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MICHELLE PIOVEZAN

EFEITO DO ÓLEO ESSENCIAL DE CANELA E CINAMALDEÍDO EM BIOFILME DE *SALMONELLA* SAINTPAUL EM AÇO INOXIDÁVEL

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências da Saúde.
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O homem é do tamanho do seu sonho.

(FERNANDO PESSOA)

Efeito do óleo essencial de canela e cinamaldeído em biofilme de *Salmonella* Saintpaul em aço inoxidável

RESUMO

A formação de biofilmes bacterianos nas superfícies tem importantes consequências para a saúde e para a indústria alimentar, podendo ser fontes de contaminação levando a deterioração de alimentos e transmissão de doenças. Este estudo avaliou o efeito do óleo essencial de canela (OE) e cinamaldeído contra biofilmes de *Salmonella enterica* sorotipo Saintpaul em superfície de aço inoxidável. Os efeitos do óleo essencial de canela e cinamaldeído em biofilmes foram avaliados em microplacas de 96 poços utilizando a coloração de *MTT* (brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil) tetrazólio). Ensaios de contagem de Unidades Formadoras de Colônias (UFC) e microscopia eletrônica de varredura (MEV) foram utilizados para avaliar o efeito dos compostos em biofilmes de *S.* Saintpaul em aço inoxidável. Os resultados do ensaio com *MTT* demonstraram que o óleo essencial de canela e cinamaldeído nas concentrações de 312 µg/mL e 624 µg/mL reduziram aproximadamente 50% dos biofilmes de *S.* Saintpaul em microplaca de poliestireno. A maior redução das contagens bacterianas na superfície de aço inoxidável foi obervada com a adição de óleo essencial de canela nas concentrações de 156 µg/mL e 234 µg/mL durante a formação do biofilme. O óleo essencial de canela e cinamaldeído reduziram o número de células bacterianas no aço inoxidável, tornado-os potenciais compostos no controle de *Salmonella* spp.

Palavras-chave: *Salmonella* spp; adesão bacteriana; sanitizantes naturais; atividade antimicrobiana; patógenos transmitidos por alimentos.

Effect of cinnamon essential oil and cinnamaldehyde on *Salmonella* Saintpaul biofilm on stainless steel surface

ABSTRACT

Biofilm formation on surfaces has important consequences for health and food industry, since they can be a source of food contamination leading to food spoilage and foodborne diseases. This study evaluated the effect of cinnamon essential oil (EO) and cinnamaldehyde against *Salmonella enterica* serotype Saintpaul biofilms on stainless steel surface. The effects of cinnamon EO and cinnamaldehyde on biofilms was evaluated in 96-well plates using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining. Colony forming units (CFU) counting assays and scanning electron microscopy (SEM) were used to evaluate the effect of the compounds on *S.* Saintpaul biofilms on stainless steel surface. The MTT assay results showed that the cinnamon EO and cinnamaldehyde at 312 µg/mL and 624 µg/mL respectively could decrease approximately 50% of *S.* Saintpaul biofilms in polystyrene microplate. The highest reduction of bacterial counts on stainless steel surface was observed with addition of 156 µg/mL and 234 µg/mL of cinnamon EO during biofilm formation. Cinnamon EO and cinnamaldehyde reduced the number of bacterial cells on stainless steel surface, making it a potential compound for *Salmonella* spp. control.

***Keywords*:** *Salmonella* spp; bacterial adhesion; natural sanitizers; antimicrobial activity; foodborne pathogens.

LISTA DE ILUSTRAÇÕES

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CAPÍTULO I

INTRODUÇÃO

As doenças transmitidas por alimentos representam uma importante causa de morbidade e mortalidade em todo o mundo. De acordo com a Organização Mundial da Saúde (OMS), muitos países têm documentado, ao longo das décadas, aumento significativo da incidência de doenças transmitidas por alimentos. Estima-se que a cada ano, uma em cada três pessoas de países industrializados é afetada por doenças de origem alimentar (WHO, 2013).

Foram reportados pelo *Center for Disease Control and Prevention* (CDC), no período de 2009-2010, 1.527 surtos de origem alimentar, resultando em 29.444 casos de doenças, 1.184 hospitalizações e 23 mortes, sendo que 30% dos surtos foram causados por *Salmonella* spp. (CDC, 2013a). Segundo dados da União Europeia, a cada ano são reportados mais de 100.000 casos de salmonelose e os gastos com a doença estão estimados em mais de 3 bilhões de euros por ano (EFSA, 2012). No Brasil, durante os anos de 1999 a 2008, foram notificados mais de 6.000 surtos de origem alimentar, sendo que 41,1% foram causados por bactérias e *Salmonella* spp. foi agente mais frequente (BRASIL, 2008).

Salmonella spp. está amplamente distribuída na natureza e pode contaminar uma grande variedade de alimentos de origem animal e também vegetal (CDC, 2013b). Além disso, estudos demonstram que estas bactérias são capazes de aderir e formar biofilmes em diferentes superfícies que entram em contato com alimentos. Uma vez formados, estes biofilmes podem tornar-se potenciais fontes de contaminação de produtos alimentares, levando a transmissão de doenças (SHI e ZHU, 2009).

Visto a grande ocorrência de surtos por *Salmonella* spp. e a importância dos biofilmes como fontes de contaminação, destaca-se o desafio de encontrar novas formas de controle dos micro-organismos envolvidos com a produção de biofilmes. Os óleos essenciais são fontes potenciais de compostos antimicrobianos frente a diversos micro-organismos patogênicos e vem sendo estudados em formulações de sanitizantes para aplicação em diferentes superfícies de contato com alimentos (SIMÕES et al., 2009).

SALMONELLA spp.

Salmonella spp. são bastonetes Gram negativos anaeróbios facultativos pertencentes à família *Enterobacteriaceae* (INGRAHAM e INGRAHAM, 2011). O gênero *Salmonella* é constituído pelas espécies *S. bongori* e *S. enterica*. *S. enterica* divide-se em seis subespécies: *enterica* (I), *salamae* (ll), *arizonae* (lla), *diarizonae* (Illb), *houtenae* (IV) e *indica* (VI). Mais recentemente foi identificada *Salmonella subterranea*, porém ainda não há um consenso se é uma nova espécie. De acordo com Guibourdenche et al. (2010) foram identificados mais de 2.600 sorotipos pertencentes gênero *Salmonella,* a maioria deles pertencentes à espécie *enterica.* Os sorotipos de *Salmonella* encontram-se amplamente distribuídos na natureza e são capazes de infectar tanto o homem quanto animais (STEENACKERS et al., 2012). Na infecção humana, a salmonelose se manifesta por gastroenterite, com sintomas de diarreia, dor abdominal, febre, náusea e vômito de 12 a 72 horas após a infecção. A doença geralmente dura de 4 a 7 dias e em adultos saudáveis normalmente evolui sem complicações, porém a susceptibilidade à salmonelose difere de pessoa a pessoa, sendo mais severa em crianças e idosos, podendo evoluir para infecção sistêmica (INGRAHAM e INGRAHAM, 2011; CDC, 2012).

As infecções em humanos são associadas, principalmente, a alimentos de origem animal (MUKHOPADHYAY e RAMASWAMY, 2012). No entanto, alimentos de origem vegetal como melões, mangas, mamões, pistaches, amendoins e tomates também estão relacionados com a transmissão da doença (CDC, 2013b).

Uma importante fonte de contaminação é o ambiente de processamento dos alimentos. Segundo Jun et al. (2010), os micro-organismos podem crescer rapidamente nos resíduos de alimentos que podem permanecer nos equipamentos de processamento resultando na contaminação cruzada das superfícies contaminadas para os produtos alimentares.

A contaminação de alimentos por *Salmonella* spp. tem despertado grande preocupação por parte das autoridades sanitárias em todo mundo e pela indústria alimentícia, visto que estes micro-organismos podem formar biofilmes em praticamente todos os tipos de superfícies que entram em contato com os alimentos durante o seu processamento (STEENACKERS et al., 2012).

BIOFILMES

O termo biofilme foi criado para descrever a forma séssil de vida microbiana, caracterizado pela adesão irreversível de micro-organismos a superfícies bióticas ou abióticas, com consequente produção de substâncias poliméricas extracelulares (EPS) (PRAKASH et al., 2003).

A estrutura dos biofilmes pode variar de acordo com o micro-organismo e com as condições ambientais (PRAKASH et al., 2003). Basicamente é formada de células microbianas e exopolissacarideos (EPS) cujos principais componentes são os polissacarídeos, as proteínas, os fosfolipídios, os ácidos teicóicos e os ácidos nucléicos, sendo que as proteínas e os polissacarídeos participam de 75-89% da composição dos EPS de um biofilme (SIMÕES et al., 2010; JAHID e HA, 2012). A função frequentemente atribuída ao EPS é o seu efeito de proteção dos micro-organismos do biofilme contra condições adversas.

Estas características conferem ao biofilme maior resistência ambiental e principalmente aos agentes antimicrobianos como aqueles utilizados na limpeza dos equipamentos. De acordo com Gilbert et al. (2002) um biofilme pode ser 10 a 100 vezes mais resistente do que quando suas células estão dispostas em suspensão na forma livre.

O processo de formação de biofilmes inclui uma sequência complexa de eventos, envolvendo interações físicas e químicas entre os micro-organismos e a superfície em que estão aderidos (SHI e ZHU, 2009). Resumidamente, inicia-se com a interação de células planctônicas com a superfície mediada pelas forças de Van der Walls, ligações químicas e interações hidrofóbicas. A fase seguinte caracteriza-se pela produção de moléculas de sinalização célula-célula, transporte de substratos para dentro do biofilme e formação dos apêndices celulares como flagelos, fimbrias, *pili* e exopolissacarideos. Surgem forças de ligação mais fortes como as dipolo-dipolo, pontes de hidrogênio, ligações iônicas e covalentes, alem das interações hidrofóbicas, responsáveis pela adesão irreversível que ocorre nesta etapa de formação do biofilme. Após a adesão inicial, a maturação completa se dá pelo aumento da densidade populacional e produção de EPS. Por fim, após a maturação completa, ocorre o desprendimento programado de células microbianas que é regulado fisiologicamente. (PRAKASH et al., 2003).

A contaminação cruzada de alimentos tem se tornado uma grande preocupação na indústria alimentar, sendo este um importante fator que contribui para as doenças transmitidas

por alimentos. Os biofilmes microbianos presentes nos equipamentos podem desprender-se e contaminar o alimento, tornando o seu controle um desafio (JUN et al., 2010). Outro fator importante é o notório aumento da resistência dos micro-organismos na forma de biofilmes aos desinfetantes convencionais (JAHID e HA, 2012). Diversos patógenos alimentares, como *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella* spp., *Clostridium perfriengens* e *Staphylococcus aureus* são comprovadamente produtores de biofilmes e estão relacionados a vários surtos de doenças transmitidas por alimentos (JAHID e HA, 2012). Devido à extensão dos problemas causados pelos biofilmes, tem havido um esforço significativo no desenvolvimento de novas estratégias de controle. Um campo novo que vem sendo explorado é o uso de compostos naturais, como os óleos essenciais na formulação de sanitizantes para serem aplicados em superfícies de contato com alimentos.

ÓLEOS ESSENCIAIS

Recentemente, pesquisadores têm focado na busca de novas substâncias para a eliminação de biofilmes, entre elas os óleos essenciais e seus constituintes que apresentam potencial antimicrobiano e podem se tornar alternativas naturais aos sanitizantes químicos comumente usados na indústria.

Os óleos essenciais são metabólitos secundários de plantas aromáticas, caracterizados por possuírem compostos orgânicos voláteis, lipofílicos, geralmente odoríferos (BAKKALI et al., 2008) e localizam-se em partes específicas das plantas como cascas, flores, folhas, sementes e raízes (BURT, 2004).

Atualmente mais de 3.000 diferentes óleos essenciais são conhecidos e têm sido amplamente usados devido a suas propriedades já observadas na natureza como, por exemplo, a atividade antimicrobiana (BAKKALI et al., 2008).

As propriedades antimicrobianas dos óleos essenciais têm sido demonstradas em diversos estudos. A variedade de plantas das quais eles são obtidos inclui o tomilho, cravo-da-índia, hortelã, orégano, sálvia, camomila, capim-limão, hortelã-pimenta, pimenta, canela, segurelha, gengibre (PRABUSEENIVASAN et al., 2006; OUSSALAH et al., 2007; SHAN et al., 2007; GUTIERREZ et al., 2009; SOKOVIĆ et al., 2010; SIVASOTHY et al., 2011; MANDAL et al.,

2011). Estes óleos mostraram-se efetivos tanto para bactérias Gram-positivas quanto Gram-negativas e também contra fungos e vírus (TAJKARIMI et al., 2010).

O óleo essencial de canela é extraído de cascas e folhas de árvores do gênero *Cinnamomum, pertencente* à família *Lauraceae* que contém aproximadamente 250 espécies, sendo as principais: *C. zeylanicum*, *C. loureirii*, *C. burmanni*, and *C. cassia* (JANTAN et al., 2008). Os constituintes importantes do óleo essencial de canela são cinamaldeído e eugenol e suas concentrações podem variar de acordo com a localização da planta em que foram obtidos (GUPTA et al., 2008).

O cinamaldeído, um aldeído aromático é o composto majoritário do óleo essencial de canela e está presente nas concentrações de 60-80%, quando obtido das cascas do gênero *Cinnamomum* (SHAN et al., 2007). Estudos comprovaram a sua atividade antimicrobiana contra diversas bactérias, como *L. monocytogenes*, *Pseudomonas aeruginosa*, *E. coli*, *Enterococcus faecalis*, *S. aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Vibrio parahemolyticus* e também *Salmonella* spp. (CHANG et al., 2001; ALI et al., 2005; SHAN et al., 2007; GUPTA et al., 2008; JIA et al., 2011; SANLA-EAD, 2012).

O provável mecanismo da ação antimicrobiana do cinamaldeído está relacionado a sua hidrofobicidade, que lhe permite desestabilizar a bicamada lipídica da membrana celular, causando aumento da permeabilidade aos prótons e saída de moléculas e íons da célula bacteriana, levando a morte da mesma (JIA et al., 2011).

JUSTIFICATIVA

A presença de biofilmes nos ambientes de produção de alimentos tem se tornado comum e podem ser encontrados em diferentes superfícies. Um dos problemas da presença destes biofilmes que eles se tornam fonte de contaminação dos alimentos por micro-organismos patogênicos aumentando o risco para a saúde. Outro problema está na dificuldade de redução ou eliminação das bactérias em biofilmes, visto que bactérias nesta forma são mais resistentes a condições adversas, mais difíceis de remover mecanicamente e também mais resistentes aos desinfetantes convencionais.

A busca de compostos eficazes no controle de biofilmes é preocupação constante para reduzir os problemas decorrentes da presença dos mesmos. Na literatura encontra-se uma vasta

gama de compostos naturais que apresentam propriedades antimicrobianas, entretanto existem poucos estudos sobre a inibição da formação de biofilmes de *Salmonella* spp. em superfícies pelo uso de óleos essenciais e seus compostos ativos. Desse modo, a pesquisa de compostos alternativos para controlar a formação de biofilmes indesejáveis tem se tornado uma área promissora.

OBJETIVOS

GERAL

O objetivo do presente estudo foi avaliar o efeito antibacteriano do óleo essencial de *Cinnamomum zeylanicum* e seu composto majoritário (cinamaldeído) em biofilmes de *Salmonella enterica* sorotipo Saintpaul na superfície de aço inoxidável.

ESPECÍFICOS

- Avaliar a composição química do óleo essencial de canela (*Cinnamomum zeylanicum*).
- Determinar a concentração inibitória mínima e concentração bactericida mínima do óleo essencial de canela e do cinamaldeído frente à *S.* Saintpaul.
- Avaliar o efeito do óleo essencial de canela e cinamaldeído em biofilmes formados por *S.* Saintpaul pela coloração de *MTT* (brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil) tetrazólio) em poliestireno.
- Avaliar a formação *in vitro* de biofilmes de *S.* Saintpaul em superfície de aço inoxidável.
- Avaliar a ação do óleo essencial de canela e do cinamaldeído na formação de biofilmes e em biofilmes pré-formados de *S.* Saintpaul em aço.

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CAPÍTULO II

Artigo: “EFFECT OF CINNAMON ESSENTIAL OIL AND CINNAMALDEHYDE ON *SALMONELLA* SAINTPAUL BIOFILM ON STAINLESS STEEL SURFACE

EFFECT OF CINNAMON ESSENTIAL OIL AND CINNAMALDEHYDE ON *SALMONELLA* SAINTPAUL BIOFILM ON STAINLESS STEEL SURFACE

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ABSTRACT: Biofilm formation on surfaces has important consequences for health and food industry, since they can be a source of food contamination causing food spoilage and foodborne diseases. This study evaluated the effect of cinnamon essential oil (EO) and cinnamaldehyde against *Salmonella* Saintpaul biofilms on stainless steel surface. The effects of cinnamon EO and cinnamaldehyde on biofilms was evaluated in 96-well plates using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining. Colony forming units (CFU) counting assays and scanning electron microscopy (SEM) were used to evaluate anti-biofilm effect on stainless steel surface. The MTT assay results showed that cinnamon EO or cinnamaldehyde at 312 µg/mL and 624 µg/mL could decrease approximately 50% of *S.* Saintpaul biofilms. The highest reduction of bacterial counts on stainless steel surface was observed with addition of 156 µg/ml and 234 µg/mL of cinnamon EO during biofilm formation. Cinnamon EO and cinnamaldehyde reduced the number of bacterial cells on stainless steel surface, making it a potential compound for *Salmonella* spp. control.

KEYWORDS: *Salmonella* spp; bacterial adhesion; natural sanitizers; antimicrobial activity; foodborne pathogens.

PRATICAL APPLICATION: The search for alternatives to control foodborne pathogens is concern for public health and for food industry. Biofilm can be a potential source of food contamination and the use of natural antimicrobial agents could be a good alternative to chemical agents commonly used for disinfection of surfaces.

INTRODUCTION

Salmonella spp. is a major cause of foodborne illness throughout the world (WHO – World Health Organization 2013) and attracts considerable concern of public health and the food industry. According to Center for Disease Control and Prevention (CDC) (2013) approximately 40.000 cases of salmonellosis are reported, every year, in the United States with an estimate of 400 deaths. In Brazil, during the years of 1999 to 2008, were reported more than 6.000 foodborne outbreaks, 41.1% were caused by bacteria, and *Salmonella* spp. was the most frequent agent (Brasil, 2008).

Salmonella spp. can adhere to different food contact surfaces in the food processing environment (Giaouris and Nychas 2006; Chia and others 2009) and form biofilms (Kim and Wei 2007). The biofilm development has important health and economic consequences, since they can be a potential source of contamination in food products, which may lead to food spoilage, reduction of shelf life of products or transmission of diseases (Stepanovic and others 2003; Ortega and others 2010; Speranza and others 2011).

Several strategies for controlling bacterial adhesion to surfaces have been proposed, including the use of essential oils (Nostro and others 2007; Chorianopoulos and others 2010; Oliveira and others 2010; Budzyñska and others 2011; Valeriano and others 2012). Recently, scientists have focused on the antimicrobial activity of cinnamon essential oil and cinnamaldehyde against various pathogens, including *Salmonella* spp (Baskaran and others 2010; Sanla-Ead 2012; Mandal and others 2011), however, few studies have evaluated its effect on bacterial biofilms. The effects of cinnamon EO or cinnamaldehyde against *L. monocytogenes* and *E. coli* (EPEC) biofilms on stainless steel surface has been demonstrated (Oliveira and others 2012). The same compounds were also evaluated against *E. coli* and *Pseudomonas* spp. on polystyrene (Niu and Gilbert 2004). Nuryastuti and others (2009) evaluated the effect of cinnamon oil on *Staphylococcus epidermidis* adhered to polystyrene while Jia and others (2011) evaluated the effect of cinnamaldehyde on the adherence of *Staphylococcus aureus* on the same surface. However, to our knowledge, no studies have reported the effects of cinnamon EO or cinnamaldehyde on *Salmonella* spp. biofilms on stainless steel surface. In the current study, the antibacterial activity of the *Cinnamomum zeylanicum* EO and its major constituent, cinnamaldehyde, against *Salmonella* Saintpaul biofilms on stainless steel surface was evaluated.

MATERIAL AND METHODS

Extraction of the essential oil

Barks from *Cinnamomum zeylanicum* were purchased from local market of Maringá, Paraná State. The essential oil was obtained by hydrodistillation using a Clevenger-type apparatus. Approximately 96 g the crushed bark was submitted to steam distillation by 2 hours. The oil were collected and dried over sodium sulphate and stored in amber flask at 4°C. The yield of the oil were 0,92 % v/w.

Chemical identification

The essential oil chemical composition was investigated by gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR).

Gas chromatographic (GC) analysis was performed with a Thermo Electron Corporation, Focus GC model, under the following conditions: DB-5 capillary column (30 m x 0.32 mm, 0.50 mm), column temperature, 60°C (1 min) to 180°C at 3°C/min; injector temperature 220°C; detector temperature 220°C; split ratio 1:10; carrier gas He; flow rate: 1.0 mL/min. Volume injected 1 μL diluted in acetone (1:10). The GC-MS analysis were performed in a Quadrupole mass spectrometer (Thermo Electron Corporation, DSQ II model), operating at 70V. Identification of the oil components was performed using the retention indices (RI) obtained with reference to n-alkane series C_8H_{18} – $C_{20}H_{44}$ on DB-5 column, and comparison with mass spectra of authentic standard purchased from Sigma-Aldrich literature data (Adams 2007). The retention index (RI) of the oil components were obtained by co-injection of the essential oil with a standard of the n-alkane series C_8–C_{20}, using the Van den Dool and Kratz equation (Van den Dool and Kratz 1963).

Nuclear Magnetic Resonance (NMR):^{1}H (300.06 MHz) and ^{13}C NMR (75.45 MHz) spectra were recorded in $CDCl_3$ solution in a Mercury-300BB spectrometer, with δ (ppm) and spectra referred to $CHCl_3$ (δ 7.27 for ^{1}H and 77.00 for ^{13}C) as internal standard.

Test Microorganisms

Salmonella Saintpaul (510/03) was isolated from raw material used for animal feed and stored in Tryptic Soy Broth (TSB, Difco, Le Pont de Claix, France) with glycerol at -20ºC at the Laboratory of Food Microbiology, Department of Biomedicine and Clinical Analysis, State University of Maringá. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603 was used as controls. Prior to use, an aliquot of frozen isolates was transferred to Bain and Hearth Infusion (BHI, Difco, Le Pont de Claix, France) and incubated at 37ºC for 24h. Subsequently, the culture was plated on Hektoen agar (Difco, Le Pont de Claix, France) and incubated at 37ºC for 24h.

Efficacy of cinnamon essential oil and cinnamaldehyde on planktonic cells

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for cinnamon EO and cinnamaldehyde (Sigma-Aldrich, China, purity 93%) were determined using a micro broth dilution method according to M7-A8 document as recommended by the Clinical and Laboratory Standards Institute (CLSI 2009). *S.* Saintpaul inoculum was padronized according to 0.5 Mc Farland scale and diluted to obtain 5.10^5 CFU/mL (unit forming colony per milliliter). Then, ten microliters of bacterial suspension was inoculated into each well of a 96-well cell culture plate (TPP, Trasadingen, Switzerland) containing 200 µL of the cinnamon EO or cinnamaldehyde diluted in Mueller Hinton Broth (MHB, Difco, Le Pont de Claix, France) at concentrations ranging from 19 to 5000 µg/mL. After 24 h incubation at 37°C, MIC was determinate by microplate reader (Asys Expert Plus) at 620 nm. The MIC was defined as the lowest cinnamon EO or cinnamaldehyde concentration that inhibited bacterial growth. Control of bacterial growth in MHB with dimetilsulfoxide (DMSO, J.T. Baker, USA), control of cinnamon EO or cinnamaldehyde diluted in MHB with DMSO were included in the test. Ampicillin (Sigma-Aldrich, Saint Louis) was used as a standard drug at concentrations of 0.5 to 128 µg/mL against *E. coli* ATCC 25922.

Following MIC assay, MBC was determinate by plating ten microliters of each well where there was no microbial growth on Hektoen agar. The MBC was defined as the lowest

concentration of cinnamon EO and cinnamaldehyde that did not allow bacterial growth following incubation at 37°C for 24 h. All tests were performed in triplicate in two different experiments.

The MIC results were classified according to Aligiannis and others (2001), considering strong activity: MIC up to 500 µg/mL; moderate inhibitors: MIC between 600 and 1500 µg/mL and weak inhibitors: MIC above 1600 µg/mL.

MTT assay

The determination of biofilm metabolic activity was performed by MTT staining assay according to Walencka and others (2005) with some modifications. Overnight cultures of *S.* Saintpaul were diluted in TSB 1:100 to obtain 10^7 CFU/mL confirmed by counting on Hektoen agar and 200 µL were added in a 96-well cell culture plate. After 24 h of incubation at 37ºC, the medium was replaced by fresh TSB and re-incubated at 37ºC for 24h. Wells were rinsed two times with 0.85% sterile saline solution and 200 µl of cinnamon EO or cinnamaldehyde at concentrations of 312 and 624 µg/mL (MIC and 2MIC respectively) was added. The microplate was incubated at 37°C for 1 h. Then, the medium was gently aspirated and the wells were rinsed three times with 0.85% sterile saline solution. Biofilm viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Saint Louis). Briefly, 200 µl of 0.05% MTT in phosphate buffered saline was added in the wells and incubated for 2 h at 37°C. The MTT solution was replaced by 200 µl of 5 mol/L HCl in isopropanol, mixed for 15 min on a micro-oscillator to dissolve all formed formazan crystals and the absorbance was measured at 550 nm. The results were expressed comparing the absorbance of treated bacterial biofilm with biofilm without treatment. Each assay was performed in quadruplicate and repeated two times.

Biofilm formation on stainless steel surface

Stainless steel surface

The cleaning procedure was performed according to Valeriano and others (2012) with some modifications. AISI 304 stainless steel coupons (1×8×8 mm) were cleaned with 100%

acetone, rinsed with distilled water, dried and cleaned with 70% ethanol (v/v). They were rinsed again with distilled water, dried for 2 h at 60ºC and autoclaved at 121ºC for 15 min.

Biofilm formation

The stainless steel surface coupons were place individually into sterile microtubes containing 1.500 µL of overnight culture of *S.* Saintpaul diluted 1:100 in TSB to yield 10^7 CFU/mL confirmed by counting on Hektoen agar. The microtubes were incubated at 37ºC for 24 h, the contents was replaced by new TSB and incubated for another 24 h at 37ºC. After incubation, the microtube contents were aspirated and the coupons were rinsed with 0.85% sterile saline solution to remove planktonic cells. The coupons were submitted to ultrasonic bath at 25 KHz for 5 min (Ultra Cleaner 750A, Unique) to detach the sessile cells. Serial dilutions were performed in 0.85% sterile saline solution, plated on Mueller Hinton agar (MHA; Difco, Le Pont de Claix, France) and incubated at 37°C for 24 h. The results were expressed in log CFU/cm^2. Sterile TSB was used as negative control and *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603 as positive controls. The tests were performed in triplicate in two different experiments.

Effect of cinnamon EO and cinnamaldehyde on established Biofilm

After biofilm formation on stainless steel for 48 h, the coupons were washed with 0.85% sterile saline solution and treated with cinnamon EO or cinnamaldehyde at concentrations of 312 and 624 µg/mL (MIC and 2MIC, respectively). After 1 hour at room temperature, the coupons were rinsed with 0.85% sterile saline solution to remove planktonic cells and the quantification of biofilm bacterial cells was performed.

Effect of cinnamon EO and cinnamaldehyde on biofilm formation

Initially, cinnamon EO or cinnamaldehyde at subMIC concentrations 234, 156 and 78 µg/mL (0.75, 0.5 and 0.25 MIC, respectively) was added microtubes containing the coupons and overnight culture of *S.* Saintpaul diluted 1:100 in TSB (10^7 CFU/mL). After incubation at 37ºC

for 24 h, the coupons were replaced by new TSB with the same concentrations of cinnamon EO or cinnamaldehyde followed by incubation at 37°C for 24 h. The biofilm quantification followed the same procedure described in biofilm formation.

Scanning electron microscopy

Biofilm formation on stainless steel was analyzed using scanning electron microscopy (SEM) according to Wong and others (2010) with modifications. Coupons were rinsed in 0.85% sterile saline solution, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and left for 48 h at 4°C. After fixing, coupons were washed twice with cacodylate buffer and dehydrated in ethanol series (50, 70, 80, 90 and 100% twice). The coupons were dried at critical-point in CO_2, coated with gold, and examined by scanning electron microscopy (Shimadzu SS-550).

Statistical analysis

A one-way analysis of variance (ANOVA) was employed to determine significant differences between means for each group, following by Tukey's test with a statistical significance of $P<0.05$. The statistical analysis was performed using the software GraphPad Prism (version 5.0).

RESULTS AND DISCUSSION

Chemical identification of cinnamon EO

The results of chemical analyses of the cinnamon bark oil and its relative amounts were determined by CG-MS analysis. The components, the percentage of each constituent and their retention indexes were summarized in Table 1. The *Cinnamomum zeylanicum* EO composition showed a predominance of E-cinnamaldehyde (94.9%). Chemical analysis to determine the ratios of active compounds present on cinnamon EO has been described previously. Cinnamaldehyde was also a predominant compound (83.6%) obtained from sticks of *C. burmanni* (Shan and others 2007). Friedman and others (2002) showed that the barks of cinnamon contained 62% of cinnamaldehyde. Other study investigating the cinnamon essential oils composition obtained from different species of *Cinnamomum* showed an amount of 44.2% of cinnamaldehyde in *C. zeylanicum* barks (Jantan and others 2008). The variability of chemicals composition may be related to differences as geographic origin, harvest time and methodology used on essential oil extraction.

Effect of cinnamon EO and cinnamaldehyde on planktonic cells

The cinnamon EO and cinnamaldehyde was able to inhibit the *S.* Saintpaul growth showing MIC of 312 µg/mL, indicating strong antimicrobial activity according to the classification proposed by Aligiannis and others (2001). Both compounds exhibited bactericidal activity at a concentration of 624 µg /mL.

Some reports indicate that cinnamon EO and cinnamaldehyde exhibit strong antimicrobial activity against *Salmonella* spp. Cinnamon EO inhibited the *Salmonella* spp. growth at 500 µg/mL in a research performed by Chang and others (2001). Other authors also observed strong antimicrobial activity of cinnamaldehyde against several serotypes of *Salmonella* as *S.* Thyphimurium, *S.* Enteritidis and *S.* Cholerasuis (Oussalah and others 2007; Sanla-Ead and others 2012; Andrade and others 2012). Another study with *Salmonella* spp. related strong antimicrobial activity (MIC 125 µg/mL) for cinnamaldehyde, while cinnamon EO presented MIC

above 2.500 µg/mL (Shan and others 2007). These results show the antimicrobial effect of cinnamon EO and cinnamaldehyde against different serotypes of *Salmonella*.

Effect of cinnamon EO and cinnamaldehyde on biofilms

Cinnamon EO and cinnamaldehyde have been proven to be an effective inhibitor against planktonic *Salmonella* spp. (Chang and others 2001; Friedman and others 2002; Shan and others 2007; Sanla-Ead and others 2012). Although there are many reports on antimicrobial activity, its capacity to inhibit biofilm formation has rarely been described.

Initially, the effect of cinnamon EO and cinnamaldehyde on biofilms by *S.* Saintpaul was evaluated by MTT staining in polystyrene microtiter plate. The susceptibility of *S.* Saintpaul biofilms to cinnamon EO or cinnamaldehyde was assayed by exposing 48-h-biofilms to the compounds at two different concentrations: 312 µg/mL (MIC) and 624 µg/mL (2MIC) for 1 hour. The MTT reduction assay results showed that the cinnamon EO and cinnamaldehyde at 312 µg/mL could decrease approximately 50% of *S.* Saintpaul biofilms activity ($P<0.05$) (Figure 1). Interestingly, the effect of 2MIC concentration for both compounds was similar to the MIC concentration with no significant differences ($P<0.05$). According to our results, the use of natural compounds in the bactericidal concentration for planktonic cells (624 µg/mL) was not able to inactivate bacteria as biofilms.

Bacteria on biofilm form are more difficult to be removed mechanically and also have greater resistance against chemical agents than planktonic form. Joseph and others (2001) showed that was necessary double exposure time and greater amount of sanitizers to inactivate *Salmonella* spp. biofilms on stainless steel than compared to planktonic cells. Moretro and others (2009) found that disinfectants were more effective against *Salmonella* spp. in suspension compared with bacteria adhered to stainless steel surfaces.

Studies demonstrating the effect of cinnamon EO and cinnamaldehyde on Gram-positive bacteria biofilms has already been described, however, to the best of our knowledge, there have been no studies evaluating the effect of cinnamon EO or cinnamaldehyde on Gram-negative bacteria biofilms by MTT staining. Nuryastuti and others (2009) observed that the treatment of *S. epidermidis* biofilms with cinnamon EO at 1% in 24 h, results in reduction of the metabolic activity and when biofilms were exposed to 2% cinnamon EO resulted in complete disappearance

of metabolic activity. According to Jia and others (2011), the complete reduction of biofilms of *S. aureus* was possible only after treatment with cinnamaldehyde at a concentration equivalent of five times the MIC for planktonic cells.

Biofilm formation on stainless steel

The number of *S.* Saintpaul viable cells on stainless steel after 48 h was about 8 log CFU/cm^2 (Tables 2 and 3). Similar results were observed by Kim and Wei (2007) and by Jun and others (2010) with *Salmonella* spp. biofilm on stainless steel. Furthermore, other authors found lower counts of bacteria on stainless steel. Valeriano and others (2012) obtained 5.78 log CFU/cm^2 after 48 h of incubation, while Chorianopoulos and others (2010) and Joseph and others (2001) also observed counts of approximately 5 CFU/cm^2 in longer time of biofilm formation. In other studies, from an initial inoculum similar to ours, it was necessary incubation for a long time, ranging from 2 to 14 days to reach approximate 6 to 7 log CFU/cm^2, (Knowles and others 2005; Giaouris and Nychas 2006; Moretro and others 2009; Chorianopoulos and others 2010). The differences in biofilm counts can be explained because several factors influence the biofilm formation on surfaces, like culture medium, bacterial cells growth phase, properties of the inert material, temperature, pH, contact time, production of EPS and cell-to-cell communication (Giaouris and Nychas 2006; Kim and Wei 2007, Speranza and others, 2011).

Salmonella Saintpaul biofilm on stainless steel surface was observed by SEM as shown in Figure 2a. Under 3000x magnification, bacteria as shown as micro-colonies with an extracellular matrix, similar to the pattern found by Kim and Wei (2007) when *Salmonella* Typhimurium was cultivated in lettuce broth. It is emphasized that the visualization of the EPS by SEM may be prejudiced due to the treatment of samples with dehydration series in ethanol, which may disrupt the biofilm and destruction of EPS, showing a simpler view of biofilms (Jia and others 2011).

Effect of cinnamon EO and cinnamaldehyde in *Salmonella* Saintpaul biofilms on stainless steel surface.

Although the antimicrobial potential of cinnamon EO and cinnamaldehyde is well documented (Oussalah and others 2007; Gupta and others 2008; Mandal and others 2011), there are no studies reporting its effect in *Salmonella* spp. biofilms on stainless steel surfaces, to the best of our knowledge.

The efficacy of cinnamon EO or cinnamaldehyde was evaluated quantifying viable *S.* Saintpaul cells during and after biofilm formation on stainless steel. Significant reductions ($P<0.05$) was observed after treatment for one hour with 312 and 624 μg/mL (MIC and 2MIC, respectively) of cinnamon EO or cinnamaldehyde on established biofilm (Table 2). Sub-inhibitory concentrations of cinnamon EO and cinnamaldehyde during the biofilm formation also proved to be efficient in preventing biofilm formation on stainless steel (Table 3). The greatest reduction of bacterial counts was observed with 156 μg/mL (0.5MIC) and 234 μg/mL (0.75MIC) of cinnamon EO in which was observed a decrease of approximately 2 log cycles in bacterial counts ($P<0.05$). SEM (Figure 2) also showed that bacterial biofilm in the presence of 156 μg/mL and 234 μg/mL of cinnamon EO was not as evident compared with the untreated control.

Studies demonstrating the effectiveness of cinnamon EO or cinnamaldehyde in bacterial biofilms have been described. Cinnamaldehyde at concentration of 0.125% reduced the number of viable biofilm cells of methicilin-resistant *S. aureus* about 2 log CFU/mL and a reduction of the colony mass of biofilm was observed by SEM (Jia and others 2011). Oliveira and others (2012) observed that cinnamon EO and cinnamaldehyde had a significant effect on *E. coli* and *L. monocytogenes* biofilms on stainless steel, comparable with the commercially available chemical sanitizers.

The exact mechanism of action of cinnamon EO and cinnamaldehyde in biofilms is still not well understood, but it is known that the essential oils and their components are hydrophobic, which enables them to partition in the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable, resulting in loss of cell contents or the exit of critical molecules and ions and bacterial death (Burt 2004).

There is a need for the development of alternative to control bacterial pathogens, especially in situation where biofilm are involved. Essential oils and their constituents have been shown to be effective to control bacterial biofilms.

CONCLUSIONS

The findings of the present study suggest that cinnamon essential oil and cinnamaldehyde are able to inhibit *Salmonella* Saintpaul biofilms on stainless steel surfaces. Our study demonstrates that these natural compounds can be successfully used as alternatives for sanitizers commonly used in the food industry.

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Table 1 - Chemical composition of the essential oil from the bark of *Cinnamomum zeylanicum*.

Compound	RI[a]	Percentual (%)	Identification[b]
α-pinene	935	0.2	GC/MS, NMR
β-pinene	979	0.2	GC/MS, NMR
p-cymene	1026	0.1	GC/MS, NMR
Limonene	1030	0.5	GC/MS, NMR
1,8-cineole	1033	0.5	GC/MS, NMR
4-terpineol	1179	0.2	GC/MS, NMR
α-terpineol	1193	0.4	GC/MS
Z-cinnamaldeyde	1222	0.3	GC/MS
E-cinnamaldeyde	1272	94.9	GC/MS, NMR
Isobornyl acetate	1288	1.3	GC/MS
α-ylangene	1378	0.4	GC/MS
Coumarin	1437	0.3	GC/MS
E-Cinnamyl acetate	1448	0.7	GC/MS
α-selinene	1503	0.1	GC/MS
δ-cadinene	1527	0.1	GC/MS

[a]RI=Retention Index, obtained with reference to *n* -alkane series C_8H_{18} – $C_{20}H_{42}$ on DB-5 , using the Van den Dool and Kratz equation (Van den Dool Kratz 1963).

[b]NMR= Nuclear Magnetic Ressonance and GC/MS = Gas Chromatography-Mass Spectrometry

Table 2 - Effect of cinnamon EO and cinnamaldehyde on 2-days-old *S.* Saintpaul established biofilm on stainless steel surface.

Concentration (µg/mL) [a]	Cinnamon EO [b]	Cinnamaldehyde [b]
0	8.05 ± (0.151)	7.81 ± (0.119)
312	7.42 ± (0.0907)*	6.85 ± (0.215)*
624	7.78 ± (0.0587)*	7.31± (0.0472)*

[a] 312 µg/mL (MIC) and 624 µg/mL (2MIC)

[b] Mean Log CFU/cm^2 with respective SD are given in brackets

* *P* value <0.05 compared without treatment

Table 3 - Effect of sub-MIC concentration of cinnamon EO and cinnamaldehyde on *S.* Saintpaul biofilm formation on stainless steel surface.

Concentration (µg/mL) [a]	Cinnamon EO	Cinnamaldehyde
0	8.41 ± (0.428)	8.25 ± (0.0750)
78	7.10 ± (0.780)	7.48 ± (0.145)*
156	6.27 ± (1.07)*	7.52 ± (0.0866)*
234	6.28 ± (1.23)*	7.19 ± (0.145)*

[a] 234 µg/mL (0.75 MIC); 156 µg/mL (0.5 MIC) and 78 µg/mL (0.25 MIC)

[b] Mean Log CFU/cm^2 with respective SD are given in brackets

* *P* value <0.05 compared without treatment

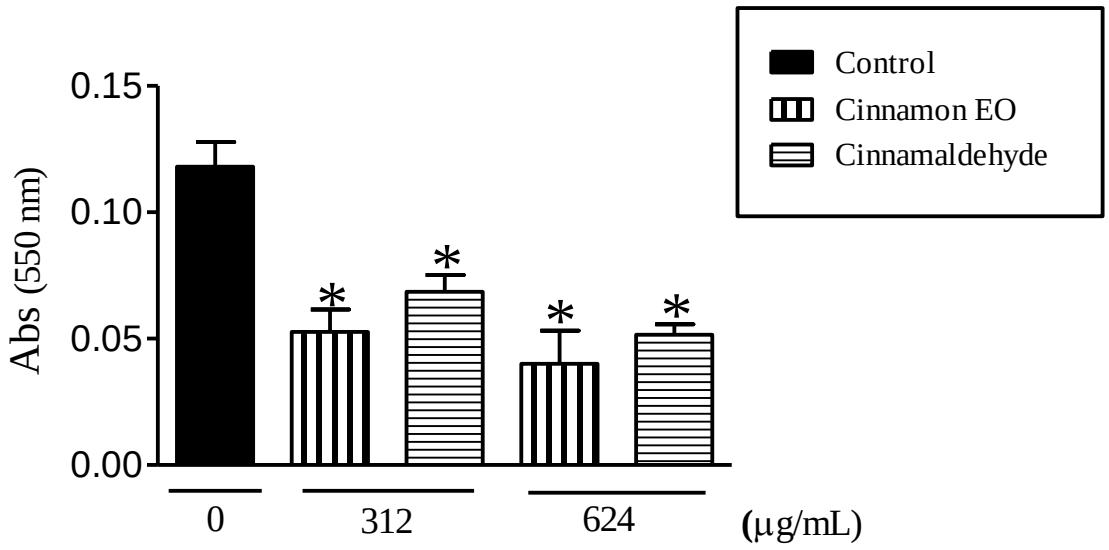

Figure 1 - Effect of different concentrations of cinnamon EO and cinnamaldehyde on *S.* Saintpaul biofilm. Viability was determinate by MTT staining. Data are expressed as mean with SD. All groups were statistically different from the control (**P* <0.05).**

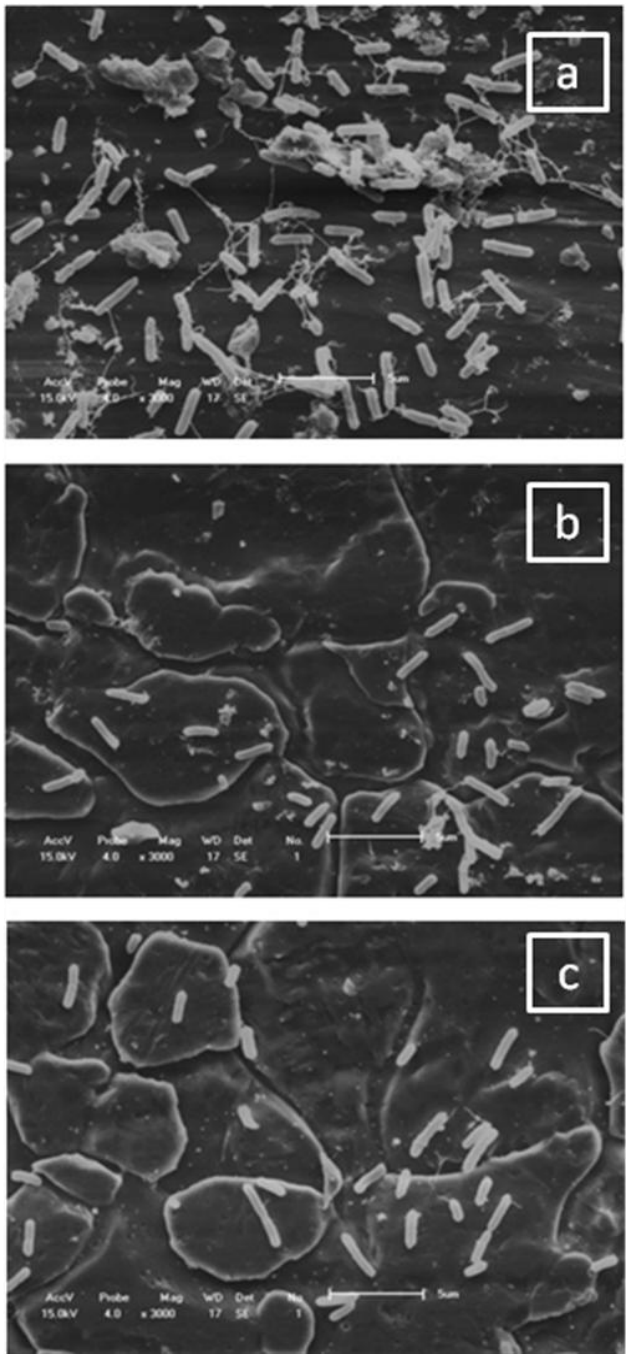

Figure 2 - Scanning electron microscopy images of *Salmonella* Saintpaul biofilm on stainless steel surface. Without treatment (a); treated with cinnamon EO during the biofilm formation at 156 µg/mL (b) and 234 µg/mL (c).

CAPÍTULO III

CONCLUSÕES

Tendo em vista os problemas de contaminação de alimentos e de erradicação de biofilmes no processamento de alimentos, além da resistência dos micro-organismos aos desinfetantes convencionais, avaliou-se a eficiência do óleo essencial de canela e do cinamaldeído na redução de biofilmes de *S.* Saintpaul em aço inoxidável. Os resultados obtidos demonstraram atividade antimicrobiana do óleo de canela e do cinamaldeído contra *S.* Saintpaul. Foi também observado que os dois compostos foram eficazes na redução do número de *S.* Saintpaul no aço inox, mostrando-se uma alternativa promissora no controle de bifilmes desta bactéria.

PERSPECTIVAS FUTURAS

- Testar maior número de isolados bacterianos.
- Alargar o estudo a outras superfícies utilizadas na produção de alimentos.
- Testar novas substâncias com potencial antibiofilme.
- Avaliar a combinação entre sanitizantes químicos e compostos naturais em biofilmes bacterianos.
- Utilizar outras metodologias para avaliar biofilmes bacterianos.