




Activity of rosemary (*Rosmarinus officinalis* - Family: Lamiaceae) essential oil compared to peracetic acid in *Listeria monocytogenes* biofilms

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ABSTRACT. The objective of this study was to evaluate the activity of *Rosmarinus officinalis* essential oil (EO) compared to peracetic acid (PA) regarding formation and elimination of *Listeria monocytogenes* biofilms on polystyrene surface. The minimum inhibitory concentration (MIC) was determined according to standard protocol. Isolates were inoculated according to MIC standards polystyrene plate wells, which were then incubated at 37°C/96 hours for evaluation of biofilm formation. Regarding the evaluation of biofilm elimination, the biofilms were treated under MIC for 10 minutes. The MIC obtained were 2.0 and 3.0 mg mL⁻¹ for EO and 0,015% for PA. Therefore, the results showed a reduction in the formation of biofilm with the presence of EO and PA, EO being more efficient ($p < 0.05$). Both compounds had a good capacity of eliminating biofilms, however the EO reduced the biofilm formation when compared to PA, highlighting its potential as an antibacterial agent and antibiofilm.

Keywords: control methods; industrial surface; rosemary; sanitization.

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Introduction

Listeria monocytogenes is an important pathogen that can cause listeriosis, an infection that in its severe form may result in a mortality rate of 20 to 30%, mainly affecting risk groups (Li et al., 2020). Most listeriosis outbreaks are associated with Read-To-Eat foods, in which the risk of contamination is considered to be higher as cooking would not be required while processing these products (Smith et al., 2019; Gray et al., 2021). *Listeria monocytogenes* has long-term persistence in processing equipment and facilities in the food industry (Serenio et al., 2019). This behavior is related to their high tolerance to stress conditions such as refrigeration temperatures, high salt concentrations and their ability to form biofilms on several surfaces (Ziech et al., 2016; Kocot & Olszewska, 2017; Papaioannou, Giaouris, Berillis, & Boziaris, 2018). Biofilms are also able to facilitate the persistence of *L. monocytogenes* for long periods in food processing plants (Gray et al., 2021) and play an important role in the survival of the pathogen in industrial environments (Kocot & Olszewska, 2017).

The biofilm formation is a major problem for the food industry, as it can cause serious technological issues that lead to uselessness of equipment and contamination of products that reach final consumers, being an important public health problem (Brasileiro et al., 2016; Smith et al., 2019).

With respect to biofilms arrangement, sessile cells have a higher resistance to antimicrobial agents than planktonic cells, due to their structure and presence of extracellular polymeric substances (EPS), sublethal concentrations, metabolic heterogeneity, activation of adaptive responses to stress, and horizontal gene transfer (Olszewska, Zhao, & Doyle, 2016; Campana, Ciandrini, & Baffone, 2018).

To reduce the likelihood of developing microbial resistance and expand the range of antimicrobial activity in the food industry, new approaches to biofilm prevention and control have been explored (Olszewska et al., 2016; Pang, Wong, Chung, & Yuk, 2019), new approaches on their use in the food industry have been

researched in order to prevent and eliminate microbial contamination by inhibiting biofilm formation. Thus, EO and its components become promising alternatives (Falcó, Verdeguer, Aznar, Sánchez, & Randazzo, 2019). The effect of EO on the elimination of biofilm was described against certain pathogenic microorganisms, such as *L. monocytogenes*, *Staphylococcus aureus* and *Salmonella* sp., as well as the association of conventional methodologies and alternative combinations of these (Vázquez-Sánchez, Galvão, Ambrosio, Gloria, & Oetterer, 2018; Kang et al., 2019; Rossi et al., 2019).

The EO are extracted from different volatile oily liquids, they are mainly composed of mono and sesquiterpenes and phenylpropanoids, metabolites that give their organoleptic characteristics being are currently used as food flavorings (Falcó et al., 2019). The rosemary EO (*Rosmarinus officinalis* - Family: Lamiaceae) an aromatic oil extracted from the leaves of the plant is widely used in food preservation and has antibacterial activities due to its side effects by being a broad-spectrum bactericidal, biodegradable, safe and non-toxic substance (Amaral et al., 2018; Borges, Ortiz, Pereira, Keita, & Carvalho, 2019). This compound works under the integrity of cell membranes, interfering in protein synthesis. However, there are no reports on the antibiofilm effect of rosemary EO on *L. monocytogenes* cells. The present study aims to evaluate the action of rosemary EO during formation and elimination of *L. monocytogenes* biofilm on a polystyrene surface compared to PA, a sanitizer commonly used in the food industry.

Material and methods

Bacterial isolates and growth condition

Listeria monocytogenes used in the biofilm assays were isolated from surfaces of equipment and utensils used in the meat processing area of pig slaughterhouses. The isolate was previously identified as *L. monocytogenes* serotype IVb (LAC/LM/P.SUI1/28), containing the virulence genes *inlA*, *inlB*, *inlC*, *inlJ*, *hlyA*, *actA*, and *plcA*, which had 100% genetic similarity with other isolates of *L. monocytogenes* (Sereno et al., 2019). The isolate was stored at -18°C in Tryptic Soy Broth added from 0.6% of Yeast Extract (TSB-YE) containing 20% (V/V) of glycerol. Then, it was cultivated aerobically at 37°C/24 hours.

Preparation of test substances: rosemary EO (*R. officinalis*) and PA solution

The rosemary EO (FERQUIMA, São Paulo, Brazil), was obtained by steam distillation of the leaves. In its composition there were 1.8 cineole (43%), camphor (15%), alpha-pinene (13%), beta-pinene (8%), and limonene (3%). The concentrations of all analyzed substances were prepared prior to each repetition of the experiment, stored at room temperature, and not exposed to light. To avoid concentration changes of main components, the same batch of EO was used throughout the experimental work. The EO was solubilized in Dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) along with TSB-YE containing 0,5% of polysorbate (Sigma-Aldrich, St. Louis, MO, USA), and homogenized for five minutes in order to produce a stable emulsion. The solution of PA (Chesiquímica LTDA) was diluted with sterile distilled water.

Determination of the minimum inhibitory concentration (MIC)

The determination of MIC was performed by the diffusion method in Agar according to CLSI (CLSI, 2018) and Silva, Camargo, Todorov, and Nero (2017) with modifications. The bacterial culture was prepared and adjusted to the 0.5 scale of McFarland and sown with sterile swabs on Mueller-Hinton agar (MH). EO solutions were diluted in concentrations from 0,1 to 30 mg mL⁻¹ (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0, 5.0, 10, 15, 20, 25, and 30). PA solutions were diluted in concentrations between 0.001 and 0.3% (0.001, 0.005, 0.007, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.12, 0.15, 0.17, 0.2, 0.25 and 0.3). Subsequently, 10 µL of each EO or PA concentration were inoculated on the previously prepared MH surface, and the plates were incubated at 37°C/24 hours. MIC is determined as the lowest concentration capable of inhibiting visible bacterial growth (CLSI, 2018). As negative controls, the EO was added to the TSB without bacterial culture, as well as the DMSO solution and sterile distilled water to evaluate the antibacterial effect. The experiment was performed in triplicate with two replications.

Biofilm formation on polystyrene surface under different conditions

The isolate was evaluated for biofilm formation and elimination on polystyrene coupons under the following conditions. Prior to the bacterial adhesion tests, the polystyrene coupons (1.0 x 1.0 x 0.1 cm) (n = 30) were washed with 70% w/w alcohol (70° INPM) and rinsed three times in distilled water and sterilized in an

autoclave for 15 min. at 121°C. Following that, 60 mL of TSB-YE supplemented with 1% meat extract were prepared and adjusted to 0.5 on the McFarland scale, from which an aliquot of 1.0 mL was used to confirm the initial inoculum. Under these conditions, three flasks containing bacterial culture were prepared, one flask used as a positive control, a flask with an aliquot of MIC of EO Rosemary and a flask with an aliquot of MIC of PA. The flasks were incubated for up to 96 hours at 37°C under orbital shaking at 100 rpm.

Regarding the quantification of sessile cells, we followed the methodology of Reis-Teixeira, Sousa, Alves, Furtado, and De Martinis (2019) with modifications. Briefly the coupons were washed with 10 mL of phosphate buffered saline pH 7.2 (1X PBS). The sessile cells were detached from the surface of the coupons by friction with sterile swabs, immersed in tubes containing 0.85% saline solution and homogenized by vortexing for two minutes. After this procedure, applicable serial dilutions were performed from each coupon and 10 µL aliquots were used to quantify the sessile cells (Herigstad, Hamilton, & Heersink, 2001) and inoculated on the surface of tryptone soy agar (TSA, KASVI). The plates were incubated at 37°C/24 hours and the results expressed in log CFU cm⁻².

Action of rosemary (*R. officinalis*) EO and PA in the elimination of *L. monocytogenes* biofilms on polystyrene surface

To evaluate the activity of rosemary EO and PA on the formed biofilm (Positive Control), four coupons from the control condition were removed, rinsed with 10 mL of 1X PBS solution to remove free cells, and exposed to 2 mL of the 10X MIC concentration obtained for EO (20 mg mL⁻¹) and PA (0.15%) for 10 minutes. After the exposed time, the coupons were rinsed again and the remaining sessile cells were detached from the surface by friction with sterile swabs, immersed in tubes containing 0.85% saline solution and homogenized in a vortex for two minutes. Subsequently, appropriate decimal dilutions were made, plated on plates containing TSA-YE and incubated at 37°C/24 hours; results were expressed in CFU cm⁻².

Statistical analysis

The experiment was performed in quadruplicate and with two distinct repetitions. Results were expressed as mean ± standard deviation. To assess whether there was a difference concerning the action of EO and AP during formation and elimination of the biofilm when compared to the control, the data were submitted to statistical tests by Shapiro-Wilk and Kolgomorov-Sminorv to verify normality. After carrying out the normality tests, the non-parametric Mann-Whitney test was applied for statistical comparison of mean counts of the different treatments. All analyzes were performed using the statistical program IBM® SPSS® Statistics Version 2.0, with a 0.05 significance level.

Results and discussion

Determination of the minimum inhibitory concentration (MIC)

The results indicated a MIC of 2 mg mL⁻¹ for EO and 0.015% for PA. Differences in the chemical composition of the tested substances may explain the observed variation on their antimicrobial activity, as well as individual genetic factors of the evaluated isolate. The antimicrobial activity of EO may be associated with active constituents, of which 90-95% consists of monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives, in addition to aldehydes, alcohols, and esters. The non-volatile portion constitutes 5-10% of its composition, which mainly includes hydrocarbons, sterols, carotenoids, and flavonoids (Miranda, Cardoso, Batista, Rodrigues, & Figueiredo, 2016). Studies attribute the antimicrobial activity of EOs against *L. monocytogenes* to the presence of phenolic compounds attributed to 1.8-cineol, which is considered to have efficient anti-anti-listerial action (Mourey & Canillac, 2002; Karadag et al., 2019). The EO used in this study has the 1.8-cineol as its major component, thus contributing to the results obtained, in which the rosemary EO had antibacterial effect on the isolate evaluated not only by MIC, but also regarding formation and elimination of biofilm.

Action of rosemary (*R. officinalis*) EO and PA in the formation and elimination of the biofilms of *L. monocytogenes* on polystyrene surface

As a result, there was a reduction in biofilm formation with the use of OE and PA treatments when compared to control biofilm (Figure 1). However, no analyzed substance was able to prevent the complete formation of the bacterial biofilm. Regarding the individual action of the compounds, the EO was more

effective than the PA in reducing the average count for *L. monocytogenes*. EO contains phenolic compounds that have antimicrobial activity by disrupting the cytoplasmic membrane, which results in excessive cell permeability, thus preventing microbial adhesion to biotic and abiotic surfaces (Miranda et al., 2016). This characteristic explains the results obtained in this study, in which there was a decrease in biofilm formation when subjected to interaction with rosemary EO. It can be endorsed that the use of sanitizing solutions made with natural compounds is effective in turning the environment where it was applied more hostile and, thus, hindering the ability of microorganisms to adhere. Hence, it becomes a useful approach in the biofilm formation phase, making hygienic control more effective and improving the action of conventional sanitizers used during the elimination process. In recent years, researches aiming at evaluating the effectiveness of essential oils has been promising, and these actions are due to two main factors, consumer market demand and an attempt to reduce microbial tolerance to conventional sanitizers. As in our study, Lagha, Ben Abdallah, Al-Sarhan, and Al-Sodany (2019) demonstrated the action of *R. officinalis* essential oil against the biofilm produced by *Escherichia coli*. In recent years, the action of rosemary essential oil has also been demonstrated on pathogens of importance in the food industry, such as *Salmonella* Enteritidis (Lira et al., 2020) and *Staphylococcus* sp., (Bezerra et al., 2023) In this work, the antibiofilm activity of the essential oil was better than the elimination activity, showing that the mechanisms of these actions are different.

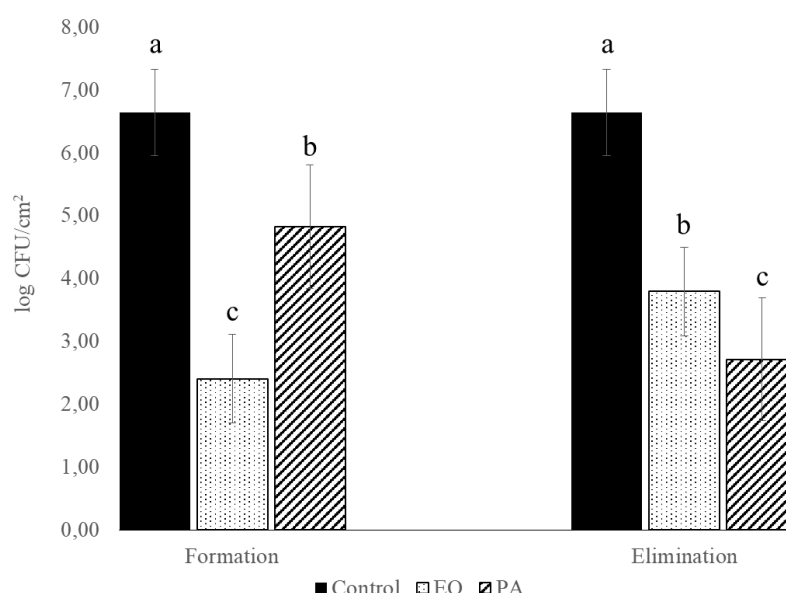


Figure 1. Evaluation of Rosemary EO and PA in *Listeria monocytogenes* biofilm formation and elimination.

Different letters in the same group (formation and elimination) represent statistically significant difference ($p < 0.05$) between treatments.

The treatments employed were effective in eliminating the *L. monocytogenes* biofilm ($p < 0.05$). There was a significant difference between the action of EO and AP ($p < 0.05$) in sessile cells (Figure 1). In contrast to biofilm formation stage, the lowest sessile cell counts were observed when PA treatment was used. PA is known as an effective sanitizer to control *L. monocytogenes* in the food industry. However, its antimicrobial action may be influenced by external factors, such as concentration and temperature of use, which is a limiting factor in industrial hygiene (Farjami, Hatami, Siahi-Shadbad, & Lotfipour, 2022). In this study, the results show a lower MIC value than the recommended concentration of this principle for surface disinfection (0.2%), demonstrating an effective antimicrobial activity of PA on the evaluated isolates. However, it does not maintain the same effectiveness when evaluating its action on sessile cells, having its activity reduced. This behavior can be explained by the fact that organic acids (acetic acid) act by altering intracellular pH, resulting in changes in cell membrane permeability, but not inhibiting cell-cell communication, a decisive tool for biofilm formation (Zhang et al., 2019).

Previous studies have shown that PA can induce sublethal lesions in pathogenic organisms (Olszewska et al., 2016; Akinbobola, Amaeze, Mackay, Ramage, & Williams, 2021). Thus, even with an MIC lower than recommended, its action in controlling bacterial biofilms may be limited.

Conclusion

In conclusion, the sessile cells of *L. monocytogenes* used in the study proved to be more resistant to the evaluated solutions than planktonic cells, which represents a major challenge in eliminating this pathogen from industrial food processing facilities. Thus, this study showed the action potential of PA as a sanitizer commonly used in the food industry in microbiological control and also highlights the action of rosemary EO as an antibacterial and antibiofilm agent for *L. monocytogenes* isolates as an alternative way to be evaluated in the control of bacterial biofilm on polystyrene surface. However, further studies are needed to evaluate the interaction of antibacterial compounds in the elimination of *L. monocytogenes* biofilms, as well as the evaluation of the regulation of gene expression of these microorganisms when subjected to stress conditions provided by rosemary essential oil.

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