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Research Paper

Habituation of enterotoxigenic *Staphylococcus aureus* to *Origanum vulgare* L. essential oil does not induce direct-tolerance and cross-tolerance to salts and organic acids

Adassa Gama Tavares[1], Daniel Farias Marinho do Monte[1],
Allan dos Reis Albuquerque[2], Fábio Correia Sampaio[2], Marciane Magnani[3],
José Pinto de Siqueira Júnior[4], Evandro Leite de Souza[1]

[1]Departamento de Nutrição, Universidade Federal da Paraíba, PB, Brazil.
[2]Laboratório de Biologia Oral, Universidade Federal da Paraíba, PB, Brazil.
[3]Departamento de Engenharia de Alimentos, Universidade Federal da Paraíba, PB, Brazil.
[4]Departamento de Biologia Molecular, Universidade Federal da Paraíba, PB, Brazil.

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Abstract

Enterotoxigenic *Staphylococcus aureus* strains that were isolated from foods were investigated for their ability to develop direct-tolerance and cross-tolerance to sodium chloride (NaCl), potassium chloride (KCl), lactic acid (LA) and acetic acid (AA) after habituation in sublethal amounts (1/2 of the minimum inhibitory concentration - 1/2 MIC and 1/4 of the minimum inhibitory concentration - 1/4 MIC) of *Origanum vulgare* L. essential oil (OVEO). The habituation of *S. aureus* to 1/2 MIC and 1/4 MIC of OVEO did not induce direct-tolerance or cross-tolerance in the tested strains, as assessed by modulation of MIC values. Otherwise, exposing the strains to OVEO at sublethal concentrations maintained or increased the sensitivity of the cells to the tested stressing agents because the MIC values of OVEO, NaCl, KCl, LA and AA against the cells that were previously habituated to OVEO remained the same or decreased when compared with non-habituated cells. These data indicate that OVEO does not have an inductive effect on the acquisition of direct-tolerance or cross-tolerance in the tested enterotoxigenic strains of *S. aureus* to antimicrobial agents that are typically used in food preservation.

Key words: *Staphylococcus*, adaptation, essential oil, oregano.

Introduction

Food processing exposes spoilage and pathogenic food-related bacteria to various stress-inducing conditions, including low pH, salts or treatments with cleaners and disinfecting agents (Cebrián *et al.*, 2010). However, the use of stressing factors in food processing can cause sublethal damage to bacterial cells, and during the injury repair process, these cells could acquire new abilities to adapt to these stress-inducing agents (direct-tolerance), leading to impacts on food safety and preservation (Silva-Angulo *et al.*, 2014). These responses can also activate the intrinsic resistance mechanisms that concomitantly decrease the susceptibility of cells to other unrelated antimicrobial compounds or procedures (cross-tolerance), meaning major implications for food processing in which multiple stresses are often applied to control microbial growth and survival (Greenacre and Brocklehurst, 2006).

Staphylococcus aureus is one of the most common causes of foodborne diseases worldwide, causing a typical intoxication through the ingestion of enterotoxins that have been pre-formed in foods by enterotoxigenic strains (Wang *et al.*, 2013). Previous studies have shown that *S. aureus* is capable of developing tolerance to heat, acidic pH and salts when exposed to sublethal stress conditions (Bikels-Goshen *et al.*, 2010; Cebrián *et al.*, 2010). The tolerance acquired by *S. aureus* to many procedures used by the food

Send correspondence to E.L. Souza. Departamento de Nutrição, Centro de Ciências da Saúde, Universidade Federal da Paraíba, Campus I, 58051-900 Cidade Universitária, João Pessoa, PB, Brasil. E-mail: evandroleitesouza@ccs.ufpb.br.

industry to control bacterial growth and survival has motivated the research and development of novel techniques to control this bacterium in foods (Gomes Neto *et al.*, 2012; Luz *et al.*, 2013).

In this context, essential oils and their active components have received attention as alternative anti-*S. aureus* compounds to use in foods (Bakkali *et al.*, 2008). Earlier investigations revealed that *Origanum vulgare* L. essential oil (OVEO) possesses broad-spectrum antimicrobial activity even at low concentration, with interesting results in inhibiting the growth of a variety of bacteria and food-related fungi when assayed alone (Nostro *et al.*, 2004; Sousa *et al.*, 2013; Souza *et al.*, 2009; Gomes Neto *et al.*, 2012) or in combination with other antimicrobial compounds or procedures used by food industry (Barros *et al.*, 2009b; Oliveira *et al.*, 2010). Studies have also revealed that OVEO possesses a strong capacity to inhibit *S. aureus* in synthetic, food based-broth and in food models, besides to suppress the action of some related virulence factors, including enterotoxin, biofilm production and synthesis of the enzymes lipase, protease and coagulase (Nostro *et al.*, 2007; Barros *et al.*, 2009a). Although the anti-*S. aureus* activity of OVEO has already been reported, little attention has been paid to the response of this bacterium when exposed to sublethal amounts of this substance.

The aim of this study was to assess the effects of exposing enterotoxigenic *S. aureus* strains that were isolated from foods to sublethal OVEO concentrations for different time points on the development of bacterial direct-tolerance and cross-tolerance to salts and organic acids typically used by the food industry. To the best of our knowledge, this is the first study on the induction of direc-tolerance or cross-tolerance in enterotoxigenic *S. aureus* strains from foods in which the strains were subjected to OVEO habituation and further assessed for modulation of the Minimum Inhibitory Concentration (MIC) values.

Materials and Methods

Antimicrobial agents

The antimicrobial agents used in this study were OVEO (Laszlo Aromaterapia Indústria e Comércio Ltda., Minas Gerais, Brazil), sodium chloride (NaCl P.A.), potassium chloride (KCl), glacial acetic acid (AA) and lactic acid 85% (LA). The NaCl, KCl, AA and LA were obtained from Vetec Química Fina Ltda. (Rio de Janeiro, Brazil). The OVEO assayed in this study present carvacrol as the most prevalent compound (66.1 g/100 mL), followed for *p*-cymene (12.4 g/100g) and γ-terpinene (8.3 g/100g), according to the technical report presented by the supplier.

OVEO solutions (40-0.3 $\mu L\ mL^{-1}$) were prepared in sterile brain heart infusion (BHI) broth (Himedia, India) with Tween 80 (1%) (Sigma Aldrich, USA) as an emulsifier. Preliminary test to ensure that the antibacterial activity was due to the OVEO and not to Tween 80 was performed, and the results demonstrated that Tween 80 at the given concentration (1%) did not inhibit the growth of the assayed bacterial strains cultivated in BHI broth. Solutions of NaCl (600-50 mg mL^{-1}), KCl (600-50 mg mL^{-1}), AA (160-1.25 $\mu L\ mL^{-1}$) and LA (160-1.25 $\mu L\ mL^{-1}$) were prepared in sterile BHI broth.

Bacterial strains

The test organisms used in this study included enterotoxigenic S. aureus strains isolated from foods (*S. aureus* FRI-S-6, producing staphylococcal enterotoxins (SE) A and B, which were isolated from frozen shrimp; *S. aureus* FRI-196-E, producing SEA and D, which were isolated from an unknown food; and *S. aureus* FRI-326, producing SEE, which was isolated from a chicken-based meal) (Bergdoll *et al.*, 1971; Wu and Bergdoll, 1971) and were generously provided by Food Research Institute (Madison, Wisconsin, USA). A standard type strain (*S. aureus* ATCC 13565, producing SEA, isolated from ham) (Johnson *et al.*, 1991) was also used as a test strain. Stock cultures were kept at 4 °C, and prior to being used in the assay, each strain was grown in BHI broth at 37 °C for 18 h (later exponential growth phase), harvested by centrifugation (4500 *g*, 15 min, 4 °C), washed twice in sterile saline solution (NaCl, 0.85%) and resuspended in sterile saline solution to obtain standard cell suspensions at which the OD reading at 660 nm (OD_{660}) was 0.1 (c.a. 10^7 cfu mL^{-1}) (McMahon *et al.*, 2008).

Determining the Minimum Inhibitory Concentration (MIC)

A modified microtiter plate assay was used to determine the MIC of OVEO, NaCl, KCl, acetic acid (AA) and lactic acid (LA) (17). The 96-well plates were prepared by dispensing 90 μL of OVEO (40 to 0.3 $\mu L\ mL^{-1}$), salt (600-50 mg mL^{-1}) or acid (160 to 1.25 mL mL^{-1}) solutions into 90 μL of doubly concentrated BHI broth in each well. Finally, 10 μL of a bacterial suspension (c.a. 10^7 cfu mL^{-1}) was added to each well. The microplate was wrapped loosely with cling film to ensure the bacteria would not become dehydrated and the OVEO would not volatilize. Each plate included a set of controls without the antimicrobial test agents. The plates were prepared in triplicate, and they were incubated statically at 37 °C for 24 h in a microplate incubator/reader (EON model, Biotek Inc., USA). After the incubation period, MIC values were confirmed as the lowest concentrations of OVEO, NaCl, KCl, AA or LA at which the OD_{660} was < 0.01 (McMahon *et al.*, 2008).

Assaying the induction of direct-tolerance

The induction of direct-tolerance was performed by exposing the test strains to sublethal OVEO concentrations in broth for different time intervals, followed by a determination of the MIC values for the same stressing agent. For this assay, 4 mL of BHI broth was inoculated with 1 mL of bacterial suspension (c.a. 10^7 cfu mL^{-1}); thus, OVEO was added at the appropriate amount to obtain the desired final concentration (1/2 MIC or 1/4 MIC), followed by static incubation at 37 °C. An aliquot of each system was taken after 24, 48 and 72 h of incubation (and standardized again to OD_{660} values of 0.1, c.a. 10^7 cfu mL^{-1} of habituated cells) and used as inoculum (10 μL) to determine the MIC of OVEO by using the same microtiter plate assay before described (McMahon *et al.*, 2008). The induction of direct tolerance in the bacteria was assessed by comparing the MIC of OVEO against those of the tested strains before and after the habituation treatment with the same stressing agent. Control systems without exposure to OVEO were assayed similarly (by non-habituation treatment).

Assaying the induction of cross-tolerance

The induction of bacterial cross-tolerance was performed by exposing the test strains to sublethal amounts of OVEO in broth for different time intervals, followed by determination of MIC values of the assayed heterologous stressing agents (NaCl, KCl, AA and LA). For this assessment, 4 mL of BHI broth was inoculated with 1 mL of bacterial suspension (c.a. 10^7 cfu mL^{-1}); thus, the OVEO was added at an appropriate amount to obtain the desired final concentration (1/2 MIC or 1/4 MIC), followed by static incubation at 37 °C. After 24, 48 and 72 h of incubation, an aliquot of each system was taken (standardized again to OD_{660} values of 0.1, c.a. 10^7 cfu mL^{-1} of habituated cells) and used as an inoculum (10 μL) to determine the MIC of the NaCl, KCl, AA and LA by using the same microtiter plate assay before described (McMahon *et al.*, 2008). The induction of bacterial cross-tolerance was assessed by comparing the MIC values of NaCl, KCl, AA and LA against the tested strains before and after the habituation treatment with sublethal amounts of OVEO. Control systems without OVEO exposure were assayed similarly (non-habituation treatment).

The assays were performed in triplicate on three separate experiments, and the results were expressed as modal or median values; where the values were the same, only the modal values were presented (McMahon *et al.*, 2008).

Results and Discussion

The habituation effects of some enterotoxigenic *S. aureus* strains on the development of bacterial direct-tolerance and cross-tolerance after different intervals of exposure to sublethal concentrations of OVEO with regards to the modulation of MIC values were assessed in this study. The MIC values of OVEO against the test strains ranged from 2.5 to 10 μL mL^{-1} (Table 1). NaCl, KCl, AA and LA yielded MIC values of 200 mg mL^{-1}, 300 mg mL^{-1}, 2.5 μL mL^{-1} and 10 μL mL^{-1}, respectively, against all the assayed strains.

Table 1 - The minimum inhibitory concentration of the essential oil from *O. vulgare* L. against different enterotoxigenic strains of *S. aureus* that were isolated from foods.

Strains	MIC of OVEO (μL mL^{-1})
S. aureus FRI-S-6	2.5
S. aureus FRI-196-E	2.5
S. aureus FRI-326	10
S. aureus ATCC 13565	10

MIC: Minimum Inhibitory Concentration; OVEO: *Origanum vulgare* L. essential oil.

The OVEO MIC values against the habituated cells were maintained or decreased up to five-fold when compared with the previously determined MIC values (10 μL mL^{-1} to 0.6 μL mL^{-1}) (Table 2), indicating that there was no induction of direct-tolerance in these cells following OVEO habituation up to 72 h. The decreased MIC of OVEO against habituated enterotoxigenic *S. aureus* cells was related to time of exposure to the sublethal concentrations of this substance because the smaller MIC values were generally found against cells that were pre-exposed to OVEO for 72 h, when compared with non-habituated cells (control assay). During all of the assessed time intervals, the MIC values of OVEO against non-habituated cells ranged from 5 to 10 μL mL^{-1}.

This lack of direct-tolerance induction in the test strains following different OVEO habituation times is interesting; previous studies showed that *S. aureus* was able to develop tolerance after being exposed to other sublethal environmental conditions. The habituation of *S. aureus* CECT 4459 from 5 min to 2 h to stress conditions caused by acid (hydrochloric acid pH 2.5), alkali (sodium hydroxide pH 12.0), hydrogen peroxide (50 mM) and heat (58 °C) in tryptone soy broth resulted in increased direct-tolerance to all tested antimicrobial agents when the survivor/death curves (viable cell counts) were observed. The development of bacterial cross-tolerance to hydrogen peroxide and acid after submitting the cells to heat shock, in addition to their increased tolerance to heat and hydrogen peroxide after acid shock, was already reported (Cebrián *et al.*, 2010).

Existing literature on the development of tolerance by *S. aureus* when exposed to sublethal amounts of essential oils regarding the modulation of MIC values is scarce, making any extensive comparative discussion of the results difficult. The susceptibility of methicillin-resistant/-sensitive *S. aureus* isolates to tea tree (*Melaleuca alternifolia*) essential oil (TTEO) and to antibiotic were determined by modulating the MIC values following a 72 h habituation to sublethal TTEO concentrations in Luria-Bertani broth. This habituation led to stress-hardening with a subsequent increase in the MIC values (≥ 2-fold increase) of TTEO and of different clinically important antibiotics (mupirocin, chloramphenicol, linezolid and vancomycin) (McMahon *et*

Table 2 - The minimum inhibitory concentration of the essential oil from *O. vulgare* L. against different enterotoxigenic strains of *S. aureus* that were isolated from foods, with or without habituation to the same stressing agent up to 72 h.

Strains	Treatment	MIC (μL mL^{-1})		
		24 h*	48 h*	72 h*
S. aureus FRI-S-6	Control (0 μL OVEO mL^{-1})	5.0	5.0	2.5
	1/2 MIC OVEO (1.25 μL OVEO mL^{-1})	2.5	1.25	0.6
	1/4 MIC OVEO (0.6 μL OVEO mL^{-1})	2.5	1.25	0.6
S. aureus FRI-196-E	Control (0 μL OVEO mL^{-1})	5.0	2.5	2.5
	1/2 MIC OVEO (1.25 μL OVEO mL^{-1})	0.6	0.6	0.6
	1/4 MIC OVEO (0.6 μL OVEO mL^{-1})	0.6	0.3	0.6
S. aureus FRI-326	Control (0 μL OVEO mL^{-1})	10	5	5
	1/2 MIC OVEO (5 μL OVEO mL^{-1})	1.25	0.6	0.6
	1/4 MIC OVEO (2.5 μL OVEO mL^{-1})	0.6	0.6	0.6
S. aureus ATCC 13565	Control (0 μL OVEO mL^{-1})	10	5	5
	1/2 MIC OVEO (5 μL OVEO mL^{-1})	1.25	0.6	0.6
	1/4 MIC OVEO (2.5 μL OVEO mL^{-1})	1.25	0.6	0.6

*hours of previous habituation or not in the assayed sublethal concentrations of *O. vulgare* L. essential oil; MIC: Minimum Inhibitory Concentration; OVEO: *O. vulgare L.* essential oil.

al., 2008). Another study assessed the increased resistance (by employing viable cell counts) of four enterotoxigenic strains of *S. aureus* (CECT 976, CECT 4459, CECT 4465 and CECT 4466 that produced SEA, B, C and D, respectively) after habituating to a high temperature (58 °C) in McIlvaine citrate phosphate buffer, and the development of heat tolerance was observed upon the entry of cells into the stationary phase of growth (Cebrián *et al.*, 2007).

In accordance with the direct-tolerance results, the MIC values for NaCl, KCl, AA and LA against the OVEO-habituated cells were the same or decreased (two- to six-fold) in each assessed exposure time interval when compared with MIC values against non-habituated cells (control cells) (Table 3). However, for most of the assessed time intervals, the MIC values remained the same. There was no clear effect of the time-of-habituation with OVEO in relation to the sensitivity of habituated cells to NaCl, KCl and LA. Otherwise, the decrease in the MIC values of AA against habituated-cells always occurred after 48 h (*S. aureus* ATCC 13565) or 72 h (*S. aureus* FRI-S-6) of exposure to sublethal amounts of OVEO.

The overnight cultivation of *S. aureus* ATCC 6538 in meat broth containing the essential oil from *Rosmarinus officinalis* L. (ROEO), and its majority compound 1,8-cineole (CIN), at sublethal amounts (ROEO 10 and 5 μL mL^{-1}; CIN 20 and 10 μL mL^{-1}), induced no direct-tolerance or cross-tolerance (NaCl 100 g l^{-1}; lactic acid pH 5.2; high temperature 45 °C) in the tested bacte-ria when assessed by viable cell count and growth/sur-vival behavior. The cells subjected to pre-habituation with ROEO or CIN revealed an increased sensitivity to LA, high temperature and NaCl when compared with the non-habituated cells. The repeated exposure of *S. aureus* cells to amounts of essential oils (or related compounds) lower than their MICs could cause an imbalance between the anabolism and catabolism that was sufficient to stop growth and cause the cells to be unable to maintain their viability (Gomes Neto *et al.*, 2010).

The sublethal injury caused by phenolic compounds in essential oils, such as the carvacrol or thymol present in OVEO (Barros *et al.*, 2009a; Luz *et al.*, 2013), can result in a damaged bacterial cell membrane, with changes in its structure and permeability (Espina *et al.*, 2013). Furthermore, an injury of the microbial cell membrane provided by sublethal concentrations of antimicrobial compounds may affect the ability of the membrane to osmoregulate the cell adequately or to exclude toxic materials (Carson *et al.*, 2002), and consequently, the decreased tolerance to salts or acids caused by OVEO may be related to membrane damage in sublethally injured bacteria. The cultivation of *S. aureus* strains isolated from foods in nutrient broth containing sublethal concentrations of OVEO (0.3 and 0.15 μL mL^{-1}) for 24 h interfered with the metabolic activity of the assayed strains, inhibiting the activity of the enzymes lipase and coagulase and enterotoxin production (Barros *et al.*, 2009b). The ability of OVEO to suppress enzyme synthesis and/or activity in *S. aureus* result in blocked protein synthesis (Nostro *et al.*, 2001; Oliveira *et al.*, 2010; Gomes Neto *et al.*, 2012), and this action could also be related to the difficulty of the different enterotoxigenic strains of *S. aureus* in developing direct-tolerance or cross-tolerance under the conditions used in this study.

The results from this study confirm that OVEO is an effective anti-staphylococcal substance because exposing enterotoxigenic *S. aureus* strains to sublethal amounts of

Table 3 - The minimum inhibitory concentrations of sodium chloride, potassium chloride, acetic acid and lactic acid against enterotoxigenic strains of *S. aureus* that were isolated from foods, with or without habituation to the essential oil from *O. vulgare* L. up to 72 h.

Strains	Treatment	Sodium chloride MIC (mg mL^{-1})			Potassium chloride MIC (mg mL^{-1})			Acetic acid MIC (µL mL^{-1})			Lactic acid MIC (µL mL^{-1})		
		24 h*	48 h*	72 h*	24 h*	48 h*	72 h*	24 h*	48 h*	72 h*	24 h*	48 h*	72 h*
S. aureus FRI-S-6	Control (0 µL OVEO mL^{-1})	200	200	200	300	200	300	2.5	2.5	2.5	10	5	5
	1/2 MIC OVEO (1.25 µL OVEO mL^{-1})	150	75	100	200	200	300	2.5	2.5	1.25	10	5	5
	1/4 MIC OVEO (0.6 µL OVEO mL^{-1})	150	75	75	200	300	300	2.5	2.5	1.25	10	5	5
S. aureus FRI-196-E	Control (0 µL OVEO mL^{-1})	200	200	150	300	300	300	2.5	2.5	2.5	10	5	5
	1/2 MIC OVEO (1.25 µL OVEO mL^{-1})	150	150	75	300	300	150	2.5	2.5	2.5	10	5	5
	1/4 MIC OVEO (0.6 µL OVEO mL^{-1})	150	200	150	300	300	300	2.5	2.5	2.5	10	5	5
S. aureus FRI-326	Control (0 µL OVEO mL^{-1})	200	150	150	300	300	300	2.5	2.5	2.5	10	5	5
	1/2 MIC OVEO (5 µL OVEO mL^{-1})	50	50	100	50	50	100	2.5	2.5	2.5	5	5	5
	1/4 MIC OVEO (2.5 µL OVEO mL^{-1})	100	150	100	200	200	200	2.5	2.5	2.5	10	5	5
S. aureus ATCC 13565	Control (0 µL OVEO mL^{-1})	150	150	200	300	300	300	2.5	2.5	2.5	10	5	5
	1/2 MIC OVEO (5 µL OVEO mL^{-1})	50	50	100	50	50	50	2.5	1.25	1.25	5	5	5
	1/4 MIC OVEO (2.5 µL OVEO mL^{-1})	100	100	150	150	200	200	2.5	1.25	1.25	10	5	5

*hours of previous habituation (or not) to *O. vulgare* L. essential oil at the assayed sublethal concentrations; MIC: Minimum Inhibitory Concentration; OVEO: *O. vulgare* L. essential oil.

OVEO caused no direct-tolerance and cross-tolerance induction to stressing agents, such as NaCl, KCl, LA an AA. Exposing the test strains to sublethal concentrations of OVEO maintained or increased susceptibility to the same stressing agent and to the assayed heterologous stressing agents, suggesting that OVEO had no impact on the induction of tolerance in enterotoxigenic strains of *S. aureus* as assessed by the modulation of MIC values. These findings reinforce the possible rational use of OVEO by food industry to control the growth and survival of enterotoxigenic *S. aureus* in foods when considered their efficacy to inhibit the growth of this bacterium besides the low capacity to induce bacterial tolerance.

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