

## COMBINATION OF *ORIGANUM VULGARE* L. ESSENTIAL OIL AND LACTIC ACID TO INHIBIT *STAPHYLOCOCCUS AUREUS* IN MEAT BROTH AND MEAT MODEL

Jefferson C. de Barros, Maria Lúcia da Conceição, Nelson Justino Gomes Neto, Ana Caroliny Vieira da Costa, Evandro Leite de Souza\*

Laboratório de Microbiologia de Alimentos, Departamento de Nutrição, Centro de Ciências da Saúde, Universidade Federal da Paraíba, João Pessoa, PB, Brasil.

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### ABSTRACT

This study assessed the occurrence of an enhancing inhibitory effect of the combined application of *Origanum vulgare* L. essential oil and lactic acid against *Staphylococcus aureus* by the determination of Fractional Inhibitory Concentration (FIC) index and cell viability in meat broth and meat model. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the oil was 0.6 and 1.25  $\mu\text{L}\cdot\text{mL}^{-1}$ , respectively. Lactic acid showed MIC and MBC of 2.5 and 5  $\mu\text{L}\cdot\text{mL}^{-1}$ , respectively. FIC indices of the combined application of the oil and lactic acid were 0.5 showing a synergic interaction. The essential oil and lactic acid showed similar ( $p>0.05$ ) anti-*S. aureus* effect in meat broth over 96 h of exposure. Treatment with essential oil or lactic acid presented a smaller anti-staphylococcal effect in meat in comparison to meat broth. No significant difference ( $p>0.05$ ) was found for the microbial counts in meat treated with each antimicrobial alone or in mixture. These results could arise as an interesting approach for the improvement of food preservation using more natural procedures, considering the current demand of consumer and sensory quality of foods.

**Key words:** essential oil, organic acid, hurdle technology, combined effect.

### INTRODUCTION

*Staphylococcus aureus* is a pathogen bacterium commonly associated with serious outbreaks of food poisoning attributed to contaminated meat products (8, 23). Staphylococcal enterotoxins form a family of major serological types of heat-stable enterotoxins that cause vomiting and diarrhoea when ingested and are responsible for staphylococcal food poisoning

(26). Since staphylococcal foodborne intoxication is established as one of the most common bacterial food-borne diseases causing problems in the food sector in many countries, strategies to control *S. aureus* in foods are of particular interest.

Food conservation is based on a search for foods with a high nutritional quality and microbial stability and it has been reached by the use of various physical and/or chemical procedures (9). However, there is a current demand from

\*Corresponding Author. Mailing address: Universidade Federal da Paraíba, Centro de Ciências da Saúde, Departamento de Nutrição, Laboratório de Microbiologia de Alimentos, Campus I, 58051-900, Cidade Universitária, João Pessoa, Paraíba, Brasil.; E-mail: [evandroleitesouza@ccs.ufpb.br](mailto:evandroleitesouza@ccs.ufpb.br)

consumers for new methods of reducing or eliminating foodborne pathogens, possibly in combination with existing methods. Consumers are requesting additive-free, fresher, more natural tasting foods and with a smaller impact on the environment, while maintaining the microbiological safety (7, 17, 29). One such possibility is the use of essential oils as antimicrobial additives (18, 31). It has long been recognized that essential oils have antimicrobial properties but the recent enhancement of interest in “green” consumerism has led to a renewed scientific interest in these substances (30).

It is reported that the application of natural compounds for controlling food-related pathogenic microorganisms demands the evaluation of efficacy with food model media and food products since the food components may reduce their antimicrobial effect (14). Others researchers have purposed that results obtained in liquid and/or solid food model media may be more useful prior to further application in/on food matrices, rather than those obtained in laboratorial media. Smith-Palmer *et al.* (28) and Gutierrez *et al.* (16) stated that these food-based models would also better reflect the nutrient availability and composition of foods, optimizing the final application of these compounds.

The main drawback of using essential oils as antimicrobial in foods is the high concentration needed to inhibit spoilage and pathogen bacteria in food matrices which often exceeds the flavor threshold acceptable to consumers. Combination of essential oils with existing physical or chemical treatments (Hurdle Technology concept) could rise as effective approach for reinforcing their natural antimicrobial effects. The addition of small amounts of other natural preservatives may be a way to provide the balance between sensory acceptability and antimicrobial efficacy (10).

The use of essential oils with other food ingredients, such as sodium chloride, sugar and organic acid might provide an enhancing effect for control the microbial growth in foods (11, 20). Organic acids are either naturally present as constituents of the food or added to the products through the food formulation.

Lactic acid lacks in acute and chronic toxicity, which has led to its widespread employment as a classical food preservative and decontamination agent (13).

Among many plants scientifically studied regarding their antimicrobial properties, *Origanum vulgare* L. (oregano), Lamiaceae, has showed prominent results (27, 29). *O. vulgare* essential oil presented interesting results in inhibiting the growth of bacteria and synthesis of microbial metabolites, including the pathogen *Staphylococcus aureus* (2, 3, 22, 30). Although, some researchers have found anti-*Staphylococcal* activity in *O. vulgare* essential oil, there is a lack of information about its antimicrobial effect when applied in combination with other antimicrobial compounds.

This study was undertaken to investigate the occurrence of an enhancing anti-*S. aureus* effect on the basis of FIC index and cell viability assay. Potential synergy between *O. vulgare* essential oil and the classical and widely applied food preservative lactic acid in meat based broth and in a meat model was investigated. To our knowledge, the effect of the combined application of *O. vulgare* essential oil and an organic acid at sub-inhibitory concentrations against the food-related pathogen *S. aureus* has not been reported to date.

## MATERIALS AND METHODS

### Antimicrobial agents

Lactic acid (85 – 90%) was obtained from Vetec Química Fina Ltda. (Rio de Janeiro, Brazil). Stock solutions of the acid were made in sterile distilled water.

The essential oil from *O. vulgare* L. (Batch 209) extracted by hydrodistillation was supplied by Ferquima Ind. e Com. Ltda. (Vargem Grande Paulista, São Paulo, Brazil). Stock solutions of the essential oil were prepared in distilled water added of bacteriological agar (0.15 g.100mL<sup>-1</sup>) as stabilizing agent (5). According to Barros *et al.* (2) the essential oil assayed here possesses carvacrol (57.71%), *p*-cymene (10.91%),  $\gamma$ -terpinene (7.18%) and terpinen-4-ol (6.68%) as its

major compounds.

Lactic acid and essential oil were assayed at concentrations of 80, 40, 20, 10, 5, 0.5, 0.25, 0.125, 0.06, 0.03 and 0.0015  $\mu\text{L}\cdot\text{mL}^{-1}$ .

### Bacterial strains

*S. aureus* QCD, *S. aureus* QCE and *S. aureus* QCF obtained from the Microorganism Collection, Laboratory of Food Microbiology, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil were used as test microorganisms. These strains were isolated from different unripened cheese samples by the standard procedures (1, 4). Stock cultures were kept on Nutrient Agar – NA (Sigma, France) slants under refrigeration.

Inocula used in antimicrobial assays were obtained from overnight cultures grown on NA slants at 37°C. A loopfull of the culture was diluted in sterile saline solution (0.85 g.100mL<sup>-1</sup>) to have a final concentration of approximately 10<sup>8</sup> colony forming unity per mL (cfu.mL<sup>-1</sup>) adjusted according to the turbidity of 0.5 McFarland standard.

### Preparation of meat broth

Bovine meat steaks were trimmed for all external fat and cut in pieces of uniform sizes (3 x 3 x 3 cm). Meat pieces were boiled in distilled water during 20 min at 90 °C. About 500 mL of meat broth were obtained and vacuum filtered using Whatman n°1. The filtrate was sterilized using autoclave for 15 min (1.21 atm). After that, the broth was stored at – 20°C in aliquots of 50 mL. When required one aliquot was thawed under refrigeration (7°C,  $\pm$  1°C) and used for the experimental analysis.

### Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC values of the essential oil and lactic acid were determined using the macrodilution in broth procedure. 5 mL of double strength Nutrient Broth-NB (Sigma, France) was inoculated with 1 mL of the bacterial inocula (approximately

10<sup>7</sup> cfu.mL<sup>-1</sup>). After that, 4 mL of the essential oil or lactic acid solution at appropriate concentration was added, followed by shaking for 30 s, and then incubated for 24 h at 37°C without shaking. MIC was defined as the lowest concentration of the assayed antimicrobial agent required to completely prevent visible bacterial growth in NB. An aliquot (100  $\mu\text{L}$ ) of the tubes with no visible bacterial growth was subcultured on sterile NA Petri dishes at 37°C for 48 h to determine if the inhibition was reversible or permanent. MBC was defined as the lowest concentration which no growth was noted on NA. Control flasks without essential oil or lactic acid were tested in the same way (12).

### Synergy assays

The study of synergy of *O. vulgare* essential oil and lactic acid was carried out by determining Fractional Inhibitory Concentration (FIC). For this, was used the macrodilution procedure using NB as previously described. FIC was calculated as follow: FIC: MIC of the oil in combination of lactic acid / MIC of the oil alone. The essential oil and lactic acid were combined at MIC + MIC; MIC + ½ MIC; MIC + ¼ MIC; ½ MIC + ½ MIC; ¼ MIC + ¼ MIC; and ½ MIC + ¼ MIC. Synergy was FIC  $\leq$  0.5; indifference was FIC > 0.5 to 4; and antagonism was FIC > 4 (14).

### Cell viability assay

The effect of the antimicrobials alone and in combination on the cell viability of *S. aureus* QCE along 96 h was evaluated by the viable cell count procedure. For this, 5 mL of meat broth was inoculated with 1 mL of the bacterial inocula (approximately 10<sup>7</sup> cfu.mL<sup>-1</sup>) and 5 mL of the antimicrobial (alone or in combination) solution at appropriated concentration were added to the system, gently shaken for 30 s, and then incubated at 7°C ( $\pm$  1°C). At different time intervals (0, 24, 48, 72 and 96 h), 1 mL of the suspension was serially diluted (10<sup>-1</sup> – 10<sup>-5</sup>) in sterile peptone water (0.1 g.100mL<sup>-1</sup>) and inoculated on NA Petri dishes for 24 h at 37°C. Control flasks with no antimicrobial were tested in the same way. The

mean number of colonies was counted and compared with that found in the control assay. The results were expressed in log of cfu.mL<sup>-1</sup>.

### Meat model

Bovine meat steaks were trimmed for all external fat and cut in pieces of uniform sizes (3 x 3 x 3 cm). The meat pieces were put in glass flasks and sterilized using autoclave (121°C for 15 min, 1.21 atm). After that, meat pieces were inoculated with a staphylococcal suspension according to the following procedure: the pieces were individually submerged in 50 mL of the bacterial inoculum (*S. aureus* QCE, approximately 10<sup>7</sup> cfu.mL<sup>-1</sup>) prepared in sterile saline solution, rotated with sterile glass stick for 1 min to ensure even inoculation, air dried for 30 min in a bio-safety cabinet before washing with the antimicrobials. The pieces were randomly divided into four groups and immersed for 30 s (1:4 w/v) as follow: (1) control – dipped in sterile distilled water; (2) dipped in the essential oil MIC solution; (3) dipped in lactic acid MIC solution; and (4) dipped in essential oil (MIC x ¼) + lactic acid (MIC x ¼) solution. The pieces were put in sterile sealed sterile polypropylene cups and stored under refrigeration (7°C, ± 1°C). At 0, 24, 48, 72 and 96 h of storage the meat samples were submitted to count of *S. aureus* according to procedure described for Vanderzant and Splittstoesser (32). The results

were described in log of cfu per gram of meat (log cfu.g meat<sup>-1</sup>).

All antimicrobial assays were carried out in triplicate and the results are expressed as average of the three parallel assays.

### Statistical analysis

Statistical analysis was performed to determine significant differences (p<0.05) by one-way ANOVA followed for Tukey test in the bacteria kill time assays. For this the Sigma stat 2.03 computer program was used.

## RESULTS AND DISCUSSION

All test strains were sensitive to *O. vulgare* essential oil and lactic acid, although the oil presented four-fold smaller MIC and MBC values in comparison to the acid. MIC value of *O. vulgare* essential oil was 0.6 µL.mL<sup>-1</sup> for all strains, while MBC was 1.25 µL.mL<sup>-1</sup>. Lactic acid showed MIC and MBC of 2.5 and 5 µL.mL<sup>-1</sup>, respectively.

FIC indices of the combined application of *O. vulgare* essential oil and lactic acid were 0.5 for all *S. aureus* strains revealing a synergic activity of the assayed antimicrobials. Test strains presented capability to grow at sub-inhibitory concentrations (½ MIC and ¼ MIC) of both antimicrobials when applied alone (Table 1).

**Table 1.** FIC indexes of the combined action of *O. vulgare* L. essential oil and lactic acid to *S. aureus* strains.

Strains	FIC	<i>O. vulgare</i> essential oil		Lactic acid	
		MIC x 1/2	MIC x 1/4	MIC x 1/2	MIC x 1/4
<i>S. aureus</i> QCD	0.5	+	+	+	+
<i>S. aureus</i> QCE	0.5	+	+	+	+
<i>S. aureus</i> QCF	0.5	+	+	+	+

(+): capability of the strain to grow in nutrient broth added of the essential oil or lactic acid at each assayed sub-inhibitory concentrations

Synergy is observed when the effect of the combined compounds is greater than the sum of the individual effects (6). Gutierrez *et al.* (15) found no synergy by FIC index when

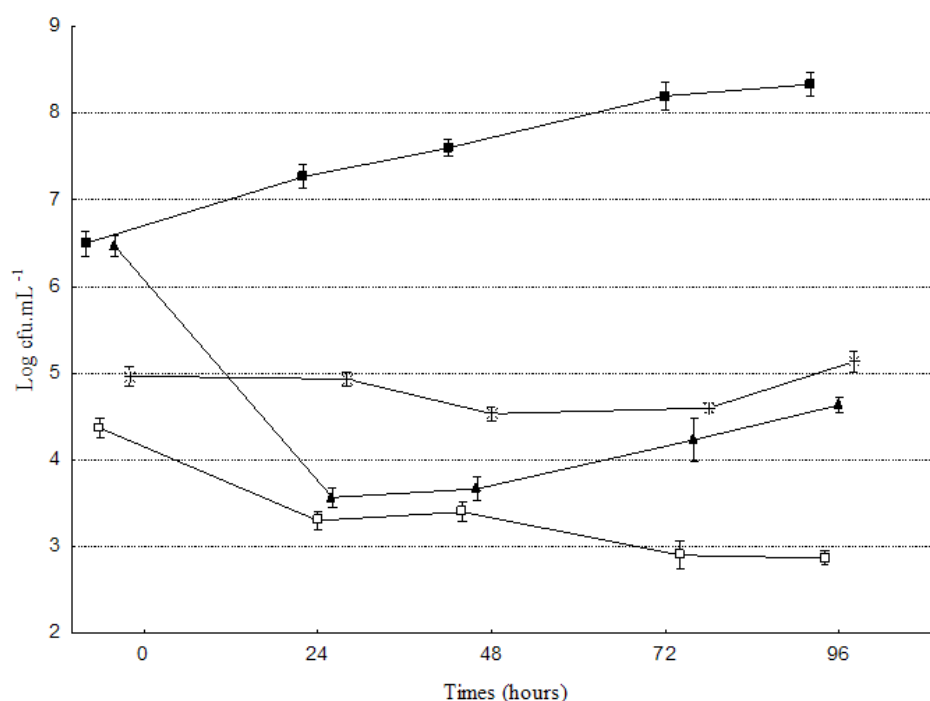
oregano essential oil was applied in combination to thyme or marjoram essential oil toward *P. aeruginosa*, *B. cereus*, *E. coli* and *L. monocytogenes*. According to the authors as the assayed

essential oils possess similar composition their combination may exhibit addition rather than a synergistic effect. Nazer *et al.* (21) found that thymol in combination with other aromatic compounds, lead to improved inhibition, but no real synergistic effect was demonstrated toward *Salmonella*.

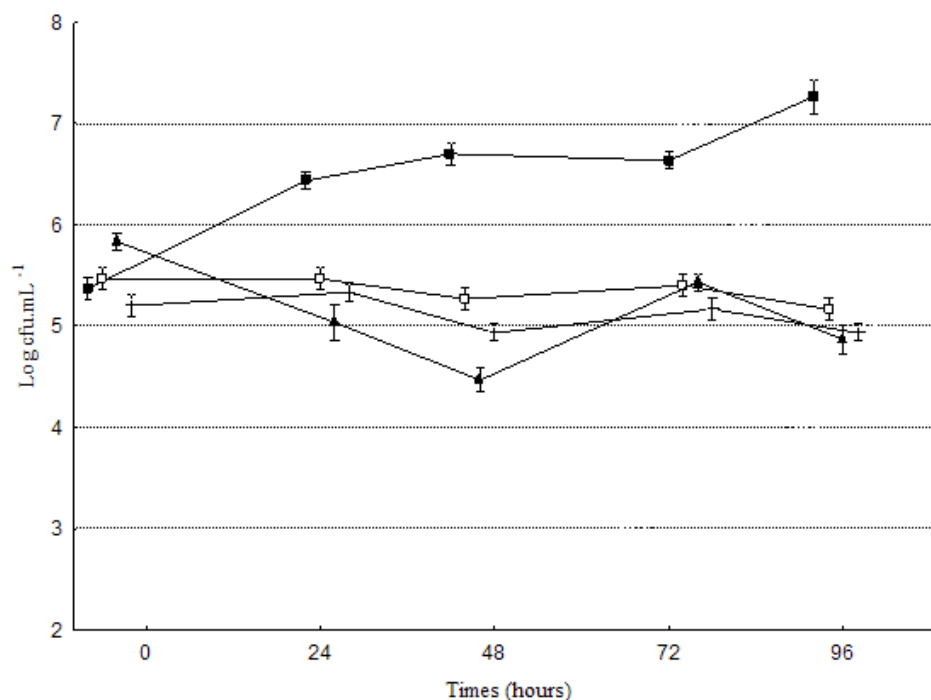
When cell viability assay is combined with FIC assay, much more kinetic information can be generated giving a dynamic picture of the antimicrobial interactions (14). Results of the effect of *O. vulgare* essential oil (MIC), lactic acid (MIC) and *O. vulgare* essential oil ( $\frac{1}{4}$  MIC) in combination with lactic acid ( $\frac{1}{4}$  MIC) in meat broth at 7°C on the cell viability of *S. aureus* QCE are given in Figure 1. The temperature of 7°C was chosen regarding the use of a more realistic temperature in respect of the most common conservation systems applied to meat products. In meat broth the essential oil provided a prominent drop in the bacterial inoculum after 24 h, followed for a steady and smaller decrease in the remainder evaluated times. Lactic acid caused a sharply

drop in bacterial inoculum after 24 h, however a slight increase in the bacterial count occurred in the remainder intervals. Along the evaluated intervals the count found for the media added of essential oil and lactic acid at sub-inhibitory concentrations were significantly lower ( $p < 0.05$ ) than those found for the control assay. No significant difference ( $p < 0.05$ ) was noted between the microbial counts found for the broth added of lactic acid at MIC and added of both antimicrobials at subinhibitory concentrations.

Results of the effect of *O. vulgare* essential oil (MIC), lactic acid (MIC) and *O. vulgare* essential oil ( $\frac{1}{4}$  MIC) in combination with lactic acid ( $\frac{1}{4}$  MIC) on the cell viability of *S. aureus* QCE in meat model at 7°C are given in Figure 2. Anti-staphylococcal effect of essential oil and lactic acid alone was smaller in meat in comparison to their addition in meat broth. No significant difference ( $p > 0.05$ ) was found for the microbial counts in meat treated with each antimicrobial alone or in mixture.



**Figure 1.** Viable cell counts of *S. aureus* QCE in meat broth at 7 °C ( $\pm 1^\circ\text{C}$ ) as a function of antimicrobial concentration: (■): control ( $0 \mu\text{L.mL}^{-1}$ ); (○): essential oil (MIC:  $0.6 \mu\text{L.mL}^{-1}$ ); (▲): lactic acid ( $2.5 \mu\text{L.mL}^{-1}$ ); (+): essential oil (MIC  $\times \frac{1}{4}$ :  $0.15 \mu\text{L.mL}^{-1}$ ) + lactic acid (MIC  $\times \frac{1}{4}$ :  $0.62 \mu\text{L.mL}^{-1}$ ).



**Figure 2.** Viable cell counts of *S. aureus* QCE in meat model at 7 °C ( $\pm$  1°C) as a function of antimicrobial concentration: (■): control (0  $\mu\text{L.mL}^{-1}$ ); (□): essential oil (MIC: 0.6  $\mu\text{L.mL}^{-1}$ ); (▲): lactic acid (2.5  $\mu\text{L.mL}^{-1}$ ); (○): essential oil (MIC x 1/4: 0.15  $\mu\text{L.mL}^{-1}$ ) + lactic acid (MIC x 1/4: 0.62  $\mu\text{L.mL}^{-1}$ ).

*O. vulgare* essential oil and lactic acid showed a stronger kill-effect in meat broth in comparison to the food model (meat pieces). Lower antimicrobial effect of phenolics-compound-containing essential oils, when applied in food models has been attributed to their solubility in the aqueous phase of the foods and distribution in the substrate. Carvacrol, majority compound of the essential oil studied here, is much more soluble in the other constituents of foods than in aqueous phase, where the bacterial proliferation takes place. Moreover, the physical structure of a food may limit the antibacterial activity of an essential oil (6).

It has generally been found that a greater concentration of essential oils is needed to achieve the same antibacterial effect in foods (24, 28). Not only are the intrinsic properties of food (fat/protein/water content, antioxidants, pH, salt and other additives) relevant in this respect, the extrinsic determinants (temperature, packaging in vacuum/gas/air, characteristics of

microorganisms) can also influence bacterial sensitivity (21).

Since higher concentrations of essential oils are generally required when added to food, the application of essential oils in food may be limited due to changes in organoleptic and textural quality (9, 12). A challenge for practical application of essential oils in food is to develop optimized low dose combinations to maintain product safety and shelf-life, thereby minimizing the undesirable flavor and sensory changes associated with the addition of high concentrations of essential oils (15).

Dimitrijević et al. (10) noted that the antilisterial effect of *Thymus vulgaris* and *Rosmarinus officinalis* essential oil (50, 100, 200 and 300  $\mu\text{L.mL}^{-1}$ ) was noticeably increased by lactic acid (50  $\mu\text{L.mL}^{-1}$ ). On the other hand, the same study reported a smaller enhancing effect to *O. vulgare* essential oil. Naveena et al. (20) found that the combination of *Syzygium aromaticum* essential oil and lactic acid provided a decrease of

psychrotrophic and coliform counts in buffalo meat.

According to Dimitrijević *et al.* (10) it is difficult to understand what the way of establishment of the enhancing antimicrobial effect from the combined application of essential oils and organic acids. Studies on the antibacterial mechanism of phenolic compounds found in essential oils focused on their effects on the cellular membrane, changing its structure and permeability (26). Lin *et al.* (19) state that the damage to cell membrane might explain the observed effects, since phenolics could cause sublethal injury to cell membranes, causing disruption of proton motive force due loss of H<sup>+</sup>-ATPase. This could make bacteria more susceptible to acid environment. Moreover, at low pH the hydrophobicity of an essential oil increases, enabling it to more easily dissolve in the lipids of the cell membrane of target bacteria (17).

The results presented in this study showed a synergic effect of *O. vulgare* essential oil and lactic acid on the base of FIC index. Our results encourage further researches focusing the application of *O. vulgare* essential oil in low doses and other preservative hurdles, in order to control the growth of *S. aureus* in selected foods, particularly meat products. Such a strategy could be used by food industry as natural barrier to control the growth of *S. aureus* in foods providing the balance between sensory acceptability and antimicrobial efficacy.

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