International Journal of Food Microbiology 293 (2019) 79–86

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Inhibition of *Listeria monocytogenes* by *Melaleuca alternifolia* (tea tree) essential oil in ground beef

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ARTICLE INFO

Keywords:
Essential oil
Food preservation
Minimum bactericidal concentration
Minimum inhibitory concentration

ABSTRACT

The food industry has been valuing the quest for natural substances for use in food preservation aiming to meet consumer demand for safer, more natural foods with preserved nutrients. This study aimed to assess the antimicrobial potential of essential oil of *Melaleuca alternifolia* (EOMA) in the inhibition of *Listeria monocytogenes* (ATCC 7644) (*L. monocytogenes*) in ground beef. An in vitro screening in solid phase was performed and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined through microdilution test. The time of action of EOMA was assessed through the death-time curve at 2 × MIC and MBC. The effects of MBC on bacterial morphology were verified under scanning electron microscopy (SEM). The meat samples were inoculated with four different suspensions of *L. monocytogenes* (1.5×10^8 CFU/mL, 4.6×10^4 CFU/mL, 9.2×10^3 CFU/mL, and 1.2×10^2 CFU/mL) and stored at 4 ± 1 °C for up to 14 days. The test samples were added with 1.5% v/w EOMA. The test of diffusion in solid medium showed *L. monocytogenes* ATCC 7644 was extremely sensitive to EOMA. MIC and MBC values were 0.10 μL/g and 0.15 μL/mL, respectively. The death-time curve revealed a reduction of viable cells after 1 h of contact with the oil. SEM showed that the treated cells had wrinkled surface and some cells had lower size and diameter when compared to control ones. The food matrix test indicated EOMA had antimicrobial activity in all samples except for the one inoculated with the suspension at 1.5×10^8 CFU/mL. Thus, the use of essential oil of *Melaleuca alternifolia* as a potential natural antimicrobial agent to preserve ground beef was promising as it was effective at low concentration. The data lay bases for new tests to be carried out in other food matrices.

1. Introduction

Worldwide, around two million people die from foodborne diseases (FBD) every year, most of whom children (WHO, 2010). According to the Center for Disease Control and Prevention (CDC, 2011), contaminated foods are responsible for approximately 48 million cases of diseases and 3000 deaths each year in the USA. The main symptoms of FBDs are diarrhea, nausea, vomit, abdominal pain, fever, headache, and, in more severe cases, hemorrhagic colitis, septicemia, meningitis, articular infection, kidney failure, paralysis, miscarriage, and cancer (Scallan et al., 2011).

Among the major food bacteria, *Listeria monocytogenes* (*L. monocytogenes*) is considered a global health issue since, despite its low occurrence, it causes listeriosis, a very serious illness with high mortality rate (WHO, 2018). At least 1470 cases, with mortality rate of 12.7%, were recorded in the European Union in 2011 (EFSA, 2018). *Listeria monocytogenes* also stands out for its ability to survive and multiply at low temperatures and to tolerate saline environments (EFFSA, 2018).

The use of essential oils for natural food preservation seems to be a promising alternative in the prevention of FBDs. Essential oils are chosen for being safer to preserve foods compared to synthetic chemical additives. In addition, they add nutritional value and have antioxidant activity (Calo et al., 2015; Gutiérrez-del-Río et al., 2018).

From the antimicrobial standpoint, these oils act by penetrating the cell wall and membrane of gram-positive bacteria through passive diffusion and changing their permeability to cations H^+ and K^+, while the external membrane of gram-negative bacteria is disintegrated (Gutiérrez-del-Río et al., 2018; Tongnuanchan and Benjakul, 2014).

That causes an increase in permeability and expansion of the cytoplasmic membrane of the microorganism, leading to loss of important

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https://doi.org/10.1016/j.ijfoodmicro.2019.01.004
Received 10 September 2018; Received in revised form 8 January 2019; Accepted 9 January 2019
Available online 11 January 2019
0168-1605/

ions depending on contact time. Higher essential oil concentrations can result in loss of DNA and proteins and cause cell death (W. Li et al., 2016).

However, essential oils tend to have greater antimicrobial action against gram-positive bacteria (Gutiérrez-del-Río et al., 2018).

Due to their hydrophobic nature, the penetration of essential oils seems to be facilitated by the interaction with the lipophilic extremities of lipoteichoic acids in the cell wall of gram-positive bacteria, while, in gram-negative ones, the proteins in the external membrane or their lipopolysaccharide constitution may hamper the diffusion of hydrophobic compounds (Gutiérrez-del-Río et al., 2018; Tongnuanchan and Benjakul, 2014).

Another important characteristic of essential oils as antibacterial agents is related to the anti-quorum sensing activity, important in reducing the virulence and pathogenicity of bacteria that have acquired resistance against in vivo drugs (Duarte et al., 2016; Khan et al., 2009). Essential oil from *Melaleuca alternifolia* (tea tree) has been used by Aboriginal people in Australia as anti-septic and disinfectant since before white colonization and its use became widespread after 1920 (Thomsen et al., 2011). Repellent, acaricide, insecticide, and antifungal properties are also attributed to this oil (Souza et al., 2016). Another broad use of *Melaleuca alternifolia* oil is in dermatology, being the most common product in the treatment of acne (Hammer, 2015), and also in many other medicinal products in several countries (ATTIA, 2015; Carson et al., 2006; Castro et al., 2005; Thomsen et al., 2011). It is widely distributed in South America, western India, Australia and southern China (Felipe et al., 2018), with Australia being the world's largest oil producer (Castro et al., 2005). According to the Rural Industries Research and Development Corporation (2013), the production of essential oil of tea tree (*Melaleuca alternifolia*) in Australia grew 4900% from 1982 to 2010, corresponding to a lucrative market, in which the oil price per 1 Kilogram is up to 35 dollars. The production of essential oil of *Melaleuca alternifolia* occurs mainly by steam distillation of leaves and branches (ATTIA, 2015; Carson et al., 2006; Zhang et al., 2018), in which an oil yield of 1 to 2% (on wet basis) can be reached (Carson et al., 2006).

Although it has been certified by the American Food and Drug and Administration as generally regarded as safe (GRAS) and as a food additive, no report has been done on its use in food matrices. As a function of its antiseptic power, *Melaleuca alternifolia* essential oil may have antimicrobial activity when added to food matrices. Thus, this study aimed to assess the antimicrobial activity effectiveness of essential oil of *Melaleuca alternifolia* (EOMA) against *Listeria monocytogenes* experimentally inoculated in ground beef.

2. Materials and methods

2.1. Essential oils

In order to use essential oil of standardized origin and extraction process, the product was purchased from the company Ferquima Indústria e Comércio LTDA (São Paulo, Brazil). The tea tree oil was extracted through steam distillation of the leaves. The oil composition was determined using an AGILENT 7820A gas chromatograph (GC) equipped with a capillary column HP-5 (30 m × 0.32 mm × 0.25 μm), coupled to an Agilent 5973 N mass spectrometer. The GC operating conditions were as follows: hydrogen as the carrier gas at a flow rate of 1.5 mL/min; column temperature was set to 70 °C (0 min) and then increased to 240 °C at a rate of 3 °C/min; injector and FID detector temperatures of 240 °C and 250 °C, respectively. 1 μL of a 1% oil-chloroform solution was injected. Mass spectra were obtained in Agilent 5973N system, equipped with a HP5MS capillary column (30 m × 0.25 mm × 0.25 mm), using helium as the carrier gas (1.0 mL/min). The detector was operated in electron impact mode, with ionization energy of 70 eV. The retention indices were calculated after the injection of a series of n-alkanes under the same conditions used for the samples (Castelo et al., 2013). The identification of the oil constituents was performed by comparing their retention indices with literature values (Adams, 2001), and patterns of the major compounds, and also by comparing the mass spectra obtained with those in the Wiley data library (6th Edition). It should be noted that the oil was used without filtration because preliminary tests indicated its sterility.

2.2. Preparation of microbial suspensions

The standard strain used in the study (*Listeria monocytogenes ATCC 7644*) was kindly provided by the Laboratory of Food Analysis Experimentation (LEAAL) of the Nutrition Department of the Federal University of Pernambuco, Brazil.

The strains were thawed and the inoculum was prepared in 0.85% saline solution as described by Carvalho et al. (2015) from cultures grown overnight in BHI agar (HiMedia, India) at 37 ± 1 °C and then in BHI broth (HiMedia, India) at 37 °C for 18 to 20 h (late exponential growth phase). The inoculum was adjusted to turbidity of 0.5 on the McFarland scale or approximately 1.5×10^8 CFU/mL, as described by Radaelli et al. (2016), and standardized using a spectrophotometer (KAZUAKI, IL-592-LC), as described by Carvalho et al. (2015).

2.3. Agar disk diffusion assay

The disk diffusion method was employed to verify the inhibitory effect of EOMA against strain *Listeria monocytogenes* ATCC 7644. The bacterial suspension containing approximately 1.5×10^8 CFU/mL was inoculated on the surface of Mueller Hinton agar (MHA) (Kasvi, Italy) as described by Ben Hsouna et al. (2017). After medium solidification, sterile filter paper disks (Oxoid) 6 mm in diameter soaked in 10 μL EOMA, one disk soaked in sterile deionized water, and another containing the antibiotic meropenem (10 mg, Oxoid) were added. The latter two disks served as negative and positive controls, respectively. The plates were incubated at 37 ± 1 °C for 24 h. After incubation, the diameter of the incubation zones was measures in mm, including the diameter of the disks. The sensitivity of the strain to the oil was classified by the diameter of the inhibition zones according to Ponce et al. (2003) as not sensitive (total diameter under 8 mm), sensitive (total diameter from 9 to 14 mm), very sensitive (total diameter from 15 to 19 mm), and extremely sensitive (total diameter above 20 mm). Each assay was carried out in triplicate with three separate experimental runs.

2.4. Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) was determined according to the methodology of microdilution in flat-bottom 96-well microplates using Muller Hinton broth (MHB) (Kasvi, Italy) and incubation temperature of 37 ± 1 °C for 24 h (Radulović et al., 2013). For the negative control, 100 μL of the medium were added (Mazzarrino et al., 2015) while the positive control used 80 μL of the medium added with 20 μL of the inoculum (approximately 1.5×10^8 CFU/mL). The microplates were prepared so that each well had final volume of 100 μL (Ben Hsouna et al., 2017). To that end, different proportions of MHB and the test substance proportions were used, which varied from 2 to 25 μL, and the suspension (approximately 1.5×10^8 CFU/mL) of the test strain was maintained constant (20 μL). The stock solution was prepared with distilled water using tween 80 (1% v/v; Sigma–Aldrich, USA) as emulsifier (Rodrigues et al., 2018). The MIC was considered the minimum oil concentration able to inhibit visible microbial growth (Carvalho et al., 2015; Miladi et al., 2016; Santos Rodrigues et al., 2017; Silva et al., 2011), while the minimum bactericidal concentration (MBC) was the lowest oil concentration able to kill the inoculum, which was determined through a 20 μL subculture in MHA in each well where visible growth was prevented (Bubonja-Sonje et al., 2011; Silva et al., 2011). Each assay was carried out in triplicate with three independent experimental replicates.

2.5. Death-time curve

An *L. monocytogenes* suspension was prepared at 1.5×10^7 CFU/mL to determine the death-time curve. Based on MIC and MBC values, concentrations of 1 x MBC and 2 × MIC were used (Diao et al., 2014; Mirzaei-najafgholi et al., 2017) in nutrient broth (NB) (Merck, Germany) through the microdilution method followed by incubation at 37 ± 1 °C for 24 h. The controls consisted of adding 20 µL suspension to 80 µL NB for the positive control and, in another well, 100 µL NB for the negative control. At 1, 3, 6, and 24 h (Medeiros Barbosa et al., 2016; Diao et al., 2014), 100 µL aliquots were collected and inoculated in plates containing nutrient agar (NA) (Merck, Germany). These aliquots were incubated at 37 ± 1 °C for 24 h for viable cell count. The analyses were carried out in triplicate and the means were calculated and expressed as log CFU/mL versus time (Bubonja-Sonje et al., 2011). The experiments were repeated three times independently.

2.6. Scanning electron microscopy

Scanning electron microscopy (SEM) was employed to observe the morphological effects of EOMA at the MBC on *L. monocytogenes* strains. A 1.5×10^7 CFU/mL bacterial suspension was prepared and 20 µL were added to the solution containing 15 µL EOMA and 65 µL NB. Next, a 5 × 5 × 1 mm stainless steel plate was inserted and the suspensions were incubated for 3 h at 37 °C. After this period, the plates were removed from the microwells and fixated by immersion in 1% glutaraldehyde in 0.1 M PBS (pH 7.2) for 2.5 h at room temperature (25 °C) in a laminar flow hood. Next, the samples were dehydrated by sequential submersion in a graded ethanol series (50, 70, 80, 95, and 100%) for 20 min at each concentration. Finally, the coupons were submerged in 100% acetone for 5 min. After this process, the samples were kept in a desiccator until being gold coated in an ion sputter coater for 10 min followed by examinations in a scanning electron microscope (Tescan, VEGA 3, Kohoutovice, Czech Republic).

2.7. Preparation of bacterial strains and meat samples

A suspension containing approximately 1.5×10^8 CFU/mL (concentration 1) was prepared in 0.85% saline solution, as described by Carvalho et al. (2015), from which serial dilution was performed. 1 mL aliquots were taken from each dilution series, inoculated in triplicate with trypticase soy agar (Merck, Germany), and incubated for 24 h at 37 ± 1 °C for later count, as described by Pesavento et al. (2015).

The ground beef samples were prepared from a piece of meat whose surface had been removed and discarded. Next, the meat was ground in a previously sterilized food processor.

After grinding, 25 g portions were added in triplicate to Stomacher bags and 1.5% v/w EOMA were added to the test samples. The samples were then homogenized in a stomacher 400 device (Stomacher® 400 Circulator, Seward, Worthing, United Kingdom) for 60 s at room temperature (25 °C) for the oil to be evenly distributed. All samples, including the control, were added with 1 mL of previously standardized microbial suspensions and homogenized again. They were then stored under refrigeration (4 ± 1 °C) for microbiological analysis at times 20 min, 2 days, 4 days, 7 days 11 days, and 14 days after inoculation. Thus, for each time to be analyzed, there existed 12 treated samples and 12 control samples since four suspensions were used with distinct *L. monocytogenes* concentrations. In total, 72 treatment samples and 72 control samples were used.

2.8. Microbiological analysis

In order to assess the viability of the strains and effectiveness of EOMA after treatment, in addition to verifying the positivity of the control (meat inoculated with the suspension with no added oil), method 11290-2, 1998, Amendment 1:2004 by the International Organization for Standardization was employed using Oxford agar medium (Neogen Corporation, Acumedia, USA) whose typical colonies were better defined than Listeria Ottavani & Agosti agar. Furthermore, Gram staining tests were performed to identify the colonies, followed by confirmatory tests using the API system.

2.9. pH

The samples were experimentally contaminated by adding 1 mL of a *L. monocytogenes* suspension at different concentrations as described in Section 2.7. The pH of the non-treated samples (control) and the treated ones (added with 1.5% v/w EOMA) were measured through potentiometry according to the methodology described by the AOAC (AOAC, 1995) using a digital pH meter (Tecnopon, MPA 210). The readings were performed at 20 min and 2, 4, 7, 11, and 14 days.

Acidity was measured from pH as this is a more precise method to indicate the concentration of hydrogen ions, a crucial parameter in the inhibition of microorganism growth (Catarino et al., 2017).

2.10. Statistical analyses

The statistical analyses were carried out in the software Prism version 5.0 (GraphPad, USA) using descriptive statistics (mean and standard deviation).

One-way analysis of variance (ANOVA) was applied to analyze the influence of the treatment in comparison to the control samples in maintaining the pH during the storage period, whereas two-way ANOVA was used to analyze the effect of the different treatments on the death time of the analyzed strains. This test was also applied to evaluate the effect of the treatment on the viability of the strains experimentally inoculated in the meat samples, and their action during the time of storage. The unpaired *t*-test was applied to compare the mean lengths of the treated strains vs. the control.

3. Results and discussion

3.1. Essential oil composition

The chemical composition analysis of EOMA indicated content of 43.1% terpinen-4-ol (Table 1), which is in accordance with the standard of identity and quality established by ISO 4730:2004 (30% to 48%).Several studies have indicated terpinen-4-ol as the main constituent responsible for the antimicrobial activity of EOMA against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) (Cuaron et al., 2013; D'Arrigo et al., 2010; Lee et al., 2013).

Table 1
Chemical composition (%) of the essential oils of *Melaleuca alternifolia* (Tea Tree).

Melaleuca alternifolia	PubChem[b] CID[a]	Composition[c] (%)
Terpinen-4-ol	11,230	43.1
γ-Terpinene	7461	22.8
α-Terpinene	7462	9.3
α-Terpineol	17,100	5.2
Terpinolene	11,463	3.5
α-Pinene	6654	3.0

[a] Compound identification number.
[b] PubChem Structure Search, 2018.
[c] Constituents with percentage < 3 were not mentioned.

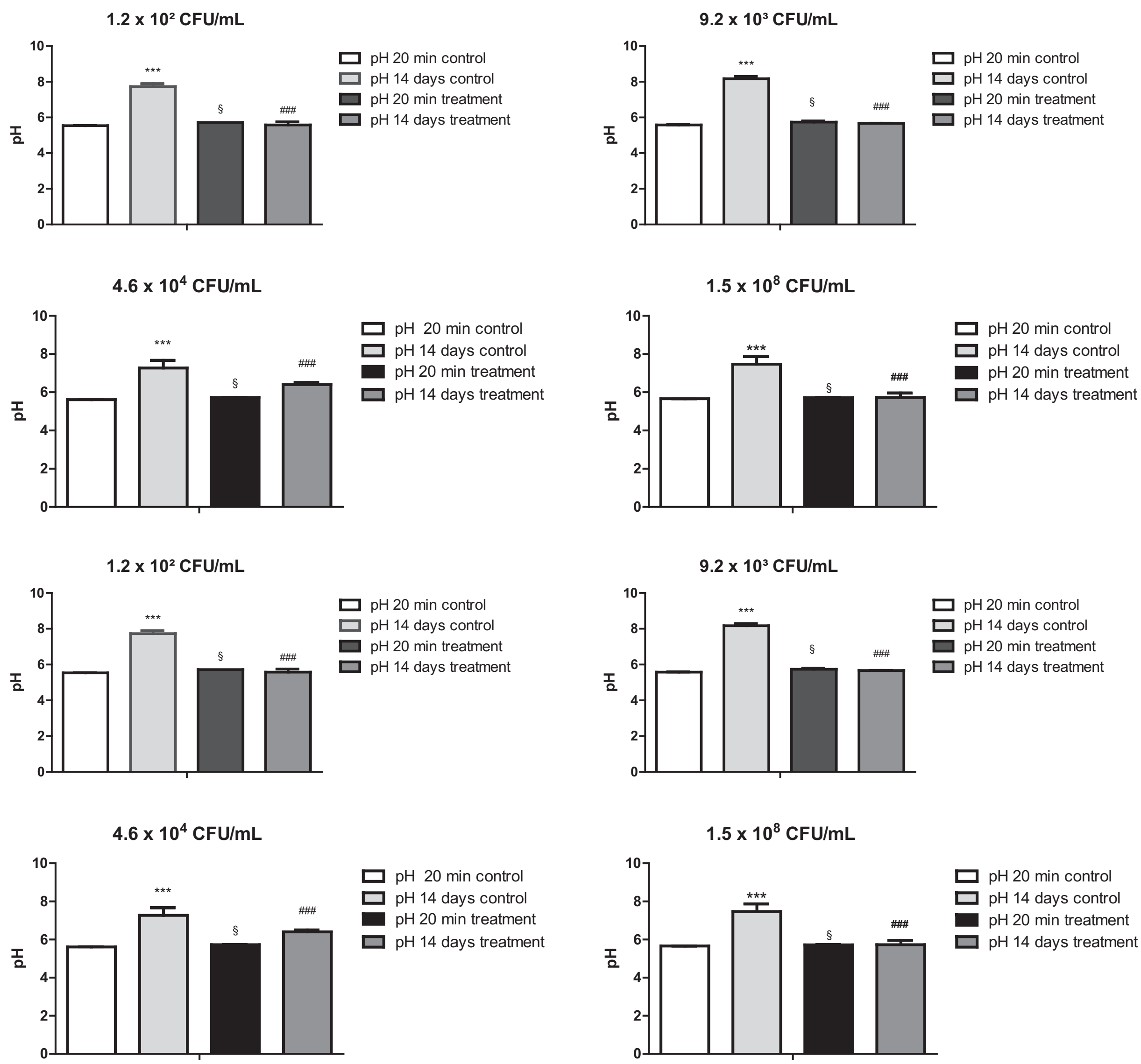

Fig. 1. pH at 20 min and 14 days of storage at 4 °C of ground beef samples experimentally contaminated with *Listeria monocytogenes*, both control and treated with 0.15 µL/mL *Melaleuca alternifolia* (tea tree) essential oil.
one-way ANOVA ($p < 0.0001$), Bonferroni posttest: significant differences - *** Ph 20 min control vs. pH 14 days control; ### pH 14 days control vs. ph 14 days treatment; § pH 14 days control vs. pH 20 min treatment.

3.2. pH

The pH of samples treated with EOMA, regardless of the concentration of the microbial suspension inoculated, did not significantly differ over the storage period studied (20 min to 14 days) (Fig. 1). That indicates that adding EOMA contained the spoilage of ground beef throughout storage. The control sample, in turn, had significant ($p < 0.0001$) increases in pH at all suspension concentrations inoculated (Fig. 1). Such increase can be justified by the breakdown of proteins in the meat to produce free amino acids, which forms alkaline reaction compounds such as NH_3 and amines (Karabagias et al., 2011; Smaoui et al., 2016). The maintenance of pH in the treated samples can be attributed to the elimination of spoilage bacteria since microbial proteases and lipases may increase volatile bases over extended storage and, thus, change pH (Chaijan et al., 2005; Hadian et al., 2017).

3.3. Agar disk diffusion assay

EOMA proved effective by causing an inhibition zone mean of 30 ± 8.8 mm diameter, which classifies *L. monocytogenes* strain ATCC 7644 as extremely sensitive to EOMA. This inhibition zone is larger than the one reported by Mazzarrino et al. (2015), who classified EOMA as having low antimicrobial activity against *L. monocytogenes* strains of different lineages in a similar assay.

However, Abdollahzadeh et al. (2014), when assessing the antimicrobial activity of essential oils from cinnamon, thyme, and rosemary against *L. monocytogenes* strain PTCC 1163, found that thyme oil was more effective than the others.

Son et al. (2017) reported that safflower seed meal extract was had effective antimicrobial activity against *L. monocytogenes* strains ATCC 19115, ATCC 19115, and KCTC 13064. That study used suspension of 10^6 CFU/mL, whose inhibition diameter was 15.91 mm.

Al-Reza et al. (2010), when assessing the antimicrobial activity of oil and organic extracts of *Zizyphus jujube* seeds against four gram-positive strains, among which *L. monocytogenes* ATCC 19166, and five gram-negative ones using suspensions of 10^7 CFU/mL, found that the essential oil had the highest activity against *L. monocytogenes*, yielding inhibition zone diameter of 17.3 ± 0.9 mm, similar to the activity found for tetracycline (17.4 ± 0.7 mm).

The results of such studies highlight the activity of EOMA against *L. monocytogenes*.

3.4. MIC and MBC determination

The microdilution showed that the MIC and MBC values were 0.10 μL/mL and 0.15 μL/mL, respectively. Thus, EOMA had potential against *L. monocytogenes*, which confirms the result found in the test of solid medium diffusion.

The concentrations differed from those found by Mazzarrino et al. (2015), who reported MIC of 10 μL/mL using the same reference strain (ATCC 7644) and EOMA, although the bactericidal activity found was higher than that of antibiotic tetracycline hydrochloride. This result can be related to the higher concentration of terpinen-4-ol (43%) present in the oil used in our work, when compared to the one used by the cited author (38.5%). The activity of essential oils is related to the capacity of destructuring the bacterial cell wall and membrane, thus increasing its permeability through changes in the conformation of some polysaccharides, fatty acids, and phospholipid layer (Gutiérrez-del-Río et al., 2018). Such alterations cause ion leaks, reduction of membrane potential, collapse of proton pumps, and great loss of content, which leads to cell death (Diao et al., 2014; Gutiérrez-del-Río et al., 2018). This effect was confirmed by Patra et al. (2015) using markers to verify how the antimicrobial activity of bio-oil from *Pinus densiflora* works against *Bacillus cereus* (*B. cereus*) and *L. monocytogenes* strains. Damage to the plasma membrane was confirmed by the leakage of electrolytes and K+ ions, reduced osmoregulation capacity under high saline concentration, and morphological alteration verified under SEM.

The use of microdilution to determine CIM and CBM proved efficient, convenient, economic, and easy to read (Bona et al., 2014). Furthermore, the use of this procedure enables reducing the inconvenience of the difficult diffusion of oils into solid media, which might underestimate their antimicrobial action (Bona et al., 2014; Mazzarrino et al., 2015).

3.5. Death-time curve

After 1 h of contact with EOMA, a significant reduction (two-way ANOVA, $p < 0.0001$; Bonferroni posttest, $p < 0.0001$, 1 h: 2 × MIC vs. MBC) was seen in the number of viable cells when treated with the dosage of 2 × MIC. No growth was seen at 3, 6, and 24 h for either dosage (Fig. 2).

Such results corroborate the reports by Bubonja-Sonje et al. (2011), who saw no growth (< 2log10 CFU/mL) when assessing the death-time curve of *L. monocytogenes* strain EGD (isolated from guinea pigs by E.G.D. Murray et al. in 1926) (serotype 1/2a) after 3 h of contact with 0.8 mg/mL polyphenols from olive oil. This effect may be explained by the mechanism of action of phenolic extracts, which is related to the damage to bacterial cell membranes, the inhibition of their enzymes, or the inhibition of the production of essential amino acids for bacterial growth (Bubonja-Sonje et al., 2011). This mechanism is similar to that attributed to essential oils, which can also be compared to lactic acid, known for its bactericidal action. Wang et al. (2015) investigated the action of lactic acid against strains *Salmonella enteritidis* (*S. enteritidis*) ATCC 14028, *L. monocytogenes* ATCC 19115, and *E. coli* CGMCC 1.2385 and found that the bactericidal action was seen in the first minutes of contact of *L. monocytogenes* with 2% lactic acid. However, when the lactic acid concentration was lowered to 0.5%, the death time for this strain was 2 h, which is close to the time found in the present study of 3 h. Rasooli et al. (2006) also reported that, when *L. monocytogenes* strain PTCC 1298 was submitted to 250 ppm thyme essential oil, growth was inhibited 20 min after contact.

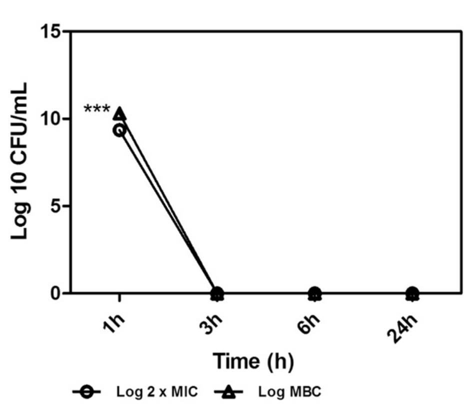

Fig. 2. Death-time curve of *Listeria monocytogenes* (1.5×10^7 CFU/mL) treated with MBC[a] and 2 × MIC[b] of essential oil from *Melaleuca alternifolia* (tea tree). Two-way ANOVA, $p < 0.0001$. Bonferroni posttest $p < 0.0001$, *** 1 h: 2 × MIC vs. MBC.
[a] minimum bactericidal concentration; [b] minimum inhibitory concentration.

The bactericidal effect of EOMA was faster with increasing volume used in the treatment, and proved to be very effective compared with most researches since it shows low MBC when using a suspension at 1.5×10^7 CFU/mL.

3.6. Scanning electron microscopy

The photomicrograph clearly showed the difference in morphology between the control and treated strains. The untreated strains were robust, well-contoured, and measured up to 1.53 μm, whereas the treated ones showed irreversible deformations or reduced size (0.38 to 1.05 μm in length) compared with the control (unpaired *t*-test, p < 0,0007) (Fig. 3). These findings show the toxicity with consequent morphological impact of the oil on these microorganisms.

Similar results were also reported by Wang et al. (2015) when studying the action of lactic acid on *L. monocytogenes* strain ATCC 19115, with the treated cells also being smaller than the control ones.

Rasooli et al. (2006) also found small, thick-walled cells close to one another after contact with a 1/16 dilution of *Thymus x-porlock* essential oil. In this study, very small and very close cells were also detected (Fig. 3). According to Rasooli et al. (2006), this would be a way of the cells organizing in an attempt to survive.

The treated cells had completely wrinkled and misshapen surfaces (Fig. 3 B), which are similar to those found by Liu et al. (2017) when analyzing the antimicrobial effect of bifidocin A against *L. monocytogenes* strain ATCC 35152. This marked deformation could be seen after 3 h of contact with the antimicrobial agent and is related to the loss of cellular material, such as organelles and cytoplasm.

Other studies (Liu et al., 2017; Lü et al., 2014; Yi et al., 2016) have used transmission electron microscopy to find that, within the first hours of contact with the antimicrobial agent, the genetic material condenses, followed by the appearance of pores and loss of cellular content, which, over time, is evidenced by the alteration in morphological structure.

The action of several antibacterial agents, as well as of essential oils, takes place through access to the cytoplasm enabled by the formation of pores in the plasma membrane, which impacts cell integrity and function and leads to its death (Bajpai et al., 2012; M. Li et al., 2016). This is because the plasma membrane acts as a barrier that controls the passage of small ions and of compounds produced by the cell itself and by organelles, thus maintaining cellular functions such as energy status, regulation of membrane and turgor pressure, and other processes (Cox et al., 2001; Patra et al., 2015). Cox et al. (2001) demonstrated this process using EOMA against strains *E. coli* AG100, *S. aureus* NCTC 8325, and *Candida albicans* (*C. albicans*) KEM H5. That study showed potassium ions leaving *E. coli* and *S. aureus* cells immediately at contact and 5 min later, respectively, when using 0.25% v/v oil.

The action of the oil on the plasma membrane has been attributed to

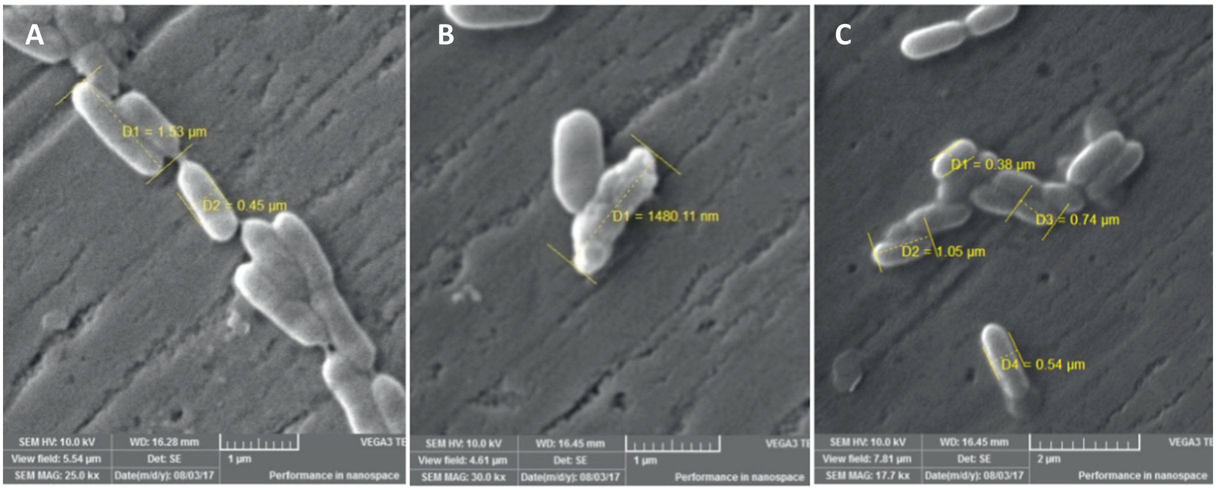

Fig. 3. Scanning electron photomicrograph of *Listeria monocytogenes*. A = control; B = bacteria treated with 0.15 µL/mL of essential oil of *Melaleuca alternifolia* (tea tree), evidencing morphological alteration. C = strain treated, reduction in size compared to control.
Unpaired *t*-test ($p < 0{,}0007$).

its lipophilic nature and to the fact that cyclic monoterpenes have affinity with the aqueous phase inside the membrane, which causes expansion, increases fluidity, and inhibits an enzyme incorporated into the membrane (Sikkema et al., 1995; Gutiérrez-del-Río et al., 2018).

Such data show the mechanism of action and explain the short time for making cells unviable and the structural modifications found in the present study.

3.7. Microbiological analysis

In order to verify the effectiveness of EOMA against the *L. monocytogenes* strain experimentally inoculated into ground beef, the control and treated samples were compared. The samples inoculated with suspension at 1.5×10^8 CFU/mL had high counts (> log 8.17 CFU/mL) of *L. monocytogenes* in the control samples and those treated with 1.5% v/w EOMA throughout storage. The control samples had high counts of *L. monocytogenes* at all concentrations and storage times, thus, count of > log 8.17 CFU/mL was considered for the suspension at 1.5×10^8 CFU/mL; > log 4.66 for suspension at 4.6×10^4 CFU/mL, > log 3.96 CFU/mL for suspension at 9.2×10^3 CFU/mL; and > log 3.06 CFU/mL for suspension at 1.2×10^2 CFU/mL.

The suspensions with concentrations of 1.2×10^2 CFU/mL and 9.2×10^3 CFU/mL showed no growth after the first 20 min, whereas the suspension at 4.6×10^4 CFU/mL caused reduction by 2.41 log CFU/mL in the first 20 min of sample contact with EOMA (ANOVA one-way, $p < 0.0001$).

This matches the report by Abed et al. (2014) when assessing the antimicrobial capacity and preservative effect of 1.25% (v/w) essential oil of *Thymus capitata* against *L. monocytogenes* ATCC 19118. When the bacteria was inoculated in diced beef stored for three days, those authors found a reduction by 4 × log/g in the treated samples compared to the control. A study by Ben Hsouna et al. (2017) tested *Citrus limon* essential oil against fungi and bacteria and *L. monocytogenes* ATCC 19117 in experimentally contaminated meat samples treated with 2 and 3 × MIC/g and stored at 4 °C and found a reduction by 2.5 cycles after four and six days of storage.

Similar results were found by Pesavento et al. (2015) when analyzing the antimicrobial activity of oregano, *Rosmarinus*, and *Thymus* essential oils against *S. aureus* and *L. monocytogenes* in experimentally contaminated beef meatballs. Those authors found a reduction in pathogen viability starting at time zero, i.e., after 2 h of contact with the oil. This is similar to the findings in the present research for suspensions at 4.6×10^4 CFU/mL, 9.2×10^3 CFU/mL, and 1.2×10^2 CFU/mL, with reduction in the number of viable cells in the first 20 min of contact with the oil, which suggests that the essential oil had immediate action after contact with microbial cells (Pesavento et al., 2015). In the case of EOMA, the bactericidal mechanism is related to the compromised cellular membrane integrity in as early as the first minutes of contact, causing loss of cellular material and, therefore, preventing maintenance of homeostasis and inhibiting respiration (Carson et al., 2006; Cox et al., 2001).

However, for samples inoculated with suspension at 1.5×10^8 CFU/mL, EOMA was not effective regardless of storage time. That can be explained by the possible interaction of a percentage of oil with the food matrix, thus reducing its efficiency, as it could be below the minimum concentration required to act against a larger number of colonies inoculated in sample. However, with suspensions starting at 4.6×10^4 CFU/mL, i.e., at lower suspension concentration, the number of viable cells decreases (Fig. 4). That indicates compensation between the interaction of the oil with the matrix and the free volume to act against the smaller number of strains inoculated in the sample, showing similar relation to that evidenced in the death-time curve. The same occurred with the samples inoculated with suspensions at

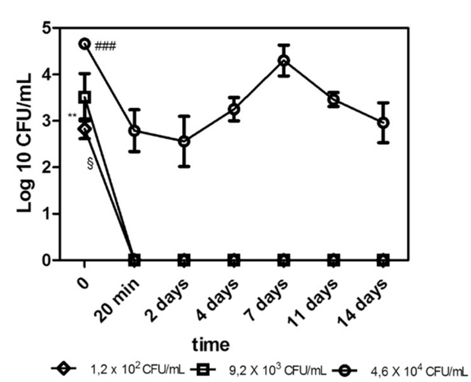

Fig. 4. Antimicrobial activity of essential oil of *Melaleuca alternifolia* (tea tree) in samples of ground beef experimentally contaminated with different suspensions of *Listeria monocytogenes* and different storage times.
Two-way ANOVA ($p < 0.0001$), Bonferroni posttest: significant differences - **time 0: $1{,}2 \times 10^2$ CFU/mL vs. $9{,}2 \times 10^3$ CFU/mL; § time 0 at 14 days: $1{,}2 \times 10^2$ CFU/mL vs. $4{,}6 \times 10^4$ CFU/mL
One-way ANOVA ($p < 0.0001$), Bonferroni posttest: significant differences - ### 4.6×10^4 CFU/mL: time 0 vs. 20 min; time 0 vs. 2 days; time 0 vs. 4 days; time 0 vs. 11 days; time 0 vs. 14 days; 20 min. vs. 7 days; 7 days vs. 14 days.

9.2×10^3 CFU/mL and 1.2×10^2 CFU/mL stored for 20 min, which showed no growth (Fig. 4). Over storage, some treated samples inoculated with the suspension at 4.6×10^4 CFU/mL showed a slight increase in the number of cells followed by their decay (ANOVA one-way, $p < 0.0001$) (Fig. 4). The resurgence may be related to the attempt to develop oil tolerance in a sublethal dose (Liu et al., 2016; Rodríguez-López et al., 2018), which can occur due to mutations in existing genes or can be acquired through new genes horizontally (Allen et al., 2016; Buchanan et al., 2017). Liu et al. (2016) showed that this result may be dose-dependent, when evaluating the effect of the treatment using essential oil of *Melaleuca alternifolia* in different concentrations against pathogenic strains of L. *monocytogenes*. In their study, they found that the secretion of exotoxin proteins listeriolysin O and p60, responsible for the virulence of pathogenic strains of L. *monocytogenes*, varied according to the dose used in the treatment. Despite this growth, the other samples inoculated with suspension at 4.6×10^4 CFU/mL stored for 11 and 14 days had a reduction in the number of CFUs. This late growth inhibition can be explained by exposure to essential oil in a closed system, consequently increasing the time of contact of the strains, which ended up suffering the action of EOMA. This is supported by the results of the SEM.

The growth after the initial reduction in bacterial growth was also observed by Khaleque et al. (2016) when assessing the use of clove and cinnamon essential oils to inactivate *L. monocytogenes* in ground beef. Those authors found that the antimicrobial activity of clove essential oil, both commercial and crude at 10%, inactivated the strains irrespective of temperature right after the three days of storage. However, when 5% crude clove essential oil was used with storage at 0 °C, slight growth was seen after five days.

Pesavento et al. (2015), when analyzing the bactericidal effect of oregano essential oil against *L. monocytogenes* experimentally inoculated in beef meatballs, also found a similar behavior. When the inoculated sample was submitted to 0.0156% oregano essential oil, the number of viable cells slightly increased after 24 h of refrigerated storage, followed by a reduction over storage time.

Another noteworthy piece of data in the present study was the efficiency of EOMA food matrix since the dosage used was not high. Several authors (Hoquea et al., 2008; Khaleque et al., 2016; Mazzarrino et al., 2015) have reported that visualizing food matrix antimicrobial activity requires two-fold concentration or more than the in vitro activity as a way to correct for the loss with the interaction of part of the oil with the food matrix. This need to increase the volume in relation to that determined in vitro is because the proteins and fats present in foods bind to and/or solubilize phenolic compounds, which reduces their antimicrobial activity (Barbosa et al., 2009). Other factors such as water activity, pH, and enzymes may decrease the efficacy of the oil (Firouzi et al., 2007; Friedly et al., 2009). On the other hand, low pH may potentialize the solubility and stability of essential oils, thus contributing to better antimicrobial action. The increase in salt concentration and reduction in storage temperature also contributes to potentializing the action of essential oils (Friedly et al., 2009). Therefore, the result found in the present study may also be related to the low-fat content of the meat used. According to Firouzi et al. (2007), when the food has high content of lipids, they dilute the essential oil, which ends up losing efficiency and efficacy.

Hence, EOMA was effective use in food matrix and matched the findings in the literature.

4. Conclusion

The essential oil of *Melaleuca alternifolia* presented antimicrobial activity in ground beef at the concentration 1.5% v/w against *L. monocytogenes* strain ATCC 7644. The results were promising and created new perspectives for the application of this essential oil as antimicrobial agent in food matrices, and in the possible control of other foodborne pathogens.

Declarations of conflict of interest

None.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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