

# Antimicrobial Potential of Biodegradable Film with Addition of clove (*Syzygium aromaticum*) Essential Oil

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## Abstract

The objective of the research was to test the antimicrobial potential of biodegradable film of soy protein isolate with locust bean gum and clove essential oil. It was tested 0.1, 0.3 and 0.5 % concentrations of clove essential oil the film. The results of the study indicate that there was inhibitory activity against the bacteria *Listeria monocytogenes* and *Salmonella Typhimurium* and inhibition of sporulation of the fungi *Aspergillus oryzae* and *Penicillium oxalicum* of soy protein isolate films containing 0.5 % of clove essential oil. From the observations, it is possible to affirm the applicability of clove essential oil as a natural preservative incorporated in biodegradable packaging, in addition to being used with the function of packaging, prevention and control of contaminants in food. There is a tendency to reduce the use of chemical additives in the food industry and the use of biodegradable packaging with natural preservative is a sustainable substitute for conventional plastics.

## Keywords

Biofilms, Food safety, Antimicrobials, Food preservation, Biocontrol

## Introduction

Most of the world's packaging consumption comes from synthetic polymers which, because of their high durability and physico-chemical stability, remain for a long time in the environment until their total degradation, which contributes to increase environmental pollution and current global warming [1].

In order to reduce environmental impacts resulting from the accumulation of conventional plastics, researches are being developed for the production of packages derived from polymers from renewable sources (biopolymers) that degrade over time through natural mechanisms such as biodegradable packaging [2]. Biopolymers can be found in nature within the group of polysaccharides, proteins and lipids and their combinations allow the production of blends of improved characteristics [3]. Plasticizers are also employed in the manufacture of films with the purpose of reducing their brittleness [4].

Soy protein is a product obtained from residues generated by soybean oil producing industries [5] and it is therefore characterized as a cheap and abundant raw material. Biodegradable films made with soy protein isolate (SPI) have good oxygen barrier properties when compared to films made with polysaccharides and lipids [6]. Despite the low oxygen permeabilities, the soy protein isolate film is highly hydrophilic, which makes it susceptible to oxygen in environments

with high relative humidity, in addition, it has no satisfactory mechanical properties [7].

Locust Bean Gum (LBG) is a type of galactomannan found in carob fruit pod, used to increase elasticity and gel strength [8]. The gum is partially soluble in water at room temperature, but it is better solubilized at higher temperatures, achieving better water binding [9]. The edible films in which the LBG gum is added present improvement in mechanical property, but high permeability to water vapor [10, 11].

An alternative to improve water vapor barrier properties of biodegradable films is the lipids addition in their formulation [12]. The incorporation of essential oils (EOs) to edible films can be an interesting option, because can increase water vapor barrier beyond act as natural bactericides and present antioxidant activity [13, 14].

EOs have antimicrobial activity because present active biologic compounds, tested in various scientific research against microorganisms in vitro [15], with success in the inhibition of microbial growth, besides bactericidal effect and inhibitory activity against fungi and yeasts [16].

The perspective of the use of EOs in the production of biodegradable packaging enables the use of natural products in food preservation and reduces environmental impacts through the use of alternative packaging to conventional plastics, making it possible to increase shelf life and add value to the food product [17].

The EOs in biodegradable packages are released in a controlled way for the food and act mainly on the surface of the product [18], not interfering significantly in the sensorial characteristics of the food.

The objective of this work was to evaluate the antimicrobial potential of biodegradable film of soy protein isolate and clove EO against bacteria associated with Foodborne Diseases and deteriorating fungi of foods.

## Material and Methods

The clove (*Syzygium aromaticum* L.) EO were extracted by steam-dragging distillation (model MA480, Marconi) in the Laboratory of Natural Products of Microbiology and Immunology Department from the Biosciences Institute of Botucatu at Paulista State University "Júlio de Mesquita", according to Beraldo et al. [19].

### Film preparation

The films were prepared conform Silva et al. [11] with minor modifications. Aqueous solutions containing 5 % soy protein isolate (SPI), 2% glycerol, 2% LBG gum and different concentrations of clove essential oil (0.1, 0.3, and 0.5 %) were prepared. To dissolve the soy protein, the pH of the solution was raised to 11 with NaOH solution (40 % w/w). The solution was then stirred for 1 hour and then heated for 70 minutes at 70 °C for 20 minutes. After treatment, the solution was poured into polystyrene dishes and routed to an oven at 30 °C for 72 hours until all solvent was evaporated.

### Characterization of films

The moisture of the samples was gravimetrically determined in triplicate on conventional oven at 105 °C until constant weight.

The film solubility (S) in water was determined in triplicate according to the method reported in the literature [11, 20]. The solubilization was made under agitation (60 rpm) at 25 °C and the solubility was determined according to Eq. 1

$$S = \frac{W_0 - W_f}{W_0} \times 100 \quad (1)$$

Where ( $W_0$ ) is the weight of disks from the dried films before immersion in water and ( $W_f$ ) is the weight of these disks after 24 h of solubilization and drying oven at 105 °C/24h.

The color of the films was determined in triplicate using a colorimeter (Konica Minolta, model CR-400) previously calibrated on white surface. The parameters L\* (lightness), a\* and b\* were obtaining.

### Antimicrobial activity of films

Antimicrobial activity was obtained in four replicates of films, cut in circular format with diameter of 7 mm.

The pathogenic bacteria associated with Foodborne Diseases were tested, *Listeria monocytogenes* INCQS 00266 (ATCC 7644) and *Salmonella enterica* subsp. *enterica* serovar. Typhimurium INCQS 00084 (ATCC 13311), from the Collection of Reference Microorganisms in Sanitary Surveillance - CMRVS, FIOCRUZ-INCQS, Rio de Janeiro, RJ, Brazil.

Bacterial cultures kept in stock under freezing at -20 °C were activated in 3 mL Tryptic Soy Broth (TSB) and incubated at 35 °C for 24 hours. After activation, the cultures were standardized on the 0.5 MacFarland scale, with an approximate cell concentration of  $1.0 \times 10^8$  CFU.mL<sup>-1</sup>, with absorbance reading (0.08 to 0.10) in a spectrophotometer with a length of wave of 625 nm.

For each standardized bacterial culture, 100 µL were spread in Petri dishes containing Plate Count Agar (PCA). Four film disks were then placed on the culture medium. For each treatment a plaque was prepared from each bacterium. The plates were incubated at 37 °C for 24 hours, then the diameters of the inhibition halos around the film were measured.

Filamentous fungi *Aspergillus oryzae* INCQS 40068 (ATCC 1003) and *Penicillium oxalicum* INCQS 40103 (ATCC 24784) from the Collection of Reference Microorganisms in Sanitary Surveillance - CMRVS, FIOCRUZ-INCQS, Rio de Janeiro, RJ, Brazil, were tested.

From the segments of mycelia stored in saline solution at room temperature (approximately 25 °C), the filament fungi were activated, the segments were arranged in the center of Petri dishes containing Potato Dextrose Agar (PDA) and incubated at 25 °C for 5 to 7 days.

The spores produced during activation were dissolved in saline solution and standardized on the 0.5 MacFarland scale, with an approximate cell concentration of  $5.0 \times 10^6$  CFU.mL<sup>-1</sup>, with absorbance reading (0.08 to 0.10) in spectrophotometer, with wavelength of 530 nm.

For each spore standardization, 100 µL were spread by scattering in Petri dishes containing PDA. Four film discs were then placed on the culture medium. For each treatment a plaque was prepared from each filamentous fungi. The plates were incubated at 25 °C for 3 days, then the diameters of the inhibition halos around the film were measured.

### Statistical analysis of data

The data were statistically analyzed by an analysis of variance (ANOVA) and Tukey's test at a 5 % significance level.

## Results and Discussion

The moisture and solubility values of the films at different concentrations of clove EO are presented in Table 1.

**Table 1:** Moisture and solubility of biodegradable films with and without clove essential oil (EO).

Treatments	Moisture (%) *	Solubility (%) *
0.0% EO	50.77 ± 0.07 <sup>a</sup>	30.33 ± 1.15 <sup>a</sup>
0.1% EO	46.32 ± 1.33 <sup>a</sup>	29.00 ± 1.00 <sup>a</sup>
0.3% EO	50.56 ± 1.45 <sup>a</sup>	33.33 ± 0.58 <sup>a</sup>
0.5% EO	51.26 ± 3.01 <sup>a</sup>	33.33 ± 3.51 <sup>a</sup>

\*Mean ± SD. Means with the same lowercase letter, in the same column, did not differ significantly at  $p \leq 0.05$  according to Tukey test.

It was found that increasing the concentration of clove EO in the film formulation did not change significantly the moisture and solubility of films.

The colorimetric analysis is presented in table 2. The clove EO addition reduced the lightness of film. The 0.1% EO addition did not influence parameter a\* significantly, however, concentrations above this value change significantly redness color of film. In relation to parameter b\*, 0.1 % EO addition increased 7% yellowish color of films.

**Table 2:** Parameters L, a and b of the biodegradable films with clove essential oil (EO).

Treatments	L*	a*	b*
0.0% EO	75.68 ± 0.50 <sup>a</sup>	3.53 ± 0.09 <sup>a</sup>	34.08 ± 1.10 <sup>a</sup>
0.1% EO	74.86 ± 0.37 <sup>a</sup>	3.57 ± 0.17 <sup>a</sup>	36.45 ± 0.07 <sup>b</sup>
0.3% EO	73.94 ± 0.72 <sup>a</sup>	2.21 ± 0.25 <sup>b</sup>	36.46 ± 0.17 <sup>b</sup>
0.5% EO	70.28 ± 1.35 <sup>b</sup>	5.07 ± 0.57 <sup>c</sup>	37.50 ± 1.02 <sup>b</sup>

\*Mean ± SD. Means with the same lowercase letter, in the same column, did not differ significantly at  $p \leq 0.05$  according to Tukey test.

The measurements of the *Listeria monocytogenes* and *Salmonella* Typhimurium inhibition halos are presented in table 3.

**Table 3:** Averages of bacterial inhibition halos of the biodegradable films with clove essential oil (EO).

Treatments	Inhibition halos (mm)*	
	<i>Listeria monocytogenes</i> ATCC 7644	<i>Salmonella</i> Typhimurium ATCC 13311
0.0% EO	12.0 ± 1.6 <sup>a</sup>	6.0 ± 0.0 <sup>a</sup>
0.1% EO	13.0 ± 1.1 <sup>a</sup>	6.0 ± 0.0 <sup>a</sup>
0.3% EO	16.0 ± 0.0 <sup>b</sup>	12.0 ± 0.0 <sup>b</sup>
0.5% EO	18.5 ± 1.0 <sup>b</sup>	14.0 ± 0.0 <sup>b</sup>

\*Mean ± SD. Means with the same lowercase letter, in the same column, did not differ significantly at  $p \leq 0.05$  according to Tukey test.

Biodegradable films with of 0.3 and 0.5 % clove EO addition showed significant antimicrobial activity against the two bacteria tested. The 0.1 % EO concentration of clove was not enough to exert the antimicrobial effect around the film. Although the averages of 0.3 and 0.5 % inhibition halos were not statistically different, it was possible to observe an indicative increase of possible continuity of inhibition at concentration above 0.5 %.

Pelissari et al. [21] studied cassava starch chitosan films incorporated with oregano EO and found that the zones of inhibition for the bacteria *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella* Enteritidis and *Escherichia coli* increased significantly when higher concentrations of EO were used.

Viuda-Martos et al. [22] also verified the antibacterial activity of EOs on the growth of food contaminating bacteria, observing that the OEs of oregano, thyme, rosemary, sage, cumin and clove have an inhibitory effect on the growth of some bacteria commonly associated with the food industry, such as *Lactobacillus curvatus*, *L. sakei*, *Staphylococcus carnosus* and *S. xylosus*, or related to food deterioration such as *Enterobacter gergoviae* and *E. amnigenus*. Oregano EO showed the highest antibacterial activity against the six bacteria tested, with zones of inhibition varying from 35.29 mm in *S. xylosus* to 57.90 mm in *E. amnigenus*. For the clove EO there was also an effect where the halos were around 26.03 and 29.5 mm for *L. sakei* and *E. gergoviae*, respectively.

Pereira et al. [23] reported an inhibitory effect on *Staphylococcus aureus* and *Escherichia coli* with the use of EO of lemongrass, oregano and clove, being that clove EO presented the best effect on bacteria in small concentrations, and thus, higher inhibition halos. For *E. coli* clove EO showed inhibition diameters of 8 to 12 mm at concentrations of 0.1 and 50% EO, respectively. For the oregano and lemongrass EOs, the means of inhibition halos were between 7 and 12 mm and 0 and 9 mm, respectively. Similarly, to *E. coli* the inhibitory effect on *S. aureus*, with larger inhibition halo averages with clove EO, from 10 mm to 15 mm at concentrations of 0.1 and 50 % of oil, respectively, was also observed. The consistency between the results probably occurred due to the influence of ethanol, solvent used in the dilution of the oils.

In chicken sausages stored under refrigeration clove EO inhibited *Listeria monocytogenes*, with significant reduction of contamination, when applied in 1 and 2 % contractions

[24]. Treatment with 1% clove EO before storage reduced *L. monocytogenes* populations in inoculated sausages with low bacterial concentrations, reducing from 2.4 to 1.1 log<sub>10</sub> CFU.g<sup>-1</sup>. In contaminations induced with high bacterial concentrations the reduction was significant from 5.4 to 2.0 log<sub>10</sub> CFU.g<sup>-1</sup>. In this case, treatment with 2 % EO of clove reduced from 5.4 to 1.1 log<sub>10</sub> CFU.g<sup>-1</sup>.

Menon and Garg [25] observed that there was a significant decrease in the amount of *Listeria monocytogenes* in meat and cheese stored at 7 and 30 °C, at concentrations of 0.5 and 1.0 % clove EO. In meat samples clove EO exerted antimicrobial activity against *L. monocytogenes* when applied at 0.5% concentration, decreasing from 1.8x10<sup>5</sup> CFU.g<sup>-1</sup> to 7.7x10<sup>3</sup> CFU.g<sup>-1</sup> after one day of storage the temperature of 30 °C. The meat samples were submitted to a concentration of 0.5 % EO at a temperature of 7 °C, a reduction in the value, ranging from 4.2x10<sup>5</sup> CFU.g<sup>-1</sup> to 6.1x10<sup>3</sup> CFU.g<sup>-1</sup>. In the cheese samples the clove EO decreased from 1.5x10<sup>6</sup> CFU.g<sup>-1</sup> to 1x10<sup>1</sup> CFU.g<sup>-1</sup>, at concentrations of 0.5 and 1.0 %, respectively.

In this study there was no inhibition of filamentous fungi at any tested concentration of clove EO. However, there was sporulation inhibition, by the difference in coloration (white) around films, different from the rest of the mycelium. The averages of the fungal sporulation inhibition halos of clove EO films are presented in table 4.

**Table 4:** Means of the fungal sporulation inhibition halos of biodegradable film with clove essential oil (EO).

Treatments	Inhibition halos (mm)*	
	<i>Aspergillus oryzae</i> ATCC 1003	<i>Penicillium oxalicum</i> ATCC 24784
0.0% EO	6.0 ± 0.0 <sup>a</sup>	6.0 ± 0.0 <sup>a</sup>
0.1% EO	6.0 ± 0.0 <sup>a</sup>	6.0 ± 0.0 <sup>a</sup>
0.3% EO	6.0 ± 0.0 <sup>a</sup>	6.0 ± 0.0 <sup>a</sup>
0.5% EO	20.0 ± 0.0 <sup>b</sup>	18.0 ± 0.0 <sup>b</sup>

\*Mean ± SD. Means with the same lowercase letter, in the same column, did not differ significantly at p ≤ 0.05 according to Tukey test.

Concerning the interference of film treatments with clove EO on the growth of filamentous fungi, uniform inhibition halos were not obtained around the films with even 0.5% clove EO. Treatments with 0.1 % clove EO had no difference for the two fungi in comparison to the control treatments. However, the 0.3% concentration of clove EO presented onset of sporulation inhibition on the films themselves. It was possible to clearly visualize that there was no sporulation around the replicates for the films with 0.5 % clove EO. The non-sporulation of the fungi was observed by the color difference of the mycelium, with the development of only white hyphae around the films in both fungi tested, a pattern different from the growth in the rest of the plaques.

Guynot et al. [26] verified the ability of clove essential oil to inhibit in vitro growth of fungi found in baking such as *Eurotium amstelodami*, *E. herbariorum*, *E. repens*, *E. rubrum*,

*Aspergillus flavus*, *A. Niger* and *Penicillium corylophilum*. The authors obtained satisfactory results, and for *E. repens* the mean inhibition diameters were 7 mm to 41 mm, after 42 days of incubation at 25 °C.

Pierre et al. [27] evaluated the inhibitory effect of clove extract, which showed that the 10 % extract showed a fungicidal effect totally inhibiting the mycelial growth of *Glomerella cingulata* and *Colletotrichum gloeosporioides* isolated from guava fruits. Similarly, Rozwalka et al. (2008) [28] observed the inhibition of 100% mycelial growth of *C. gloeosporioides* isolated from guava fruits, when using clove extracts at 1.0% and 10% concentrations, besides clove EO.

Mariath et al. [29] obtained inhibition of all strains of the *Aspergillus*, *Cladosporium*, *Curvularia*, *Exophiala werneckii*, *Fonsecaea compacta* and *Piedraie hortae* fungi on the application of clove EO in the concentrations of 2, 4 and 8 %, producing inhibition halos that varied from 10 mm to 24 mm.

Amaral and Bara [30] studied the antifungal activity of clove EO and other EOs at different concentrations (0.01, 0.025, 0.05, 0.2, 0.3 and 0.5 %), observing that the minimum inhibitory concentration for the fungi *Fusarium oxysporum*, *F. solani*, *Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii*, added to the culture medium of these fungi or present in seeds such as rice, corn, soybeans and beans, was 0.05% where the clove EO presented a relevant antifungal potential against phytopathogens.

In order to apply the EOs in foods as natural preservatives, several studies show the antimicrobial activity against pathogens and deteriorating microorganisms. However, it is emphasized that the concentrations of EOs employed that have efficacy, drastically interfere in the appreciation of the food. Thus, the possibility of applying EOs in effective concentrations, even if restricted to the surface, but with less effect on the odor and flavor of the food, allows new proposals such as the use in films for viable applications of EOs as natural food preservatives incorporated in biodegradable packages to be used with the function of packaging, prevention and control of contaminants in food.

## Conclusions

Significant inhibitory activity of *Listeria monocytogenes* and *Salmonella Typhimurium* and inhibition of sporulation of *Aspergillus oryzae* and *Penicillium oxalicum* fungi from films based soy protein isolate + LBG containing 0.5 % clove EO was observed in this study. These notes indicate an applicability of clove EO as a natural preservative incorporated in biodegradable packages to be used with the function of packaging, prevention and control of contaminants in food.

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