DANILO FLORISVALDO BRUGNERA

EFEITO ANTILISTERIAL DE COMPOSTOS MAJORITÁRIOS DE ÓLEOS ESSENCIAIS CONTRA CÉLULAS PLANCTÔNICAS E SÉSSEIS

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência dos Alimentos, para a obtenção do título de Doutor.

Orientadora

Dra. Roberta Hilsdorf Piccoli

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APROVADA em 28 de fevereiro de 2014.

Dra. Carolina Valeriano de Carvalho	UFLA
Dr. Cleube Andrade Boari	UFVJM
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LAVRAS – MG

2014

À vovó Umbelina, certamente está muito feliz por mais um conquista de quem sempre lhe deu orgulho

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RESUMO

Biofilmes microbianos representam um grande problema para indústrias de alimentos e sua eliminação tem sido alvo de diversas pesquisas. A utilização de óleos essenciais e seus compostos majoritários tem se mostrado eficiente na remoção de biofilmes de diversas espécies, como *Listeria monocytogenes* (LM). No entanto, pouco se conhece sobre o efeito de concentrações subletais destes compostos nas células bacterianas. A primeira etapa desta pesquisa foi conduzida com o objetivo de avaliar a atividade antilisterial de constituintes de óleos essenciais citral (CIT), timol, cinamaldeído (CIN), eugenol, α-terpineol, carvacrol e terpinen-4-ol, contra células planctônicas das cepas ATCC 19117, 7644 e 15313. Concentrações Mínimas Inibitórias (CMIs) foram determinadas por microdiluição em caldo, posteriormente determinaram-se as Concentrações Mínimas Bactericidas (CMBs). Todos os constituintes apresentaram atividade antibacteriana. Soluções de citral e cinamaldeído, constituintes que apresentaram as menores CMBs, foram testadas contra biofilmes formados sobre aço inoxidável AISI#304. Os valores de CMB_BS variaram de 0,42 a 1,2% (v/v). Citral e cinamaldeído apresentaram efeito antibacteriano também contra células sésseis, podendo ser novas alternativas para elaboração de sanificantes. A segunda etapa desta pesquisa foi conduzida com o objetivo de avaliar o efeito da exposição do biofilme de LM, durante sua formação, a concentrações subletais de (CIN) e (CIT). Os biofilmes foram formados em aço inoxidável AISI 304, em contato com caldo triptona de soja suplementado com 0,6% (p/v) de extrato de levedura (TSB-YE) e incubado a 37 °C, sendo, a cada 48 horas, tratados com soluções contendo concentrações subletais (1/3 da concentração letal) de CIN e CIT durante 15 minutos, além de uma solução controle (sem antimicrobianos). Para cada cepa, foram desenvolvidos três tipos de biofilmes: BC (biofilme controle), BECIN (biofilme exposto a concentrações subletais de cinamaldeído durante a sua formação) e BECIT (biofilme exposto a concentrações subletais de citral durante a sua formação). Ao final de 240 horas o número de células viáveis foi determinado imediatamente após o tratamento com as soluções, sendo calculado o Log de redução. Pelo número de células sésseis após 240 horas, verificou-se que a exposição a concentrações subletais de CIN e CIT afetou a formação dos biofilmes. A exposição a concentrações subletais de CIN e CIT afetou o biofilme de LM, podendo torná-lo mais resistente a estes compostos como também mais sensível, de maneira cruzada ou não, sendo a ocorrência destes efeitos dependente da cepa e do composto utilizado.

Palavras-chave: Biofilmes. *Listeria monocytogenes*. Resistência microbiana. Citral. Cinamaldeído.

ABSTRACT

Microbial biofilms represent a major problem for the food industry, and their disposal has been the subject of several studies. The use of essential oils and their major compounds has shown to be effective in removing biofilms of several species, such as *Listeria monocytogenes* (LM). However, little is known about the effect of sublethal concentrations of these compounds in bacterial cells. The first stage of this research was conducted with the objective of evaluating the antilisterial activity of citral (CIT) essential oils' constituents, thymol, cinnamaldehyde (CIN), eugenol, α-terpineol, carvacrol and terpinen-4-ol against planktonic cells of ATCC 19117, 7644 and 15313 strains. Minimum Inhibitory Concentrations (MICs) were determined by broth microdilution, subsequently determining the Bactericidal Minimum Concentrations (BMC_S). All constituents show antibacterial activity. Citral and cinnamaldehyde solutions, constituents which showed the lowest BMC_S, were tested against biofilms formed on # AISI 304 stainless steel. The CMB_Bs values ranged from 0.42 to 1.2% (v/v). Citral and cinnamaldehyde also showed antibacterial effect against sessile cells, and may be new alternatives for sanitizer development. The second stage of this research aimed at evaluating the effect of the exposure of the LM biofilm during its formation, to sublethal concentrations of CIN and CIT. The biofilms were formed on a AISI 304 stainless steel, in contact with tryptone soy broth supplemented with 0.6 % (w /v) of yeast extract (TSB-YE) and incubated at 37 °C, treated every 48 hours with solutions containing sublethal concentrations (1/3 of lethal concentration) of CIN and CIT for 15 minutes, in addition to a control solution (without antibiotics). We developed three types of biofilm for each strain: BC (control biofilm), BECIN (biofilm exposed to sublethal concentrations of cinnamaldehyde during its formation) and BECIT (biofilm exposed to sublethal concentrations of citral biofilm during its formation). After 240 hours, the number of viable cells was determined immediately after treatment with the solutions, and the log reduction was calculated. With the number of sessile cells after 240 hours, we verified that the exposure to sublethal concentrations of ash and CIT affect biofilm formation. Exposure to sublethal concentrations of CIN and CIT affected biofilm formation. The exposure to sublethal concentrations of CIN and CIT affected the LM biofilm, and may cause it to be more resistant to these compounds as well as more sensitive, of cross- way or not, with the occurrence of these effects dependent on the strain and the compound used.

Keywords: Biofilms. *Listeria monocytogenes.* Bacterial resistance. Citral. Cinnamaldehyde.

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PRIMEIRA PARTE

1 INTRODUÇÃO

De forma a garantir a qualidade microbiológica dos alimentos, toda superfície, utensílios e colaboradores que entram em contato com o alimento a ser produzido devem passar pelo processo de higienização, que consiste nas etapas de limpeza e sanitização.

A sanitização é uma etapa que visa eliminar os microrganismos patogênicos e reduzir o número de deteriorantes, sendo que atualmente existem diversos produtos disponíveis. No entanto, devido ao problema de aquisição de resistência dos microrganismos a estes sanitizantes comerciais, novas alternativas de controle vêm sendo estudadas a fim de melhorar a eficiência dos processos de sanitização.

Quando o processo de sanitização é ineficiente, acaba permitindo a presença de microrganismos na superfície ou equipamentos e se as condições da superfície forem favoráveis, ocorre a adesão desses na superfície e a consequente formação de um biofilme microbiano. Quando em biofilme, estas células se tornam mais resistentes, reduzindo a eficiência dos sanitizantes, bem como a quantidade de antimicrobiano que entra em contato com a célula, resultando em exposições subletais, o que acaba tornando as células mais resistentes aos santificantes (ou aos sanitizantes) utilizados.

Atualmente diversas pesquisas vêm sendo realizadas, procurando novas alternativas de antimicrobianos para serem utilizados no processo de sanificação. A utilização de óleos essenciais e de alguns compostos que compõem estes óleos tem se mostrado promissora em testes realizados em laboratório simulando biofilmes em superfícies utilizadas na indústria de alimentos.

Diversos microrganismos têm a capacidade de formar biofilmes, e *Listeria monocytogenes* tem sido alvo de inúmeras pesquisas devido a sua alta patogenicidade, capacidade de persistir na planta de processamento e mecanismos de resistências que a torna resistente aos sanificantes comerciais.

Considerando o exposto, esta Tese foi desenvolvida com o objetivo geral de avaliar a capacidade antibacteriana do citral e do cinamaldeído contra células planctônicas e sésseis de cepas de *L. monocytogenes* e, posteriormente, estudar o efeito da exposição de células sésseis a concentrações subletais destes agentes antibacterianos durante a formação de biofilmes.

Os objetivos específicos foram: (i) determinar as Concentrações Mínimas Inibitórias (CMIs) e Concentrações Mínimas Bactericidas (CMBs) do citral, timol, cinamaldeído, eugenol, α-terpineol, carvacrol e terpinen-4-ol contra células planctônicas de *L. monocytogenes* ATCC 19117, 7644 e 15313, com intuito de verificar quais os melhores antibacterianos para serem testados contra células sésseis; (ii) determinar as concentrações mínimas do citral e do cinamaldeído necessárias para reduzir as células viáveis do biofilme de *L. monocytogenes* (Concentrações Mínimas Bactericidas do Biofilme, CMB_Bs); (iii) com base nas CMB_Bs, determinar as concentrações subletais; e (iv) avaliar os comportamentos das células sésseis após exposição frequente a doses subletais de citral e cinamaldeído durante a formação de biofilmes.

2 REFERENCIAL TEÓRICO

2.1 Higienização na indústria de alimentos e formação de biofilmes microbianos

A preocupação das indústrias de alimentos com a segurança microbiológica é cada vez maior e a higienização dos equipamentos, superfícies e colaboradores é de fundamental importância para evitar a contaminação dos alimentos e garantir sua inocuidade.

Nas indústrias de alimentos, a higienização inclui as etapas de limpeza e sanitização das superfícies de alimentos, ambientes de processamento, equipamentos, utensílios, colaboradores e ar de ambientes de processamento (ANDRADE; PINTO; ROSADO, 2008), e deve levar em consideração tanto a composição orgânica e inorgânica dos resíduos de alimento como também a microbiota constitutiva da superfície (FLINT; BREMER; BROOKS, 1997; MITTELMAN, 1998).

A limpeza tem como objetivo principal a remoção de resíduos orgânicos e minerais aderidos às superfícies, constituídos principalmente por carboidratos, proteínas, gorduras e minerais. Os detergentes ácidos controlam depósitos minerais, como fosfato de cálcio. Os detergentes alcalinos apresentam a função de saponificar ácidos graxos, pela liberação de íons hidroxila (OH^-). A solubilização de proteínas também é função dos agentes alcalinos e ocorre devido ao aumento do pH ocasionado pela liberação dos íons hidroxila (ANDRADE; PINTO; ROSADO, 2008).

A limpeza é a primeira etapa da higienização, sendo de realização obrigatória e de fundamental importância para o sucesso do processo de sanitização dos equipamentos (FORSYTHE; HAYES, 1998), que tem como objetivo eliminar microrganismos patogênicos para níveis considerados seguros

e reduzir o número de microrganismos deterioradores. Os sanitizantes químicos mais utilizados em superfícies de equipamentos e utensílios nas indústrias alimentícias brasileiras são aqueles que possuem princípios ativos dos grupos: quaternários de amônio, compostos inorgânicos liberadores de cloro ativo, compostos orgânicos liberadores de cloro ativo, compostos à base de ácido peracético, iodo e derivados (ANDRADE; PINTO; ROSADO, 2008; BRASIL, 1988, 1993, 1999).

No entanto, caso não haja implantação de sistemas de qualidade nas indústrias alimentícias e aplicação de efetivos agentes sanitizantes, microrganismos podem não ser completamente removidos das superfícies e instalações que entram em contato com os alimentos. A retenção e o acúmulo de microrganismos em tais ambientes contribuirão para o desenvolvimento de biofilmes (BOS et al., 2000).

Em ambientes naturais, cerca de 95% a 99% dos microrganismos existem na forma de biofilmes (KIM; FRANK, 1995; NIKOLAEV; PLAKUNOV, 2007), de maneira que, a forma de vida bacteriana livre ou planctônica é observada, simplesmente, como mecanismo de translocação entre superfícies (WATNICK; KOLTER, 2000). Biofilmes podem ser definidos como formas de existência microbiana espacialmente e metabolicamente estruturadas em comunidades embebidas em matrizes de substâncias poliméricas extracelulares (EPS) (NIKOLAEV; PLAKUNOV, 2007) e aderidas a superfícies bióticas ou abióticas (DUNNE JUNIOR, 2002; LASA et al., 2005).

A formação de biofilmes microbianos em ambiente de processamento de alimentos é um processo complexo que pode ser resumidamente descrito nas etapas a seguir. Inicialmente, moléculas orgânicas provenientes do alimento são depositadas sobre a superfície de equipamentos formando o filme condicionante. Em seguida, microrganismos ativos biologicamente aderem à superfície condicionada, atraídos pelas moléculas orgânicas. Algumas células microbianas

persistem mesmo após a limpeza e sanitização e iniciam o crescimento do biofilme. Por último, forma-se o biofilme maduro com a ajuda da expressão de genes específicos e *quorum sensing* (SHI; ZHU, 2009).

A adesão a superfícies pode ser realizada por microrganismos deteriorantes ou patogênicos, resultando em sérios problemas de saúde pública ou de ordem econômica (NORWOOD; GILMOUR, 1999). Uma das consequências mais graves da higienização inadequada nas indústrias de alimentos é a possível ocorrência de doenças de origem alimentar. Cerca de 200 doenças podem ser veiculadas pelos alimentos. Elas são provocadas por bactérias, fungos, vírus, parasitas, agentes químicos e substâncias tóxicas de origem animal e vegetal. Bactérias representam o grupo de maior importância, sendo responsáveis pela ocorrência de cerca de 70% dos surtos e 90% dos casos (ANDRADE; MACÊDO, 1996).

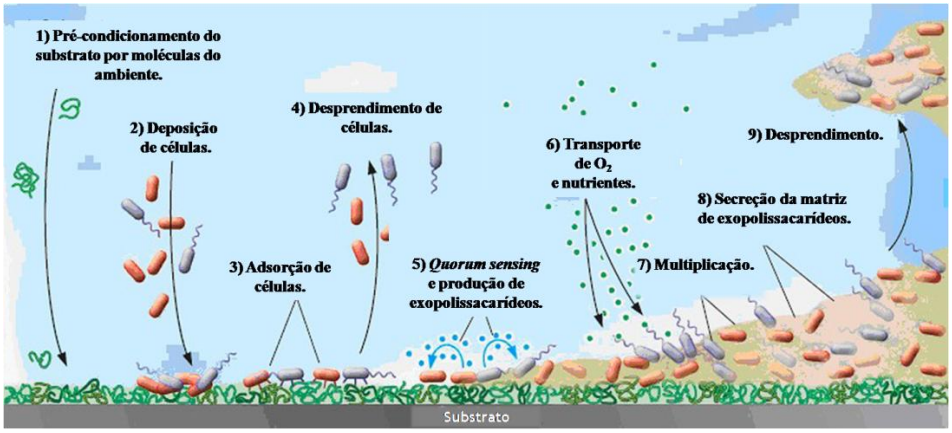

Figura 1 Processos que governam a formação de biofilmes
Fonte: Breyers e Ratner (2004), Oliveira (2011) e Simões, Simões e Vieira (2010).

Dentre todos os microrganismos, são as bactérias que mais frequentemente formam biofilme, ainda que umas apresentem, naturalmente, maior aptidão que outras. Os seus reduzidos tamanhos, elevadas taxas de

multiplicação, grande capacidade de adaptação e de produção de substâncias e estruturas extracelulares que as protegem do meio circundante são as principais características que fazem das bactérias excelentes microrganismos formadores de biofilmes (CHARACKLIS, 1990). *Alcaligenes*, *Bacillus*, *Enterobacter*, *Flavobacterium*, *Pseudomonas* e *Staphylococcus* são alguns dos gêneros mais comuns de bactérias formadoras de biofilmes (MATTILA-SANDHOLM; WIRTANEN, 1992).

Na indústria de alimentos, dentre os microrganismos deteriorantes destacam-se: *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Micrococcus* sp. e *Enterococcus faecium* (ANDRADE; BRIDGEMAN; ZOTTOLA, 1998; LERICHE; CARPENTIER, 1995). Como exemplos de microrganismos patogênicos, encontram-se: *Pseudomonas aeruginosa*, *L. monocytogenes*, *Yersinia enterocolitica*, *Salmonella* entérica Typhimurium, *Escherichia coli* O157:H7, *Staphylococcus aureus* e *Bacillus cereus* (LERICHE; CARPENTIER, 1995; SMITH; FRATÂMICO, 1995; SURMAN; MORTON; KEEVIL, 1996).

2.1.1 *Listeria monocytogenes*

L. monocytogenes é uma bactéria Gram-positiva, anaeróbica facultativa, catalase positiva, oxidase negativa, halotolerante, não formadora de esporos e desprovida de cápsula. Possui motilidade, morfologia de bacilo, diâmetro de 0,4-0,5 µm, comprimento de 0,5-2,0 µm, extremidades arredondadas e flagelos peritríquios que permitem sua motilidade (ADAMS; MOSS, 2004; ROCOURT, 1999). Os sorotipos encontrados são 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e e 7 (JAWETZ; MELNICK; ADELBERG, 1998). A faixa de pH para crescimento situa-se entre 5,6 a 9,6 (SWAMINATHAN, 2001), embora tenha sido constatado crescimento em pH abaixo de 4,0. A atividade de água (a_w) ótima é maior que 0,97, porém, a mínima varia entre 0,90 - 0,93 (LOU;

YOUSEF, 1999). Possui capacidade de se desenvolver em temperaturas que variam de -0,4ºC a 50ºC, apresentando crescimento ótimo entre 30ºC e 37ºC (SEVERINO, 2006). Sua natureza psicrotrófica permite seu desenvolvimento em alimentos refrigerados (BORUCKI et al., 2003).

Denomina-se listeriose a doença causada pela ingestão de alimentos contendo *L. monocytogenes* (SWAMINATHAN, 2001). O período de incubação da listeriose varia de horas a semanas, contudo, a dose infectante necessária para causar a doença ainda não está bem definida. A dose aproximada, relatada de casos, varia de 10^3 a 10^9 UFC/g ou mL (DALTON et al., 1997), mas há relatos de surtos com contaminação muito baixa (MEAD et al., 1999). A manifestação clínica da doença é descrita de duas formas, listeriose invasiva e listeriose gastrintestinal (não invasiva). A listeriose invasiva é uma doença severa, pois a taxa de mortalidade é alta (20% a 30%), principalmente, para pessoas susceptíveis a adquirir a infecção, como gestantes, recém-nascidos, idosos, pacientes submetidos à hemodiálise e a terapias prolongadas e indivíduos com sistema imunológico deprimido (SWAMINATHAN, 2001). As manifestações mais comuns são meningite, meningoencefalite, septicemia e aborto (DONELLY, 2001). Já a listeriose não invasiva pode causar infecções brandas, semelhantes a uma gripe, até surtos de gastrenterite febril em indivíduos saudáveis, mas, normalmente, não evolui para óbito (CARRIQUE-MAS et al., 2003; GAHAN; HILL, 2005).

Espécies de *Listeria* foram inicialmente isoladas do solo, poeira, água, esgoto, vegetais e silagem (HOFER; PÓVOA, 1984). Entretanto, na atualidade, os representantes patogênicos do gênero *Listeria* adquiriram extraordinária importância devido à sua veiculação por alimentos (HOFER, 2001; SEVERINO, 2006). Apesar de ser frequentemente encontrada em alimentos crus de origem vegetal e animal, *L. monocytogenes* também pode ser isolada de alimentos cozidos, devido a contaminações durante o processamento industrial. Mesmo

quando o patógeno está presente em pequenas quantidades no alimento inicialmente contaminado, sua capacidade de se multiplicar durante a estocagem sob refrigeração permite que seu número atinja doses infectantes. Dentre os principais alimentos associados à listeriose de origem alimentar estão o leite cru ou pasteurizado, queijos, sorvetes, vegetais crus, carnes e derivados, aves, peixes e frutos do mar (BUCHANAN et al., 1989; FARBER; PETERKIN, 1991).

Pesquisas realizadas visando detectar *L. monocytogenes* em alimentos no Brasil indicam sua presença em leite cru e leite pasteurizado (CATÃO; CEBALLOS, 2001), queijo de coalho (DUARTE et al., 2005), queijo artesanal (ZAFFARI; MELLO; COSTA, 2007), queijo minas frescal (SILVA et al., 2003); blanquet e presunto de peru fatiados (ARAÚJO et al., 2002), carne bovina crua moída (MANTILLA et al., 2007), linguiça frescal (SILVA et al., 2004), salame (DEGENHARDT; SANT'ANNA, 2007), mortadela (BERSOT et al., 2008), carcaça suína (SANTOS et al., 2005), salmão gravlax (CRUZ et al., 2008) e dieta enteral (PINTO; CARDOSO; VANETTI, 2004).

Alimentos crus não são a maior fonte de contaminação por *L. monocytogenes*. Ela ocorre durante o processamento e equipamentos agem como os principais reservatórios (DAUPHIN; RAGIMBEAU; MALLE, 2001; VOGEL et al., 2001). Assim, a ocorrência de surtos, bem como de casos esporádicos causados por esta bactéria, pode ser atribuída ao aumento dos fatores de virulência, melhor adaptação à sobrevivência em alimentos, ampla distribuição no ambiente e ou aumento da habilidade de sobrevivência em ambientes de processamento de alimentos através de biofilmes (CHAE et al., 2006).

Neste contexto, *L. monocytogenes* é de grande interesse na indústria alimentícia, uma vez que inúmeros estudos têm demonstrado sua capacidade de persistência no ambiente, algumas vezes durante anos (BAGGE-RAVN et al., 2003; MIETTINEN; BJORKROTH; KORKEALA, 1999). Sabe-se que esta

persistência pode ser resultado da adaptação ambiental de certos subtipos do microrganismo, de limpeza e sanitização inadequadas dos equipamentos ou da habilidade do microrganismo em desenvolver tolerância a alguns dos produtos utilizados no procedimento de higienização (GRAM et al., 2007).

2.2 Resistência bacteriana

A exposição subletal a determinadas condições ambientais e substâncias antimicrobianas pode resultar no desenvolvimento de maior resistência e promoção de resistência cruzada aos compostos antimicrobianos (GANDHI; CHIKINDAS, 2007; RUSSELL, 1984; YUK; MARSHALL, 2004).

Muitos microrganismos são capazes de desenvolver resposta adaptativa ao estresse subletal, permitindo-lhes tolerar e sobreviver a subsequente exposição a níveis letais do mesmo estresse ou até mesmo um tipo diferente de estresse (ROLLER, 2003).

A resistência de dado microrganismo à determinada droga pode ser classificada inicialmente como intrínseca ou adquirida. A resistência intrínseca é aquela que faz parte das características naturais, fenotípicas do microrganismo, transmitida apenas verticalmente à prole. Faz parte da herança genética do microrganismo. O maior determinante de resistência intrínseca é a presença ou ausência do alvo para a ação da droga. A resistência ainda pode ser não natural ou adquirida. Ocorre quando há o aparecimento de resistência em uma espécie bacteriana anteriormente sensível à droga em questão. Essa nova propriedade é resultado de alterações estruturais e/ou bioquímicas da célula bacteriana, determinada por alterações genéticas cromossômicas ou extra cromossômicas (plasmídios). A aquisição de resistência pode aparecer originária de uma mutação ou ainda transferível. É importante enfatizar também o termo "pressão seletiva" comumente utilizado para antibióticos, que estabelece que

antimicrobianos em contato com microrganismos exerçam sua atividade, levando à morte as cepas sensíveis sobrevivendo então as resistentes. Com o uso frequente, essa seleção leva ao predomínio das cepas que de alguma forma sobreviveram, multiplicaram-se e agora são maioria (DEL FIO; MATTOS FILHO; GROPPO, 2013).

O uso de sanitizantes pode levar à seleção de linhagens de bactérias intrinsicamente resistentes ao agente antibacteriano aplicado. Inúmeros relatos indicam que a exposição bacteriana a concentrações subletais de sanitizantes pode ter como consequência um aumento de resistência (LANGSRUD et al., 2003). O desenvolvimento de susceptibilidade reduzida em populações bacterianas, seja por adaptação fenotípica, alteração genética ou aquisição genética, aumenta a probabilidade de uma posterior falha na sanitização, tendo como consequência sérios problemas na preparação industrial de alimentos e na área da saúde humana (CHAPMAN et al., 2003).

Após a exposição regular à ação dos sanitizantes, bactérias Gram-positivas, como *S. aureus*, demonstram adaptação e tolerância a estes compostos químicos. As bactérias Gram-negativas, como *Pseudomonas* sp., também podem se adaptar aos sanitizantes utilizados nas plantas de processamento de alimentos (TO et al., 2002). Já *L. monocytogenes* tem mostrado capacidade de adaptar-se a compostos quaternários de amônio (QCA), que são amplamente utilizados nas indústrias de alimentos (AASE et al., 2000), a maioria dos estudos de resistência de *L. monocytogenes* vêm sendo feitos com este grupo de sanitizantes químicos.

Respostas adaptativas se desenvolvem quando as bactérias são expostas a concentrações subletais de sanitizantes (AASE et al., 2000; GANDHI et al., 1993), sendo que estas condições podem ocorrer como resultado do processo de limpeza e sanitização ineficiente (AASE et al., 2000). Adaptação de *L. monocytogenes* aos QACs e possível adaptação a outros sanitizantes utilizados nas indústrias de alimentos pode levar a aumento da sobrevida (LUDÉN et al.,

2003), permitindo que esta bactéria se mantenha na planta de processamento, podendo vir a contaminar os alimentos que entrem em contato com superfícies contaminadas.

Muitos estudos já foram realizados para determinar a susceptibilidade de *L. monocytogenes* a QACs. Mereghetti et al. (2000) estudaram a sensibilidade a QACs de noventa e sete cepas de *L. monocytogenes* não relacionadas epidemiologicamente. As cepas foram isoladas de ambiente, produtos alimentícios, animais e humanos. Sete dos isolados que eram de origem ambiental ou proveniente de alimentos apresentaram altas concentrações mínimas inibitórias (CMIs) para os QACs testados, tais como cloreto de benzalcônio e cetrimida. Os autores concluíram que a resistência destas cepas podia explicar a persistência de alguns destes microrganismos em plantas de processamentos de alimentos. Além disso, todos os isolados continham o gene *mdrL*, que codifica para uma bomba de efluxo responsável por conferir resistência aos QACs. Os autores sugeriram que a rotação entre dois tipos diferentes de sanitizantes poderia ser um procedimento útil para prevenir o desenvolvimento de cepas persistentes e resistentes.

Contudo, segundo Lundén et al. (2003), a adaptação cruzada de cepas de *L. monocytogenes* a sanitizantes relacionados ou não levanta a questão sobre a real eficiência da prática de rotação de produtos sugerida por Mereghetti et al. (2000) no controle do desenvolvimento da resistência a antimicrobianos.

O mecanismo mais comum de resistência bacteriana a sanitizantes é reduzir sua absorção ou bombeá-lo ativamente para fora da célula (RUSSELL; CHOPRA, 1996). Sabe-se que os QACs podem ser substrato do mesmo sistema de efluxo em bactérias Gram-positivas (HEIR; SUNDHEIM; HOLCK, 1998, 1999; MIDGLEY, 1994; TAKIFF et al., 1996) e em Gram-negativas. Estes sistemas são chamados bombas de efluxo de multidrogas, uma vez que os mesmos, frequentemente, apresentam resistência específica a uma gama de

antibióticos, corantes e surfactantes estruturalmente relacionados. A disseminação de genes que codificam para bombas de efluxo de QACs é responsável pela pressão seletiva que tem sido sugerida como a causa de ampla ocorrência deste mecanismo de resistência (PAULSEN; BROWN; SKURRAY, 1996).

A maior resposta adaptativa das células é manter a fluidez de suas membranas em um valor constante, independente das condições ambientais do momento. Tal estabilização da fluidez da membrana constitui a resposta predominante de bactérias a substâncias que atuam em membranas ou a mudanças nas condições ambientais (HEIPIEPER; MEINHARDT; SEGURA, 2003), prevenindo a perda das propriedades químicas e mecânicas da bicamada lipídica (RUSSELL; FUKANAGA, 1990), se a perturbação da integridade da membrana ocorrer, então suas funções como barreira, sítio de atividade enzimática e local de produção de energia serão comprometidas (HEIPIEPER; MEINHARDT; SEGURA, 2003).

Existem diversos agentes antibacterianos que possuem como alvo a membrana citoplasmática bacteriana. Segundo Andrade, Pinto e Rosado (2008), QACs interferem nas propriedades de permeabilidade da membrana celular, levando ao extravasamento de metabólitos. Além disso, pode ocorrer interferência no metabolismo de proteínas, causando a desnaturação proteica e inibição enzimática. Outros compostos que possuem modo de atuação semelhante são os constituintes dos óleos essenciais. Di Pasqua et al. (2006) observaram mudanças na composição de ácidos graxos na membrana de células bacterianas como mecanismo de adaptação das células em resposta a concentrações subletais de timol, carvacrol, limoneno, cinamaldeído e eugenol

A formação de biofilmes também pode levar ao aumento da resistência das células microbianas aos agentes antimicrobianos, sendo causada por vários fatores. O EPS limita a difusão de sanitizantes e pode causar a inativação dos

mesmos e a densidade bacteriana no interior do biofilme parece também estar envolvida (VIDAL; RAGOT; THIBAULT, 1997; WIRTHLIN; CHEN; HOOVER, 2005). A resistência intensificada das células no interior dos biofilmes também é atribuída a fatores como baixa taxa de crescimento das bactérias e neutralização de sanitizantes pela matriz polimérica, já que a mesma é constituída por matéria orgânica (ROSSI; PORTO, 2009). Doses subletais de agentes biocidas aplicadas em células em biofilmes podem ocasionar resistência (GILBERT; ALLISON; MCBAIN, 2002). Biofilmes formados por diferentes espécies são perigosos porque elas podem proteger umas as outras durante a aplicação de agentes químicos. Este fato é causado pela diferente resistência de uma respectiva espécie microbiana contra os agentes utilizados (VIDAL; RAGOT; THIBAULT, 1997; WIRTHLIN; CHEN; HOOVER, 2005).

Tem sido sugerido que a resistência de *L. monocytogenes* a antimicrobianos ou agentes sanitizantes em ambientes de processamento de alimentos resulta da habilidade das células em formar biofilmes (BLACKMAN; FRANK, 1996; KUMAR; ANAND, 1998; WONG, 1998). Biofilmes de *L. monocytogenes* têm se mostrado mais resistentes ao estresse e a agentes sanitizantes do que células planctônicas (CHAVANT; GAILLARD-MARTINIE; HEBRAUD, 2004; HOLAH et al., 2002; VATANYOOPAISARN et al., 2000).

2.3 Óleos essenciais e seus constituintes no controle de biofilmes microbianos

Devido à aquisição de resistência bacteriana a sanitizantes químicos utilizados em indústrias de alimentos, novas alternativas vêm sendo pesquisadas. Neste contexto, alternativas não convencionais de sanitização ou até mesmo novas ferramentas de controle de biofilmes também vêm sendo estudadas, tais

como: ruptura da matriz de substâncias poliméricas extracelulares pela ação de enzimas (XAVIER et al., 2005), adsorção de biossurfactante aniônico a superfícies para auxiliar a sanitização por agentes químicos (MEYLHEUC; RENAULT; BELLON-FONTAINE, 2006), exclusão competitiva de patógenos por bactérias ácido lácticas (ZHAO et al., 2006), aplicação de irradiação gama (BYUN et al., 2007), óleo essencial e hidrolato de *Satureja thymbra* (CHORIANOPOULOS et al., 2008), óleo essencial de *Cymbopogon citratus* e *Cymbopogon nardus* (OLIVEIRA et al., 2010), extratos de condimentos e plantas medicinais (SANDASI; LEONARD; VILJOEN, 2010), nisina (CABO et al., 2009) e ácidos graxos esterificados com sacarose (FURUKAWA et al., 2010).

Neste contexto, destacam-se os óleos essenciais, que são formados a partir de vias metabólicas secundárias e podem ser definidos como misturas complexas de substâncias voláteis, lipofílicas, geralmente odoríferas e líquidas, que ocorrem em estruturas secretoras especializadas, tais como pelos glandulares, células parenquimáticas diferenciadas, canais oleíferos ou em bolsas lisígenas ou esquizolisígenas. Podem ser estocados nas flores, folhas, casca do caule, madeira, raízes, rizomas, frutos e sementes, podendo variar na sua composição de acordo com a localização em uma mesma espécie (SIMÕES; SPITZER, 2004).

Os componentes dos óleos essenciais incluem dois grupos de origem biossintética distinta (BETTS, 2001; BOWLES, 2003; CROTEAU; KUTCHAN; LEWIS, 2000; PICHERSKY; NOEL; DUDAREVA, 2006). O grupo principal é composto pelos terpenos e terpenoides, e o outro por constituintes aromáticos e alifáticos, todos caracterizados por baixo peso molecular (BAKKALI et al., 2008).

Considerando que os óleos essenciais são formados por diversos constituintes, torna-se difícil atribuir a eficácia da atividade antimicrobiana a

apenas um deles. Neste contexto, o mecanismo de ação de um dado constituinte pode diferir quando comparado ao de outros, sendo relatados vários alvos na célula microbiana (AYALA-ZAVALA; GONZÁLEZ-AGUILAR; DEL-TORO-SÁNCHEZ, 2009). De acordo com Bakkali et al. (2008), óleos essenciais são compostos tipicamente lipófilos e, por isso, são capazes de passar através da parede celular e membrana citoplasmática bacteriana, causando aumento da permeabilidade por danificar a estrutura de diferentes camadas de polissacarídeos, ácidos graxos e fosfolipídeos. Segundo Ayala-Zavala, González-Aguilar e Del-Toro-Sánchez (2009) e Burt (2004), o mecanismo de ação dos óleos essenciais inclui: destruição da parede celular e membrana citoplasmática, danificação de proteínas de membrana, liberação de conteúdo celular, coagulação do citoplasma, depleção da força próton motiva, inativação de enzimas essenciais e perturbação da funcionalidade do material genético.

Os óleos essenciais, geralmente, apresentam dois ou três compostos majoritários, com concentrações bastante elevadas em comparação aos demais, presentes em quantidades vestigiais. São os constituintes majoritários que, na maioria das vezes, determinam as propriedades biológicas (BAKKALI et al., 2008). Com base nesta afirmação, alguns estudos têm utilizado os compostos majoritários como antimicrobianos, tanto para o controle de células planctônicas (DI PASQUA et al., 2006; ETTAYEBI; YAMANI; ROSSI-HASSANI, 2000; LA STORIA et al., 2011; SADDIQ; KHAYYAT, 2010), quanto células sésseis (LEONARD et al., 2010; NOSTRO et al., 2007; SANDASI; LEONARD; VILJOEN, 2008).

Vários estudos já testaram óleos essenciais e/ou seus constituintes contra biofilmes bacterianos, enfatizando seu uso em indústrias de alimentos. A Tabela 1 demonstra os estudos já realizados por nosso grupo de pesquisa, coordenado pela Profa. Dra. Roberta Hilsdorf Piccoli, do Departamento de Ciência de Alimentos (DCA) da Universidade Federal de Lavras (UFLA). Enfatiza-se que

os trabalhos já realizados comprovaram o efeito de diferentes óleos essenciais e seus constituintes contra bactérias Gram-positivas e Gram-negativas, em sua maioria, patogênicas. Contudo, pouco se sabe sobre o comportamento bacteriano após a exposição a doses subletais destes compostos, o que assegura a importância e o ineditismo da presente Tese.

Óleos essenciais podem apresentar variações quanto à concentração dos compostos majoritários, devido a quimiotipos diferentes, efeito das épocas do ano, localização geográfica, o que torna a composição química variável e dificulta a difusão da utilização destes como antibacterianos. Por outro lado, atualmente, diversos constituintes dos óleos essenciais são sintetizados ou isolados e estão disponíveis comercialmente, o que torna a concentração dos mesmos padronizada, permitindo a utilização com maior segurança. Dentre os diversos constituintes disponíveis, destaca-se o citral e o cinamaldeído.

Citral (3,7-dimetil-2,6-octadienal*)* é o constituinte majoritário do óleo essencial extraído das folhas de *Cymbopogon citratus* (capim-limão), estando presente em níveis de, aproximadamente, 65-85%. Na verdade, citral é o nome dado a uma mistura isomérica de dois aldeídos monoterpênicos acíclicos: geranial (trans-citral ou citral A) (Figura 1 A) e neral (cis-citral, citral B) (Figura 1B) (SADDIQ; KHAYYAT, 2010).

Já o cinamaldeído (3-fenil-2-propenal), também conhecido como aldeído cinâmico, é o componente majoritário do óleo essencial da casca de espécies de canela, como a canela da China (*Cinnamomum cassia*). Ooi et al. (2006), por exemplo, encontraram 85,06% de trans-cinamaldeído no óleo essencial da casca de *C. cassia*. A estrutura química do principal constituinte do óleo essencial de *C. cassia* encontra-se na Figura 1C

Tabela 1 Estudos já realizados na Universidade Federal de Lavras, sob orientação da Profa. Dra. Roberta Hilsdorf Piccoli, que comprovaram o efeito antibacteriano de óleos essenciais e/ou de seus constituintes contra células em biofilme

Autores	**Bactéria**	**Superfície de formação do biofilme**	**Óleos essenciais e/ou constituintes**
Oliveira et al. (2010)	*Listeria monocytogenes*	Aço inoxidável	*Cymbopogon citratus* e *Cymbopogon nardus*
Oliveira et al. (2012a)	*Listeria monocytogenes* e *Escherichia coli* enteropatogênica (EPEC)	Aço inoxidável	*Cinnamomum cassia* e cinamaldeído
Oliveira et al. (2012b)	*Listeria monocytogenes* e *Escherichia coli* enteropatogênica (EPEC)	Polipropileno e aço inoxidável	*Melaleuca alternifolia,Cymbopogon flexuosus* e *Cinnamomum cassia*
Millezi et al. (2012)	*Staphylococcus aureus* e *Escherichia coli*	Polipropileno	*Cymbopogon nardus* e *Citrus limonia*
Valeriano et al. (2012)	*Salmonella enterica* sorotipo Enteritidis	Aço inoxidável	*Mentha piperita* e *Cymbopogon citratus*
Millezi et al. (2013)	*Aeromonas hydrophila*	Aço inoxidável	*Thymus vulgaris* e *Cymbopogon citratus*

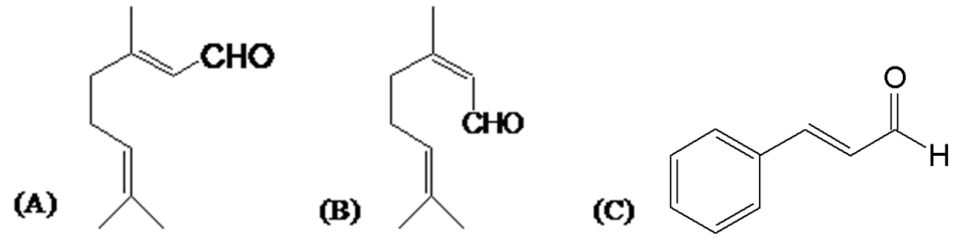

Figura 2 Estrutura química do geranial (A), neral (B) e cinamaldeído (C)

3 CONSIDERAÇÕES FINAIS

A afirmação de que bactérias patogênicas causam enormes prejuízos à saúde pública é indiscutível. *L. monocytogenes* é uma espécie que se destaca por causar uma infecção com alta taxa de mortalidade, contaminando os alimentos frequentemente devido à sua capacidade de formar biofilmes e persistir em ambientes de processamento de alimentos.

É evidente o problema que os biofilmes microbianos podem ocasionar e os óleos essenciais têm se mostrado uma alternativa e um possível método de controle. No entanto, devido a problemas de variação entre a composição dos óleos essenciais, a utilização dos compostos majoritários torna mais fácil a difusão desse novo método de controle, embora pouco se conheça sobre o risco que a utilização de concentrações subletais pode ocasionar.

Diante disto, estudos que avaliem a utilização de concentrações subletais devem ser realizados avaliando diferentes cepas do mesmo microrganismo, a fim de obter informações sobre um possível aumento da resistência ou até mesmo aumento da susceptibilidade de microrganismo quando em contato com concentrações subletais.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 Antibacterial activity of essential oils major compounds against planktonic and sessile cells of *Listeria monocytogenes*

Artigo submetido ao periódico *International Journal of Food Science* & *Technology*.

Antibacterial activity of essential oils major compounds against planktonic and sessile cells of *Listeria monocytogenes*

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Summary - *Listeria monocytogenes* (LM) is a foodborne pathogen of great importance nowadays and has been used as a target microorganism in studies about antimicrobials. The antilisterial activity of essential oils constituents (EOCs) was evalueted. Citral, thymol, cinnamaldehyde, eugenol, α-terpineol, carvacrol and terpinen-4-ol were tested against planktonic cells of LM strains ATCC 19117, 15313 and 7644. Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) were determined. All the EOCs showed antibacterial activity. Solutions of citral and cinnamaldehyde, EOCs that showed the lowest MBCs, were tested against biofilms formed on stainless steel surface. Besides the control treatment (without EOCs), were used the following concentrations: MBC, 2xMBC, 4xMBC, 6xMBC, 8xMBC and 10xMBC. After plate count, the results were obtained in log-reduction and the Minimum Bactericidal Concentrations of Biofilms (MBCBs) were determined. The MBCBs values varied from 0.42 to 1.2% (v/v). Citral and cinnamaldehyde may be new sanitizers alternatives.

Keywords - Natural Antimicrobials, antilisterial activity, biofilms, sanitizer effect.

Introduction

Currently, different microbial pathogens have emerged as food contaminants and outbreaks of foodborne illness causers. Among them is *Listeria monocytogenes* (LM) (Scallan *et al.*, 2011; Hoffmann *et al.*, 2012), psychrotrophic species with ubiquitous distribution in the nature, able to multiply on different substrates and tolerate adverse situations (Gandhi & Chikindas, 2007). LM is the causative agent of the foodborne infection listeriosis, that is characterized by high mortality (Scallan *et al*., 2011; Silk *et al.*, 2012) and can contaminate food, mainly due to biofilms formation (Møretrø

& Langsrud, 2004; Vorst *et al.*, 2006; Rodríguez *et al.*, 2007; Oliveira *et al*., 2010a).

The concern with the formation of microbial biofilms due to an ineffective and/or improper use of chemical sanitizers available commercially, added to the possible presence of resistant microorganisms, have driven researches that seeks new antimicrobials for be used in food industries (Simões *et al.*, 2010). In the specific case of LM, studies have shown resistance to chemicals agents commonly used in food industries, especially for sessile cells (Romanova *et al.*, 2002; Chavant & Gaillard-Martinie, 2004; Pan *et al.*, 2006; Cruz & Fletcher, 2012; Nakamura *et al.*, 2013).

In order to ensure the microbiological safety of foods, effective methods to inactivate planktonic and sessile cells of LM should be developed to reduce the probability of occurring listeriosis. Alternative antimicrobials for biofilms control have emerged and highlighted, such as essential oils (EOs) and EO compounds (EOCs) (Oliveira *et al.*, 2010b, 2012a, 2012b; Desai *et al.*, 2012; Valeriano *et al.*, 2012; Millezi *et al*., 2012, 2013; Jadhav *et al.*, 2013; Kerekes *et al.*, 2013; Upadhyay *et al*., 2013; Szczepanski & Lipski, 2014). EO and EOCs are able to act in microbial cells, damaging their structure, physiology and metabolism, causing his death. According to Carson & Hammer (2011), the initial interaction between EOCs and microbial cell seems to be the passive diffusion through the cell wall of Gram-positive bacteria and fungi or outer membrane of Gram-negative bacteria.

This study evaluated the antibacterial effect of citral, thymol, cinnamaldehyde, eugenol, α-terpineol, carvacrol and terpinen-4-ol, by determining the Minimum Inhibitory Concentrations (MICs) and the Minimum Bactericidal Concentrations (MBCs) against planktonic cells of LM strains, besides verifying the Minimum Bactericidal Concentrations of Biofilms (MBCBs) of the most effective EOCs.

Material and methods

Bacterial strains

Were used three LM strains: ATCC 19117 (serotype 4d), ATCC 7644 (serotype 1/2c) and ATCC 15313 (serotype 1/2a). The strains, kindly provided by the Reference Materials Laboratory of the National Institute of Quality Control in Health of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), were kept frozen at -20 °C in Tryptone Soy Broth supplemented with 0.6% (w/v) yeast extract (TSB-YE) and added 20% glycerol.

Activity against planktonic cells

Standardization and inoculum preparation

For reactivation, cells (10 µL) of the stock cultures was transferred to test tubes containing TSB-YE and two consecutive subcultures were performed with incubation at 37 °C/24 hours. The cultures were striated on Tryptone Soy Agar supplemented with 0.6% (w/v) yeast extract (TSA-YE) and incubated at 37 °C/24 hours. Cells (10 µL) from each culture was taken and transferred to 150 mL of TSB-YE, which was incubated at 37 °C until the desired cell density, based on standard growth curves previously performed at $OD_{620\ nm}$. The absorbances used to obtain the concentration of about 8 Log CFU/mL were: 0.305 for the ATCC 19117, 0.385 for the ATCC 7644 and 0,254 for the ATCC 1531. The strains were cultured and standardized individually.

Minimal Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) determination

For the MICs determination, citral, thymol, cinnamaldehyde, eugenol, α-terpineol, carvacrol and terpinen-4-ol (Sigma Aldrich) were used and the broth microdilution technique was employed. Flat bottom polystyrene microplates with 96 well were used and EOCs concentrations (0 to 1% v/v) were prepared in

TSB-YE with 0.5% (v/v) Tween 80 added. The liquid volume in the wells was 150 µL. TSB-YE containing the bacterial inoculum was added to the wells (10 µL). Incubation was carried out at 37 °C/24 hours. The absorbance was read at 620 nm in a microplate reader (Anthos 2010) before and after the incubation period. The lowest concentrations that resulted in complete bacterial growth inhibition were called MICs (Oliveira et al., 2012a).

To determine the MBCs, after the incubation period, aliquots of 10 µL were removed from the wells that containing the EOCs tested and were inoculated in petri dishes containing TSA-YE, which were incubated at 37 °C/24 hours. The lowest concentrations with no bacterial growth were established as MBCs.

Activity against sessile cells

Based on the MBCs results, were selected two EOCs that showed better results for the three strains tested, these being citral and cinnamaldehyde.

Standardization and inoculum preparation

The strains were reactivated and standardized based on the absorbances according to the methodology described above. The absorbances used to obtain the concentration of about 9 Log CFU/mL were: 0.895 for the ATCC 19117, 0.590 for the ATCC 7644 and 0.670 for the ATCC 1531.

Biofilms formation

For the biofilms formation, coupons (1x10x20 mm) of AISI 304 (#4) stainless steel were used, these being hygienized and sterilized according to Oliveira *et al.* (2010a). The coupons were placed in test tubes (16x125 mm) and 5 mL of TSB-YE 8 Log CFU/mL were added. The tubes were incubated at 37 °C/48 hours under static conditions.

Minimum Bactericidal Concentrations of Biofilms (MBCBs) determination

After the incubation period, the coupons were removed, washed three times in 0.1% peptone water (w/v) to remove the non-adherent cells and transferred to test tubes (16x125 mm) containing 5 mL of the sanitizing solutions containing the antimicrobial agent under test (cinnamaldehyde or citral) for 15 minutes at room temperature (25 °C).

The EOCs concentrations of the sanitizing solutions used were determined based on the MBCs of each strain, and the values used were: MBC, 2xMBC, 4xMBC, 6xMBC, 8xMBC and 10xMBC, plus a control solution (without the antibacterial agent).

For the strains ATCC 19117 and 7644 the EOCs concentrations ranging from 0 to 1.2% (v/v), and for the strain ATCC 15313 the concentrations varied from 0 to 0.6% (v/v). The sanitizing solutions were prepared in sterile distilled water containing 0.5% Tween 80 (v/v).

Sessile bacterial cells count

For removal and quantification of the remaining viable sessile cells, the coupons were removed from the test tubes, washed three times in 0.1% peptone water (w/v), for the sanitizing residues removed, and transferred to plastic tubes (50 mL) with conical bottom containing 10 mL of peptone water. The plastic tubes were shaken on vortex at maximum speed for 2 minutes.

To represent the biofilms counts, coupons not subject to any solution were also sampled directly from TSB-YE after the incubation period, washed three times in 0.1% (w/v) peptone water to remove any non-adherent cell and placed in plastic tubes with conical bottom for vortexing.

After stirring, serial dilutions were performed in peptone water 0.1% (w/v), with subsequent plating on TSA-YE using the microdrop technique (10

µL). The incubation was performed at 37 °C/24 hours. The results were expressed as log-reduction, obtained by the difference between the log of the initial cell count (biofilm) and the log of the surviving cells after the antibacterial treatment and the control treatment.

Statistical analysis

For each strain was used a completely randomized design with three repetitions. The results were transformed to log-reduction and subjected to variance analysis. When relevant, regression analysis and mean test (Skott-Knott) were done. Statistical analyzes were performed using the statistical program Sisvar 5.3.

Results and discussion

Minimal Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)

All constituents showed antibacterial activity, being observed different sensitivity patterns among the strains. The MICs ranged from 0.12% (v/v) (citral and cinnamaldehyde) to 0.5% (v/v) (eugenol, carvacrol and terpinen-4-ol) for the ATCC 19117, from 0.12% (v/v) (citral and cinnamaldehyde) to 0.5% (v / v) (α-terpineol, carvacrol and terpinen-4-ol) for the ATCC 7644, and from 0.06% (v/v) (citral and cinnamaldehyde) to 0.5% (v/v) (eugenol, α-terpineol, carvacrol and terpinen-4-ol) for the ATCC 15313. With respect to MBCs, all EOCs tested showed bactericidal activity. Except for eugenol, that for the ATCC 7644 showed a MBC greater than the MIC, for most of the tested EOCs MBC was equal MIC. Citral and cinnamaldehyde presented the lowest values of MICs and MBCs, showing greater antimicrobial effect when compared to the other compounds tested (Table 1).

Table 1 Minimal Inhibitory Concentrations (MICS) and Minimum Bactericidal Concentrations (MBCs) of essential oils constituents obtained against *Listeria monocytogenes* strains, and expressed in % (v/v)

Constituents	*Listeria monocytogenes* strains					
	ATCC 19117		ATCC 7644		ATCC 15313	
	MIC	MBC	MIC	MBC	MIC	MBC
Cinnamaldehyde	0.12	0.12	0.12	0.12	0.06	0.06
Carvacrol	0.50	0.50	0.50	0.50	0.50	0.50
Citral	0.12	0.12	0.12	0.12	0.06	0.06
Eugenol	0.50	0.50	0.25	0.50	0.50	0.50
Terpinen-4-ol	0.50	0.50	0.50	0.50	0.50	0.50
Thymol	0.25	0.25	0.25	0.25	0.25	0.25
α-terpineol	0.25	0.50	0.50	0.50	0.50	0.50

EOs are volatile and complex natural compounds characterized by a strong odor and formed by aromatic plants as secondary metabolites (Bakkali *et al.*, 2008). Its constituents include mainly two groups of distinct biosynthetic origins. The main group is composed of terpenes and other by phenylpropanoids (Bowles, 2004; Bakkali *et al.*, 2008.).

EOs usually have two or three major compounds with very high concentrations compared to the others, present in trace amounts. The major constituents, in most cases, are the responsible for the biological properties (Bakkali *et al.*, 2008). In the early part of this study, it was found that the EOCs citral and cinnamaldehyde have stood out in terms of antimicrobial activity (Table 1).

Citral (3,7-dimethyl-2 ,6-octadienal) is the major constituent of the EO extracted from the leaves of some species of *Cymbopogon,* such as *C. citratus*

(lemongrass). This EOCs is present at levels of approximately 65-85%. In fact, citral is the name given to a mixture of two isomeric acyclic monoterpene aldehydes: geranial (trans-citral or citral A) and neral (cis-citral, citral B) (Saddiq & Khayyat, 2010). On the other hand, cinnamaldehyde (3-phenyl-2-propenal), also known as cinnamic aldehyde, is the major component of the EO of cinnamon bark species, such as Chinese cinnamon (*Cinnamomum cassia*). Ooi *et al.* (2006), for example, found 85.06% trans-cinnamaldehyde in the EO from the bark of *C. cassia*.

EOs are compounds typically lipophilic and therefore are able to pass through the cell walls and accumulate in the bacterial cytoplasmic membrane, causing increased permeability due to damage the structure of the different layers of polysaccharides, fatty acids and phospholipids (Bakkali *et al.*, 2008). The increase in membrane fluidity appears to be among the first antimicrobial effects caused by EOs treatment. The expansion and increased fluidity of the plasma membrane can lead to breakage of the integrity with consequent loss of small intracellular components, such as hydrogen, sodium and potassium. The loss of these ions are associated with the decrease of the membrane potential, intracellular pH and ATP pool, caused by damage to the ion gradient that occurs between the interior and exterior of the cell. High concentrations of EOs or long exposure times may lead to higher damage to the cytoplasmic membrane, causing loss of macromolecules such as DNA and proteins, a factor closely related to cell death (Hammer & Carson, 2011).

As in this study, the effect of citral and cinnamaldehyde against various microorganisms has been reported in the literature. Moleyar & Narasimham (1992) studied the antibacterial activity of EOCs and found that cinnamaldehyde was the most active compound, followed by citral, geraniol, eugenol and menthol. Friedman *et al.* (2002) have noted that the most active EOCs against LM were cinnamaldehyde, eugenol, thymol, carvacrol, citral, geraniol,

perillaldehyde, carvone S, estragole and salicylaldehyde. Lis-Balchin & Deans (1997) reported that strong anti-*Listeria* activity was often correlated with EOs containing a high percentage of monoterpenes, eugenol, cinnamaldehyde, thymol, and sometimes with citronellol, limonene and geraniol. However, in a general way, it is emphasized that the antimicrobial activity of EOs and their constituents against planktonic cells should be the initial step to select the most active agents to be further evaluated as natural preservatives in food or as constituents of sanitizing solutions. The second phase of this study used citral and cinnamaldehyde for the preparation of sanitizing solutions to be used in the control of biofilms formed by strains of LM under stainless steel.

Biofilms formation

The strains of LM used were able to adhere on the surface of stainless steel coupons and form biofilms, exhibiting the following counts: 5.73±0.06 Log CFU/cm^2 for the ATCC 19117, 5.61±0.12 Log CFU/cm^2 for the ATCC 7644, and 5.67±0.35 Log CFU/cm^2 for the ATCC 15313. In study performed by Upadhyay *et al.* (2013) three strains of LM (ATCC 19115, Scott and Presque-598) were also able to adhere and form biofilm in microtiter plates and stainless steel coupons at temperatures of 37, 25 and 4 °C, indicating ability to adapt to different surfaces and temperatures environments. Oliveira *et al.* (2010a), using the ATCC 19117 strain, observed by scanning electron microscopy and bacterial counts on plates that LM was capable of quickly adhere to stainless steel, with 4.08 Log CFU/cm^2 counts after 48 hours.

After biofilms formation on industrial areas, LM may be able to survive in the food processing environment for long periods of time (Fatemi & Frank, 1999; Koffi & Frank, 1990). The persistence of LM in industrial facilities constitutes a significant threat to food security, since microbial biofilms presenting an increase resistance against the action of sanitizers (Borucki *et al.*,

2003; Folsom & Frank, 2007), besides serving as a continuous source of microbiological contamination of food.

According to Oliveira *et al.* (2010a), one of the great biofilm formation problem in the food industry or other areas is cell detachment, which makes it a constant source of microorganism contamination in food, water, or new infection processes. Study conducted by Oliveira *et al.* (2010a), verified that the detachment of LM ATCC 19117 cells from stainless steel surface was practically constant during the biofilm formation stages analyzed (96, 144, 192 and 240 hours). Thus, control of LM in food processing facilities is critical to ensuring food security and reducing the incidence of listeriosis in humans (Upadhyay *et al*., 2013). Routine cleaning and sanitation are essential to control the persistence of LM onto surfaces (Tompkin *et al*., 1999). In this context, there is an increasing interest in the identification of safe and effective antimicrobials for LM control in food processing plants (Upadhyay *et al*., 2013).

Minimum Bactericidal Concentrations of Biofilms (MBCBs)

As noted, citral and cinnamaldehyde showed the better MBC results and were selected to assess their activities against sessile cells of LM attached to stainless steel.

For LM ATCC 19117, there was no significant interaction ($p>0.05$) and no significant difference ($p>0.05$) between citral and cinnamaldehyde. However, significant difference ($p<0.05$) was verified between the concentrations tested, therefore was generated a regression based on the average value of the two compounds (Figure 1). The concentration of greater reduction estimated by the regression equation was 0.48% (v/v) for both EOCs tested (Table 2).

Table 2 Regression equations used to determine the concentrations of citral and cinnamaldehyde required to obtain the highest log-reductions values for *Listeria monocytogenes* biofilms formed on stainless steel coupons.

ATCC	Constituints	Regression equations	R^2
19117	Citral and cinnamaldehyde	$y = 0.827442 – 3.891555x + 157.200581x^2 – 433.053313x^3 + 429.044112x^4 – 143.939805x^5$	92.24%
7644	Citral	$y = 1.259021 – 0.913726x + 3.630148x^2$	95.00%
7644	Cinnamaldehyde	$y = 1.184294 – 0.043230x – 14.246709x^2 + 41.401318x^3 – 22.444615x^4$	99.81%
15313	Citral and cinnamaldehyde	$y = 1.127475 + 53.63486x – 963.173x^2 + 5190.407x^3 – 10402.4x^4 + 7021.117x^5$	99.01%

y (log-reduction) and x (citral and/or cinnamaldehyde concentrations).

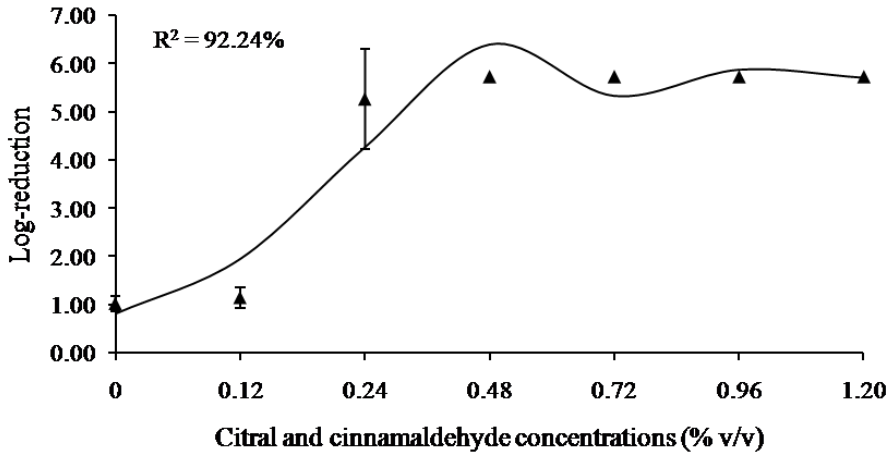

Figure 1 Log-reductions of *Listeria monocytogenes* ATCC 19117 biofilm formed under stainless steel, obtained as a function of different concentrations of citral and cinnamaldehyde. Vertical bars represent the standard deviation from the mean. (▲) Observed averages and (-) estimated averages.

In the case of LM ATCC 7644, significant difference ($p<0.05$) and significant interaction ($p<0.05$) between concentration and compound used were obtained, so the unfolding was made and the averages of each compound were compared by Skott-knot test. For concentrations of 0, 0.12, 0.24 and 1.2% (v/v) there was no significant difference between citral and cinnamaldehyde ($p>0.05$). For the concentration of 0.48% (v/v) citral was more effective than cinnamaldehyde ($p<0.05$), and for the concentrations of 0.72 and 0.96% (v/v) cinnamaldehyde was more effective than citral ($p<0.05$) (Figure 2). Based on regression equations the concentrations with higher reductions was 1.2% (v/v) to citral and 1.1% (v/v) to cinnamaldehyde (Table 2).

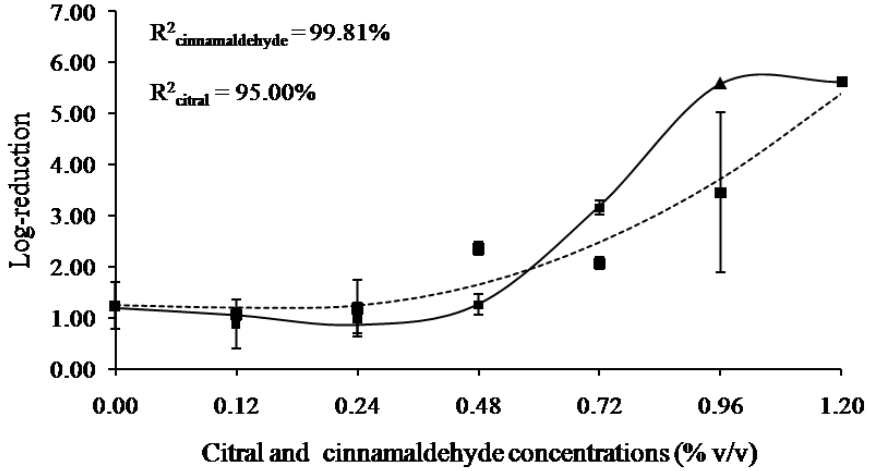

Figure 2 Log-reductions of *Listeria monocytogenes* ATCC 7644 biofilm formed under stainless steel, obtained as a function of different concentrations of citral and cinnamaldehyde. Vertical bars represent the standard deviation from the mean. Within each concentration, the same letter do not differ by Skott-Knott test at 5% probability. (▲) Observed averages of cinnamaldehyde, (-) estimated averages of cinnamaldehyde, (■) observed averages of citral and (---) estimated averages of citral.

Regarding LM ATCC 15313, there was no significant interaction ($p>0.05$) and no significant difference ($p>0.05$) between citral and cinnamaldehyde; however a significant difference ($p<0.05$) between the concentrations being tested was noted, and was generated a regression based on the average value of the two compounds (Figure 3). The concentration of greater reduction estimated by the regression equation was 0.42% (v/v) for both compounds tested (Table 2).

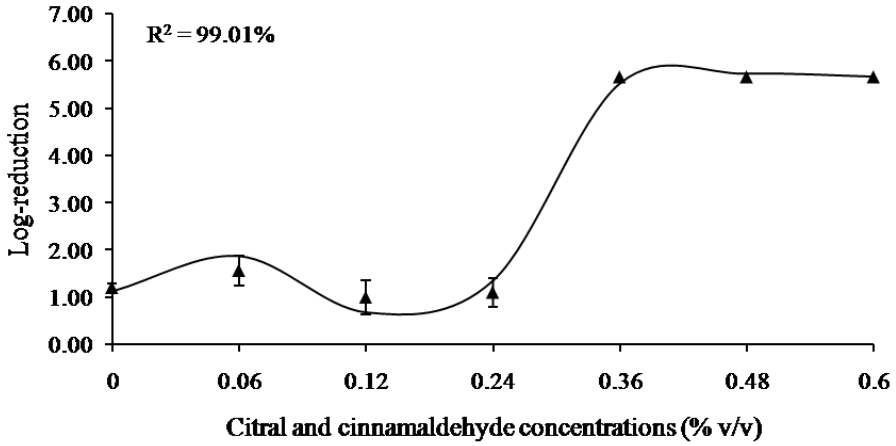

Figure 3 Log-reductions of *Listeria monocytogenes* ATCC 15313 biofilm formed under stainless steel, obtained as a function of different concentrations of citral and cinnamaldehyde. Vertical bars represent the standard deviation from the mean. (▲) Observed averages and (-) estimated averages.

EOs and EOCs are among the new alternatives for the development of sanitizers to control microbial biofilms in food industry, representing a promising line of research in increasing expansion. Oliveira *et al.* (2012b) found that EOs of *Cinnamomum cassia* bark and *Cymbopogon flexuosus* leaves, which have cinnamaldehyde and citral, respectively, as major compounds, were effective against planktonic cells of LM ATCC 19117, as well as against sessile cells adhered to the wells of polystyrene microplates. Oliveira *et al.* (2010b) found that the EO of *Cymbopogon citratus* is a new alternative for the sanitizing of industrial stainless steel surfaces contaminated with LM. Recently, Upadhyay *et al.* (2013) demonstrated that bactericidal concentrations of trans-cinnamaldehyde, carvacrol, thymol and eugenol inactivate mature biofilm of LM formed of stainless steel. Similarly, Oliveira *et al.* (2012a) found that the EO of

C. cassia and cinnamaldehyde may be effective alternatives to replace commercial chemicals sanitizers used for the reduction or elimination of sessile bacterial cells of LM present on stainless steel surfaces. However, no other studies that evaluated the antilisterial effect of sanitizing solutions containing citral against biofilms formed on stainless steel were observed, being the first time that this biological activity was noted.

In both phases of the study, testing with planktonic cells (Table 1) and with sessile cells (Figures 1, 2 and 3), there were differences in sensitivity to antibacterials between the three strains of LM used. For citral and cinnamaldehyde, LM ATCC 15313 was more sensitive than the other strains when in planktonic state (Table 1), a fact that was repeated when rated the antilisterial effect on their sessile cells (Figure 3). This demonstrates the necessity of using different strains of the same species for decisions regarding the biological activity of a compound. For Aureli *et al*. (1992), the antibacterial activity of 32 essential oils against four strains of LM and one strain of *L. innocua* was strain dependent. However, for Upadhyay *et al*. (2013), the antibiofilm effect of trans-cinnamaldehyde, carvacrol and thymol was not significantly different ($p>0.05$) between the three strains of LM studied (ATCC 19115, Scott A and Presque-598).

Observing MBCs (Table 1) and MBCBs (Figures 1, 2 and 3) values, it was found that the sessile cells of the LM strains used were more resistant than the planktonic ones. This fact ranged from 4 times more resistant for the ATCC 19117, when both compounds were used, to 10 times more resistant for the ATCC 7644, when was used citral. Several factors have been cited as responsible for resistance of biofilms to antimicrobial agents. Bacteria on biofilms, particularly those found on the innermost layers, present reduced metabolic and growths rates, and are less susceptible to antimicrobials,the high population density of biofilm also appears to be involved in the increased

resistance, the matrix of extracellular polymers acts as an adsorbent, reducing the amount of antimicrobial agent available to interact with the biofilm cells. In addition, the matrix of extracellular polymeric substances could physically reduce the penetration of the antimicrobial agent or cause its inactivation, and cells on the biofilm are physiologically distinct from planktonic cells and express specific protection factors (Vidal *et al*., 1997; Gilbert *et al*., 2002). Thus, it is often necessary to use efficient sanitizers in industrial surfaces, aiming at that mature biofilms, microbial communities with a high degree of resistance, are not formed.

Citral and cinnamaldehyde are Generally Recognized as Safe (GRAS),and its use is approved in food industry, usually as flavoring agents (Ress *et al*., 2003; Johny *et al*., 2010), which demonstrates the potential of using the same as sanitizers, being safe for operators and the environment, characterizing, according Botticella *et al.* (2013), an eco-friendly sanitizing alternative. According to Oliveira *et al*. (2012a), in a general way, the use of solutions containing EOs or their constituents to sanitize surfaces will not significantly alter the usual routine of hygiene within the food industry. Parameters such as contact time and temperature must be adjusted so that the antibacterial agent (EO or constituent) is applied at the lowest possible concentration. Low and effective concentrations of EO or their constituents will be easily removed after the hygiene procedure and will not cause residual odor problems. A final rinse under pressure with water containing a surfactant compound and using temperatures between 40 and 50 °C may be sufficient for the removal of EO or their constituents, but more studies should be done under industrial conditions to support this fact.

The antimicrobial activity of EOs has been proven in various studies (Burt, 2004; Bakkali *et al*., 2008.). However, the use of its constituents to elaborate sanitizers is a new and more promising alternative. Major EOs

compounds concentration can vary according to the chemotype, effect of seasons and/or geographical location, which makes the chemical composition variable and hinders the diffusion of the use of these as antibacterial. On the other hand, nowadays, many EOCs are synthesized or isolated and commercially available, making the concentration of the same in a sanitizer solution standardized, allowing the use with greater safety.

Conclusion

All tested compounds showed antibacterial activity against LM; cinnamaldehyde and citral showed the better effects, with satisfactory results at concentrations above 0.12% (v/v) against the planktonic cells of all strains used. With respect to the antibacterial effect of citral and cinnamaldehyde against sessile cells of LM, based on the results of the three strains tested, concentrations of 1.2% (v/v) to citral and 1.1% (v/v) to cinnamaldehyde proved to be effective for bacterial cells inactivation and may be new methods to control biofilms formed by LM in food industries.

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(VERSÃO PRELIMINAR)

ARTIGO 2 **Sensibility of *Listeria monocytogenes* biofilms after formation with frequente exposure to sublethal concentrations of essential oils constituents**

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Sensibility of Listeria monocytogenes biofilms after formation with frequente exposure to sublethal concentrations of essential oils constituents

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Abstract

Essential oils (EOs) and their constituents have been studied as new alternatives for biofilms control, especially against pathogens of great risk to public health, such as *Listeria monocytogenes* (LM). However, little is known about the effect of the frequent use of sublethal concentrations of the same in the sensitivity of microorganisms in biofilms. This study evaluated the effect of biofilms exposure, during their formation, to sublethal concentrations of cinnamaldehyde (CIN) and citral (CIT). The strains ATCC 19117, 15313 and 7644 were used. The biofilms were formed under stainless steel coupons in contact with Tryptone Soy Broth supplemented with 0.6% (w/v) yeast extract (YE-TSB) and incubated at 37 °C. Every 48 hours, the biofilms were treated with solutions containing sublethal concentrations (1/3 of lethal concentration) of CIN and CIT for 15 minutes, and a control solution (without antibacterial agents). For each strain,

three types of biofilms were developed: CB (control biofilm), BECIN (biofilm exposed to sublethal concentrations of CIN during its formation) and BECIT (biofilm exposed to sublethal concentrations of CIT during its formation). At the end of 240 hours (10 days), the number of viable cells was determined immediately after treatment with the solutions, the log-reduction was calculated. Analyses were also conducted to determine possible cross-resistance occurrence, as well as to evaluate the biofilms formation. Was no difference between the strains ability of biofilm formation, as well as between the counting of the same during the study period. Observing the number of sessile cells after 240 hours was found that exposure to sublethal concentrations of CIN and CIT affect biofilm formation. BECIN (ATCC 19117) became more resistant to treatment with CIT and CIN after 240 hours; for the ATCC 7644 strain, in a general way, biofilms have become more sensitive to CIN and CIT; whereas for ATCC 15313, BECIN has become more sensitive to the CIN and CIT BECIT and more sensitive to CIT. Exposure to sublethal concentrations of CIN and CIT can affect the biofilm LM, both can make it much more resistant to these compounds as well as more sensitive, cross-way or not, with the occurrence of these effects and EO compound dependent. Exposure to sublethal concentrations of CIN and CIT can affect LM biofilm, both can make it much more resistant to these compounds as well as more sensitive, in a cross-way or not, and the occurrence of these effects is EO and EO compound- dependent. The use of EOs constituents as sanitizers must be done cautiously, since improper use of the same, as regards the concentration, may cause bacterial resistance to antimicrobials used or even cross-resistance.

Keywords: Natural antibacterials, citral, cinnamaldehyde, bacterial resistance.

Highlights

- *Listeria monocytogenes* biofilms were exposed to sublethal concentrations of antimicrobials.
- Exposure to sublethal concentrations of cinnamaldehyde and citral affected biofilms formation.
- Exposure to cinnamaldehyde sublethal concentrations increased the ATCC 19117 biofilms resistance.
- Increased sensitivity was observed in strains ATCC 15313 and 7644 biofilms.
- Essential oils constituents should be used carefully to control biofilms.

1 Introduction

Listeria monocytogenes (LM) is an important foodborne pathogen that is the causative agent of listeriosis, a severe disease with high hospitalization and case fatality rates (Gandhi and Chikindas, 2007; Faezi-Ghasemi and Samaneh Kazemi, 2014). It plays a significant role in food safety control due to its wide distribution in nature and its capacity to survive and grow on the food products in spite of exposure to stressful conditions associated with food processing and preparation (Lou and Yousef, 1997). LM isolates have been described as 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).

Microorganisms can exist in the environment either as planktonic cells or as communities in biofilms, where they are attached to a surface and enclosed in a matrix predominantly made up of polysaccharide material (Gandhi and Chikindas, 2007). With regard specifically to the food industry, the biofilms

formation in processing plants highlights as a significant problem. When contamination of food occurs, microbial biofilms are often major contributors. This fact is even more alarming since a large number of studies have indicated the persistence of food-borne pathogens on surfaces that come in contact with food, which affects the safety of food products (Simões et al., 2010). LM biofilms has proven more resistant to stress and sanitizing agents than planktonic cells and this makes their elimination from food processing facilities a big challenge (Vatanyoopaisarn et al., 2000; Mah and O'Toole, 2001; Lewis, 2001; Holah et al., 2002; Chavant et al., 2004;).

To ensure consumer safety, it is essential for the food industry to control microorganisms both in food products and on food-processing equipment (Lelieveld, 1985; Pontefract, 1991). An effective sanitizing program is necessary to neutralize microorganisms that may contaminate food and lead to spoilage. Typically, chemical sanitizers are used in the sanification process and are applied to food-contact and non food-contact surfaces in food-processing plants, food service kitchens, day-care centers, and formula preparation areas in hospitals (Marriott and Gravani, 2006). However, given the great need to control these microbial communities, studies on alternative sanitizing agents have emerged (Oliveira et al., 2010a). Beyond this fact, the emergence of bacteria resistant to sanitization conventional processes clearly demonstrates that new control strategies are needed (Simões et al., 2010). Growing negative consumer perception against synthetic compounds has led to the development of natural alternatives (Davidson, 1997; Roller, 1995). Promising and natural alternatives, for the chemical sanitizers commonly used, are essential oils (EOs) and their constituents (Oliveira et al., 2012a).

EOs are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. As typical lipophiles, they pass through the bacterial cell wall and cytoplasmic membrane,

disrupt the structure different layers of polysaccharides, fatty acids and phospholipids and permeabilize them (Bakkali et al., 2008).

Many studies have been done in order to assess the potential for sanitizing of EOs and their constituents (Oliveira et al., 2010a, 2012a, 2012b; Desai et al., 2012; Valeriano et al., 2012; Millezi et al., 2012, 2013; Jadhav et al., 2013; Szczepanski and Lipski, 2014); however is extremely scarce the informations about a possible acquisition of resistance after a period of continuous treatment with sublethal concentrations of these natural antimicrobials. According to McDonnell and Russell (1999), the mishandling of the sanitizers regarding the concentration, time and temperature may lead to the regular exposure of the contaminants to sub-lethal conditions and consequently to the emergence of persistent strains that are difficult to eradicate. Aase et al. (2000) mentioned that inadequate sanification process may produce resistance to the sanitizer as a result of selection or adaptation through regular exposure to sublethal concentrations. Lourenço et al. (2009) observed that LM strains, after being progressively exposed to increased sub-inhibitory concentrations of a sanitizer agent, doubled their initial MICs for this product. Lourenço et al. (2009) also observed that some strains doubled the MICs of an agent after being pre-exposure to another; however, in some cases after the progressive exposure, the MIC value was half of the original one. Aase et al. (2000) reported that serial subcultivation LM strains that were initially sensitive or resistant to benzalkonium chloride in sublethal concentrations of the same agent resulted in enhanced and approximately equal resistance of all strains to the compound. In this study, we evaluated the effect of LM biofilm exposure to sublethal concentrations of cinnamaldehyde (CIN) and citral (CIT) during its formation on stainless steel surfaces.

2 Material and methods

2.1 Bacterial strains

Were used three LM strains: ATCC 19117 (serotype 4d), ATCC 7644 (serotype 1/2c) and ATCC 15313 (serotype 1/2a). The strains, kindly provided by the Reference Materials Laboratory of the National Institute of Quality Control in Health of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), were kept frozen at -20 °C in Tryptone Soy Broth supplemented with 0.6% (w/v) yeast extract (TSB-YE) and added 20% glycerol.

2.2 Standardization and inoculum preparation

For reactivation, an aliquot (10 µL) of the stock cultures was transferred to test tubes containing TSB-YE and two consecutive subcultures were performed with incubation at 37 °C/24 hours. The cultures were striated on Tryptone Soy Agar supplemented with 0.6% (w/v) yeast extract (TSA-YE) and incubated at 37 °C/24 hours. An aliquot of each culture was taken and transferred to 150 mL of TSB-YE, which was incubated at 37 °C until the desired cell density, based on standard growth curves previously performed at $OD_{620\ nm}$. The strains were cultured and standardized individually. The absorbances used to obtain the concentration of about 9 Log CFU/mL were: 0.895 for the ATCC 19117, 0.590 for the ATCC 7644 and 0.670 for the ATCC 1531.

2.3 Antimicrobial agents

As antimicrobial agents were used CIN and CIT, both acquired from Sigma Aldrich.

2.4 Biofilms formation and exposure to sublethal concentrations of CIN and CIT

For the biofilms formation, coupons (1x10x20 mm) of AISI 304 (#4) stainless steel were used, these being hygienized and sterilized according to Oliveira *et al.* (2010a). The coupons were placed in test tubes (16x125 mm) and 5 mL of TSB-YE 8 Log CFU/mL were added. The tubes were incubated at 37 °C under static conditions. Every 48 hours the coupons were removed, washed three times in 0.1% peptone water (w/v) to remove non-adherent cells and transferred to test tubes containing 5 mL of a solution containing sublethal concentration of an antimicrobial agent (CIN or CIT) for 15 minutes. The sublethal concentrations accounted for one third of the lethal concentrations were obtained in a previous study (data not shown). The compositions of the solutions are shown in Table 1.

Thus, for each strain, three types of biofilms were grown: CB (control biofilm, not exposed to sublethal concentrations of antimicrobial agents, only being exposed to a solution containing only distilled water and Tween 80 during its formation), BECIN (biofilm exposed to CIN sublethal concentrations during its formation) and BECIT (biofilm exposed to CIT sublethal concentrations during its formation).

Table 1. Composition of solutions used for treatment of stainless steel coupons containing *Listeria monocytogenes* biofilms.

Solutions	Composition		
	Distilled water with 0.5% Tween 80 (% v/v)	**Cinnamaldehyde (% v/v)**	**Citral (% v/v)**
CS	100.00	0.00	0.00
CINS 1	99.84	0.16	0.00
CINS 2	99.64	0.36	0.00
CINS 3	99.86	0.14	0.00
CITS 1	99.84	0.00	0.16
CITS 2	99.60	0.00	0.40
CITS 3	99.86	0.00	0.14

CS (control solution, without essential oil constituents). CINS 1 (solution containing sublethal concentration of cinnamaldehyde, prepared for the ATCC 19117 strain). CINS 2 (solution containing sublethal concentration of cinnamaldehyde, prepared for the ATCC 7644 strain). CINS 3 (solution containing sublethal concentration of cinnamaldehyde, prepared for the ATCC 15313 strain). CITS 1 (solution containing sublethal concentration of citral, prepared for the ATCC 19117 strain). CITS 2 (solution containing sublethal concentration of citral, prepared for the ATCC 7644 strain). CITS 3 (solution containing sublethal concentration of citral, prepared for the ATCC 15313 strain).

2.5 Evaluation of the effect of exposure to CIN and CIT sublethal concentrations

After 240 hours (10 days), the coupons were removed, washed three times with peptone water and transferred to test tubes containing 5 ml of sanitizing solutions according to the strain used, as shown in Table 1, for 15

minutes. Subsequently, the coupons were rinsed 3 times for sanitizers removal and transferred to conical bottom plastic tubes of 50 mL capacity containing 10 mL of peptone water for remaining cells removal and quantification. The tubes were vortexed at maximum speed for 2 minutes. After stirring, serial dilution was done with plating on TSA-YE and 37 °C for 24 hours incubation. The results were expressed as log-reduction obtained by the difference between the log of the initial cell count (biofilm) and the log of the surviving cells after the antibacterial treatment and the control treatment.

Even after 240 hours, the biofilms treatment was performed with sublethal doses for further evaluation of the sensitivity thereof. This was done because previous studies (data not shown) demonstrated that it was not interesting to use the lethal concentration of CIN and CIT since these solutions would cause the total elimination of viable cells quantified by plate count in biofilms that have become more sensitive, which unfeasible a satisfactory comparison of the solutions used effects.

2.6 Assessment of cross-resistance

To evaluate a possible cross-resistance after 240 hours (10 days) of biofilms formation, coupons treated with CIN sublethal doses were treated with solutions containing CIT and the coupons treated with sublethal CIT doses were treated with solutions containing CIN. The biofilm cell count and the log-reduction calculation were performed as described above.

2.7 Evaluation of biofilms development

To evaluate the biofilms development of the LM strains, every 48 hours three coupons not subjected to any treatment were removed, washed in peptone water three times to remove non-adherent cells and transferred to a 50 mL plastic tubes containing 10 mL of peptone water.

For removal of cells and quantification tubes were vortexed at maximum speed for 2 minutes. After stirring, serial dilution and plating was done on TSA-YE and incubated at 37 °C for 24 hours. The results were expressed as log CFU/cm².

2.8 Statistical analyzes

In the assessing of biofilms development was made a completely randomized factorial 3 x 5 (strains x formation times) in three repetitions. The results were subjected to variance analysis, regression analysis and mean test (Skott-Knott).

To study the behavior of cells after sublethal exposure, for each tested strain was made a completely randomized factorial 3 x 3 (sanitizing solutions x formation conditions) in three repetitions. The results were submitted to variance analysis and mean test (Skott-Knott).

Statistical analyzes were performed using Sisvar 5.3program.

3 Results and discussion

3.1 Formation of biofilms for 240 hours (10 days)

LM strains used were able to adhere to the stainless steel coupons surface and form biofilms. The biofilms development was evaluated for 240 hours. It was found that there was significant difference between the ability of biofilm formation of the three strains used, as well as between the counting of the same during the study period ($p<0.05$) (Figure 1).

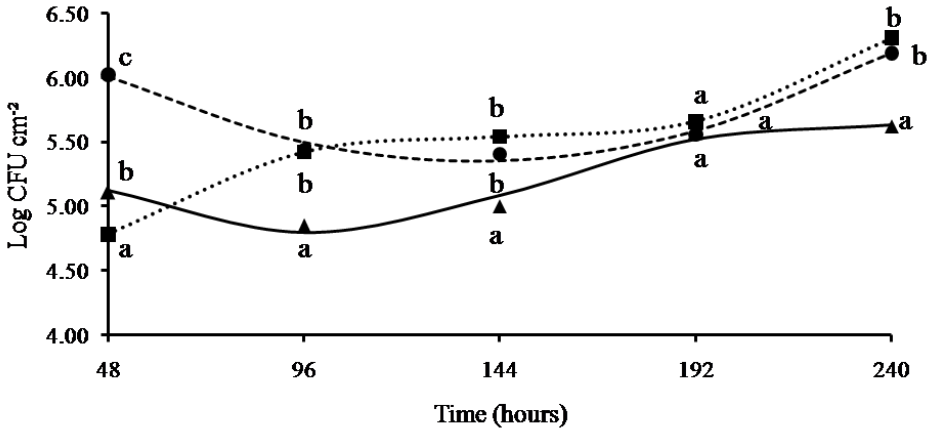

Figure 1. Biofilm formation on stainless steel surfaces by strains of *Listeria monocytogenes*. Within each step of biofilm formation, the same letters do not differ by Skott-Knott test at 5% probability. (▲) Observed averages for the ATCC 19117 strains, (-) estimated average for the ATCC 19117 strain, (■) observed average for the ATCC 7644 strain, (....) estimated average for the ATCC 7644 strain, (●) observed averages for the ATCC 15313 strain, and (----) estimated averages for the ATCC 15313 strain.

Numerous studies demonstrated the ability of LM to adhere to surfaces and form biofilms (Adrião et al., 2008; Rieu et al., 2008; Somers and Wong, 2004; Takahashi et al., 2010), and some have shown that LM strains vary in their ability to adhere to surfaces and form biofilms (Chae and Schraft, 2000; Kalmokoff et al., 2001; Borucki et al., 2003). Chae and Schraft (2000), for example, grew thirteen LM strains on glass surfaces and observed that there was a significant difference in the strains adherence. The research also showed that

biofilm growth for 24 h presented significant differences in cell numbers among LM strains.

In a general way, the number of cells in LM strains biofilms increased between the initial stage (48 hours) and the final stage (240 hours) (Figure 1). The biofilm formation is complex and involves many events, which can be summarized in the following stages: conditioning film formation, initial microbial adhesion, microcolonies formation, adhesion of secondary colonizers and mature biofilm formation with high population density and production of extracellular polymers (Malone & Caldwell, 1983; Kumar & Anand, 1998; Christensen & Characklis, 1990; Watnick & Kolter, 2000). According to Oliveira et al. (2010b), in all these stages of biofilm formation sessile cells can break off and contaminate the substrate that flowing through the site, which shows the risk that these microbial communities to the quality of food and consumer safety.

3.2 Effect of biofilms exposure to sublethal concentrations of CIN and CIT and cross-resistance

After 240 hours (10 days) sessile cell count was determined in the LM biofilms which were often exposed to sublethal concentrations of CIN and CIT as well as in CBs (not exposed to any antimicrobial). The mean CBs count was higher than the counts obtained in biofilms exposed to sublethal concentrations of CIT and CIN ($p<0.05$). In turn, the average BECINs count was higher than the ones of BECITs ($p<0.05$). Among the strains, it was found that the average count of LM ATCC 19117 biofilm was lower than the other ones ($p<0.05$) (Table 2).

Table 2. *Listeria monocytogenes* cells count in the biofilms formed during 240 hours (10 days) exposed or not to cinnamaldehyde and citral sublethal concentrations.

Formation conditions	*Listeria monocytogenes* counts (Log CFU cm^{-2})			Averages
	ATCC 19117	ATCC 7644	ATCC 15313	
CB	5.62±0.18	6.31±0.24	6.19±0.16	6.04±0.36a
BECIN	5.56±0.13	5.73±0.53	5.80±0.07	5.70±0.29b
BECIT	5.25±0.11	5.37±0.05	5.42±0.39	5.35±0.22c
Averages	5.48±0.21A	5.80±0.50B	5.80±0.40B	

Results expressed as mean±the standard deviation. Same lowercase letters in the same column and the same capital letters on the same line do not differ by the Scott-Knott test at 5% probability. CB (control biofilm, not exposed to sublethal concentrations of antimicrobial agents, only being exposed to a solution containing only distilled water and Tween 80 during its formation), BECIN (biofilm exposed to CIN sublethal concentrations during its formation) and BECIT (biofilm exposed to CIT sublethal concentrations during its formation).

According to the results of Table 2, it was observed that frequent exposure to sublethal concentrations of CIN or CIT adversely affect biofilm formation by LM strains studied. However, despite the apparently interesting industrial point of view, this should be seen in a careful way, as a possible acquisition of resistance by the microorganisms in question should always be taken into consideration. Thus, after 240 hours, the biofilms were exposed to solutions containing sublethal concentrations of the antimicrobials and a control solution, the number of viable cells was determined and log-reduction was calculated. The results (Tables 3 to 5) indicated that the exposure to sublethal concentrations of CIT and CIN during the biofilms formation resulted in effects that vary according to the strain concerned, making LM biofilms more resistant

or sensitive, sometimes with cross-resistance or cross-sensitivity.

Table 3 shows log-reduction values of the biofilms formed by the ATCC 19117 strain after 240 hours obtained after treatment with solutions containing sublethal concentrations and control solution. When observing the effects obtained in each condition of biofilm formation, it was found that BECIN became more resistant to treatment with sanitizing solutions containing CIT and CIN and after 240 hours with lower values of log-reductions after exposure to sanitizing solutions containing these antimicrobials when compared to the control solution (without antimicrobials) exposure ($p<0.05$). Furthermore, with regard to the effects obtained by taking into consideration the type of solution applied, it was found that BECIN was the biofilm formation condition more resistant to CITS, followed by CB and BECIT ($p<0.05$). BECIN and BECIT were more sensitive to CS when compared to CB ($p<0.05$). BECIT was more sensitive to CIN solution than CB and BECIN ($p<0.05$).

Table 3. Log-reduction values of *Listeria monocytogenes* biofilms formed by ATCC 19117 strain after 240 hours (10 days) obtained after treatment with sublethal concentrations of solutions containing cinnamaldehyde and citral and a control solution.

Solutions	**Formation conditions**					
	CB		**BECIN**		**BECIT**	
	Log-reduction	**%**	**Log-reduction**	**%**	**Log-reduction**	**%**
CINS	0.44±0.16aA	7.83	0.40±0.18aA	7.19	1.32±0.44aB	25.14
CS	0.67±0.33aA	11.92	1.36±0.52bB	24.46	1.24±0.52aB	23.62
CITS	0.96±0.34aB	17.08	0.32±0.07aA	5.76	1.72±0.32aC	32,76

SC (control solution without essential oils constituents). CINS (solution containing sublethal concentrations of cinnamaldehyde). CITS (solution containing sublethal concentration of citral). CB (control biofilm, not exposed to sublethal concentrations of antimicrobial agents, only being exposed to a solution containing only distilled water and Tween 80 during its formation), BECIN (biofilm exposed to CIN sublethal concentrations during its formation) and BECIT (biofilm exposed to CIT sublethal concentrations during its formation). The log-reduction was the difference between the log of initial cells counts (Table 2) and the log of survival cells following antibacterial treatments. % (log-reductions x 100)/viable cell count of untreated coupons. Same lowercase letters in the same column and the same capital letters on the same line do not differ by the Scott-Knott test at 5% probability.

In this study, were used solutions containing CIN and CIT, constituents of EOs classified as Generally Recognized as Safe (GRAS); their use has been adopted in food industry, usually as flavoring agents (Ress et al., 2003; Johny et al., 2010). CIN (3-fenil-2-propenal), also known as cinnamic aldehyde, is the major component of cinnamon bark species essential oils, such as China cinnamon (*Cinnamomum cassia*). Ooi et al. (2006), for example, found 85.06% trans-cinnamaldehyde in the bark essential oil of *C. cassia*. CIT (3,7-dimethyl-2,6-octadienal) is the major constituent of the essential oil extracted from the leaves of some species of *Cymbopogon*, such as *C. citratus* (lemongrass); present at levels of approximately 65-85%. In fact, CIT is the name given to a mixture of two isomeric acyclic monoterpene aldehydes: geranial (trans-citral or citral A) and neral (cis-citral, citral B) (Saddiq; Khayyat, 2010). The antimicrobial effect of both has been shown in other studies (Moleyar and Narasimham, 1992; Lis-Balchin and Deans, 1997; Friedman et al, 2002), some of them using biofilms (Oliveira et al, 2012a; Upadhyay et al. 2013).

It was observed that after exposure to CIN sublethal concentrations the ATCC 19117 biofilm has become more resistant to this antimicrobial (CIN) and acquired cross-resistance to CIT (Table 3). According to Luz te al. (2012a), the literature regarding the assessment of tolerance development by LM when exposed to sublethal amounts of EOs or their compounds is still limited, and most of the past studies focused on the development of direct resistance and cross resistance by LM have involved assays in which bacteria were exposed to classical chemical and physical food preservative procedures (Skandamis et al., 2009; Soni et al., 2011). Even less is known about the exposure of bacterial biofilms to sublethal concentrations of sanitizing solutions containing EOs or their constituents. In a study by Sandasi et al. (2008), the effect of five EOs compounds on biofilms were investigated on two pathogenic LM isolates and treatment of a 6 h preformed biofilm with each of the EOs compounds at a

concentration of 1 mg/ml enhanced growth of the biofilm.

Using disinfectants at concentrations recommended for the food industry LM will be completely inactivated; however different factors can significantly reduce the efficiency of disinfectants (Best et al., 1990; Pan, Breidt, & Kathariou, 2006; Saa Ibusquiza, Herrera, & Cabo, 2011). Inadequate cleaning and disinfection procedures like insufficient cleaning before disinfection, disinfection of wet surfaces and dosage failure in food processing plants can expose bacteria regularly to sublethal concentration of disinfectants (Rajkovic et al., 2009; Rakic-Martinez, Drevets, Dutta, Katic, & Kathariou, 2011). Particularly hard-to-reach places, such as complex processing machines of poor hygienic design, have been suggested to create good conditions for adherence and adaptive responses of LM (Lundén et al., 2002). In this study, frequent exposure to sublethal concentrations of antimicrobial agents during the LM biofilms formation aimed to simulate a improper use (at concentrations below those needed) of sanitizing solutions containing the constituents of EOs CIN and CIT. According to Langsrud et al. (2003), residual sublethal concentrations of the disinfectants in the environment could lead to a selective pressure for retention or acquisition of resistance genes among microorganisms, or to adaptation of initially sensitive bacteria. Many microorganisms are able to develop adaptive response to sublethal stress, allowing them to tolerate and survive subsequent exposure to lethal levels of the same stress or even a different type of stress (ROLLER, 2003). Adaptation to a disinfectant may lead to crossadaptation to other disinfectants, enhancing the survival of the bacteria (Jones et al., 1989; Gandhi et al., 1993). But, the regular exposure to sublethal concentration of disinfectants) might not only lead to resistance to the used disinfectants, but also to a resistance to a range of other antimicrobial compounds including antibiotics (Rajkovic et al., 2009; Rakic-Martinez, Drevets, Dutta, Katic, & Kathariou, 2011). Combined resistance to disinfectants

and other types of antibacterial agents may also become a challenge to the food processing industries. Cross-resistance between disinfectants and antibiotics in food pathogens could lead to serious consequences for the public health (Langsrud et al., 2003). Exposure of LM to sublethal concentrations of disinfectants supports the survival and growth of intracellular bacteria, underlining the importance of proper disinfection in food processing environment to limit the number of LM foodborne illnesses (Pricope et al., 2013). In addition to these risks, the development of reduced susceptibility in bacterial populations increases the probability of subsequent failure sanitization, resulting in serious problems in industrial food preparation (Chapman et al. 2003).

The biggest adaptive response of the cells is to maintain the fluidity of the membrane at a constant value, independent of the environmental conditions at the time. This stabilization of membrane fluidity is the predominant response of bacteria to substances that act on membranes or changes in environmental conditions (HEIPIEPER; MEINHARDT; SAFE, 2003), preventing the loss of the chemical and mechanical properties of the lipid bilayer (RUSSELL; FUKANAGA, 1990); if the disruption of membrane integrity occurs, then its functions as a barrier, site of enzymatic activity and local energy production will be compromised (HEIPIEPER; MEINHARDT; SAFE, 2003). There are many antibacterial agents which have as target the bacterial cytoplasmic membrane. The increase in membrane fluidity appears to be among the first antimicrobial effects caused by treatment with EOs. The expansion and increased fluidity of the plasma membrane can lead to breakage with consequent loss of the integrity of small intracellular components, such as hydrogen, sodium and potassium. High concentrations of EOs or long exposure times may lead to greater damage to the cytoplasmic membrane, causing loss of macromolecules such as DNA and proteins, a factor closely related to cell death (HAMMER; CARSON, 2011). Di

Pasqua et al. (2006) observed changes in the composition of fatty acids in the bacterial cell membrane as a mechanism of adaptation of cells in response to sublethal concentrations of thymol, carvacrol, limonene, cinnamaldehyde and eugenol.

Situations to acquire greater sensitivity to antimicrobials after exposure to sublethal doses of these were also observed in this study. Table 4 shows the log-reduction values of the biofilms formed by ATCC 7644 strain obtained after 240 hours after treatment with solutions containing sublethal concentrations and a control solution. It was found that, in general, biofilms have become more sensitive solutions with CIT and CIN ($p<0.05$), with higher values of log-reduction for the same when compared to the control treatment effect (no antimicrobial).

Table 4. Log-reduction values of *Listeria monocytogenes* biofilms formed by ATCC 7644 strain after 240 hours (10 days) obtained after treatment with sublethal concentrations of solutions containing cinnamaldehyde and citral and a control solution.

Solutions	**Formation conditions**						**Averages**
	CB		**BECIN**		**BECIT**		
	Log-reduction	**%**	**Log-reduction**	**%**	**Log-reduction**	**%**	
CS	0.68±0.25	10.78	0.19±0.08	3.32	1.22±0.39	22.72	0.70±0.50a
CITS	2.21±0.18	35.02	2.77±1.50	48.34	3.03±0.41	56.42	2.67±0.86b
CINS	3.16±0.44	50.07	2.86±1.36	49.91	2.90±1.03	54.00	2.97±0.89b

SC (control solution without essential oils constituents). CINS (solution containing sublethal concentrations of cinnamaldehyde). CITS (solution containing sublethal concentration of citral). CB (control biofilm, not exposed to sublethal concentrations of antimicrobial agents, only being exposed to a solution containing only distilled water and Tween 80 during its formation), BECIN (biofilm exposed to CIN sublethal concentrations during its formation) and BECIT (biofilm exposed to CIT sublethal concentrations during its formation). The log-reduction was the difference between the log of initial cells counts (Table 2) and the log of survival cells following antibacterial treatments. % (log-reductions x 100)/viable cell count of untreated coupons. Same lowercase letters in the same column do not differ by the Scott-Knott test at 5% probability.

Table 5 shows the log-reduction values of biofilms formed by ATCC 15313 strain obtained after 240 hours after treatment with solutions containing sublethal concentrations and control solution. When observing the effects obtained in each condition of biofilm formation, it was found that BECIN became more sensitive to treatment with CIN and sanitizing solutions containing CIT after 240 hours (10 days), with higher values of log-reduction after exposure the sanitizing solutions containing these antimicrobial agents when compared to the control solution (without antimicrobials) exposure ($p<0.05$).

This result indicates, in addition to a reduction in resistance, increased cross-sensitivity to CIT caused by exposure to sublethal concentrations of CIN. BECIT became more sensitive to treatment with the CIT sanitizing solution after 240 hours ($p<0.05$). Concerns the effects obtained by taking into consideration the type of solution applied, it was found that BECIT was the condition of biofilm formation that was more resistant to CINS, followed by BECIN and CB ($p<0,05$).

Table 5. Log-reduction values of *Listeria monocytogenes* biofilms formed by ATCC 15313 strain after 240 hours (10 days) obtained after treatment with sublethal concentrations of solutions containing cinnamaldehyde and citral and a control solution.

Solutions	**Formation conditios**					
	CB		**BECIN**		**BECIT**	
	Log-reduction	**%**	**Log-reduction**	**%**	**Log-reduction**	**%**
CITS	0.64±0.36aA	10.34	0,50±0.26bA	8.62	0,64±0.05bA	11.80
CS	0.88±0.06aA	14.22	0,14±0.07aB	2.41	0,37±0.34aB	6.83
CINS	0,93±0.07aA	15.02	0,55±0.10bB	9.48	0,18±0.09aC	3.32

SC (control solution without essential oils constituents). CINS (solution containing sublethal concentrations of cinnamaldehyde). CITS (solution containing sublethal concentration of citral). CB (control biofilm, not exposed to sublethal concentrations of antimicrobial agents, only being exposed to a solution containing only distilled water and Tween 80 during its formation), BECIN (biofilm exposed to CIN sublethal concentrations during its formation) and BECIT (biofilm exposed to CIT sublethal concentrations during its formation). The log-reduction was the difference between the log of initial cells counts (Table 2) and the log of survival cells following antibacterial treatments. % (log-reductions x 100)/viable cell count of untreated coupons. Same lowercase letters in the same column and the same capital letters on the same line do not differ by the Scott-Knott test at 5% probability.

Similar results to those observed for ATCC 7644 (Table 4) and ATCC 15313 (Table 5) strains, as regards the non-alteration of the microbial susceptibility upon exposure to sublethal concentrations of antimicrobials were obtained in other studies. Gomes Neto et al. (2012) verified that the overnight exposure of *Staphylococcus aureus* to sublethal amounts of both *Rosmarinus officinalis* EO or 1,8-cineole in meat broth did not result in direct or cross protection. Cells progressively subcultured (24-h cycles) in meat broth with increasing amounts of EO or 1,8-cineole showed no increased direct tolerance. According to Luz et al. (2012a), *Origanum vulgare* EO and carvacrol have little effect on the acquisition of direct resistance or cross resistance by LM ATCC 7644. Luz et al. (2012b) observed that the overnight exposure of *Salmonella enterica* serovar Typhimurium to sublethal amounts of *O. vulgare* EO and carvacrol did not result in direct and cross-bacterial protection; the study revealed few significant changes in bacterial susceptibility.

By comparing the results obtained for different strains of LM used (Tables 3, 4 and 5), it appears that the sensitivity of LM after exposure to sublethal doses of CIN and CIT was strain and EO compound dependent. This demonstrates the risk that the decision based on the analysis of only one strain or just an EO constituint can lead.

Conclusions

Exposure to sublethal concentrations of CIN and CIT can affect the biofilm of LM, which can make it much more resistant to these compounds as well as more sensitive, cross-way or not, with the occurrence of these effects strain and EO compound dependent. The use of EOs constituents as sanitizers must be performed very carefully, since improper usage can cause bacterial resistance. The results are important in a new line of research to be followed; further studies to elucidate the mechanisms responsible for increased bacterial

resistance or susceptibility should be performed, as well as trials involving persistent strains or not isolated directly from the food industry.

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