



# Combined effects of oregano essential oil and salt on the growth of *Escherichia coli* in salad dressing

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## ABSTRACT

There is a broad research interest in the search for alternatives to chemical additives for use as natural food preservatives. Although many natural compounds have biological *in vitro* properties evidenced, *in situ* studies are still scarce. This study evaluated the effect of oregano essential oil (OEO) and salt (NaCl) concentrations against *Escherichia coli* (ATCC 8739), in salad dressing, using the response surface methodology. The experiment included a 2<sup>2</sup> central composite rotatable design (CCRD) in a total of 11 formulations of salad dressings. Oregano essential oil was characterized by gas chromatography and salad dressings by ash, lipids, proteins and moisture. OEO was composed mainly by carvacrol (65.1%) and p-cymene (12.0%). Salad dressings showed similar chemical profiles. A mathematical model for the prediction of the antibacterial activity in salad dressing was obtained. The results revealed that the interaction between OEO and salt showed effect on the bacterial count. However, the effect of salt was negative suggesting that the highest NaCl concentrations decreases the bacterial count. Therefore, within the parameters studied, the use of OEO to control *E. coli* in salad dressing can be considered promising and allows reduction in the levels of salt to be incorporated in food.

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## 1. Introduction

Outbreaks of foodborne diseases have been the focus of discussions over the last years due their negative and economic impacts (Chen et al., 2015). Changes in the epidemiology of these diseases occur due to the expansion of the consumer market, economic globalization, dietary habits and increased consumption of purchased foods or taking food outside the home (Shinohara et al., 2008). Scallan et al. (2011) reported that in the United States occur on average 228,744 annual hospitalizations due to foodborne illness. Of these, 64% of cases are caused by bacteria.

Suspensions about the toxicity of some chemical additives and

abuse in the use of compounds as food preservatives have strongly pushed the governments of the most developed countries to control food production via legislation. Therefore, there is an increasing interest in research for natural food preservatives (Gutierrez et al., 2009; Haberbeck et al., 2012). Furthermore, the discovery of new natural antimicrobials also provides applications in the medical field, due to concerns about bacterial antibiotic resistance (Sagdic, 2003).

The global burden of foodborne diseases is currently unknown but the World Health Organization (WHO) launched an initiative to provide better estimates (Newell et al., 2010). In Brazil, the epidemic profile of the foodborne diseases remains limited as the result of the lack of official and available data of foodborne outbreaks, the most common etiologic agents and often involved food besides the contributing factors (Van Amson et al., 2006). Among the commonly involved in foodborne microorganisms, *Escherichia coli* can be highlighted. *Escherichia coli* are ordinarily commensal organisms, classified in six diarrhea genic pathotypes according to

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virulence characteristics and specific phenotypic traits (Rasko et al., 2011).

New approaches and technologies of efficient natural preservatives for foods include the use of essential oils from herbs and spices. These compounds have demonstrated antimicrobial activity against a broad spectrum of microorganisms, particularly when employed in combination with other technologies (Burt, 2004; Castilho et al., 2012; Haberbeck et al., 2012). Several essential oils from spices already are categorized as safe or GRAS by the US Food and Drug Administration (Burt, 2004; Benavides et al., 2012).

Although many studies evidence the *in vitro* properties of essential oils, its information about the antimicrobial effects in food products and their interaction with food components are still scarce (Pesavento et al., 2015). To evaluate the influence of distinct variables in a response of interest the response surface methodology (RSM) can be applied. It consists in a mathematical and statistical method whose experimental results indicate a combination of factor levels within an optimal region. The RSM is usually employed to the improvement and optimization of a process (Azevedo et al., 2014). Therefore, the RSM was employed to evaluate the individual and combined effects of the oregano essential oil (OEO) and salt (sodium chloride) against *Escherichia coli* (ATCC 8739) in salad dressing.

## 2. Material and methods

### 2.1. Material

Culture media was manufactured by HiMedia Laboratories (Mumbai, India) and Tween 80 from Sigma Aldrich Corporation (St. Louis, USA). Raw material was purchased from the local market of São José do Rio Preto, São Paulo State, Brazil, for the preparation of oregano salad dressing: soybean oil (Vila Velha, Louis Dreyfus Commodities Brasil), soy protein powder (Mãe Terra Produtos Naturais Ltd.), whole milk powder (Itambé Alimentos Corporation), sodium chloride (Lebre, Norte Salineira Corporation), pure oregano (*Origanum vulgare*) essential oil - OEO (Laszlo Aromatherapy Ltd.) and mineral water.

### 2.2. Microbial culture

*Escherichia coli* (ATCC 8739) was held in Plate Count Agar (PCA), at 4 °C, and reactivated overnight in PCA at 35 °C. The bacterial culture was the standard concentration of 10<sup>4</sup> CFU/mL. The microbial count was standardized from preliminary studies in which the different treatments of salad dressing were contaminated with *E. coli* and the microbial count was verified using the Eosin Methylene Blue Agar after serial decimal dilution for counting the forming colony units.

### 2.3. Oregano salad dressing processing

The mineral water (30.53 g), whole milk powder (3.8 g), soybean oil (64.12 g) and soy protein powder (1.55 g) were constantly stirred with the aid of a mixer at low speed (Walita – RI2044). At the end of the mixture, OEO and sodium chloride (independent variables) were added in order to observe the influence of these ingredients in bacterial count (dependent variable), as described in the experimental design shown in Table 1, a Central Composite Rotable Design (CCRD).

### 2.4. In situ antibacterial activity

Oregano salad dressings were analyzed in accordance with current legislation in Brazil, designated by the Microbiological

**Table 1**

The matrix of the central composite rotatable design with the coded and real values for the response bacterial count to assess the *in situ* antibacterial activity.

Run	Coded variables		Real variables <sup>a</sup>	
	X <sub>1</sub>	X <sub>2</sub>	Salt (%)	OEO (%)
1	−1	−1	1.14	0.20
2	+1	−1	1.30	0.20
3	−1	+1	1.14	0.40
4	+1	+1	1.30	0.40
5	−1.41	0	1.11	0.30
6	+1.41	0	1.33	0.30
7	0	−1.41	1.22	0.16
8	0	+1.41	1.22	0.44
9	0	0	1.22	0.30
10	0	0	1.22	0.30
11	0	0	1.22	0.30

<sup>a</sup> Percentages of OEO and salt in relation to 100 g of mixture of mineral water, whole milk powder, soybean oil and soy protein powder (weight/weight).

Standards of Technical Regulation for Food (Brasil, 2001), whose legislative limits are designated for coliforms at 45 °C, *Staphylococcus aureus* and *Salmonella* sp. After salad dressings microbiological control, samples were contaminated with *E. coli* (10<sup>4</sup> CFU/g). For *E. coli* counts, it was employed the methodology described by Kornacki and Johnson (2001), in which the Eosin Methylene Blue Agar was used to investigate the colonies of *E. coli* by spread plate inoculation. Microbiological analyses were performed immediately after the inoculation and were conducted at the Food Microbiology Laboratory, Department of Food Engineering and Technology, São Paulo State University.

### 2.5. Oregano salad dressings composition

The oregano salad dressings compositions were determined in accordance with the AOAC (1997) for ash, moisture, lipids and proteins. Total carbohydrates content was estimated by difference.

### 2.6. Chromatographic analysis of the oregano essential oil

Gas chromatographic of the oregano essential oil was performed using HP 5890 (GC Hewlett-Packard; Palo Alto, USA) equipped with flame ionization detector. A HP-1 (Hewlett-Packard; Palo Alto, USA) column 20 m × 0.25 mm, 0.25 µm film thicknesses was employed. The following temperature program was applied: 40 °C for 3 min, risen to 150 °C at the range of 3 °C/min. Injector temperature: 200 °C. Carrier gas: hydrogen (2 ml/min). FID temperature: 200 °C. Oregano essential oil replicates were diluted to 0.5% in tetrahydrofuran. Samples were analyzed in duplicate.

### 2.7. Experimental design

The chemical composition of different oregano essential oil salad dressings (carbohydrates, ash, lipids, proteins and moisture) was evaluated using descriptive statistics and non-parametric Kruskal-Wallis test followed by Dunn's test at 5% significance level. The response surface methodology was applied in the *in situ* antibacterial study, using a rotational central composite design for two independent variables (Rodrigues and Iemma, 2014), namely: the oregano essential oil concentration and the salt concentration. The dependent variable used was bacterial count. Eleven tests were performed: four of factorial points (2<sup>2</sup>) (two levels for each factor), four axial points (two for each variable) and three repetitions of the central point (Table 1). Central points provide additional degrees of freedom for error estimation, which increases power when testing the significance of effects, according to Carvalho et al. (1997). All

experiments were carried out in a randomized way and each run of the experimental design was conducted in triplicate. The regression was evaluated by means of analysis of variance; the regression was considered to be significant when  $p$ -value  $\leq 0.05$  and no lack of fit at  $p$ -value  $> 0.05$ . Linear and quadratic models were tested to explain the influence of independent variables on the response variable. The data were treated with the aid of Statistica software 7.0 (StatSoft Inc., Oklahoma, USA).

### 3. Results and discussion

Oregano salad dressings were in accordance with current Brazilian legislation on Microbiological Standards for Foods (Brasil, 2001) and the samples were considered suitable for human consumption. No *E. coli* was detected on the salad dressings employed in this research.

The formulations (treatments) of oregano salad dressings differed from each other in the concentrations of OEO and salt, except for the 9 to 11 treatments, that were replicates of the center point. Salad dressings presented moisture ranging between 31.99 and 32.33%, protein between 1.48 and 1.70%, lipids ranging between 57.89 and 60.09%, ash between 1.35 and 1.55% and carbohydrate between 5.01 and 6.70%. Oregano salad dressings samples did not differ ( $p$ -value  $> 0.05$ ) compared to the levels of carbohydrates, lipids, proteins and moisture. However, for the ash content, treatment 2 (1.30% salt and 0.2% OEO) was lower ( $p$ -value  $\leq 0.05$ ) than treatment 8 (1.22% salt and 0.44% OEO). Therefore, the different oregano essential oil salad dressings showed similar characteristics and conducive to microbial growth, such as high moisture content, lipids, proteins and carbohydrates.

The main component of OEO was carvacrol (65.1%), followed by  $p$ -cymene (12.0%),  $\gamma$ -terpinene (6.8%) and thymol (3.4%), confirming results of Silva et al. (2010), in an analysis of five brands of OEO commercialized in Brazil. The authors reported that carvacrol was present in the range from 61.66 to 93.42%, thymol between 1.88 and 23.85% and  $p$ -cymene in the range from 0.63 to 15.95%. In addition, in one of the brands evaluated, the authors evinced the presence of  $\gamma$ -terpinene. Souza et al. (2016), in a study about the inhibitory effects of the essential oil from *Origanum vulgare* L. showed that the major components of the samples were carvacrol (69.0%), thymol (14.12%),  $\gamma$ -terpinene (3.71%) and  $p$ -cymene (3.67%). Furthermore, Siroli et al. (2015) report that the scientific literature evidences  $p$ -cymene, carvacrol, thymol and  $\gamma$ -terpinene as the main components present in oregano, as verified in the present study.

According to Hernández-González et al. (2017), the antimicrobial activity of oregano essential oil is due to thymol and carvacrol, which are capable of disintegrating microorganism's outer membrane. The major compounds present in plants are those that determine the biological properties of the essential oils (Bakkali et al., 2008; Llana-Ruiz-Cabello et al., 2015). Carvacrol is usually reported as the major component of the oregano essential oil. It is a hydrophobic phenolic compound, with well documented antimicrobial activity against bacteria, fungi and yeasts (Guarda et al., 2011; Burt, 2004) and antioxidant activity (Baser, 2008), showing a high potential to promote the extension of the shelf life and safety of food products (Rubilar et al., 2013).

The composition of essential oils may be affected by intrinsic characteristics of the plant, climatic factors, cultivation and extraction method employed to obtain the essential oil. Therefore, these factors have an influence on the properties of the essential oils. However, the major components present will continue to exert its antimicrobial effect, allowing the use of essential oils to control the microbial growth (Masotti et al., 2003; Angioni et al., 2006; Teixeira et al., 2013; Calo et al., 2015).

The variation of the microbial load between treatments was at

most 12% and approximately 2 log units higher than the initial bacterial count of the samples (Fig. 1).

Estimates of the effects of parameters on the antimicrobial activity are shown in Table 2. The  $p$ -values were used as a tool to check the significance of each of the coefficients. The smaller the magnitude of  $p$ -value, the more significant is the corresponding coefficient. According to Table 3, salt concentration and the combination of oregano essential oil and salt concentrations were significant at a confidence level of 95% against *Escherichia coli*. The interaction between oregano essential oil and salt showed effect in the bacterial count, allowing to suggest that the decrease in the interaction of factors (antagonist effect) improves the reduction of the microbial count. Furthermore, the effect of salt in the *E. coli* load can be observed (Fig. 2).

Based on the analysis of variance, ANOVA, (Table 3), a second-order model (Eq. (1)) describing the *E. coli* count in function of oregano essential oil and salt concentrations was established. Non-significant terms were added to the lack of fit for the calculation of the coefficient of determination ( $R^2$ ) and the F-test. The closer the  $R^2$  value is to 1, the better is the fit of the model to experimental data. The coefficient of determination was 0.8610, indicating that the proposed mathematical model explains 86.10% of the variability in the response variable count of *E. coli*. The F-test was higher than the  $F_{(0.95; 3; 7)}$ . Therefore, the mathematical model is predictive and adjusted, showing no lack of fit ( $p = 0.0538$ ).

$$\log \frac{CFU}{g} = 6.07 - 0.07 \text{ Salt} + 0.19 \text{ Salt}^2 + 0.36 \text{ Salt} \times \text{OEO} \quad (1)$$

The encoded mathematical model was employed to generate de response surface and contour diagram for *E. coli* count (Fig. 3A–B). The lowest bacterial counts can be observed with an oregano essential oil concentration range from 0.4 to 0.5% in combination with salt concentration range from 1.1 to 1.2% and also with an oregano essential oil range from 0.1 to 0.2% and a salt concentration range from 1.3 to 1.4%. The essential oil and the salt exert effect on *E. coli* load when used in concentrations inversely proportional.

Azevedo et al. (2014) applied the response surface methodology in a study about the effect of cassava starch, chitosan and *Lippia gracilis* Schauer genotype concentrations on the antimicrobial

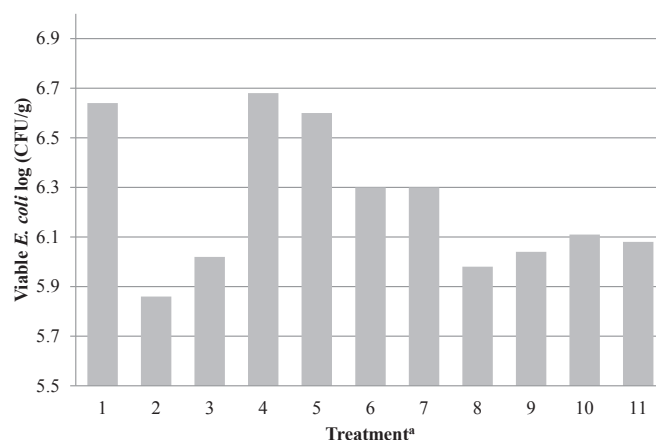


Fig. 1. Logarithmic viable *Escherichia coli* in salad dressings.

<sup>a</sup> Treatments from 1 to 11 correspond to each formulation of oregano salad dressing containing different amounts of OEO and salt. 1 = 1.14% of salt and 0.2% of OEO; 2 = 1.30% of salt and 0.2% of OEO; 3 = 1.14% of salt and 0.4% of OEO; 4 = 1.30% of salt and 0.4% of OEO; 5 = 1.11% of salt and 0.3% of OEO; 6 = 1.33% of salt and 0.3% of OEO; 7 = 1.22% of salt and 0.16% of OEO; 8 = 1.22% of salt and 0.44% of OEO; 9, 10 e 11 = 1.22% of salt and 0.3% of OEO. Percentages of oregano essential oil (OEO) and salt in relation to 100 g of mixture of mineral water, whole milk powder, soybean oil and soy protein powder (weight/weight).

**Table 2**Main effects analysis for *Escherichia coli* count (log CFU/g) in salad dressing.

Term	Effect	Std. Err.	t-value	p-value
Mean	6.0768	0.0203	299.8544	<0.001
(1) Salt (%) (L)	−0.0684	0.0126	−5.4357	0.0322
Salt (%) (Q)	0.1956	0.0154	12.7225	0.0061
(2) OEO (%) (L)	−0.0313	0.0125	−2.5093	0.1288
OEO (%) (Q)	0.0306	0.0149	2.0495	0.1769
OEO <sub>2</sub> Salt	0.3600	0.0175	20.5018	0.0024
Regression (ANOVA)	—	—	—	<0.001
Lack of fit	—	—	—	0.0538

OEO: oregano essential oil; (L): linear; (Q): quadratic.

**Table 3**Analysis of variance (ANOVA) for antimicrobial activity of oregano essential oil and salt (NaCl) against *Escherichia coli* count (log CFU/g) in salad dressing.

Source	Sum of squares	Degrees of freedom	Mean squares	F-test
Regression	0.7521	3	0.2507	4.96
Residue	0.0811	7	0.0116	
Lack of fit	0.0786	5	0.0157	0.68
Pure error	0.0025	2	0.0012	
Total	0.8332	10	—	

\* $R^2 = 0.8610$ ;  $F(0.95; 3; 7) = 4.35$ .

activity of edible coatings against eight pathogens bacteria, including *E. coli*. The authors were successful in obtaining mathematical models for the prediction of the antimicrobial activity of edible coatings according to the chitosan and essential oil concentrations. RSM methodology was also applied by Calderón-Oliver et al. (2016) to optimize a mixture of nisin (an antimicrobial) with avocado seed and peel (an antioxidant) to maximize both responses, reporting the efficacy of the technique for prediction purposes.

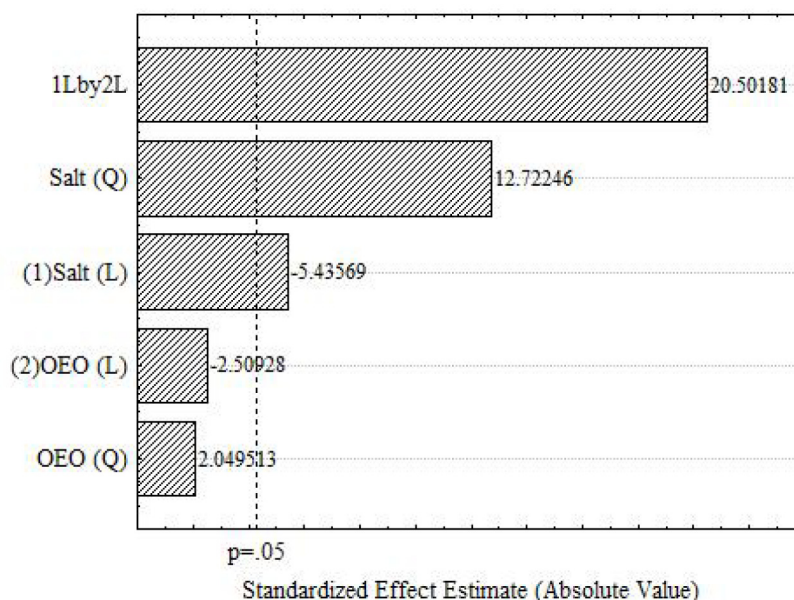
Frangos et al. (2010) reported that the combination of salt,

oregano essential oil (0.2% v/w) under vacuum-packaging led to the extension of shelf-life of fillets (11–12 days), compared to the control (kept under aerobic conditions), according to sensory data. The authors verified that the combined effect of OEO (0.4% v/p), NaCl and vacuum condition managed to inhibit the growth of *Enterobacteriaceae* during the storage period.

Karabagias et al. (2011) showed that the shelf-life of meat-based products increased when thyme and oregano essential oils were applied at 0.1 and 0.3%. Similarly, Pesavento et al. (2015) evinced that staphylococcal load in meat samples was reduced during their preservation at 4 °C, suggesting a possible use of Rosmarinus, Thymus and Oregano essential oils, as food preservatives, in concentration of 0.5% (v/w) or lower in raw meat.

The effectiveness of many antimicrobial naturally present in food or intentionally added to them may be reduced by intrinsic and extrinsic factors related to food (Burt, 2004). According to Calo et al. (2015), lipids, proteins, water activity, pH and enzymes can potentially reduce the efficacy of essential oils. Smith-Palmer et al. (1998) reported that higher lipid content requires higher concentrations of essential oils to inhibit bacterial growth. Other researchers also suggest that lipids and proteins are capable of absorbing the extracts of spices, reducing the antimicrobial effect of these compounds (Shekarforoush et al., 2014). In this study, oregano salad dressing formulations presented lipids ranging between 57.89 and 60.09%, which is an extremely important factor for the evaluated bacterial inhibition. In turn, sodium chloride reduces the water activity of the food and, thus, led to the prevention of the development of microbial strains.

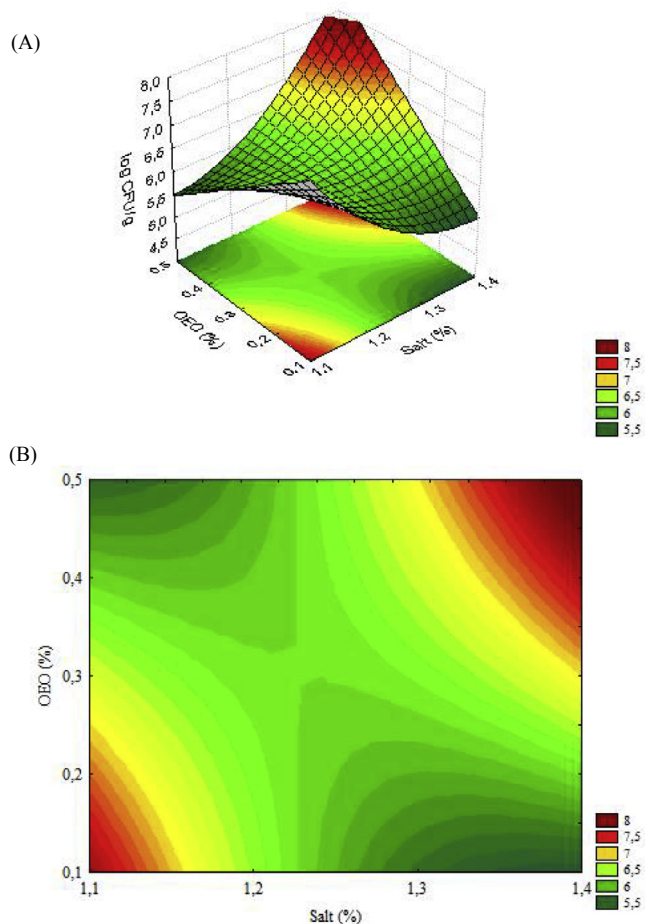
Although several biological properties of natural compounds have been evidenced in *in vitro* studies, they usually fail to be effective when employed in complex foods. It is known that the concentration and the components present in foods give rise to molecular and colloidal interactions that impact the antimicrobial effect of essential oils. Some factors that contribute to the antimicrobial paradox are the polarity, molar mass and charge of the



1Lby2L represents the effect of the interaction between oregano essential oil (OEO) and salt.

**Fig. 2.** Pareto chart for *Escherichia coli* count in salad dressings containing oregano essential oil and salt (NaCl). Linear (L) and quadratic (Q) coefficients. 1Lby2L represents the effect of the interaction between oregano essential oil (OEO) and salt.





**Fig. 3.** Surface response (A) and contour plot (B) for *Escherichia coli* count (log CFU/g) as function of oregano essential oil (OEO) and salt concentrations in salad dressing. CFU: colony-forming unity.

antimicrobial compound, besides the pH, the ionic strength and the temperature of the medium. The strategies employed to improve the biological effects of natural compounds on food involve the modification of their physical properties by encapsulation (Weiss et al., 2015).

In addition essential oils have a green labelling and natural image (Olmedo et al., 2013), it is of paramount importance to pay attention to the amount of essential oil to be applied in foods systems, as this is a determining factor in sensory acceptance of products, influencing the flavor and aroma (Chouliara et al., 2007). Cattelan et al. (2015) evaluating the sensory acceptability of salad dressing formulated with different concentrations of oregano essential oil and salt showed that the formulation with intermediate quantities of salt and OEO (1.22 and 0.3%, respectively) was preferred by the consumers in relation to flavour and overall liking. Furthermore, salad dressings containing lower concentrations of salt, independent of OEO content, had greater purchase intention.

An inherent analysis of the essential oils used individually or in combination with other substances allows using them to achieve synergy of lethal effects against micro-organisms, employing low concentrations of these compounds, and resulting in no adverse sensory characteristics to foods. It is known that the most successful treatments used in the food industry are those that, in combination, can provide excellent barriers to microbial growth. Knowledge of the barrier mechanisms of action helps in optimizing the most effective treatment conditions (Ait-Ouazzou et al., 2013).

#### 4. Conclusion

The scientific data obtained in this study allowed building a mathematical model describing the *Escherichia coli* count as a function of oregano essential oil and salt concentrations, using the response surface methodology. The results revealed that the interaction between oregano essential oil and salt exerted effect on the bacterial count. However, the effect of salt was negative suggesting that the highest NaCl concentrations decreases the bacterial count. The essential oil and the salt exert antibacterial effect against *E. coli* strain when used in concentrations inversely proportional. Therefore, within the parameters studied, the use of OEO in combination with NaCl to control *E. coli* in salad dressing can be considered promising.

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