Inhibitory Effect of Mexican Oregano (Lippia berlandieri Schauer) Essential Oil on Pseudomonas aeruginosa and Salmonella Thyphimurium Biofilm Formation

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Biofilms are difficult to eliminate with standard antimicrobial treatments due to their high antibiotic resistance. Therefore, some plants contain natural compounds with antimicrobial activity that can control biofilm formation. The aim of this work was to evaluate the effect of Mexican oregano (Lippia berlandieri Schauer) essential oil (EO) on Pseudomonas aeruginosa and Salmonella Typhimurium biofilm formation on stainless steel surfaces. EO was added to achieve final concentrations of 0, 200, 250, 300, 400, 600, 800, and 1,600 mg/L. Growth data were modeled using the modified Gompertz model. EO antimicrobial activity against biofilm can be observed for both microorganisms. Moreover, at an EO concentration below inhibition, there is a delay in biofilm formation, thus decreasing the number of microorganisms that are part of the biofilm. The maximum growth rate in biofilm formation showed an inversely proportional behavior in relation to EO concentration. P. aeruginosa requires nearly 1 h to start forming biofilms. There is a delay in biofilm formation, thus decreasing the number of microorganisms that are part of the biofilm (24 h for 400 mg/L and 18 h for 600 mg/L). Salmonella Typhimurium biofilm formation is affected at 200 mg/L of Mexican oregano EO, causing film detachment from the stainless steel surface after 18 h, while at 250 mg/L biofilm formation was inhibited. Mexican oregano EO could inhibit the development of P. aeruginosa and Salmonella Typhimurium biofilm formation on stainless steel surfaces. Salmonella was more susceptible than Pseudomonas to Mexican oregano EO according to the concentrations required to inhibit biofilm growth.

Keywords: biofilms, foodborne pathogens, inactivation, Mexican oregano, essential oil

INTRODUCTION

Biofilms are defined as adherent, matrix-enclosed bacterial populations attached with physical appendages to a surface, followed by coating with extracellular polysaccharides (Furukawa et al., 2010; Laird et al., 2012). In nature, microorganisms usually attach to solid surfaces, especially at liquid–solid interfaces. Biofilms mediate bacteria persistence and shield from hostile environments.
The initial microbial adhesion to surfaces is a complex process dependent on non-specific interactions between bacteria and the surface to form slimy layers. Once bacteria firmly established a biofilm, it is very difficult to eradicate because it exhibits an increase of resistance to chemical compounds (Nostro et al., 2010; Ibusquiza et al., 2012; Roy et al., 2011). Many foodborne pathogens are able to survive at low temperatures and attach to, form resistant biofilms, and colonize different surfaces. This resistance is related on the three-dimensional film structure which has enhanced tolerance to physical and chemical treatments and considered a potential hazard, a source of food contamination, and a cause of economic losses by technical failure in water systems, heat exchangers, product quality deterioration, and biocorrosion (Dheilly et al., 2008; Bae et al., 2012; Bodur and Cagri-Mehmetoglu, 2012; Ibusquiza et al., 2012).

Besides that, biofilms are difficult to eliminate with standard antimicrobial treatments due to their high antibiotic resistance relative to free-living cells (Kavanaugh and Rübsbeck, 2012). Some plants contain natural compounds with antimicrobial activity; in their natural state, these compounds can play the role of prolonging the shelf-life of food. Many of them have been studied for their potential as food antimicrobials (Rivera-Caló et al., 2015; Swamy et al., 2016; Ambrosio et al., 2017; Rakmai et al., 2018). The use of natural compounds as food additives involves isolation, purification, stabilization, and incorporation of these compounds into food for antimicrobial purposes while maintaining the food's organoleptic and nutritive properties (Pandey et al., 2017; Rakmai et al., 2017a; Navarro-Cruz et al., 2018). The antimicrobial activity of these natural additives is due to the presence of their chemical compounds which can alter and penetrate the microorganism's cell wall lipid structure, disturbing cellular structures and leading to protein denaturation and cell membrane destruction, increasing their permeability and the likelihood of cytoplasmic ruptures, leaks, or cell lysis and eventually microbial death (Rakmai et al., 2017b; Reyes-Jurado et al., 2019). Therefore, natural compounds as essential oils (EOs) are an alternative to be used as antimicrobial in the bacterial biofilm formation.

Mexican oregano is one of the aromatic plants used as a food additive to enhance food flavor; carvacrol and thymol are the major chemical compounds present in this EO and are the responsible for their antimicrobial action (Avila-Sosa et al., 2010; Anaya-Castro et al., 2017; Cid-Pérez et al., 2019). The aim of this study was to evaluate the inhibitory effect of Mexican oregano (Lippia berlandieri Schauer) EO on Pseudomonas aeruginosa and Salmonella Typhimurium biofilm formation.

MATERIALS AND METHODS

Essential Oil, Microorganisms, Culture Preparation, and Stainless Steel Surfaces

Mexican oregano EO was obtained and processed at CIReNa (Natural Resources Research Center, DGTA, Salaices, Chihuahua), where it was characterized based on its carvacrol and thymol contents. The oregano was grown on site, and the EO was obtained by vapor extraction for 4 h using a Clevenger-type apparatus (Silva-Vazquez and Dunford, 2005). Pseudomonas aeruginosa (ATTC 14,210) and Salmonella enterica serovar Typhimurium (ATCC 14028) were obtained from Facultad de Ciencias Químicas Microbiology Laboratory and maintained on tryptone soy agar (TSA, Merk, Mexico, Mexico). At the time of performing the biofilm formation assay, the bacteria were incubated on tryptone soy broth (TSB, Merk, Mexico, Mexico) at 37°C for 18 h until exponential phase was observed. Stainless steel plates (3.0 × 6.0 cm) were prepared according to Speranza et al. (2011) by washing in acetone (Sigma-Adrich, Milwaukee, WI, USA) for 30 min, rinsing in distilled water, then soaking in NaOH (1 N for 1 h), and final rinsing in distilled water prior to use.

Biofilm Formation Assay

Stainless steel plates were placed vertically into jars containing 20 mL of TSB and autoclaved at 121°C for 15 min. EO was added to achieve final concentrations of 0, 200, 250, 300, 400, 600, 800, and 1,600 mg/L and mechanically (using a vortex shaker) incorporated under sterile conditions. Then, 500 μl of bacteria was inoculated to obtain an initial bacterial population of about 10^3 CFU/ml. The jars were incubated at 37°C during 0, 1, 2, 4, 6, 12, 36, 48, 60, and 72 h. For all the samples, incubation was done without agitation. At these times, the stainless steel plates were aseptically removed and rinsed with sterile distilled water in order to eliminate the unattached cells. The biofilm cells were collected and enumerated according to Xu et al. (2010) by swabbing over the area with a sterilized water-moistened cotton swabs and immersing in tubes with 1 ml of 0.1% sterile peptone water. These were vortexed vigorously with 9 ml of 0.1% sterile peptone water to suspend the adhering cells. In order to ensure the viability of the organisms, a growth control was performed with broth cells. Both counts (biofilm and broth) were determined by the pour plating method on TSA and were incubated at 37°C for 48 h. Every test was performed in triplicate.

Data Modeling and Statistical Analysis

The growth data were modeled using the modified Gompertz model (van Boekel, 2008):

\[
\log \left( \frac{N}{N_0} \right) = A \exp \left[ - \exp \left( \frac{v_{\text{max}} \cdot \exp (1/A) (\lambda - t) + 1} \right) \right] \]

where \( N \) is the number of microorganisms (UFC/ml) at time \( t \) (h), \( N_0 \) (UFC/ml) is the number of microorganisms at initial time, \( A \) is the asymptotic value of the maximum number of microorganisms, \( v_{\text{max}} \) is the maximum growth rate \((\log N/N_0)/h\), and \( \lambda \) is the lag phase or the adaptation time period (h) that microorganisms take under new conditions.

Statistical analyses were performed with the general linear model procedure in Minitab 15 (LEAD Technologies Inc., NJ, USA). Significantly \( (p < 0.05) \) different means were separated with Tukey’s test. Student \( t \)-tests \( (p < 0.05) \) were performed in order to compare biofilm and broth growth.
RESULTS

Mexican oregano EO was obtained from plants grown in Chihuahua. Several compounds are related to its chemical composition—thymol (1.991 g/ml) and carvacrol (0.353 g/ml) and their precursors (p-cymene, 1,8-cineole, and γ-terpinen)—which were detected in very low concentrations. EO antimicrobial activity against biofilm formation is remarkable; it can be observed that, for both microorganisms (Figures 1, 2), the minimal inhibitory concentration (MIC) values are lower (50%) than the MIC values in broth culture. All bacterial curves presented an adequate data fit to Gompertz model equation ($R^2_{adj} = 0.906 \pm 0.04$).

Figure 1 shows significant differences ($p < 0.05$) between Gompertz growth parameters on P. aeruginosa development in broth culture, with MIC values of 800 and 1,600 mg/L. It can be observed that it has the largest number of microorganisms that adhere on stainless steel surfaces, forming the biofilm. It requires approximately near 8 log($N/N_o$) and near 1 h to start forming biofilms. Moreover, at EO concentration values below MIC (Figure 3A), there is a delay in biofilm formation, thus decreasing the number of microorganisms that are part of the biofilm (24 h for 400 mg/L and 18 h for 600 mg/L). The maximum growth rate in biofilm formation showed an inversely proportional behavior in relation to the EO concentration.

A comparison between the statistical analyses of the Gompertz parameters on the development of Salmonella Typhimurium in broth and on stainless steel is shown in Figure 2. In culture broth, Salmonella does not require an adaptation time since they start to grow from the start, while biofilm formation requires ~6 h of lag phase. Significant differences ($p < 0.05$) are detected in $A$ and $\nu_{max}$ parameters, being higher in culture broth. Furthermore, the biofilm-forming bacteria's stationary phase (Figure 3B) is shorter. This may be due to biofilm formation's last phase (phase 5: colonizing cell dispersion). The Salmonella Typhimurium biofilm formation is affected at 200 mg/L of Mexican oregano EO, causing film detachment from the stainless steel surface after 18 h, while at 250 mg/L biofilm formation was inhibited.

DISCUSSION

The Mexican oregano EO antimicrobial activity is due to the presence of thymol and carvacrol along with other minor components. The main component is thymol, and its concentrations are greater when the extracts are obtained from younger plants; in contrast, in European oregano EO, carvacrol concentration is higher (88%) than thymol (2%) (Chorianopoulos and Kalpoutzakis, 2004). In addition, it is common to find variations on EO chemical composition. It depends on the climate and the soil conditions as well as the phenological plant stage (Avila-Sosa et al., 2014).

Biofilm formation ability has been associated with bacterial virulence colonization, environmental survival, antibiotic resistance, surface characteristics, and growth medium (Ferreira et al., 2013; Di Ciccio et al., 2015). During this process, the bacteria can change their phenotype and morphology, affecting the structural changes necessary to for the biofilm to develop and causing the detachment shortly after starting its formation or avoiding their formation (Chmielewski and...
**FIGURE 2** Modified Gompertz model parameters for *Salmonella Typhimurium* biofilm growth (□) and broth growth (■) curves subjected to selected concentrations of Mexican oregano (*Lippia berlandieri* Schauer) essential oil. (A) Maximum number of microorganisms. (B) Maximum growth rate. (C) Lag phase. Each bar treatment with different letters is significantly different (*p* < 0.05). Asterisk denotes significant difference between growth conditions (*p* < 0.05).

**Frank, 2003**). *P. aeruginosa*’s fast biofilm forming is due to cell surface characteristics such as flagella, pili, adhesion, and capsule proteins that also influence adhesion. **Kumar and Anand (1998)** mentioned that pili acts as a velcro to anchor bacteria to some surfaces and also acts as chemoreceptors, directing to some specific sites. By possessing these surface characteristics in combination with a nutrient-rich medium, biofilm formation is adapted quickly on stainless steel surfaces. **Srey et al. (2013)** suggested that *Salmonella* strains preferably adhere to hydrophobic surface materials that depend on surface interfacial free energies. Moreover, bacterial cell walls (conferring by peptidoglycan anionic teichoic acids) and cell surface hydrophobicity (enhanced by the presence of lactic acid), which affect the electrostatic charge, are the main factors that cause biofilms to attach to stainless steel surfaces (**Bridier et al., 2015**).

**Burt (2004)** reported that the oregano EO antimicrobial effect is not attributed to a single mechanism but to several, due to multiple cell targets, since it can act on cytoplasmic membrane proteins and on lipophilic compounds, specifically with the protein’s hydrophobic parts. As a consequence, they can modify enzyme activities that regulate energy production or structural compound synthesis. It can be observed that...
the biofilm formation initial phases would be avoided where the exopolysaccharide matrix is synthesized to establish an irreversible physical contact between them and the surface. Ultee et al. (1998) also reports that carvacrol interacts with bacterial membranes, changing its permeability for cations (H\(^+\) and K\(^-\)), where ion gradient dissipation leads to a deterioration in the essential processes in the cell and finally to cell death. Biological effects may depend on the concentration and the exposure time, modifying the composition of the cell membrane.

Furthermore, Dat et al. (2012) found that biofilm formation might be enhanced by low pH through cell-to-cell signaling. Moreover, pH changes may trigger numerous signaling substances. These substances could contribute to the overall acid tolerance response and promote biofilm formation, which is known as an adaptation to external stressful environments. The biofilms with low pH can allow the higher antimicrobial action of thymol and carvacrol (whose pH dissociation values are acidic). According to Martínez-Graci et al. (2015), optimal pH should be considered at which the antimicrobial agent may have a state of dissociation that allows better antimicrobial activity. Rivera-Caló et al. (2015) mentioned that the EO antimicrobial effect may be attributed to their ability to penetrate through the bacterial membrane to the cell interior and to exhibit an inhibitory activity on the cell's functional properties. Because biofilm formation is an active process that requires energy for various extracellular components, Wang et al. (2015) suggested that the inhibitory effect of biofilm formation may be due to an increase of ATP permeability in the cytoplasmic membrane. *Salmonella* Thymphium growth in broth suggests ATP use to maintain its viability, leaving behind the biofilm production.

The Mexican oregano EO could inhibit the development of *P. aeruginosa* and *Salmonella* Typhimurium biofilm formation on stainless steel surfaces. *Salmonella* was more susceptible than *Pseudomonas* to Mexican oregano EO according to the concentrations required to inhibit biofilm growth. The Mexican oregano EO could be an alternative for cleaning stainless steel surfaces and equipment.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.

**AUTHOR CONTRIBUTIONS**

RA-S, FR-J, and CO-V wrote the manuscript. FR-J, TC-P, and PH-C designed and performed the experiments. RM-P provided and characterized the microorganisms. All the authors approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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