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Interaction of *Salmonella* sp. and essential oils: bactericidal activity and adaptation capacity

Interacción entre *Salmonella* sp. y aceites esenciales: actividad bactericida y adaptabilidad

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Abstract

Bacteria of the genus *Salmonella*, responsible for many foodborne disease outbreaks, are capable of forming biofilms on various surfaces in the food industry. The constant exposure of these bacteria to sublethal concentrations of sanitizers has made them tolerant to several of them. Seeking alternatives to control of bacterial biofilms and adaptation under sublethal exposure, this study tested the antimicrobial activity of *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano) essential oils (EOs) and their major compounds, thymol and carvacrol, against of *Salmonella* enterica serovars Enteritidis and Typhimurium. Carvacrol 0.25% (v/v) was the most efficient antimicrobial agent against planktonic cells of *S*. Enteritidis and biofilm were more susceptible to oregano EO at 2.0% (v/v). Differently, *S.* Typhimurium planktonic was inhibited at 0.25% (v/v) of thyme EO and biofilm was more susceptible to carvacrol (2.5% v/v). Adaptation of *S.* Enteritidis and Typhimurium was observed on all tests ($p < 0.05$). This study confirms the potential of EOs and its major compounds as alternative sanitizers in the food industry against pathogenic bacteria such as *Salmonella* spp. and of possible adaptation due to sublethal exposure.

Keywords: biofilm, planktonic cells, carvacrol, thymol, stress response.

Resumen

La bacteria *Salmonella*, responsable de numerosos brotes de intoxicación alimentaria, puede formar biopelículas en varias superficies utilizadas en la industria alimentaria. La exposición constante de estas bacterias a concentraciones subletales de agentes desinfectantes los hace tolerantes a muchos de estos agentes. Buscando alternativas para el control de biofilms adaptadas a condiciones subletales, este estudio evaluó la actividad antimicrobiana de los aceites esenciales *Thymus vulgaris* (tomillo) y *Origanum vulgare* (orégano) y sus componentes principales timol y carvacrol contra *Salmonella enterica* serovares Enteritidis y Typhimurium. Carvacrol 0.25% (v/v) fue más eficiente contra las células planctónicas de *S.* Enteritidis y su biopelícula fue más sensible al 2.0% (v/v) de orégano EO. *S.* Typhimurium en forma planctónica fue inhibida por el tomillo EO a una concentración de 0.25% (v/v) y su biopelícula fue más susceptible al carvacrol (2.5% v/v). *S.* Enteritidis y *S.* Typhimurium se adaptaron a todas las concentraciones subletales de los antimicrobianos probados. Este estudio confirma el uso potencial de EO y sus componentes principales como desinfectantes en la industria alimentaria para controlar bacterias patógenas como *Salmonella* spp. y su capacidad para adaptarse a concentraciones subletales.

Palabras clave: biopelículas, células planctónicas, carvacrol, timol, respuesta al estrés.

Introduction

It is well known that the implementation of health control measures, cleaning and sanitization in food industries can prevent economic loss. Aiming at the safety of their products, the food industry use antimicrobial agents with varied modes of action, exposure time and chemical composition. However, the hygienic programs have been inefficient and often unable to completely remove bacterial biofilms that accumulate on surfaces and equipment of food processing environments, even considering all the misfortunes that these contaminations can cause regarding microbiological aspects. Several factors contribute to this situation, mainly the loss of susceptibility to antimicrobial agents due to the frequent exposure of pathogenic bacteria, such as *Salmonella*, to sublethal concentrations of sanitizers during cleaning sessions.

Bacteria of the genus *Salmonella* are foodborne pathogens of global coverage and cause the most outbreak-related in the world (CDC, 2018). The contamination caused by these Gram-negative rod-shaped microorganisms can bring great losses to the food industry through embargoes and taxes established by importing countries (Shinohara et al., 2008), and Brazil is a major exporter of meat and poultry (Brasil, 2015). Salmonellosis is considered a disease of major importance for human health by the high risk of mortality and morbidity. It typically causes gastroenteritis in humans and the infection is related to precarious hygiene conditions in poultry farms (Pui et al., 2011).

Among all *Salmonella enterica* serotypes, Typhimurium and Enteritidis are the two most frequently observed in salmonellosis-recorded outbreaks (Mor-Mur and Yuste, 2010). *Salmonella enterica* serotypes can survive in a broad range of temperature (7 to 48 °C), pH (4.3 to 9.3) and frequently associated to resistance to common antibiotics and sanitizers (D'Aousts, 1997, McLaren *et al*., 2011). In addition, *Salmonella* spp. has great ability to form biofilms on surfaces and equipment of food industries (Steenackers *et al*., 2012, Fuente-Núñez *et al*., 2013). Biofilms are cells aggregates in which increase significantly the prevalence of pathogenic strains in various food environments (Yaron & Romling, 2014). Due to the difficulty to control the development of biofilms formed by *Salmonella* spp. and other microorganisms, the food industry is in need for new products with active ingredients with antimicrobial efficiency and non-toxic to humans.

Essential oils (EOs) and their components are renowned to be effective against a wide range of microorganisms, including pathogenic bacteria (Burt, 2004). EOs are distinguished by high antimicrobial activity and, in appropriate concentrations, generally recognized as safe. Thus, it is considered exempt from food additive tolerance requirements for its use (Smith *et al*., 2005). Essential oils of *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme) contain, among other compounds, thymol and carvacrol, which are considered powerful bactericides and fungicides (Kalemba and Kunicka, 2003). Induced stress conditions as exposure to sublethal concentrations of bactericidal compounds are an important evaluation to indicate adaptive capacity of microorganisms. However, information on possible effects of using EOs or its compounds at sublethal concentrations on microbial sensitivity to antimicrobials or physical processes is still scarce (Souza, 2016).

The investigation of bacteria challenged with sublethal stresses reveal significant physiological changes that may enhance their ability to survive the imposed hostile conditions. In recent literature, a plenty of evidences shows that exposure of bacteria to sublethal stresses may induce decrease of sensitivity to food antimicrobials, biocides and other food preservation techniques (Erickson and Doyle, 2017). Furthermore, relevant increase of virulence and lower infectious doses in pathogenic bacteria brings even more concern to food antimicrobials, such as essential oils (Gadea et al., 2017).

The aim of this work was to evaluate the antimicrobial effect of *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano) EOs and its major compounds, thymol and carvacrol, against *Salmonella Enteritidis* and *Typhimurium serovars* testing the adaptive response of their biofilms to sublethal concentrations of these substances and classifying their biofilm formation capacity.

Material and methods

Essential oils and major compounds

The essential oils of *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme) were acquired by Ferquima Indústria e Comercio Ltda (Vargem Grande Paulista, São Paulo, Brazil). Oregano EO composition was specified by the supplier pointing carvacrol (71%), γ-terpinene (4.5%), β-cariofilene (4.0%); p-cimene (3.5%), thymol (3.0%), while thyme EO contained thymol (47.3%), p-cimene (26.8%), γ-terpinene (6.0%), linalol (5.2%), carvacrol (3.1%), α-pinene (2.2%), mircene (1.4%), 1.8-cineole (1.3%), borneol (0.9%), canfene (0.8%) and β-cariofilene (0.8%). In addition, the high-purity major compounds of thymol (99.5%) and carvacrol (98%) were purchased from Sigma-Aldrich®.

Microorganisms

Salmonella enterica subspecies *enterica* serovars Enteritidis S64 and Typhimurium S190 were donated by the Laboratory of Enterobacteria (LABENT) at Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, Brazil). Stock culture was stored in preservation culture medium and reactivation occurred in Brain Heart Infusion broth (BHI) (HIMEDIA) incubation at 37°C for 24h. Standard inoculum was obtained by growth curve and tests were carried out using 10^8 CFU/mL. All analysis were performed in Laboratory of Food Microbiology of Federal University of Lavras, Minas Gerais.

Formation and classification of biofilms

Biofilms were formed by inoculation of 50 µL aliquots of standard cultures into wells containing 150 µL of TSB followed by incubation at 37°C for 48 hours. Biofilm formation was determined by absorbance measures of crystal violet (0.1% w/v) added into each well at 600 nm in a microplate reader Anthos 2010 (Biochrom®), after wash/dry periods and addition of ethanol 95% (v/v) (Merritt *et al*., 2005). Classification of biofilms followed Stepanović *et al*. (2000) proposal where "Dob" is optical density of biofilm and "Donc" is optical density of negative control: no biofilm former (Dob ≤ Donc), weak biofilm former (Donc < Dob ≤ 2x Donc), moderate biofilm former (2x Donc < Dob ≤ 4x Donc) strong biofilm former (4x Donc < Dob). Final measures were obtained by arithmetic mean of absorbance of eight replicates. Statistical analyses were performed using Kruskall-Wallis test and SPSS 19.0 program ($p < 0.05$).

Minimal bactericidal concentration of essential oils and major compounds against planktonic and sessile cells

The minimum bactericidal concentration against planktonic cells (MBC) and biofilms (MBCB) of oregano and thyme EOs, thymol and carvacrol was determined using microdilution technique with 96-well polystyrene microplates according to CLSI-M100 (Clinical and Laboratory Standards Institute, 2019) with modifications. EOs and major compounds solutions were diluted in Tryptic soy broth (TSB) (HIMEDIA®), with addition of 0.5% Tween 80, in concentrations of (%): 0.03; 0.06; 0.12; 0.25; 0.50 and 1.00 (v/v). Then, microplates with 10 µL of standard cultures and solutions were sealed and incubated at 37ºC for 24h, followed by Tryptic soy agar (TSA) (HIMEDIA®) plating by microdrop technique using 10 µL of each well and 37°C/24h incubation to obtain the MBC of substances.

After biofilm formation, cultures were removed, washed, and EOs and major compounds solutions with 0.5% Tween 80 were added in such concentrations (%) (v/v): 0.12; 0.25; 0.50; 1.00; 2.00; 2.50; 3.00; 3.50; 4.00; 4.50; 5.00 and 6.00. After 20 min, tested cultures were washed and incubated with addition of TSB during 24h followed by TSA plating 37°C for 24h in order to obtain the MBCB. Tests were performed in triplicate and three repetitions using negative (TSB with 0.5% Tween 80 and EO or major compounds) and positive (TSB with 0.5% Tween 80 and inoculum) control.

Adaptation homologue of sessile cells to antimicrobials

Solution of TSB with 0.5% Tween 80 and sublethal concentrations (1/4 MBCB) of thyme (0.06%) or oregano (0.12%) EOs, carvacrol (0.12%) or thymol (0.12%) were added into the wells and inoculated with 50 µL of standard cultures. The microplates were sealed and incubated at 37ºC for 48h. After this period, exposed cultures were removed, washed and tested against new concentrations of EOs and major compounds: 2.00; 2.50; 3.00; 3.50; 4.00; 4.50; 5.00 and 6.00 % (v/v). After 20 minutes, solutions were removed and washed. Then, TSB was added in order to incubate adapted biofilms at 37°C for 24h, followed by TSA plating during 24h at 37°C.

Results and discussion

Table 1 displays the minimal bactericidal concentrations against planktonic (MBC) and sessile (MBCB) cells, and adapted biofilms ($MBCB_A$) of EOs and major compounds. Susceptibility tests revealed that MBC of EOs and major compounds varied from 0.25 to 1.00 (% v/v) against planktonic cells of both *Salmonella* serovars ($p < 0.05$) and all MBCB were above 1.00% (v/v).

EOs and major compounds tested against *S.* Enteritidis and Typhimurium biofilms showed higher minimal bactericidal concentrations (MBCB) than those obtained against planktonic cells (MBC) and significant differences between them were found ($p<0.05$). Several factors are involved in this increased tolerance of cells in biofilms to antimicrobial agents, including the matrix of extrapolymeric substances (EPS) in which the cells are embedded limiting the diffusion of antimicrobials. Various substances are also found embedded in the EPS reacting with these agents and reducing their efficiency (Bridier *et al*., 2011). In addition to EPS, it is known that when in biofilm, cells multiply more slowly, increasing tolerance to antimicrobials, which is a major concern in food safety standards (Srey *et al*,. 2013).

It was observable a significant difference between carvacrol and thymol MBC and is well known in literature that inactivation of microbial

Table 1. Essential oils and major compounds minimal bactericidal concentrations (% v/v) against planktonic and sessile cells and adapted biofilms of *Salmonella* serovars

Biocidal		***S.* Enteritidis**			***S.* Typhimurium**		
	%	**MBC**	**MBCB**	**$MBCB_A$**	**MBC**	**MBCB**	**$MBCB_A$**
Oregano EO		0.5 ±0.07	2.0 ±0.12	5.0 ±0.13	1.0 ± 0.13	3.0 ±0.29	6.0 ±0.25
Thyme EO		0.5 ±0.07	2.5 ±0.12	4.5 ±0.13	0.25 ±0.13	2.5 ±0.29	3.0 ±0.25
Thymol		0.5 ±0.07	3.0 ±0.12	6.0 ±0.13	0.5 ±0.13	5.0 ±0.29	6.0 ±0.25
Carvacrol		0.25 ±0.07	2.5 ±0.12	4.5 ±0.13	0.5 ±0.13	2.5 ±0.29	5.0 ±0.25

enzymes is also related to the presence of the hydroxyl group in monoterpenes (Bakkali *et al*., 2008). The group can interact with the cell membrane causing leakage of cellular components through membrane. Thymol (4-isopropyl-2-metylphenol) and carvacrol (2-isopropyl-5-metylphenol) are isomers differing only by the position of hydroxyl group. This difference in the positions changes the reactivity of each compound since most of the reactions must occur by interaction with the hydroxyl group. It is possible that, in carvacrol tests, the steric hindrance performed by methyl is much smaller than propyl performs on thymol, due to its size and number of present atoms. In methyl, there is only one carbon and three hydrogen atoms to hinder interaction with the hydroxyl group while in thymol, propyl offers three carbon and seven hydrogen atoms to that effect (Mastelic *et al*,. 2008, Hyldgaard *et al*., 2012, Meeran *et al*., 2017).

Both serotypes were capable to adapt to tested antimicrobial compounds. The comparison between MBCB and $MBCB_A$ shows significant differences ($p<0.05$) among biofilm adaptational conditions. Much higher concentrations were required to inhibit *S.* Enteritidis biofilm adapted in sublethal doses of EOs and major compounds solutions, suggesting an increased tolerance to antimicrobial agents when exposed to mild-stress conditions. Statistically significant values in different concentrations compared by Kruskall-Wallis test ($\alpha = 0.05$) revealed that thyme EO showed no significant difference between biofilm and adaptation to sublethal concentrations to *S.* Typhimurium.

Table 2 displays the optical densities of biofilms exposed to sublethal concentrations of EOs and major compounds in order to classify them. According to Stepanović *et al*. (2000) classification, it can be observed that both strains, even after culturing in the presence of sublethal concentrations of antimicrobials, remain considered as "strongly biofilm forming".

The response to environmental stress of microorganisms is well-known by factors such as temperature, pH, osmolality, antibiotics and sanitizers. In addition, *Salmonella* sp. activates regulators in response to environmental stress that promote increased and / or decreased gene expression leading to higher tolerance to this or other types of stress (e.g. thermal), allowing this pathogen to survive in food processing environments. This adaptation can also lead to increased virulence and resistance to several antimicrobial agents (Spector & Kenyon, 2012). However, it is not completely understood in regard of the adaptability of bacteria to essential oils and their compounds both in planktonic and sessile forms (Oloketuyi & Khan, 2017; Rossi *et al.,* 2017).

Phenotypic changes caused in *Salmonella* by exposure to sublethal concentrations of oils and their compounds have been reported. The exposure to sub-lethal concentrations of thyme and oregano EO and carvacrol, thymol, trans-2-hexenal and citral of *Listeria monocytogene*s, *S.* Enteritidis and *Escherichia coli* induced a marked increase of some membrane associated fatty acids, particularly unsaturated fatty acids, trans-isomers, and specific released free fatty acids (Siroli *et al*. 2015). *S.* Enteritidis 86 (SE86) grown in sublethal concentrations of oregano EO and carvacrol exhibited alteration in gene expression associated with repair of cell damage caused by osmotic, oxidative, acid stress and thermal shock (Cariri *et al*., 2019). However, the study was not evaluated if increased tolerance to antimicrobial has occurred. In another study, *Salmonella* Senftenberg, isolated from an outbreak linked to the herb *Ocimum basilicum* L. (basil) adapted to linalool with a minimal inhibitory concentration increasing of at least 8-fold and conferred heterologous adaptation to the antibiotics trimethoprim, sulfamethoxazole, piperacillin, chloramphenicol and tetracycline (Kalily *et al*., 2017)isolated from an outbreak linked to the herb Ocimum basilicum L. (basil. These information shows that exposure to inadequate concentrations of EOs or major compounds can also increase bacterial tolerance to other stressors in the processing environment, leading to bacterial persistence in industry and food.

For biofilm cells, similar results to this study were obtained by Zou *et al*. (2012). The biofilm and dispersed cells of *S.* Typhimurium showed higher

Table 2. Biofilm formation capacity of the two serotypes of *Salmonella* grown in presence of sublethal concentration of essential oils and major components

Serotype	Sublethal stress	Conc. (%)	DOA	DOCN	Biofilm class.
Enteritidis	control	0.0	0.27±0.02	0.06±0.002	FFB
	Oregano EO	0.25	0.29±0.02	0.06±0.002	FFB
	Thyme EO	0.12	0.27±0.02	0.06±0.002	FFB
	thymol	0.12	0.28±0.02	0.06±0.002	FFB
	carvacrol	0.06	0.30±0.03	0.06±0.002	FFB
Typhimurium	control	0.0	0.29±0.02	0.06±0.002	FFB
	Oregano EO	0.12	0.30±0.02	0.06±0.002	FFB
	Thyme EO	0.06	0.28±0.03	0.06±0.002	FFB
	thymol	0.12	0.29±0.03	0.06±0.002	FFB
	carvacrol	0.12	0.34±0.02	0.06±0.002	FFB

Non biofilm forming - NF (Doa < Docn), Weakly biofilm forming - FF, (Docn < Doa ≤ 2 x Docn), moderately biofilm forming - MF MF (2 x Docn < Doa ≤ 4 x Docn), and strongly biofilm forming– FFB (4 x Docn < Doa). Where Doa is biofilm optical density and Docn, negative growth control optical density.

resistance to antimicrobials, allyl isothiocyanate, thymol, eugenol and polyphenol, than the planktonic cells after cultivation in the presence of sublethal concentrations of the compounds. In this regard, the potential use of essential oils and its major compounds as alternative sanitizers raises awareness to concentration adjustment in order to avoid sublethal exposure leading to microbial adaptation and persistence on common food industry surfaces.

Conclusions

Carvacrol and thyme EO were more efficient against planktonic cells of *S.* Enteritidis and Typhimurium, respectively, while oregano EO showed better performance against *S.* Enteritidis biofilms. Adaptation was observed on all treatments and both serovars were classified as strong biofilm formers, proving the high risk of resistance development of *Salmonella* sp. to sublethal doses of EOs and major compounds.

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