ISABELLE DA SILVA LUZ

Capacidade de adaptação e tolerância em bactérias contaminantes de alimentos frente ao óleo essencial de *Origanum vulgare* L. e carvacrol

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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BANCA EXAMINADORA

_____

Prof[a]. Dr[a]. Tânia Lúcia Montenegro Stamford

(Universidade Federal de Pernambuco - UFPE)

_____

Prof[a]. Dr[a]. Leonie Asfora Sarubbo

(Universidade Católica de Pernambuco - UNICAP)

_____

Prof[a]. Dr[a]. Regina Célia Bressan Queiroz Figueiredo

(Centro de Pesquisas Aggeu Magalhães - CPqAM)

_____

Prof[a]. Dr[a]. Roberta de Albuquerque Bento

(Universidade Federal de Pernambuco - UFPE)

_____

Prof[a]. Dr[a]. Geíza Alves Azêredo de Oliveira

(Instituto Federal de Educação, Ciência e Tecnologia de Pernambuco - IFPE)

Recife

2014

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“Os que estão plantados na casa do Senhor
florescerão nos átrios do nosso Deus.
Na velhice ainda darão frutos;
serão viçosos e florescentes,
Para anunciar que o Senhor é reto;
Ele é a minha rocha e
nele não há injustiça.”
Sl 92:13-15

RESUMO

A crescente demanda por alimentos saudáveis isentos de conservantes químicos tem estimulado a pesquisa de tecnologias alternativas para a produção de alimentos seguros. Neste cenário, os óleos essenciais surgem como possíveis agentes antimicrobianos para uso em alimentos, em especial aqueles obtidos da espécie *Origanum vulgare* L. (OEOV). A ação antimicrobiana do OEOV tem sido atribuída aos compostos fenólicos naturalmente presentes em sua constituição, sendo o carvacrol (CAR) frequentemente citado como seu constituinte majoritário. Considerando estes aspectos, este estudo foi desenvolvido com objetivo de investigar a capacidade de adaptação e de desenvolvimento de tolerância ao OEOV e CAR em algumas cepas de bactérias contaminantes de alimentos (*Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* e *Salmonella* Typhimurium) e avaliar a ocorrência de injúria e alterações na composição de ácidos graxos da membrana citoplasmática de *S.* Typhimurium quando exposta a concentrações subletais destes agentes. A determinação da Concentração Inibitória Mínima (CIM) foi realizada por macrodiluição em caldo. Para os ensaios de tolerância direta e cruzada (45^{o}C; pH 5.2; NaCl 5-15 g/100 mL), as cepas foram expostas a concentrações subletais (1/2 CIM e 1/4 CIM) do OEOV ou do CAR em caldo base carne (18 h) e em modelo base carne (72 h). Para os ensaios de verificação de injúria subletal, *S.* Typhimurium foi exposta a concentrações subletais do OEOV ou CAR e semeadas em meios adicionados de agentes seletivos. O perfil de ácidos graxos da membrana citoplasmática foi determinado por meio de cromatografia gasosa. Os valores da CIM frente às bactérias testadas variaram entre 0.62 µL/mL e 5.0 µL/mL para OEOV e entre 0.62 µL/mL e 2.5 µL/mL para CAR. Ensaios de indução de tolerância direta e cruzada em caldo base carne revelaram ausência do desenvolvimento de tolerância em todas as cepas bacterianas ensaiadas. Resultados semelhantes foram observados para *P. aeruginosa* em modelo base carne. Ensaios adicionais de indução de tolerância direta nas cepas bacterianas quando em caldo base carne adicionado de concentrações crescentes (1/16 CIM - 2 CIM) dos compostos também não evidenciaram desenvolvimento de tolerância bacteriana. A exposição de *S.* Typhimurium a concentrações subletais de OEOV ou CAR revelou injúria à membrana citoplasmática e à membrana externa, bem como alterações em sua composição de ácidos graxos, especialmente relacionadas ao aumento da razão ácidos graxos insaturados:ácidos graxos saturados. Os resultados obtidos neste estudo demonstram que a exposição das cepas bacterianas a concentrações subletais do OEOV ou CAR não induziu o desenvolvimento de tolerância bacteriana direta e cruzada quando cultivadas em meio base carne. Ainda, sugeriram que estes compostos podem induzir uma resposta adaptativa em *S.* Typhimurium, relacionada à mudanças no perfil de ácidos graxos de membrana. Estes achados revelam o potencial antimicrobiano do OEOV e do CAR em sistemas de conservação de alimentos, quando considerada a sua destacável capacidade de inibição do crescimento das cepas utilizadas no estudo, concomitante ao não desenvolvimento de tolerância bacteriana direta e cruzada, considerando avaliações de uso em concentrações subletais.

Palavras-chave: Adaptação biológica. Testes de sensibilidade microbiana. *Origanum.* Compostos fenólicos. Conservação de alimentos.

ABSTRACT

The growing demand for healthy foods free of chemical preservatives has encouraged the search for alternative technologies for the production of safe food. In this scenario, the essential oils appear as potential antimicrobial agents for use in foods, especially that obtained from the species *Origanum vulgare* (OEOV). The antimicrobial action of OEOV has been attributed to phenolic compounds naturally present in its constitution, and carvacrol (CAR) often cited as its major constituent. Considering these aspects, this study was conducted in order to investigate the ability of adaptation and tolerance development to OEOV and CAR in some strains of bacteria contaminating food (*Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* Typhimurium) and assess the occurrence of injury and changes in fatty acid composition of the cytoplasmic membrane of *Salmonella* Typhimurium when exposed to sublethal concentrations of these agents. Determination of Minimum Inhibitory Concentration (MIC) was performed by broth macrodilution. For the tests of direct and cross tolerance (45^{o}C; pH 5.2; NaCl 5-15 g/100 mL), the strains were exposed to sublethal concentrations (1/2 MIC and 1/4 MIC) of OEOV or CAR in meat broth (18 h) and model based meat (72 h). For the verification tests of sublethal injury, *S.* Typhimurium was exposed to sublethal concentrations of OEOV or CAR and plated on media added of selective agents. The fatty acid profile of the cytoplasmic membrane was determined by gas chromatography. The MIC values of the tested bacteria ranged from 0.62 µL/mL and 5.0 µL/mL for OEOV and between 0.62 µL/mL and 2.5 µL/mL for CAR. The tests of direct and cross tolerance induction in broth based meat revealed the absence of tolerance development in all tested bacterial strains. Similar results were observed for *P. aeruginosa* in model based meat. Additional tests for induction of direct tolerance in the bacterial strains in broth based meat added of increasing concentrations (1/16 MIC - 2 MIC) of the compounds also showed no development of bacterial tolerance. Exposure of *S.* Typhimurium to sublethal concentrations of OEOV or CAR revealed injury to the cytoplasmic membrane and to the outer membrane, as well as changes in its fatty acid composition, especially related to the increase of the unsaturated:saturated fatty acids. The results of this study demonstrated that exposure of bacterial strains to sublethal concentrations of OEOV or CAR did not induce the development of bacterial direct and cross tolerance when grown in medium based meat. Also, they suggested that these compounds can induce an adaptive response at *S.* Typhimurium, related to changes in the fatty acid membrane profile. These findings reveal the antimicrobial potential of OEOV and CAR in food preservation systems, when considering their detachable capacity to inhibit growth of strains used in the study, concomitant with no development of bacterial direct and cross tolerance, considering use evaluations in the sublethal concentrations.

Keywords: Adaptation, biological. Microbial sensitivity tests. *Origanum*. Phenolic compounds. Food preservation.

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LISTA DE ABREVIATURAS E SIGLAS

ATCC - American Type Culture Collection (Coleção Americana de Cultura Tipo)

ATP - Adenosina trifosfato ou trifosfato de adenosina

B. - Blume

BHI - Brain Heart Infusion (Infusão de Cérebro e Coração)

C. - Cheel

CAR - Carvacrol

CBM - Concentração Bactericida Mínima

CIM - Concentração Inibitória Mínima

CS - Cloreto de sódio

D.C. - De Candolle

DVA - Doenças Veiculadas por Alimentos

FDA - Food and Drug Administration (Administração de Alimentos e Medicamentos)

Food-grade - De grau alimentício

GRAS - Generally Recognized as Safe (Geralmente Reconhecido como Seguro)

HCl - Ácido clorídrico

HPP - High Pressure Processing (Processamento por Alta Pressão)

L. - Linnaeus

MAP - Modified Atmosphere Packaging (Embalagem com Atmosfera Modificada)

NaCl - Cloreto de sódio

OEOV - Óleo essencial de *Origanum vulgare* L.

pH - Potencial hidrogeniônico ou potencial de hidrogênio

SB - Sais biliares

SEA - Staphylococcal Enterotoxin A (Enterotoxina Estafilocócica A)

SEB - Staphylococcal Enterotoxin B (Enterotoxina Estafilocócica B)

UFC - Unidade Formadora de Colônia

SUMÁRIO

1 APRESENTAÇÃO

A qualidade dos alimentos pode ser adversamente afetada por alterações ocasionadas pela ação de fatores físicos, químicos ou microbiológicos. O fator microbiológico é o mais importante pela sua capacidade de contaminação, variedade de efeitos que origina e intensidade de alterações que provoca (LÜCK; JAGER, 2000; FORSYTHE, 2002; ÁLVAREZ-ORDÓÑEZ et al., 2009; DUBOIS-BRISSONNET et al., 2011).

Entretanto, frente ao conhecimento de que alguns conservantes químicos são suspeitos ou são tóxicos aos consumidores, somado aos resultados encontrados por estudos sobre 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 consequente adoção de alternativas mais naturais para obtenção dos seus propósitos (SOUZA et al., 2005; BARROS et al., 2009).

Ainda, com a mudança nas exigências do consumidor que demandam por alimentos mais naturais e/ou com níveis baixos de aditivos químicos, alta qualidade organoléptica e com a conveniência de uma longa vida de prateleira, do ponto de vista industrial, agentes antimicrobianos naturais têm se mostrado interessantes pelo aspecto da segurança alimentar que sugerem (BRUL; COOTE, 1999; SOKOVIC et al., 2010).

Os óleos essenciais, entre os variados compostos estudados com vistas a um potencial uso na conservação de alimentos, têm mostrado resultados destacáveis na inibição do crescimento e sobrevivência de micro-organismos patogênicos e deteriorantes (BUSATTA et al., 2008; BARBOSA et al., 2009; SOUZA et al., 2010; DA SILVA MALHEIROS et al., 2010; ZARAI et al., 2012; KEREKES et al., 2013). Também chamados de óleos voláteis, estes compostos são constituídos por misturas complexas, lipofílicas (BAKKALI et al., 2008), cujos componentes incluem hidrocarbonetos terpênicos, alcoóis simples, aldeídos, cetonas, fenóis, ésteres e ácidos orgânicos fixos em diferentes concentrações (SANTURIO et al., 2007).

Dentre os óleos essenciais com propriedades antimicrobianas, aquele obtido da espécie *Origanum vulgare* L., conhecida tradicionalmente como orégano, destaca-se pela inibição de micro-organismos contaminantes de alimentos, tais como *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* enterica sorovar Typhimurium (*Salmonella* Tyhimurium) e *Listeria monocytogenes* (SOUZA et al., 2010; OLIVEIRA et al., 2010; SOKOVIC et al., 2010; LUZ et al., 2012a). As propriedades biológicas e a atividade

antimicrobiana deste óleo essencial têm sido atribuídas a seus compostos fenólicos, tais como o carvacrol e o timol, os quais são frequentemente relatados como seus constituintes majoritários (SEYDIM; SARIKUS, 2006; BASER, 2008).

Estes compostos têm características hidrofóbicas e interagem com diferentes sítios da célula microbiana, especialmente com a membrana citoplasmática e a parede celular, promovendo colapso da estrutura de membrana, perda de constituintes celulares e, consequentemente, morte celular. Componentes presentes em menor quantidade também podem contribuir para o estabelecimento das propriedades antibacterianas do óleo essencial, provavelmente envolvendo interações sinérgicas com os compostos fenólicos presentes (BURT, 2004).

Embora um óleo essencial tenha diferentes componentes que podem agir sobre mecanismos celulares distintos resultando em uma dificuldade de adaptação microbiana à sua ação, existem relatos sobre a capacidade de bactérias relacionadas a alimentos exibirem respostas adaptativas a condições ambientais adversas, tais como temperatura, ácido, sal, compostos terpenos e peróxido (SKANDAMIS et al., 2008; ÁLVAREZ-ORDÓÑEZ et al., 2009; DUBOIS-BRISSONNET et al., 2011; CONDELL et al., 2012).

Considerando os aspectos acima citados, este estudo teve como propósito avaliar a eficácia do óleo essencial de *O. vulgare* L. (OEOV) e de seu constituinte majoritário carvacrol (CAR) em inibir o crescimento/sobrevivência de cepas bacterianas de importância em alimentos, bem como investigar a indução de tolerância/adaptação bacteriana a estes compostos.

2 PERGUNTA CONDUTORA E HIPÓTESE

Para o desenvolvimento desta pesquisa, foram geradas as seguintes pergunta condutora e hipótese:

2.1 PERGUNTA CONDUTORA

As bactérias contaminantes de alimentos *L. monocytogenes, S. aureus, P. aeruginosa* e *S.* Typhimurium são capazes de se adaptar e desenvolver tolerância direta e/ou cruzada quando expostas a concentrações subletais do OEOV e de seu constituinte majoritário, CAR?

2.2 HIPÓTESE

As bactérias contaminantes de alimentos *L. monocytogenes, S. aureus, P. aeruginosa* e *S.* Typhimurium não são capazes de se adaptar e desenvolver tolerância direta e cruzada quando expostas a concentrações subletais do OEOV e de seu constituinte majoritário, CAR.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar a capacidade de adaptação e desenvolvimento de tolerância em bactérias contaminantes de alimentos quando expostas a concentrações subletais do OEOV e do CAR.

3.2 OBJETIVOS ESPECÍFICOS

a) Analisar a influência da exposição ao OEOV ou CAR sobre o crescimento das cepas bacterianas ensaiadas em caldo base carne;

b) Avaliar a capacidade de desenvolvimento de tolerância direta e cruzada (termotolerância, ácidotolerância e osmotolerância) das cepas bacterianas cultivadas em modelo base-carne adicionado de concentrações subletais do OEOV e CAR;

c) Verificar o efeito da exposição a concentrações subletais do OEOV e CAR sobre as membranas citoplasmática e externa de *S.* Typhimurium.

4 REVISÃO DA LITERATURA

Nesta revisão de literatura, focalizaram-se os principais tópicos relacionados ao tema estudado.

4.1 Abordagem sobre resistência, tolerância e/ou adaptação bacteriana a processos ou compostos antimicrobianos

A produção de alimentos em escala industrial facilita o aparecimento e a disseminação da resistência através do uso intensivo de agentes antimicrobianos e do comércio internacional de animais e produtos alimentícios, cuja principal rota de transmissão entre animais e seres humanos ocorre através de produtos alimentares, embora outras formas de transmissão, como contato direto e ambiente, também possam ocorrer (AARESTRUP; WEGENER; COLLIGNON, 2008).

Uma grande variedade de antimicrobianos é utilizada para controlar infecções e doenças em humanos devido ao consumo de alimentos contaminados com micro-organismos patogênicos ou toxinas produzidas por estes micro-organismos (SOKOVIC et al., 2010). Entretanto, as bactérias têm desenvolvido mecanismos múltiplos para a evolução eficiente e disseminação de resistência aos antimicrobianos.

Devido ao aumento da prevalência de resistência em isolados patogênicos, há uma maior exigência por meios alternativos de desinfecção para prevenir infecções e surtos. Estes mecanismos dependem do uso efetivo de compostos antimicrobianos, uma vez que há preocupação de que o aumento resultante da utilização destes compostos em concentrações subletais na agricultura, ambientes hospitalares e na produção de alimentos, venha a contribuir para a seleção de cepas resistentes a antibióticos (RUSSELL, 2003). A susceptibilidade diminuída a diversos antimicrobianos seguida da exposição a concentrações subletais do mesmo, ou de diferentes agentes utilizados como antibióticos tem sido demonstrada *in vitro* (FUANGTHONG et al., 2011; HAMMER; CARSON; MEE; RILEY, 2012).

A resistência 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 resistência a antimicrobianos é principalmente adquirida através de três mecanismos principais: (1) transferência de genes de resistência 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).

Alguns micro-organismos são capazes de se adaptar e crescer na presença de concentrações tóxicas de agentes antimicrobianos e estas mudanças podem alterar tanto propriedades físico-químicas da membrana celular bacteriana, afetando sua permeabilidade, como a atividade das enzimas de membrana e, consequentemente, a viabilidade da célula. A resposta pode depender das condições de adaptação específica impostas durante a fase estacionária com subsequente estresse subletal (FERREIRA et al., 2003).

Uma das respostas de adaptação ao estresse mais estudadas e relevantes é a resposta de tolerância ao ácido, induzida para proteger as bactérias contra o estresse ácido severo. Estudos de Álvarez-Ordóñez et al. (2009) demonstraram que *S. enterica* sorovar Typhimurium CECT 443 e *S. enterica* sorovar Senftenberg CECT 4384 apresentaram uma maior tolerância ao ácido quando foram expostas ao pH letal (2.5) no suco de laranja ou maçã do que aquelas que cresceram em BHI tamponado (células não adaptadas ao ácido).

A tolerância ao ácido parece conferir também proteção cruzada a outros tipos de estresse. Por exemplo, a resposta de tolerância ao ácido induzida por ácido clorídrico (HCl) protege a bactéria contra a exposição a grandes quantidades de sais, temperaturas elevadas, peróxidos (HUMPHREY, 2004; LEYER; JOHNSON, 1993; XU; LEE; AHN, 2008) e ácidos orgânicos, podendo também induzir uma resposta de tolerância ao ácido diferente de acordo com a temperatura de incubação do microrganismo teste (KWON; RICKE, 1998; GREENACRE et al., 2003).

Assim, é possível que células microbianas possam desenvolver resposta de tolerância ao ácido, que protegeria contra outros fatores de preservação de alimentos, tais como o sal (GREENACRE; BROCKLEHURST, 2006). Isto sugere que a presença de um mecanismo de resposta comum ao estresse é responsável pelo desenvolvimento de proteção cruzada a outros agentes potencialmente encontrados no mesmo ambiente (GRUZDEV et al., 2011).

Tem sido relatado que algumas bactérias patogênicas desenvolvam susceptibilidade reduzida a concentrações subletais de antimicrobianos através de processos de adaptação que incluem mutações ou regulação genética (YUK; MARSHALL, 2006). Modificações na fluidez da membrana citoplasmática também 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].

A membrana citoplasmática bacteriana separa a célula do ambiente externo, sendo que esta barreira também desenvolve um papel na transdução de energia, na manutenção de transporte de solutos e atua na regulação do metabolismo e crescimento celular (DENICH et al., 2003). Os lipídios da membrana são as moléculas mais adaptáveis em resposta às perturbações físico-químicas. Quando células microbianas são expostas ao estresse subletal, a membrana celular é capaz de mudar sua composição de ácidos graxos com a finalidade de lidar com o novo ambiente (ÁLVAREZ-ORDÓÑEZ et al., 2008).

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 (BAYSSE et al., 2005; PATRIGNANI et al., 2008).

Em estudo realizado por Di Pasqua et al. (2006), foi relatado que um mecanismo adaptativo ao estresse induzido por concentrações subletais de compostos ativos de óleos essenciais resultou em uma maior concentração de alguns ácidos graxos insaturados na membrana de *Escherichia coli* O157:H7, *Salmonella* e *Brochothrix thermosphacta*. Aparentemente, alterações na susceptibilidade a óleos essenciais poderiam ser explicadas por adaptação fenotípica, o que resultaria em um baixo nível de tolerância devido a mudanças reversíveis na composição dos lipídios de membrana (ULTEE et al., 2000; DI PASQUA et al., 2006), e efluxo (PAPADOPOULOS et al., 2008).

A indução de resistência a óleos essenciais ou seus constituintes mostrou baixa ou inexistente redução na susceptibilidade microbiana (ALI et al., 2005; FERRINI et al., 2006; SHAPIRA; MIMRAN, 2007; McMAHON et al., 2008). Considerando tais aspectos, o interesse no possível uso de alternativas naturais como aditivos alimentares para prevenir o crescimento bacteriano tem aumentado notavelmente. Plantas e produtos vegetais podem representar uma fonte alternativa para prover um incremento no tempo de conservação e segurança dos alimentos (PATRIGNANI et al., 2008).

De acordo com Hammer et al. (2012), o óleo essencial de *Melaleuca alternifolia* C. tem pouco impacto sobre o desenvolvimento de resistência a antibióticos em *E. coli* e *S. aureus*, e a exposição ao constituinte majoritário terpinen-4-ol não alterou significativamente a susceptibilidade destes micro-organismos. Ali et al. (2005) investigaram cinco cepas de

Helicobacter pylori sensíveis ou resistentes aos antibióticos metronidazol, amoxicilina e claritromicina quanto ao desenvolvimento de resistência a concentrações subletais (0.25 e 0.5 µg/mL) de eugenol e cinamaldeído, e sugeriram que estes compostos preveniram o crescimento bacteriano *in vitro* sem induzir a aquisição de resistência pela bactéria.

Nos resultados obtidos por Ferrini et al. (2006), foi visto que a atividade anti-estafilocócica do óleo essencial de *M. alternifolia* C. e seu principal componente terpinen-4-ol foi superior à de diversos antibióticos, sendo ativos na inibição de cepas resistentes a mupirocina, ácido fusídico, vancomicina, meticilina e linezolida. McMahon et al. (2008) relataram que a exposição repetida de cepas de *S. aureus* resistentes ou sensíveis à meticilina, à concentração subletal (0.25% por 72 h) do óleo essencial de *M. alternifolia* C. reduziu sua susceptibilidade a maiores concentrações do óleo essencial (1% e 10%), fato este observado pelo aumento de pelo menos quatro vezes no valor da CIM.

Em contrapartida, Dubois-Brissonnet et al. (2011) observaram que o timol, CAR, eugenol e citral podem induzir a adaptação bacteriana e tolerância em *S.* Typhimurium ATCC 13311, cultivada em concentrações subletais (variando de 0.3 a 3 mM) destes compostos. No estudo, os pesquisadores observaram aumento de tolerância da cepa testada aos desinfetantes ácido peracético (3 ppm por 5 min a 20^{o}C) e brometo de dimetil dodecil (10 mg por 5 min a 20^{o}C).

4.2 Óleos essenciais: características e mecanismos de ação antimicrobiana

Os óleos essenciais são compostos complexos, naturais, voláteis e límpidos formados como metabólitos secundários de plantas aromáticas geralmente cultivadas em países tropicais e de climas temperados. Estes compostos, que são responsáveis pela fragrância das plantas e por muitas de suas atividades biológicas, podem ser sintetizados por todos os órgãos da planta como flores, folhas, caules, ramos, sementes, frutos, raízes ou cascas, e são armazenados em células secretoras, cavidades, canais, células epidérmicas ou tricomas glandulares. Na natureza, os óleos essenciais possuem papel importante na proteção das plantas devido aos efeitos antibacterianos, antivirais, antifúngicos, inseticidas (repelindo insetos indesejáveis) e também contra herbívoros por meio da redução do apetite em relação às plantas produtoras. Além disso, podem atrair insetos para favorecer a dispersão de pólens e sementes (BAKKALI et al., 2008).

As propriedades bioativas dos óleos essenciais 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, isto é, bactericida, virucida e fungicida, além da sua fragrância, os óleos essenciais são usados na produção de embalagens ativas, como analgésicos, sedativos, antiinflamatórios, espasmolíticos, anestésicos tópicos e como antimicrobianos (BAKKALI et al., 2008). Devido sua atividade antimicrobiana, sugere-se que estes compostos sejam agentes promissores para uso na conservação de alimentos (BURT, 2004; BOUHDID et al. 2010), dos quais alguns foram aprovados pela *Food and Drug Administration* (FDA) para uso em alimentos e bebidas (USFDA, 2009).

O perfil químico dos produtos de óleos essenciais 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 do óleo essencial. 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. Existem diversos métodos para extração de óleos essenciais, 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 óleos essenciais de composição constante, estas substâncias devem ser extraídas sob as mesmas condições a partir do mesmo órgão da planta, que apresente crescimento no mesmo solo, sob o mesmo clima e na mesma estação do ano (ANGIONI et al., 2006; ABAD et al., 2012).

Os óleos essenciais podem conter entre 20 a mais de 60 componentes em proporções diferentes e são caracterizados por dois ou três constituintes majoritários em concentrações mais elevadas (20-70%) quando comparadas às concentrações de outros componentes presentes (BAKKALI et al., 2008; ABAD et al., 2012). Entre constituintes encontrados no OEOV, o CAR tem sido citado como o componente mais frequentemente detectado como sendo seu componente majoritário (AZEREDO et al., 2011) [Figura 1]. O CAR já apresentou efetividade comprovada na inibição de *B. subtilis*, *E. coli*, *Proteus mirabilis*, *P. aeruginosa*, *S. enterica*, *S.* Typhimurium e *S. aureus* (SOKOVIC et al., 2010).

Em relação a suas propriedades biológicas, pode-se dizer que a atividade antimicrobiana dos óleos essenciais não depende apenas de sua composição química, mas também de sua configuração estrutural, grupos funcionais e possíveis interações aditivas e sinérgicas entre seus componentes, que basicamente se dividem em 2 grandes grupos: o grupo principal é representado pelos terpenos e terpenóides (terpenos que contém oxigênio em sua estrutura) e o outro grupo, por constituintes aromáticos e alifáticos, todos de baixo peso molecular (BAKKALI et al., 2008).

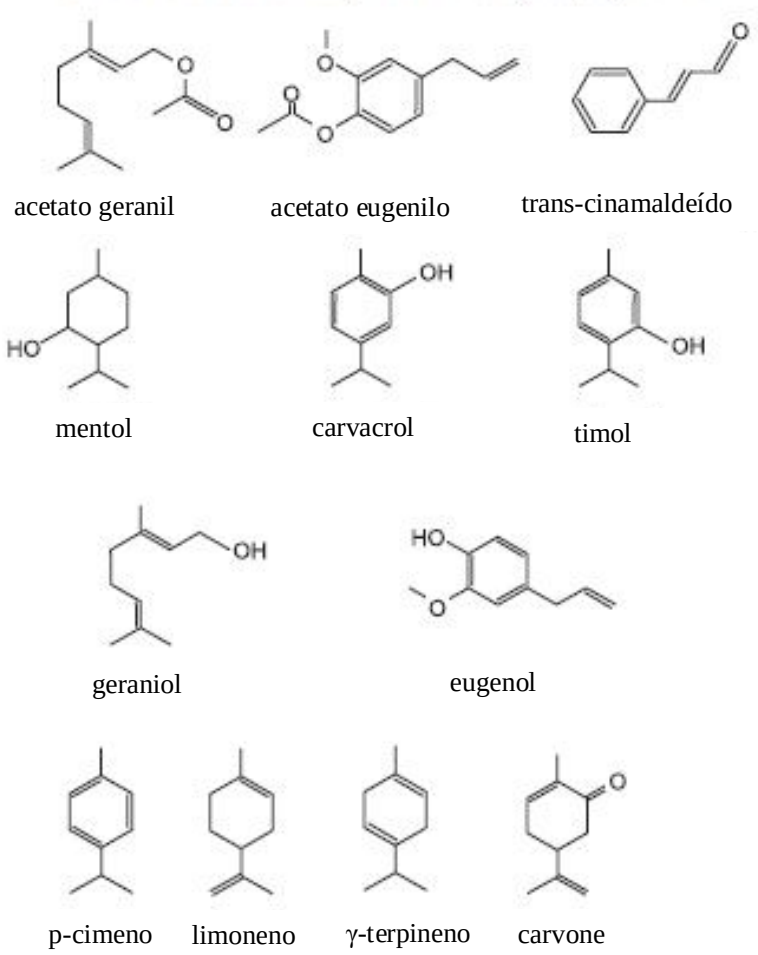

Figura 1- Fórmulas estruturais dos componentes selecionados de óleos essenciais.
Fonte: Adaptado de Burt (2004).

Os mecanismos pelos quais os óleos essenciais inibem o crescimento e/ou sobrevivência dos micro-organismos podem em parte ser devido a sua hidrofobicidade, uma vez que estes compostos conseguem passar pela parede celular e membrana citoplasmática, destruindo a estrutura dos polissacarídios, lipídios e fosfolipídios, o que provoca um dano à membrana, fator responsável, em parte, pela citotoxicidade que apresentam na célula alvo (CARSON, MEE; RILEY, 2002; BURT, 2004).

Além disso, em decorrência da ação de óleos essenciais, moléculas lipofílicas podem se acumular na bicamada lipídica e distorcer a interação lipídio-proteína (BAKKALI et al., 2008). As células também poderiam alterar a permeabilidade da membrana, como um mecanismo de reação, para evitar a entrada de algumas moléculas (SIKKEMA; de BONT; POOLMAN, 1995).

A depender dos compostos majoritários presentes no óleo essencial, uma sequência de eventos envolvendo diferentes modos de ação poderá ocorrer, conduzindo ao estabelecimento da inviabilidade ou morte do micro-organismo, sendo relatados vários alvos na célula microbiana (Figura 2). Entre estes eventos podem ser citados: sensibilização da dupla camada fosfolipídica, com perturbação na função e na composição da membrana citoplasmática; aumento da permeabilidade e perda de componentes intracelulares vitais; inativação do mecanismo enzimático, inibindo o transporte de elétrons para produção de energia, interrompendo a força motriz de prótons, a translocação proteica e a síntese de componentes celulares; extravasamento de material citoplasmático; lise e eventual morte celular (BURT, 2004).

O caráter lipofílico do esqueleto hidrocarbônico e o caráter hidrofílico dos grupos funcionais são de extrema importância na ação antimicrobiana dos óleos essenciais. Os possíveis modos de ação dos compostos fenólicos de óleos essenciais têm sido analisados (ULTEE; BENNINK; MOEZELAAR, 2002; APOSTOLIDIS; KWON; SHETTY, 2008; ADORJAN; BUCHBAUER, 2010), porém o mecanismo de ação destas substâncias é complexo e ainda pouco conhecido, sendo provável que estas propriedades antimicrobianas não sejam atribuídas a um único mecanismo (KOROCH; JULIANI; ZYGADLO, 2007). Sabe-se, porém, que em baixa concentração, os fenóis afetam a atividade enzimática, particularmente aquela associada com produção de energia, enquanto em altas concentrações, causam desnaturação de proteínas (TIWARI et al., 2009).

Geralmente, óleos essenciais são mais efetivos contra bactérias Gram-positivas do que contra bactérias Gram-negativas (GUTIERREZ; BARRY-RYAN; BOURKE, 2008), porém alguns, a exemplo do OEOV, canela (*Cinnamomum zeylanicum* B.), cravo (*Syzygium aromaticum* L.) e capim-santo (*Cymbopogon citratus* D.C. Stapf) são efetivos contra ambos os grupos (KIM; FUNG, 2004). Os óleos essenciais de algumas plantas, tais como manjerona (*O. majorana* L.) e manjericão (*Ocimum basilicum* L.), apresentam destacável atividade inibitória contra *B. cereus*, *Enterobacter aerogenes*, *E. coli* e *Salmonella* spp., e os óleos essenciais de erva-cidreira (*Melissa officinalis* L.) e sálvia (*Salvia officinalis* L.) parecem ter atividade interessante contra *L. monocytogenes* e *S. aureus* (GUTIERREZ; BARRY-RYAN; BOURKE, 2008).

Alguns estudos avaliaram a atividade antimicrobiana de óleos essenciais e de seus constituintes majoritários testados isoladamente. Em seu estudo, Burt (2004) observou que os constituintes majoritários CAR e timol do OEOV provocaram alterações na estrutura física da célula, causando expansão e consequente desestabilização na membrana citoplasmática,

modificando sua permeabilidade, desnaturando enzimas essenciais e alterando a força motriz de prótons, por meio de variações no pH e potencial elétrico. Lambert et al. (2001) relataram que o CAR causou alterações na membrana celular e desintegrou a membrana externa de *E. coli* e *S.* Typhimurium, enquanto Ultee, Kets e Smid (1999) observaram depleção da quantidade de ATP intracelular e dissipação de componentes da força motriz de prótons em *B. cereus* na presença de CAR.

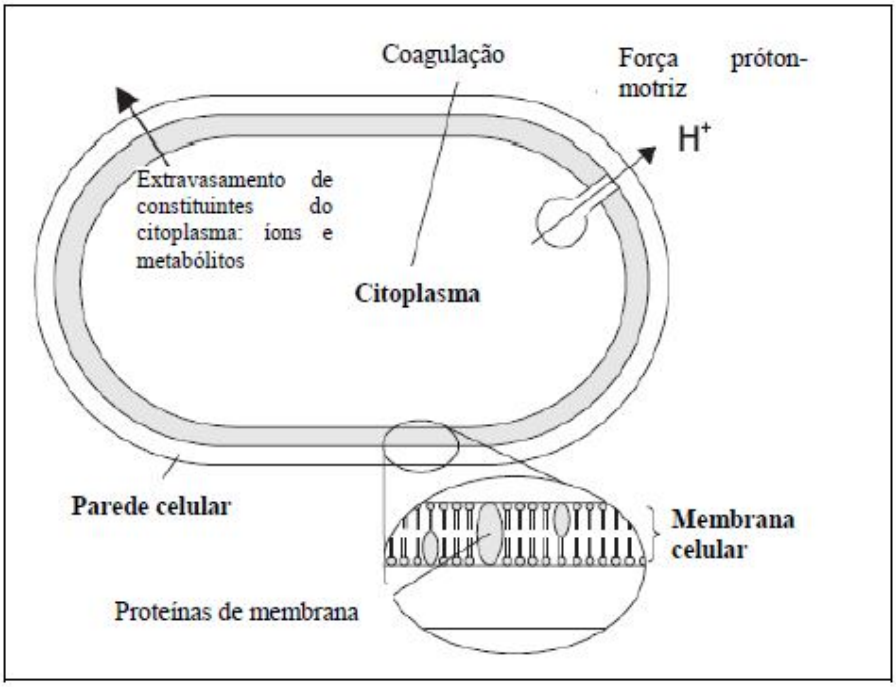

Figura 2- Possíveis modos de ação dos óleos essenciais contra diferentes alvos na célula bacteriana. Fonte: Adaptado de Burt (2004).

4.3 Potencial uso de óleos essenciais na conservação de alimentos

A contaminação bacteriana deve ser considerada tanto sob os aspectos de deterioração, e consequentes perdas econômicas, como pela ocorrência de Doenças Veiculadas por Alimentos (DVA) contaminados com micro-organismos patogênicos, que representam um problema de saúde pública (NAVAJAS et al., 2008).

As condições impostas aos micro-organismos durante o processamento de alimentos podem conduzir ao desenvolvimento de respostas adaptativas em decorrência à exposição a processos que causem injúrias (estresse) subletais à célula microbiana. Portanto, a avaliação da resposta à injúria subletal em micro-organismos pode auxiliar na redução da incerteza acerca do possível risco microbiano, associado à falta de conhecimento concernente ao

comportamento fisiológico das células microbianas sobreviventes desse processo (SKANDAMIS et al., 2008; 2012).

Para o alcance da produção de alimentos microbiologicamente estáveis, diversos procedimentos de natureza física ou química, a exemplo de tratamentos térmicos intensos, acidificação, refrigeração, congelamento, desidratação, adição de agentes químicos e de sal podem ser empregados. Além disso, algumas tecnologias de conservação de alimentos têm incluído a inativação não térmica, como radiação por ionização, alta pressão hidrostática e campos elétricos pulsados, aplicação de ácido orgânico ou dióxido de cloro, embalagem ativa e de atmosfera modificada, e uso de compostos antimicrobianos naturais (DEVLIEGHERE; VERMEIREN; DEBEVERE, 2004; SILVA; LOPES; VALENTE-MESQUITA, 2006; GOMEZ-LOPEZ et al., 2009; CHOI et al., 2010; RAJKOVIC; SMIGIC; DEVLIEGHERE, 2011).

A crescente demanda por parte dos consumidores por alimentos mais saudáveis que apresentem a conveniência de possuir ampla vida útil e, principalmente, de serem seguros ao consumidor, impõe à indústria a necessidade de buscar alternativas para garantir a qualidade microbiológica dos alimentos (DIMIRTIEVIAE et al., 2007; RAJKOVIC; SMIGIC; DEVLIEGHERE, 2011; CONDELL et al., 2012).

Conservantes naturais, particularmente em adição ou combinação sinergística com outros fatores e técnicas que já estão em uso, podem desempenhar um papel importante, e de acordo com Lado e Yousef (2002), estas tecnologias alternativas poderiam exercer uma ação eficaz de inibição do crescimento microbiano em alimentos, prevenindo ou minimizando a adaptação de patógenos às condições de estresse aplicadas para o controle de micro-organismos.

Neste contexto, opções inovadoras e emergentes para o alcance da segurança microbiológica dos alimentos, como o uso de bacteriocinas, culturas protetoras, quitosana e compostos vegetais (extratos e óleos essenciais) têm sido avaliadas (BURT; REINDERS, 2003; DEVLIEGHERE; VERMEIREN; DEBEVERE, 2004; CHORIANOPOULOS et al., 2004; TIWARI et al., 2009; DA SILVA MALHEIROS et al., 2010).

Dentre os diversos compostos estudados com vistas a um potencial uso na conservação de alimentos, os óleos essenciais têm mostrado resultados destacáveis na inibição do crescimento e sobrevivência de diferentes espécies de micro-organismos (PEREIRA et al., 2008; SOUZA et al., 2009; AZEREDO et al., 2011; MUTHAIYAN et al., 2012). Höferl et al. (2009) mostraram potente atividade do OEOV contra *E. coli* e Santurio et al. (2007) avaliaram a atividade antimicrobiana dos OEOV, de tomilho (*Thymus vulgaris* L.) e de canela

(*C. zeylanicum* B.) e constataram para o OEOV forte atividade antimicrobiana frente a diversos sorovares de *S. enterica* de origem avícola.

Em alguns estudos, é sugerido o uso combinado de óleos essenciais com outros métodos, 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 (PERIAGO; PALOP; FERNANDEZ, 2001; NAZER et al., 2005; NAVEENA et al., 2006; DIMIRTIEVIAE et al., 2007; GUTIERREZ; BARRY-RYAN; BOURKE, 2008). Por outro lado, é relatado que o NaCl pode apresentar efeito antagônico sobre a atividade antimicrobiana de alguns constituintes de óleos essenciais utilizados na indústria de alimentos (ULTEE et al., 2000).

Avaliação do efeito antimicrobiano do óleo essencial de segurelha (*Satureja montana* L.) contra *Clostridium perfringens* tipo A inoculado em mortadelas formuladas com diferentes níveis de nitrito de sódio evidenciou um efeito sinérgico entre o óleo essencial testado (0.78, 1.56 e 3.12%) e o nitrito (100 ppm), sugerindo que a associação entre os dois agentes poderia permitir uma redução nos níveis de nitrito utilizados para controlar o desenvolvimento de *C. perfringens* no produto (OLIVEIRA et al., 2011).

A efetividade dos óleos essenciais de cravo (*Syzygium aromaticum* L.), canela (*C. zeylanicum* B.), noz moscada (*Myristica fragans* H.) e tomilho (*Thymus vulgaris* L.) na inibição da produção das enterotoxinas estafilocócicas A (SEA) e B (SEB) e da α-toxina por *S. aureus* foi avaliada em estudo de Smith-Palmer, Stewart e Fyfe (2004). Estes pesquisadores observaram que a hemólise devido à α-toxina foi significativamente reduzida após a cultura em presença dos óleos essenciais testados, e que a produção de SEA e de SEB foi diminuída quando a bactéria foi cultivada na presença dos óleos essenciais de cravo e canela.

Embora a atividade de óleos essenciais e seus constituintes seja comprovada contra diversos patógenos relacionados a alimentos, geralmente estes compostos são empregados como agentes flavorizantes (BURT, 2004), e sua aplicação em matrizes alimentares com o objetivo de controle microbiano pode requerer a aplicação de quantidades superiores ao que seria aceitável sensorialmente (NAVEENA et al., 2006; SOUSA et al., 2012). Assim, a adição de quantidades subletais de óleos essenciais pode ser uma opção para fornecer o balanço entre aceitabilidade sensorial e eficácia antimicrobiana (AZEREDO et al., 2011).

4.3.1 Propriedade antimicrobiana do óleo essencial de Origanum vulgare *L.*

A espécie *Origanum vulgare* L. (orégano) é nativa de regiões montanhosas do Sul da Europa. Pertencente à família *Lamiaceae*, é uma planta herbácea, perene, ereta, aromática, de hastes algumas vezes arroxeadas, de 30-50 cm de altura. Apresenta folhas simples, esparso-pubescentes, de 1-2 cm de comprimento e as flores são esbranquiçadas, rosas ou violáceas, dispostas em glomérulos e reunidos em inflorescências paniculadas terminais. Os seus óleos essenciais são ricos em polifenóis, que protegem as células e inibem o envelhecimento da planta. Além disso, esta espécie se destaca devido às propriedades biológicas que têm sido exploradas em particular pelas indústrias farmacêutica, de cosmético, culinária, agricultura, como substâncias aromatizantes em alimentos e perfumes em decorrência de sua fragrância picante (ALIGIANIS et al., 2001; LORENZI; MATOS, 2002; NEPOMUCENO, 2007).

O OEOV tem sido conhecido como uma fonte interessante de compostos antimicrobianos alternativos para serem aplicados na conservação de alimentos (CHUN et al., 2005; SOUZA et al., 2007), tendo apresentado resultados significantes na inibição do crescimento de bactérias, fungos e sobre a síntese de metabólitos microbianos (BAYDAR et al., 2004; NOSTRO et al., 2004), sendo Geralmente Reconhecidos como Seguros (GRAS) [PITTMAN et al., 2011].

O OEOV apresenta uma grande variação na composição, principalmente em compostos ativos, em que os fenóis monoterpenos, tais como timol e CAR, alcançaram 80.2 a 98% de composição total deste óleo essencial (SIMÕES et al., 2003; ARCILA-LOZANO et al., 2004; REHDER et al., 2004; RODRIGUES et al., 2004; CLEFF et al., 2008).

O CAR parece não mostrar efeitos tóxicos marginais ou significantes *in vivo* (DEMIRCI et al., 2004) e tem sido conhecido como um marcador de atividade antimicrobiana em óleos essenciais (FARIAS-ALVES; SICCHIROL-LAVRADOS; PEREIRA-DE-MARTINIS, 2003; CHUN et al., 2005). O CAR aumenta a permeabilidade passiva da membrana citoplasmática microbiana, devido a sua capacidade de dissolução na bicamada de fosfolipídio, se alinhando entre os ácidos graxos e resultando em distorção da estrutura física da membrana citoplasmática (DORMAN; DEANS, 2000; LAMBERT et al., 2001).

Resultados obtidos por Tsigarida, Skandamis e Nychas (2000) revelaram que o OEOV (adição de 0.8% v/w) é capaz de inibir *L. monocytogenes* e flora microbiana nativa em redução inicial de 2-3 $\log_{10}$ UFC/g durante estocagem de carne bovina em embalagem com atmosfera modificada (MAP; 40% CO_2/30% O_2/30% N_2) e a vácuo.

A adição de OEOV na concentração de 0.8% (v/p) em filés de carne bovina, juntamente com o uso de embalagens com diferentes concentrações de CO_2, O_2, N_2 e a vácuo, resultou em uma redução inicial da população de *S.* Typhimurium de 1 a 2 log_{10}, partindo-se o ensaio de um inóculo inicial de aproximadamente 5 log_{10} UFC/g a uma temperatura de 5°C, como observado por Skandamis, Tsigarida e Nychas (2002). Também foi observada uma redução da flora microbiana nativa dos produtos cárneos. Os autores destacaram que o uso em conjunto de atmosferas modificadas em baixas temperaturas e OEOV poderia ser discutido com finalidades práticas de emprego na conservação de produtos cárneos.

Marino, Bersani e Comi (2001) em estudo sobre o efeito antimicrobiano de várias especiarias, mostraram que o OEOV foi o mais efetivo na inibição de bactérias Gram-positivas e Gram-negativas, causando um alongamento da fase lag. Além disso, este óleo essencial apresentou-se como eficiente substância bactericida, detectado pela não recuperação de tais micro-organismos após reincubação em meio de recuperação. Entre as cepas Gram-negativas, *E. coli* O157:H7 foi a mais sensível, enquanto *L. monocytogenes* e representantes da família *Bacilaceae* foram os mais sensíveis entre as bactérias Gram-positivas ensaiadas.

Análises realizadas por Baydar et al. (2004) sobre a efetividade antibacteriana do OEOV em concentrações distintas (1/50, 1/100, 1/200, 1/300 v/v) sobre 15 bactérias envolvidas na deterioração e/ou como agentes causadores de DVA, mostraram que na concentração de 1/50 (v/v), tal composto foi eficaz na inibição de todas cepas bacterianas ensaiadas, entre as quais podendo-se citar *Aeromonas hydrophila, Bacillus amyloliquefaciens, B. brevis, Corynebacterium xerosis, Klebsiella pneumoniae, L. monocytogenes, Micrococcus luteus, M. smegmatis, S. aureus, Yersinia enterocolitica*, entre outros.

A eficiência do OEOV em combinações com outros compostos com propriedades antimicrobianas também tem sido avaliada. Apostolidis, Know e Setty (2008) observaram esta atividade com o OEOV, frente a *L. monocytogenes* em caldo e em carne moída cozida, quando combinado com extrato de *cranberry* na proporção 50:50, e lactato de sódio a 2%. Em outro estudo, Lin, Labbe e Shetty (2004) relataram atividade antimicrobiana aumentada quando o OEOV e o extrato de *cranberry* foram misturados na proporção de 75% de OEOV e 25% do extrato, sendo a eficácia desta combinação aumentada pelo uso adicional de ácido lático.

Du et al. (2009) avaliaram os óleos essenciais de pimenta da Jamaica (*Pimenta dioica* L.), alho (*Allium sativum* L.) e de orégano incorporados em filmes comestíveis frente *E. coli* O157: H7, *S. enterica* e *L. monocytogenes*, encontrando que o OEOV foi o mais efetivo entre os óleos essenciais testados, seguido do óleo essencial de pimenta da Jamaica e do óleo

essencial de alho. A atividade do OEOV e do CAR também foi maior quando comparada a outros óleos essenciais em ensaios com *S. enterica* e *E. coli* em suco de maçã (FRIEDMAN et al., 2004).

Resultados obtidos por Barros et al. (2009) mostraram que o cultivo da cepa padrão *S. aureus* ATCC 6538 em caldo nutriente suplementado com concentrações subletais de OEOV (1/2 CIM, 0.3 µL/mL e 1/4 de CIM, 0.15 µl/mL) durante 24 h interferiu na atividade metabólica bacteriana, sendo observada redução de tolerância ao sal (NaCl), da atividade de lipase e da coagulase, bem como da produção de enterotoxinas.

Posteriormente, Serio et al. (2010) avaliaram o efeito do OEOV sobre a viabilidade de *L. monocytogenes* ATCC 7644 e observaram que após 30 min de exposição em concentrações variando de 0 a 1.25%, o óleo essencial na concentração de 0.25% reduziu a viabilidade celular em 1.70 log UFC.mL^{-1}, sendo que o efeito aumentou gradualmente até a concentração de 1.25%, onde foi observada redução de 5 log UFC.mL^{-1}.

A atividade do OEOV também tem sido avaliada frente a isolados clínicos, e/ou de origem epidêmica. Becerril, Nerín e Gómez-Lus (2012) verificaram a susceptibilidade de 48 isolados clínicos e de 12 cepas padrão de bacilos Gram-negativos aos óleos essenciais de orégano e canela, e combinações de ambos. Os óleos essenciais testados foram ativos contra todos os isolados testados e na análise de efeitos do uso dos óleos essenciais combinados, não foi observado sinergismo ou antagonismo. Ainda, dentre os bacilos testados, as espécies *Serratia marcenscens*, *Morganella morganii*, *Proteus mirabilis* e *P. aeruginosa* foram expostas a cultivo *overnight* em meio adicionado de concentrações subletais dos óleos essenciais e foi constatado que *M. morganii* e de *P. mirabilis* tiveram aumento de quatro vezes nos valores de CIM e CBM frente ao OEOV. Em relação ao óleo essencial de canela, não houve alteração nos valores de CIM para as mesmas espécies.

No estudo de Karatzas et al. (2001), detectou-se uma ação sinérgica nos tratamentos combinados de CAR e alta pressão hidrostática (HHP) em diferentes temperaturas, utilizando células de *L. monocytogenes* em crescimento exponencial. Os efeitos antimicrobianos foram mais relevantes a 1^{o}C do que a 8^{o}C ou a 20^{o}C. Além disso, a adição de CAR para células expostas ao tratamento subletal pela alta pressão hidrostática causou reduções similares em números viáveis, assim como no tratamento simultâneo com CAR e HHP. O sinergismo também foi observado entre CAR e HHP em leite semi-desnatado, que foi artificialmente contaminado com *L. monocytogenes*.

Morente et al. (2010) estudaram a ação de CAR e 2-nitro-1-propanol em diferentes concentrações, frente a *E. coli* O157:H7, *S.* Enteritidis, *B. cereus* e *S. aureus* inoculadas em

uma massa de farinha de milho adicionada destes compostos. Após 24 h, foi observada completa inibição das bactérias testadas, quando a concentração final dos agentes foi de 5%, porém a inibição variou conforme as concentrações testadas e tipo de inóculo (co-cultura ou cultura pura).

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.

5 Materiais e Métodos

Os experimentos foram conduzidos no Laboratório de Microbiologia e Bioquímica dos Alimentos (Centro de Ciências da Saúde) e no Laboratório de Ácidos Graxos (Centro de Tecnologia) da Universidade Federal da Paraíba, Campus I, João Pessoa-PB.

5.1 Óleo essencial de *O. vulgare* L. e carvacrol

O óleo essencial de *O. vulgare* L. (OEOV), tipo *food-grade*, foi obtido da Aromalândia Ind. Com. Ltda. (Minas Gerais, Brasil), atendendo 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. O constituinte carvacrol (CAR) foi obtido da Empresa Sigma Aldrich Brasil Ltda. As soluções do OEOV e do CAR (160-0.075 μL/mL) foram preparadas em caldo Nutriente (Sigma, França) usando Ágar Bacteriológico (0.15g/100mL) como agente estabilizante (BENNIS et al., 2004).

5.2 Cepas bacterianas

As cepas padrão de *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027 e *S.* Typhimurium ATCC 14028 utilizadas como micro-organismos testes nos ensaios antimicrobianos foram obtidas através 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 testes foram cultivadas em caldo Infusão de Cérebro e Coração (BHI, Himedia, Índia) por 18 h a 35°C. Feito isso, as células bacterianas foram separadas através de centrifugação (4000 rpm por 12 min, 4°C), lavadas duas vezes com tampão fosfato-salino (PBS, 0.1 g/100mL, Oxoid, Brasil) e resuspendidas em tampão fosfato-salino (PBS, 0.1 g/100mL, Oxoid, Brasil). As suspensões bacterianas foram ajustadas em espectrofotômetro para uma densidade óptica de 1,5 a 620 nm, correspondente a um inóculo de aproximadamente 9 log UFC/mL (CARSON; MEE; RILEY, 2002). Culturas estoque foram mantidas em tubos de ensaio com ágar Nutriente (Himedia, Índia) inclinado a 7^{o}C.

5.3 Preparação do caldo base carne

Os ensaios de dinâmica de crescimento, investigação da capacidade de adaptação e desenvolvimento de tolerância (direta e cruzada) das cepas bacterianas foram realizados utilizando caldo carne como substrato de cultivo. Para isso, carne bovina do tipo patinho foi separada da gordura externa e cortada em pedaços de tamanho uniforme (3 x 3 x 3 cm), que foram fervidos em água destilada durante 30 min a 90°C. Em seguida, a carne foi retirada, obtendo-se cerca de 500 mL de caldo que foram submetidos à filtração a vácuo através de papel filtro Whatman No. 1. O filtrado foi esterilizado em autoclave durante 15 min (1,21 atm), sendo posteriormente armazenado a -20°C em alíquotas de 50 mL. Quando necessário, aliquota foi descongelada sob refrigeração (7 ± 1°C) e utilizada para os ensaios (OLIVEIRA et al., 2010).

5.4 Determinação da Concentração Inibitória Mínima

A Concentração Inibitória Mínima (CIM) do OEOV ou CAR foi determinada através da técnica de macrodiluição em caldo (NOSTRO et al., 2001). Inicialmente, 1 mL da suspensão bacteriana foi inoculado em 4 mL de caldo Nutriente (Sigma, França). Em seguida, foram adicionados 5 mL da solução do OEOV ou do CAR em concentrações variando de 40 a 0.62 µL/mL. A mistura foi agitada durante 30 seg utilizando aparelho tipo Vortex, e incubada a 35°C por 24 h. A menor concentração do óleo essencial ou constituinte capaz de inibir o crescimento microbiano visível (turvação) foi considerada como a CIM. Suspensões sem o óleo essencial ou constituinte foram testadas similarmente.

5.5 Ensaios de viabilidade bacteriana

Os ensaios de influência do OEOV e do CAR (1/4 CIM, 1/2 CIM e CIM) na cinética de crescimento microbiano foram realizados pelo 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 do OEOV ou do CAR. O sistema foi incubado a 35°C, e nos intervalos de 0, 15, 30, 45, 60 e 120 min pós-incubação, uma alíquota de 1 mL da suspensão foi diluída seriadamente (1:9 v/v) em PBS (0.1 g/100mL, Oxoid, Brasil) estéril e inoculada em Ágar Nutriente (Himedia, Índia). O experimento controle foi constituído por caldo carne

inoculado sem adição do OEOV ou do CAR. A contagem do número de células viáveis foi expressa em log UFC/mL (BARROS et al., 2009).

5.6 Indução de tolerância bacteriana direta

A capacidade das cepas bacterianas desenvolverem tolerância direta ao OEOV ou CAR foi avaliada através da sua exposição continuada em cultivo *overnight* em caldo carne adicionado de concentrações subletais destes compostos.

Para isso, 18 mL de cada substrato de crescimento contendo inóculo inicial de 5 log UFC/mL (2 mL) foram adicionados do OEOV ou de CAR em quantidade suficiente para obtenção de uma concentração final de 1/4 CIM e 1/2 CIM. Sistemas de cultivo das cepas bacterianas sem adição do OEOV ou do CAR foram usados como ensaio controle. O material foi incubado a 35ºC durante 18 h (LUZ et al., 2012b; GOMES-NETO et al., 2012) e posteriormente uma alíquota de 2 mL foi utilizada como inóculo para a análise de dinâmica de crescimento das cepas adaptadas (tratadas) e não adaptadas (controle) em caldo carne (20 mL) adicionado do OEOV na concentração ajustada à CIM correspondente.

A análise de dinâmica de crescimento microbiano foi realizada nos intervalos de 0, 30, 60, 120, 180 e 240 min através da contagem de células viáveis em Ágar Nutriente (Himedia, Índia) de acordo com metodologia descrita por Sagdiç (2003), sendo os resultados expressos em log UFC/mL.

5.7 Indução de tolerância bacteriana cruzada

A indução de tolerância cruzada nas cepas bacterianas foi realizada por exposição *overnight* a concentrações subletais do OEOV ou CAR em caldo de carne, seguida por exposição a fatores ambientais (temperatura moderada, baixo pH e NaCl) como descrito por Luz et al. (2012b) e Gomes-Neto et al. (2012).

Para isso, testes preliminares foram realizados para avaliar a máxima condição não inibitória para as cepas ensaiadas 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), e caldo carne sem os compostos antimicrobianos. Após o estabelecimento das condições de estresse, uma alíquota de 2 mL de suspensão bacteriana foi inoculada em 18 mL de caldo carne adicionado do OEOV ou CAR (concentrações finais de 1/4 CIM ou 1/2 CIM; tratamento de adaptação).

Os ensaios foram estaticamente incubados *overnight* a 35°C (contagens de células viáveis finais para células pré-adaptadas foram sempre entre 5 e 6 log UFC/mL, e as contagens de células viáveis para os ensaios controle estiveram compreendidos entre 6 e 7 log UFC/mL) e, posteriormente, uma alíquota de 2 mL de cada tratamento foi inoculada em 18 mL de caldo carne (incubada estaticamente a 35°C), acidificada 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 a indução de ácidotolerância e osmotolerância, respectivamente.

Para analisar a indução de termotolerância, uma alíquota de 2 mL de cada tratamento foi inoculada em 18 mL de caldo carne incubado estaticamente 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 UFC/mL (SAGDIÇ, 2003).

Para determinar se a tolerância bacteriana cruzada foi induzida, as contagens de células viáveis ao longo do tempo sujeitas à adaptação ao OEOV ou CAR foram calculadas e comparadas com as obtidas a partir de suspensões bacterianas não adaptadas.

5.8 Indução de tolerância através de ciclos de habituação

A capacidade das cepas bacterianas desenvolverem tolerância ao OEOV ou CAR foi também avaliada através de ciclos de habituação por exposição sucessiva a diferentes concentrações dos antimicrobianos testados (1/16 CIM, 1/8 CIM, 1/4 CIM, 1/2 CIM, CIM e 2 CIM) ao longo de cultivo em caldo carne (20 mL) de acordo com o procedimento descrito por To et al. (2002) com modificações. Inicialmente, as cepas foram expostas à menor concentração do OEOV ou CAR 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 crescimento em Ágar Nutriente (Himedia, Índia), seguido por incubação a 35°C por 24 h. Em seguida, uma alíquota deste sistema (2 mL) foi inoculada ao meio de cultivo adicionado do OEOV ou CAR na concentração 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 (2 CIM).

5.9 Análise dos ácidos graxos da membrana citoplasmática bacteriana

Culturas de *S.* Typhimurium ATCC 14028 foram expostas ao OEOV ou CAR em concentrações subletais (1/4 CIM, 1/2 CIM) e avaliadas quanto ao seu perfil de ácido graxo. Dois litros de caldo carne com um pré-inóculo (5 log UFC/mL) de *S.* Typhimurium foram tratados com concentrações subletais de cada composto e cultivados a 35°C por 18h sob agitação (130 rpm). Após este período, as células foram sedimentadas por centrifugação (8000 x g, a 4°C, 10 min) e resuspensas em 50 mL de água ultra-purificada.

A extração de lipídios e ésteres metílicos dos ácidos graxos a partir de sedimentos de células foi realizada de acordo com o procedimento descrito por Hartman e Lago (1973). Suspensões sem o óleo essencial ou constituinte (aproximadamente 6.5 log UFC/mL) foram testadas similarmente.

O perfil de ácidos graxos das amostras foi avaliado utilizando um cromatógrafo gasoso Varian 430-GC com detector de ionização de chama (FID) e uma coluna de 60 m x 0.25 mm de capilar de sílica fundida com um mícron de espessura de filme 25 (Varian CP WAX 52 CB). Hélio foi utilizado como gás transportador, a um caudal de 1 mL/min. A temperatura do forno foi aumentada de 100^{o}C para 240^{o}C numa taxa de 2,5^{o}C/min e mantida a 240^{o}C durante 20 min, com um tempo total de 76 min. As temperaturas do injetor e do detector foram mantidas a 250°C e 260°C, respectivamente.

Uma alíquota de 1 µL do extrato esterificado foi injetada em um tipo de injetor de separação, e os cromatogramas foram registrados usando o software Sistema de Dados de Cromatografia de Galaxie. Os ácidos graxos foram identificados por comparação de tempos de retenção de ésteres metílicos com os padrões da Supelco ME19-Kit (ésteres metílicos de ácidos graxos C6-C24). Os resultados foram quantificados por área de normalização dos ésteres metílicos e expressa como percentagem (%) da área.

5.10 Ensaios de dano à membrana citoplasmática e externa

A verificação de danos à membrana citoplasmática e externa após exposição ao OEOV ou CAR em concentrações subletais (1/4 CIM e 1/2 CIM) foi avaliada em *S.* Typhimurium ATCC 14028 cultivada em caldo carne por 18 h a 35^{o}C. Para tanto, as células foram semeadas em Ágar BHI (Himedia, Índia), Ágar BHI (Himedia, Índia) suplementado com cloreto de sódio (NaCl, 3 g/100 mL; BHI-CS) ou Ágar BHI (Himedia, Índia) suplementado com sais biliares (0.35 g/100 mL; BHI-SB), cujas concentrações foram estabelecidas previamente

como a concentração máxima não inibitória, e incubadas durante 48 h a 35ºC (ESPINA et al., 2011). A extensão da lesão subletal foi expressa como a diferença entre as contagens em log no meio não seletivo (BHI) e as contagens em log em meio seletivo (BHI-CS e BHI-SB).

5.11 Irradiação da carne para o modelo base carne

Carne moída magra e crua, adquirida em supermercado local na cidade de João Pessoa-PB, foi utilizada para avaliação de indução de tolerância bacteriana direta em um micromodelo alimentar. Para isto, a carne moída foi distribuída em sacos a vácuo (30g/saco), para as concentrações subletais (1/4 CIM, 1/2 CIM) bem como para o ensaio controle, congeladas a -20^{o}C e irradiadas (25 kGy; 2h) para eliminar a microflora nativa. As amostras foram então armazenadas a -20^{o}C e quando requerido, foram descongeladas sob refrigeração (7± 1^{o}C) e utilizadas para os ensaios (JUNEJA; HWANG; FRIEDMAN, 2010). Amostras aleatórias foram testadas previamente para verificar a eliminação ou inativação de microflora por diluição (1:1) da carne moída irradiada em PBS (0.1 g/100mL, Oxoid, Brasil), seguida por plaqueamento direto em superfície da suspensão (0.1 mL e 1 mL) em Ágar Nutriente (Himedia, Índia) e incubadas aerobicamente a 35^{o}C por 48 h.

5.12 Indução de tolerância bacteriana direta em modelo base carne

A indução de tolerância direta em *P. aeruginosa* ATCC 9027 utilizando um modelo base carne foi realizada por exposição a concentrações subletais do OEOV ou CAR (concentrações finais de 1/4 CIM e 1/2 CIM) em amostras de carne irradiada, armazenadas previamente a 7°C. Para isto, as amostras de carne moída (30g/saco) contendo o OEOV ou CAR foram inoculadas com 3 mL da suspensão bacteriana e homogeneizadas em Stomacher (Modelo MA440, Marcone Ltda., Piracicaba, Brasil) por 5 min para assegurar uma distribuição de micro-organismos nas amostras de carne (contagem final de células viáveis de células pré-adaptadas finais entre 5 e 6 log UFC/mL, e as contagens de células viáveis para o ensaio controle compreendidas entre 6 e 7 log UFC mL) e incubadas *overnight* a 7ºC.

Depois disso, as amostras de carne moída inoculadas foram adicionadas do OEOV ou CAR (concentração final ajustada à CIM), e novamente misturadas em Stomacher (Modelo MA440, Marcone Ltda., Piracicaba, Brasil) por 5 min e incubadas a 7ºC. Células viáveis nos sistemas foram enumeradas ao longo do tempo (60, 120, 180 e 240 min, e 24, 48 e 72 h) por diluição em série (10^{-1} - 10^{-5}) em PBS (0.1 g/100mL, Oxoid, Brasil), plaqueadas em Ágar

Nutriente (Himedia, Índia) e incubadas por 24-48 h a 35°C. Suspensões sem o óleo essencial ou constituinte foram testadas similarmente e os resultados expressos em log CFU/mL (SAGDIÇ, 2003).

5.13 Análises estatísticas

Os ensaios de investigação de indução de tolerância das cepas bacterianas foram realizados em triplicata, sendo os resultados expressos como médias dos três ensaios paralelos. As análises estatísticas foram realizadas utilizando o teste de Tukey para determinação da diferença significativa entre as médias obtidas, considerando $p < 0,05$ e o software Sigma Stat. 3.1. Os resultados das análises de ácidos graxos foram analisados no software SAS 9.1 e submetidos à análise de variância (ANOVA) e teste de Tukey para a comparação de médias, com $p < 0,05$ para significância.

6 RESULTADOS E DISCUSSÃO

Os resultados e discussão produzidos durante o desenvolvimento da tese de doutorado estão expostos em formato de artigos científicos submetidos (constam no item Apêndices) ou publicados (presentes no item Anexos) em revistas científicas indexadas, e formatados de acordo com as normas dos periódicos. Por sua vez, a comprovação de submissão dos artigos científicos enviados para publicação também está incluída no item Anexos.

7 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O desenvolvimento deste estudo poderá auxiliar a corroborar uma possível aplicação futura do OEOV e CAR em sistemas de conservação de alimentos, considerando a influência de concentrações subletais destes compostos não apenas sobre o controle do crescimento microbiano como também sobre a capacidade de adaptação e/ou desenvolvimento de tolerância em bactérias contaminantes de alimentos. Desta forma, a aplicação do OEOV e CAR em matrizes alimentares poderá alcançar equilíbrio entre eficácia antimicrobiana e aceitação sensorial.

Além disso, a partir da análise dos dados obtidos nesta pesquisa, podem ser sugeridas algumas perspectivas para estudos posteriores. Estas perspectivas poderiam ser assim mencionadas:

a) Investigar os efeitos da exposição a concentrações subletais (1/4 CIM, 1/2 CIM) do OEOV e CAR sobre novos sítios alvo nas células bacterianas de origem alimentar, como: efeito sobre o perfil da mureína (envelope composto por peptidoglicano que mantem a rigidez ou a integridade da parede celular e contorno bacteriano); efeito nas respostas mediadas por regulação gênica que envolvam a síntese de ácidos graxos da membrana e lipídio A; efeito sobre a expressão de bombas de efluxo;

b) Investigar a capacidade de desenvolvimento de tolerância bacteriana cruzada a concentrações subletais do OEOV e CAR frente aos principais grupos de antibióticos em bactérias de origem alimentar sensíveis ou resistentes a estes antimicrobianos;

c) Ampliar a investigação sobre o potencial antimicrobiano do OEOV e CAR, em concentrações subletais, sobre a capacidade de adaptação e indução de tolerância em bactérias inoculadas e cultivadas em matrizes alimentares de composição distinta (culturas puras ou mistas).

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APÊNDICES

APÊNDICE A - Sublethal amounts of *Origanum vulgare* L. essential oil and carvacrol cause injury and changes in membrane fatty acid of *Salmonella* Typhimurium cultivated in a meat broth

APÊNDICE B - Study of tolerance induction in *Pseudomonas aeruginosa* in artificially contaminated meat added of *Origanum vulgare* L. essential oil or carvacrol

APÊNDICE A - Sublethal amounts of *Origanum vulgare* L. essential oil and carvacrol cause injury and changes in membrane fatty acid of *Salmonella* Typhimurium cultivated in a meat broth

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Sublethal amounts of *Origanum vulgare* L. essential oil and carvacrol cause injury and changes in membrane fatty acid of *Salmonella* Typhimurium cultivated in a meat broth

Running-title: *Salmonella* challenged with oregano essential oil

Isabelle da Silva Luz[1], Adma Nadja Ferreira de Melo[2], Taliana Kênia Alves Bezerra[3], Marta Suely Madruga[3], Marciane Magnani[2], and Evandro Leite de Souza[1]

[1] Laboratory of Food Microbiology, Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil

[2] Laboratory of Biochemistry of Foods, Department of Food Engineering, Center of Technology, Federal University of Paraíba, João Pessoa, Brazil

[3] Laboratory of Flavour, Department of Food Engineering, Center for Technology, Federal University of Paraíba, João Pessoa, Brazil

Corresponding author: Marciane Magnani

Federal University of Paraíba

Laboratory of Biochemistry of Foods

Department of Food Engineering

Campus I, 58051-900, João Pessoa, Paraíba, Brazil

email: magnani2@gmail.com

Telephone number: + 55 83 3216 7576

Fax number: + 55 83 3216 7094

Abstract

This study aimed to evaluate whether sublethal concentrations of the essential oil of *Origanum vulgare* L. (OVEO) and its major compound carvacrol (CAR) cause injury to the cell membrane and outer membrane of *Salmonella enterica* serovar Typhimurium ATCC 14028 grown in a meat broth and to assess the effect of these substances on membrane fatty acid (FA) composition. Exposure of *S.* Typhimurium ATCC 14028 to sublethal concentrations of OVEO or CAR caused damage to the cytoplasmic membrane and outer membrane. OVEO and CAR-treated cells showed lower amounts of saturated FA than non-treated cells. Changes in membrane FA composition were mainly related to an increase of C16:1ω7c, C16:1ω7t and C18:2ω6c; and decrease of C16:0, C17:0 cyclo and C19:0 cyclo. These results indicate that exposure to sublethal concentrations of OVEO or CAR caused sublethal injury *S.* Typhimurium ATCC 14028 and suggest that an adaptive response to these stresses is related to increased synthesis of unsaturated FA and *cis-trans* isomerization.

Keywords: *Salmonella*; injury; adaptive response; plant compounds

Introduction

Stress response development in bacteria has received much attention in recent years, including concern from a food safety perspective (Di Pasqua *et al.*, 2006). The stressful conditions that microorganisms face during food processing could lead to sublethal injury and subsequent development of physiological adaptive responses (Luz *et al.*, 2012a). *Salmonella enterica* serovar Typhimurium is one of the most frequent causes of food-borne disease around the world (Silva *et al.*, 2011). Resistance of many enteropathogenic bacteria, such as *Salmonella*, to antibiotics and other biocides raises

interest about the use of natural compounds, such as essential oils, as a possible alternative for the control of these pathogens (Dubois-Brissonnet *et al.*, 2011).

Acquired tolerance to antimicrobial compounds can be related to various cellular physiological changes such as genetic changes that occur through plasmid acquisition or mutation, synthesis of stress proteins and modification of lipid membrane composition (Patrignani *et al.*, 2008). Some researchers have reported the implications of changes in membrane fatty acid composition, to maintain both membrane integrity and functionality, as a primary response of bacteria to several sublethal stresses (Álvarez-Ordóñez *et al.*, 2008).

In an early study, it was found a Minimum Inhibitory Concentration (MIC) value of both *Origanum vulgare* L. essential (OVEO) and its major compound carvacrol (CAR) of 1.25 µL/mL against *Salmonella enterica* serovar Typhimurium ATCC 14028 (*S*. Typhimurium ATCC 14028). It was also observed that cells of this strain sub-cultured (24-h cycles) with increasing concentrations of OVEO or CAR survived a one fold increase over MIC, suggesting minor changes in sensitivity to OVEO and CAR (Luz *et al.*, 2012a). Here, it is reported the effects of OVEO and CAR at sublethal concentrations on the cell membrane and outer membrane of *S*. Typhimurium ATCC 14028 grown in a meat broth and the effects of OVEO and CAR exposure on bacterial membrane fatty acid composition.

Material and Methods

Essential oil and CAR

OVEO (batch OREORG01; density at 20 °C, 0.90; refractive index at 20 °C, 1.47), obtained by steam distillation, was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil). CAR was purchased from Sigma Aldrich (Sigma, France). Solutions of

OVEO and CAR were prepared in nutrient broth (Himedia, India) with a range of concentrations (160 - 0.075 μL/mL) using bacteriological agar (0.15 g/100 mL) as a stabilizing agent (Luz *et al.*, 2012b).

Test strain

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, India) under refrigeration (7 ºC). Unless stated otherwise, the inocula (c.a. 8 log cfu/mL) used in the assays were obtained from suspensions of the strain at the end of the exponential phase of growth according to a previously described procedure (Di Pasqua *et al.*, 2006). The suspension was serially diluted in PBS (10^{-1}) to provide a viable cell count of approximately 7 log cfu/mL.

Preparation of meat broth

Bovine meat steaks were trimmed for all external fat and cut into pieces of uniform size (3 x 3 x 3 cm). Meat pieces were boiled in distilled water for 30 min at 90 ºC. Approximately 500 mL of meat broth was obtained and vacuum filtered using Whatman nº 1. The filtrate was sterilized by autoclaving (121 ºC, 1.1 atm, for 15 min). The filtered broth was stored at – 20 ºC in 50 mL aliquots. When required, one aliquot was thawed under refrigeration (7 ± 1 ºC) and used for the assays (de Oliveira *et al.*, 2010).

Cytoplasmic and outer membrane damage assays

To assess whether OVEO and CAR cause cytoplasmic membrane damage and/or outer membrane damage, *S.* Typhimurium cultures (final viable cell count of

approximately 6 log cfu/mL) were exposed to OVEO or CAR at 1/4 MIC (0.3 µL/mL) or 1/2 MIC (0.6 µL/mL) in meat broth for 18 h under stirring (130 rpm). Cells were then plated on BHI (Brain Heart Infusion) agar, BHI agar supplemented with sodium chloride (NaCl, 3 g/100 mL; BHI-SC) or BHI agar supplemented with bile salts (0.35 g/100 mL; BHI-BS) and incubated for 48 h at 35 ºC (Espina *et al.*, 2013). These levels of NaCl and bile salts were previously determined to be the maximum non-inhibitory concentration for the tested strain. The extent of sublethal injury was expressed as the cell viability inhibition regarding the log counts on the non-selective medium (BHI) and the log counts on the selective medium (BHI-SC or BHI-BS), according the formula: Cell viability inhibition (%) = log cfu/mL on the non-selective medium - log cfu/mL on the selective medium. The detection limit of the test was 2 log cfu/mL.

Analysis of fatty acid profile

S. Typhimurium cultures (viable count of approximately 6 log cfu/mL) were exposed to OVEO or CAR at sublethal concentrations (1/4 MIC, 1/2 MIC) and tested for their fatty acid (FA) profile. Two liters of a pre-inoculum (5 log cfu/mL) of *S.* Typhimurium was treated with sublethal concentrations of each compound by cultivation at 35 °C for 18 h under stirring (130 rpm) (final viable count of approximately 4.5 log cfu/mL). After this period, cells were pelleted by centrifugation (8000 x g, 4 °C, 10 min) and res-suspended in 50 mL of ultra-purified water. Extraction of lipids and obtainment of fatty acid methyl esters from bacterial cells pellets was performed as previously described (Hartman and Lago, 1973). Control flasks without the OVEO or CAR (final viable count of approximately 7.5 log cfu/mL) were similarly performed.

The fatty acid profile of samples was evaluated using a Varian 430-GC gas chromatograph with a flame ionization detector (FID) and a 60 m x 0.25 mm fused silica

capillary column with a 25 micron thick film (Varian CP WAX 52 CB). Helium was used as carrier gas at a flow rate of 1 mL/min. The oven temperature was raised from 100 °C to 240 °C at a rate of 2.5°C/min and maintained at 240°C for 20 min, with a total time of 76 min. The injector and detector temperatures were maintained at 250 °C and 260 °C, respectively. A 1 µL aliquot of the esterified extract was injected into a split-type injector, and the chromatograms were recorded using Galaxie Chromatography Data System software. Fatty acids were identified by comparing methyl ester retention times with standards from the Supelco ME19-Kit (Fatty Acid Methyl Esters C6-C24). Results were quantified by area normalization of the methyl esters and expressed as percent (%) area.

Reproducibility and statistical analysis

Assays were performed in triplicate with two independent trials, and the results were expressed as an average of the assays. Fatty acid profiles were analyzed using SAS 9.1 software and submitted to analysis of variance (ANOVA) and Tukey test for the comparison of averages, utilizing $p < 0.05$ for significance.

Results and Discussion

S. Typhimurium cells challenged with sublethal concentrations of OVEO or CAR in meat broth for 18 h displayed a ≥ 99.9% rate of cell viability loss (viable cell counts ≤ 2 log cfu/mL, which corresponding to the detection limit of the test) when further cultivated in BHI-SC and BHI-BS. However, cells challenged with sublethal concentrations of OVEO or CAR were able to grow further in BHI, suggesting a repair of sublethal injury. Non-exposed cells maintained cell viability in BHI-SC and BHI-BS (c.a. 7.5 log cfu/mL). These findings suggest that cells of *S.* Typhimurium exposed to OVEO or CAR at 1/4 and

1/2 MIC sustained sublethal injuries to their cytoplasmic membranes and outer membranes (Espina *et al.*, 2013).

A total of 21 FA were found in the analysis of *S*. Typhimurium ATCC 14028 cells (Table 1). OVEO- and CAR-treated cells showed lower amounts ($P < 0.05$) of saturated fatty acids (SFA), notably C16:0 (palmitic acid), C14:0 (myristic acid) and C20:0 (araquidic acid), compared with untreated control cells. With regard to unsaturated fatty acids (UFA), OVEO- and CAR-treated cells presented higher amounts ($P < 0.05$) of UFA (1.76 and 3.30; 2.21 and 3.62 fold-increase, respectively) compared with control cells. Challenging the cells with OVEO or CAR resulted in increased synthesis ($P < 0.05$) of the unsaturated fatty acids C16:1 (palmitoleic acid; C16:1ω7c), C16i (palmitoleic acid isomer; C16:1ω7t) and C18:1ω9c (oleic acid).

The isomerization in double bond of fatty acids in bacterial membranes, resulting in an increase ($P < 0.05$) of isomer fatty acids (such as the C16:1ω7t), is reported to confer a membrane chemical stability and protection against toxic molecules (Patrignani *et al.*, 2008, Di Pasqua *et al.*, 2006). The presence of high amounts of the isomer C16:1ω7t in bacterial cells challenged with OVEO or CAR could be related to the increase of UFA in membrane of these cells; there are evidences that the change in the membrane fluidity is a precondition for the isomer formation between other FA alterations (Di Pasqua *et al.*, 2006; Kim *et al.*, 2005). Moreover, the higher amounts ($P < 0.05$) of the isomer C16:1ω7t in pre-adapted cells could be a consequence of the absence of exponential bacterial growth caused by the presence of the tested compounds in cultivation media over the exposure time; in fact exponentially growing bacterial cells are characterized by lower degree or absence of isomer fatty acids (Härtig *et al.*, 2005).

Only the hydroxy-fatty acid 3-OHC14:0 (3-hydroxi-myristic acid) was found and its content was similar in cells exposed and non-exposed to OVEO and CAR. 3-

OHC14:0 has been commonly found in bacterial membranes, such as of *Salmonella* species (Dubois-Brissonnet *et al.*, 2011; Kim *et al.*, 2005). The cyclopropane fatty acids (CFA) C17:0 cyclo (cis-11,12-methylenehexadecanoato) and C19:0 cyclo (cis-9,10-methyleneneoctadecanoato) were found in both treated and non-treated cells, however the content of these fatty acids was lower ($P < 0.05$) in cells exposed to OVEO or CAR when compared to control cells. Usually, only minor amounts of CFA are observed in bacterial membranes during bacterial growth. Increasing amounts of CFA in bacterial cells are detected during the transition of the exponential to the stationary growth phase (Kim *et al.*, 2005), as possibly occurred in control experiments, since the cells were sampled and analyzed immediately after an 18 h cultivation period. Although the synthesis of CFA is reported to be increased by a change in environmental conditions (Álvarez-Ordóñez *et al.*, 2008), a decrease of relative amounts of CFA in *E. coli* and *S.* Typhimurium cells exposed to plant products, such as 2-(*E*)-hexenal and hexanal, was already reported (Patrignani *et al.*, 2008).

The main FA modifications observed in cells challenged with OVEO or CAR were the decrease of CFA and the increase of UFA, particularly in the isomer C16:1ω7t. CFA are produced by the addition of a methyl group from *S*-adenosyl methionine across a UFA *cis*-double bond. Essential oils-related compounds are able to accumulate between acyl chain of fatty acids, limiting the access of *S*-adenosyl methionine to the UFA *cis*-double bond and hence the cyclization of UFA. Since *S.* Typhimurium exposed to phenolic compounds showed decreased CFA synthesis (Di Pasqua *et al.*, 2006), the observed increase in UFA in cells of *S.* Typhimurium cultivated in the presence of OVEO or CAR could be related to the decrease of CFA. A previous study found that the gene of the cyclopropane fatty acid acyl phospholipid synthase *cfa* was downregulated, but not inhibited, in the presence of high amounts of phenolic compounds (Wu *et al.*, 2009).

Similarly, the findings of this study suggest that the cyclization pathway was not completety inhibited when *S.* Typhimurium was exposed to OVEO or CAR at sublethal concentrations because C17:0 cyclo and C19:0 cyclo were still detected, although in small amounts.

The CFA decrease in *S*. Typhimurium cells with the increase of OVEO or CAR amounts in the cultivation broth could also be related with the increasing amounts of C16:1ω7t found in these cells. The formation of *trans* FA is catalyzed by a nonreversible *cis-trans* isomerase in the double bond position (Dubois-Brissonnet *et al.*, 2011). It is interesting to note that the *cis-trans* isomerization occurred in cells exposed to OVEO or CAR in a concentration dependent manner. There is evidence that changes in membrane fluidity are the primary signal eliciting the isomerization reaction, being known as a precondition for *cis-trans* conversion (Härtig *et al.*, 2005).

In bacteria, the UFA:SFA ratio is known to be the predominant mechanism for controlling membrane fluidity, and the saturation level of carbon chains is a critical parameter involved in membrane state (Dubois-Brissonnet *et al.*, 2011). Cells exposed to OVEO or CAR at sublethal concentrations increased the UFA:SFA ratio in a dose-dependent manner, indicating an increase of UFA synthesis in opposite to a decrease in SFA synthesis (mostly in C16:0, C14:0, myristic acid; and C20:0, araquidic acid). Interestingly, it should be noted that an increase in SFA synthesis, considered an important adaptation related to resistance development in bacteria, by hindering the penetration of biocide compounds and their activity (Casadei *et al.*, 2002; Yuk and Marshall, 2004) was not observed in the present study for cells challenged with OVEO or CAR.

The increase in the UFA:SFA observed in *S*. Typhimurium ATCC 14028 due to the of treatment with OVEO or CAR could be resulted mainly from increased synthesis of C16:1ω7c, C16:1ω7t, C18:1ω9c and C18:1ω9c. Studies have suggested that the increased

activity of an oxygen-consuming desaturase system, with a consequent increase in fatty-acid desaturation, changes UFA ratios to maintain an optimal level of membrane fluidity (Guerzoni *et al.*, 2001).

The results reveal that exposure to sublethal concentrations of OVEO or CAR in a meat broth caused sublethal injury to the cytoplasmic membrane and outer membrane of *S.* Typhimurium ATCC 14028. This injury induced changes in membrane FA composition, particularly an increase in UFA synthesis and *cis-trans* isomerization, suggesting an adaptive response related to changes in membrane FA profile. From these results, essential oils and related-compounds, particularly OVEO and CAR, could induce bacterial adaptation and therefore their use in foods must be cautiously considered, especially at sublethal concentrations.

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Disclosure Statement

No competing financial interests exist.

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TABLE 1. MEMBRANE FATTY ACID COMPOSITION OF *S.* TYPHIMURIUM ATCC 14028 EXPOSED AND NON-EXPOSED TO SUBLETHAL AMOUNTS OF *ORIGANUM VULGARE* L. ESSENTIAL OIL OR CARVACROL IN A MEAT-BASED BROTH FOR 18 H AT 35 °C.

FATTY ACIDS	CONTROL	OVEO		CAR	
		1/4 MIC	1/2 MIC	1/4 MIC	1/2 MIC
SFA (% of area)					
C7:0	0.10 (±0.01)	ND	ND	0.03 (±0.02)	0.01 (±0.01)
C8:0	0.31 (±0.04)^{b}	ND	ND	0.02 (±0.01)^{a}	0.04 (±0.01)^{a}
C9:0	0.30 (±0.02)^{b}	0.39 (±0.03)^{b}	0.51 (±0.02)^{b}	0.06 (±0.01)^{a}	0.04 (±0.01)^{a}
C12:0	0.16 (±0.01)^{a}	1.27 (±0.06)^{d}	0.60 (±0.01)^{b}	0.60 (±0.02)^{b}	0.96 (±0.03)^{c}
C14:0	3.81 (±0.17)^{b}	2.76 (±0.07)^{a}	1.59 (±0.45)^{a}	2.23 (±0.62)^{a}	2.23 (±0.21)^{a}
C16:0	37.28 (±1.95)^{c}	23.29 (±1.58)^{b}	16.34 (±2.15)^{ab}	20.22 (±1.95)^{b}	14.39 (±1.63)^{a}
C17:0	0.29 (±0.04)^{b}	0.35 (±0.02)^{c}	0.11 (±0.01)^{a}	0.26 (±0.02)^{b}	0.15 (±0.03)^{a}
C18:0	1.00 (±0.06)^{c}	0.91 (±0.04)^{c}	0.47 (±0.03)^{b}	0.44 (±0.03)^{a}	0.38 (±0.04)^{a}
C19:0	0.36 (±0.02)^{c}	0.41 (±0.03)^{cd}	0.49 (±0.04)^{d}	0.29 (±0.02)^{b}	0.18 (±0.03)^{a}
C20:0	5.34 (±0.85)^{b}	NF	NF	0.74 (±0.01)^{a}	NF
C21:0	0.81 (±0.07)^{c}	NF	NF	0.35 (±0.02)^{b}	0.23 (±0.04)^{a}
C23:0	1.13 (±0.09)^{b}	NF	NF	0.24 (±0.06)^{a}	NF
UFA (% of area)					
C15:1ω5c	NF	2.25 (±0.07)^{a}	3.90 (±0.52)^{a}	NF	NF
C16:1ω7t	NF	27.78 (±2.21)^{a}	50.66 (±2.86)^{c}	38.87 (±2.23)^{b}	57.78 (±2.78)^{c}
C16:1ω7c	0.50 (±0.08)^{a}	5.76 (±0.05)^{c}	3.32 (±0.12)^{b}	5.04 (±0.85)^{b}	2.98 (±0.26)^{b}
C18:1ω9c	1.23 (±0.06)^{a}	15.83 (±1.10)^{c}	8.43 (±0.95)^{b}	12.39 (±1.08)^{c}	6.68 (±1.01)^{b}
PUFA (% of area)					
C18:2ω6c	15.81 (±1.85)^{c}	5.80 (±0.06)^{b}	4.36 (±0.66)^{a}	5.88 (±0.55)^{b}	4.23 (±0.07)^{a}
C20:4ω6c	2.04 (±0.09)	NF	NF	NF	NF
HFA (% of area)					
3-OH (C14:0)	0.41 (±0.02)^{a}	0.43 (±0.05)^{ab}	0.45 (±0.01)^{b}	0.37 (±0.05)^{a}	0.48 (±0.02)^{b}
CFA (% of area)					
C17:0 cyclo	23.11 (±2.15)^{c}	11.33 (±1.42)^{b}	8.13 (±0.89)^{ab}	10.96 (±1.12)^{b}	7.74 (±0.83)^{a}
C19:0 cyclo	6.03 (±0.95)^{c}	1.44 (±0.04) ^{b}	0.43 (±0.02)^{a}	1.01 (±0.23)^{b}	1.52 (±0.32)^{b}
SFA	50.89	29.38	20.11	25.48	18.61
UFA	1.73	51.62	66.31	56.30	67.44
UFA/SFA	0.03	1.76	3.30	2.21	3.62

Results expressed as average (± standard deviation); NF:not found; MIC: Minimum Inhibitory Concentration; OVEO: *Origanum vulgare* L. essential oil; CAR: Carvacrol; OVEO 1/4 MIC: 0.3 µL/mL; OVEO 1/2 MIC: 0.6 µL/mL; CAR 1/4 MIC: 0.3 µL/mL; CAR 1/2 MIC: 0.6 µL/mL; Control: 0 µL/mL of OVEO or CAR;SFA: saturated fatty acids; UFA: unsaturated fatty acids; HFA: hydroxylated fatty acid; CFA: cyclopropane fatty acid; UFA/SFA: ratio of unsaturated fatty acids to saturated fatty acids; $^{a-d}$ Different superscripts letters on the same raw indicate difference by the Tukey test ($P < 0.05$)

APÊNDICE B - Study of tolerance induction in *Pseudomonas aeruginosa* in artificially contaminated meat added of *Origanum vulgare* L. essential oil or carvacrol

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Study of tolerance induction in *Pseudomonas aeruginosa* in artificially contaminated meat added of *Origanum vulgare* L. essential oil or carvacrol

Running title: Oregano oil and carvacrol added to meat

ISABELLE DA SILVA LUZ[1], NELSON JUSTINO GOMES NETO[1], MARCIANE MAGNANI[2]*, EVANDRO LEITE DE SOUZA[1]

[1] *Laboratory of Food Microbiology, Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil*

[2] *Laboratory of Biochemistry of Foods, Department of Food Engineering, Center of Technology, Federal University of Paraíba, João Pessoa, Brazil*

* Author for correspondence

Universidade Federal da Paraíba

Centro de Tecnologia

Departamento de Engenharia de Alimentos

Campus I, 58051-900, Cidade Universitária, João Pessoa, Paraíba, Brasil.

E-mail adresses: magnani2@gmail.com (Magnani, M)

Telephone number: + 55 83 3216 7576; Fax number: + 55 83 3216 7269

ABSTRACT

A meat based model artificially contaminated with *Pseudomonas aeruginosa* ATCC 9027 was used to assess the interference of the *Origanum vulgare* essential oil (OEOV) and carvacrol (CAR) in the bacterial growth and survival and whether overnight exposure (18 h) to sublethal amounts of this compounds induced direct or cross bacterial tolerance . When exposed to the Minimum Inhibitory Concentration (MIC), 1/2 MIC and 1/4 MIC for 120 min, the OVEO or CAR inhibited the viability of *P. aeruginosa* in meat-based broth. Direct or cross-protection was not induced after exposure to sublethal amounts of OVOE or CAR in meat-based broth and in an artificially contaminated ground-beef. Bacteria progressively subcultured in meat broth with increasing amounts of OVEO or CAR were able to survive up to the MIC for OVOE and to 1/2 MIC for CAR. The results revealed a lack of induction of tolerance in *P. aeruginosa* when exposed to OVEO or CAR in a meat-based model.

***Keywords*:** oregano, carvacrol, *Pseudomonas*, tolerance, meat

1. INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous bacterium that is highly adaptable and metabolically versatile and able to grow in low nutrient environments (Legnani, Leoni, Rapuano, Turin, & Valenti, 1999). *P. aeruginosa* is found in soil, animal feces and water (Aoki et al., 2004), and a microbiological examination of foodstuffs often reveals the presence of *P. aeruginosa*. This bacterium is possibly involved in enteritis transmitted by foods and water (Kim & Wei, 2007) and associated with the spoilage of a variety of foods, including meat (Yi, Zhu & Fu, 2010).

P. aeruginosa has long been regarded as an antibiotic-resistant organism, and the low permeability of its outer membrane is known to prevent many agents from accessing their sites of action (Loughlin, Jones & Lambert, 2002). *Pseudomonas* species are known to adapt to disinfectants when serially passaged in increasing concentrations of a biocide (Jones, Herd & Christie, 1989; Tattawasart, Maillard, Furr & Russell, 1999). Its intrinsic resistance to many antimicrobials and ability to adapt to various sublethal stress conditions gives *P. aeruginosa* a physiological advantage to survive and grow in meat products and makes it a significant threat for the food industry and consumers.

The essential oils of plants and their constituent compounds that have antimicrobial properties have been proposed as an interesting alternative to control this bacterium in foods and food-processing plants. Early studies showed that the essential oil from *Origanum vulgare* L. (OVEO) and its majority compound carvacrol (CAR) have a strong and broad antimicrobial activity against food-related bacteria, including *Pseudomonas* genera [Sousa et al., 2012; Azerêdo et al. 2011].

Although essential oils and their compounds are known as potential antimicrobials for use in foods, little information is available regarding the possibility for the development of direct-protection and or cross-protection by *P. aeruginosa* when exposed to these substances in sublethal amounts using meat-model based. This study aimed to evaluate in a meat based model artificially

contaminated, the efficacy of OVEO and CAR in inhibiting the *P. aeruginosa* ATCC 9027 and to assess the development of direct and or cross tolerance when the strain is challenged with sublethal amounts of these substances.

2. MATERIAL AND METHODS

2.1 Strain test and antimicrobial compounds

P. aeruginosa ATCC 9027 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, India) under refrigeration (7 ± 1ºC). Unless otherwise stated, all used inocula were obtained from stationary-phase cultures according to a previously described method (Gomes-Neto et al., 2012). The obtained bacterial suspensions (c.a. 9 log of CFU/mL) were serially diluted in PBS (10^{-1} - 10^{-3}) to provide a viable cell count of approximately 7 log of cfu/mL.

The OVEO (batch OREORG01; density at 20°C: 0.90; refractive index at 20°C: 1.47) obtained by steam distillation, was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil) CAR was purchased from Sigma Aldrich (Sigma, France). Solutions of OVEO and CAR 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) as a stabilizing agent (Souza et al. 2009). A previous study has demonstrated that CAR (66.9 g/100 g) is the main constituent of the OVEO assayed here (Azêredo et al., 2011).

2.2 Preparation of meat models

Bovine meat steaks were trimmed for all external fat and cut in pieces of uniform sizes (3 x 3 x 3 cm). Meat pieces were boiled in distilled water during 30 min at 90ºC. About 500 mL of meat broth were obtained, and vacuum filtered using Whatman nº1 filter paper. The filtrate was sterilized

using autoclave for 15 min (1.21 atm). Afterwards, the broth was stored at – 20ºC in aliquots of 50 mL, and when required one aliquot was thawed under refrigeration (7 ± 1ºC) and used for the assays (Oliveira, Stamford, Gomes-Neto & Souza, 2010).

Raw lean ground beef, purchase from a local retail supermarket in João Pessoa (Brazil), was used for evaluation of development of bacterial direct-tolerance in artificially contaminated model. For this, the ground beef was separated by batches into Stomacher bags (30 g/bag) 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 – 20ºC, and when required the bags were thawed under refrigeration (7 ± 1ºC) and used for the assays (Juneja, Hwang & Friedman, (2010). Random samples were tested to verify elimination of microflora by diluting (1:1) the irradiated ground beef in PBS, followed by direct surface plating of the suspension (0.1 and 1mL) onto TSA and incubating aerobically at 37°C for 48 h.

2.3 Minimum Inhibitory Concentration (MIC) and time-kill assay

MIC values of OVEO and CAR were determined using macrodilution in broth according procedure described by Nostro et al. (2001). Control flasks without OVEO or CAR were tested similarly. Time-kill assays and assays to determine the development of direct-protection and bacterial cross-protection were performed using a meat-based broth as a substrate for bacterial cultivation. The effect of OVEO or CAR (MIC, 1/2 MIC and 1/4 MIC) on the bacterial viability of *P. aeruginosa* in meat broth over 120 min was evaluated by the viable cell count procedure. For this, 4 mL of meat broth was inoculated with 1 mL of the bacterial inocula, and 5 mL of the solution of OVEO or CAR were added to the system and gently shaken for 30 s using a vortex (viable cells count of approximately 6 log CFU/mL). The system was statically incubated at 37°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 and inoculated on sterile nutrient agar for 24 h at 37°C [19]. Control flasks without the OVEO or CAR were tested similarly. The results were expressed as log of CFU/mL.

2.4 Induction of direct protection in meat based-broth

The induction of direct protection in *P. aeruginosa* was performed by overnight exposure to sublethal amounts of OVEO or CAR in meat broth (Gomes-Neto et al., 2012). For this, 2 mL of the bacterial suspension was inoculated in 18 mL of the meat broth added of OVEO or CAR (final concentration of 1/2 MIC or 1/4 MIC) and vortexed for 30 s (adaptation treatment with viable cells count of approximately 6 log CFU/mL). Control flasks without the OVEO or CAR were assayed similarly (non-adaptation treatment). The systems were statically incubated overnight (18 h) at 37ºC (final bacterial counts for the adapted cells were always between 5 and 6 log CFU/mL; counts for control assays were always between 6 and 7 log CFU/mL). After the incubation period, an aliquot (2 mL) of each treatment was inoculated in a fresh meat broth (18 mL) added of OVEO or CAR (final concentration of the MIC previously determined), shaken for 30 s using a vortex and statically incubated at 37°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 by plating in nutrient agar for 24-48 h at 37°C. The results were expressed as log of CFU/mL. The induction of bacterial direct-protection was assessed by comparing the counts of viable cells in adapted and non-adapted systems.

2.5 Induction of bacterial cross protection meat based-broth

The induction of cross protection in *P. aeruginosa* was performed by an overnight exposure of the bacterium to sublethal amounts of OVEO or CAR in meat broth, followed for exposure to other stressing agents (high temperature, low pH and NaCl) (Luz et al., 2012). To determine the salt tolerance, acid tolerance and thermotolerance of the test bacterial strain, untreated cultures were inoculated in meat broth containing NaCl (5 - 15 g/100mL, at 37°C), lactic acid (pH 4.5 - 6.0, at

37°C) and in meat broth incubated at different high temperatures (40 - 55°C). After the establishment of these conditions, a 2 mL aliquot of a newly obtained bacterial suspension was inoculated in 18 mL of the meat broth added of OVEO or CAR (final concentration of the 1/2 MIC or 1/4 MIC) and vortexed 30 s (adaptation treatment). Control flasks without the OVOE or CAR were assayed similarly (non-adaptation treatment). The systems were statically incubated overnight (18 h) at 37ºC (final viable cell counts for adapted cells were always between 5 and 6 log CFU/mL, and the viable cell counts for the control assays were always between 6 and 7 log CFU/mL). After the incubation period, an aliquot (2 mL) of each treatment was statically inoculated in a fresh meat broth (18 mL) acidified with lactic acid (VETEC Química Fina Ltda., Brazil) to a pH of 5.2 and in a fresh meat broth added of NaCl (Qeel, Brasil) at 10 g/100mL for evaluation of induction of acid tolerance and osmotolerance, respectively, and incubated at 37°C. For analysis of induction of thermotolerance, an aliquot (2 mL) of each treatment was inoculated in a fresh meat broth (18 mL) and statically incubated at 45°C. Viable cells in the systems were enumerated over time (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 37°C. The results were expressed as log of CFU/mL. The induction of bacterial cross-protection was assessed by comparing the counts of viable cells in adapted and non-adapted systems

2.6 Induction of bacterial direct-protection in an artificially contaminated meat-model

The induction of direct protection in *P. aeruginosa* using an artificially contaminated meat-based model was performed by an exposure of the bacterium to sublethal amounts of OVEO or CAR in samples of irradiated ground beef stored at 7°C. For this, samples of ground beef (30 g/bag) containing the OVEO or CAR (final concentrations of 1/2 MIC or 1/4 MIC) was inoculated with 3 mL of the bacterial suspension and blended with a Stomacher (Model MA440, Marcone Ltda., Piracicaba, Brazil) for 5 min to ensure even distribution of microorganism in the meat sample and incubated overnight at 7ºC (final viable cell counts for preadapted cells were always between 5 and

6 log CFU/mL, and the viable cell counts for the control assays were always between 6 and 7 log CFU/mL). Thereafter, the inoculated ground beef samples were added of OVEO or CAR (final concentration of MIC) and again blended with a Stomacher for 5 min and incubated at 7ºC. Systems (bags) without the OVEO or CAR were assayed similarly (non-adaptation treatment). Viable cells in the systems were enumerated over time (0: just after the homogenization in Stomacher, 60, 120, 180 and 240 min; 24, 48 and 72 hours) 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 log of CFU/mL. The induction of bacterial direct- and cross protection was assessed by comparing the counts of viable cells in adapted and non-adapted systems

2.7 Successive habituation in meat based-broth

The capacity of *P. aeruginosa* to develop tolerance to OVEO and CAR was also assessed by exposure of the bacterium to increasing amounts of these substances (1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC and 2 x MIC) throughout successive 24-h habituation cycles in meat broth, regarding a more prolonged time of exposure [Gomes-neto et al., 2012; Luz et al., 2012). For this, 2 mL of the bacterial suspension was inoculated in 18 mL of the meat broth added of OVEO or CAR (final concentration of the 1/16 MIC), shaken for 30 s using a vortex, and statically incubated for 24 h at 37ºC (viable cells counts of approximately 6 log CFU/mL). After the incubation period, a 100µL aliquot of the system was serially diluted (10^{-1} - 10^{-5}) in PBS and inoculated on sterile nutrient agar for viable cell detection (37ºC for 24 h). At the same time, a 2 mL aliquot of the meat broth added of OVEO or CAR at 1/16 MIC (allowing the bacterial growth) was inoculated in a fresh meat broth added of the OVEO or CAR in the next tested higher concentration (1/8 MIC), followed for static incubation at 37°C and viable cell detection according to the condition above cited. This procedure was repeated through the exposition of the bacteria to the other increasing concentrations of OVEO or CAR (1/4 MIC - 2 x MIC), or up to no viable cell was detected.

2.8 Reproducibility and statistics

The assays were made in triplicate on three 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 Tukey test using the Sigma stat 3.1 computer program (Jandel Scientific Software, San Jose, CA).

3. RESULTS

For *P. aeruginosa* ATCC 9027 (*P. aeruginosa*), the OVEO or CAR both had a MIC value of 0.6 µL/mL. After 120 min of exposure at all the tested concentrations (MIC, 1/2 MIC and 1/4 MIC), OVEO and CAR decreased the number of viable cells of *P. aeruginosa*. Decrease in cell viability counts occurred as early as 15 min after exposure to OVEO or CAR and no recovery in the cell viability was observed for the remainder of the exposure time. The OVEO or CAR at 1/2 and 1/4 MIC decreased the viable cell counts of *P. aeruginosa* 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 concentrations of either OVEO or CAR (1/2 MIC or 1/4 MIC) revealed no induction of bacterial direct-protection in the cells exposed for over 240 min in the meat broth supplemented with the same stressing agent at the MIC (Figure. 1A-1B). Compared with the non-adapted cells, *P. aeruginosa* that was previously challenged with sublethal concentrations of OVEO presented lower viable cell counts ($P < 0.05$) when further cultivated in the growth medium containing the same compound at the MIC. Otherwise, no difference ($P > 0.05$) was found in the viable counts of cells adapted and non-adapted when further cultivated in growth medium containing the same compound at MIC.

An examination of the different viable cells cell counts determined after the overnight cultivation of *P. aeruginosa* in both meat broth supplemented with OVEO at sublethal

amounts (4 to 5 log CFU/mL) and in meat broth not supplemented with OVEO (5 to 6 log CFU/mL) revealed similar survival-curve (or growth-curve) shapes over the most assessed time intervals for cells cultivated in both systems (adapted and non-adapted). Although the behavior of the cells adapted to CAR was different (survival-curve shape) from that observed for the non-adapted cells at the initial exposure times (up to 60 min) to the MIC, the viable cell counts found for adapted and non-adapted cells were similar ($P > 0.05$) at the later assessed exposure times.

According to the viable cell counts over 240 min of exposure, *P. aeruginosa* exposed overnight and adapted to sublethal concentrations (1/4 MIC and 1/2 MIC) of OVEO or CAR showed no induction of cross protection to lactic acid (pH 5.2), salt (NaCl at 10 g/100 mL) and high temperature (45°C) (Figure. 2A-2C and Figure. 3A-3C). These results are consistent with the results obtained in the assays for induction of bacterial direct protection. Compared with the non-adapted counterparts, the systems submitted to pre-adaptation to the OVEO or CAR at 1/2 and 1/4 of the MIC revealed lower viable cell counts ($P < 0.05$) when they were further exposed to the heterologous stressing agents (high temperature, low pH and NaCl). The exception was the cells adapted to CAR that were further exposed to high temperature, which had similar viable cell counts ($P > 0.05$) to those of the control.

Still, the survival behavior (survival-curve or growth-curve shape) presented by the adapted and non-adapted cells when further exposed to the heterologous stressing agents was similar for most of the assessed systems. We found fewer viable cell counts (approximately 4 log CFU/mL) of adapted *P. aeruginosa* cells in meat broth containing added NaCl (Figure 2C and 3C) compared with the viable cell counts in the meat broth incubated at high temperature (Figure. 2A and 3A) or containing added lactic acid (Figs. 2B and 3B); although this behavior was also found in the control assay, suggesting that there was no change in the intrinsic sensitivity of the strain with respect to NaCl.

In order to evaluate the behavior of *P. aeruginosa* for the ability to develop direct protection to the OVEO and CAR tolerance in food matrix, we also evaluated the induction of direct protection in this bacterium when it was exposed to these compounds at sublethal amounts (1/2 MIC and 1/4 MIC) for a longer exposure time (72 h) in samples of previously irradiated ground beef. It was observed that, as in the assays of induction of direct bacterial protection using meat broth, there was no development of this feature in *P. aeruginosa* over 72 h of exposure for OVEO or CAR in ground beef (Figure 4A-4B). Compared with the non-adapted, the cells adapted to the OVEO or CAR at 1/2 or 1/4 of the MIC in ground beef revealed lower viable cells counts ($P < 0.05$) when they were further cultivated in the same food matrix containing the OVEO or CAR at the MIC over 72 h. Moreover, no significant difference was observed ($P > 0.05$) between the viable counts of cells adapted with 1/4 MIC or 1/2 MIC of both tested compounds. The survival behavior (kill-curve or growth-curve shape) presented by the adapted and non-adapted cells to OVEO or CAR when further exposed to the same stressing agents at MIC in the tested meat model was similar for most of the assessed systems.

Experiments performed to assess the induction of bacterial tolerance in *P. aeruginosa* ATCC 9027 in meat broth when exposed to consecutive 24-h cycles of increasing concentrations of OVEO or CAR (1/16 MIC to 2 x MIC) showed that *P. aeruginosa* was able to survive (as detected by the viable cell count) in the broth containing concentrations of OVEO or CAR up to the MIC and 1/2 MIC, respectively, revealing only a one-fold increase in the values of the MIC previously determined for the OVEO.

4. DISCUSSION

P. aeruginosa is highly resistant to many biocides, and this intrinsic resistance has often been associated with the nature of its outer membrane, which is composed primarily of a lyposaccharide, which forms a hydrophilic permeability barrier that protects against the effects of toxic agents [Mann, Cox & Markham, 2000; Mayaud, Carricajo, Zhiri, & Aubert, 2008). Because of its hydrophobic nature, the OEOV and CAR are able to interfere with the structural and functional properties of bacterial membranes, which in turn become increasingly permeable to protons and ultimately leading to the loss of integrity [Lambert, Skandamis, Coote & Nychas, 2001; Nostro et al., 2001). This interference could be explain why there was a decrease in viable cell counts when *P. aeruginosa* was cultivated in meat broth containing OVEO or CAR, (over 120 min). In addition, early studies reported that CAR was able to damage the outer envelope of *P. aeruginosa*, uptaking through the outer membrane [Cox et al., 2001; Lambert et al., 2001]. In their study Longbottom et al. (2004) found that *P. aeruginosa* was resistant to *Melaleuca alternifolia* essential oil (rich in terpinen-4-ol and γ-terpinen), and this intrinsic tolerance was associated with the barrier and energy functions of the outer membrane and may involve efflux systems. The ATP-dependent MexAB-OprM and MexXY-OprM efflux systems are known to be involved in *P. aeruginosa* tolerance to antibiotics, organic solvents and detergents [Chuanchuen, Karkhoff-Schweizer & Schweizer, 2003, Li, Poole & Nikaido, 2003). However the capacity of the CAR to penetrate the outer membrane may interfere in the efflux systems.

For most systems, the monitoring of cell viability or survival/growth behavior of adapted and non-adapted cells of *P. aeruginosa* ATCC 9027 to OVEO or CAR had similar shapes over the evaluated interval times, suggesting that there was no change in the intrinsic sensitivity of *P. aeruginosa* to the homologous and heterologous stressing agents. It is interesting that direct and cross protection in *P. aeruginosa* ATCC 9027 was not induced when the cells were pre-challenged for 18 h with sublethal concentrations of OVEO or CAR in a food-base medium because of the

documented development of homologous and heterologous resistance of *P. aeruginosa* cells that were challenged with sublethal conditions during a short time exposure to other antimicrobial compounds or procedures [Loughlin et al., 2002; Jones et al., 1989; Chuanchuen et al., 2001; Hassani, Mañas, Pagán & Condón, 2007). .

The cultivation of *P. aeruginosa* in irradiated ground beef samples containing sublethal amounts of OVEO or CAR for a longer exposure time (72 h) also revealed no increased tolerance to these compounds, reinforcing the findings of no changes in tolerance observed in assays with meat-broth. The literature about the tolerance of *P. aeruginosa* exposed to sublethal amounts of essential oils or their compounds is limited; however overnight cultivation of *P. aeruginosa* in meat based broth containing essential oil of *Rosmarinus officinalis* L. and its majority compound, 1,8-cineole (CIN) at sublethal amounts (ROEO 20 and 10 µL/mL; CIN 40 and 20 µL/mL) showed results similar to those present here (Gomes-Neto et al., 2012).

The cultivation of *P. aeruginosa* to increasing concentrations of the CAR for successive habituation 24 h-cycles revealed no increasing adaptive tolerance because the bacterium was able to survive (detected by the presence of viable cells) in meat broth containing CAR up to 1/2 MIC; *P. aeruginosa* was able to survive in meat broth containing up to the MIC of OVEO (one-fold increase in MIC), suggesting minor changes in antimicrobial susceptibility when evaluated by the standard MIC assessment criteria (Hammer, Carson & Riley, 2012); and if adaptive measures were induced they were not sufficient to substantially alter antimicrobial susceptibility. These findings are in agreement with the results of a previously published study, which reported minor changes in susceptibility of *Salmonella enterica* serovar Typhimurium ATCC 14028 (one-fold increase decrease in original MIC value) after exposure to increasing sublethal amounts of OVOE or CAR (Oliveira et al., 2010). Otherwise, a previous study found a decreased susceptibility of *P. aeruginosa* ATCC 9027 (one-fold decrease in original MIC value) after exposure to increasing sublethal amounts of ROEO or CIN in meat-broth (Gomes-Neto et al., 2012).

Essential oils and their compounds have been cited to suppress the synthesis and activity of enzymes in a member bacteria, including *Pseudomonas* even at levels lower than the MIC (Azerêdo et al., 2011; Sousa et al., 2012) resulting in a protein synthesis block (Oliveira et al., 2010; Barros et al., 2009; Nostro et al., 2001). A possible disturbance in protein synthesis and enzymatic activity could be related to the difficulty that bacteria encounters in developing direct or cross adaptation in the conditions generated by the presence of essential oils or their compounds in growth media, as caused by OVEO or CAR in this study (Luz et al., 2012). Previous study has noted that the concomitant presence of protein synthesis inhibitors, such as rifampicin and chloramphenicol, in the growth media with the other stressing agents completely abolished the increase in homologous resistance to pH and hydrogen peroxide (Cebrian, Sagarzazu, Pagán, Condón & Mañas). To the best of our knowledge, this is the first study that examined the development of tolerance by *P. aeruginosa* when exposed to sublethal amounts of OVEO or CAR, in an artificially contaminated meat-based model. The results demonstrate that exposure to sublethal concentrations of OVEO or CAR did not induce direct and cross protection (high temperature, lactic acid and NaCl) in *P. aeruginosa* ATCC 9027 grown even in a meat-based medium or in meat-based food model.

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FIGURE LEGEND

FIGURE. 1. Survival curve of *Pseudomonas aeruginosa* ATCC 9027 in meat broth (at 37°C, and added of the essential oil at MIC - 0.6 µL/mL) after overnight exposure to sublethal concentrations of *O. vulgare* L. essential oil (A) or carvacrol (B). (■): control - non-adapted cells; (+): cells adapted at MIC/2 - 0.3 µL/mL; (Δ): cells adapted at MIC/4 - 0.15 µL/mL.

FIGURE. 2. Survival curve of *Pseudomonas aeruginosa* ATCC 9027 in meat broth at 45°C (A), containing lactic acid - pH 5.2 (B) or NaCl - 10 g/100 mL (C) after an overnight exposure to sublethal concentrations of *O. vulgare* L. essential oil. (■): control - non-adapted cells; (+): cells adapted at MIC/2 - 0.3 µL/mL; (Δ): cells adapted at MIC/4 - 0.15 µL/mL.

FIGURE 3. Survival curve of *Pseudomonas aeruginosa* ATCC 9027 in meat broth at 45°C (A), containing lactic acid - pH 5.2 (B) or NaCl - 10 g/100 mL (C) after overnight exposure to sublethal concentrations of carvacrol. (■): control - non-adapted cells; (+): cells adapted at MIC/2 - 0.3µL/mL; (Δ): cells adapted at MIC/4 - 0.15 µL/mL.

FIGURE. 4. Survival curve of *Pseudomonas aeruginosa* ATCC 9027 in irradiated ground beef (at 7°C, and added of the essential oil and carvacrol separately at MIC - 0.6 µL/mL) after exposure to sublethal concentrations of *O. vulgare* L. essential (A) and carvacrol (B). (■): control - non-adapted cells; (+): cells adapted at MIC/2 - 9 µL/mL; (Δ): cells adapted at MIC/4 - 4.5 µL/mL.

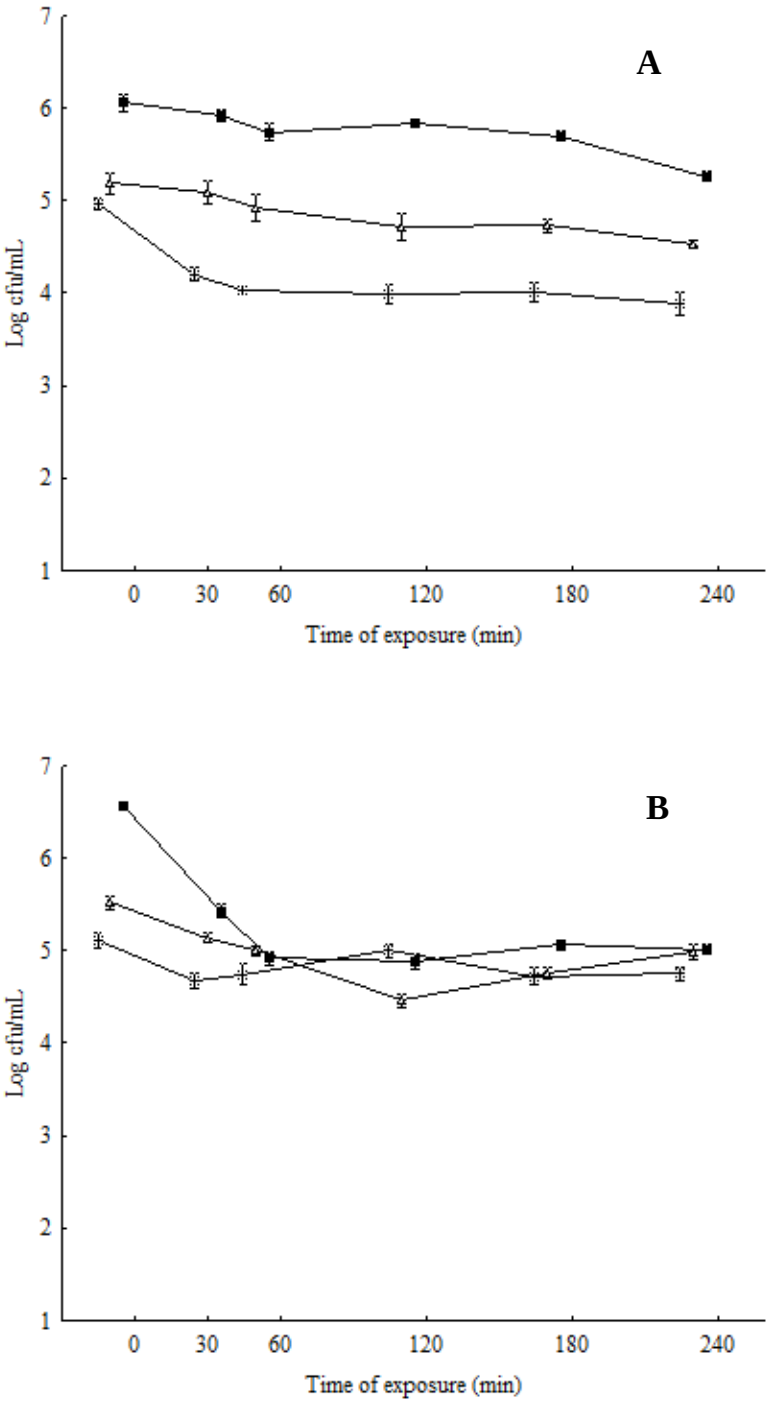
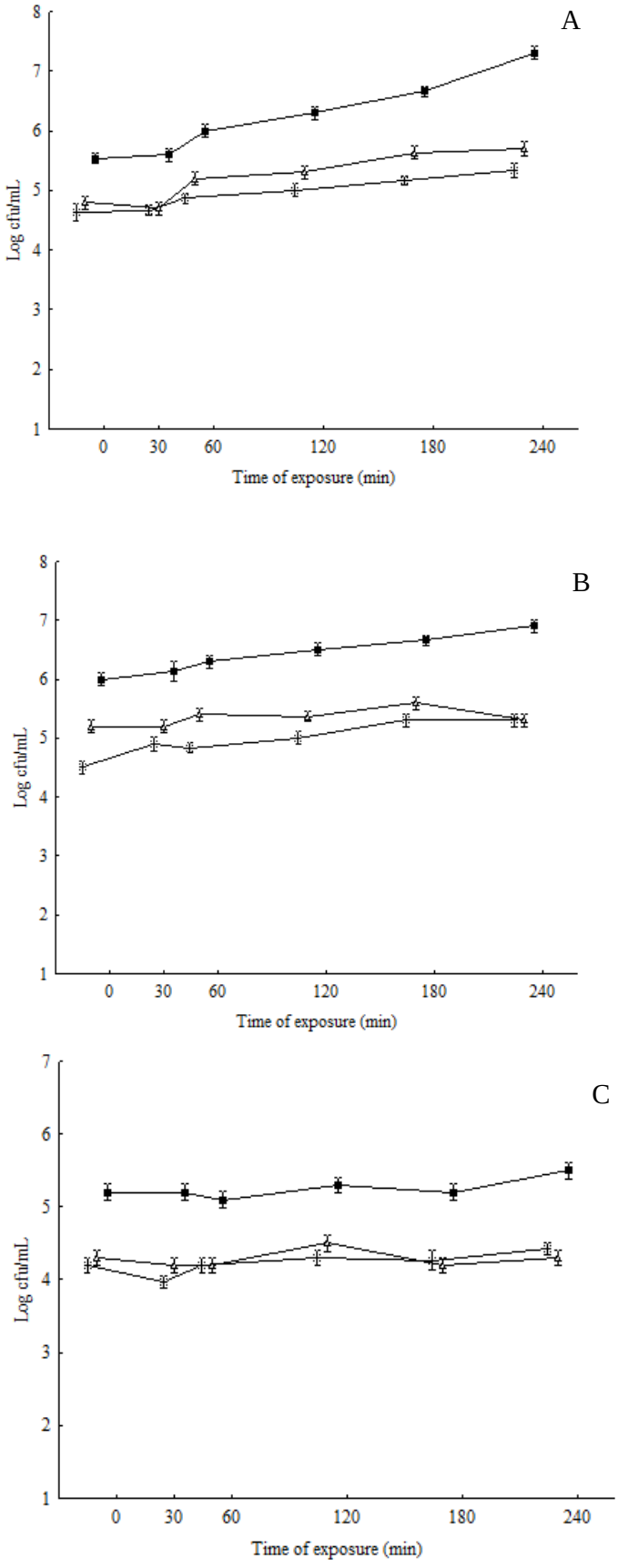

A
Log cfu/mL
Time of exposure (min)
B
Log cfu/mL
Time of exposure (min)

Figure 1.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C
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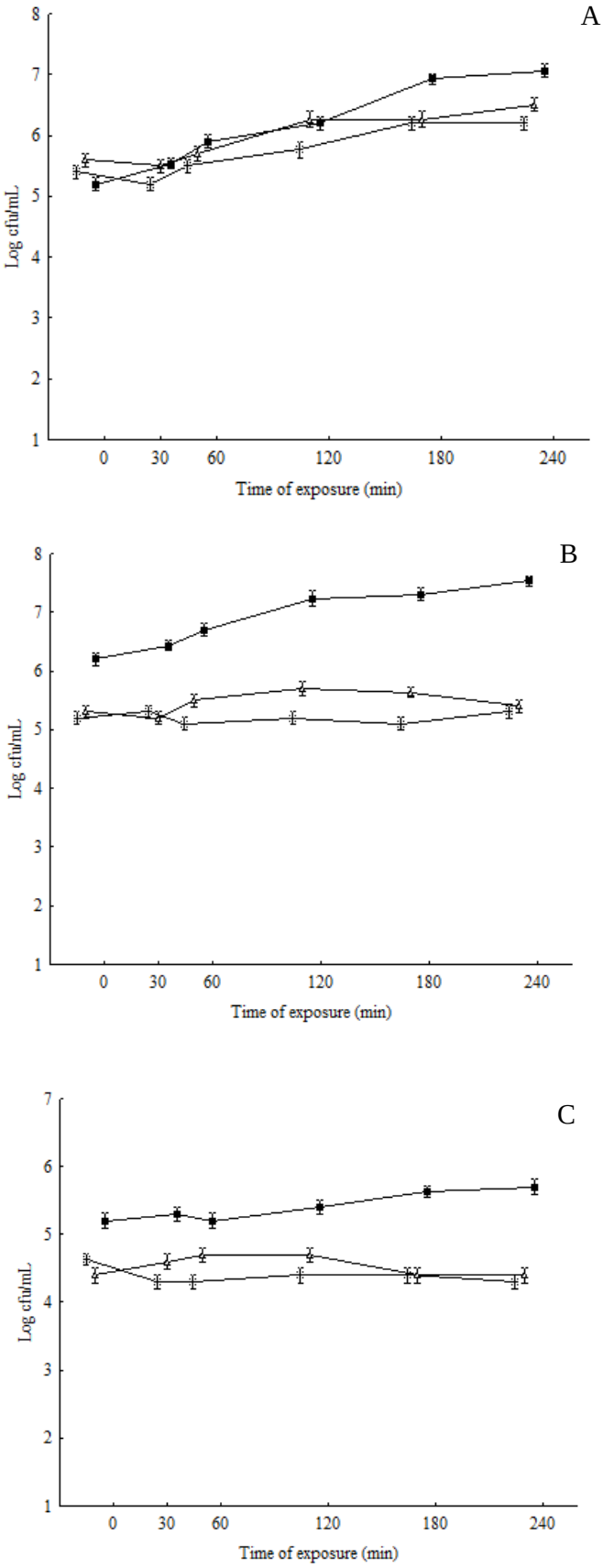
Time of exposure (min)

Figure 2.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Log cfu/mL
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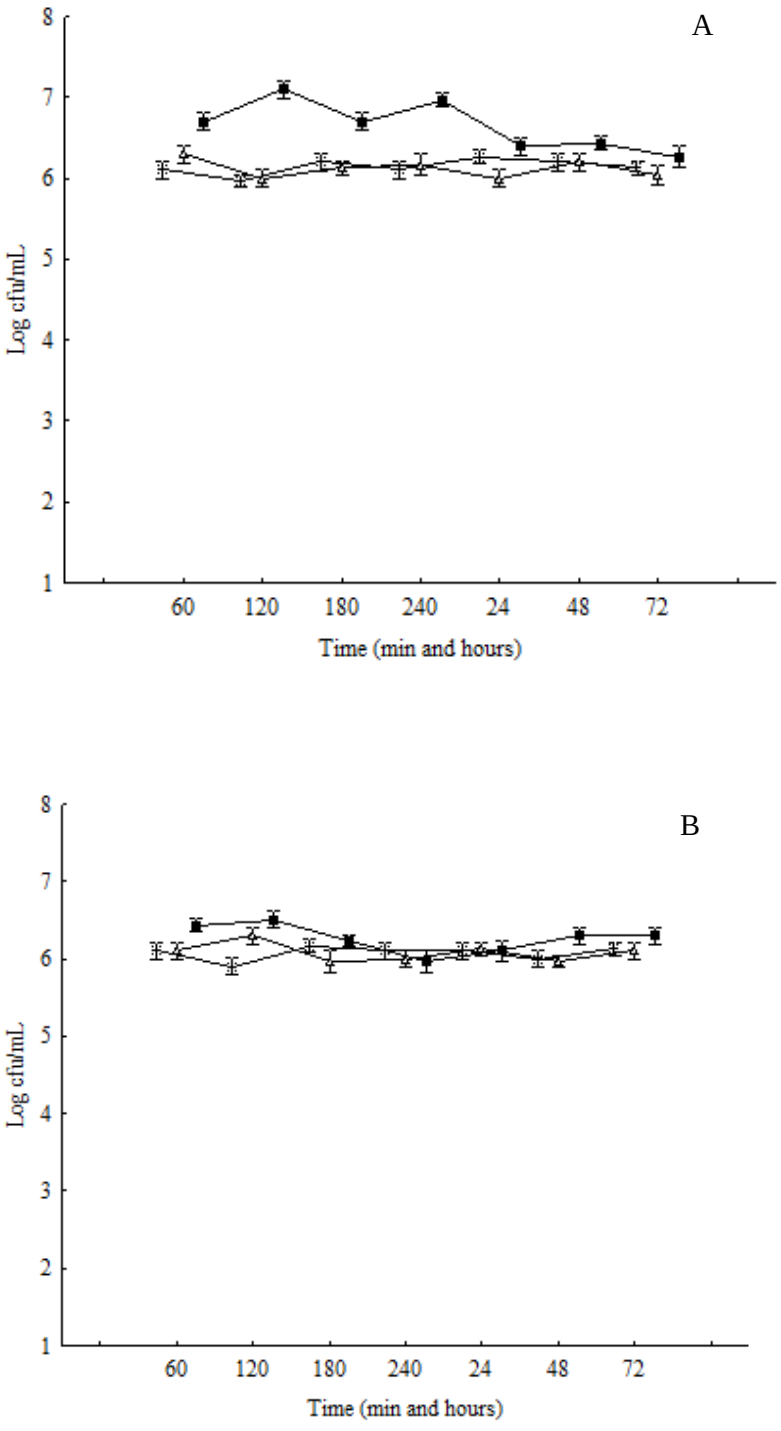
Time of exposure (min)

Figure 3.


A
8
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Log cfu/mL
60
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180
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72
Time (min and hours)
B
8
7
6
5
4
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2
1
Log cfu/mL
60
120
180
240
24
48
72
Time (min and hours)

Figure 4.

ANEXOS

ANEXO A - Evidence for Lack of Acquisition of Tolerance in *Salmonella enterica* Serovar Typhimurium ATCC 14028 after Exposure to Subinhibitory Amounts of *Origanum vulgare* L. Essential Oil and Carvacrol

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Isabelle da Silva Luz, Nelson Justino Gomes Neto, Adassa Gama Tavares, Pollyana Campos Nunes, Marciane Magnani and Evandro Leite de Souza

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Evidence for Lack of Acquisition of Tolerance in *Salmonella enterica* Serovar Typhimurium ATCC 14028 after Exposure to Subinhibitory Amounts of *Origanum vulgare* L. Essential Oil and Carvacrol

Isabelle da Silva Luz,[a] Nelson Justino Gomes Neto,[a] Adassa Gama Tavares,[a] Pollyana Campos Nunes,[a] Marciane Magnani,[b] and Evandro Leite de Souza[a]

Laboratory of Food Microbiology, Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil,[a] and Laboratory of Biochemistry of Foods, Department of Food Engineering, Center of Technology, Federal University of Paraíba, João Pessoa, Brazil[b]

Overnight exposure of *Salmonella enterica* serovar Typhimurium to sublethal amounts of *Origanum vulgare* essential oil (OV) and carvacrol (CAR) did not result in direct and cross-bacterial protection. Cells subcultured with increasing amounts of OV or CAR survived up to the MIC of either compound, revealing few significant changes in bacterial susceptibility.

Stress response development in bacteria has received much attention in recent years, including concern from a food safety perspective (11). The main reason for investigating the stress response of food-related bacteria is based on the fact that traditional methods used for food preservation impose physical and chemical stress upon bacterial cells to limit their growth and survival (12).

Salmonella enterica serovar Typhimurium is among the major food-borne pathogens that are of public concern with respect to food safety, causing gastroenteritis outbreaks with different severities (19). *S.* Typhimurium encounters many diverse and extreme environments that induce a bacterial developmental response to combat the stress, such as extreme pH, salt, and reactive oxygen intermediates (12). This acquired tolerance can be related to various cellular physiological changes that occur through plasmid acquisition, mutation, synthesis of stress proteins, and modification of lipid membrane composition (16, 19).

Early studies showed that *Origanum vulgare* L. essential oil (OV) possesses strong antimicrobial activity against food-related pathogenic bacteria (3, 9, 10, 13), including *Salmonella* (1, 8, 14). Despite the use of essential oils (and their components) as potential antimicrobials in foods, there is a lack of information regarding the potential development of direct and cross-protection by bacteria following exposure to subinhibitory amounts of these compounds. This study assessed the development of direct and cross-protection in *S.* Typhimurium ATCC 14028 when the strain was exposed to subinhibitory concentrations of OV and of the predominant component of this essential oil, carvacrol (CAR) (2), in a meat-based medium.

OV was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil). The compound CAR was purchased from Sigma-Aldrich (Sigma, France). Solutions (160 to 0.075 μl ml^{-1}) of OV and CAR were prepared in nutrient broth (NB) using bacteriological agar (0.15 g 100 ml^{-1}) as a stabilizing agent (4). Inocula of *S.* Typhimurium ATCC 14028 used in the assays (ca. 7 log CFU ml^{-1}) were obtained as previously described (7). MIC values of OV and CAR were determined by macrodilution in NB (18). OV and CAR both display a MIC value of 1.25 μl ml^{-1} against *S.* Typhimurium ATCC 14028.

Development of direct and cross-bacterial protection was measured using cultures grown in a meat-based broth prepared as previously described (10). All assays were performed in triplicate on three separate occasions, and the results were expressed as an average of results of the assays. The significant differences ($P < 0.05$) were calculated by analysis of variance (ANOVA) followed by Tukey tests using the software SigmaStat 3.1.

For assessing the induction of bacterial direct protection, the strain was exposed overnight to subinhibitory concentrations (1/2 MIC and 1/4 MIC) of OV or CAR in meat broth at 35°C (6, 16). The induction of direct protection was determined by comparing the viable cell counts (log CFU ml^{-1}) in the treated and untreated (cells not previously exposed to OV or CAR) suspensions upon further inoculation into meat broth at 35°C, to which the same stress agent was added at its MIC values. The overnight exposure of *S.* Typhimurium to subinhibitory amounts of either OV or CAR did not reveal an induction of bacterial direct protection, as demonstrated by the viable cell counts over 240 min of exposure (Fig. 1A and B). *S.* Typhimurium that had been previously challenged with sublethal concentrations of OV displayed a decrease, compared to nonadapted cells, 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. Otherwise, there was no difference ($P > 0.05$) between the viable counts of untreated cells and cells adapted to CAR at 1/2 MIC and 1/4 MIC following further cultivation in growth medium to which the same compound (at its MIC) was added.

Measurements of cross-protection induction were performed as previously described (5). *S.* Typhimurium cells were exposed overnight at 35°C to subinhibitory amounts of OV or CAR (1/2 MIC and 1/4 MIC), followed by exposure to other stress agents separately (45°C, pH 5.2, 5 g of NaCl 100 ml^{-1}, conditions that modestly inhibited the growth of the assayed strain). The induction of bacterial cross-protection was measured by comparing viable cell counts (log CFU ml^{-1}) in the treated and untreated samples following inoculation into growth medium either containing stressful additives or exposed to environmental stressful condi-

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Address correspondence to Evandro Leite de Souza, evandroleitesouza@ccs.ufpb.br.

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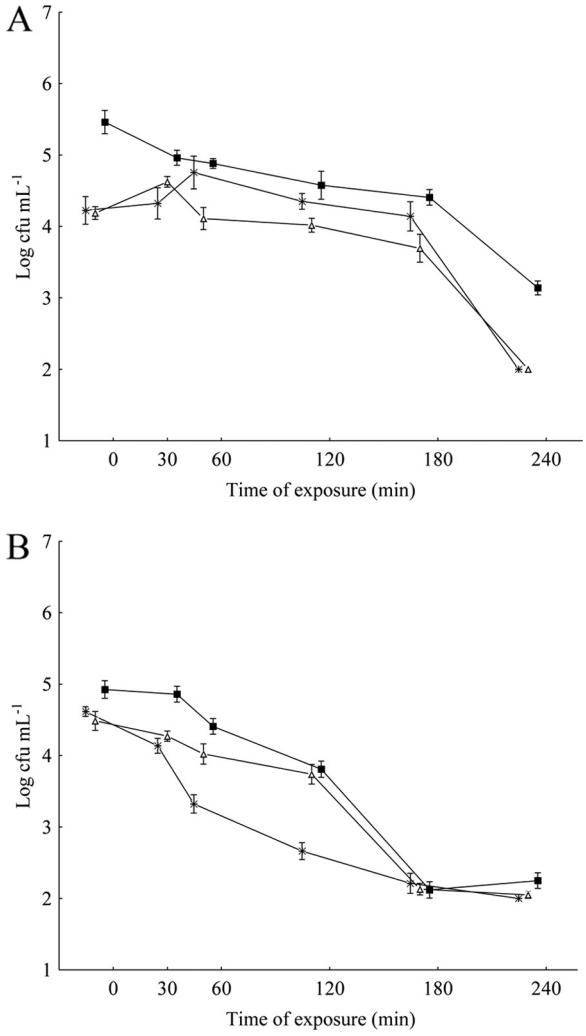

FIG 1 Viable counts of *S.* Typhimurium ATCC 14028 in meat broth to which the essential oil (at its MIC) was added following overnight exposure at 35°C to subinhibitory concentrations of *O. vulgare* L. essential oil (A) and carvacrol (B). ■, control (nonadapted cells); +, cells preadapted at an MIC of 2 to 0.625 μl ml^{-1}; Δ, cells preadapted at an MIC of 4 to 0.312 μl ml^{-1}.

tions (45°C). Consistent with the results of the bacterial direct protection assays, *S.* Typhimurium cells that were exposed overnight to sublethal concentrations of OV or CAR showed no induction of cross-protection to high temperature, lactic acid, or salt as determined by the viable cell counts throughout the 240 min of exposure (Fig. 2A to C and 3A to C). The cells submitted to preadaptation with OV or CAR at their 1/2 and 1/4 MICs revealed a decrease in viable counts ($P < 0.05$) upon further exposure to heterologous stressing agents in comparison with cells not previously adapted. These findings are interesting, as the development of homologous and heterologous resistance in *S.* Typhimurium has been well documented following the challenge of these cells with sublethal exposure to compounds or procedures classically used to control microorganisms in foods and food processing plants (11, 12, 16).

S. Typhimurium cells were exposed to increasing amounts of OV or CAR (1/16 MIC to 4× MIC) throughout successive 24-h habituation cycles (35°C) and monitored for viable cell detection.

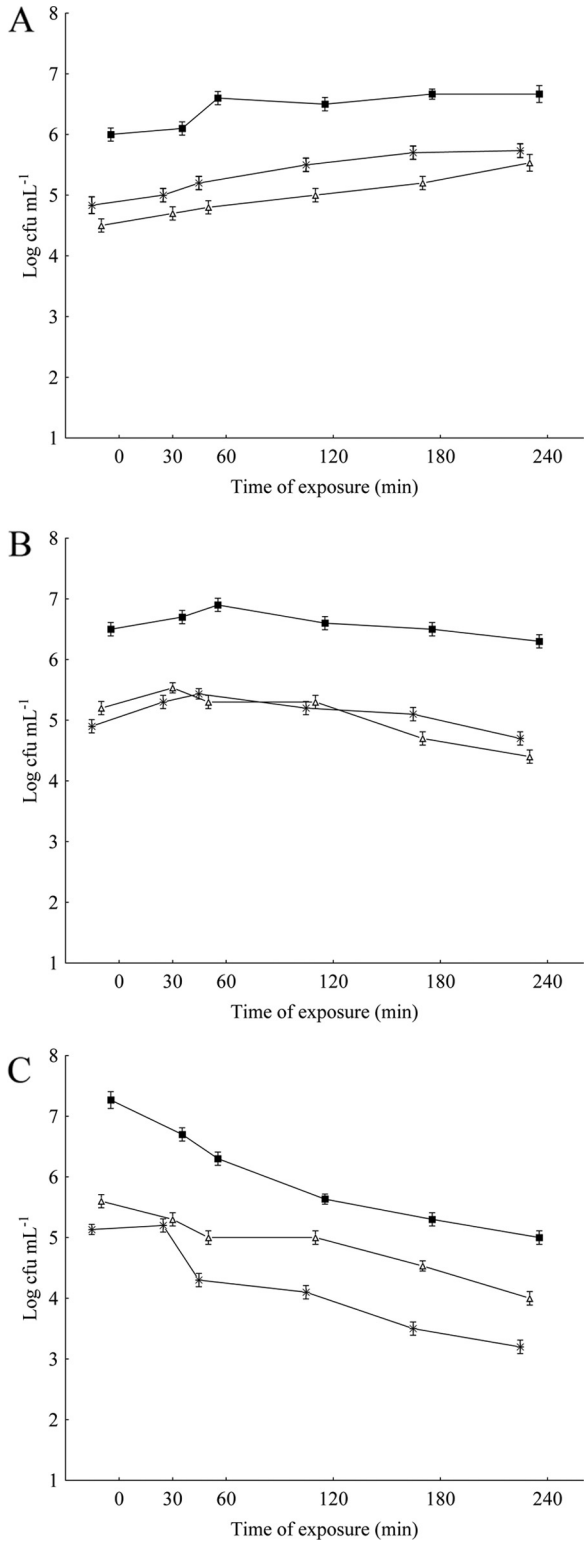

FIG 2 Viable cell counts of *S.* Typhimurium ATCC 14028 grown in meat broth incubated at high temperature (45°C) (A) or to which lactic acid (pH 5.2) (B) or NaCl (5 g 100 ml^{-1}) (C) was added following overnight exposure at 35°C to subinhibitory concentrations of *O. vulgare* L. essential oil. ■, control (nonadapted cells); +, cells preadapted at an MIC of 2 to 0.625 μl ml^{-1}; Δ, cells preadapted at an MIC of 4 to 0.312 μl ml^{-1}.

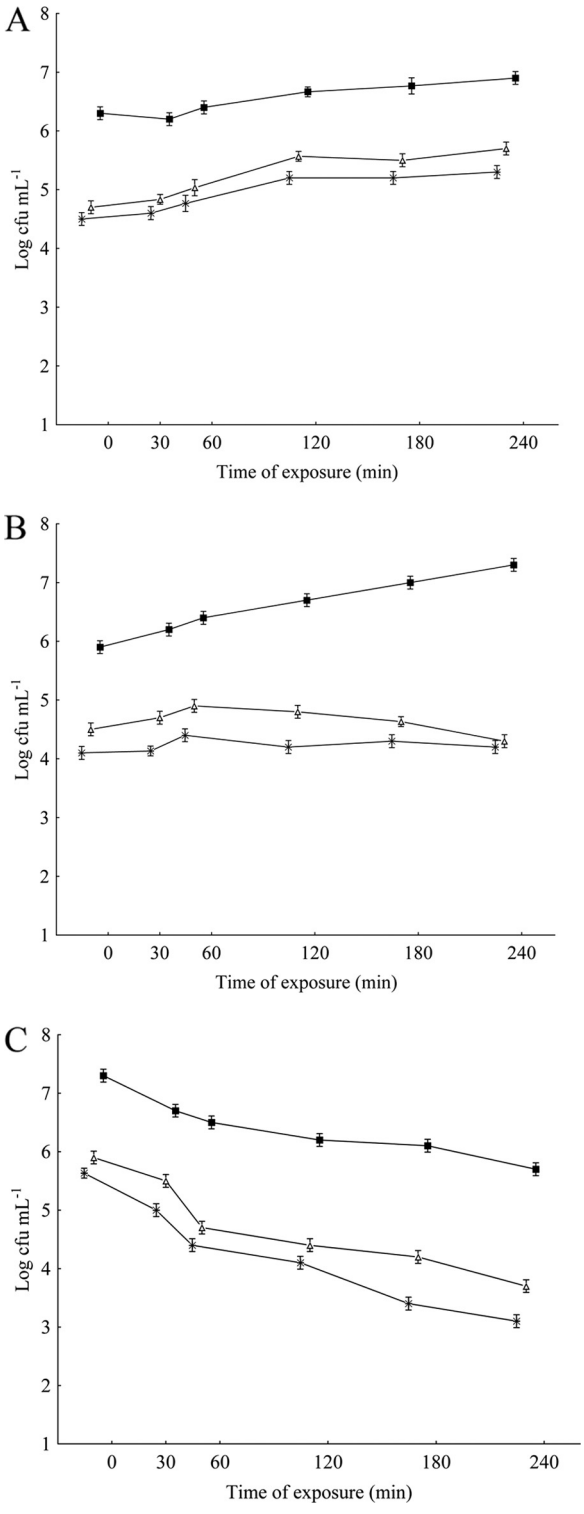

FIG 3 Viable counts of *S.* Typhimurium ATCC 14028 grown in meat broth incubated at high temperature (45°C) (A) or to which lactic acid (pH 5.2) (B) or NaCl (5 g 100 ml^{-1}) (C) was added following overnight exposure at 35°C to subinhibitory concentrations of carvacrol. ■, control (nonadapted cells); +, cells preadapted at an MIC of 2 to 0.625 μl ml^{-1}; Δ, cells preadapted at an MIC of 4 to 0.312 μl ml^{-1}.

This procedure was repeated with bacteria exposed to increasing concentrations of the compounds until a concentration was reached at which no viable cells were detected (20). The assays of induction of tolerance following exposure to 24-h cycles in meat broth showed that *S.* Typhimurium was able to survive in broth to which the OV or CAR was added in concentrations up to their respective MIC values (1-fold increase in the original detected MIC value). These findings suggest minor changes (15, 17) in susceptibility in *S.* Typhimurium when cells are exposed to sublethal amounts of OV and CAR for a more prolonged time, and if adaptive measures were induced they were not sufficient to substantially alter antimicrobial susceptibility.

To the best of our knowledge, this is the first study investigating the development of adaptation or tolerance by a strain of *S.* Typhimurium, as determined by monitoring cell viability or growth behavior, following exposure to sublethal amounts of OV or CAR after short and more prolonged exposure times. These studies revealed that exposure to sublethal concentrations of these compounds did not induce direct or cross-protection to high temperature, lactic acid, or NaCl in *S.* Typhimurium ATCC 14028 when the cells were cultivated in a meat-based medium.

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ANEXO B - Exposure of *Listeria monocytogenes* to sublethal amounts of *Origanum vulgare* L. essential oil or carvacrol in a food-based medium does not induce direct or cross protection

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Short communication

Exposure of *Listeria monocytogenes* to sublethal amounts of *Origanum vulgare* L. essential oil or carvacrol in a food-based medium does not induce direct or cross protection

Isabelle da Silva Luz [a], Nelson Justino Gomes Neto [a], Adassa Gama Tavares [a], Marciane Magnani [b], Evandro Leite de Souza [a,*]

[a] Laboratory of Food Microbiology, Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil
[b] Laboratory of Biochemistry of Foods, Department of Food Engineering, Center of Technology, Federal University of Paraíba, João Pessoa, Brazil

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ABSTRACT

In this study, the effects of *Origanum vulgare* L. essential oil (OVEO) and carvacrol (CAR) on the growth and survival of *Listeria monocytogenes* ATCC 7644 were evaluated. The induction of direct protection against OVEO and CAR and of cross protection against various stresses (high temperature 45 °C; lactic acid, pH 5.2; NaCl 10 g/100 mL) after exposure to sublethal amounts of OVEO and CAR was also evaluated. Both OVEO and CAR decreased the cell viability of *L. monocytogenes* in meat broth over 120 min of exposure at all assayed concentrations (MIC, 1/2 MIC and 1/4 MIC). The overnight exposure of *L. monocytogenes* to sublethal amounts of OVEO or CAR did not induce direct protection or cross protection against high temperature, lactic acid or NaCl. Cells that were subcultured (24 h cycles) in meat broth containing progressively increasing amounts of the antimicrobials were able to survive exposure to up to 2×MIC (two-fold increase) of OVEO and up to the MIC (one-fold increase in MIC) of CAR, suggesting that there were only minor changes in the antimicrobial susceptibility to these substances. Overall, these data indicate that OVEO and CAR have little effect on the acquisition of direct resistance or cross resistance by *L. monocytogenes*.

1. Introduction

Listeria monocytogenes causes listeriosis, a foodborne disease that occurs predominantly in pregnant woman, the elderly and immunosuppressed individuals and can lead to miscarriages and death (Kim & Kathariou, 2009; Swaminathan & Gerner-Smidt, 2007). *L. monocytogenes* can be found in raw and processed foods such milk, dairy products, meat products, seafood and vegetables (Franklin, Cooksey, & Getty, 2004; Nguyen, Gidley, & Dykes, 2008) causing many times the recall of foods with substantial economic losses to the food industry worldwide (Gandhi & Chikindas, 2007).

Studies have found a number of isolates of *L. monocytogenes* that are resistant to one or more antimicrobial compounds or procedures applied by the food industry to control the growth and survival of microorganisms in foods (Karatzas & Bennik, 2002; Rajkovic et al., 2009). Food processing conditions resemble natural environmental stresses that bacteria may encounter, such that sublethal conditions may induce changes in the cellular physiology of bacteria, causing the bacteria to mount adaptive responses to the antimicrobial interventions used to treat foods (Hill, Cotter, Sleator, & Gahan, 2002; Shadbolt, Ross, & McMeekin, 2001).

The threat posed by this anticipated adaptive response of *L. monocytogenes* to antimicrobials compounds used in foods has prompted studies on the development of novel technologies to control the survival of this pathogen; such technologies must have broad-spectrum antimicrobial activity, be of low toxicity for consumers and involve a low risk of increasing the prevalence of microbial resistance (Nascimento, Locatelli, Freitas, & Silva, 2000; Pazhani et al., 2004). In this context, essential oils and the compounds contained therein have received the attention of researchers and industry for use as alternative antimicrobials in foods (Marino, Bersani, & Comi, 2001).

Early studies showed that *Origanum vulgare* L. essential oil (OVEO) possesses strong and broad-spectrum antimicrobial activity against spoilage-related and pathogenic food-related bacteria (D'Antuono, Galletti, & Bochinni, 2000; Oliveira, Stamford, Gomes Neto, & Souza, 2010; Souza, Barros, Conceição, Gomes Neto, & Costa, 2009). The antimicrobial property of OVEO has been shown to be related primarily to the phenolic compound carvacrol (CAR), which is often the major component of this oil (Azerêdo, Stamford, Nunes, Gomes Neto, & Souza, 2011; Seydim & Sarikus, 2006).

Despite the fact that essential oils (and their compounds) are potential antimicrobials that can be used in foods, there is a lack of reports about the development of direct protection and/or cross

* Corresponding author at: Federal University of Paraíba, Department of Nutrition, Campus I, 58051-900, João Pessoa, Paraíba, Brazil. Tel.: +55 83 3216 7807; fax: +55 83 3216 7094.
E-mail address: evandroleitesouza@ccs.ufpb.br (E.L. de Souza).

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protection by *L. monocytogenes* when exposed to these compounds at sublethal concentrations. This study assessed the ability of OVEO and the major component thereof, CAR, to inhibit the growth and survival of *L. monocytogenes* ATCC 7644 and evaluated the development of direct protection and cross protection when this strain was exposed to sublethal concentrations of these substances in a meat-based medium.

2. Material and methods

2.1. Essential oil and carvacrol

OVEO (batch OREORG01; density at 20 °C, 0.90; refractive index at 20 °C, 1.47), as obtained by steam distillation, was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil). CAR was purchased from Sigma Aldrich (Sigma, France). Solutions of OVEO and CAR 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) as a stabilizing agent (Bennis, Chami, Chami, Bouchikhi, & Remmal, 2004).

2.2. 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 (Himedia, India) 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 agar (Himedia, India) at 37 °C. Then, liquid cultures were prepared by inoculating 100 mL of Brain Heart Infusion broth with two bacterial colonies from the overnight plates, and 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 cfu/mL colony forming units per milliliter (cfu/mL) (Azerêdo, Figueiredo, Souza, & Stamford, 2012). Suspensions were serially diluted in PBS (10^{-1}–10^{-3}) to provide a viable cell count of approximately 7 log of cfu/mL.

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 as a substrate for bacterial cultivation. Bovine meat steaks were trimmed of all external fat and cut into uniformly sized pieces (3 × 3 × 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 paper. The filtrate was sterilized by autoclaving for 15 min (1.21 atm). 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 (Oliveira et al., 2010).

2.4. Determination of the Minimum Inhibitory Concentration (MIC)

MIC values for OVEO and CAR were determined using macrodilution in broth. Four milliliters of double-strength nutrient broth (Himedia, India) were inoculated with 1 mL of bacterial culture, mixed with 5 mL of OVEO or CAR solutions 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 essential oil or carvacrol that prevented visible bacterial growth (Nostro et al., 2001). Control flasks without the tested compounds were similarly tested.

2.5. Time-kill assay

The effect of OVEO and CAR 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 OVEO or CAR solutions 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 and incubated for 24 h at 35 °C (Barros et al., 2009). Control flasks without the tested compounds were tested similarly. The results were expressed as the log of cfu/mL.

2.6. Induction of bacterial direct protection

The induction of direct protection in *L. monocytogenes* was performed by exposing bacteria overnight to sublethal concentrations of OVEO and CAR in meat broth as previously described (Brown, Ross, McMeekin, & Nichols, 1997; Leyer & Johnson, 1993). Meat broth (18 mL) containing the essential oil or carvacrol (final concentrations of 1/2 MIC or 1/4 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 (non-adaptation 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 OVEO or CAR (final concentration of the MIC was determined previously), shaken for 30 s using a vortex and incubated at 37 °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 plating on nutrient agar for 24–48 h at 35 °C. The results were expressed as the log of cfu/mL. To determine if direct protection was induced, the viable cell counts over time of bacteria subjected to adaptation treatments were compared to the counts of non-adapted bacteria when both groups were inoculated into growth media containing the antimicrobials at their MIC values.

2.7. Induction of bacterial cross protection

The induction of cross protection in *L. monocytogenes* was performed by exposing bacteria overnight to sublethal concentrations of OVEO and CAR in meat broth followed by exposure to other environmental stressors (high temperature, low pH and NaCl) as previously described (Boziaris, Chorianopoulos, Haroutounian, & Nychas, 2011), but with minor modifications. Preliminary experiments were performed for evaluating the thermotolerance, acid tolerance and salt tolerance of the bacterial test strain. Untreated bacterial cultures were inoculated into normal meat broth and into meat broth incubated at different high temperatures (40–60 °C) or containing lactic acid (pH 4.5–6.0, at 35 °C) or NaCl (1 g–15 g/100 mL, at 35 °C) to determine the temperature, pH value and NaCl concentration 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 OVEO or CAR (final concentrations of 1/2 MIC or 1/4 MIC) and shaken for 30 s using a vortex (adaptation treatment); the same was done for control flasks without antimicrobials (non-adaptation treatment). The assays were incubated overnight (18 h) at 35 °C, after which a 2-mL aliquot of each treatment was inoculated into 18 mL of fresh meat broth acidified with lactic acid (VETEC Química Fina Ltda., Brazil) to pH of 5.2, or into fresh meat broth containing NaCl (Qeel, Brazil) at 10 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 of cfu/mL. To determine if bacterial cross protection was induced, the viable counts over time of the bacterial suspensions subjected to the adaptation treatments were compared to the counts of non-adapted bacteria following inoculation into growth media exposed to different environmental stressors.

2.8. Induction of bacterial direct tolerance throughout successive habituation 24 h-cycles

The capacity of *L. monocytogenes* to develop direct tolerance to OVEO and CAR was assessed in meat broth by exposing the bacteria 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 to prolong the time of exposure according to the procedure described by To, Favrin, Romanova, and Griffiths (2002), but with minor modifications. Thus, 2 mL of the bacterial suspension was inoculated into 18 mL of meat broth containing the OVEO or CAR (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 (1/8 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 tested compounds 1/4 MIC — 2 × MIC), or until no viable cells were detected. The detection limit for the viable cell count method used was 2 log of cfu/mL for all assays.

2.9. Reproductibility and statistics

All assays were performed in triplicate on three 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.

3. Results and discussion

3.1. MIC and time-kill assays

OVEO and CAR both exhibited MIC values against *L. monocytogenes* ATCC 7644 of 0.62 μL/mL. In a previous study, Azerêdo et al. (2011) found an MIC value for OVEO of 1.25 μL/mL against this same *L. monocytogenes* strain. The differences in the observed MIC values of essential oils and their compounds are thought to be related to the test strain or isolate used, to the composition of the growth medium and/or to other intrinsic and extrinsic characteristics (Burt, 2004).

OVEO and CAR decreased the cell viability of *L. monocytogenes* ATCC 7644 at all assayed concentrations (MIC, 1/2 MIC and 1/4 MIC). The compounds decreased the bacterial cell viability after only 15 min of exposure, and no recovery in the viable count was noted for the remainder of the evaluated time interval. The exposure of *L. monocytogenes* to the antimicrobials at concentrations of 1/2 MIC, 1/4 MIC and MIC caused a significant decrease ($P<0.05$) in the viable count in comparison with the control treatment. *L. monocytogenes* grown in broth containing both tested compounds at their MICs exhibited viable counts ≤ 2.0 log cfu/mL after 45 min of exposure, and no recovery in the viable counts was observed over the remainder of the evaluated time interval. OVEO and CAR at 1/2 and 1/4 MIC decreased the viable count of *L. monocytogenes* 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.

Azerêdo et al. (2011) noted that the OVEO (1.25 μL/mL) used in this study caused a decrease in viable count of *L. monocytogenes* ATCC 7644 to 2.3 log cfu/mL over 120 min of exposure in a vegetable-based broth. In the same study, the authors identified carvacrol (66.9 g/100 g), *p*-cymene (13.9 g/100 g) and γ-terpinene (7.8 g/100 g) as the main constituents of OVEO. In other study, the same OVEO (1.25 μL/mL) caused release of intracellular material in *L. monocytogenes* and 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 (Azerêdo et al., 2012).

3.2. Induction of bacterial direct protection after overnight exposure to OVEO and CAR

The overnight exposure of *L. monocytogenes* to sublethal amounts of both OVEO and CAR (1/2 MIC and 1/4 MIC) did not induce direct protection (Fig. 1). The kill-curves of *L. monocytogenes* cells that were previously challenged with a sublethal amount of OVEO or CAR exhibited similar viable counts ($P>0.05$) when further cultivated in growth medium containing the same antimicrobial at the MIC. The viable counts found for the control cells (non-adapted cells) were higher ($P<0.05$) than those found for cells that have been previously treated with CAR (at 1/2 MIC and 1/4 MIC).

Although no difference ($P>0.05$) in the counts of *L. monocytogenes* treated with sublethal amounts of OVEO for adaptation or left untreated was found when the strain was exposed to the essential oil at its MIC for 240 min, the cells that were pre-adapted in broth containing 1/4 MIC or 1/2 MIC of OVEO exhibited a slight increase in the viable count between 0 and 180 min of exposure, followed by a sharp decrease in the counts at 240 min. This survival pattern was different from the linear decrease in the viable counts found for the control non-adapted cells over the same time interval. Cells submitted to pre-adaptation with CAR exhibited an initial drop in the viable counts when further exposed to the MIC, followed by the maintenance of the number of viable cells over the remainder of the evaluated time interval.

The literature regarding the assessment of tolerance development by *L. monocytogenes* when exposed to sublethal amounts of essential oils or their compounds is still limited, and most of the past studies focused on the development of direct resistance and cross resistance by *L. monocytogenes* have involved assays in which bacteria were exposed to classical chemical and physical food preservative procedures (Skandamis, Stopforth, Yoon, Kendall, & Sofos, 2009; Soni, Nannapaneni, & Tasara, 2011). Koutsoumanis, Kendall, and Sofos (2003) evaluated the acid tolerance response (ATR) of three- and five-strain mixtures of *L. monocytogenes* previously grown in Tryptic Soy Broth (TSB) containing glucose (1 g/100 mL — assumed to be acid-adapted cells) and found an enhanced ATR in adapted cultures relative to cells grown in TSB without glucose (non-acid-adapted cells). Pagán, Condón, and Sala (1997) reported an adaptive heat response (tolerance to 64–65 °C) in *L. monocytogenes* after a heat shock (48 °C) for 1–2 h. Skandamis, Yoon, Stopforth, Kendall, and Sofos (2008) reported that exposure to sublethal stress did not affect the thermotolerance of *L. monocytogenes*, whereas simultaneous exposure to multiple stresses (NaCl at 10 g/100 mL, HCl at pH 5.0, high temperature of 46 °C) simultaneously for 1.5 h in TSB resulted in increased tolerance of the bacterium to acidic environments (HCl at pH 3.5).

3.3. Induction of bacterial cross protection after overnight exposure to OVEO and CAR

In agreement with the results obtained in the assays of the induction of direct protection, the exposure of *L. monocytogenes* cells overnight to sublethal concentrations (1/4 MIC and 1/2 MIC) of OVEO or CAR did not

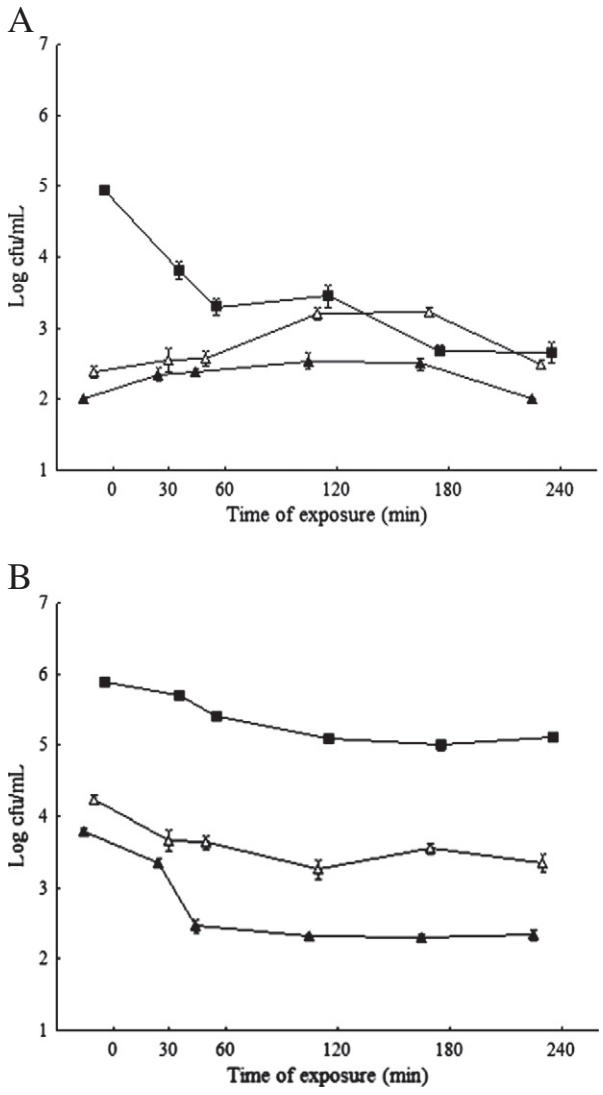

Fig. 1. Viable counts of *L. monocytogenes* ATCC 7644 in meat broth to which the *O. vulgare* L. essential oil or carvacrol (at its MIC) were added following overnight exposure at 35 °C to sublethal concentrations of *O. vulgare* L. essential oil (A) and carvacrol (B): (■) Control, non-adapted cells; (+): cells pre-adapted at 1/2 MIC — 0.3 μL/mL; (Δ): cells pre-adapted at 1/4 MIC — 0.15 μL/mL.

induce cross protection against high temperature (45 °C), lactic acid (pH 5.2) or salt (NaCl at 10 g/100 mL) (Fig. 2A–C and Fig. 3A–C). Cells of *L. monocytogenes* that were pre-adapted with the antimicrobials exhibited smaller counts ($P<0.05$) in media incubated at high temperature or containing lactic acid and NaCl relative to the cells that were not submitted to the pre-adaptation protocol (control assay) with the exception of cells that were pre-adapted with 1/2 MIC of OVEO and then challenged with lactic acid. Moreover, no difference ($P>0.05$) was found between the counts of viable *L. monocytogenes* cells submitted to pre-adaptation with OVEO or CAR at 1/2 MIC and 1/4 MIC when later exposed to the heterologous stressing agents (high temperature, low pH and NaCl).

In general, lower counts (3–4 log cfu/mL) of pre-adapted cells of *L. monocytogenes* were found when the cells were subsequently cultivated in broth containing NaCl relative to the counts observed for cells grown in broth incubated at high temperature or containing lactic acid. In the NaCl system, the viable counts of cells pre-adapted with 1/2 MIC or 1/4 MIC of OVEO or CAR were lower ($P<0.05$) than the counts for the control cells (non-adapted cells).

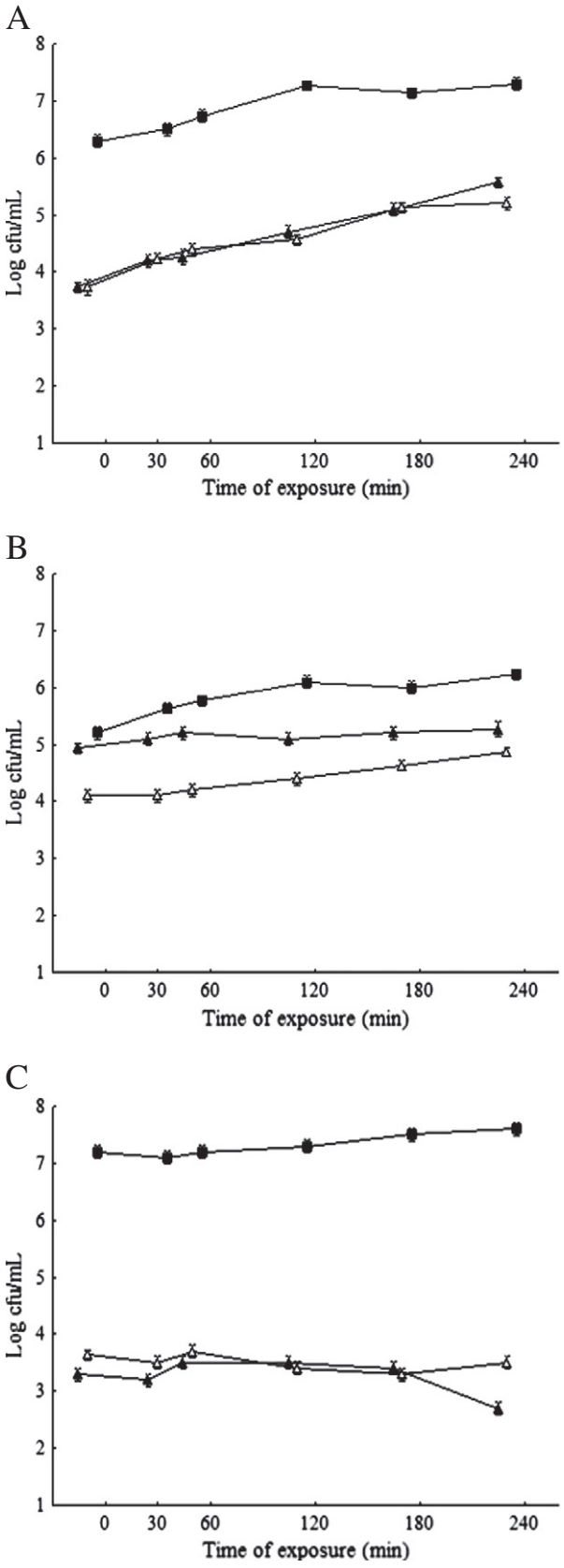

Fig. 2. Viable counts of *L. monocytogenes* ATCC 7644 grown in meat broth incubated at high temperature — 45 °C (A) or to which lactic acid — pH 5.2 (B) or NaCl — 10 g/100 ml (C) was added following overnight exposure at 35 °C to sublethal concentrations of *O. vulgare* L. essential oil. (■) Control, non-adapted cells; (+): cells pre-adapted at 1/2 MIC — 0.3 μL/mL; (Δ): cells pre-adapted at 1/4 MIC — 0.15 μL/mL.

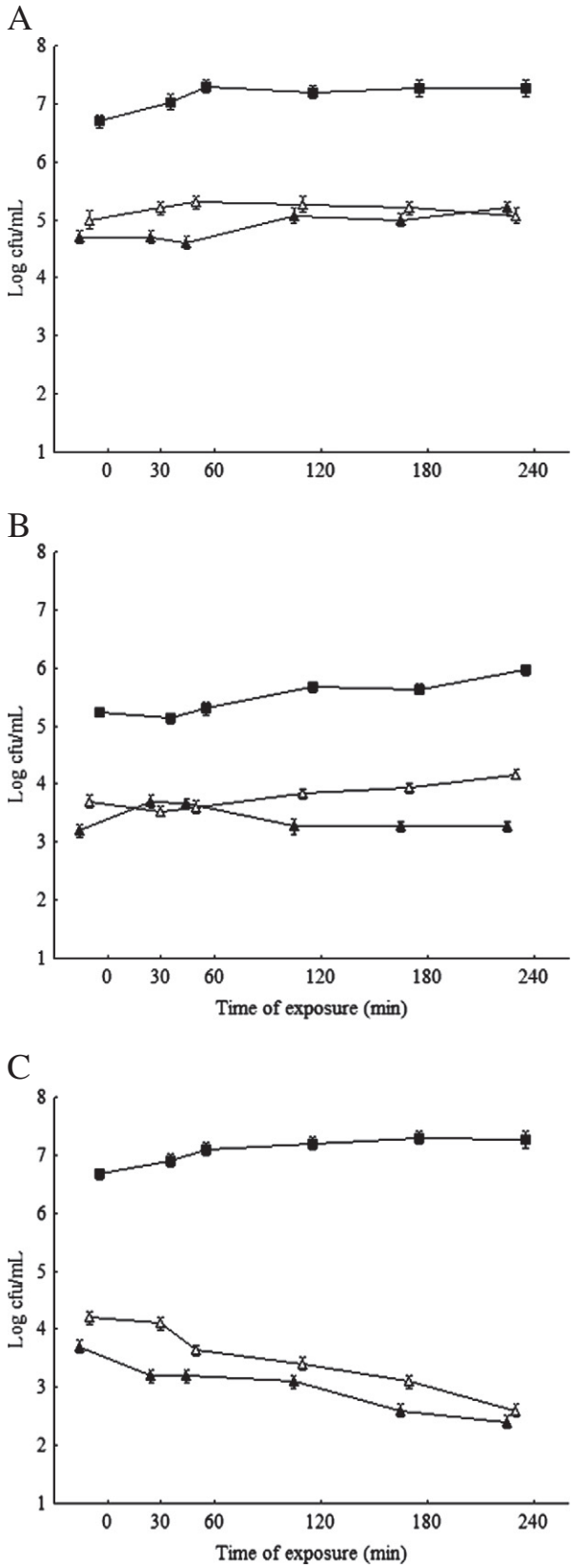

Fig. 3. Viable counts of *L. monocytogenes* ATCC 7644 grown in meat broth incubated at high temperature — 45 °C (A) or to which lactic acid — pH 5.2 (B) or NaCl — 10 g/100 ml (C) was added following overnight exposure at 35 °C to sublethal concentrations of carvacrol. (■) Control, non-adapted cells; (+): cells pre-adapted at 1/2 MIC — 0.3 μL/mL; (Δ): cells pre-adapted at 1/4 MIC — 0.15 μL/mL.

3.4. Induction of bacterial direct tolerance to OVEO and CAR throughout successive habituation 24 h-cycles

To address the possible limitations of the assays carried out with the two tested sublethal amounts of OVEO and CAR and the chosen exposure times for pre-adaptation (18 h), further experiments were performed to assess the induction of bacterial tolerance in *L. monocytogenes* ATCC 7644 when the bacteria were subcultured for 24 h cycles in meat broth containing progressively increasing amounts of OVEO or CAR (1/16 MIC – 2×MIC). The results of these assays showed that *L. monocytogenes* was able to survive (as determined by the count of viable cells) in meat broth containing up to 2×MIC (2-fold increase in MIC value) of OVEO and up to the MIC (one-fold increase in MIC value) of CAR.

The repeated exposure of *L. monocytogenes* to increasing amounts of OVEO and CAR did not induce significant changes in the bacterial susceptibility when evaluated by the standard MIC assessment criteria (Hammer, Carson, & Riley, 2012). These findings are largely in agreement with the results of previously published studies that indicated minor changes in susceptibility (2-fold increase or less in the MIC) of *Staphylococcus aureus* and *Escherichia coli* after exposure to increasing sublethal amounts of the essential oil from *Melaleuca alternifolia* and/or compounds contained therein (Hammer et al., 2012; McMahon, Blair, Moore, & McDowell, 2007); and of *Salmonella typhimurium* when exposed to sublethal amounts of OVEO and CAR (Luz et al., in press).

4. Conclusions

The results of this study reveal that *L. monocytogenes* ATCC 7644 exhibited no clear induction of direct protection or cross protection (high temperature, pH and NaCl) after an overnight exposure (one-step adaptation) to sublethal concentrations of OVEO or CAR in a meat-based medium. Moreover, the exposure of the cells to increasing sublethal amounts (multi-step adaptation) of both OVEO and CAR for a longer time resulted in no significant global effects on the acquisition of direct tolerance by *L. monocytogenes*. These findings reinforce the possible rational use of OVEO and CAR by food industry to control *L. monocytogenes* in foods regarding their efficacy to establish a fast and steady inhibitory effect, besides the low capacity to induce bacterial tolerance.

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ANEXO C - Lack of induction of direct protection or cross-protection in *Staphylococcus aureus* by sublethal concentrations of *Origanum vulgare* L. essential oil and carvacrol in a meat-based medium

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SHORT COMMUNICATION

Lack of induction of direct protection or cross-protection in *Staphylococcus aureus* by sublethal concentrations of *Origanum vulgare* L. essential oil and carvacrol in a meat-based medium

Isabelle Silva da Luz · Nelson Justino Gomes Neto · Adassa Gama Tavares · Pollyana Campos Nunes · Marciane Magnani · Evandro Leite de Souza

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Abstract The capacity of *Origanum vulgare* L. essential oil (OVEO) and its majority compound, carvacrol (CAR), to induce direct tolerance and cross-tolerance in *Staphylococcus aureus* against high temperature (45 °C), lactic acid (pH 5.2) and NaCl (10 g/100 mL) was assessed. Overnight exposure of *S. aureus* to sublethal concentrations (1/2 MIC, 1/4 MIC) of either OVEO or CAR in meat broth revealed no induction of direct protection. *S. aureus* cells pre-adapted to OVEO or CAR showed no induction of cross-protection to high temperature, lactic acid or NaCl. Cells subjected to 24 h cycles of adaptation in increasing amounts (1/2 MIC to 2 × MIC) of OVEO or CAR showed no increase in direct tolerance. These results revealed a lack of induction of direct protection or cross-protection in *S. aureus* exposed to sublethal amounts of OVEO or CAR in meat-based broth, as determined by monitoring cell survival and growth behavior.

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I. Silva da Luz · N. J. Gomes Neto · A. G. Tavares · P. C. Nunes · E. L. de Souza
Laboratory of Food Microbiology, Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil

M. Magnani
Laboratory of Biochemistry of Foods, Department of Food Engineering, Center of Technology, Federal University of Paraíba, João Pessoa, Brazil

M. Magnani (✉)
Departamento de Engenharia de Alimentos, Centro de Tecnologia, Universidade Federal da Paraíba, Campus I, Cidade Universitária, João Pessoa, Paraíba 58051-900, Brazil
e-mail: magnani2@gmail.com

Keywords Oregano · Carvacrol · *Staphylococcus* · Bacterial adaptation · Tolerance

Introduction

Stress response development in bacteria has received much attention in recent years, including concern from a food safety perspective (Aarestrup et al. 2008). The conditions that microorganisms face during food processing can lead to the development of physiologically adaptive responses and tolerance development following exposure to stressors that are able to cause sublethal injury to the microbial cells (Luz et al. 2012). It is well known that sublethal exposure to particular environmental conditions and antimicrobial substances may result in the development of increased tolerance to the same (homologous) stress agents or cross-tolerance (heterologous) to distinct stress agents (Álvarez-Ordóñez et al. 2008).

The assessment of these responses to sublethal injury in food-related microorganisms could be helpful in reducing the uncertainty surrounding the actual microbiological risks posed by tolerance that arise from a lack of knowledge about the physiological behavior of microbial cells that survive sublethal stress caused by an antimicrobial compound or procedure (Greenacre and Brocklehurst 2006). Concerns over negative consumer perception of chemical preservatives have led to an increased interest in the use of natural compounds to control the growth and survival of bacteria in foods. Thus, essential oils of plants and their constituents that possess broad-spectrum antimicrobial effects have been proposed as alternatives to synthetic antimicrobials in foods (Patrignani et al. 2008; Dubois-Brissonet et al. 2011).

Earlier studies have shown that the essential oil of *Origanum vulgare* L. (OVEO) possesses strong- and

broad-spectrum antimicrobial activity against food-related bacteria (Souza et al. 2009; Oliveira et al. 2010; Luz et al. 2012), specifically against the classical pathogenic bacteria *Staphylococcus aureus* (Souza et al. 2010). The antimicrobial activity of this essential oil has been attributed to its majority phenolic compound, carvacrol (CAR). CAR possesses hydrophobic characteristics and interacts with different targets in the bacterial cell, particularly with the cytoplasmic membrane and the cell wall, where it causes the loss of cell constituents, the collapse of membrane structures and, ultimately, cell death (Burt 2004).

Although essential oils and their compounds are known as potential antimicrobials for use in foods, little information is available regarding the possibility for the development of direct protection and/or cross-protection by *S. aureus* when exposed to these substances in sublethal amounts. This study assessed the efficacy of OVEO and its majority compound, CAR, in inhibiting the growth and survival of *S. aureus* ATCC 6538, and the development of direct protection and/or cross-protection when the strain was exposed to sublethal concentrations of these substances in a meat-based medium.

Materials and methods

Essential oil and CAR

OVEO (batch OREORG01; density at 20 °C, 0.90; refractive index at 20 °C, 1.47), as obtained by steam distillation, was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil), and the quality parameters were described in an accompanying technical report. CAR was purchased from Sigma Aldrich (Sigma, France). Solutions of OVEO and CAR 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) as a stabilizing agent (Mann and Markham 1998; Souza et al. 2010). A previous study has demonstrated that CAR (66.9 g/100 g) is the main constituent of the OVEO assayed in this study (Azêredo et al. 2011).

Test microorganism

S. aureus ATCC 6538 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 (Himedia, India) under refrigeration (7 ± 1 °C). Unless otherwise stated, all assays used inocula obtained from stationary-phase cultures according to a previously described method (Carson et al. 2002). The obtained bacterial suspensions (c.a. 9 log of colony-forming units per milliliters—cfu/mL) were serially diluted in PBS (10^{-1} to 10^{-3}) to provide a viable cell count of approximately 7 log of cfu/mL.

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 as a substrate for bacterial cultivation. Bovine meat steaks were trimmed of all external fat and cut into uniformly sized pieces (3 × 3 × 3 cm). Meat pieces were then boiled in distilled water for 30 min at 90 °C, yielding approximately 500 mL of meat broth, which was then vacuum filtered using Whatman No. 1 filter paper. The filtrate was sterilized by autoclaving for 15 min (1.21 atm). Afterward, 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 (Oliveira et al. 2010).

Determination of the minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) values for OVEO and CAR were determined using macrodilution in broth. Four milliliters of double-strength nutrient broth (Himedia, India) was inoculated with 1 mL of bacterial culture, mixed with 5 mL of the antimicrobial solution and vortexed for 30 s (viable cells count of approximately 6 log cfu/mL). The assay was statically incubated for 24 h at 37 °C. The MIC was defined as the lowest concentration of OVEO or CAR that prevented visible bacterial growth (Nostro et al. 2001). Control flasks without OVEO or CAR were similarly tested.

Time-kill assay

The effect of OVEO or CAR (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; then, 5 mL of OVEO or CAR was added to the system, and the culture was gently shaken for 30 s using a vortexer (viable cell count of approximately 6 log cfu/mL). The system was statically incubated at 37 °C for increasing time intervals (0, 15, 30, 45, 60 and 120 min). One mL of the suspension was serially diluted (10^{-1} to 10^{-5}) in PBS, plated on nutrient agar and incubated for 24 h at 37 °C (Nostro et al. 2001). Control flasks without OVEO or CAR were tested similarly. The results were expressed in log cfu/mL.

Evaluation of induction of bacterial direct protection

The induction of direct protection in *S. aureus* was performed by exposing bacteria overnight to sublethal

concentrations of OVEO or CAR in meat broth as previously described (Luz et al. 2012; Gomes Neto et al. 2012). Meat broth (18 mL) containing OVEO or CAR (final concentrations of 1/2 MIC or 1/4 MIC) was inoculated with 2 mL of the bacterial suspension and shaken for 30 s using a vortexer (adaptation treatment with viable cell count of approximately 6 log cfu/mL). The control system without antimicrobials was assayed similarly (non-adaptation treatment). The assays were statically incubated overnight (18 h) at 37 °C (final bacterial counts for the adapted cells were always between 5 and 6 log cfu/mL; counts for control assays were always between 6 and 7 log cfu/mL). Subsequently, a 2 mL aliquot of each treatment was inoculated into fresh meat broth (18 mL) containing OVEO or CAR (the final concentration of the MIC was determined previously), shaken for 30 s using a vortexer and statically incubated at 37 °C. Viable cells were enumerated over time (0, 30, 60, 120, 180 and 240 min) by serial dilution (10^{-1} to 10^{-5}) in PBS, plating on nutrient agar and incubation for 24–48 h at 37 °C. The results were expressed as the log of cfu/mL. To determine if direct protection was induced, the cell viability counts over time of the bacterial suspensions subjected to adaptation to OVEO or CAR were calculated and compared to those obtained with non-adapted bacteria when both groups were inoculated into growth media containing OVEO or CAR at their MIC values.

Evaluation of induction of bacterial cross-protection

The induction of cross-protection in *S. aureus* was performed by exposing bacteria overnight to sublethal concentrations of OVEO or CAR in meat broth followed by exposure to other environmental stressors (high temperature, low pH and NaCl) as previously described (Luz et al. 2012; Gomes Neto et al. 2012). Preliminary experiments were performed for evaluating the salt tolerance, acid tolerance and thermotolerance of the bacterial test strain. Untreated bacterial cultures were inoculated into meat broth and into meat broth containing NaCl (5–15 g/100 mL, at 37 °C) or lactic acid (pH 4.5–6.0, at 37 °C), or meat broth and statically incubated at various 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 OVEO or CAR (final concentrations of 1/2 MIC or 1/4 MIC) and shaken for 30 s using a vortexer (adaptation treatment). The same procedure was done for the control flasks without antimicrobials (non-adaptation treatment). The assays were statically incubated overnight (18 h) at 35 °C (final viable cell counts for preadapted cells were always between 5 and 6 log cfu/mL, and the viable cell counts for the control assays were always between 6 and 7 log cfu/mL); subsequently, a 2 mL aliquot of each treatment was inoculated into 18 mL of fresh meat broth (statically incubated at 37 °C) acidified with lactic acid (VETEC Química Fina Ltda., Brazil) to a pH of 5.2 or into fresh meat broth (statically incubated at 37 °C) containing NaCl (Qeel, Brasil) at 10 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 statically incubated at 45 °C. Viable cells for all assays were enumerated (0, 30, 60, 120, 180 and 240 min) as described above. The results were expressed as the log of cfu/mL. To determine whether bacterial cross-protection was induced, the viable cell counts over time of the bacterial suspensions subjected to the adaptation to OVEO or CAR were calculated and compared to those obtained from the non-adapted bacteria following inoculation into the growth media exposed to different environmental stressors.

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

The capacity of *S. aureus* to develop tolerance to OVEO or CAR was assessed in meat broth by exposing the bacteria 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 to prolong the time of exposure according to a previously described procedure (Luz et al. 2012; Gomes Neto et al. 2012). Thus, 2 mL of the bacterial suspension was inoculated into 18 mL of meat broth containing OVEO or CAR (final concentrations of 1/16 MIC), shaken for 30 s using a vortexer (viable cells counts of approximately 6 log cfu/mL) and statically incubated for 24 h at 37 °C. A 100 μL aliquot of the culture was then serially diluted (10^{-1} to 10^{-5}) in PBS, plated on nutrient agar and incubated for 24 h at 37 °C. 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 (1/8 MIC). This assay was statically incubated at 37 °C, and viable cells were detected according to the conditions cited above. The procedure was repeated with increasing concentrations of OVEO or CAR (1/4 MIC, 2 × MIC) or until no viable cells were detected.

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

Reproducibility and statistics

All assays were performed in triplicate on three 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. All statistical analyses were performed using Sigma Stat 3.1 (Jandel Scientific Software, San Jose, CA).

Results and discussion

The MIC values for OVEO or CAR against *S. aureus* ATCC 6538 were determined to be 5.0 and 2.5 μL/mL, respectively. For all of the concentrations tested (MIC, 1/2 MIC and 1/4 MIC), OVEO and CAR were found to inhibit ($P < 0.05$) 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. OVEO and CAR at 1/2 or 1/4 MIC decreased the viable cell count of *S. aureus* to approximately 5 log cfu/mL after 180 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* to sublethal concentrations of either OVEO or CAR (1/2 MIC or 1/4 MIC) revealed no induction of bacterial direct protection in the viable cells exposed for over 240 min in the meat broth supplemented with the same stressing agent at the MIC (Fig. 1). The kill-curves for *S. aureus* subjected to preadaptation in the meat broth containing OVEO at sublethal concentrations and further exposed to the MIC of the same stressing agent revealed viable counts similar ($P > 0.05$) to those obtained in the control assay (no preadapted cells) after 120 min of exposure; these counts exhibited similar trends ($P > 0.05$) at the later assessed time intervals (Fig. 1a). Although the behavior of the cells preadapted to OVEO was different (kill-curve shape) from that observed for the non-adapted cells at the initial exposure times (up to 30 min) to the MIC, the viable cell counts found for adapted and non-adapted cells were similar ($P > 0.05$) at the later assessed exposure times.

S. aureus previously cultivated in meat broth containing 1/2 MIC or 1/4 MIC of CAR revealed lower viable cell counts ($P < 0.05$) when inoculated into broth containing the MIC of CAR for up to 120 min of exposure in comparison with the viable cell counts found in the non-adapted control assay; however, the viable counts of cells cultivated in the adapted and non-adapted systems were similar ($P > 0.05$) after 240 min of exposure. Cells adapted and non-adapted to CAR exhibited similar kill-curve shapes over the assessed time periods.

Notably, after 60 min of exposure to the MIC of OVEO or CAR, the adapted and non-adapted cells presented viable cell counts close to 2 and 3 log cfu/mL, respectively, and no recovery in viable count was detected in the remaining

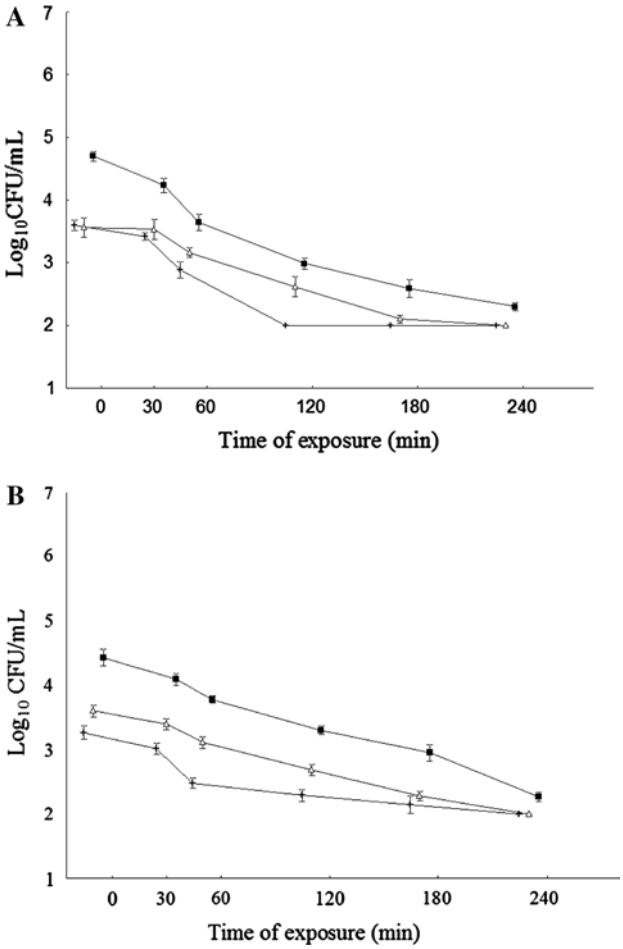

Fig. 1 Survival curve of *S. aureus* ATCC 6538 in meat broth (37 °C, containing the essential oil at MIC) after overnight exposure to sublethal concentrations of *O. vulgare* L. essential oil. **a** (*square* control, non-adapted cells; +, cells preadapted at 1/2 MIC, 2.5 μL/mL; *triangle*, cells preadapted at 1/4 MIC, 1.25 μL/mL) or carvacrol. **b** (*square*, control, non-adapted cells; +, cells preadapted at 1/2 MIC, 1.25 μL/mL; *triangle*, cells preadapted at 1/4 MIC, 0.6 μL/mL) (*bars* represent standard deviation)

assessed exposure times. An examination of the different cell counts determined after the overnight cultivation of *S. aureus* in both meat broth supplemented with OVEO or CAR at sublethal amounts (4–5 log cfu/mL) and meat broth not supplemented with these substances (6–7 log cfu/mL) revealed similar kill-curve shapes over the most assessed time intervals for cultivated cells found in both systems (adapted and non-adapted).

According to the results obtained from the assays on the induction of bacterial direct protection, *S. aureus* preadapted to sublethal amounts (1/4 MIC and 1/2 MIC) of OVEO and CAR showed no induction of cross-protection to high temperature (45 °C), lactic acid (pH 5.2) or NaCl (10 g/100 mL) (Figs. 2, 3), as determined by the viable cell counts throughout the 240 min of exposure. The survival

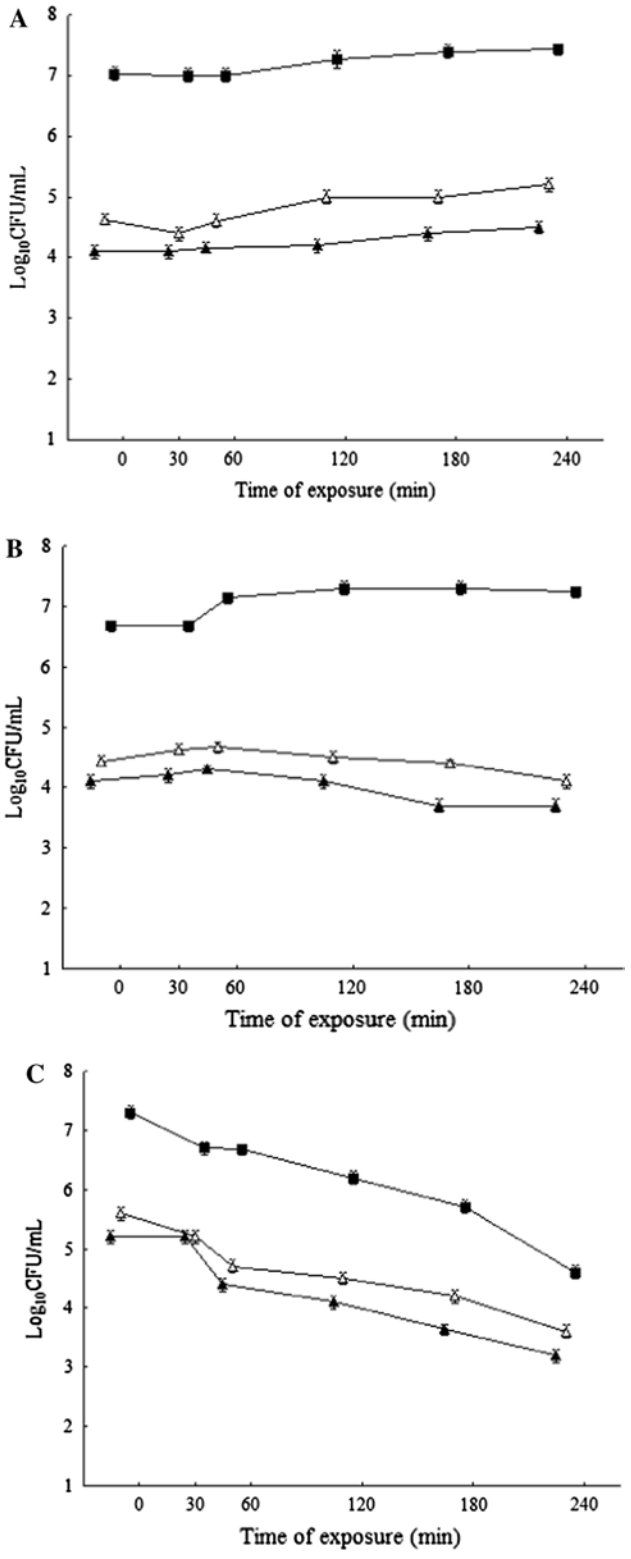

Fig. 2 Survival curve of *S. aureus* ATCC 6538 in meat broth at 45 °C (**a**), with lactic acid at pH 5.2 (**b**) and with 10 g/100 mL NaCl (**c**) after overnight exposure to sublethal concentrations of *O. vulgare* L. essential oil. *Square,* Control, non-adapted cells; *black triangle*, cells preadapted at 1/2 MIC, 2.5 μL/mL; and *white triangle,* cells preadapted at 1/4 MIC, 1.25 μL/mL (*bars* represent standard deviation)

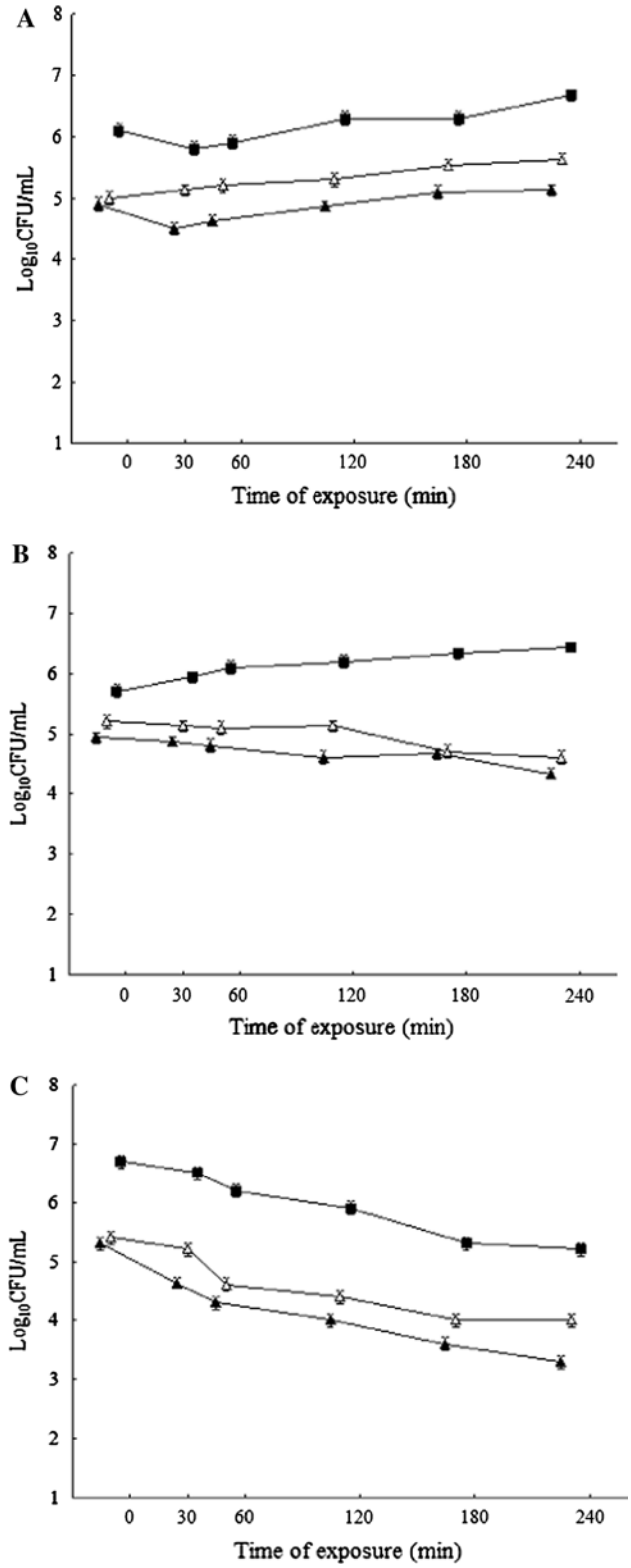

Fig. 3 Survival curve of *S. aureus* ATCC 6538 in meat broth at 45 °C (**a**), with lactic acid at pH 5.2 (**b**) and with 10 g/100 mL of NaCl (**c**) after overnight exposure to sublethal concentrations of carvacrol. *square,* Control, non-adapted cells; *black triangle*, cells preadapted at 1/2 MIC, 1.25 μL/mL; and *white triangle*, cells preadapted at 1/4 MIC, 0.6 μL/mL (*bars* represent standard deviation)

behavior (kill-curve shape) presented by the adapted and non-adapted cells when further exposed to the heterologous stressing agents was similar for most of the assessed systems. Nevertheless, the adapted cells presented smaller values ($P < 0.05$) of viable cells in comparison with the non-adapted cells at all evaluated time intervals.

When *S. aureus* was assayed for acid tolerance (Figs. 2b, 3b) and osmotolerance (Figs. 2c, 3c), the strain showed a decrease in viable cell count over the assessed exposure times. When the strain was assayed for the development of cross-protection to high temperature, however, a slight increase in viable cell count was observed over time, although this behavior was also found in the control assay, suggesting that there was no change in the intrinsic sensitivity of the strain with respect to the high temperature assay. Higher values ($P < 0.05$) for the viable cell counts of *S. aureus* previously adapted to 1/2 or 1/4 MIC of both OVEO or CAR were observed when the strain was further grown at 45 °C and also when it was exposed to lactic acid (pH 5.2) or NaCl (10 g/100 mL) for 240 min upon comparison with the control assay (non-adapted cells).

Cultivation of *S. aureus* in Muller Hinton broth (for 120 min) containing a sublethal concentration (20 μg/mL) of Epigallocatechin gallate (EGCC), the major phenolic compound of green tea extract, led to adaptation and cross-resistance to vancomycin, ampicillin and oxacillin with increased MIC values (2- to 8-fold increase); nevertheless, adaptation to EGCG increased heat resistance in the preadapted cells (Sing et al. 2007). The overnight cultivation of *S. aureus* ATCC 6538 in meat broth with the addition of the essential oil of *Rosmarinus officinalis* L. (ROEO) and its majority compound, 1, 8-cineole (CIN), at sublethal amounts (ROEO 10 and 5 μL/mL; CIN 20 and 10 μL/mL) induced no direct or cross-protection (NaCl 10 g/L; lactic acid pH 5.2; high temperature 45 °C) in the tested bacterium when assessed by viable cell count and growth/survival behavior (Gomes Neto et al. 2012).

Results of the experiments used to assess the induction of bacterial tolerance in *S. aureus* ATCC 6538 in meat broth when exposed to consecutive 24-h cycles of increasing concentrations of OVEO or CAR (1/16 MIC to 2 x MIC) revealed that *S. aureus* was able to survive (based on the detection of viable cells) in broth containing these substances in concentrations of up to 1/2 MIC, suggesting that the exposure of the cells to increasing amounts of either OVEO or CAR did not stimulate the development of tolerance. These findings are largely in agreement with the results of a previously published study, which reported decreased susceptibility of *S. aureus* ATCC 6530 (onefold decrease in original MIC value) after exposure to increasing sublethal amounts of ROEO or CIN (Gomes Neto et al. 2012) However, minor changes in susceptibility (twofold increase or less in the original MIC value) of *S. aureus* to *Melaleuca alternifolia* essential oil were observed after a 72-h exposure to sublethal concentrations (McMahon et al. 2008); these changes have been cited to be most likely related to reversible alterations in the bacterial membrane lipid composition (Hammer et al. 2012).

The inhibition of the *S. aureus* already present in the meat broth upon the addition of 1/2 MIC of the OVEO or CAR could be related to the manifestation of cell injury; when bacterial cells are continuously exposed in a stressing but non-lethal environment, the cells may lose viability and the capacity to survive over time (Gomes Neto et al. 2012). The cultivation of *S. aureus* ATCC 6538 in nutrient broth supplemented with sublethal concentrations of OVEO (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, reducing salt (NaCl) tolerance, lipase and coagulase activity and enterotoxin production (Barros et al. 2009). Previous research has stated that the changes in the metabolic activity of the bacterium could be related to the sublethal injury of the cell induced by the sublethal concentrations of OVEO, which could alter the ability of the cell to perform adequate osmoregulation, exclude toxic materials or synthesize some physiological determinants, such as proteins and enzymes. An earlier study reported the ability of *S. aureus* CECT 44459 to develop direct and cross-resistance when exposed (5 min–2 h) to sublethal conditions of acidic (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 and 48 °C) in Tryptone Soy Broth (Cebrián et al. 2010). In the same study, however, researchers noted that the concomitant presence of the antibiotics rifampicin and chloramphenicol in the growth media with the other stressing agents completely abolished the increase in homologous resistance to pH and hydrogen peroxide. Given that essential oils and their compounds have been cited to suppress the synthesis and activity of enzymes even at levels lower than the MIC in a number of *S. aureus* strains, resulting in a protein synthesis block (Barros et al. 2009; Nostro et al. 2001; Oliveira et al. 2010), a possible disturbance in protein synthesis and enzymatic activity could be related to the difficulty *S. aureus* ATCC 6538 encounters in developing direct or cross-adaptation in the conditions generated by the presence of OVEO or CAR at sublethal amounts in meat broth, as assayed in this study.

To the best of our knowledge, this is the first study examining the development of adaptation or tolerance by *S. aureus* exposed to sublethal amounts of OVEO or CAR after both short and more prolonged exposures in a food-based broth as a substrate for bacterial cultivation. Our results revealed that the exposure to sublethal concentrations of OVEO and CAR provided no induction of direct protection or cross-protection in *S. aureus* ATCC 6538 grown in a meat-based broth. Nevertheless, the exposure

of the cells to successively increasing sublethal amounts of either OVEO or CAR resulted in no significant effect on the acquisition of tolerance in *S. aureus*.

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ANEXO D - Comprovação de submissão e aceite do artigo científico intitulado: Sublethal amounts of *Origanum vulgare* L. essential oil and carvacrol cause injury and changes in membrane fatty acid of *Salmonella* Typhimurium cultivated in a meat broth"

ANEXO E - Comprovação de submissão do artigo científico intitulado: Study of tolerance induction in *Pseudomonas aeruginosa* in artificially contaminated meat added of *Origanum vulgare* L. essential oil or carvacrol"

ANEXO D

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To: magnani2@pq.cnpq.br

23-Oct-2013

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Title: Study of tolerance induction in Pseudomonas aeruginosa in artificially contaminated meat added of Origanum vulgare L. essential oil or carvacrol
Meat Science

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