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MICROBIOLOGY

Combined effect of carvacrol, thymol and nisin against *Staphylococcus aureus* and *Salmonella Enteritidis*

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Abstract: The aim of this study was to investigate the combined effect of carvacrol, thymol and nisin against *Staphylococcus aureus* and the combined effect of carvacrol and thymol against *Salmonella* Enteritidis. Minimum inhibitory concentrations (MIC) of carvacrol, thymol, and nisin for *S. aureus* were 200, 150 and 30 µg/mL, respectively. MIC of carvacrol and thymol for *Salmonella* Enteritidis was 200 µg/mL. A factorial method of independent variables was then used to study the combined effect of antimicrobials. Results showed that combinations of carvacrol-thymol-nisin (reduction of 1.2 log CFU/mL for MIC and 4.98 log CFU/mL for 2MIC), carvacrol-thymol (reduction of 1.33 log CFU/mL for 2MIC), nisin-thymol (reduction of 3.52 log CFU/mL for 2MIC) and nisin-carvacrol (reduction of 3.41 log CFU/mL for 2MIC) attained a significant inhibition of *S. aureus*. Similarly, there was significant reduction of *Salmonella* Enteritidis due to combined effect of thymol-carvacrol (reduction of 4.5 log CFU/mL for MIC and inhibition below detection limit for 2MIC). Therefore, the combinations of natural antimicrobials described in this work showed potential to be used as an additional barrier for food safety.

Key words: antimicrobial, factorial design, pathogens, response surface analysis.

INTRODUCTION

Recently, consumer demand for safe foods prepared without addition of chemical preservatives and extended shelf life motivates the food industry and research institutions to develop new conservation technologies. Alternatives to meet this demand include natural antimicrobial ingredients, which can be used individually or in combination (Mathur et al. 2017). The choice of a natural antimicrobial should be based on its chemical, nutritional and sensorial compatibility with the target food. Moreover, the effectiveness against undesirable bacteria, antimicrobial resistance, safety, among other characteristics should be considered (Davidson et al. 2013, Pisoschi et al. 2018).

To this end, several antimicrobial compounds present in plant essential oils have been widely studied and can inhibit or control multiplication of undesirable microorganisms in food (Zheng et al. 2013, D'Amato et al. 2018). Essential oils are aromatic compounds with high volatility and hydrophobicity obtained from various parts of plants *e.g.*, leaves, flowers, buds, seeds, herbs, and others (Tariq et al. 2019). These compounds have been classified as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA), and represent a "green alternative" to classic fungicides and synthetic antimicrobials (Calo et al. 2015). Carvacrol and its isomer thymol are major components of oregano and thyme essential oils, both are phenolic compounds with activity against

bacteria, filamentous fungi and yeasts (Barbosa et al. 2009, Miladi et al. 2016). These compounds can cause the collapse of the plasma membrane structure, loss of essential cell components and consequently cell death (Burt 2004, Barbosa et al. 2009). Therefore, carvacrol and thymol have the potential to prolong shelf life and improve food protection.

Another GRAS-approved antimicrobial for food use is the bacteriocin nisin, an antimicrobial peptide produced by some strains of *Lactococcus lactis*, which is authorized for use in more than 40 countries (Cleveland et al. 2001). Nisin was the first commercially produced bacteriocin approved as food preservative, especially dairy products (De Martinis et al. 2002, Balciunas et al. 2013). Its action can be explained by interactions with membrane phospholipids, creating pores and causing efflux of essential constituents of the microbial cell (Balciunas et al. 2013). Nisin has antimicrobial activity especially against Gram-positive bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* (Balciunas et al. 2013).

The inhibitory effect of combining two or more antimicrobials can be higher than an antimicrobial alone. In the food industry, this approach can increase the spectrum of action, reduce the required antimicrobial concentration, decrease antimicrobial resistance and improve microbiological quality, maintaining sensorial and nutritional properties in food products (Singh et al. 2001, Churklam et al. 2020).

Since *S. aureus* and *Salmonella* are among the most common etiological agents involved in foodborne diseases outbreaks in Europe, Brazil and United States (EFSA 2018, Centers for Disease Control and Prevention 2018, Ministry of Health of Brazil 2018), they were chosen for this research. Although studies have evaluated the individual effect and/or the combination of natural antimicrobials against *S. aureus* and *Salmonella* spp., the combination of thymol, carvacrol and nisin is underexplored. Therefore, the aim of this study was to investigate the combined effect of carvacrol, thymol and nisin against *S. aureus* and the combined effect of carvacrol and thymol against *Salmonella* Enteritidis through a factorial design method.

MATERIALS AND METHODS

Bacterial cultures

S. aureus ATCC 2998 and *Salmonella* Enteritidis SE86 were selected as representatives of Gram-positive and Gram-negative bacteria, respectively. Both strains are part of the microorganism collection of the Food Control and Microbiology Laboratory of the Federal University of Rio Grande do Sul (UFRGS, Porto Alegre, Brazil). *Salmonella* Enteritidis SE86 was isolated from cabbage involved in a foodborne outbreak, exhibiting the same genetic profile of more than 90% of *Salmonella* Enteritidis related to outbreaks reported in Rio Grande do Sul State, Southern Brazil (Capalonga et al. 2014). These strains were chosen from a preliminary study that defined them as more resistant to the studied antimicrobials (data not shown). The bacteria were kept at -18°C in 10% (v/v) glycerol. For activation, 20 µL of each culture was transferred to 3 mL of Brain Heart Infusion broth (BHI; HiMedia, Mumbai, India) incubated at 37°C for 24 h. Each bacterial suspension (*S. aureus* or *Salmonella* Enteritidis SE86) was adjusted to approximately 10^8 CFU/mL by diluting the inoculum in sterile BHI broth to obtain ~0.5 absorbance units (OD_{630nm}) using an Ultrospec 3100 Pro UV/Visible spectrophotometer (Amersham Biosciences, UK). For confirmation, plate counting was performed using BHI agar.

Antimicrobial solutions

Thymol (≥ 98.5% purity) and carvacrol (98% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solutions of these compounds (10.6 mg/mL) were prepared in 10% (v/v) DMSO (dimethyl sulfoxide).

Commercial nisin (Nisaplin) was acquired from Danisco Brasil Ltda. (Cotia, Brasil). According to the manufacturer, the formulation contains 77.5% NaCl, 20% denatured milk solids and 2.5% pure nisin. For the preparation of the solution, 1 g of Nisaplin was diluted in 10 mL of 0.01 M HCl and filtered through 0.22 µm membranes (Millipore). Then, 2 mL of this solution was diluted in 3 mL of 10 mM phosphate buffer, pH 7.0 (Malheiros et al. 2010).

Minimum Inhibitory Concentration (MIC)

MIC was determined for thymol, carvacrol and nisin using 96-well microplates for *S. aureus* and *Salmonella* Enteritidis SE86. To this end, 100 µL of Mueller Hinton broth (HiMedia, Mumbai, India) was added to each well. Next, 100 µL of antimicrobial solutions were added to the first well and homogenized; then, 100 µL of this mixture were transferred to the next well, and so forth. After, 100 µL of each bacterial culture was added to each well. Controls were performed using BHI broth only and diluent solutions without antimicrobials. Subsequently, the plates were incubated at 37°C for 18-24 h and the turbidity of each well was visually observed. The MIC was considered the lowest concentration without turbidity in the well.

Factorial design for evaluation of combined antimicrobial effect

To evaluate the combined effect of the antimicrobials, a factorial design methodology was used. This methodology allows us to evaluate the effect of a large number of variables from a small number of experimental trials (Myers et al. 2016). To analyze this effect on the *S. aureus* population, the experimental procedures were organized through a 2^3 factorial design with three repetitions of the central point (defined as the MIC values). To describe the response surface, complete factorial designs with three coded levels, with their increased two points (coded values -1 and +1) with three repetitions of the center points (all factors at level 0), and three variables were used to study the combined influence of concentration of nisin (N), thymol (T) and carvacrol (C). The coded experimental design and the real values of the independent variables are shown in Table I.

The following polynomial equation was fitted for each factor evaluated at each experimental point:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

where Y is the dependent variable representing the population of *S. aureus*, x_1, x_2, x_3 are the independent variables representing the single components in mixtures; b_0, b_1, b_2, b_3 are independent coefficients and b_{12}, b_{13}, b_{23} interaction coefficients.

Nisin did not show antimicrobial activity (MIC) against *Salmonella* Enteritidis and thus, it was not included in the experimental design. For this reason, to analyze the combined effect of antimicrobials on the *Salmonella* Enteritidis population, the experimental procedures were organized through a 2^2 factorial design using thymol and carvacrol, with three repetitions of the central point.

To describe the response surface, complete factorial designs with three coded levels, with their two increased points (coded values -1 and +1) with three repetitions of the central points (all factors at level 0), and two variables were used to study the combined influence of thymol (T) and carvacrol (C) concentration. The coded

Table I. Combination effect of nisin (N), thymol (T) and carvacrol (C) against *S. aureus*.

Assay*	T (x_1)	C (x_2)	N (x_3)	*S. aureus* (log CFU/mL) (Y)		
				Test 1	Test 2	Test 3
1	-1	-1	-1	9.37 ± 0.01	8.94 ± 0.05	9.13 ± 0.10
2	+1	-1	-1	8.89 ± 0.16	9.09 ± 0.44	9.12 ± 0.08
3	-1	+1	-1	9.03 ± 0.21	9.55 ± 0.61	9.31 ± 0.12
4	+1	+1	-1	8.04 ± 0.19	9.47 ± 0.24	9.32 ± 0.03
5	-1	-1	+1	8.70 ± 0.00	9.00 ± 0.00	9.43 ± 0.16
6	+1	-1	+1	5.85 ± 0.21	8.72 ± 0.40	9.07 ± 0.47
7	-1	+1	+1	5.96 ± 0.55	9.08 ± 0.00	9.50 ± 0.21
8	+1	+1	+1	4.39 ± 0.01	5.64 ± 0.45	8.81 ± 0.17
9	0	0	0	8.34 ± 0.06	8.89 ± 0.27	8.84 ± 0.20
10	0	0	0	8.16 ± 0.23	8.06 ± 0.17	9.42 ± 0.25
11	0	0	0	8.01 ± 0.01	8.60 ± 0.48	9.29 ± 0.15

Note: * Randomized prior to experimentation. Mean values ± standard deviation. Test 1: +1 (N = 60 mg/mL, T = 300 mg/mL, C = 400 mg/mL); -1 (without antimicrobial); 0 (N = 30 mg/mL, T = 150 mg/mL, C = 200 mg/mL). Test 2: +1 (N = 30 mg/mL, T = 150 mg/mL, C = 200 mg/mL); -1 (without antimicrobial); 0 (N = 15 mg/mL, T = 75 mg/mL, C = 100 mg/mL). Test 3: +1 (N = 15 mg/mL, T = 75 mg/mL, C = 100 mg/mL); -1 (without antimicrobial); 0 (N = 7.5 mg/mL, T = 37.5 mg/mL, C = 50 mg/mL).

experimental design and the real values of the independent variables are shown in Table II.

The following polynomial equation was fitted for each factor evaluated at each experimental point:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 \quad (2)$$

where Y is the *Salmonella* Enteritidis population; $x_1 x_2$ are the independent variables representing the single components in mixtures; b_0, b_1 e b_2 are independent coefficients and b_{12} the interaction coefficient.

Combined effect of thymol, carvacrol and nisin

The assays were performed in glass test tubes closed with a screw cap (1×10 cm, 3 mL) containing Mueller Hinton broth. Thymol, carvacrol and nisin were added to each tube at the final concentration of ½ MIC, MIC and 2MIC. Then, the bacterial culture was added (to 10^8 CFU/mL), mixed and the tubes were incubated at 37°C for 24 h. Controls without antimicrobials were performed. After incubation, dilutions were performed and 20 µL of each dilution were plating in Xylose Lysine Deoxycholate agar (XLD; Oxoid, Basingstoke, UK) for *Salmonella* Enteritidis SE86 and Baird-Parker agar (BP; Oxoid) for *S. aureus*. The effects of the antimicrobial combinations were measured as the decrease of viable counts of target microorganisms.

Statistical analysis

The results were performed using Protimiza Experimental Design software (http://experimental-design.protimiza.com.br) through two independent experiments. Analysis of variance (ANOVA) was performed. Response surfaces and contour diagrams were designed to illustrate the effects of independent variables on the populations of the bacteria studied.

Table II. Combination effect of carvacrol (C) and thymol (T) against *S.* Enteritidis SE86.

Assay*	T (x_1)	C (x_2)	*S. Enteritidis* (log CFU/mL) (Y)		
			Test 1	Test 2	Test 3
1	-1	-1	9.79 ± 0.07	9.25 ± 0.07	9.50 ± 0.07
2	+1	-1	5.51 ± 0.13	8.43 ± 0.24	9.40 ± 0.28
3	-1	+1	4.65 ± 0.92	8.69 ± 0.18	9.49 ± 0.24
4	+1	+1	0.00 ± 0.00	4.63 ± 0.16	7.83 ± 0.18
5	0	0	5.38 ± 0.14	8.60 ± 0.20	9.43 ± 0.04
6	0	0	5.47 ± 0.18	8.60 ± 0.01	9.05 ± 0.18
7	0	0	5.41 ± 0.01	8.47 ± 0.01	9.41 ± 0.18

Note: * Randomized prior to experimentation. Mean values ± standard deviation. Test 1: +1 (T,C = 400 mg/mL); -1 (without antimicrobial); 0 (T,C = 200 mg/mL). Test 2: +1 (T,C = 200 mg/mL); -1 (without antimicrobial); 0 (T,C = 100 mg/mL). Test 3: +1 (T,C = 100 mg/mL); -1 (without antimicrobial); 0 (T,C = 50 mg/mL).

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration

The MIC for *S. aureus* was 150 and 200 µg/mL for thymol and carvacrol, respectively. Therefore, even being isomers, MIC was different for both antimicrobial agents. These results are in agreement with Guarda et al. (2011), who observed MICs values of 250 and 225 µg/mL for thymol and carvacrol against *S. aureus* ATCC 25923, respectively. The MIC of nisin against *S. aureus* in this study was 30 µg/mL. Similarly, MIC values of nisin between 16 and 32 µg/mL were described against 13 different isolates of *S. aureus* (Shi et al. 2017).

For *Salmonella* Enteritidis, MIC was 200 µg/mL using thymol and carvacrol as antimicrobial agents. Similarly, a MIC value of 250 µg/mL for carvacrol against *Salmonella.* Typhimurium was previously reported (Kim et al. 1995). Nisin was not able to cause inhibitory effect on Gram-negative bacteria. The inability of nisin to inhibit *Salmonella* is possibly due to the presence of an outer membrane that protects the cell wall of Gram-negative bacteria, which limit the passage of some antimicrobial compounds (Schved et al. 1994, Helander & Mattila-Sandholm 2000, Olasupo et al. 2003).

Model fitting

An adjustment of the model was made for each factorial design. The independent and dependent variables were adjusted for the nonlinear first order equation and examined according to the quality of the fit. The reparameterization of the model was carried out to the desired significance level. ANOVA was used to evaluate the adequacy of the adjusted model. The R^2 value indicates the response values variability. A good fit is considered by values above 0.9 (Haaland 1989). The *F* test indicates if the model is predictable, which occurs when the calculated *F* value is higher than the critical *F* value (Rodrigues & Iemma 2014).

Determination of combined effect of nisin, thymol and carvacrol against *S. aureus*

Table I shows the results of factorial design for the combined effect of nisin, thymol and carvacrol against *S. aureus*. Contour plots of the results are shown in Figure 1, which are graphical representation of experimental data fitted to

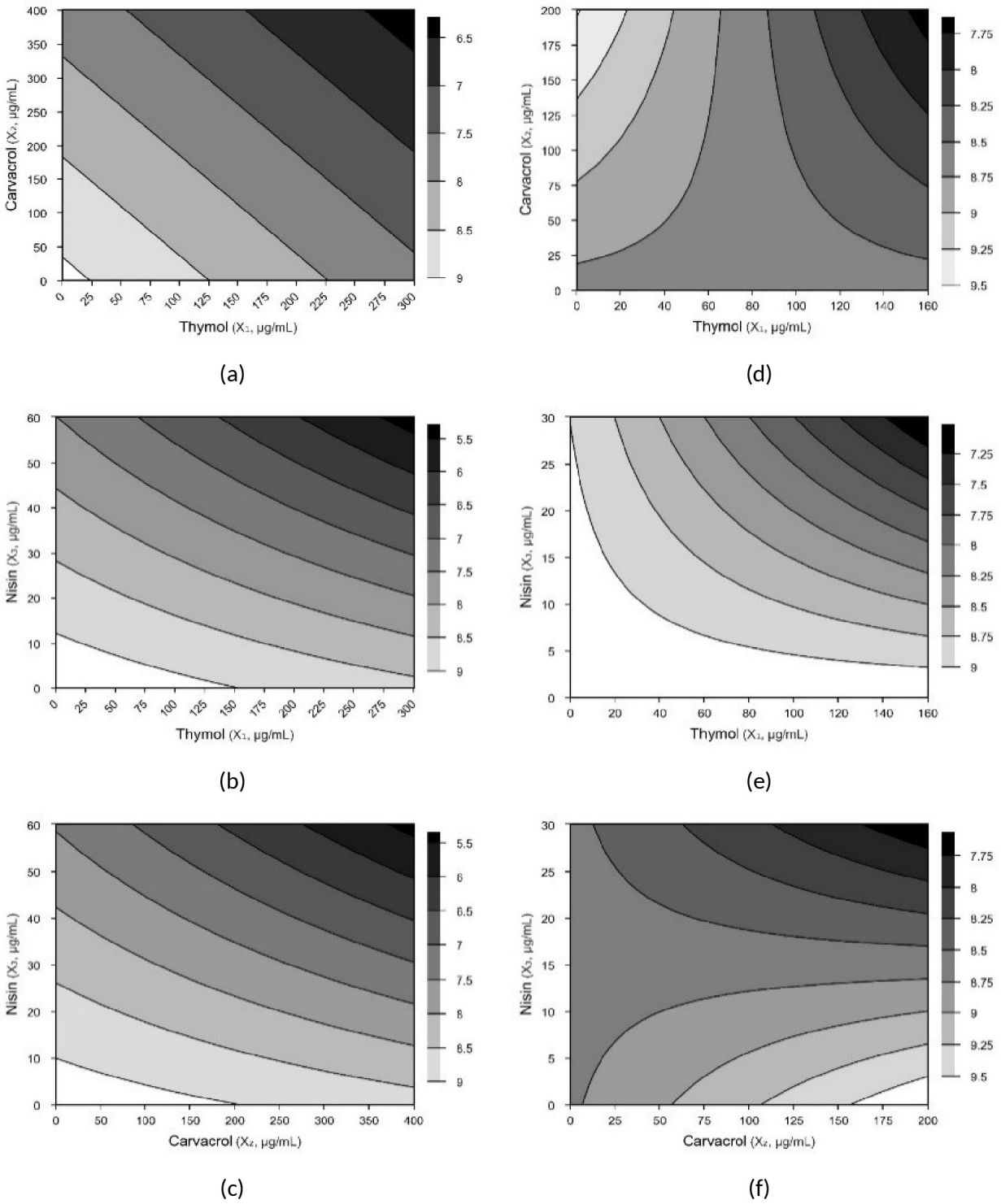

Figure 1. Contour plots of Test 1 and Test 2, for the combined effect of thymol-carvacrol (a, d), thymol-nisin (b, e) and carvacrol-nisin (c, f) against *S. aureus* (log CFU/mL; lateral scale).

Equation 1. In Equations 3 and 4, resulting from tests 1 and 2, respectively, *Y* is the response of *S. aureus* population; x_1, x_2, x_3 are the codded independent variables (thymol, carvacrol and nisin coded values, respectively).

In test 1 for thymol, nisin and carvacrol against *S. aureus*, the statistical analysis showed good fit of the experimental data to the mathematical model (Equation 3; R^2=0.9434, indicating that 94.34% of the experimental data can be explained by the model; *F*=16.67, being higher than the *F*=5.05 at 5% of significance), which allows the plot and critical analysis of the factorial design results. The use of individual antimicrobials (nisin, thymol and carvacrol) caused a significant (p<0.05) reduction in the population of *S. aureus* (Figure 1a,b,c). Also, the carvacrol-nisin and thymol-nisin interactions were significant (p<0.10) to reduce the population of *S. aureus* under tested conditions. Interaction between thymol and carvacrol was not significant (p>0.10).

Results from test 1 showed that thymol, nisin and carvacrol reduced, respectively, 0.48, 0.67 and 0.34 log CFU/mL the bacterial population compared to the experiments without antimicrobials. Combination of thymol (300 µg/mL) and carvacrol (400 µg/mL) reduced the bacterial population by 1.33 log CFU/mL. When nisin (60 µg/mL) was combined with thymol (300 µg/mL), there was a reduction of 3.52 log CFU/mL and when combined with carvacrol (400 µg/mL) the reduction was 3.41 log CFU/mL. The solution containing nisin (60 µg/mL), thymol (300 µg/mL) and carvacrol (400 µg/mL) reduces 4.98 log CFU/mL compared to the control.

$$Y = 7.70 - 0.74x_1 - 0.67x_2 - 1.30x_3 - 0.37x_1x_3 - 0.38x_2x_3 \quad (3)$$

In test 2, the contour diagrams of Equation 4 (Figure 1d,e,f), which was statistically significant (R^2=0.8276, indicating that 82.76% of the experimental data are explained by the model; *F*=4.80, being higher than the F_c=3.45 at 10% of significance) showed that individual concentrations of thymol (p<0.10) and nisin (p<0.05) were significant to reduce *S. aureus* population. Moreover, the combinations thymol-carvacrol, thymol-nisin and carvacrol-nisin caused significant (p<0.10) reduction of *S. aureus* population under tested conditions. The effect of carvacrol individually was not significant (p>0.10). Results showed that when nisin (30 µg/mL) was combined with thymol (150 µg/mL), there was a reduction of 0.22 log CFU/mL. Combination of thymol (75 µg/mL), carvacrol (100 µg/mL) and nisin (15 µg/mL) reduced the bacterial population by 0.42 log CFU/mL. By using double concentration of thymol, carvacrol and nisin, viable counts of *S. aureus* were reduced by about 3.30 log CFU/mL.

$$Y = 8.64 - 0.46x_1 - 0.58x_3 - 0.42x_1x_2 - 0.47x_1x_3 - 0.50x_2x_3 \quad (4)$$

For test 3, although the Fisher's test indicates significance of the equation (*F*=4.80 > F_c=3.45 at 10% of confidence), experimental data does not fit adequately to the regression model, since R^2 was quite low (R^2=0.5200, which indicates that 52% of the experimental data can be explained by the model). This situation does not allow a correct representation of contour or response surfaces plots. Analysis of the obtained results (Table I) indicates that thymol concentration individually is significant (p<0.10) to reduce the population of *S. aureus*. Moreover, the interaction between thymol-nisin was significant (p<0.10) to reduce the population of *S. aureus* under tested conditions. On the other hand, the interactions between nisin-carvacrol and thymol-carvacrol were not significant (p>0.10).

The thymol-carvacrol interaction was also evaluated by Netopilova et al. (2018) who found similar results against twelve *S. aureus* strains. They found an additive antimicrobial effect

against all strains tested at concentrations from 32 to 1,024 g/mL.

Thymol and carvacrol have similar structures with the hydroxyl group at different locations in the phenolic ring and they interact with the lipid bilayer of the plasma membrane due to their hydrophobic nature (Martínez-Graciá et al. 2015). The effects of thymol and carvacrol may occur at different bacterial cells sites, being effective against Gram-positive and Gram-negative bacteria. They can cause the collapse of the plasma membrane structure and consequently cell death (Burt 2004, Barbosa et al. 2009).

The combination of nisin with other antimicrobial compounds may be an alternative to increase the spectrum of action, to reduce the required nisin concentrations and decrease antimicrobial resistance (Singh et al. 2001, Churklam et al. 2020). Lambert et al. (2001) observed that the exposure of *S. aureus* to thymol and carvacrol caused a strong effect on pH homeostasis and on inorganic ion equilibrium of the bacteria due to damage on membrane integrity. Meanwhile, nisin mode of action against Gram-positive bacteria is related to interactions with membrane phospholipids, creating pores and causing efflux of essential constituents of microorganisms (Helander & Mattila-Sandholm 2000). This result show mathematical significant importance of combination of these antimicrobial agents against this important foodborne bacterium, commonly related to food outbreaks in Brazil.

Determination of combined effect of thymol and carvacrol against *Salmonella* Enteritidis

Table II shows the results of factorial design for the combined effect of thymol and carvacrol against *Salmonella* Enteritidis. Figure 2 represents the contour plots of Equations 5, 6 and 7, resulting from mathematical modeling of experimental data to Equation 2. In these

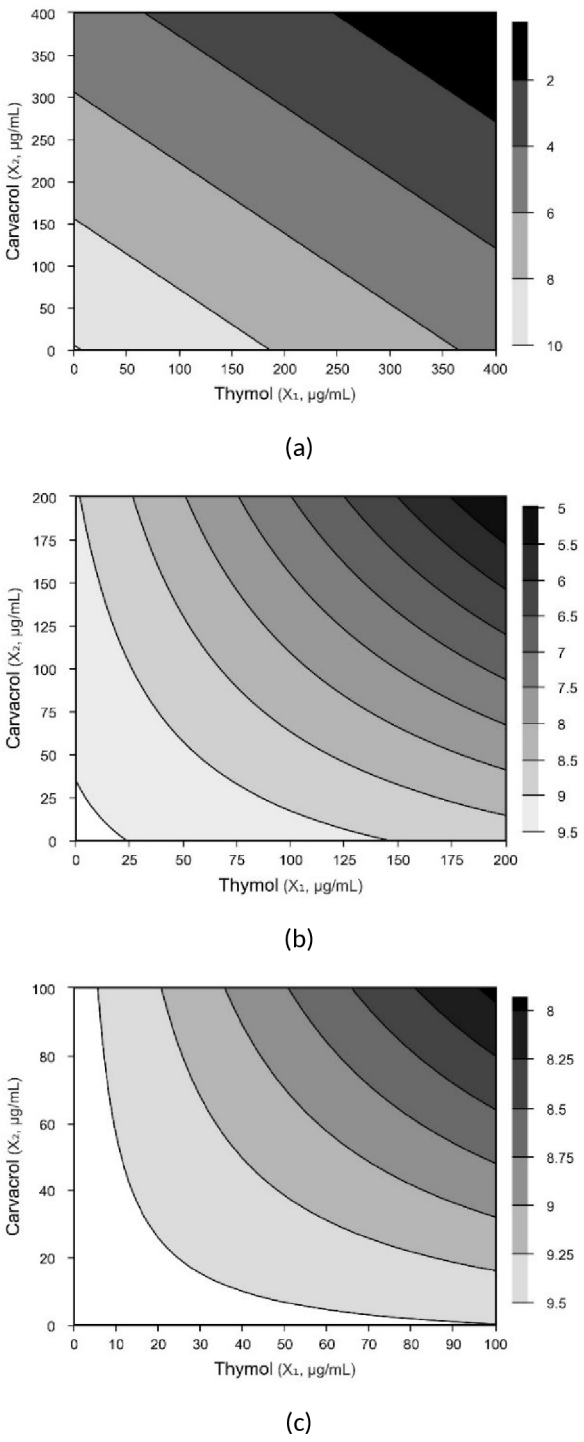

Figure 2. Response surfaces and contour diagrams of Test 1 (a), Test 2 (b) and Test 3 (c) for the combined effect of thymol-carvacrol against *Salmonella* Enteritidis (log CFU/mL, lateral scale).

equations, *Y* is the response of *Salmonella* Enteritidis population; x_1, x_2 are the independent variables, representing thymol and carvacrol coded values, respectively.

In test 1, the use of antimicrobials individually caused significant inhibition of *Salmonella* Enteritidis (p<0.05). Data analysis showed that the combination of carvacrol and thymol resulted in a significant (p<0.05) reduction of *Salmonella* Enteritidis in comparison to their individual effect (contour plot in Figure 2a, graphical representation of Equation 5, which was statistically significant at 95% of confidence - R^2 = 0.9926; F=268,97 > F_c=6,94). No viable counts were detected when *Salmonella* Enteritidis was exposed to a solution composed of thymol and carvacrol (400 µg/mL each). By using half concentration (200 µg/mL each), inhibition was also elevated, with a reduction around 4.5 log CFU/mL of *Salmonella* Enteritidis counts.

$$Y = 5.17 - 2.23x_1 - 2.66x_2 \quad (5)$$

In test 2, a solution of thymol and carvacrol (200 µg/mL each) was able to reduce the population of *Salmonella* Enteritidis by 4.62 log CFU/mL, while inhibition was approximately 1 log CFU/mL by using half concentration (100 µg/mL each). Equation 6, represented by the contour diagram in Figure 2b (which was statistically significant at 95% of confidence – R^2=0.9221; F=11.83 > F_c=9.28), indicates that the concentrations of thymol and carvacrol individually (p<0.05) and the interaction thymol-carvacrol (p<0.10) caused significant inhibition of *Salmonella* Enteritidis.

$$Y = 8.10 - 1.22x_2 - 1.09x_1 - 0.81x_2x_1 \quad (6)$$

Results obtained from test 3 were statistically significant (p<0.05) (R^2=0.9124; F=10.41 > F_c=9.28). As shown in Figure 2c (contour plot of Equation 7), a solution of thymol and carvacrol (100 µg/mL each) was able to decrease the population of *Salmonella* Enteritidis by 1.67 log CFU/mL. Figure 2c indicates that thymol and carvacrol compounds individually and their combination caused significant (p<0.05) inhibition of *Salmonella* Enteritidis.

$$Y = 9.16 - 0.44x_2 - 0.39x_1 - 0.39x_2x_1 \quad (7)$$

As discussed before, the isomers thymol and carvacrol have similar chemical structures and interact with the plasma membrane due to their hydrophobic nature (Martínez-Graciá et al. 2015). Therefore, its antimicrobial properties depend on the ability to permeabilize, depolarize and disrupt the cytoplasmic membrane of Gram-negative bacteria (Helander et al. 1998, Lambe,rt et al. 2001, Memar et al. 2017). The increased antibacterial efficacy of carvacrol and thymol when used in combination has also been observed against *E. coli*, *L. monocytogenes* and *Salmonella* Typhimurium (Du et al. 2015, Moon & Rhee 2016). Moreover, phenolic monoterpenes such as thymol and carvacrol have a tendency to increase the activity of other natural substances when used in mixtures (Bassolé & Juliani 2012). According to Ahmad et al. (2011), the positional differences of the hydroxyl group in thymol and carvacrol changes their antimicrobial activities, influencing the disruption of membrane integrity, which could explain the greater activity of thymol-carvacrol combination in comparison to their individual effects. The results of the present section show statistically the importance of combination of natural antibacterial compounds as a food safety strategy to control *Salmonella* Enteritidis, a Gram-negative bacterium, not rarely linked to inherent resistance to some antimicrobials used in food applications.

CONCLUSIONS

The combinations of thymol-nisin and nisin-carvacrol-thymol at ½ MIC, MIC e 2MIC were the most efficient in reducing the population of *S. aureus* under tested conditions. Thymol-carvacrol mixture demonstrated an additive antimicrobial effect against *Salmonella* Enteritidis. At MIC and 2MIC this combination reduced 4.62 log CFU/mL and 9.79 log CFU/mL (reduced the population to levels below the detection limit of the technique) of *Salmonella* Enteritidis, respectively. Therefore, these combinations of natural antimicrobials able to inhibit *S. aureus* and *Salmonella* Enteritidis has potential to be used as an additional barrier for food safety.

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