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

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).

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 linguíça 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.
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 nitrates and nitrates (BRASIL, 2001). The main concern with the use of nitrates 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., 2017).

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. Çação (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 çação 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 cholerae) 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 x 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 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.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Without addition of oregano essential oil</td>
</tr>
<tr>
<td>Treatment T1</td>
<td>0.08% of oregano essential oil</td>
</tr>
<tr>
<td>Treatment T2</td>
<td>0.16% of oregano essential oil</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Oregano μg mL^-1</th>
<th>CLO μg mL^-1</th>
<th>CBM μg mL^-1</th>
<th>Without oil addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio cholerae</td>
<td>200</td>
<td>100</td>
<td>1600</td>
<td>NI</td>
</tr>
<tr>
<td>Salmonella serotype Enteritidis</td>
<td>200</td>
<td>100</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>400</td>
<td>100</td>
<td>1600</td>
<td>NI</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>50</td>
<td>100</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>50</td>
<td>100</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>800</td>
<td>100</td>
<td>1600</td>
<td>NI</td>
</tr>
</tbody>
</table>

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 \(9.9 \times 10^6\) 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).

<table>
<thead>
<tr>
<th>Psychrotrophic (UFC g(^{-1}))</th>
<th>Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>9.9x10^4</td>
</tr>
<tr>
<td>Treatment T1</td>
<td>5.4x10^4</td>
</tr>
<tr>
<td>Treatment T2</td>
<td>4.2x10^4</td>
</tr>
</tbody>
</table>

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. *\((10^6\) 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.

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>
<thead>
<tr>
<th>Escherichia coli (MPN 100g(^{-1}))</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
</tr>
<tr>
<td>Control</td>
<td>2.3x10^5 aB</td>
</tr>
<tr>
<td>Treatment T1</td>
<td>3.6x10^1 aA</td>
</tr>
<tr>
<td>Treatment T2</td>
<td>&lt;3.0x10^1 aA</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>Brasil (2000)</th>
<th>Control</th>
<th>Treatment T1</th>
<th>Treatment T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ashes</td>
<td>3.0 (max.)</td>
<td>03.83±0.0*</td>
<td>2.67±0.2a</td>
<td>2.73±0.1a</td>
</tr>
<tr>
<td>Humidity</td>
<td>70 (max.)</td>
<td>64.67±1.5a</td>
<td>64.6±2.4a</td>
<td>64.6±2.4a</td>
</tr>
<tr>
<td>Lipids</td>
<td>30 (max.)</td>
<td>19.5±0.0a</td>
<td>21.1±0.3a</td>
<td>22.46±0.7a</td>
</tr>
<tr>
<td>Proteins</td>
<td>12 (min.)</td>
<td>13.52±0.4a</td>
<td>14.10±0.2a</td>
<td>13.58±0.5a</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>67.6±2.4a</td>
<td>67.6±2.4a</td>
<td>67.6±2.4a</td>
</tr>
</tbody>
</table>

*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 10⁸ 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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REFERENCES


