL) Laboratórios Difco Ltd., Brazil) as a stabilizing agent (Bennis et al. 2004).

Test microorganism

Listeria monocytogenes ATCC 7644 was obtained from the Collection of Reference Microorganisms, National Institute of Quality Control in Health (FIOCRUZ, Rio de Janeiro, Brazil). A stock culture was kept on nutrient agar (Laboratórios Difco Ltd., Brazil) under refrigeration (7 ± 1 °C). Unless otherwise stated, all assays used inocula obtained from stationary-phase cultures. To obtain stationary-phase cultures, *L. monocytogenes* was first grown overnight on brain heart infusion (BHI) agar (Laboratórios Difco Ltd., Brazil) at 37 °C. Then, liquid cultures were prepared by inoculating 100 mL of BHI broth with 2 bacterial colonies from the overnight plates, and the cultures were incubated overnight at 35 °C. After incubation, the cells were harvested from the growth medium by centrifugation at 10 000*g* for 12 min at 4 °C, washed twice with phosphate-buffered saline (PBS, pH 7.4), and suspended in PBS. Suspensions were adjusted so that the optical density at 620 nm (OD_{620}) of a 1:100 dilution was approximately 0.3, which corresponded to approximately 10 log of colony-forming units (cfu) per millilitre (de Azerêdo et al. 2012). Suspensions were serially diluted in PBS (10^{-1}–10^{-3}) to provide a viable cell count of approximately 7 log cfu/mL.

Preparation of meat broth

Time-kill assays and assays to determine the development of direct and bacterial cross-protection were performed using a meat-based broth as a substrate for bacterial cultivation. Bovine meat steaks were trimmed of all external fat and cut into uniformly sized pieces (3 cm × 3 cm × 3 cm). Meat pieces were then boiled in distilled water for 30 min at 90 °C, yielding approximately 500 mL of meat broth that was then vacuum filtered using Whatman No. 1 filter papers (Waltman Ltd., UK). The filtrate was sterilized by autoclaving for 15 min (1.21 atm (1 atm = 101.29 kPa)). Afterwards, the broth was stored at –20 °C in aliquots of 50 mL. When required, a single aliquot was thawed under refrigeration (7 ± 1 °C) and used for the assays (de Oliveira et al. 2010).

Determination of the minimum inhibitory concentration

Minimum inhibitory concentration (MIC) values for the EO and 1,8-cineole were determined using macrodilution in broth. Four millilitres of double-strength nutrient broth (Laboratórios Difco Ltd., Brazil) was inoculated with 1 mL of

bacterial culture, mixed with 5 mL of the antimicrobial solution, and vortexed for 30 s. The assay was statically incubated for 24 h at 35 °C. The MIC was defined as the lowest concentration of the EO or 1,8-cineole that prevented visible bacterial growth (Nostro et al. 2001). Control flasks without antimicrobials were similarly tested.

Time-kill assay

The effect of the EO and 1,8-cineole on bacterial viability in meat broth following 120 min of exposure was evaluated using viable cell counts. Briefly, 4 mL of meat broth was inoculated with 1 mL of bacterial suspension; 5 mL of the antimicrobial solution was then added to the assay, and the mixture was gently shaken for 30 s using a vortex. The mixture was incubated at 35 °C, and at different time intervals (0, 15, 30, 45, 60, and 120 min), 1 mL of the suspension was serially diluted (10^{-1}–10^{-5}) in PBS, inoculated onto sterile nutrient agar (Laboratórios Difco Ltd., Brazil), and incubated for 24 h at 35 °C (de Barros et al. 2009). Control flasks without antimicrobials were tested similarly. The results were expressed as the log of cfu per millilitre.

Induction of bacterial direct protection

The induction of direct protection in *L. monocytogenes* was performed by exposing bacteria overnight to sublethal concentrations of *R. officinalis* EO and 1,8-cineole in meat broth as previously described (Leyer and Johnson 1993, Brown et al. 1997). Meat broth (18 mL) containing the EO or 1,8-cineole (final concentrations of ½ MIC or ¼ MIC) was inoculated with 2 mL of bacterial suspension and shaken for 30 s using a vortex (adaptation treatment). Control broth without antimicrobials was assayed similarly (nonadaptation treatment). The assays were incubated overnight (18 h) at 35 °C, after which a 2 mL aliquot of each treatment was inoculated into fresh meat broth (18 mL) containing the EO or 1,8-cineole (final concentration of the MIC was determined previously), shaken for 30 s using a vortex and incubated at 35 °C. Viable cells were enumerated over time (0, 30, 60, 120, 180, and 240 min) by serial dilution (10^{-1}–10^{-5}) in PBS and were plated on nutrient agar for 24–48 h at 35 °C. The results were expressed as the log cfu per millilitre. To determine if direct protection was induced, the viable cell counts over the time the bacteria was subjected to adaptation treatments were compared with the counts of nonadapted bacteria when both groups were inoculated into growth media containing the antimicrobials at their MIC values.

Induction of bacterial cross-protection

The induction of cross-protection in *L. monocytogenes* was performed by exposing bacteria overnight to sublethal concentrations of the EO and 1,8-cineole in meat broth followed by exposure to other environmental stressors (high temperature, low pH, and NaCl) as previously described (Boziaris et al. 2011), but with minor modifications. Preliminary experiments were performed for evaluating the salt tolerance, acid tolerance, and thermotolerance of the bacterial test strain. Untreated bacterial cultures were inoculated into normal meat broth, into meat broth containing NaCl (1–15 g/100 mL at 35 °C) or lactic acid (pH 4.5–6.0 at 375 °C), or into meat broth incubated at different high temperatures (40–60 °C), to determine the NaCl concentration, pH value, and temperature that modestly inhibited the growth of the cell suspension.

After establishing the stress conditions, a 2 mL aliquot of fresh bacterial suspension was inoculated into 18 mL of meat broth containing the EO or 1,8-cineole (final concentrations of ½ MIC or ¼ MIC) and shaken for 30 s using a vortex (adaptation treatment); the same was done for control flasks without antimicrobials (nonadaptation treatment). The assays were incubated overnight (18 h) at 35 °C, after which a 2 mL aliquot of each treatment was inoculated into either 18 mL of fresh meat broth acidified with lactic acid (VETEC Química Fina Ltd., Brazil) to a pH of 5.2 or into fresh meat broth containing NaCl (Qeel, Brasil) at 3 g/100 mL to evaluate the induction of acid tolerance and osmotolerance, respectively. To analyze the induction of thermotolerance, a 2 mL aliquot of each treatment was inoculated into 18 mL of fresh meat broth and incubated at 45 °C. Viable cells for all assays were enumerated over time (0, 30, 60, 120, 180, and 240 min) by serial dilution (10^{-1}–10^{-5}) in PBS followed by plating on nutrient agar for 24–48 h at 35 °C. The results were expressed as the log cfu per millilitre. To determine if bacterial cross-protection was induced, the viable counts over time of the bacterial suspensions subjected to the adaptation treatments were compared with the counts of nonadapted bacteria following inoculation into growth media exposed to different environmental stressors.

Induction of bacterial direct tolerance throughout successive habituation 24 h cycles

The capacity of *L. monocytogenes* to develop tolerance to the EO and 1,8-cineole was assessed in meat broth by exposing the bacteria to increasing amounts of the antimicrobials (1/16 MIC, ⅛ MIC, ¼ MIC, ½ MIC, MIC, and 2× MIC) throughout successive 24 h habituation cycles to prolong the time of exposure as previously described (To et al. 2002), but with minor modifications. Thus, 2 mL of the bacterial suspension was inoculated into 18 mL of meat broth containing the EO or 1,8-cineole (final concentrations of 1/16 MIC), shaken for 30 s using a vortex, and incubated for 24 h at 35 °C. Then, a 100 µL aliquot of the culture was serially diluted (10^{-1}–10^{-5}) in PBS and inoculated onto sterile nutrient agar to detect viable cells (35 °C for 24 h). Concurrently, a 2 mL aliquot from the broth containing antimicrobials at 1/16 MIC (and bacterial growth) was inoculated into fresh meat broth (18 mL) containing antimicrobials at the next highest concentration (⅛ MIC); this assay was incubated at 35 °C and viable cells were detected according to the conditions cited above. This procedure was repeated with increasing concentrations of the antimicrobials (¼ MIC – 2× MIC) or until no viable cells were detected.

The detection limit for the viable cell count method used was 2 log cfu/mL for all assays

Reproducibility and statistics

All assays were performed in triplicate on 3 separate occasions, and the results were expressed as averages for each of the assays. Statistical analysis was performed to determine significant differences ($P < 0.05$) using ANOVA followed by Tukey's test. The Sigma Stat 3.1 computer program was used.

Results and discussion

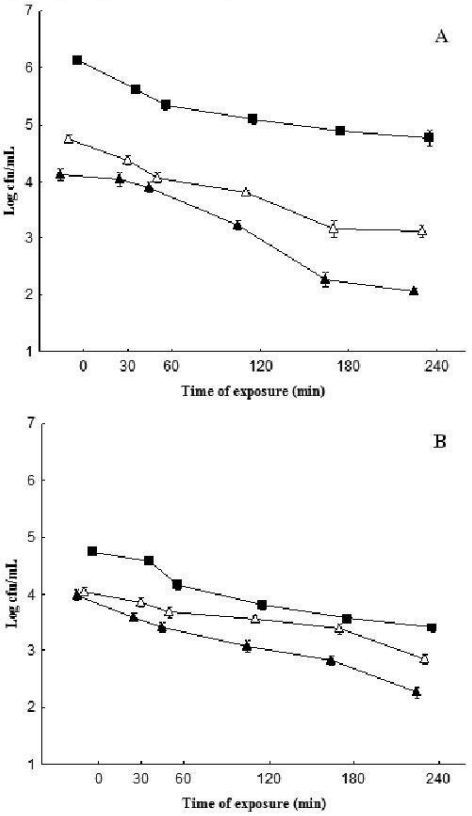
Both the *R. officinalis* EO and 1,8-cineole presented an MIC value of 40 µL/mL against *L. monocytogenes* ATCC 7644. Gutierrez et al. (2008) found an MIC of 20 µL/mL for *R. officinalis* EO against *L. monocytogenes* IL323 in tryptic soy agar, while Smith-Palmer et al. (1998) found an MIC for *R. officinalis* EO against *L. monocytogenes* 11994 of 2 µL/mL in tryptic soy broth (TSB). Although we have not found previous studies reporting the MIC value of 1,8-cineole against *L. monocytogenes*, Mulyaningsih et al. (2010) found MIC values of 32 and 64 µL/mL for this compound toward *Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 29213, respectively. Different MIC values for the EOs and their constituent compounds could be related to the microbial test strains or isolates used, the composition of the growth medium, or other intrinsic and extrinsic factors (de Souza et al. 2010).

At all concentrations tested (MIC, ½ MIC, and ¼ MIC), *R. officinalis* EO and 1,8-cineole inhibited the cell viability of *L. monocytogenes* ATCC 7644. Both tested antimicrobials caused inhibition of the bacterial cell viability after only 15 min of exposure, and no recovery in viable counts was noted after this point. The exposure of *L. monocytogenes* to ½ MIC, ¼ MIC, and the MIC of the antimicrobials caused a significant decrease ($P < 0.05$) in the viable cell count in comparison with the control assay. At the MIC and ½ MIC, *R. officinalis* EO established its bactericidal effect (≥3 $\log_{10}$ reduction of the initial inocula, i.e., ≥ 99.9% killed (LaPlante 2007)) after 60 min. At the MIC, 1,8-cineole established a bactericidal effect toward *L. monocytogenes* when assayed after 60 min of exposure. Both compounds showed dose- and time-dependent inhibitory effects on bacterial cell viability.

Karatzas et al. (2000) found that *R. officinalis* EO caused a decrease of 2.1 log cycles in the count of *L. monocytogenes* in BHI at 35 °C during 20 min of exposure. de Azerêdo et al. (2011) noted that the *R. officinalis* EO (20 µL/mL) used in our study caused a decrease in the count of viable cell of *L. monocytogenes* to 2.3 log cfu/mL over 120 min of exposure in a vegetable-based broth at 35 °C. In the same study, the authors identified 1,8-cineole (32.2 g/100 g), camphor (15.2 g/100 g), α-pinene (14.2 g/100 g), camphene (8.2 g/100 g), β-pinene (7 g/100 g), and limonene (5.6 g/100 g) as the main constituents of the *R. officinalis* EO. In another study, the same EO (20 µL/mL) caused a decrease in the glucose consumption and release of intracellular material in *L. monocytogenes* over 360 min of exposure in nutrient broth and caused marked morphological changes in the bacterial cells, including shrinkage and condensation of the cytoplasmic content and detachment of the cell wall from the plasma membrane after 6 h of exposure (de Azerêdo et al. 2012).

The overnight exposure of *L. monocytogenes* to sublethal amounts of both *R. officinalis* EO and 1,8-cineole (½ MIC and ¼ MIC) revealed no induction of direct bacterial protection over 240 min of exposure (Figs. 1A and 1B) according to the viable cell count. The kill-curves of *L. monocytogenes* previously challenged with both sublethal amounts of *R. officinalis* EO presented smaller viable counts ($P < 0.05$) when further cultivated in growth medium to which the same antimicrobial at the MIC was added compared with the nonadapted parents. This same behavior was found for the cells prechallenged with 1,8-cineole at ½ MIC. The exposure of *L. monocytogenes* to sublethal amounts of the antimicrobials resulted in no change in its intrinsic sensitivity to the homologous stressing agents because, in general, preadapted and non-preadapted cells revealed similar kill-curve shapes over the assessed time intervals.

Fig. 1. Survival curve of *Listeria monocytogenes* ATCC 7644 (at 37 °C in media to which *Rosmarinus officinalis* EO (A) and 1,8-cineole (B) was added at the MIC, 40 µL/mL) after overnight exposure to sublethal concentrations of the same stressing agent. ■, nonadapted cells (control); ▲, cells preadapted at ½ MIC, 20 µL/mL; Δ, cells preadapted at ¼ MIC, 5 µL/mL.



To the best of our knowledge, there are no studies evaluating the development of adaptation by *L. monocytogenes* exposed to sublethal amounts of EOs or their compounds. Most of the studies regarding the development of direct and cross-adaptation by *L. monocytogenes* have involved the challenge of simple or mixed cultures with classical chemical and physical antimicrobial procedures applied in food or food-processing environments (Tiganitas et al. 2009; Skandamis

et al. 2008; Soni et al. 2011). Alonso-Hernando et al. (2010) found that *L. monocytogenes* cells exposed to subinhibitory concentrations of acid decontaminants (citric acid, acidified sodium chlorite, and peroxyacids) showed higher percentages of survival than unexposed cells when cultivated in increasing amounts of the same stress agent in Mueller–Hinton broth.

To et al. (2002) assessed the behavior of *L. monocytogenes* strains that were either sensitive or resistant to benzalkonium chloride when subcultured progressively in increasing sublethal amounts (1–10 μg/mL) of the sanitizer for 24–48 h in TSB. In that study, at least a 5-fold increase in the MIC was noted in the sensitive strains; whereas, the MIC doubled for resistant strains. The adaptation of *L. monocytogenes* to decontaminant agents has been associated with changes in the fluidity and physical status of the cell membrane, the production of catalase, decreased activity of membrane-bound dehydrogenases, and decreased DNA damage (Mokgatla et al. 2002; Alonso-Hernando et al. 2010).

Koutsoumanis et al. (2003) evaluated the acid tolerance response (ATR) of 3- and 5-strain mixtures of *L. monocytogenes* previously grown in TSB with added glucose (1 g/100 mL — assumed to induce acid adaptation in cells). These authors found enhanced ATR in prechallenged cultures when compared with parent cultures grown in TSB without glucose. ATR is a known adaptive response in *L. monocytogenes* that provides protection against severe acid stress and cross-protection against heat, ethanol, oxidative, and osmotic stress (Gahan et al. 1996; O'Driscoll et al. 1996; Lou and Yousef 1997; van Schaik et al. 1999). ATR is also cited as a possible physiological advantage for *L. monocytogenes*, allowing it to overcome some of the host defense barriers, thereby enhancing its ability to cause an infection (O'Driscoll et al. 1996; Ferreira et al. 2003).

In agreement with the results obtained in the assays for the induction of direct bacterial protection, *L. monocytogenes* cells exposed overnight to sublethal concentrations (¼ MIC and ½ MIC) of *R. officinalis* EO and 1,8-cineole showed no induction of cross-protection against lactic acid (pH 5.2), salt (NaCl at 10 g/100 mL), or high temperature (45 °C) (Figs. 2A–2C, 3A–3C) according to the viable cell count over 240 min of exposure. Moreover, preadapting the bacteria with the antimicrobials at ½ MIC and ¼ MIC resulted in reduced viable counts ($P < 0.05$) following further exposure of the bacteria to the heterologous stressing agents (high temperature, low pH, and NaCl) compared with the non-preadapted cells.

Smaller counts (2–3 log cfu/mL) of preadapted *L. monocytogenes* cells were found in meat broth containing NaCl (Figs. 2B and 3B) compared with the counts noted in the meat broth containing lactic acid (Figs. 2A and 3A) or in those incubated at a high temperature (Figs. 2C and 3C). For some systems, the kill-curves of preadapted and non-preadapted cells revealed similar shapes over the evaluated interval times.

Skandamis et al. (2008) reported that exposure to sublethal stress did not affect the thermotolerance of *L. monocytogenes*; whereas, simultaneous exposure to multiple stresses (NaCl, 10 g/100 mL; HCl, pH 5.0; high temperature, 46 °C) concomitantly for 1.5 h in TSB resulted in increased tolerance of the bacterium to an acidic environment (HCl pH 3.5). Chorianopoulos et al. (2011) found that acid-adapted sessile

Fig. 2. Survival curve of *Listeria monocytogenes* ATCC 7644 in meat broth containing lactic acid, pH 5.2 (A); NaCl, 10 g/100 mL (B); or incubated at high temperature, 45 °C (C) after overnight exposure to sublethal concentrations of *Rosmarinus officinalis* EO. ■, non-preadapted cells (control); ▲, cells preadapted at ½ MIC, 20 μL/mL; Δ, cells preadapted at ¼ MIC, 10 μL/mL.

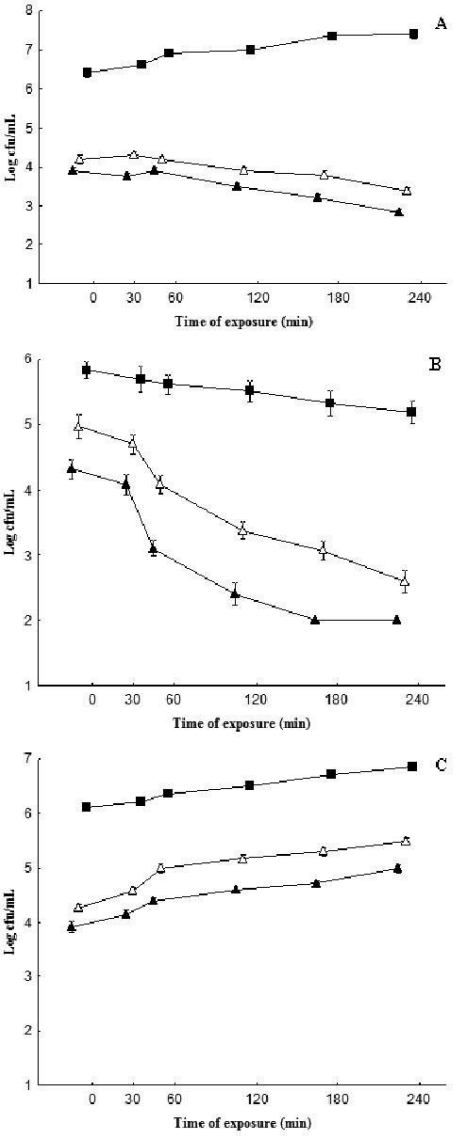
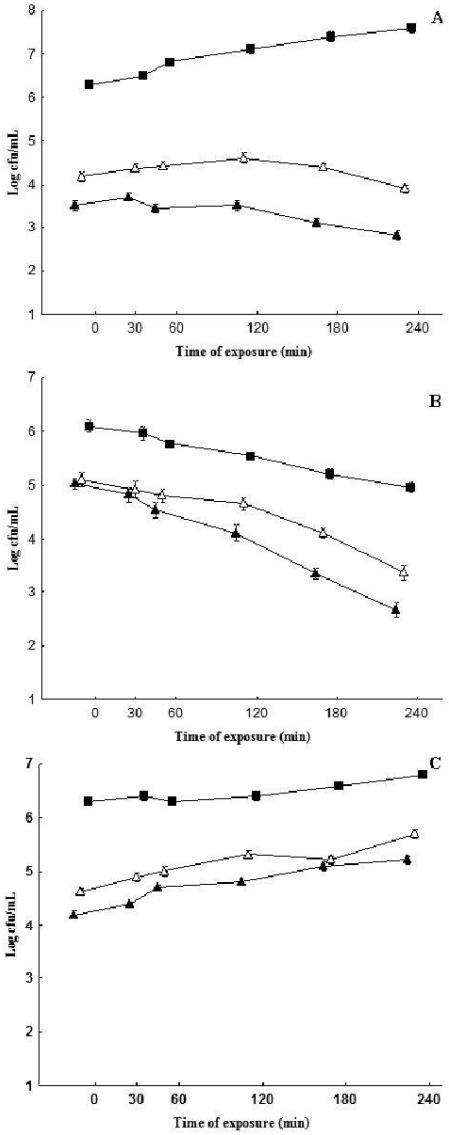


Fig. 3. Survival curve of *Listeria monocytogenes* ATCC 7644 in meat broth containing lactic acid, pH 5.2 (A); NaCl, 10 g/100 mL (B); or incubated at high temperature, 45 °C (C) after overnight exposure to sublethal concentrations of 1,8-cineole. ■, non-preadapted cells (control); ▲, cells preadapted at ½ MIC, 20 µL/mL; △, cells preadapted at ¼ MIC, 10 µL/mL.



cells of *L. monocytogenes* (named ATR cells) presented enhanced resistance to subsequent challenge with acid (exposure to pH 2.0 for 6 min; adjusted with either hydrochloric or lactic acid) and NaCl (5.5 g/100 mL) in BHI broth. In the same study, the authors found that ATR-planktonic cells demonstrated enhanced attachment to stainless steel surfaces following extended exposure to mildly acidic conditions (HCl pH 4.5).

Although the experimental conditions used in this study were applied in previous studies on the evaluation of the induction of direct or cross-adaptation in food-related bacteria (Jorgensen et al. 1999; Longbottom et al. 2004; Skandamis et al. 2008), possible limitations of the assays performed with the 2 tested sublethal concentrations of *R. officinalis* and 1,8-cineole and the chosen exposition time (18 h) for preadaptation exist. Thus, further experiments were performed to assess the induction of bacterial tolerance in *L. monocytogenes* when exposed to 24 h challenge cycles in meat broth with increasing amounts of the EO and 1,8-cineole (1/16 MIC to 4× MIC). The results of these assays showed that *L. monocytogenes* ATCC 7644 was able to survive (as demonstrated by the count of viable cells) in the broth containing antimicrobial concentrations up to ½ MIC, suggesting that the exposure of the cells to increasing sublethal amounts of both substances did not cause any development of tolerance. The addition of ½ MIC of the antimicrobials to growing cells inhibited further growth, which may have been due to cell injury. The exposure of exponentially growing bacterial cells to stressful but nonlethal conditions, such as those induced by the antimicrobials used here, has been shown to reduce bacterial viability over time (Rees et al. 1995). Bacterial cells injured by exposure to antimicrobials at concentrations lower than the MIC may develop an imbalance between anabolism and catabolism sufficient to disrupt growth, and although the metabolic rate of these cells is not affected, they are unable to form colonies on agar. This imbalance can also result in the self-destruction of the cells through free-radical attack (burst radical production), mainly due to a failure of the cell repair mechanisms (Dodd et al. 1997).

Other studies have also noted that the addition of different levels of inhibitors of protein synthesis, such as rifampicin and chloramphenicol, to growth media completely prevents the development of resistance to acid pH in *L. monocytogenes*, suggesting that protein synthesis is necessary for the development of resistance in bacteria (Flahaut et al. 1996; Hartke et al. 1996; Cebrián et al. 2010). Even at levels below the MIC, EOs and their compounds have shown the capability to suppress the synthesis and activity of enzymes in a number of food-related bacteria, causing inhibition of protein synthesis (Herbert et al. 2001; Nostro et al. 2001; Oliveira et al. 2010). Additionally, the inhibition of protein synthesis could be related to the inability of *L. monocytogenes* to develop direct or cross-adaptation to the conditions used in this study. Moreover, terpenes found in *R. officinalis* EO (such as 1,8-cineole, β-pinene, and α-terpinene) have the ability to disturb and penetrate the lipid structure of the bacterial cell wall, leading to protein denaturation, destruction of the cell membrane, cytoplasm leakage, cell lysis, and eventually death. The decrease in pH due to this terpene-induced cell membrane disruption results in a loss of control of cell pro-

cesses such as DNA transcription, protein synthesis, and enzyme activity (Oussalah et al. 2006; Raybaudi-Massilia et al. 2006; de Azerêdo et al. 2011).

In this study, there was no induction of direct or cross-protection in *L. monocytogenes* when the cells were exposed to sublethal concentrations of *R. officinalis* EO and 1,8-cineole in a food-based medium for a short (18 h) or long duration (24 h). This finding is interesting, as the development of homologous and heterologous resistance of *L. monocytogenes* has been well documented following the challenge of these cells with sublethal exposure to compounds or procedures classically used to control the growth and survival of microorganisms in foods (Bayles and Wilkinson 2000; Becker et al. 2000; Hill et al. 2002; Angelidis and Smith 2003).

The results obtained in this study reinforce the antimicrobial efficacy of the EO from *R. officinalis* and of the related compound 1,8-cineole for use in food conservation systems. Both of these antimicrobials have the ability to inhibit the growth and survival of *L. monocytogenes* ATCC 7644 and to induce nonhomologous and heterologous resistance to pH, high temperature, and salt in a food-based media.

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Anexo D

Título: Tolerance evaluation in *Salmonella enterica* serovar Typhimurium challenged with sublethal amounts of *Rosmarinus officinalis* L. essential oil or 1,8-cineole in meat model

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Tolerance evaluation in *Salmonella enterica* serovar Typhimurium challenged with sublethal amounts of *Rosmarinus officinalis* L. essential oil or 1,8-cineole in meat model

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Summary The induction of direct bacterial tolerance and cross-tolerance (NaCl, acid pH, high temperature) in *Salmonella* Typhimurium ATTC 14028 following the exposure to sublethal amounts of the essential oil from *Rosmarinus officinalis* L. (ROEO), and its major component 1,8-cineole (CIN) was evaluated in this study. Direct protection was not induced when cells were exposed to 1/2 MIC and 1/4 MIC of ROEO or CIN in meat broth and in previously irradiated meat ground-beef. Cells exposed to ROEO or CIN at sublethal amounts did not present cross-protection to high temperature, lactic acid and NaCl. Likewise, cells progressively subcultured in meat broth containing increasing amounts of ROEO or CIN were able to survive only up to 1/4 MIC for both tested substances. From these results, *S.* Typhimurium ATCC 14028 was not capable to develop direct or cross-tolerance when exposed to ROEO or CIN in a meat-based growth media and was not able to develop direct tolerance in a meat-based model.

Keywords Essential oil, pathogenic microorganism, *Salmonella*.

Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a ubiquitous bacterium that is widely spread in the nature and of great importance for public health because it is responsible for gastroenteritis outbreaks across the world (Gunduz *et al.*, 2009). The foodborne disease caused by *S.* Typhimurium mainly affects immunosuppressed and old people, and the symptoms include fever, diarrhoea, vomiting and abdominal cramps (Bajpai *et al.*, 2012). Foods of animal origin, especially meat products, are commonly the vehicles for transmission of *S.* Typhimurium, and these foods are related to most of the gastroenteritis outbreaks caused by this bacterium (Phillips *et al.*, 2008).

Food-related pathogenic bacteria have developed efficient systems for protection against a wide variety of unfavourable environmental conditions (Patrignani *et al.*, 2008). Early studies have reported the isolation of strains of *S.* Typhimurium with the capacity to survive and grow in stress conditions, such as low or high temperatures, acidic pH and high amounts of NaCl (Dubois-Brissonnet *et al.*, 2011; Luz *et al.*, 2012a).

Because of the special risk posed by *S.* Typhimurium to food safety and due the resistance of strains to classical compounds or procedures applied by industry to control microbial growth in foods (Ravishankar *et al.*, 2010), some researchers studied the possible use of essential oils and their related compounds as alternatives to control *S.* Typhimurium in different foods (Raybaudi-Massilia *et al.*, 2006). Previous studies found that the essential oil from *Rosmarinus officinalis* L. (ROEO) was effective for inhibiting food-related pathogenic bacteria, including *Salmonella* species (Tyagi & Malik, 2010; Azerêdo *et al.*, 2011). The antimicrobial property of ROEO has been attributed to the presence of the monoterpene 1,8-cineole (CIN), frequently found as the major component of this essential oil (Azerêdo *et al.*, 2011; Sousa *et al.*, 2012).

However, despite the use of ROEO or the related compound CIN as potential anti-*S.* Typhimurium compounds in food, there is a lack of information

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regarding the potential development of direct and/or cross-tolerance by this bacterium following exposure to sublethal amounts of these substances. Therefore, this study aimed to evaluate the development of tolerance in *S.* Typhimurium ATCC 14028 when the strain was challenged with sublethal amounts of these substances in meat broth and in meat-based model.

Materials and methods

ROEO and CIN

Essential oil from *Rosmarinus officinalis* L. (batch ROSTUN04; density at 20 °C: 0.94; refractive index at 20 °C: 1.51) obtained by steam distillation was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil), and its quality parameters were described in an accompanying technical report. CIN was purchased from Sigma-Aldrich (Sigma, St. Louis, France). A previous study (Azerêdo *et al.*, 2011) found CIN (32.2 g 100 g^{-1}) as the major component of the ROEO assayed here. Solutions of ROEO or CIN were prepared in nutrient broth (Himedia, Mumbai, India) in a range of concentrations (160–0.075 μL mL^{-1}) using bacteriological agar (0.15 g L^{-1}) as a stabilising agent (Mann & Markham, 1998).

Test strain

Salmonella enterica serovar Typhimurium ATCC 14028 (*S.* Typhimurium ATCC 14028) was obtained from the Collection of Reference Microorganisms at the National Institute of Control Quality in Health (FIOCRUZ, Rio de Janeiro, Brazil). A stock culture was kept on nutrient agar (Himedia) under refrigeration (7 °C). Unless stated otherwise, bacterial inoculum used in the assays was obtained from suspensions of the strain in stationary growth phase according to a previously described procedure (Luz *et al.*, 2012a). The obtained bacterial suspension was serially diluted in PBS (10^{-1}–10^{-3}) to provide a viable cell count of approximately 7 log CFU mL^{-1}.

Preparation of meat broth and meat-based model

Assays for the development of direct protection and bacterial cross-protection were carried out using a meat-based broth as the substrate for bacterial cultivation. Bovine meat steaks (200 g) were trimmed of all external fat and cut in pieces of uniform sizes (3 × 3 × 3 cm) and heated in distilled water (600 mL) for 30 min at 90 °C. Approximately 500 mL of meat broth was obtained and vacuum filtered using Whatman no 1 filters. The filtrate was sterilised via autoclave for 15 min (1.21 atm). Afterwards, the broth was stored at −20 °C in aliquots of 50 mL, and when required one aliquot was thawed under refrigeration (7 ± 1 °C) and used for the assays (Oliveira *et al.*, 2010).

Raw lean ground beef, purchased from a local retail supermarket in João Pessoa (Brazil), was used for evaluation of the development of bacterial direct tolerance in a food micro model. The ground beef was separated by batches into stomacher bags (30 g bag^{-1}) for different treatments, vacuum sealed, frozen at −20 °C and irradiated (25 kGy; 2 h) to eliminate indigenous microflora. Afterwards, the bags were stored at −20 °C, and when required the bags were thawed under refrigeration (7 ± 1 °C) and used for the assays (Juneja *et al.*, 2010). Random samples of the irradiated ground beef were tested to verify elimination or inactivation of microflora by diluting (1:1) in PBS, followed by surface plating the suspension (0.1 and 1 mL) onto nutrient agar and incubating aerobically at 37 °C for 48 h.

Determination of the minimum inhibitory concentration

Minimum inhibitory concentration values of ROEO and CIN were determined using macro-dilution in broth using screw-capped tubes. Four millilitres of double-strength nutrient broth was inoculated with 1 mL of the bacterial inocula, mixed with 5 mL of the antimicrobial solution (viable cells count of approximately 6 log CFU mL^{-1}) and vortexed for 30 s. The system was incubated statically for 24 h at 35 °C. The MIC was defined as the lowest concentration of ROEO or CIN required preventing visible bacterial growth (Nostro *et al.*, 2001). Control flasks without ROEO or CIN were tested similarly.

Evaluation of the induction of bacterial direct tolerance in meat broth

The induction of bacterial direct tolerance was performed by an overnight exposure of the bacterium to subinhibitory amounts of ROEO or CIN in meat broth according to a previously described procedure (Luz *et al.*, 2012a,b). Two millilitres of the bacterial suspension was inoculated in 18 mL of the meat broth containing ROEO or CIN (final concentration of 1/2 MIC or 1/4 MIC) in screw-capped tubes and shaken for 30 s using a vortexer (adaptation treatment with viable cells count of approximately 6 log CFU mL^{-1}). Control flasks without the antimicrobials were assayed similarly as the control (nonadaptation treatment). The systems were incubated statically overnight (18 h at 35 °C). After the incubation period, an aliquot (2 mL) of each treatment was inoculated into fresh meat broth (18 mL) containing ROEO or CIN (final concentration of the MIC previously determined), shaken for 30 s using a vortexer and incubated statically at 35 °C. Viable cells were counted at various time

points (0, 30, 60, 120, 180 and 240 min) by serial dilution (10^{-1}–10^{-5}) in PBS and by plating in nutrient agar for 24–48 h at 35 °C. The results were expressed as the log CFU mL^{-1}. The induction of bacterial direct protection was assessed by comparing the viable cells over time in the suspensions submitted and not submitted to the adaptation treatment (inoculated in growth media with the same stressing agent at the full MIC value).

Evaluation of the induction of bacterial cross-protection in meat broth

The induction of bacterial cross-protection was performed by an overnight exposure of the bacterium to sublethal amounts of ROEO or CIN in meat broth, followed by exposure to other stressing agents (high temperature, low pH and NaCl) according to previously described procedure (Gomes Neto *et al.*, 2012). Preliminary experiments were performed for evaluating the thermotolerance, acid tolerance and salt tolerance of the test bacterial strain. Untreated cultures were inoculated in meat broth containing NaCl (1–5 g L^{-1}, at 35 °C), lactic acid (pH 4.5–6.0, at 35 °C) and in meat broth incubated at different temperatures (40–55 °C) to determine the concentration of NaCl, pH value and temperature that inhibited moderately the growth of the cell suspension. After the establishment of these conditions, a 2 mL aliquot of a fresh bacterial suspension was inoculated in 18 mL of the meat broth containing ROEO or CIN (final concentration of the 1/2 MIC or 1/4 MIC) in screw-capped tubes and shaken for 30 s using a vortexer (adaptation treatment). Control flasks without the antimicrobials were assayed similarly (nonadaptation treatment). The systems were incubated statically overnight (18 h at 35 °C; final viable cell counts for pre-adapted cells were always between 5 and 6 log CFU mL^{-1}, and the viable cell counts for the control assays were always between 6 and 7 log CFU mL^{-1}). After the incubation period, an aliquot (2 mL) of each treatment was inoculated into a fresh meat broth (18 mL) acidified with lactic acid (VETEC Ltda., Rio de Janeiro, Brazil) to a pH of 5.2 and in a fresh meat broth supplemented with NaCl (Qeel, São Paulo, Brazil) at 3 g L^{-1} for the evaluation of the induction of acid tolerance and osmotolerance, respectively, and incubated statically at 35 °C. For analysis of the induction of thermotolerance, an aliquot (2 mL) of each treatment was inoculated into a fresh meat broth (18 mL) and incubated statically at 45 °C. The number of viable cells in the systems was counted at various time points (0, 30, 60, 120, 180 and 240 min) by serial dilution (10^{-1}–10^{-5}) in PBS and by plating in nutrient agar for 24–48 h at 35 °C. The results were expressed as the log CFU mL^{-1}. The induction of bacterial cross-protection was assessed by comparing the viable cells counts at various time points of the suspensions submitted or not submitted to the adaptation treatments and inoculated in growth media containing or exposed to the other stressing agent.

Evaluation of the induction of bacterial direct protection in a meat-based model

The induction of direct protection in *S.* Typhimurium using a meat-based model was performed by exposure of the bacterium to sublethal amounts of ROEO and CIN in samples of irradiated ground beef stored at 7 °C. Samples of ground beef (30 g per commercially sterile sealed bag) containing ROEO or CIN (final concentrations of 1/2 MIC or 1/4 MIC) were inoculated with 3 mL of the bacterial suspension, blended with a stomacher (Model MA440; Marconi Ltda., Piracicaba, Brazil) for 5 min to ensure even distribution of the microorganism in the meat sample and incubated overnight at 7 °C. Thereafter, the ROEO or CIN (final concentration of MIC) was added to the inoculated ground beef samples, which were blended again with a stomacher for 5 min and incubated at 7 °C. Systems (bags) without the ROEO or CIN were assayed similarly (nonadaptation treatment). The number of viable cells in the systems was counted at various time points (0, 1, 2, 3, 4, 24, 48 and 72 h) by serial dilution (10^{-1}–10^{-5}) in PBS and by plating in nutrient agar for 24–48 h at 37 °C. The results were expressed as the log CFU mL^{-1}. The induction of bacterial direct protection was assessed by comparing the viable cells counts at various time points in the suspensions submitted and not submitted to the adaptation treatments in meat samples when exposed to ROEO or CIN at their MIC values in the same food matrix.

Evaluation of the induction of bacterial direct tolerance throughout successive 24-h habituation cycles

The capacity of *S.* Typhimurium to develop tolerance to ROEO or CIN was also assessed through exposure of the bacterium to increasing amounts of the antimicrobials (1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC and 2 × MIC) throughout successive 24-h habituation cycles in meat broth, according to a previously described procedure (Gomes Neto *et al.*, 2012), including a prolonged exposure time. In this procedure, 2 mL of the bacterial suspension was inoculated into 18 mL of the meat broth supplemented with ROEO or CIN (final concentration 1/16 of the MIC) in screw-capped tubes, shaken for 30 s using a vortexer (viable cells counts of approximately 6 log CFU mL^{-1}) and incubated statically for 24 h at 35 °C. After the incubation period, a 100 μL aliquot of the system was serially diluted (10^{-1}–10^{-5}) in PBS and inoculated onto

sterile nutrient agar for viable cell detection (35 °C for 24 h). Concomitantly, a 2 mL aliquot of the meat broth containing ROEO or CIN at 1/16 MIC (allowing for bacterial growth) was inoculated into fresh meat broth containing ROEO or CIN in the next tested higher concentration (1/8 MIC). This culture was incubated statically at 35 °C, and viable cell counts were determined according to the previously described serial dilution procedures. This procedure was repeated, exposing the bacteria to increasing concentrations of ROEO or CIN (1/4 MIC–2 × MIC) until no viable cells could be detected.

The detection limit of the method used for counting the viable cells was 2 log CFU mL^{-1} for all assays.

Reproducibility and statistics

The assays were performed in triplicate on two separate occasions, and the results were expressed as an average of the assays. Statistical analysis was performed to determine significant differences ($P < 0.05$) by ANOVA followed by a Tukey's test using the SigmaStat 3.1 computer program.

Results and discussion

Essential oil from *Rosmarinus officinalis* L. and CIN presented MIC values of 20 and 80 μL mL^{-1}, respectively, against *S.* Typhimurium ATCC 14028. The exposure of this bacterium to sublethal amounts (MIC/2 and MIC/4) of ROEO or CIN for 18 h did not result in the development of direct tolerance, according to the strain's survival/growth profile when further cultivated for 240 min in meat broth containing either ROEO or CIN at the previously determined MIC (Figure S1a,b). *S.* Typhimurium cultivated in meat broth containing the tested sublethal amounts of ROEO exhibited lower viable cells counts ($P < 0.05$) compared with the control (nonadapted cells), with a linear decrease in cells counts during the assessed interval times. *S.* Typhimurium cultivated in broth without ROEO or CIN presented a smaller decrease in viable cell counts during the assessed period.

In agreement with the results found using assays for the development of direct tolerance, the development of cross-tolerance in *S.* Typhimurium to low pH, NaCl and high temperature following the exposure to the tested sublethal amounts of ROEO or CIN in meat broth was not observed (Figures S2 and S3). The survival behaviour (kill-curve shape) presented by the adapted and nonadapted cells when further exposed to the heterologous stressing agents was similar for most of the assessed systems. The systems including ROEO or CIN at sublethal amounts and further exposed to NaCl showed decrease in viable cell counts over the assessed time intervals, although these counts were always lower ($P < 0.05$) than those found in the control assays (Figures S2b and S3b). Otherwise, the cells previously treated with the sublethal amounts of ROEO or CIN when further exposed to low pH and high temperature revealed a slight, but linear, increase in viable cell counts during the assessed time intervals (Figures S2a,c and S3a,c); however, these counts were also always lower ($P < 0.05$) than those exhibited by the untreated cells (control assays) with the exception of cells pre-exposed to ROEO and further cultivated in low pH, which revealed similar counts ($P > 0.05$) to the control assays (Figure S2a). This behaviour of progressive increases in viable cells counts of *S.* Typhimurium pre-challenged with ROEO or CIN and further exposed to low pH and high temperature could be related to an intrinsic feature of the assayed strain rather than an adaptive response to these stressing agents.

These findings of no induction of direct and cross-tolerance in *S.* Typhimurium cells prechallenged with sublethal concentrations of ROEO or CIN in a food-based medium are remarkable, as the development of homologous and heterologous tolerance in *S.* Typhimurium challenged with sublethal conditions provided by others antimicrobial compounds or procedures used to control microbial growth and survival in foods is already documented (Dubois-Brissonnet *et al.*, 2011). The exposure of *S.* Typhimurium LT2 to mildly acidic conditions (pH 5.8, adjusted with HCl) in BHI broth has been demonstrated to enhance tolerance towards various stresses, including heat (50 °C), salt (NaCl 2.5 M) and the surface-active agents crystal violet (5000 mg L^{-1}) and polymyxin B (5000 mg L^{-1}; Laplante, 2007). Similarly, the cultivation of *S.* Typhimurium 14028s in the presence of short-chain fatty acids (acetate, butyrate and propionate) induced acid resistance (pH 3.0, adjusted with HCl; Kwon & Ricke, 1998).

The cultivation of *S.* Typhimurium in irradiated ground beef samples containing sublethal amounts of ROEO or CIN revealed no increased tolerance to these compounds, reinforcing the findings of no changes in tolerance observed in assays with meat broth (Figure S4a,b). Compared with the non-pre-adapted cells, the cells pre-adapted to ROEO in ground beef over 72 h revealed smaller viable cells counts ($P < 0.05$) when they were further cultivated in the same food matrix containing ROEO or CIN at the MIC (previously determined) over 72 h (Figure S4a). Cells pre-adapted to CIN at 1/4 MIC revealed viable cells counts ($P > 0.05$) similar to the control assay (nonadapted cells) when they were further cultivated in the same meat model containing CIN at the MIC over 72 h, while cells pre-adapted with CIN at MIC/2 revealed smaller viable cells counts ($P < 0.05$) compared with the control assay over the assessed time intervals (Figure S4b).

The literature on the tolerance of *S.* Typhimurium exposed to essential oils or their compounds is limited; therefore, it is difficult to make an extensive comparative discussion of the obtained results, especially regarding food matrices. Within this context, cells of *Escherichia coli* O157:H7 grown in moderately acidic medium (pH 5.0) showed increased resistance to lactic acid (pH 3.8), as well as an increased ability to survive in acidic foods, such as apple cider and fermented sausages for over 120 h (Leyer *et al.*, 1995). Cells of *S. enterica* serovar Typhimurium CECT 443 and *S. enterica* serovar Senftenberg CECT 4384 showed greater resistance when exposed to lethal pH (2.5) in orange juice and apple juice (acidic foods) in comparison with the counterparts grown in buffered BHI broth (pH 7.0, nonadapted cells; Álvarez-Ordóñez *et al.*, 2009).

Cells of *S.* Typhimurium exposed (24 h cycles) to increasing amounts of ROEO or CIN survived (as demonstrated by viable cell counts) in meat broth containing these compounds in concentrations up to MIC/4, revealing a onefold decrease in the value of the MIC previously determined. The inhibition of the cells already present in the broth upon the addition of sublethal amounts of the tested substances could be related to the manifestation of cell injury; because when bacterial cells are kept continuously exposed in a stressing but nonlethal environment, as was provided by the sublethal amounts of the ROEO or CIN, the cells may lose viability and the capacity to survive over time (Gomes Neto *et al.*, 2012). It has been hypothesised that bacterial cells injured by exposure to antimicrobials, such as essential oils and their constituents, at concentrations lower than the MIC may develop an imbalance between anabolism and catabolism sufficient to disrupt growth, making them unable to form colonies on agar (Dodd *et al.*, 1997).

Some authors have proposed that the protein synthesis is required for the development of tolerance in bacteria (Hartke *et al.*, 1996; Cebrián *et al.*, 2010) and that essential oils and their related compounds (even at levels lower than their MIC) are able to disturb (or even suppress) the synthesis and enzymatic activity in a number of food-related bacteria. The inability of *S.* Typhimurium ATCC 14028 to mount an tolerance response to the conditions used in this study could be related to the capability of ROEO (and of the terpenes found therein, such as CIN, β-pinene and α-terpinene; Azerêdo *et al.*, 2011), to cause cell membrane disruption resulting in loss of control of cell processes, such as DNA transcription, protein synthesis and enzyme activity (Raybaudi-Massilia *et al.*, 2006).

To the best of our knowledge, this study reveal for first time, that a strain of *S.* Typhimurium cannot develop direct protection or cross-tolerance (low pH, high temperature and NaCl) when exposed to sublethal amounts of ROEO or CIN for a short or prolonged exposure time in a food-based growth media and in a meat-based model. The lack of induction of homologous and heterologous tolerance by ROEO or CIN reinforces the possibility of using these substances to inhibit *S.* Typhimurium in foods, particularly in meat products.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Survival curve of *S.* Typhimurium ATCC 14028 in meat broth at 37 °C, and in meat broth containing the *R. officinallis* L. essential oil at MIC – 20 μL mL^{-1} (a) or 1,8-cineole – MIC 80 μL mL^{-1} (b) after overnight exposure to sublethal concentrations of the same stressing agent.

Figure S2. Survival curve of *Salmonella* Typhimurium ATCC 14028 in meat broth containing lactic acid – pH 5.2 (a), NaCl – 10 μL mL^{-1} (b) and incubated at high temperature −45 °C (c) after overnight exposure to sublethal concentrations of *R. officinalis* L. essential oil.

Figure S3. Survival curve of *Salmonella* Typhimurium ATCC 1428 in meat broth containing lactic acid – pH 5.2 (a), NaCl – 10 μL mL^{-1}. (b) and incubated at high temperature −45 °C (c) after overnight exposure to sublethal concentrations of 1,8-cineole.

Figure S4. Survival curve of *S.* Typhimurium ATCC 14028 in irradiated ground beef (at 7 °C, containing the *R. officinalis* L. essential oil at MIC – 20 μL mL^{-1} after exposure to sublethal concentrations of *R. officinalis* L. essential oil (a) or containing 1,8-cineole at MIC – 80 μL mL^{-1}) after exposure to sublethal concentrations of 1,8-cineole (b).

Anexo E

Título: Influence of general stress-response alternative sigma factors σ^S (RpoS) and σ^B (SigB) on bacterial tolerance to the essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. and pulsed electric fields

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Short communication

Influence of general stress-response alternative sigma factors σ^S (RpoS) and σ^B (SigB) on bacterial tolerance to the essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. and pulsed electric fields

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ABSTRACT

This study assessed the influence of general stress-response alternative sigma factors RpoS (σ^S) and SigB (σ^B) on tolerance of *Escherichia coli* (*E. coli* MG1655 and its isogenic mutant *E. coli* MG1655 Δ*rpoS*) and *Listeria monocytogenes* (*L. monocytogenes* EGD-e and its isogenic mutant *L. monocytogenes* EGD-e Δ*sigB*) to the essential oils (EOs) from *Origanum vulgare* L.—oregano (OVEO) and *Rosmarinus officinalis* L.—rosemary (ROEO), as well as the changes in tolerance of parental and Δ*rpoS* and Δ*sigB* mutant strains to OVEO, ROEO and pulsed electric fields (PEF) following overnight exposure to subinhibitory concentrations (1/2 × minimum inhibitory concentration—MIC) of each tested EO. MIC values of OVEO and ROEO against the mutant cells were usually lower than those found against the parental cells. Survivor curves showed that mutant cells were more sensitive to these EOs than parental cells. The recovery of survivors in selective media showed a greater proportion of cells sublethally injured at their cell envelopes in the mutant strains compared with the parental strains. Induction of increased direct-tolerance to OVEO and ROEO or cross-tolerance to PEF was not observed after pre-exposure of parental and mutant cells to EOs. Otherwise, parental and mutant cells of *E. coli* and *L. monocytogenes* pre-exposed to OVEO or ROEO showed decreased tolerance when further treated with the homologous stressing agent at 2 × MIC. Still, mutant cells pre-exposed to OVEO or ROEO showed lower tolerance to PEF than parental strains. These results showed the influence of σ^S and σ^B in tolerance of single strains of *E. coli* and *L. monocytogenes*, respectively, to OVEO and ROEO. Moreover, the deletion of σ^S and σ^B resulted in decreased tolerance to OVEO, ROEO or PEF in tested strains following exposure to OVEO or ROEO at a subinhibitory concentration.

1. Introduction

Food-related pathogenic bacteria have developed efficient systems for protection against a wide variety of unfavorable environmental conditions that are commonly applied during the food processing or storage, such as low or high temperatures, acidic pH and high amounts of salts (Di Pasqua et al., 2006). *Escherichia coli* and *Listeria monocytogenes* are often cited as able to mount protection responses to different stressing conditions normally applied in food conservation (Luz et al., 2012; Ryu et al., 2012). Most *E. coli* are harmless to humans, however some *E. coli* strains are pathogenic causing diarrhea or illness outside of the intestinal tract (Ryu et al., 2012; Chueca et al., 2015). *L. monocytogenes* is a foodborne pathogen that causes listeriosis, a life-threatening invasive disease in humans (Timmermans et al., 2014).

One important aspect of bacterial resistance is gene regulation by activation of stress response alternative sigma factors which leads to the transcription of the set of sigma-regulated genes (Abee and Wouters, 1999; Dodd and Aldsworth, 2002; O'Byrne and Karatzas, 2008). The stress response mediated by sigma factors RpoS (σ^S), encoded by *rpoS*, and SigB (σ^B), encoded by *sigB*, are well described in gram-negative and gram-positive bacteria, respectively, exposed to a range of harsh conditions. The σ^S factor controls transcription of different types of proteins (such as enzymes, membrane proteins and regulatory proteins) that are active in the protection and repair of damage in *E. coli* cells exposed to osmotic stress conditions, changes in pH, high temperatures and presence of antimicrobial compounds (Ait-Ouazzou et al., 2011; Somolinos et al., 2008). Similarly, it has been observed

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that expression of the alternative σ^B factor mediated the stress response in *L. monocytogenes* cells exposed to high salt concentration, low pH, high pressure, refrigeration temperatures or classical antimicrobial compounds (O'Byrne and Karatzas, 2008; Somolinos et al., 2010a; Van Schaik and Abee, 2005). Increased tolerance phenomena as a consequence of sublethal stress have also been demonstrated to be regulated, among others, by alternative sigma factors in *E. coli* (Vidovic and Korber, 2014) and *L. monocytogenes* (Chaturongakul et al., 2008; Chung et al., 2006).

Because of the development of increased bacterial tolerance to classical compounds or thermal treatment applied by industry to control microbial growth in foods, essential oils (EOs) and the non-thermal technology pulsed electric fields (PEF) have been proposed as alternatives to control these organisms by food industry (Luz et al., 2012; Timmermans et al., 2014; Chueca et al., 2015). Additionally, consumers have demanded safer, fresher and healthier foods with no or few chemical preservatives, creating a market demand for natural, non-thermal and feasible technologies for ensuring the microbial safety of foods (Sivakumar and Bautista-Baños, 2014). The EOs from *Origanum vulgare* L.—oregano (OVEO) and *Rosmarinus officinalis* L.—rosemary (ROEO) have shown broad-spectrum antimicrobial activity against a range of food-related bacteria in synthetic media and in mimicking food-models (Azerêdo et al., 2011; Gomes-Neto et al., 2014; Luz et al., 2012). PEF has presented efficacy in bacterial inactivation to guarantee food safety, and in contrast to heat treatments presents minimal impact on sensory and nutritional food properties (Espina et al., 2013; Chueca et al., 2015).

Since the incorporation of high doses of EOs may adversely affect the sensory aspects of foods (Azerêdo et al., 2011), minimization of the concentrations of these substances in foods has been strongly recommended, mainly when applied in sequence or in combination with other selected physical inactivation techniques, such as PEF (Ait-Ouazzou et al., 2011; Chueca et al., 2015). Despite the fact that EOs are known as potential antimicrobials for use in emerging non-thermal food preservation procedures (Ait-Ouazzou et al., 2012; Gomes-Neto et al., 2012), the investigations about the possible induction of increased bacterial cross-tolerance to PEF by EOs at subinhibitory concentrations (as usually possible to be applied in foods) are still scarce or inexistent. Moreover, it is interesting to carry out these studies because PEF possesses a well-known and simple mechanism of bacterial inactivation in which target structures are mainly cell envelopes, as observed for most of the EOs individual constituents (Espina et al., 2014).

Considering these aspects, this study was carried out with the following objectives: (i) to assess the influence of general stress-response alternative sigma factors σ^S and σ^B on the tolerance of single cultures of *E. coli* and *L. monocytogenes* to OVEO and ROEO, as measured through the determination of the minimum inhibitory concentration, effects on bacterial survival and the occurrence of cell injuries; (ii) to evaluate changes in tolerance of parental and *rpoS* and *sigB* mutant cells of *E. coli* and *L. monocytogenes* to OVEO, ROEO and PEF following exposure to each of the tested EOs at a subinhibitory concentration.

2. Material and methods

2.1. Test strains and inoculum

The test strains used in this study were *E. coli* MG1655 and its isogenic deletion mutant Δ*rpoS* (*E. coli* MG1655 Δ*rpoS*), and *L. monocytogenes* EGD-e and its isogenic deletion mutant Δ*sigB* (*L. monocytogenes* EGD-e Δ*sigB*). The *E. coli* MG1655 strain was tested because it has been used in physiological studies approaching the bacterial tolerance to antimicrobial compounds or procedures in Gram-negative cells (Minty et al., 2011; Fontenot et al., 2013; Chueca et al., 2015), as well as *L. monocytogenes* EDG-e has been tested representing Gram-positive cells (van der Veen and Abee, 2010; Somolinos et al., 2010a; Ait-Ouazzou et al., 2012). The *rpoS* mutant was constructed using P1 phage transduction and was derived from an *E. coli* single-gene knockout library (Baba et al., 2006). Positive P1 transductants were confirmed by acquisition of kanamycin resistance and PCR. Removal of the kanamycin-resistance cassette was accomplished using the pCP20 plasmid (Datsenko and Wanner, 2000). The mutant *L. monocytogenes* EGD-e Δ*sigB* was kindly provided by Prof. Chakraborty (Institute for Medical Microbiology, Giessen, Germany). This mutant was constructed by using the temperature-sensitive suicide plasmid pAUL-A, resulting in an in-frame chromosomal deletion (Chatterjee et al., 2006). Stock cultures were maintained in cryovials at − 80 °C. The inocula (7 $\log_{10}$ CFU/mL) used in assays were obtained from overnight (stationary phase) cultures grown on TSAYE according to a previously described procedure (Ait-Ouazzou et al., 2012). This study was performed with stationary phase cells because bacterial resistance to food preservation technologies is usually maximum at this growth stage (Mañas and Pagán, 2005).

2.2. Essential oils

OVEO (batch OREORG01) and ROEO (batch ROSTUN04) obtained by steam distillation were purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil). Emulsions of OVEO or ROEO (10–600 ppm) were prepared using TSBYE containing Tween 80 (1%, *v*/*v*; Sigma-Aldrich, USA) as an emulsifier. At the highest assayed concentration (1%, *v*/*v*), Tween 80 presented no inhibitory effect against the bacterial strains tested. The OVEO and ROEO presented carvacrol (67.1 g/100 g) and 1,8-cineole (32.2 g/100 g) as major constituent, respectively (Azerêdo et al., 2011; Luz et al., 2014).

2.3. Determination of the minimum inhibitory concentration (MIC)

MIC values of OVEO and ROEO were determined using macrodilution in broth method (Ait-Ouazzou et al., 2011). Four milliliters of double-strength TSBYE was inoculated with 1 mL of the bacterial inoculum, mixed with 5 mL of OVEO or ROEO emulsions (viable cell counts of approximately 7 $\log_{10}$ CFU/mL) and vortexed for 30 s (viable cell counts of approx. 7 $\log_{10}$ CFU/mL). The system was incubated under stirring (130 rpm) for 24 h at 37 °C or 30 °C for *E. coli* and *L. monocytogenes* strains, respectively. The MIC was defined as the lowest concentration of OVEO or ROEO at which bacteria failed to grow, with no visible changes detected in the bacterial growth medium.

2.4. Effects of OVEO and ROEO on bacterial survival

An aliquot (1 mL) of each microbial suspension was centrifuged (6000 ×*g* for 5 min) and re-suspended for a final concentration of approximately 7 $\log_{10}$ CFU/mL in sterile McIlvaine buffer at pH 7.0 containing OVEO or ROEO (final concentration of 2 × MIC) followed by incubation under stirring (130 rpm) at the appropriate temperature. Viable cells were enumerated over time (0, 10, 20, 40, 50, 60, 70 and 80 min) by serial dilution in PBS and subsequent plating in TSAYE for 24–48 h at 37 °C or 30 °C for *E. coli* and *L. monocytogenes* strains, respectively. The results were expressed as the reduction in bacterial counts ($\log_{10}$ CFU/mL) in relation to the initial bacterial population—CFU/mL at time zero ($\log_{10} N_0 - \log_{10} N$; where N_0 was the initial count at time zero and N was the count after incubation for each indicated time at appropriate temperature).

2.5. Detection of sublethal injury

To assess whether OVEO and ROEO cause sublethal injury damage in test strains, cultures (final viable cell count of approximately 7 $\log_{10}$ CFU/mL) were initially exposed to OVEO or ROEO at 2 × MIC in TSBYE for 10 min under stirring (130 rpm) at 37 °C or 30 °C for *E. coli* and *L. monocytogenes*, respectively. After this period, cells were then plated on TSAYE supplemented with sodium chloride (NaCl, 3 g/100 mL for *E. coli* and 6 g/100 mL for *L. monocytogenes*; TSAYE-SC) or TSAYE

supplemented with bile salts (0.35 g/100 mL for *E. coli*; TSAYE-BS) and incubated for 24 h longer than those containing non-selective medium at appropriate temperature for each tested bacteria. The assayed concentrations of NaCl and bile salts were previously determined to be the maximum non-inhibitory concentration for the stationary-phase cells of each of the tested strain (data not shown). Systems without OVEO or ROEO were assayed similarly as control (essential oils non-treated cells). The extent of sublethal injury in cytoplasmic and outer membrane caused by bile salts and NaCl, respectively (Mackey, 2000) was expressed as $\log_{10}$ CFU/mL reduction cycles regarding the viable cell counts ($\log_{10}$ CFU/mL) in suspensions treated with OVEO or ROEO and plated on the selective medium (TSAYE-SC or TSAYE-BS) and on the non-selective medium (TSAYE) in comparison with the viable cell counts in control assays (Mackey, 2000; Somolinos et al., 2010b).

2.6. Effects on response to homologous stressing agent

The assays to evaluate possible changes in bacterial tolerance to OVEO or OREO after exposure of the test strains to subinhibitory concentration of OVEO or ROEO in TSBYE were performed according to a previously described procedure (Gomes-Neto et al., 2012, 2014; Luz et al., 2014). For this, 2 mL of the bacterial suspension was inoculated in 18 mL of TSBYE containing OVEO or ROEO (final concentration of 1/2 MIC) in screw-cap tubes and vortexed for 30 s (final viable cell counts of approx. 7 $\log_{10}$ CFU/mL). The systems were incubated under stirring (130 rpm) for 18 h at 37 °C or 30 °C for *E. coli* and *L. monocytogenes* strains, respectively. After the incubation period, an aliquot (1 mL) of each microbial suspension was centrifuged (6000 ×*g* for 5 min) and re-suspended for a final bacterial population of approximately 7 $\log_{10}$ CFU/mL in sterile McIlvaine buffer at pH 7.0 containing OVEO or ROEO (final concentration of 2 × MIC) followed by incubation under stirring (130 rpm) at the appropriate temperature. Viable cells were enumerated over time (0, 10, 20, 40, 50, 60, 70 and 80 min) by serial dilution in PBS and by plating in TSAYE for 24–48 h at 37 °C or 30 °C for *E. coli* and *L. monocytogenes* strains, respectively. Control systems without OVEO or ROEO were assayed similarly as control (non-pre-exposure treatment). The results were expressed as the reduction in viable cell counts ($\log_{10}$ CFU/mL fraction) in bacterial population—CFU/mL at time zero ($\log_{10} N_0 - \log_{10} N$, where N_0 was the initial count at time zero and N was the count after incubation for each time at appropriate temperature).

2.7. Effects on response to heterologous stressing agent (PEF)

The evaluation of possible changes in bacterial tolerance to PEF after exposure of the test strains to subinhibitory concentration of OVEO or ROEO in TSBYE was performed according to previously described procedures (García et al., 2005; Gomes-Neto et al., 2012, 2014). For this, 2 mL of the bacterial suspension was inoculated in 18 mL of TSBYE containing OVEO or ROEO (final concentration of 1/2 MIC) in screw-cap tubes and vortexed for 30 s (viable cell counts of approximately 7 $\log_{10}$ CFU/mL). Control systems without OVEO or ROEO were assayed similarly as control (non-pre-exposure treatment). The systems were incubated under stirring (130 rpm) for 18 h at 37 °C or 30 °C for *E. coli* and *L. monocytogenes* strains, respectively. After the incubation period, an aliquot (2 mL) of each treatment was centrifuged (6000 rpm for 5 min, 4 °C), re-suspended in fresh TSBYE, and 1 mL was inoculated in 10 mL of sterile McIlvaine buffer at pH 7.0 (final viable cell counts standardized again to approx. 7 $\log_{10}$ CFU/mL), and the electrical conductivity of the system was adjusted to 2 mS/cm (similar to food products). For PEF treatment, an aliquot of 0.5 mL of each system tested was placed into the treatment chamber with a sterile syringe and submitted to exponential waveform pulses at electrical field strength of 30 kV/cm, with pulse repetition rate of 1 Hz and specific energy input of each pulse of 4.7 kJ/kg. Experiments started at room temperature (22 °C, ± 1 °C), and in all experiments the temperature of the samples after the application of 50 pulses (1 Hz, pulse width 2 μs) was lower than 35 °C. PEF treatment was carried out using equipment that delivered exponential-decay pulses, as previously described (García et al., 2005). After PEF treatment, viable cells were enumerated by serial dilution in PBS, plating in TSAYE for 24–48 h and incubated at appropriate temperature. The results were expressed as the reduction in bacterial counts ($\log_{10}$ CFU/mL) in relation to the initial bacterial population—CFU/mL at time zero ($\log_{10} N_0 - \log_{10} N$, where N_0 was the initial count at time zero and N was the count after exposure to each treatment).

The detection limit of the viable cell detection was 2 $\log_{10}$ CFU/mL for all experiments.

2.8. Reproducibility and statistical analysis

Assays were performed in triplicate with three independent experiments, and the results are expressed as an average of the assays. Data were analyzed using SAS 9.1 software and submitted to comparison of averages for significance ($p \leq 0.05$) using Student *t* test for data (reduction of $\log_{10}$ CFU/mL cycles) of injury in cytoplasmic and outer membrane in cells pre-exposed and non-pre-exposed to each EO tested; and analysis of variance (ANOVA) followed by *post-hoc* Tukey test for data of response ($\log_{10}$ CFU/mL of survival fraction) to homologous and heterologous stressing agents of strains pre-exposed and non-pre-exposed to each EO tested. For MIC determination assays, the results are expressed as modal values because the MIC values were the same in all repetitions.

3. Results

MIC values of OVEO were 100 and 50 ppm against parental and mutant strains, respectively, in both *E. coli* and *L. monocytogenes* (Table 1). The growth inhibitory activity of ROEO was much lower than that of OVEO. MIC values of OVEO were 30,000 and 25,000 ppm towards parental and Δ*rpoS E. coli* strains, respectively; while MIC values were 35,000 ppm against parental and Δ*sigB L. monocytogenes* strains (Table 1). Thus, the tolerance of *E. coli* and *L. monocytogenes* tested strains to OVEO and ROEO was, in most cases, influenced by absence of *rpoS* and *sigB* genes, respectively.

Survival curves of the parental and mutant strains of *E. coli* and *L. monocytogenes* treated with 2 × MIC of OVEO or ROEO at room temperature (closed symbols in Fig. 1) showed a linear decrease in viable counts (counts) over time. After an 80 min-treatment, the decreases in counts of parental and mutant cells were in a range of 2–3 and 3–5 $\log_{10}$ UFC/mL cycles, respectively. Despite the more severe treatment applied in most cases against the parental cells—due to their corresponding higher MIC—mutant cells showed a greater sensitivity to the presence of both EOs tested, which reveals the influence of sigma factors expression in the bacterial tolerance to killing concentrations of both EOs.

Evaluating bacterial counts using non-selective and selective (containing NaCl or bile salts) recovery media indicated that both EOs injured cell envelopes of parental and mutant strains of both *E. coli* and *L. monocytogenes*. The number of $\log_{10}$ cycles of inactivation of the parental and mutant cells treated with 2 × MIC of OVEO or ROEO for 10 min and recovered in non-selective and selective media is presented in Table 2. The degree of inactivation reached in the selective media was

Table 1
Minimal inhibitory concentration (ppm) of the essential oil from *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* L. (ROEO) against *Escherichia coli* (MG1655 and MG1655 Δ*rpoS*) and *Listeria monocytogenes* (EGD-e and EGD-e Δ*sigB*).

Essential oils	*Escherichia coli* MG1655		*Listeria monocytogenes* EGD-e	
	Parental	Δ*rpoS*	Parental	Δ*sigB*
OVEO	100	50	100	50
ROEO	30,000	25,000	35,000	35,000

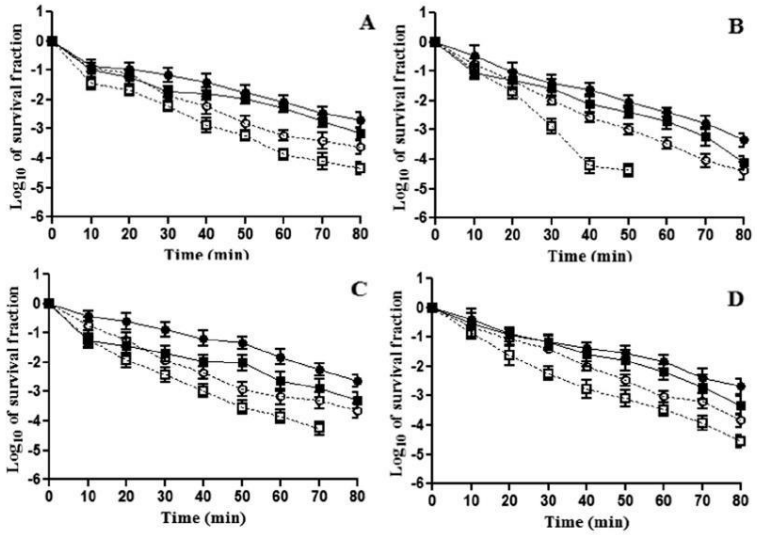

Fig. 1. Viable cells counts (Log_{10} CFU/mL) of *Escherichia coli* strains—MG1655 and MG1655 Δ*rpoS*—(A and C), and *Listeria monocytogenes* strains—EGD-e and EGD-e Δ*sigB*—(B and D) in McIlvaine buffer at pH 7.0 containing *Origanum vulgare* L. (OVEO) (A and B) or *Rosmarimus officinalis* L. (ROEO) (C and D) at 2 × MIC following pre-exposure or non-pre-exposure to OVEO (A and B) or ROEO (C and D) at 1/2 MIC. (●) essential oil non pre-exposed parental cells; (■) essential oil non pre-exposed mutant cells; (○) essential oil pre-exposed parental cells; (□) essential oil pre-exposed mutant cells.

greater than in non-selective media ($p \leq 0.05$), which showed the occurrence of cytoplasmic membrane injured cells for *L. monocytogenes* and on both the cytoplasmic and outer membrane for *E. coli* parental and mutant strains. Nevertheless, the differences between the counts obtained in selective and non-selective media were higher in the mutant strains in most cases, showing the occurrence of a greater proportion of sublethally injured cells in mutant than in parental strains. After pre-exposure to the EOs, sublethally injured parental and mutant cells were able to grow in high numbers (approximately 6 log_{10} CFU/mL) when cultivated in non-selective media, showing a capacity to repair the imposed (sublethal) injury. Cells non-pre-exposed to ROEO or OVEO maintained their viability when cultivated in TSB-SC and TSB-BS (approximately 6 log_{10} CFU/mL) (data not shown).

Fig. 1 also includes the survival-fraction curves of cells pre-exposed to a subinhibitory concentration (1/2 × MIC) of OVEO or ROEO and further challenged with the same stressing agent at 2 × MIC (opened symbols). The overnight exposure of parental and mutant strains of *E. coli* and *L. monocytogenes* to subinhibitory concentration of OVEO or ROEO caused a reduction of bacterial cell tolerance to the subsequent homologous stressing agent (2 × MIC). Therefore, no induction of increased direct-tolerance but a decreased bacterial tolerance was observed due to the previous exposure to the sublethal stressing conditions imposed by the EOs. Pre-exposed or non-pre-exposed cells presented similar survival-fraction curves shapes (linear decrease in survivor counts) over the assessed time period, although the degree of decreased tolerance was greater for sigma mutants than for parental strains. In fact, Δ*rpoS* and Δ*sigB* strains pre-exposed and non-pre-exposed to subinhibitory concentration of OVEO or ROEO always showed lower counts (or survival fractions) ($p \leq 0.05$) when compared with parental strains (differences of up to 2 log_{10} CFU/mL) treated in media containing the OVEO or ROEO at 2 × MIC.

Parental and mutant strains of *E. coli* and *L. monocytogenes* non-pre-exposed to OVEO and ROEO showed similar ($p > 0.05$) log_{10} CFU/mL reductions when subjected to PEF treatment (Fig. 2). Nevertheless, Δ*rpoS* and Δ*sigB* cells pre-exposed to subinhibitory concentration of OVEO or ROEO showed higher ($p \leq 0.05$) log_{10} CFU/mL reductions when further subjected to PEF treatment than the pre-exposed parental strains, showing that the absence of σ^S and σ^B factors expression is related with the higher sensitivity to PEF under the experimental conditions tested. Still, both Δ*rpoS* and Δ*sigB* cells pre-exposed to ROEO or OVEO presented higher log_{10} CFU/mL reductions ($p \leq 0.05$) when subjected to PEF than the non-pre-exposed counterparts (Fig. 2). As previously observed for the response to a homologous stressing agent, the overnight exposure to subinhibitory concentration of either OVEO or ROEO did not cause induction of increased bacterial cross-tolerance

Table 2
Reduction of log_{10} CFU/mL cycles of *Escherichia coli* (MG1655 and MG1655 Δ*rpoS*) and *Listeria monocytogenes* (EGD-e and EGD-e Δ*sigB*) strains treated with OVEO or ROEO at 2 × MIC for 10 min and further recovery in non-selective media (TSBYE), selective media with NaCl (TSBYE-SC) and selective media with bile salts (TSBYE-BS).

Strains	Cells pre-exposed to OVEO		
	TSAYE	TSAYE−SC	TSAYE−BS
E. coli MG1655	1.1 (±0.5)[a]	2.0 (±0.4)[b]	3.0 (±0.6)[b]
E. coli Δ*rpoS*	1.1 (±0.3)[a]	3.3 (±0.5)[b]	3.8 (±0.2)[b]
L. monocytogenes EGD-e	1.0 (±0.3)[a]	2.6 (±0.4)[b]	–
L. monocytogenes Δ*sigB*	1.2 (±0.2)[a]	3.1 (±0.5)[b]	–
	Cells pre-exposed to ROEO		
	TSAYE	TSAYE−SC	TSAYE−BS
E. coli MG1655	0.6 (±0.2)[a]	2.0 (±0.3)[b]	2.7 (±0.4)[b]
E. coli Δ*rpoS*	1.2 (±0.3)[a]	2.2 (±0.3)[b]	2.9 (±0.2)[b]
L. monocytogenes EGD-e	1.4 (±0.6)[a]	2.2 (±0.2)[b]	–
L. monocytogenes Δ*sigB*	1.4 (±0.6)[a]	2.8 (±0.5)[b]	–

(–): not tested.
[a–b]Different superscript lowercase letters, in the same row, denote differences ($p \leq 0.05$) between counts obtained for parental and mutant cells in each selective and non-selective media according to the student t test ($p \leq 0.05$).

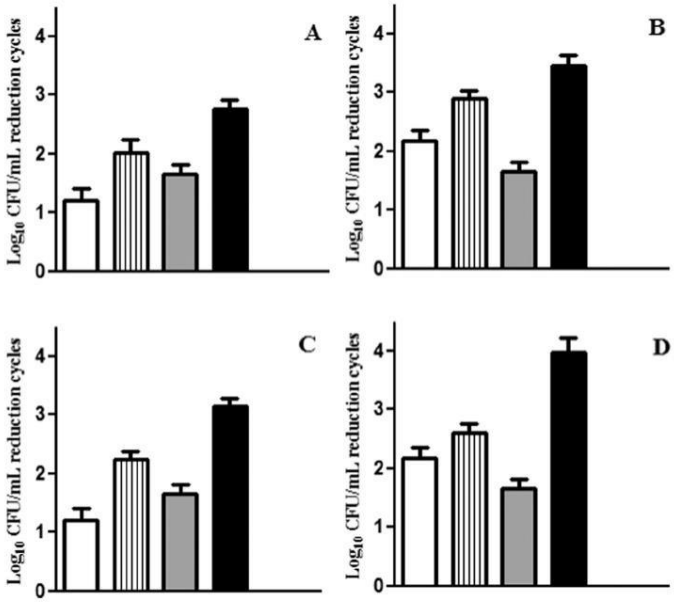

Fig. 2. Viable cells counts (Log_{10} CFU/mL) reduction cycles of *Escherichia coli* strains—MG1655 and MG1655 Δ*rpoS*—(A and C), and *Listeria monocytogenes* strains—EGD-e and EGD-e Δ*sigB*—(B and D) when subjected to PEF at 30 kV/cm in McIlvaine buffer at pH 7.0 following overnight pre-exposure or non-pre-exposure to OVEO (A and B) or ROEO (C and D) at 1/2 MIC. Essential oil non-pre-exposed parental cells: white bars; essential oil pre-exposed parental cells: dashed bars; essential oil non-pre-exposed mutant cells: grey bars; essential oil pre-exposed mutant cells: black bars.

but a decrease of tolerance to the heterologous stressing agent PEF in both parental and mutant cells of *E. coli* and *L. monocytogenes*.

4. Discussion

The strongest inhibitory effect of OVEO against *E. coli* and *L. monocytogenes* in comparison to ROEO, considering their MIC values, has been previously reported (Azerêdo et al., 2011; Luz et al., 2012). These findings have been related with the differences in composition between these EOs. Moreover, this study also shows the ability of both EOs (2 × MIC) to cause microbial inactivation, showing a similar survival curve shape, and the decrease of more than 2 $\log_{10}$ CFU/mL cycles (99%) after 80 min of treatment. On the other hand, both the inhibitory (MIC determination) and the survival assays showed the lower tolerance to OVEO and ROEO of Δ*rpoS* and Δ*sigB* mutant strains when compared with parental strains, revealing, for the first time, the influence of *rpoS* and *sigB* genes expression in the tolerance of *E. coli* and *L. monocytogenes* to the EOs tested.

The *E. coli* and *L. monocytogenes* mutant cells were more severely injured through exposure to OVEO or ROEO (at 2 × MIC) than the parental strains. Thus, the absence of *rpoS* and *sigB* in mutant cells resulted in either a lower resistance of the cell envelopes (cytoplasmic membrane in *L. monocytogenes*; and cytoplasmic and outer membranes in *E. coli*) to the OVEO or ROEO or an impaired cell ability to repair sublethal injuries after exposure to these substances. On the other hand, pre-exposure of parental and mutant cells of *E. coli* and *L. monocytogenes* to subinhibitory concentration of OVEO or ROEO caused no induction of increased direct-tolerance. The observed decreased tolerance to OVEO and ROEO in parental, Δ*rpoS* and Δ*sigB* strains after exposure to subinhibitory concentration of the same EO, when compared with non-pre-exposed cells, could be associated with the ability of these EOs to inflict sublethal injuries in the cell envelope structure (as observed in assays with NaCl and bile salts), which might increase the membrane permeability (Azerêdo et al., 2012; Luz et al., 2014). This alteration in membrane structure caused by pre-exposure to EOs may affect the ability of the bacterial membrane to adequately osmoregulate the bacterial cell or to exclude toxic materials (e.g., EOs individual constituents), decreasing the tolerance of the assayed bacterial strains to the subsequent exposure to higher concentrations of OVEO or ROEO. Still, the decreased tolerance to both OVEO and ROEO in non-pre-exposed mutant cells when compared with non-pre-exposed parental cells shows that the sigma factors σ^S and σ^B could be involved in tolerance changes of *E. coli* and *L. monocytogenes* cells subjected to stressing conditions imposed by subinhibitory concentrations of EOs. This influence of alternative sigma factors in tolerance of *E. coli* and *L. monocytogenes* to the tested EOs was also revealed by findings of lower viability and ability to repair injury over time in Δ*rpoS* and Δ*sigB* cells pre-exposed to OVEO and ROEO and subsequent challenge with the same EO at higher concentration, when compared with parental cells (data not shown).

This study demonstrated that the *rpoS* and *sigB* genes expression does not have influence in tolerance induction to PEF treatment in *E. coli* and *L. monocytogenes* tested strains pre-exposed to OVEO or ROEO at subinhibitory concentration. Otherwise, decreased tolerance to PEF was observed in Δ*rpoS* and Δ*sigB* strains pre-exposed OVEO or ROEO when compared to either the non-pre-exposed counterparts or the pre-exposed parental cells. The mechanism of inactivation by PEF (electroporation) is intimately associated with the formation of pores in the cytoplasmic membrane (Espina et al., 2014). Considering that the structure and composition of the membrane could determine the

susceptibility of the bacterial cells to the electroporation (Álvarez et al., 2002), the capability of EOs to damage this cell structure (Mackey, 2000; Somolinos et al., 2010b) could favor the enhancement of the antimicrobial effects of PEF against ROEO or OVEO pre-exposed cells.

The results of this study revealed the influence of the sigma factors σ^S and σ^B in tolerance of single strains of *E. coli* and *L. monocytogenes*, respectively, to OVEO and ROEO. The deletion of σ^S and σ^B resulted in decreased tolerance to OVEO, ROEO or PEF in the strains tested following exposure to the OVEO or ROEO at subinhibitory concentration. Overall, these findings suggest that the understanding of environmental conditions, including intrinsic and extrinsic food-related parameters, involved in activation of sigma factors in bacteria cells would be helpful to design more effective strategies to control bacterial survival by application of EOs in sequential steps with PEF.

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