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CONTROL OF PSYCHROTROPHIC BACTERIA AND *Escherichia coli* IN FRESCAL TYPE FISH SAUSAGE USING OREGANO ESSENTIAL OIL

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ABSTRACT

The aim of this work was to prepare *frescal* fish sausage using oregano essential oil as a preservative. For this, Minimum Inhibitory Concentration (MIC) of oregano essential oil against six food-borne pathogens was determined. The antimicrobial activity of oregano oil (0.08% and 0.16% concentrations) was tested in fish sausage kept under refrigeration, by counting the cultivable psychrotrophic bacteria (CPB) after 20 days and by intentionally inoculation with *Escherichia coli* (72 h). The oregano essential oil exhibited a strong antibacterial activity (MIC 50-800 μg mL^{-1}), with bactericidal action (MBC 1600 μg mL^{-1}). The development of *E. coli* was inhibited in the *frescal* fish sausage prepared with 0.08% oregano oil, and the addition of 0.16% oregano essential oil resulted in a better control of CPB. Oregano essential oil can, therefore, be used as an additional barrier against the development of spoilage bacteria and *E. coli* in food.

Key words: built; spices; antimicrobial activity; refrigeration.

CONTROLE DE BACTERIAS PSICROTRÓFICAS E *Escherichia coli* EM LINGUIÇA DE PEIXE TIPO FRESCAL ADICIONADA DE ÓLEO ESSENCIAL DE ORÉGANO

RESUMO

O objetivo deste trabalho foi elaborar linguiça de peixe frescal adicionada de óleo essencial de orégano como conservante natural. Para isso foi determinado o valor da Concentração Inibitória Mínima (CIM) do óleo essencial de orégano frente a seis patógenos de interesse alimentar. A atividade antimicrobiana do óleo de orégano (0,08 e 0,16%) na linguiça de peixe mantida sob refrigeração foi verificada por meio da contagem de bactérias psicotróficas cultiváveis (BPC) durante 20 dias e após contaminação intencional com *Escherichia coli* (72 h). O óleo essencial de orégano apresentou forte atividade antibacteriana (CIM 50-800 μg mL^{-1}), com ação bactericida (CIB 1600 μg mL^{-1}). A linguiça de peixe frescal adicionada do óleo de orégano (0,08%) inibiu o desenvolvimento de *E. coli* no sistema alimentar, enquanto a concentração de 0,16% do óleo de orégano se mostrou mais efetivo no controle de BPC. O óleo essencial de orégano pode ser usado como barreira adicional em alimentos para o desenvolvimento de bactérias deteriorantes e *E. coli*.

Palavras-chave: embutidos; especiarias; atividade antimicrobiana; refrigeração.

INTRODUCTION

In recent years, a demand for healthy food has led to a change in consumer habits, reducing the consumption of red meats and resulting in seafood becoming more popular. In 2014, the global consumption of fish was 146 million tons, corresponding to consumption of 20 kg per capita consumption (FAO, 2016).

The nutritional benefits of fish are mainly attributed to its constituent proteins (15-20%), which have high digestibility, vitamins, minerals, and the presence of EPA (eicosapentaenoic) and DHA (docosahexaenoic) type fatty acids. Among the various physiological effects of these acids in humans can be cited as the prevention and treatment of cardiovascular diseases, hypertension, inflammation in general, asthma, arthritis, psoriasis, and various types of cancer (MACIEL *et al*., 2015).

Although varying with the country, the dietary habits such as consumption of fresh produce and the lack of diversification of products obtained from fish meat imply that fish consumption is nevertheless low when compared to the other sources of animal protein (OKSUZ *et al*., 2008). This fact has encouraged the stakeholders of fish processing industries to explore new products other than just the frozen form of fish fillet and whole fish.

The manufacture of sausages of the fresh type stands out among the manufacture of meat products, embedded by its acceptance and ample commercialization. In the manufacturing process, preservatives are used for maintenance of color and curing, and salts are used for bacteriostatic action, both of which help in increasing the shelf life of the product (IAMARINO *et al*., 2015). Several synthetic preservatives can be used for food preservation, such as sorbic acid, calcium carbonate, sodium benzoate, and nitrites and nitrates (BRASIL, 2001). The main concern with the use of nitrites and nitrates as food preservatives is the toxic effects caused by their excess quantities in diet, by the endogenous formation of n-nitroso compounds, such as n-nitrosodimethylamine and monomethylnitrosamine, which have carcinogenic, teratogenic, and mutagenic effects (IAMARINO *et al*., 2015). As a result, foods processing industries, as well as consumers, have signaled a demand for reduction in the use of synthetic chemicals in food preservation (HAYOUNI *et al*., 2008).

Essential oils are aromatic compounds of plant origin that exhibit broad- spectrum biological activity against Gram-negative and Gram-positive bacteria. The antibacterial, antiviral, and antifungal action of essential oils has encouraged the food industry to use these oils as natural antimicrobial agents in food products and beverages (DONSI and FERRARI, 2016; BALLESTEROS *et al*., 2016). Essential oils, when compared to powdered condiments, guarantee a longer shelf life, maintenance of aroma and a lower risk of microbiological contamination, ensuring good quality of the processed product (TRAJANO *et al*., 2009; ZAMPIERI *et al*., 2017).

Essential oils are present in plants as a natural component. They are defined as the by-products of plant metabolism, composed of terpenes, which may or may not be associated with other components, and are mostly volatile in nature (BANDONI and CZEPAK, 2008). These compounds are generally composed of terpene hydrocarbons, alcohols, aldehydes, ketones, phenols, esters, ethers, oxides, peroxides, furans, organic acids, lactones, coumarins, and even compounds such as sulfur (BAKKALI *et al*., 2008; DONSI and FERRARI, 2016).

Given the increase in demand for food products, especially the demand for foods with high nutritional value and protein content, the formulation of fish products, such as fish sausages, using species of low commercial value or the by-products of their industrialization, have gained interest. Also, the use of natural preservatives has emerged as a promising alternative to the chemical counterparts, in order to add value to the product. Cação (*Carcharhinus* spp.) is a species of fish having intermediate commercial value and high-fat content; it is widely marketed in the Recôncavo Baiano. Based on the above information, the present work was aimed at elaboration of *frescal* type sausage using cação with different concentrations of oregano essential oil added as a natural preservative.

METHODS

Microorganisms

Six bacteria species isolated from food were obtained from the collection of cultures at the Laboratory of Food Microbiology and Environmental, in Nucleus of Studies in Fisheries and Aquaculture. Of these, three were Gram-negative bacteria (*Escherichia coli, Salmonella* serotype Enteritidis, and *Vibrio cholera*) and three were Gram-positive bacteria (*Staphylococcus aureus, Listeria monocytogenes,* and *Bacillus cereus*).

Oregano essential oil

The essential oil of oregano was obtained commercially from Terra Flor Aromaterapia, Chapada dos Veadeiros, Alto Paraíso, GO.

Determination of Minimum Inhibitory Concentration (MIC)

In order to prepare the dilutions of oregano essential oil, 1 g of oil was weighed and dissolved in methanol until it reached the concentration of 640 mg mL^{-1} (Solution I). After that, 1:100 dilution in Muller-Hinton broth was performed, obtaining a concentration of 6400 μg mL^{-1} (Solution II) (SANTURIO *et al*., 2007). Based on the document M7-A6 (from NCCLS, 2005), the determination of Minimum Inhibitory Concentration (MIC) consisted of distribution of 200 μL of Muller-Hinton broth into the wells of a microtiter plate; following which, 200 μL of Solution II was added to the first well and after homogenization, was transferred to the second well and so on, obtaining the final concentrations of 3200, 1600, 800, 400, 200, 100, 50 and 25 μg mL^{-1}. To these wells, 10 μL (1×10^6 CFU mL^{-1}) inoculum of each microorganism (bacterial species) was added Muller-Hinton broth without addition oregano oil was used as negative control, and a solution of antimicrobial chloramphenicol at a concentration of 100 μg mL^{-1} was used as positive control. The microtiter plates were incubated at 35 °C for 24 h. The results of the assay were read using the colorimetric method, by adding 20 μL resazurin sodium dye (Sigma, Aldrich) at a concentration of 0.01% (w/v) to all the wells of the microtiter plate. This dye is recognized as a colorimetric oxide-reduction indicator (PALOMINO *et al*., 2002). The change in the color from blue to pink indicated microbial multiplication. All assays were performed in triplicate. The MIC was determined as the lowest concentration of the essential oil capable of causing total inhibition of bacterial growth.

Determination of Minimum Bactericidal Concentration (MBC)

For the determination of CBM, 10 μL of the lowest three concentrations of the essential oil containing the strains tested with the absence of microbial growth were plated in a Petri dish containing Muller-Hinton agar and incubated at 35 °C for 24 h. The concentration that did not allow the reactivation of the microorganism in the medium, without the addition of essential oil, was considered bactericidal. For the microorganisms that exhibited multiplication, the bacteriostatic action was considered (SANTURIO *et al*., 2007). All assays were performed in triplicate.

Elaboration of frescal fish sausage

The *frescal* sausage was handcrafted using 85% fish meat. Of the frozen fish obtained commercially from Cruz das Almas, BA, 3 Kg was used. For thawing, the fish were first refrigerated until the temperature of 10 °C was reached; the frozen fish were then were cut into small pieces and ground in a manual meat grinder (8 mm diameter) to obtain ground fish meat. The sausages were elaborated in the Laboratory Multifunctional 04 - Laboratory Block O, UFRB. The *frescal* cação sausage was prepared using the following ingredients (%): fish meat (85), lard (15), iodized salt (1.0), seasoning complete (0.5*), english sauce (0.5), urucum (0.2), wheat flour (0.5), corn cream (0.5), sugar (2.0) and water (8.0). *salt, onion, garlic, chives, parsley, basil.

The fish meat and the bacon, after being ground, were mixed manually with the other ingredients. For the treatments, oregano essential oil was used in two concentrations (Table 1), based on the concentration values identified in the MIC assay. For each treatment, the mass was separated into two portions: one for counting the cultivable psychrotrophic bacteria (CPB) and the other for intentional *E. coli* contamination. The sausages were embedded in the gut (caliber 45 mm) and cut into tubes of approximately 10 cm in length.

Microbiological analysis

The antimicrobial activity of oregano essential oil in fish sausage was verified using two microbiological assays. In the first assay, the CPB count (SILVA *et al*., 2010) was evaluated using a limit of 10^7 CFU g^{-1} (ICMSF, 2015). The CPB count was performed on the sausage mass immediately after preparation (Time T0), as well as later on at T4, T8, T12, T16, and T20 times of the storage days at 7 °C. All analyses were performed in duplicate.

In the second microbiological assay, intentional contamination of the fish sausage with an *E. coli* culture (1 x 10^4 CFU g^{-1}) was performed. The antimicrobial action of oregano oil against *E. coli* bacteria in fish sausages kept at refrigeration at 7 °C was monitored at times T0, T24, T48 and T72 h (SILVA *et al*., 2010). All samples were analyzed in duplicate.

Table 1. Treatments used in the elaboration of the *frescal* cação sausage.

Treatment	Description
Control	Without addition of oregano essential oil
Treatment T1	0.08% of oregano essential oil
Treatment T2	0.16% of oregano essential oil

Physicochemical analysis

For the characterization and determination of the centesimal composition of each formulation of the *frescal* cação sausage, the following analyses were carried out in triplicate: moisture, ashes, lipids, protein, and carbohydrates. The analyses were performed in accordance with the manual of the ADOLFO LUTZ INSTITUTE (IAL, 2008).

Statistical analysis

Statistical analyses were performed using the statistical program SISVAR 4.3 (FERREIRA, 2011). The means of the treatments groups, when significant, were compared by Scott-Knott test at 5% probability.

RESULTS

Antimicrobial activity

The MIC results for oregano essential oil demonstrated that the concentrations ranging from 50 µg mL^{-1} to 800 µg mL^{-1} were efficient in inhibiting both Gram-positive and Gram-negative bacteria that were being tested. The lowest MIC (50 µg mL^{-1}) was obtained for the Gram-positive pathogens *L. monocytogenes* and *S. aureus*, while the highest MIC was obtained for the Gram-positive pathogen *B. cereus* (Table 2).

Oregano essential oil exhibited bactericidal action against *B. cereus, E. coli* and *V. cholera:* whereas *S.* serotype Enteritidis, *L. monocytogenes* and *S. aureus* did not exhibit cell death even at the highest concentration of oregano oil that was for testing (3200 µg mL^{-1}), therefore, exhibiting bacteriostatic action.

Table 2. The Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of oregano essential oil against Gram-negative and Gram-positive bacteria.

Microorganisms	Oregano µg mL^{-1}	CLO µg mL^{-1}	CBM µg mL^{-1}	Without oil addition
Vibrio cholerae	200	100	1600	NI
Salmonella serotype Enteritidis	200	100	NI	NI
Escherichia coli	400	100	1600	NI
Listeria monocytogenes	50	100	NI	NI
Staphylococcus aureus	50	100	NI	NI
Bacillus cereus	800	100	1600	NI

NI: There was no inhibition; CLO: Chloramphenicol; CBM: Minimum Bactericidal Concentration.

Microbiological analysis

Microbiological analysis of cultivable psychrotrophic bacteria

The CPB count for different treatments groups exhibited a significant difference between the times of evaluation (Table 3). All treatments exhibited a statistically significant increase ($p < 0.05$) in the number of bacterial cells over the course of 20 days. The control test (without oil) exhibited a significant increase in the number of bacterial cells (CFU), reaching a concentration greater than 9.9×10^6 CFU g^{-1} at the end of 20 days. The difference between the control and treatments groups was observed mainly after 12 days, when a significant increase ($p < 0.05$) was observed in the bacterial development in the control group, reaching uncountable values.

The refrigerated *frescal* cação sausage had CPB counts below 10^7 CFU g^{-1} up to 16 days of treatment T2; four days longer than that for the T1 treatment and eight days longer than that for the control group. From time T0 to T12, there was no statistical difference in the microbial densities (CPB) between the treatment groups (T1 and T2). Treatments groups began to differ from day 16, when a significant increase ($p < 0.05$) was observed in the bacterial development in the T1 treatment group, reaching the values above 10^6 CFU g^{-1} (Table 3).

Antimicrobial effect of oregano essential oil on frescal cação sausage contaminated intentionally

The analysis of the intentionally contaminated sausage demonstrated that there was no increase in the number of bacterial cells in the control group (without the presence of essential oil) during the 72 h. The values obtained for the control group (2.3×10^3 NMP $100g^{-1}$) served as a reference for assessing the development of *E. coli* bacteria in the treatments groups (Table 4).

The action of the essential oil could be observed from time T0 when the same amount of *E. coli* was inoculated in all treatments. Even though the control exhibited no increase in the microbial density of *E. coli* with time, it was nonetheless higher than the values obtained for the fish sausage treated with oregano essential oil. During 72 h, no significant difference was observed in the development of bacteria between the two treatments groups containing oregano essential oil, demonstrating that the increase in oil quantity did not result in greater efficiency in reducing the number of inoculated cells.

Physicochemical analysis

The three products developed had similar amounts of all ingredients except the amount of essential oil (Table 5). The *frescal* fish sausage met the physicochemical standards, according to the Technical Regulation of Identity and Product Quality (BRASIL, 2000).

Table 3. Cultivable psychrotrophic bacteria count (CPB) in *frescal* cação sausage containing oregano essential oil stored at 7 °C for 20 days.

Psychrotrophic (UFC g^{-1})	Time (days)					
	0	4	8	12	16	20
Control	9.9×10^1 aA	1.6×10^5 bB	2.9×10^{6}* cA	>9.9×10^6 dB	>9.9×10^6 dB	>9.9×10^6 dA
Treatment T1	5.4×10^1 aA	8.6×10^4 bA	1.6×10^6 cA	2.4×10^{6}* cA	>9.9×10^6 dB	>9.9×10^6 dA
Treatment T2	4.2×10^1 aA	4.5×10^4 bA	1.2×10^6 cA	1.5×10^6 cA	2.2×10^{6}* cA	>9.9×10^6 dA

Means followed by equal letters do not differ by Scott-Knott's test ($p < 0.05$), comparing lowercase letters in rows and capitals in columns. *(10^7 CFU g^{-1}) Limit according to ICMSF (2015). T1 = sausage containing 0.08% of oregano essential oil. T2 = sausage containing 0.16% of oregano essential oil.

Table 4. Development of intentionally added *Escherichia coli* in *frescal* cação sausage samples containing oregano essential oil and stored at 7 °C for 72 h.

Escherichia coli (MPN $100g^{-1}$)	Time (hours)			
	T0	T24	T48	T72
Control	2.3×10^3 aB	2.3×10^3 aB	2.3×10^3 aB	2.3×10^3 aB
Treatment T1	3.6×10^2 aA	<3.0×10^1 aA	<3.0×10^1 aA	<3.0×10^1 aA
Treatment T2	<3.0×10^1 aA	<3.0×10^1 aA	<3.0×10^1 aA	<3.0×10^1 aA

Means followed by equal letters do not differ from one another by the Scott-Knott test ($p < 0.05$), comparing lowercase letters in rows and capitals in columns. T1 = sausage containing 0.08% of oregano essential oil. T2 = sausage containing 0.16% of oregano essential oil.

Table 5. Centesimal composition (%) of the *frescal* cação sausage containing oregano essential oil.

Composition (%)	Brasil (2000)	Control	Treatment T1	Treatment T2
Ashes	3.0 (max.)	02.80±0.0a*	2.67±0.2a	2.73±0.1a
Humidity	70 (max.)	67.67±1.5a	67.6±2.4a	68.47±0.2a
Lipids	30 (max.)	19.84±0.0a	21.12±0.3a	22.46±0.7a
Proteins	12 (min.)	13.52±0.4a	14.10±0.2a	13.58±0.5a
Carbohydrates	-	03.83±2.0c	5.49±2.4b	7.24±0.6a

*Means followed by equal letters do not differ by Scott-Knott test ($p < 0.05$); - There is no limit of values established in the current legislation. T1 = sausage containing 0.08% of oregano essential oil. T2 = sausage containing 0.16% of oregano essential oil.

DISCUSSION

The bactericidal and bacteriostatic activity of oregano essential oil in the Gram-negative and Gram-positive bacteria (SANTURIO *et al*., 2007; VAN HAUTE *et al*., 2016) are due to the major constituents of the oil, mainly carvacrol and thymol (BASSANETTI *et al*., 2017), demonstrating the potential use of this essential oil in processed foods, by adding in formulation or by coating in the form of a film.

The CPB count assessment was based on the values suggested by the International Commission on Microbiological Specifications for Foods (ICMSF, 2015), considering 10^7 CFU g^{-1} as the limit value for the evaluation of the microbiological quality of the product. Although Brazilian legislation (BRASIL, 2001) does not establish limits for psychrotrophic microorganisms in sausages, their presence in refrigerated foods contributes to the reduction in the shelf life of the product because of the deteriorating action of the microorganisms present.

The growth pattern observed with CPB was different from that observed with the intentional inoculation of *E. coli*. This could be due to the fact that CPB comprise a range of different bacteria, which may not be the same as the ones observed in the in vitro assays. The variable microbiota observed in sausages, when compared to other processed meats, is due to the condiments used, as almost all the condiments contribute with their own microbial load (JAY, 2005).

In spite of the proven action of essential oils in the in vitro microbial inhibition, when applied in the food systems, it may be necessary to use concentrations which are 2 to 100 times the MIC values obtained in the in vitro assays, in order to achieve the same efficiency. This is due to the higher availability of nutrients in the food system, which allows the bacteria to repair the cell damage rapidly (BUSSATTA *et al.,* 2007; OUEDRHIRI *et al*., 2016). Moreover, food components such as proteins and fats are known to bind or solubilize the phenolic compounds, thereby reducing the antimicrobial activity. These factors can, however, be circumvented through synergism with the intrinsic parameters, such as low water activity, and temperature and oxygen reduction (HAYOUNI *et al*., 2008).

The reduction in the *E. coli* microbial load at the concentrations below the bactericidal MIC (1600 μg mL^{-1}) [up to two decimal places compared to the reference value (control)] in 72 h demonstrated that it is feasible to replace the use of synthetic additives with the addition of oregano essential oil as a preservative associated with refrigeration of fish sausages. Similar results were reported by BUSSATTA *et al.* (2007) who observed that the development of *E. coli* intentionally added in the batches of *frescal* fish sausages containing concentrations of oregano essential oil was not perceptible over 30 days.

The centesimal analysis of the three sausage formulations demonstrated no significant difference, as all the formulations contained the same concentration of animal protein. For the lipids percentage, variation in the concentrations of oregano essential oil in the sausages was not sufficient to cause a significant statistical difference between the treatments groups.

In Brazil, there is no specific legislation for fish sausages; however, the legislation for Tuscan sausages (raw) recommends a maximum amount of 70% moisture and 30% fat, and a minimum of 12% protein, with the cured sausages served at normative in force (BRASIL, 2000). Similar results were reported by BARBOSA *et al*. (2015) while preparing *frescal* sausage from bearded fish, and by MARQUES *et al*. (2012) during the elaboration of Nile tilapia sausage.

CONCLUSIONS

Oregano essential oil exhibits food-grade antimicrobial activity against food-borne Gram-negative and Gram-positive pathogens. The addition of 0.08% oregano oil to fish sausage was efficient in inactivating *E. coli* and hence, it could be used as a natural preservative in sausages.

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