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# Ação antimicrobiana de óleos essenciais de *Thymus vulgaris* L. e *Satureja hortensis* L. e de seus componentes contra *Salmonella* spp. na forma planctônica e em biofilme.

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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. Área de concentração: Saúde Humana.

Orientador: Profa. Dra. Jane Martha Graton Mikcha.

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FOLHA DE APROVAÇÃO

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DEDICATÓRIAS

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# EPÍGRAFE

*Se quiseres conhecer uma pessoa,*

*não lhe pergunte o que pensa,*

*mas sim o que ama.*

*(SANTO AGOSTINHO)*

Ação antimicrobiana de óleos essenciais de *Thymus vulgaris* L. e *Satureja hortensis* L. e de seus componentes contra *Salmonella* spp. na forma planctônica e em biofilme


**RESUMO**

Adesão e formação de biofilme bacteriano em superfícies de polipropileno podem ser fontes de contaminação cruzada em instalações de processamento de alimentos, uma ameaça constante para a saúde pública e a qualidade dos alimentos. Várias estratégias para controlar a aderência bacteriana em superfícies têm sido propostas, incluindo o uso de compostos naturais. Neste estudo foi avaliada a Concentração Inibitória Mínima (MIC) e Concentração Bactericida Mínima (MBC) dos óleos essenciais (OEs) de *Thymus vulgaris* L. (Tomilho) e *Satureja hortensis* L. (Segurelha) e seus compostos majoritários contra *Salmonella* spp., bem como a avaliação da combinação dos compostos mais ativos carvacrol e timol. Também foi investigado o efeito do carvacrol e do timol contra biofilmes de *Salmonella* spp. formados e em formação em superfície de polipropileno. Os OEs foram obtidos por hidrodestilação e analisado por Cromatografia Gasosa acoplada à Espectrometria de Massas e ressonância magnética. A CIM e CBM foram avaliadas pelo método de microdiluição em caldo e o efeito sinérgico foi avaliado pelo método checkerboard. A ação do carvacrol e do timol em biofilmes de *Salmonella* spp. em polipropileno foi avaliada utilizando diferentes concentrações dos compostos pela contagem de Unidades Formadoras de Colônias e por microscopia eletrônica de varredura. Foi observado que os óleos essenciais e seus compostos apresentaram efeito anti-*Salmonella* spp. e a combinação dos compostos demonstrou sinergismo (FIC 0,141). Carvacrol e timol também reduziram o número de células na superfície do polipropileno em todas as concentrações testadas. O ácido peracético foi utilizado foi como sanitizante padrão e apresentou CIM e CBM de 100 ppm e reduziu drasticamente e números de bactérias em polipropileno. Concluímos que o carvacrol e timol foram efetivos contra biofilmes de *Salmonella* spp. já formados e durante a formação em polipropileno, entretanto a efetividade do sanitizante controle foi superior. Assim, os compostos naturais avaliados aparecem como uma alternativa no controle de biofilmes de *Salmonella* spp. em superfícies com pouca contaminação.



**Palavras-Chave: *Salmonella* spp; Biofilme; Óleos essenciais; Carvacrol; Timol, Ácido peracético.**

# Antimicrobial action of *Thymus vulgaris* L. and *Satureja hortensis* L. essential oils and its compounds against *Salmonella* spp. as planktonic and biofilm cells.


## *ABSTRACT*

Adesion and biofilm formation on polypropylene surfaces can be source of cross-contamination in food processing plants, leading major threat to public health and food quality. Several strategies to control bacterial adhesion to surfaces have been proposed, including the use of natural compounds. In this study Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Thymus vulgaris L. (Thyme) and Satureja hortensis L. (Savory) essential oils (EOs) and its predominant compounds against *Salmonella* spp. was evaluated and the combination of the active compounds of carvacrol and thymol . It was also evaluated the effect of carvacrol and thymol against *Salmonella* spp. biofilms on polypropylene. The EOs were obtained by hydrodistillation apparatus and the chemical composition was analyzed by mass spectrometry gas chromatography and nuclear magnetic resonance. MIC and MBC were determined using a broth microdilution method and the synergistic effect was assessed by the checkerboard method. The effect of carvacrol and thymol on *Salmonella* spp. biofilms on polypropylene was evaluated using different concentrations of the compounds by Colony Forming Units (CFU) counting assay and by scanning electron microscopy. It was observed that essential oils and their compounds showed anti-*Salmonella* spp. activity and the combination of the compounds showed synergism (0,141 FIC). Carvacrol and thymol also reduced the number of bacterial cells on polypropylene the surface at all concentrations tested. Peracetic acid, a standard sanitizer, was used as control, showed MIC and MBC of 100 ppm and drastically reduced bacterial counts on polypropylene. We conclude that carvacrol and thymol were effective against *Salmonella* spp. established biofilms and during its formation on polypropylene; however the effectiveness of control sanitizing was superior. Thus, natural compounds tested appear to be an alternative to control *Salmonella* biofilms on surfaces with low contamination.



***Keywords*: *Salmonella* spp; Biofilm; Essential oil; Carvacrol; Thymol; Peracetic acid.**

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# CAPÍTULO I

## Introdução

A transmissão de patógenos de origem alimentar é preocupante uma vez que resulta em altas taxas de morbidade e mortalidade e significativas perdas econômicas. De acordo com a Organização Mundial de Saúde, houve um aumento significativo da incidência de doenças transmitidas por alimentos nas ultimas décadas em vários países (WHO; 2013). Estima-se que o custo econômico relacionados com as doenças transmitidas por alimentos nos Estados Unidos ultrapassa 50 bilhões de dólares por ano, onde 48 milhões de pessoas são afetadas (Bermudez-Aguirre e Barbosa Canovas, 2013).

*Salmonella* spp. é uma das principais bactérias transmitidas por alimentos, sendo responsável pela maioria dos surtos de origem alimentar em vários países (Mead et al., 1999; Callaway et al., 2008). Segundo o CDC (Center for Disease Control and Prevention, 2012), estima-se que nos Estados Unidos, *Salmonella* spp. seja a principal bactéria causadora de doença transmitida por alimentos, sendo responsável por mais de 1 milhão de casos, cerca de 19.000 hospitalizações e 378 mortes a cada ano. Segundo a Secretaria de Vigilância em Saúde – Ministério da Saúde, *Salmonella* spp. foi o agente etiológico em 39,39% dos surtos de doenças transmitidas por alimentos causadas por micro-organismos no Brasil de 2000 a 2013 (Brasil, 2013). No Estado do Paraná - Brasil, os dados epidemiológicos de 1998 a 2010 revelaram que 53% dos surtos de doenças transmitidas por alimentos foram causados por bactérias, sendo *Salmonella* spp. responsável por 57,1% dos casos (Kottwitz et al., 2010).

Além disso, estudos demonstram que *Salmonella* spp. é capaz de aderir e formar biofilmes em diferentes superfícies que entram em contato com alimentos na indústria e nos domicílios (Steenackers et al., 2012). Com a grande ocorrência de surtos causados por *Salmonella* spp. no Brasil e no mundo e a importância dos biofilmes como fontes de contaminação, surge a necessidade de encontrar novas formas de controle dos micro-organismos envolvidos na produção de biofilmes. Assim, 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., 2010).

## *Salmonella* spp.

Existem mais de 2500 sorotipos de *Salmonella* amplamente distribuídos na natureza, dos quais aproximadamente 200 são responsáveis pela maior parte das infecções tanto no homem quanto em animais (Guibourdenche et al., 2010; Steenackers et al., 2012). A maioria destas ocorre pela ingestão de alimentos de origem animal embora nos últimos anos tenham sido descritos surtos envolvendo produtos de origem vegetal (Center for Disease Control and Prevention, 2014).

No homem, a salmonelose se manifesta por gastroenterite, com sintomas de diarreia, dor abdominal, febre, náusea e vômito em 12 a 72 horas após a infecção. A doença geralmente dura de 4 a 7 dias e em adultos saudáveis, a doença 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, 2014).

A contaminação de alimentos por *Salmonella* spp. torna-se uma 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 e por aumentar a resistência à limpeza e sanitização (Stepanovic et al., 2004; Steenackers et al., 2012).

## Biofilmes

Biofilme é definido como uma comunidade de microrganismos sésseis desenvolvido com origem em uma única espécie ou múltiplas espécies. É caracterizado

por células que se aderem a superfícies bióticas e ou abióticas, incorporadas a uma matriz extracelular formada por exopolissacarídeos (EPS). (Costerton et al, 1999; Donlan et al, 2002, Aparna et al, 2008). A estrutura dos biofilmes pode variar de acordo com o micro-organismo e com as condições ambientais (Prakash et al. 2003). Os principais componentes do biofilme são os polissacarídeos, as proteínas, os fosfolipídios, os ácidos teicóicos e os ácidos nucléicos (Jahid e Ha, 2012).

A formação do biofilme é caracterizada por uma etapa reversível; onde a bactéria adere à superfície, seguida de uma etapa irreversível onde ocorre multiplicação celular e continuidade da aderência e por fim a formação de glicocálix (Ciston et al., 2008). Os biofilmes microbianos presentes nos equipamentos podem desprender-se e contaminar o alimento, tornando o seu controle um desafio para a indústria.

Outro fator importante é a resistência dos micro-organismos na forma de biofilmes aos desinfetantes convencionais tornando-se motivo de preocupação para a tanto para saúde pública quanto para a indústria (Jahid e Ha, 2012; Gilbert et al., 2002 ; Stepanovic et al., 2004).

Devido à necessidade de controle de biofilmes bacterianos, a utilização de compostos naturais, como os óleos essenciais, na formulação de sanitizantes para serem aplicados em superfícies de contato com alimentos aparecem como novas estratégias no controle de biofilmes.

## Óleos essenciais

As plantas por meio de seu metabolismo secundário dão origem aos óleos essenciais, compostos voláteis naturais, obtidos por hidrodestilação a partir de folhas, caules ou sementes (Oliveira et al., 2006; Bakkali et al., 2008). Atualmente mais de 3.000 diferentes óleos essenciais são conhecidos e têm sido amplamente utilizados. Dentre esses 300 são comercialmente importantes devido a suas propriedades já observadas na natureza (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). Os óleos essenciais de *Thymus vulgaris* L. (tomilho) e *Satureja hortensis* L. (segurelha) apresentam atividade antimicrobiana devido seus componentes fenólicos carvacrol e timol (Cosentino et al., 1999; Lambert et al., 2001).

O carvacrol é um fenol monoterpenoide quimicamente denominado 2-metil-5-(1-metiletil)-fenol ou isopropyl-0-cresol. Essa substância apresenta-se como um dos principais constituintes dos óleos essenciais de orégano, tomilho e segurelha. Estudos demonstram efeito antimicrobiano de carvacrol contra *Streptococcus mutans* (Botelho et al., 2007), *Listeria monocytogenes* (Lim,et al., 2010), e *Salmonella enterica* (Knowles et al., 2005).

O timol é um fenol monoterpenoide com nomenclatura quimica 5-metil-2-(1-metiletil)-fenol, encontrado nos óleos essenciais de tomilho, segurelha, Lippia e orégano. (Hudaib et al., 2002). Possui alta atividade *in vitro* contra bactérias Gram negativas e Gram positivas (Dorman e Deans, 2000), inibindo o crescimento de *Pseudomonas aeruginosa*, *Staphyloccus aureus*, *Salmonella* spp. e *Listeria monocytogenes e Escherichia coli* (Burt, 2004; Al-Bayati; 2008; Nikolic et al.,2014).

O provável mecanismo da ação antimicrobiana desses compostos está relacionado a uma característica importante, denominada 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).

A busca por alimentos seguros, livres de contaminantes de natureza química e biológica que não exponha a saúde da população ao risco, estimula novas pesquisas com substâncias naturais, como óleos essenciais, na conservação de alimentos.Óleos essenciais contendo carvacrol e timol, bem como estas substâncias isoladas, também

apresentam a capacidade de reduzir biofilmes bacterianos em diversas superfícies (Nostro et al., 2007; Perez-Conesa, 2011; Soumya et al., 2011; Szczepanski et al., 2014; Santos Junior.,2011; Millezi et al., 2012).

## Justificativa

Biofilmes são importantes fontes de contaminação de alimentos, ocasionando um problema de saúde pública pelos riscos que trazem para a saúde humana e animal e também para a indústria.

A fim de controlar esses biofilmes e reduzir a utilização de sanitizantes químicos evitando quaisquer contaminantes no alimento, os pesquisadores têm desenvolvido estudos a partir de produtos naturais como novas estratégias para o controle do desenvolvimento de patógenos em alimentos, bem como de biofilmes (Bakkali et al., 2008).

Entre os produtos naturais, o uso do carvacrol e timol, isolados ou combinados, pela indústria farmacêutica e de alimentos, como conservantes já é relatado. Porém, há poucos estudos sobre a utilização destes compostos na inibição de biofilmes bacterianos em superfícies.A pesquisa de compostos alternativos para controlar o desenvolvimento microbiano nos alimentos e a formação de biofilmes tem se tornado uma área promissora. Os óleos essenciais são uma possível alternativa não somente para utilização em escala industrial como para uso domiciliar garantindo a inocuidade dos alimentos e menor risco à saúde.

## Objetivos

### Geral

O objetivo do presente estudo foi avaliar o efeito dos óleos essenciais de *Thymus vulgaris* L. (tomilho) e *Satureja hortensis* L. (segurelha) e seus compostos

majoritários no desenvolvimento de *Salmonella* spp. e avaliar a ação do carvacrol e timol em biofilmes de *Salmonella* spp. em polipropileno.

## Específicos

1. Avaliar a composição química do óleo essencial de *Thymus vulgaris* L. (tomilho) e *Satureja hortensis* L. (segurelha).

2. Determinar a concentração inibitória mínima e concentração bactericida mínima dos óleos essenciais de tomilho e segurelha, de seus compostos majoritários e de ácido peracético, como sanitizante padrão, contra *Salmonella* spp.

3. Avaliar o efeito antimicrobiano da combinação dos compostos ativos contra *Salmonella* spp.

4. Avaliar a formação *in vitro* de biofilmes de *Salmonella* spp. em superfície de polipropileno.

5. Avaliar a ação do carvacrol e timol durante a formação de biofilmes e em biofilmes formados de *Salmonella* spp. em polipropileno

6. Avaliar o efeito do ácido peracético em biofilme formado de *Salmonela* spp. em polipropileno.

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# CAPÍTULO II

**Artigo1:** Antibacterial activity of *Satureja hortensis* L. and *Thymus vulgaris* L. essential oils and the synergistic effect of its major components against *Salmonella* spp.

# Antibacterial activity of *Satureja hortensis* L. and *Thymus vulgaris* L. essential oils and the synergistic effect of its major components against *Salmonella* spp.

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**Abstract**

This study evaluated the antibacterial activity of *Satureja hortensis* L. essential oil (SEO), *Thymus vulgaris* L. essential oil (TEO) and their major components against *Salmonella* spp. as well as to investigate the effect of the combination of active components. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using a broth microdilution method against

eighteen *Salmonella* spp. isolates. The synergistic effect was performed in a checkerboard method and the fractional inhibitory concentration index (FIC) was determined. The SEO and TEO demonstrated active against all *Salmonella* spp. isolates investigated (MIC and MBC = 2500 µg/mL). Among the tested compounds the best results were obtained with thymol and carvacrol. The MIC and MBC obtained with carvacrol range from 156 µg/mL to 312µg/mL. Thymol showed MIC of 312µg/mL and MBC ranges from 312µg/mL to 625 µg/mL. P-cymene and borneol not exhibit anti-*Salmonella* activity (MIC and MBC ≥ 5.000µg/mL). Effect of combination was evaluated with carvacrol and thymol, which are the most active compounds. Synergistic effect was observed (FIC = 0,141). SEO, TEO, carvacrol and thymol were able to inhibit the *Salmonella* spp. growth. The most potent activity was verified with carvacrol, thymol and with the combination of them. These compounds may be an alternative to *Salmonella* spp. control.

**Keywords**

*Satureja hortensis* L. essential oil, *Tymus vulgaris* L. essential oil, Carvacrol, Thymol, *Salmonella* spp.

## INTRODUCTION

Salmonellosis is major cause of foodborne illness worldwide (WHO, 2014). In the United States, it is estimated that about one million cases of salmonellosis occur each year with more than 19,000 hospitalizations and nearly 400 deaths (Scallan et al., 2011). In Brazil *Salmonella* spp. was the leading cause of foodborne illness during 2000 to 2013 (BRASIL, 2013). Thus, there is a concern about the need of *Salmonella* spp. control in food and in food processing environments.

The essential oils are complex natural mixtures of volatile secondary metabolites isolated from plants that have been associated to different antimicrobial properties such as antibacterial (Djabou et al., 2013; Jallali et al., 2014; Stefanakis et al., 2013), antifungal (Kedia et al., 2014; Yamamoto-Ribeiro et al., 2013) and antiviral (Astani et al., 2011; Elizaquível et al., 2013).

The antibacterial activity of *Satureja hortensis* L. essential oil (SEO) has been demonstrated against plant pathogenic bacteria (Kotan et al., 2013); periodontal pathogens (Gursoy et al., 2009); *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Listeria monocytogenes* (Oussalah et al., 2007) and *Pseudomonas putida* (Oussalah et al., 2006). The *Thymus vulgaris* L. essential oil (TEO) showed active against several bacteria including *Pseudomonas aeruginosa; Staphylococcus aureus* (Nikolic´ et al., 2014); *Salmonella* Typhi, *Salmonella* Typhimurium (Al-Bayati, 2008); *Listeria monocytogenes* and *Escherichia coli* (Burt et al., 2004).

Some reports verified a synergistic effect involving the essential oils and their major components against foodborne pathogens and food spoilage bacteria (Sousa et al., 2012; Turgis et al., 2012; Ultee et al., 2000). The use of essential oils and their components as an antimicrobial alternative in food conservation has been shown (Azeredo et al., 2011; Sousa et al., 2012).

In these work we evaluate the effect of TEO, SEO and their major components against different *Salmonella* serotypes isolated from food incriminated in outbreaks of foodborne illnesses and animal feed as well as the antibacterial effect of the major and active components in combination.

## MATERIAL AND METHODS

### Plant material and pure compounds

The fresh leaves of *Thymus vulgaris* L. was collected from the Prof. Irenice Silva Medicinal Plant Garden in the State University of Maringá – Paraná - Brazil. The leaves were identified and a voucher specimen was deposited in the Herbarium of the Botanical Department of the State University of Maringá. The leaves of *Satureja hortensis* L. were purchased from Ingá Alimentos® store at the same city.

### Extraction of the essential oils

The essential oils were extracted by conventional steam distillation using a Clevenger-type apparatus for 2 h. in the Department of Chemistry, State University of Maringá. The obtained essential oil were dried over sodium sulphate and stored at 4 ºC in dark vials until use. The yields of *Satureja hortensis* L. and *Thymus vulgaris* L. essential oils were 0,97% v/w and 1,4% respectively.

### Analysis of the essential oils and compound identification

The essential oil chemical composition was investigated by gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). Gas chromatography was performed using a Thermo Electron Focus GC model under the following conditions: DB-5 capillary column (30 m×0.32 mm x 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. A sample of 0,2µL of essential oil diluted in acetone was injected. The GC-MS analysis was performed using a Quadrupole mass spectrometer (Thermo Electron, DSQ II model) that operated at 70 eV.

The identification of the oil components was performed using retention indices (RIs) obtained with reference to *n*-alkane series $C_8H_{18}$ – $C_{20}H_{44}$ on a DB-5 column and comparisons with mass spectra of authentic standards purchased from Sigma-Aldrich (Adams et al., 2007). The RIs of the oil components were obtained by co-injecting the

EO with a standard of the *n*-alkane series $C_8$–$C_{20}$ using the Van den Dool and Kratz equation (1963). $^1$H NMR (300.06 MHz) and $^{13}$C NMR (75.45 MHz) spectra were recorded in a $CDCl_3$ 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 the internal standard.

**Bacterial isolates**

Seventeen *Salmonella* isolates belonging to different serotypes obtained from foods incriminated in outbreaks of foodborne illness and raw materials for animal feed and one *Salmonella* typhimurium strain ATCC 14028 (Table 3) were analyzed. The isolates were stored in Brain and Heart Infusion broth (BHI; Difco, Le Pont de Claix, France) with glycerol 20% at -20 ºC in the Laboratory of Food Microbiology, Department of Clinical Analyses and Biomedicine, State University of Maringá.

Prior to use, an aliquot of frozen isolates was transferred to BHI and incubated at 37 ºC for 24 h. The culture was plated on Hektoen agar (Difco, Le Pont de Claix, France) and incubated at 37 ºC for 24 h.

**Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

The pure compounds thymol, carvacrol, p-cymene and borneol were purchased from Sigma. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the essential oils and compounds were determined using a broth microdilution method according to the protocol M07-A9/2012 established by the CLSI (Clinical and Laboratory Standards Institute). Previously, the TEO, SEO and the compounds thymol, p-cimene and borneol were prepared in dimethyl sulfoxide (DMSO) (J.T. Baker, Center Valley, Pennsylvania, USA). Carvacrol was diluted in ethanol absolute (FMaia®).

For MIC determinations, bacterial suspensions were standardized according to the 0.5 McFarland scale and diluted to obtain $5.10^5$ CFU/mL. An aliquot of 10µl of bacterial suspensions were inoculated into each well of a 96-well cell culture plate

(TPP, Trasadingen, Switzerland) that contained 100 µl of the essential oils and compounds diluted in Mueller Hinton Broth (MHB, Difco, Le Pont de Claix, France) at concentrations of 19, 39, 78, 156, 312, 625, 1250, 2500, 5.000 µg/mL. After 24 h of incubation at 37 °C, the MIC was determined using a microplate reader (Asys Expert Plus) at 620 nm. The MIC was defined as the lowest of essential oil and compounds concentration that inhibited bacterial growth.

For the determination of the MBC, an aliquot at 10 µl where there was no microbial growth in the MIC assay was plated on Hektoen agar (Difco, Le Pont de Claix, France). The plates were incubated at 37 °C for 18-24 h and the MBC was defined as the lowest concentration that did not allow bacterial growth on agar plates. Each test was performed in duplicate and repeated four times.

**Synergy assay**

The effect of the combination of thymol and carvacrol was tested against *Salmonella* Saintpaul (510-02) and *Salmonella* Typhimurium ATCC 14028. The synergy assay was performed by determining the fractional inhibitory concentration (FIC) index in MHB broth using the microdilution method in a checkerboard scheme in 96-well microtiter plates (Moody et al., 2003). FIC was calculated as FIC (A) + FIC (B) where FIC.A = (MIC.A combination/MIC.A alone) and FIC.B = (MIC.B combination/MIC.B alone). The results were interpreted as synergy (FIC $< 0.5$), addition ($0.5 \leq$ FIC $\leq 1$), indifference ($1 <$ FIC $\leq 4$) or antagonism (FIC $> 4$) according to Schelz et al., (2006). All experiments were done in triplicate.

**RESULTS**

**Chemical identification of *Satureja hortensis* L. and *Thymus vulgaris* L. essential oils.**

The chemical composition of essential oils and compounds were investigated using GC-MS and NMR. The GC-MS analysis for SEO showed a predominance of carvacrol (43,04%), thymol (34,60%), β-caryophyllene (5,97%), p-cymene (4,98%) and

the borneol (3,95%) as demonstrated in Table 1.The profile of TEO analysed by GC-MS showed a predominance of p-cymene (34,4%), thymol (19,9%), borneol (16,2%) and carvacrol (6,7%) as observed in Table 2.

**Table 1.** Percentual of the chemical composition of *Satureja hortensis* L. essential oil.

| Retention time | Compounds | Percentual (%)* | Identification |
|---|---|---|---|
| 8.06 | Solvente | — | GC/MS, NMR |
| 9.79 | *p*-Cymene | 4.89 | GC/MS, NMR |
| 11.09 | *γ*-Terpinene | 0.79 | GC/MS, NMR |
| 12.74 | Linalool | 1.38 | GC/MS, NMR |
| 14.68 | Camphor | 0.38 | GC/MS, NMR |
| 15.55 | Borneol | 3.95 | GC/MS, NMR |
| 16.08 | *α*-Terpineol | 0.91 | GC/MS, NMR |
| 18.57 | Carvacrylmethylether | 0.78 | GC/MS |
| 21.03 | Thymol | 34.60 | GC/MS, NMR |
| 21.44 | Carvacrol | 43.04 | GC/MS, NMR |
| 24.62 | Eugenol | 0.24 | GC/MS, NMR |
| 26.45 | *β*-Caryophyllene | 5.97 | GC/MS, NMR |
| 30.28 | *γ*-Muurolene | 0.60 | GC/MS |
| 30.65 | *β*-Bisabollene | 1.08 | GC/MS |
| 32.97 | Caryophylleneoxide | 1.24 | GC/MS, NMR |
| 35.76 | *γ*-Cadinene | 0.15 | GC/MS |

* Relative percentage of chemical constituents

**Table 2.** Percentual of the chemical composition of *Thymus vulgaris* L. essential oil.

| Retention time | Compound | Percentual (%)* | Identification |
|---|---|---|---|
| 6,21 | *α*-pinene | 3,0 | GC/MS, NMR |
| 6,37 | camphene | 2,4 | GC/MS, NMR |
| 7,02 | — | 0,5 | GC/MS, NMR |
| 8,47 | *p*-cymene | 34,4 | GC/MS, NMR |
| 8,75 | — | 2,1 | GC/MS, NMR |
| 9,73 | — | 1,1 | GC/MS, NMR |
| 11,26 | Linalool | 2,6 | GC/MS, NMR |
| 12,26 | — | 0,3 | GC/MS, NMR |
| 12,94 | — | 0,9 | GC/MS, NMR |
| 13,87 | Borneol | 16,2 | GC/MS, NMR |
| 14,37 | — | 1,4 | GC/MS, NMR |
| 14,91 | *α*-terpineol | 3,0 | GC/MS, NMR |
| 16,76 | — | 1,5 | GC/MS, NMR |
| 18,93 | Carvacrolmethylether | 1,8 | GC/MS, NMR |
| 19,21 | Thymol | 19,9 | GC/MS, NMR |
| 19,57 | Carvacrol | 6,7 | GC/MS, NMR |
| 22,83 | — | 0,5 | GC/MS, NMR |
| 24,55 | Caryophyllene | 1,8 | GC/MS, NMR |

* Relative percentage of chemical constituents

**Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of SEO, TEO and their major compound.**

As observed in Table 3, SEO, TEO, thymol and carvacrol, showed effective against all *Salmonella* serotypes investigated. The SEO showed the same value for MIC and MBC, 2,500 µg/mL. The MIC and MBC for TEO varies from 1,250 to 2,500 µg/mL.

The compound Thymol showed MIC of 312 µg/mL for all *Salmonella* spp. isolates while MBC was 312 µg/mL for 16 isolates and 625 µg/mL to another two. Carvacrol showed MIC of 156 µg/mL for 16 isolates and 312 µg/mL to another two, while MBC was 156µg/mL for 15 isolates and 312 µg/mL for 3 isolates. Borneol and p-cymene not exhibit anti- *Salmonella* activity as verified by MIC and MBC (≥ 5,000 µg/mL).

**Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (µg/mL) of the SEO, TEO, thymol, carvacrol, borneol and *p*-cymene against *Salmonella* spp.**

| | | | SEO | TEO | Thymol | Carvacrol | Borneol | *p*-Cymene |
|---|---|---|---|---|---|---|---|---|
| IDENTIFICAÇÃO | SOROTIPO | FONTE | MIC/MBC | MIC/MBC | MIC/MBC | MIC/MBC | MIC/MBC | MIC/MBC |
| 284-95 | *S.* Mbandaka | Crysalis | 2500/ 2500 | 1250/ 1250 | 312 /625 | 156/ 312 | ≥5000 | ≥5000 |
| 730-96 | *S.* Enteritidis | Saltedchicken ("drumstick") * | 2500/ 2500 | 2500/ 2500 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 1044-97 | *S.* Rissen | Meatandbonemeal | 2500/ 2500 | 2500/ 2500 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 289-98 | *S.* Enteritidis | Mayonnaise * | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 312/ 312 | ≥5000 | ≥5000 |
| 642-98 | *S.* Schwarzengrund | Pasta with sardines and mayonnaise* | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 312/ 312 | ≥5000 | ≥5000 |
| 66-99 | *S.* Infantis | Frescalsausage | 2500/ 2500 | 2500/ 2500 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 502-00 | *S.* Enteritidis | Homemade mayonnaise* | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 906-01 | *S.* Typhimurium | Codfish* | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 1640-01 | *S.* Coeln | Pavé * | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 100-02 | *S.* Oranienburg | Bone Pet | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 406-02 | *S.* London | ScrapesOxhide | 2500/ 2500 | 2500/ 2500 | 312/ 625 | 156/ 156 | ≥5000 | ≥5000 |
| 510-02 | *S.* Saintpaul | Bloodmeal | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 1121-02 | *S.* Schwarzengrund | Soya bran | 2500/ 2500 | 2500/ 2500 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 1122-02 | *S.* Cubana | Soya bran | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 312-02 | *S.* Anatum | bran Meat and bone | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 1123-02 | *S.* Urbana | Soya bran | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 1124-02 | *S.* Mbandaka | Soya bran | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/ 156 | ≥5000 | ≥5000 |
| 14028 | *S.* Typhimurium | ATCC 14028 | 2500/ 2500 | 1250/ 1250 | 312/ 312 | 156/156 | ≥5000 | ≥5000 |

* isolated from food incriminated in outbreaks of foodborne illnesses

**Synergy assay**

The FIC index obtained for the combination of carvacrol and thymol against *S.* Typhimurium and *S.* Saintpaul was 0,141 indicating the synergic effect between the compounds according to Schelz et al. (2006).

## DISCUSSION

Essential oils (TEO and SEO) evaluated in this study showed a predominance of carvacrol and thymol, which was also demonstrated in other studies (Amiri, 2012;. Oussalah et al, 2007;. Soković et al, 2010; Skocibusíc, 2004). However, Nikolic 'et al. (2014) and Costa-Ballester et al. (2013) analyzed TEO that carvacrol and thymol is either not present or are present as minor compounds. This variability of the composition of the essential oil may be the result of differences in geographical origin, time of harvest, and the methodology used for their extraction (Isman et al., 2007). This variability can influence the antimicrobial effect.

In the present work the antibacterial activity of the SEO and TEO was verified against all *Salmonella* spp. investigated, in accordance with previous reports. Oussalah et al. (2007) reported the effect of SEO against *S.* Typhimurium with MIC at 0,5 ml, while Mihajilov-Krstev et al. (2010), demonstrated activity of SEO against *S.* Enteritidis.

The effect of TEO against *Salmonella* spp. has also been reported. Ivanovic et al. (2012) obtained MIC at 640 µg/mL for TEO against *S.* Enteritidis. Another study showed that TEO present MIC at 125µg/mL and 250µg/mL for *S.* Typhimurium and *S.*

Typhi, respectively (Al-Bayati, 2008). These differences in MIC could be due to differences in essential oils composition. (Aligiannis et al.,2001).

The antibacterial properties against foodborne pathogens attributed to essential oils are associated to the high percentage of phenolic compounds such as carvacrol, eugenol and thymol (Cosentino et al., 1999; Lambert et al., 2001). A previous report indicated that TEO with not contain carvacrol and thymol as predominant compound was not effective against *S.* Typhimurium (Turgis et al., 2012).

In this study the concentration of carvacrol and thymol was smaller in TEO than in SEO, nevertheless, TEO showed to be more active than SEO. Some authors related that minority components have an important role in antibacterial activity in *Thymus* spp., maybe producing a synergistic effect among other components (Lattaoui and Tantaoui-Elaraki, 1994; Marino et al., 1999; Paster et al., 1995).

According Chorianopoulos et al., (2004) the antimicrobial activity of the essential oils of *Satureja* spp., *Thymu*s spp. and *Origanum* spp. is not only due to the presence of carvacrol and thymol, but as a result of the presence of other components in low concentrations, that can cause synergistic, additive or antagonist interactions. It is known that *p*-cymene alone is not an effective antibacterial agent, but when combined with carvacrol shown a synergistic effect (Burt, 2004).

We observed that *p*-cymene was found in greater concentration in TEO than in SEO, thus, *p*-cymene can be in part responsible for the better antimicrobial property of TEO when compared with SEO.

In the present study, carvacrol demonstrated MIC that ranges from 156 to 312 µg/mL while thymol showed MIC at 312 µg/mL. These results are in agreement with other authors that reported the activity of carvacrol and thymol against *S.* Typhimurium,

*S.* Enteritidis and *S.* Typhi with MIC that ranges from 0,5 to 400 µg/mL (Cosentino et al.,1999; Kim et al., 1995; Nazer et al., 2005; Rattanachaikunsopon and Phumkhachorn, 2009; Soković et al.,2010; Zhou et al. 2007). Borneol and *p*-cymene showed ineffective against all *Salmonella* spp. investigated, in accordance with Dorman e Deans (2000) which observed that borneol was not able to inhibit *Salmonella* spp.

Our results demonstrate the synergistic effect between carvacrol and thymol against *S.* Typhimurium and *S.* Saintpaul indicating that this combination is a good alternative to *Salmonella* spp. control. A synergistic effect between these compounds against *S.* Typhimurium was previous reported by Zhou et at. (2007). This combination was also synergistic against several bacteria isolated from fresh produce (Zheng et al., 2013).

Several studies have investigated the antibacterial activity of mixtures involving carvacrol and thymol. Santiesteban-Lopez et al. (2007) associate carvacrol and thymol with potassium sorbate, a synthetic antimicrobial agent, and obtained sinergystic effect against *S.* Typhimurium.

The combination of carvacrol and thymol with chelators and organic acid against *S.* Typhimurium also indicated sinergystic effect (Zhou et al., 2007b). According to Esteban et al. (2013) the combination of carvacrol and nisin against *S.* Enteritidis and *S.* Senftenberg resulted on additive effect.

## CONCLUSION

This study demonstrates the anti-*Salmonella* activity of SEO, TEO and the major components, carvacrol and thymol, against several *Salmonella* serotypes isolated from human and animal feed. The most potent activity was verified with carvacrol, thymol and with the combination of them.

The results are particularly interesting to the food industry considering possibility to the use of a natural food preservative to extend the shelf life and control *Salmonella* spp.

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**Artigo2:** Carvacrol and thymol against *Salmonella* spp. biofilms on polypropylene**.**

# Carvacrol and thymol against *Salmonella* spp. biofilms on polypropylene.

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**Summary**

Biofilm formation on surfaces is important for the health and food industry consequences, it can be a source of food contamination. The present study evaluated the effect of carvacrol, thymol compared to peracetic acid against *Salmonella* spp. biofilm on polypropylene. The efficacy of the compounds was assessed by quantifying *Salmonella* spp. viable cells during and after the formation of biofilm on polypropylene and scanning electron microscopy. The greatest reduction in bacterial count was observed with the addition of carvacrol in 156 and 312 ug / ml, *Salmonella* typhimurium ATCC (14028) biofilms established. Carvacrol to 117 mcg / mL was also effective against S. Typhmurium ATCC (14028) during the formation of biofilm and 312 mg / ml *Salmonella* Saintpaul established biofilm. S. Enteritidis establish biofilm was effectively reduced by thymol to 624 mcg / mL. Peracetic acid bacterial cells disposed in polypropylene. Carvacrol and thymol reduced the number of *Salmonella* spp. polypropylene, becoming potential compounds for *Salmonella* spp. Control

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**Significance and Impact of the Study:** *Salmonella* spp. infection is a major public health problem in many countries and polypropylene surfaces are widely used in processes foods. Due to difficulty of eliminating *Salmonella* spp. in biofilms, studies of alternative compounds for its control are essential.

**Keywords:** biofilm; carvacrol; thymol; peracetic acid; polypropylene; *Salmonella* spp.;

**Running head:** Carvacrol and thymol on *Salmonella* spp biofilm.

**Introduction**

The impact of foodborne diseases, in especially that caused by *Salmonella* spp. is a concern worldwide. It has been estimated that 93.8 million cases of gastroenteritis due to *Salmonella* species occur worldwide each year, with 155,000 deaths, that represents a considerable problem in both developing and developed countries (Majowicz et al., 2010; Scallan et al., 2011).

*Salmonella spp.* biofilms has attracting the attention of the food processing environment, since it is a continuing source of food contamination (Van Houdt and Michiels, 2010). *Salmonella* spp. biofilms can exist on various surfaces in food processing industry such as plastic, glass, metal and wood (Steenackers et al., 2012). Surfaces of polypropylene are widely used in the food industry in tanks, and cutting surfaces and several studies have reported the occurrence of bacterial biofilms in this surface (Arutchelvi et al., 2011; Bayoumi et al., 2012; Millezi et al., 2012; Santos Júnior, 2011; Stoodley et al., 2012).

Various strategies to reduce bacterial biofilms in food contact surfaces have been considered and essential oils and their components appear to be an interesting alternative (Oliveira et al., 2010; Budzyñska et al., 2011; Kavanaugh and Ribbeck, 2012; Valeriano et al., 2012). Essential oils containing carvacrol and thymol as well as these components isolated have been reported by their ability into reduce bacterial biofilms in different surfaces (Nostro et al., 2007; Perez-Conesa, 2011; Soumya et al., 2011; Szczepanski and Lipski,A., 2014).

However, there are few reports of the action of essential oils on bacterial biofilms on polypropylene surfaces. Santos Júnior (2011) investigated the action of the thymus and clove essential oils on *Staphylococcus aureus* biofilm on polypropylene. Millezzi et al. (2012) reported the effect of the citronella and lemon grass essential oils on *S. aureus* and *Escherichia coli* biofilms on polypropylene.

According to our knowledge there are no reports of the action of the carvacrol and thymol on *Salmonella* spp. biofilms on polypropylene. So, in the present study, was evaluating the effect of carvacrol and thymol on *Salmonella* spp. biofilm on polypropylene surface.

## Materials and methods

*Test microrganisms.*

It was evaluated four *Salmonella* spp. isolates. One *Salmonella* Typhimurium strain ATTC (14028) and three isolates from food incriminated on foodborne diseases, *Salmonella* Enteritidis (502-00), *Salmonella* Saintpaul (510-02) and *Salmonella* Typhimurium (906-01).

The isolates were stored in Bain and Hearth Infusion (BHI; Difco, Le Pont de Claix, France) with glycerol 20% at -20 ºC at the Laboratory of Food Microbiology, Department of Biomedicine and Clinical Analysis, State University of Maringá. Prior to use, an aliquot of frozen isolates was transferred to BHI and incubated at 37 ºC for 24 h.

Subsequently, the culture was plated on Hektoen agar (Difco, Le Pont de Claix, France) and incubated at 37 ºC for 24 h.

*Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).*

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for thymol (purity ≥ 99,5%, Sigma-Aldrich), carvacrol (purity ≥ 98%, Sigma-Aldrich), and peracetic acid (Ecolabe - Brasil) were determined using a broth microdilution method according to the document M07-A9/2012 established by the Clinical and Laboratory Standards Institute (2012) as described below.

For MIC determinations, bacterial suspensions were standardized according to the 0.5 McFarland scale and diluted to obtain $5.10^5$ CFU/mL. Bacterial suspensions were inoculated into each well of a 96-well cell culture plate (TPP, Trasadingen, Switzerland) that contained 100 µl of thymol and carvacrol diluted in MHB Mueller Hinton broth (Difco, Le Pont de Claix, France) at concentrations of 19 to 5,000 µg/mL. After 24 h of incubation at 37 °C, the MIC was determined using a microplate reader (Asys Expert Plus) at 620 nm.

The MIC was defined as the lowest concentration of thymol and carvacrol that inhibited bacterial growth. All of the tests were performed in triplicate in two different experiments.

The peracetic acid, a chemical disinfectant, was used as control. The MIC of peracetic acid was verified adding 10µl of bacterial suspensions standardized ($5.10^5$ CFU/mL) to 100 µl of peracetic acid at concentrations of 200 ppm to 0.7815 ppm.

The microplates were maintained at room temperature for 10 minutes and then the acid was neutralized with 0.2% sodium thiosulfate. After neutralization, 10 µl of each well was plated on Hektoen agar to evaluate the MBC.

The microplates were incubated at 37 °C for 24 h of incubation and the MIC was determined using a microplate reader at 620 nm. All of the tests were performed in triplicate in two different experiments.

*Biofilm formation on polypropylene surface*

*Polypropylene surface.*

The procedure to preparing the polypropylene was performed according to Marques et al. (2007) with some modifications. Polypropylene 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 60 ºC, and autoclaved at 121 ºC for 15 min.

*Biofilm formation.*

Biofilm formation was performed according to Speranza et al., (2011) with modifications. Overnight *Salmonella* spp. cultures diluted 1:100 in TSB (Tryptic Soy

Broth- Difco®) to yield $10^7$ CFU/mL, confirmed by counting on Hektoen agar, was added to the polypropylene coupons into microtubes.

The microtubes were incubated at 37 ºC for 24 h. The contents were carefully replaced by new TSB and incubated for another 24 h at 37 ºC.

After incubation, the microtube contents were aspirated, and the coupons were rinsed with 0.85% sterile saline solution to remove planktonic cells. It was added 1500 µl of 0.85% sterile saline solution and the coupons were subjected to an ultrasonic bath at 25 KHz for 5 min (Ultra Cleaner 750A, Unique) to detach sessile cells.

These conditions were previously standardized in our laboratory. 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 as log CFU/$cm^2$. The tests were performed in triplicate in four different experiments.

*Effect of thymol and carvacrol on biofilm formation.*

Thymol and carvacrol were evaluated at sub-MIC concentrations (thymol at 234 µg/mL and 156 µg/mL and carvacrol at 117 µg/mL and 78 µg/mL). These compounds were added to microtubes with the coupons and overnight cultures of *Salmonella* spp. diluted 1: 100 in TSB ($10^7$ CFU/ml) and the microtubes were incubated at 37 ºC for 24 h. The coupons were carefully replaced with new TSB with the same concentrations of thymol or carvacrol, followed by incubation at 37 °C for 24 h. Biofilm bacterial cells were quantified as described above.

*Effect of thymol, carvacrol and peracetic acid on established biofilms.*

After biofilm formation on polypropylene for 48 h, the coupons treated with thymol at 312 µg/mL (1x MIC) and 624 µg/mL (2x MIC) or carvacrol at 156 µg/mL (1x MIC) and 312 µg/mL (2x MIC) After 1 h at room temperature, the coupons were rinsed with sterile 0.85% saline to remove planktonic cells, and biofilm bacterial cells were quantified.

The coupons were also treated with peracetic acid at 100 ppm (1x MIC) and 200ppm (2x MIC) for 10 minutes. The acid was neutralized with sodium thiosulfate 0.2% and bacterial cells were quantified as described above.

*Scanning electron microscopy.*

Biofilm formation on polypropylene surface was analyzed using scanning electron microscopy (SEM) according to Marques et al. (2007) with modification. The coupons treated with peracetic acid were previously neutralized with sodium thiosulfate 0.2% before their processing to electron microscopy.

The 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 fixation, the coupons were washed twice with cacodylate buffer and dehydrated in an increasing series of ethanol (50, 70, 80, 90, and 100% twice).

The coupons were critical-point dried in $CO_2$, coated with gold, and examined under a Shimadzu SS-550 scanning electron microscope.

*Statistical analysis.*

The data were typed in spreadsheet program Microsoft Excel 2010 and analyzed statistically with the aid of SAS 9.1 software. To evaluate the mean and standard deviation for quantitative variables, followed by the Mann-Whitney test for comparison of groups and the Kurskal-Wallis test was used to compare three or more groups, followed by Dunn test to ascertain which groups differ among them.

The significance level used in the tests was 5%, were considered significant associations whose $p < 0.05$.

## Results

*Biofilm formation on polypropylene surface.*

The number of *Salmonella* spp. viable cells on polypropylene surface was approximately 8 log CFU/cm$^2$ for all isolates evaluated (Table 1). Evident *Salmonella* spp. biofilm formation on polypropylene surface was observed by SEM (Figure 1), where bacteria were visualized as micro-colonies and the presence of different layers and exopolysaccharides, under 3,000 × magnification.

*Effect of carvacrol, thymol and peracetic acid on establish biofilm.*

Results of MIC and MBC were used to evaluated the effect of carvacrol, thymol and peracetic acid on *Salmonella* spp. biofilms. The following concentrations (1X and 2X MIC) were used: carvacrol at 156 and 312 ug / ml, thymol at 312 and 624 ug / ml and peracetic acid at 100 and 200 ppm. Significant reductions ($P < 0.05$) were observed when formed biofilms of all *Salmonella* spp. isolates were treated with carvacrol and thymol at different concentrations.

The treatment of *Salmonella* spp. isolated from food established biofilms with carvacrol and thymol (1x MIC and 2x MIC), showed reduction of approximately 1 to 4 log CFU/cm$^2$ (Table 1). The greatest reductions were observed with 312 µg/mL (2x MIC) of carvacrol and 624 µg/mL (2x MIC) of thymol, in which we observed a decrease of approximately 3 log cycles and 4 log cycles in bacterial counts, respectively. As observed in Table 1, *S.* Typhimurium ATTC 14028 biofilms on polypropylene was reduced 5 log cycles after treatment with carvacrol in both concentrations (1x MIC and 2x MIC).

Thymol could reduce only 1 log cycle of *S.* Typhimurium *(*ATTC 14028) viable counts (Table 1). The scanning electron microscopy (Figure 1) illustrates the effect of carvacrol and thymol against biofilm when compared to the untreated control. Treatment of *Salmonella* spp. biofilms established with peracetic acid at concentrations of 100 ppm (1x MIC) and 200 ppm (2x MIC) drastically reduced the bacterial count (<2 log CFU/cm$^2$).

*Effect of carvacrol and thymol during Salmonella spp. biofilm formation on polypropylene surface.*

Treatment with carvacrol and thymol at sub-MIC concentrations during biofilm formation resulted on a decrease of about 1 and 2 log cycles in the three *Salmonella* spp. isolated from food (Table 2). The greatest reduction was observed on *S.* Saintpaul treated with thymol at 156 µg/mL.

Biofilm viable cells counts of *S.* Typhimurium ATTC 14028 were reduced 2 log and 4 log cycles when treated with carvacrol at 78 µg/mL and 117 µg/mL, respectively (Table 2). Sub-MIC concentrations of thymol during biofilm also reduced bacterial counts.

A statistically significant reduction ($p < 0.05$) was observed on biofilms treated with carvacrol and thymol at all sub inhibitory concentrations tested.

The effect of carvacrol and thymol during *Salmonella* spp. biofilm formation was also observed by scanning electron microscopy, in which biofilm was not visualized (data not shown).

## DISCUSSION

*Salmonella* spp. biofilms are usually found in food processing environments, serving as a persistent reservoir of contamination, compromising food safety and human health (Steenackers et al., 2012; Van Houdt and Michiels, 2010). This study demonstrates the effect of carvacrol and thymol against Salmonella spp. biofilm on polypropylene.

It was verified by bacterial counts and by SEM that the isolates were able to form biofilms on polypropylene, as also related elsewhere (Bayoumi et al., 2012; Iibuchi et al., 2010, Oliveira et al., 2006). This result is important when we consider that polypropylene is a material currently used in the industry to build tanks, fittings, pipes and surfaces in food processing and a common source of contamination, which therefore require control measures (Millezi et al. 2012)

Few studies about the capacity of essential oils on biofilm on polypropylene can be found. Millezi et al (2012) demonstrated that the essential oils of lemon and citronella reduced *E. coli* and *S. aureus* biofilm on polypropylene. Thyme and clove essential oils were effective on *S. aureus* biofilms formed on polypropylene as demonstrated by Santos Júnior (2011). However, according to our knowledge there was no report about the effect of the carvacrol and thymol on *Salmonella* spp. biofilm on polypropylene. We only found some studies reported the effect of carvacrol on *S.* Typhimurium biofilms on polystyrene and steel surfaces (Knowles et al., 2005; Knowles and Roller, 2001; Soni et al., 2013).

The effectiveness of essential oils is frequently determined by the number of surface-adhered cells thymol on they are capable to reduce, obtained by standard plate count (Millezi et al., 2012). Our results demonstrated that treatment of *Salmonella* spp. biofilms with carvacrol and thymol reduced bacterial counts in both biofilm formation and establish *Salmonella* spp. biofilm.

Carvacrol was able to reduce the bacterial counts until 5 log cycles in established *Salmonella* spp. biofilms and 4 log cycles during biofilm formation, showing better results than those found by Knowles et al. (2005) who observed a reduction of 3 log cycles during biofilm formation and by Knowles and Roller (2001) who obtained a reduction of 2.6 log cycles on viable counts of *S*. Typhimurium adhered to stainless steel. Soni et al. (2013) reported a reduction of 7 log cycles in S. Typhimurium biofilm treated with carvacrol on the steel surface

Our results demonstrate that treatment with thymol during biofilm formation and on established *Salmonella* spp. biofilm, resulted in reduction of approximately 1 to 2 log cycles in bacterial counts. Soumya et al. (2011) and Nostro et al. (2007) demonstrated the efficacy of thymol in *Pseudomonas aeruginosa* and *Staphylococcus* spp. respectively polystyrene biofilms. According to our knowledge there has been no report in the literature about the effect of thymol on biofilms of *Salmonella* spp.

The effectiveness of carvacrol and thymol during and after *Salmonella* spp. biofilm formation was also observed by SEM (Figure 1), where we visualized a disruption of the typical structure of biofilm illustrating the results observed by viable cell counts.

In this study *Salmonella* spp. biofilm was reduced after the treatment with carvacrol and thymol. However, these treatments were unable to eliminate the bacterial cells on polypropylene. It suggests that they can be more efficient when used in surfaces that containing little bacterial contamination.

Peracetic acid, a disinfectant commonly used in food industry, eliminated *Salmonella* spp. biofilms when exposed at 100 ppm during 10 min. Castelijn et al (2013) used short exposure time (2 min) to peracetic acid at the same concentration against *S*. Typhymurium, *S*. Derby, *S*. Brandenburg and *S*. Infantis biofilms on steel and polystyrene surface and obtained a reduction that ranges from 5 to 7 log cycles.

The effectiveness of the peracetic acid was better than carvacrol and thymol, but, according to Surdeau et al. (2006) a disinfectant is considered to be effective if it reduces a microbial population attached to a surface by 3 log cycles.

Thus, carvacrol at 117 µg/mL and 156µg/mL was effective against *S.* Typhimurium ATCC (14028) during and after biofilm formation, since it reduced more than 3 log cycles. Effectiveness was also verified with carvacrol at 312 µg/mL that reduced *S.* Saintpaul established biofilm approximately 3 log cycles. Similar results were evidenced with thymol at 624 µg/mL against *S.* Enteritidis.

## Conclusion

We concluded that carvacrol and thymol were able to reduce *Salmonella* spp. biofilm on polypropylene. The use of essential oils and their components can be an effective alternative or supplement to control bacterial biofilms.

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**Table 1.** Effect of carvacrol and thymol on *Salmonella* spp. established biofilms on polypropylene.

| | | **Carvacrol** | | **Thymol** | |
|---|---|---|---|---|---|
| | | Mean Log CFU/cm$^2$ (SD) | | Mean Log CFU/cm$^2$ (SD) | |
| **Isolates** | **Controle** | **156 µg/ml** | **312 µg/ml** | **312 µg/ml** | **624 µg/ml** |
| *S*. Enteritidis (502-00) | 8.32± 0.089 | 6.23± 0.235 | 6.44± 0.151 | 6.83±0.644 | 4.95±0.742 |
| *S*. Saintpaul (510-02) | 8.54±0.111 | 7.25±0.235 | 5.38±0.224 | 7.06±0.095 | 6.63±0.378 |
| *S*. Typhimurium (906-01) | 8.28±0.154 | 6.91±0.131 | 7.41±0.134 | 6.99±0.116 | 6.28±0.252 |
| *S*. Typhimurium (ATCC 14028) | 8.43±0.047 | 3.43±0.825 | 3.71±0.370 | 7.41±0.042 | 7.27±0.062 |

Values represent the mean ± S.D. of at least two independent experiments performed in triplicate. All treatments differed from their respective controls by Kruskal-Wallis test ($p < 0.05$) followed by Dunn test.

**Table 2**. Effect of sub-MIC concentrations of carvacrol and thymol on *Salmonella* spp. biofilm formation on polyproylene.

| | | **Carvacrol** Mean Log CFU/cm$^2$ (SD) | | **Thymol** Mean Log CFU/cm$^2$ (SD) | |
|---|---|---|---|---|---|
| **Isolates** | **Controle** | **78 µg/mL** | **117 µg/mL** | **156 µg/mL** | **234 µg/mL** |
| *S*. Enteritidis (502-00) | 8.32± 0.089 | 6.85±0.274 | 6.75±0.333 | 7.42±0.095 | 7.38±0.097 |
| *S*. Saintpaul (510-02) | 8.54±0.111 | 7.57±0.247 | 7.30±0.074 | 6.29±0.145 | 6.64±0.407 |
| *S*. Typhimurium (906-01) | 8.28±0.154 | 7.01±0.190 | 7.00±0.531 | 7.15±0.073 | 7.07±0.588 |
| *S*. Typhimurium (ATCC 14028) | 8.43±0.047 | 6.23±0.296 | 4.07±0.548 | 7.15±0.156 | 6.72±0.246 |

Values represent the mean ± S.D. of at least two independent experiments performed in triplicate. All treatments differed from their respective controls by Kruskal-Wallis test ($p < 0.05$) followed by Dunn test.

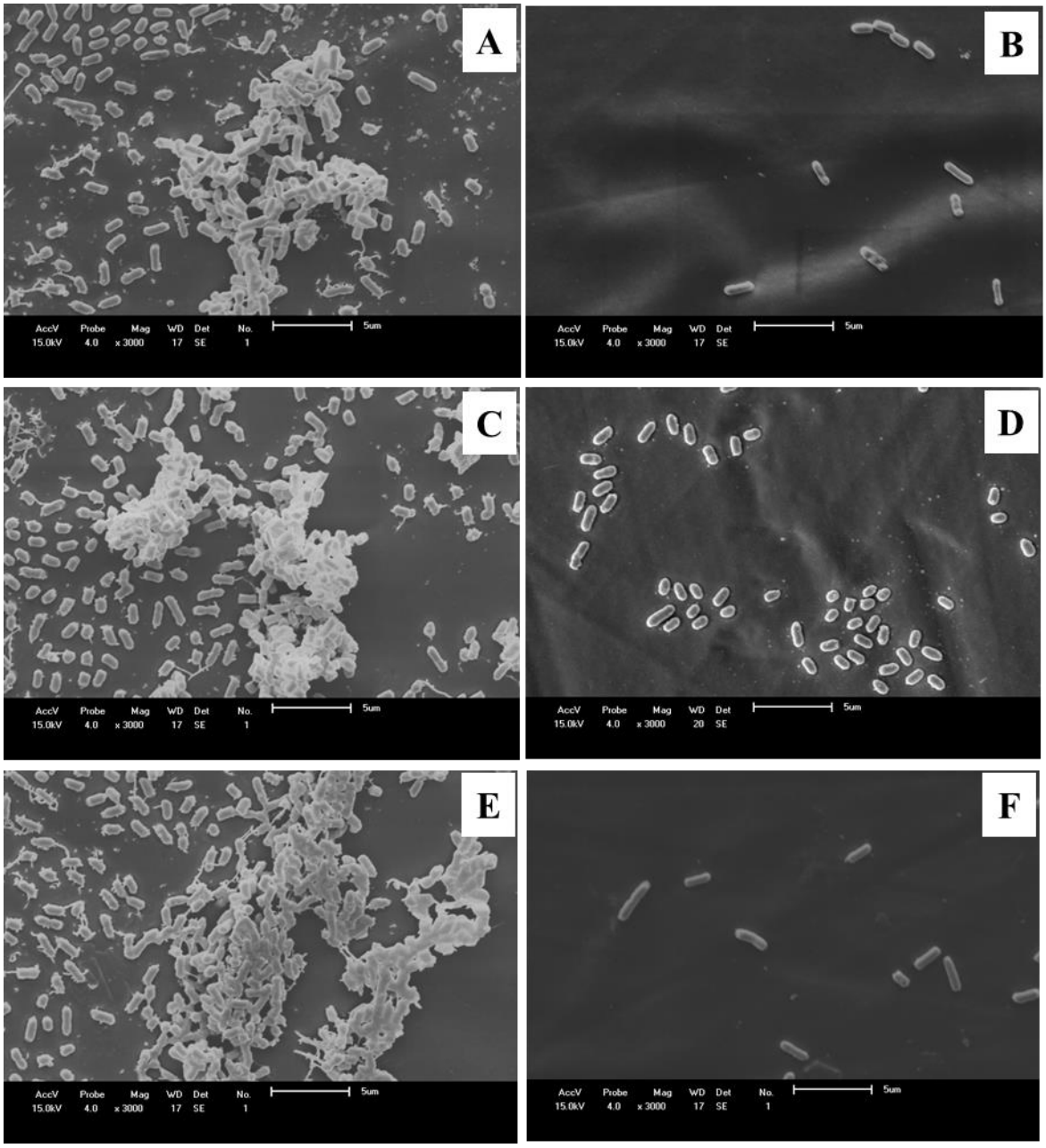


**Fig. 1**. Scanning electron microscopy (SEM): A) *S*. Saintpaul biofilm without treatment; B) *S*. Saintpaul biofilm after treatment with carvacrol at 312 µg/mL; C) *S*. Enteritidis biofilm without treatment; D) *S*. Enteritidis biofilm after treatment with thymol at 624 µg/mL; E) *S*. Typhmurium ATCC 14028 biofilm without treatment; F) *S*. Typhmurium ATCC 14028 biofilm after treatment with carvacrol at 312 µg/mL.

# CAPÍTULO III

O presente estudo sobre a avaliação do efeito dos óleos essenciais de tomilho (*Thymus vulgaris* L.) e segurelha (*Satureja hortensis* L) e seus compostos majoritários em diferentes sorotipos de *Salmonella* spp., e a ação do carvacrol e do timol em biofilmes de *Salmonella* spp. em polipropileno demonstrou que:

1) Os óleos essenciais de tomilho e segurelha, o carvacrol e o timol isoladamente apresentaram ação antimicrobiana;

2) A combinação de timol e carvacrol apresentou efeito sinérgico;

3) Os diferentes isolados de *Salmonella* spp. foram capazes de formar biofilme em polipropileno;

4) Carvacrol e timol foram efetivos na redução de biofilmes de *Salmonella* spp. em polipropileno, tanto durante quanto após sua formação;

5) O ácido peracético, utilizado como sanitizante padrão, apresentou atividade anti-*Salmonella* spp. e reduziu drasticamente o biofilme bacteriano formado.

Em seguida, são apresentadas algumas sugestões para futuros trabalhos:

- Testar o efeito dos compostos naturais em biofilmes mistos;
- Testar outras superfícies utilizadas na produção de alimentos;
- Testar outras substâncias isoladas e combinadas entre si;
- Utilizar outras metodologias para avaliar biofilmes bacterianos.