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Evaluation of PLA active biodegradable films incorporated of essential oils to inhibit microbial adhesion

Evaluación de películas biodegradables activas de PLA incorporada de aceites esenciales para inhibir adhesión microbiana

Anderson Pazmiño*[1], Ana Campuzano[2], Karina Marín[3], Jonathan Coronel[3], and Rómulo Salazar[3]

[1]*Instituto de Ciencias Básicas, Universidad Técnica de Manabí. Código Postal 130105, Portoviejo, Ecuador.*
[2]*Facultad de Ciencias Agrarias, Universidad Agraria del Ecuador, Código Postal 090104, Guayaquil, Ecuador.*
[3]*Escuela Superior Politécnica del Litoral, ESPOL, Facultad de Ingeniería en Mecánica y Ciencias de la Producción, Campus Gustavo Galindo, Km 30.5 Vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador.*

*Corresponding author: anderson.pazmino@utm.edu.ec

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Abstract

An evaluation of incorporated polylactic acid (PLA) active biodegradable films of essential oils to inhibit microbial adhesion was performed using a 2_V^{5-1} fractional exploratory design, combining the factors, amount of PEG 400 plasticizer (10 and 20 %), amount of cinnamon essential oil (0.5 % and 1 %), amount of oregano essential oil (0.5 % and 1 %), *Salmonella* spp. concentration (10^3 CFU mL^{-1} and 10^4 CFU mL^{-1}) and *Staphylococcus aureus* concentration (10^3 CFU mL^{-1} and 10^4 CFU mL^{-1}). The results of microbial adhesion inhibition test showed that the maximum inhibition percentage reached 73.82 ± 0.35 % corresponding to experiment 7 (bce), which contains 10 % of PEG 400, 1 % of cinnamon essential oil, 1 % of oregano essential oil, 10^3 CFU mL^{-1} of *Salmonella* spp. concentration, 10^4 CFU mL^{-1} of *Staphylococcus aureus* concentration. Statistical analysis determined that there is strong significant evidence ($p - value = 0.0283$) that *Staphylococcus aureus* concentration influences the inhibition percentage to microbial adhesion; as well as that the cinnamon essential oil- *Salmonella* spp. interaction has little significant evidence ($p - value = 0.0711$) that influences the inhibition percentage. Inhibition results greater than 60 % have the highest concentration of *Staphylococcus aureus* as a common factor. PLA active biodegradable films with a higher inhibition percentage can potentially be used in the food industry as a barrier mechanism to avoid bacterial contamination.

Keywords: Biopolymer, food, barrier, infectious agent.

Resumen

La evaluación de películas biodegradables activas de ácido poliláctico (PLA) incorporado de aceites esenciales para inhibir la adhesión microbiana se realizó mediante un diseño exploratorio fraccionario 2_V^{5-1}, combinando los factores, cantidad de plastificante PEG 400 (10 y 20%), cantidad de aceite esencial de canela (0,5 y 1%), cantidad de aceite esencial de orégano (0,5 y 1%), concentración en unidades formadoras de colonias (UFC) de *Salmonella* spp. (10^3 y 10^4 UFC mL^{-1}) y concentración de *Staphylococcus aureus* (10^3 y 10^4 UFC mL^{-1}). Los resultados del ensayo de inhibición a la adhesión microbiana mostraron que el porcentaje máximo de inhibición alcanzó el $73,82 \pm 0,35\%$, correspondiente al experimento 7 (bce), el cual contiene 10% PEG 400, 1% aceite esencial de canela, 1% aceite esencial de orégano, 10^3 UFC mL^{-1} de concentración de *Salmonella* spp. y 10^4 UFC mL^{-1} de concentración de *Staphylococcus aureus*. El análisis estadístico determinó que existe evidencia significativa (valor $p = 0,0283$) que indica que la concentración de *Staphylococcus aureus* influye en el porcentaje de inhibición a la adhesión microbiana; así como también, que la interacción del aceite esencial de canela-*Salmonella* spp. tiene poca evidencia significativa (valor $p = 0,0711$) que influye en el porcentaje de inhibición. Los resultados de inhibición están en función del tipo de bacteria, siendo mayor para las Gram positivas. Los resultados de inhibición superiores al 60% tienen como factor común la concentración más alta de *Staphylococcus aureus*. Por lo tanto, las películas biodegradables activas de PLA con mayor porcentaje de inhibición pueden usarse potencialmente en la industria alimentaria como mecanismo de barrera para evitar contaminación bacteriana.

Palabras clave: Biopolímero, alimento, barrera, patógenos.

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Orcid IDs:

Anderson Pazmiño: http://orcid.org/0000-0001-5333-3819
Ana Campuzano: http://orcid.org/0000-0003-0010-4267
Karina Marín: http://orcid.org/0000-0002-0706-7692
Jonathan Coronel: http://orcid.org/0000-0001-6535-0261
Rómulo Salazar: http://orcid.org/0000-0002-0172-839X

1 Introduction

The use of biodegradable packaging to replace polyolefins with a low degradability rate is one of the main challenges of the food industry, as are efforts to reduce the number of people affected by foodborne diseases by encouraging the application of biocontrols and other barrier mechanisms to ensure food safety for consumers (Tauxe et al., 2010; Jahid and Ha, 2012), especially in minimally processed or fresh food, considering for example, outbreaks of illnesses caused by *Salmonella* spp. have been linked to contamination of cucumbers, fresh papaya, cut melon and pineapple, chicken, eggs, raw tuna, ground beef, ground turkey meat, and many other food (Centers for Disease Control and Prevention, 2020).

The World Health Organization (WHO), estimates that foodborne diseases affect one in ten people globally, and cause 420,000 deaths per year, with the main etiological agents being *Norovirus*, *E. coli*, *Campylobacter* spp., and non-typhoidal *Salmonella* (WHO, 2016). The Centers for Disease Control and Prevention estimates that each year in the United States, 48 million people are affected by foodborne illness, out of which 128,000 are hospitalized and 3,000 die (Centers for Disease Control and Prevention, 2019). Out of the total affected, 9.4 million people (20%) become ill from 31 known foodborne pathogens, and 38.4 million (80%) are affected by unspecified agents that are in food whose symptoms start with acute gastroenteritis. The estimated figures indicate that foodborne illnesses are caused by viruses (59%), bacteria (39%), and parasites (2%). The pathogens causing most of the illnesses are *Norovirus* (58%), *Salmonella* spp. (11%), *Clostridium perfringens* (10%), *Campylobacter* spp. (9%), and *Staphylococcus aureus* (3%). Of the total estimated hospitalizations, 64% are caused by bacteria, 35% by *Salmonella* spp., and 15% by *Campylobacter* spp. Of the total estimated deaths, 64% are caused by bacteria, of which 28% correspond to *Salmonella* spp., 19% to *Listeria monocytogenes*, and 6% to *Campylobacter* spp. (Scallan et al., 2011).

The use of essential oils is an option to improve the properties of biodegradable films or packaging, providing antibacterial properties against pathogens that cause foodborne diseases (Calo et al., 2015). Among these essential oils are oregano oil (*Origanum vulgare*) and cinnamon essential oil (*Cinnamomum zeylanicum*), both categorized as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA) (FDA, 2020)

Cinnamon essential oil is considered useful for food preservation due to its antioxidant, antifungal and antibacterial properties. These properties are a consequence of its Cinnamaldehyde (94.65% - 65%), D-Limonene (3.55%) and Eugenol (1.8%) content (Burt, 2004; Cardoso-Ugarte et al., 2016; Echegoyen and Nerín, 2015). Studies on the mechanism of action and minimum inhibitory concentrations against *Yersinia enterolitica* (0.075 mg mL^{-1}), *Listeria monocytogenes* (2.5 mg mL^{-1}), *Bacillus cereus* (2.5 mg mL^{-1}), *Escherichia coli* (5 mg mL^{-1}), *Salmonella enterica serovar Typhimurium* (5 mg mL^{-1}) and *Staphylococcus aureus* (5 mg mL^{-1}), have determined that the release of intracellular potassium ions, reduction of metabolic and replication activity resulted as a consequence of the essential oil (Duan and Zhao, 2009; Silveira et al., 2012).

Oregano essential oil has a high antioxidant and antimicrobial power, which favors the preservation of the chemical, sensory, nutritional and microbiological quality of food, due to the effect of its main components Carvacrol (0.5-88.7%), Thymol (3.1-82%), 1,8-Cineole (0.1-14%), p-Cymene (2.7-28.0%) (Franz and Novak, 2009; Ventura et al., 2011; Teixeira et al., 2013; Rostro-Alanis et al., 2019). Gram-negative bacteria, *E. coli, Pseudomonas aeruginosa, Vibrio cholerae Salmonella enterica serovar Typhimurium, Salmonella choleraesuis*, and Gram-positive bacteria, *S. aureus, Listeria monocytogenes* and *B. cereus* have been used to evaluate the antimicrobial activity of oregano essential oil and its active principles, and out of these only *Pseudomonas aeruginosa* has shown high resistance (Albado Plaus et al., 2001; Oussalah et al., 2007; Emiroğlu et al., 2010; Sung et al., 2013; Calo et al., 2015). This bactericidal action is due to its active principles that increase the permeability of the cytoplasmic membrane of microorganisms, causing cell death (Lambert et al., 2001; Rhayour et al., 2003; Sung et al., 2013; Calo et al., 2015).

A good synergistic antibacterial effect of Cinnamaldehyde and Carvacrol was demonstrated by Ye et al. (2013), especially against *E. coli* and *S. aureus*; thus, the use of essential oils containing these compounds can lead to the development of bio-

polymers with high bactericidal activity for food preservation.

Regarding the creation of biodegradable films, the semicrystalline biopolymer polylactic acid (PLA), due to its characteristics of transparency, safety, biodegradability and ease of processing is presented as a suitable polymer for food packaging (Jamshidian et al., 2010; Lalanne et al., 2011; Burgos et al., 2013; Blanot, 2017; Salazar et al., 2014; Ruellan et al., 2015).

PLA biofilms with essential oils (bergamot, lemongrass, rosemary, clove, myrtle, thyme, cinnamon, garlic, and oregano) have been elaborated with positive results both in rheological, thermal, antioxidant and antibacterial aspect (Ahmed et al., 2016; Qin et al., 2017; Yahyaoui et al., 2016; Anuar et al., 2017; Scaffaro et al., 2018; Zeid et al., 2019), obtaining biofilms with adequate transparency, more flexible, less permeable to water vapor and oxygen, without significantly affecting the thermal degradation profile of PLA, and with antimicrobial activity against Gram-positive and Gram-negative bacteria.

However, the evaluation of the incorporation of these essential oils has been individually, so it is necessary to establish possible combinations of essential oils in biopolymers to obtain better barrier and control effects against pathogenic microorganisms that develop biofilms on different surfaces and cause food diseases, considering possible positive effects on the synergy of the essential oils used and the interactions between them and the other components of the polymer (Calo et al., 2015).

Due to the worldwide need to produce biodegradable packaging and because pathogenic microorganisms use food and packaging as transport to affect the health of consumers, this study aims to evaluate the antimicrobial activity of biodegradable active PLA films with essential oils of oregano and cinnamon.

2 Materials and Methods

Polylactic acid (PLA-Ingeo™ Biopolymer 2003D) was acquired from Nature Works®Co. LLC (USA). The plasticizer polyethylene glycol (PEG 400) and the chloroform solvent were purchased from Loba Chemie Pvt. Ltd (India). Absolute ethanol was obtained from Sigma-Aldrich (United States).

The essential oils of oregano (*Origanum vulgare*) and cinnamon (*Cinnamomum zeylanicum*) were purchased from Nowfoods (Bloomingdale, IL, USA), are 100% pure, and were obtained by the steam distillation method. According to technical specifications, oregano essential oil (specific gravity: 0.937-0.955 g mL^{-1} at 20°C; refractive index: 1.498-1.521 n_D; ≈75% Carvacrol) was extracted from dried flower herb, and cinnamon essential oil (specific gravity: 1.010-1.030 g mL^{-1} at 20°C; refractive index: 1.573-1.591 n_D; ≈60% Cinnamaldehyde) was extracted from dried inner bark of the plant.

Culture media and reagents for microbiological tests were purchased from Difco (USA). The pathogenic bacteria used in this research, *Staphylococcus aureus* (ATCC 25923) and *Samonella* spp. (ATCC 14028) were obtained from the strain bank of the Biotechnology Research Center of Ecuador (CIBE, Ecuador).

2.1 Determination of the component solubility with PLA

PLA solubility with chloroform, PEG 400, Carvacrol (main component of oregano essential oil) and Cinnamaldehyde (main component of cinnamon essential oil) was determined as described by Ruellan et al. (2015). Equation 1 and Equation 2 are used to determine the distance (Δδ) of the compound with PLA and the relative differential energy (RED), respectively. The Hansen solubility parameters needed to apply the equations were consulted in the user manual described by Hansen (2007).

$$\text{Distance}(\Delta\delta) = ((\delta_{\text{d comp}} - \delta_{\text{d PLA}})^2 + (\delta_{\text{p comp}} - \delta_{\text{p PLA}})^2 + (\delta_{\text{h comp}} - \delta_{\text{h PLA}})^2)^{0,5} \quad (1)$$

$$RED = \frac{Distance}{Ratio} \quad (2)$$

Where, δ_d, δ_p and δ_h are the components of the solubility parameters.

2.2 Minimum inhibitory concentration of essential oils

The minimum inhibitory concentration (MIC) of each essential oil was determined by the methodology described by Pazmiño et al. (2020), where the MIC was determined by microdilution assay in nutrient broth and supplemented with nutrient agar of 0.15% (w/v) concentration; the amount of 50 μL of this broth was distributed from the second to the twelfth well in each row of a 96-well polypropylene microtiter plate (HDM Cia. Ltda., Ecuador).

A 4% dilution of each essential oil was prepared with bacterial agar and nutrient broth. Independently, 100 μL of each prepared dilution was poured into the first well of each row of the microtiter plate; and then 50 μL in scalar dilution were transferred from the second to the eleventh well, thus obtaining an amount of essential oil between 2% (v/v) and 0.0019% (v/v) from the first well to the eleventh well. The twelfth well did not receive the essential oil and was considered as a growth control. Then, 50 μL of *Salmonella* spp. suspension of 10^6 - 10^7 colony forming units (CFU mL^{-1}) were added to each well, except to the eleventh well, which was considered as witness. The assay was done in triplicate, and the same procedure was performed with *S. aureus* (10^6 - 10^7 CFU mL^{-1}). The plate was incubated at 37°C for 18 h.

2.3 Experiment design

To evaluate the inhibition of microbial adhesion on the plastic film, the following factors were considered: amount of PEG 400 (A), amount of cinnamon essential oil (B), amount of oregano essential oil (C), concentration of *Salmonella* spp. (D) and concentration of *S. aureus* (E), each factor evaluated at two levels (Table 1). For this purpose, an exploratory fractional design 2_v^{5-1} with a generating ratio E = ABCD and in two blocks (AB=CDE) (Table 2) was programmed and analyzed in RStudio Version 0.99.486. Sixteen experiments were carried out, each with its replication.

Table 1. Levels of each factor to be assessed in the experimental design

Factor	Low level (-1)	High level (1)
Amount of PEG 400 (%)	10	20
Amount of cinnamon essential oil (%)	0,5	1
Amount of oregano essential oil (%)	0,5	1
Concentration of *Salmonella* spp. (CFU mL^{-1})	10^3	10^4
Concentration of *Staphylococcus aureus* (CFU mL^{-1})	10^3	10^4

2.4 Development of biodegradable active films based on PLA and essential oils

PLA pellets were dried at 40°C for 48 hours, so that the moisture content before processing was 0.29% measured on a thermobalance (KERN, Germany, Mod MLB50-3). The plate casting method was performed with the methodology described by Pazmiño et al. (2020). The amount of 0.45 g of PLA and PEG 400 (10 or 20% with respect to PLA) were dissolved in 10 mL of chloroform. After dissolving the resin in the solution at room temperature, the essential oils were added in the corresponding amount (0.5% or 1% with respect to PLA) while stirring for a few minutes until dissolved. The solution was then poured into Petri dishes, adding $5 \pm 0{,}5$ g of solution in each dish, and finally left for 24 h at room temperature inside the extraction chamber for the solvent to evaporate. Films without addition of essential oils (control films) were also prepared. The films obtained had a thickness between 27 to 30 μm, measured by a micrometer (Qualitest, United States, Mod MRG-25).

2.5 Inhibition adhesion percentage of microorganisms on active biodegradable films

The inhibition percentage to microbial adhesion was performed by modifying the method applied by Coronel-León et al. (2016). According to the experimental design, 1 mL of each microorganism

concentration (10^3 o 10^4 CFU mL^{-1}) was inoculated on the plastic film, with incubation at 37°*C* for 24 hours. At the end of the incubation period, each film was washed twice with 1 mL of distilled water to remove residues. 1 mL of absolute ethanol was added, placing the plates in the extraction chamber for 24 hours to evaporate the alcohol. The plastic film was cut into a rectangle (8.75 cm^2), and placed on a slide where the crystal violet reagent (500 μL) was added and left for one minute. Then, it was rinsed with distilled water to eliminate the excess of the reagent. Once dried with paper towel, it was cut into 8 pieces to introduce them in a test tube, adding 2 mL of absolute ethanol. Subsequently, 200 μL of each solution was deposited in the wells of the 96-well microplate to measure the concentration of the reagent by UV-Vis absorption spectrum in the Synergy™ HTX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, United States) with an absorbance of 595 nm at 24.8°*C*.

The inhibition percentage to microbial adhesion (%) was calculated with Equation 3. Where, A_c represents the absorbance of the solution in the well corresponding to the plastic film according to the experimental design, and A_0 represents the absorbance of the control film solution (absence of essential oil). The experiment was performed in triplicate, and the results are presented as an average.

$$\% = [1 - (A_c/A_0)] \times 100 \quad (3)$$

Table 2. Fractional exploratory design 2_V^{5-1} with a generating ratio E = ABCD in two blocks (AB=CDE)

Experiment	Factors					AB	Totals	Blocks
	A	B	C	D	E			
1	-1	-1	-1	-1	1	1	e	1
2	1	-1	-1	-1	-1	-1	a	2
3	-1	1	-1	-1	-1	-1	b	2
4	1	1	-1	-1	1	1	abe	1
5	-1	-1	1	-1	-1	1	c	1
6	1	-1	1	-1	1	-1	ace	2
7	-1	1	1	-1	1	-1	bce	2
8	1	1	1	-1	-1	1	abc	1
9	-1	-1	-1	1	-1	1	d	1
10	1	-1	-1	1	1	-1	ade	2
11	-1	1	-1	1	1	-1	bde	2
12	1	1	-1	1	-1	1	abd	1
13	-1	-1	1	1	1	1	cde	1
14	1	-1	1	1	-1	-1	acd	2
15	-1	1	1	1	-1	-1	bcd	2
16	1	1	1	1	1	1	abcde	1

3 Results and Discussion

3.1 Solubility of components with PLA

The values obtained with Equation 1 and Equation 2 are shown in Table 3. Based on the criterion that distances less than 5 (J $cm^{-3})^{0.5}$ indicate a better solubility of the compound with the polymer, it can then be stated that the compounds have a good solubility with PLA, especially Cinnamaldehyde from cinnamon essential oil. The RED value close to zero indicates a better compatibility of the compounds with the polymer, and a value greater than 1 indicates a non-miscibility of the compound with the polymer, and according to the results, all the compounds are completely miscible.

Table 3. Distance results and RED value of Carvacrol, Cinnamaldehyde, PEG 400 and chloroform, in relation to PLA.

Compound	Hansen solubility parameters (J cm^{-3})$^{0.5}$			Distance	RED*
	δd	Δp	Δh	$\Delta\delta$	
PLA[a]	18.60	9.90	6.00	—-	—-
PEG400[a]	17.90	4.20	14.20	10.01	0.94
Carvacrol[b]	19.00	4.50	10.80	7.24	0.68
Chloroform[b]	17.80	3.10	5.70	6.85	0.64
Cinnamaldehyde[b]	19.40	12.40	6.20	2.63	0.25

*PLA Radius = 10.7 (Ruellan et al., 2015).
a: (Ruellan et al., 2015); b: (Hansen, 2007), values of carvacrol as an isomer of thymol.

3.2 Minimum inhibitory concentration of the essential oil

The results show that both essential oils inhibit Gram-positive *S. aureus* and Gram-negative *Salmonella* spp. bacteria (Table 4). The MIC 0.016% (v/v) of oregano essential oil for *Salmonella* spp. is lower than the MIC of cinnamon essential oil for the same bacteria; while the MIC of both essential oils against *S. aureus* is equal, being 0.031% (v/v).

The MIC of oregano essential oil for *Salmonella* spp. is lower compared to the results of Oussalah et al. (2007), where the MIC for *Salmonella enterica serovar Typhimurium* was 0.05% (v/v), and also compared to the results of Pesavento et al. (2015), where the MIC found for *Salmonella enteritidis* and *S. enterica serovar Typhimurium* was 0.125% (v/v). The MIC results of oregano essential oil for *S. aureus* are within the range of values reported by Nostro (2007), where the MIC of oregano essential oil on several strains of this microorganism were between 0.015-0.062% (v/v) and in certain strains up to 0.125% (v/v).

The MIC of cinnamon essential oil for *Salmonella* spp. and *S. aureus* is higher than that found by Sheng and Zhu (2014), where the MIC of cinnamon essential oil extracted from *Cinnamomum cassia* variety was 0.025% (v/v) for both *S. enterica serovar Typhimurium* and *S. aureus*. The MIC results of oregano essential oil and cinnamon essential oil, expressed in mg mL^{-1} in (Table 4) and which were obtained in this research are higher compared to the results found in other investigations, where the MIC of oregano essential oil ranged from 0.16 - 0.2 mg mL^{-1} for *Salmonella spp.* and 0.4-0.9 mg mL^{-1} for *S. aureus*, and the MIC of cinnamon essential oil ranges from 0.2 - 0.5 mg mL^{-1} for both *Salmonella* spp. and *S. aureus* (Chang et al., 2001; Becerril et al., 2012; Boskovic et al., 2015; Martucci et al., 2015; Cui et al., 2019).

Table 4. Minimum inhibitory concentration (MIC) of cinnamon and oregano essential oils for *Salmonella* spp. and *Staphylococcus aureus*.

Essential oil	Active ingredient	MIC *Salmonella* spp.		MIC *Staphylococcus aureus*	
		(%) (v/v)	mg mL^{-1}	(%) (v/v)	mg mL^{-1}
Cinnamon	Cinnamaldehyde	0.063	64.26	0.031	31.62
Oregano	Carvacrol	0.016	15.14	0.031	29.33

The data described above demonstrate the antimicrobial activity of oregano and cinnamon essential oils. Carvacrol from oregano essential oil damages the cell wall and membrane, increases permeability, thus leading to cell lysis (Lambert et al., 2001; Rhayour et al., 2003; Lv et al., 2011; Cui et al., 2019). Cinnamaldehyde from cinnamon essential oil presents different mechanisms of action on its own or along with Carvacrol, collapsing the bacterial structure, as well as disrupting metabolic processes such as cellular energy generation, cytoplasmic material formation and cell division (Kwon et al., 2003; Burt, 2004; Becerril et al., 2007; Bouhdid et al., 2009; Ye et al., 2013; Calo et al., 2015).

3.3 Inhibition adhesion percentage of microorganisms on active biodegradable films

The results of the inhibition percentages to microbial adhesion in the active biodegradable films are detailed in Table 5. The results obtained showed that the maximum inhibition percentage reached 73.82 ± 0.35% corresponding to experiment 7 (bce) of the experimental design, and there is a significant difference with the other results, except with 13 (cde). Experiment 7 (bce) comprised 10% amount of PEG 400, 1% cinnamon essential oil, 1% oregano essential oil, 10^3 CFU mL^{-1} concentration of *Salmonella* spp, 10^4 CFU mL^{-1} concentration of *S. aureus*. This inhibition percentage to adhesion is higher than that reported in studies performed with Lichenysin (4000 μg mL^{-1}) in *S. aureus* (68.73%) (Coronel-León et al., 2016), and with Rhamnolipids (1%) in *S. aureus* (67.7%), and it was not significant in *Salmonella enteritidis* (Do Valle Gomes and Nitschke, 2012).

Table 5. Microbial adhesion inhibition rates of active biodegradable films.

Experiment	PEG 400 (%)	Cinnamon essential oil	Oregano essential oil	*Salmonella* spp. (UFC mL^{-1})	*Staphylococcus aureus* (UFC mL^{-1})	Inhibition of microbial adhesion
1 (e)	10	0.5	0.5	10^3	10^4	$55.04 \pm 1.52^{c,d}$
2 (a)	20	0.5	0.5	10^3	10^3	31.46 ± 2.96^{e}
3 (b)	10	1	0.5	10^3	10^3	$54.71 \pm 0.55^{c,d}$
4 (abe)	20	1	0.5	10^3	10^4	$60.73 \pm 0.39^{b,c}$
5 (c)	10	0.5	1	10^3	10^3	43.49 ± 0.83^{d}
6 (ace)	20	0.5	1	10^3	10^4	48.04 ± 1.96^{d}
7 (bce)	10	1	1	10^3	10^4	73.82 ± 0.35^{a}
8 (abc)	20	1	1	10^3	10^3	47.24 ± 2.40^{d}
9 (d)	10	0.5	0.5	10^4	10^3	58.21 ± 1.34^{c}
10 (ade)	20	0.5	0.5	10^4	10^4	66.11 ± 1.55^{b}
11 (bde)	10	1	0.5	10^4	10^4	$59.83 \pm 1.56^{b,c}$
12 (abd)	20	1	0.5	10^4	10^3	66.34 ± 0.53^{b}
13 (cde)	10	0.5	1	10^4	10^4	$67.73 \pm 0.66^{a,b}$
14 (acd)	20	0.5	1	10^4	10^3	42.93 ± 0.49^{d}
15 (bcd)	10	1	1	10^4	10^3	$51.46 \pm 1.95^{c,d}$
16 (abcde)	20	1	1	10^4	10^4	49.60 ± 2.07^{d}

Values for inhibition percentage of microbial adhesion are means ± SD.
Values followed by different letters are significantly different ($p \leq 0,05$).

The inhibition percentage achieved in experiment 7 (ecb) reveals the high affinity between the radicals of the active ingredients of essential oils, especially oregano essential oil, and the cell membranes of Gram-positive bacteria, causing structural alterations and loss of cell viability (Bouhdid et al., 2009; Lv et al., 2011). Thus, the higher the concentration of this type of microorganisms on a surface, the higher the inhibition percentage.

Studies of *in vitro* evaluations or food matrices (especially meat products) of PLA and other biopolymers (alginate, soy protein) with oregano essential oil and cinnamon essential oil, both employed at 1% independently, showed good results in the microbiological control of Gram-positive and Gram-negative bacteria (Oussalah et al., 2006; Emiroğlu et al., 2010; Anuar et al., 2017); however, these studies do not specify the inhibition percentage to establish more precise comparisons, because they employed inhibition halo measurements.

The results also indicate that experiments 4 (abe), 10 (ade), 12 (abd), and 13 (cde), show inhibitions to microbial adhesion of 60.73 ± 0.39%, 66.11 ± 1.55%, 66.34 ± 0.53%, and 67.73 ± 0.66%, respectively; also showing that the common factor between them and experiment 7 (bce) is the *S. aureus* concentration (10^4 CFU mL^{-1}), except in experiment 12 (abd). There are no significant differences between 7 (bce) and 13 (cde), and there is differen-

ce between the components because they differ in the amount of cinnamon essential oil and the concentration of *Salmonella* spp. with 13 (cde), having the lowest amount of cinnamon essential oil and the highest concentration of *Salmonella* spp. than in 7 (bce). However, as discussed below, at the lower amount of cinnamon essential oil when the concentration of *Salmonella* spp. is higher, the results of the inhibition percentage of microbial adhesion are as high as when 1% cinnamon essential oil is used. This situation contributes to the fact that there is no significant difference between 7 (bce) and 13 (cde).

A model was made with the data from the experiment corresponding to design 2_V^{5-1}, with two blocks of eight observations as specified by resolution V, that included the main effects and the two-way interactions, except for the PEG 400 - cinnamon essential oil (AB) interaction, because it was misled with the blocks. According to the results obtained from this initial model, the PEG 400 - *S. aureus* interaction was removed (p-value > 0.1). In the same way we proceeded with the following models, where the interactions PEG 400 - oregano essential oil, PEG 400 - *Salmonella* spp., cinnamon essential oil - oregano essential oil, cinnamon essential oil - *S. aureus*, *Salmonella* spp. - *S. aureus*, oregano essential oil - *S. aureus*, and oregano essential oil - *Salmonella* spp. were gradually eliminated, because these interactions were not statistically significant either. After eliminating the interactions, the results of the applied model are shown in Table 6.

Table 6. Main effects and two-way interactions with their corresponding effect value and p-value.

Factor or interaction	Effect	p-value
PEG 400	-6.48	0.1422
Cinnamon essential oil	6.34	0.1498
Oregano essential oil	-3.51	0.4029
Salmonella spp.	5.96	0.1727
Staphylococcus aureus	10.63	0.0283
Cinnamon essential oil-*Salmonella* spp.	-8.28	0.0711

There is strong significant evidence (p-value = 0.0283) that *S. aureus* concentration factor influences the inhibition percentage of microbial adhesion (Figure 1) with an effect value of 10.63% between the mean inhibition percentage when *S. aureus* concentration is low (49.48%) and the mean inhibition percentage when *S. aureus* concentration is high (60.11%). Hence, the inhibition percentages of microbial adhesion higher than 60% have the higher concentration of *S. aureus* as a common factor. The cinnamon essential oil - *Salmonella* spp. interaction has little significant evidence (p-value = 0.0711) that influences the inhibition percentage. There is no significant statistical evidence of their influence on the inhibition results regarding the other factors and interactions.

In the graph of cinnamon essential oil - *Salmonella* spp. interaction (Figure 2) is observed that when the amount of cinnamon essential oil and the concentration of *Salmonella* spp. are the lowest (Figure 2), the mean of the inhibition adhesion percentage is 44.51%, and when the amount of cinnamon essential oil is the highest and the concentration of *Salmonella* spp. is still the lowest, the mean of the inhibition percentage is 59.12%; when the amount of cinnamon essential oil is the lowest and the concentration of *Salmonella* spp. is the highest, the mean of the inhibition adhesion percentage is 58.74%, and when the amount of cinnamon essential oil is the highest and the concentration of *Salmonella* spp. is still high, the mean of the inhibition percentage is 56.81%.

Thus, the greatest effect is observed at cinnamon essential oil concentration of 0.5% with an increase in the inhibition percentage of 14.24% when the concentration of *Salmonella* spp. goes from low level (10^3 CFU mL^{-1}) to a high level (10^4 CFU mL^{-1}), while there is a reduction in the inhibition percentage of 2.32% at cinnamon oil concentration of 1% when the concentration of *Salmonella* spp. also changes. When the concentration of cinnamon essential oil goes from 0.5% to 1% and a low *Salmone-*

lla spp. concentration, the inhibition percentage increases by 14.62%, while at high concentration of *Salmonella* spp. the inhibition percentage reduces by 1.94%, which could indicate a tendency to decrease in the inhibition percentage at higher concentrations of *Salmonella* spp.

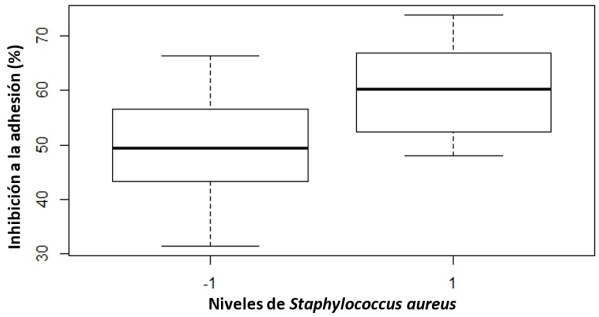

Figure 1. Adhesion inhibition percentage to different concentrations of *Staphylococcus aureus*.

Considering these two factors, it is also evident that the highest inhibition percentage of microbial adhesion 59.12% occurs when the cinnamon essential oil is at 1% and the *Salmonella* concentration is 10^3 CFU mL^{-1}; and the lowest inhibition percentage 44.51% occurs when the cinnamon essential oil is at 0.5% at the same *Salmonella* spp. concentration. Then, Figure 2 indicates that 1% of cinnamon essential oil has greater inhibition effectiveness for the different concentrations of *Salmonella* spp., represented in this research as Gram-negative bacteria.

The results of the cinnamon essential oil - *Salmonella* spp. interaction demonstate that oregano essential oil is less effective against Gram-negative bacteria because it has a lower affinity with the cell membrane of a thin layer of peptidoglycan, opening the action of cinnamon essential oil and its active ingredient on this type of bacteria (Becerril et al., 2007), which are metabolically more difficult to fight because of the external film they develop as a protection mechanism (Cabrera et al., 2007).

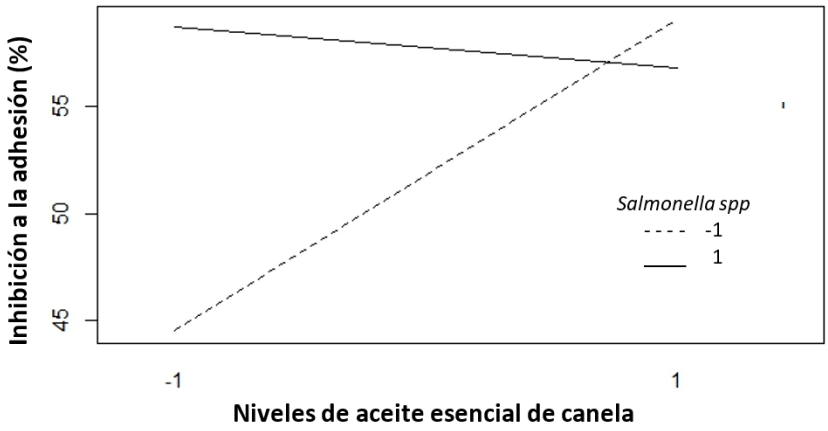

Figure 2. Cinnamon essential oil-*Salmonella* spp. interaction.

4 Conclusions

Biodegradable active films of PLA with oregano and cinnamon essential oils were obtained, with good solubility between the components. The inhibition percentage to microbial adhesion on the active biodegradable films is a function of the representative Gram-positive bacteria model for this research. Statistical analysis determined that there is strong significant evidence that the concentration of *S. aureus* influences the inhibition percentage of microbial adhesion. Thus, inhibitions higher than 60% have higher concentration of *S. aureus* as a common factor.

In addition, the amount of 1% cinnamon essential oil has greater inhibition effectiveness for the different concentrations of *Salmonella* spp. despite registering a slight inhibition decrease when going from the lowest to the highest *Salmonella* spp. concentration.

Biodegradable active PLA films with higher inhibition percentage to microbial adhesion can potentially be used in the food industry as an environmentally friendly alternative to increase additional barrier mechanisms to prevent contamination with *Salmonella* spp. and *S. aureus*, and reduce the number of people infected by these microorganisms.

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