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Single and binary applications of essential oils effectively control *Listeria monocytogenes* biofilms

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ABSTRACT

The high environmental impact and the increasing resistance of microorganisms to conventional disinfectants applied in the food industry have led to an increasing interest in the antimicrobial properties of plant essential oils (EOs). The present study aimed to develop highly effective EO-based treatments against *Listeria monocytogenes* biofilms formed on stainless steel and polystyrene surfaces under food-related conditions. EOs with an efficacy comparatively higher or similar to peracetic acid and sodium hypochlorite against *L. monocytogenes* planktonic cells were selected, including for the first time *Cordia verbenacea* and *Pimenta pseudochariophyllus* oils. Selected EOs were subsequently characterized chemically by GC/MS analysis and applied in single treatments, binary combinations and combinations with peracetic acid to evaluate their efficacy against 24-h-old *L. monocytogenes* biofilms. *Lippia sidoides, Thymus vulgaris* and *Pimenta pseudochariophyllus* oils were highly effective against planktonic cells and biofilms of *L. monocytogenes*. Thymol was the main compound of *Lippia sidoides* and *Thymus vulgaris* oils, whereas *Pimenta pseudochariophyllus* contained high concentrations of chavibetol. However, only *Lippia sidoides* oil was able to completely eradicate *L. monocytogenes* biofilms at relatively high doses (2.75% v/v). The application of selected EOs in binary combinations decreased considerably doses required to kill 99.99% of biofilm cells. Moreover, the application of peracetic acid combined with these EOs enhanced its efficacy against *L. monocytogenes* biofilms. Therefore, EO-based treatments proposed in this study, particularly those combining *L. sidoides* with *T. vulgaris* or peracetic acid, could represent an effective and environmentally-friendly strategy to control *L. monocytogenes* biofilms in food-processing environments.

1. Introduction

Listeria monocytogenes is a ubiquitous bacteria responsible of food-borne outbreaks with high rates of hospitalization and mortality (CDC, 2016; EFSA, 2016). This pathogen can cause from a febrile gastro-enteritis to a severe invasive illness (listeriosis), particularly affecting to pregnant women, the elderly and immunocompromised persons (Charlier et al., 2017; Vázquez-Boland et al., 2001). In the food industry, *L. monocytogenes* showed a long-term persistence ability on floors, drains and equipment of different food-processing facilities (Carpentier and Cerf, 2011; Ferreira et al., 2014; Jami et al., 2014). This persistence is mainly due to its high tolerance to food-related conditions that are stressful for other bacteria (e.g. desiccation, heat, high salt content, chilled temperatures), and its biofilm-forming ability on different surfaces (Doijad et al., 2015; Doyle et al., 2001; Gardan et al., 2003; Moorhead and Dykes, 2004; Møretrø and Langsrud, 2004; Zoz et al., 2017). Moreover, the application of ineffective sanitizing procedures in food-processing facilities can also increase the risk of persistence of *L. monocytogenes* and, thus, generate a continuous contamination of food products (Chmielewski and Frank, 2003; Martín et al., 2014).

Most disinfectants used nowadays in the food industry for the removal of food spoilage and pathogenic bacteria have a high environmental impact, are corrosive to metal surfaces or harmful for workers (Stanga, 2010; Zabala et al., 2011). In addition, the emergence of biocide resistances in the food industry, mainly due to the application of sub-lethal doses or short exposure times (Langsrud et al., 2003; Morente et al., 2013), have led to an increasing interest in the antimicrobial properties of plant essential oils (EOs). In fact, several EOs have demonstrated a high performance to control *L. monocytogenes* biofilms (Desai et al., 2012; Jadhav et al., 2013; Leonard et al., 2010; Nostro et al., 2016; Oliveira et al., 2010; Sandasi et al., 2010). The

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antimicrobial activity of EOs was related to the presence of high concentrations of aldehydes or phenols, followed by terpene alcohols (Bassolé and Juliani, 2012; Perricone et al., 2015). In contrast, EOs mainly composed by ketones or esters have shown a lower effectiveness, whereas those containing high amounts of terpene hydrocarbons have been commonly inactive. Nevertheless, the antimicrobial activity of EOs also depends on the interaction between the major and the minor constituents, resulting in synergistic, additive and antagonistic effects (Bassolé and Juliani, 2012). Interestingly, EO compounds with antimicrobial properties have shown diverse modes of action affecting different cell targets (Calo et al., 2015; Hyldgaard et al., 2012; Nazzaro et al., 2013), which can avoid (or at least reduce) the appearance of biocide resistances.

Therefore, the present study aimed to distinguish EO-based treatments highly effective for the removal of *L. monocytogenes* biofilms from stainless steel and polystyrene surfaces, two materials commonly present in the food industry. With this aim, the antimicrobial activity of eleven EOs were firstly evaluated against *L. monocytogenes* planktonic cells, including for the first time *Cordia verbenacea* and *Pimenta pseudochariophyllus* oils. Subsequently, the most effective EOs were then characterized chemically and evaluated against *L. monocytogenes* biofilms in single applications, binary combinations and combinations with peracetic acid.

2. Material and methods

2.1. Bacterial strains and culture conditions

Two *L. monocytogenes* strains (L2 and L8) persistent in fish-processing facilities were investigated. They have been previously identified as *Listeria* by genus-specific *prs* gene, classified in the *L. monocytogenes* serogroups I (L2) and II (L8) and genotyped as different strains by RAPD-PCR (Vázquez-Sánchez et al., 2017). In addition, these strains showed a higher biofilm-forming ability on stainless steel (L2) and polystyrene surfaces (L8) than the reference strain *L. monocytogenes* ATCC 15313. Bacterial stocks of each strain were maintained at −80 °C in tryptic soy broth (TSB) (Kasvi, Brazil) containing 20% glycerol (v/v). Strains were thawed and sub-cultured twice in TSB at 37 °C for 24 h under static conditions prior to each experiment.

2.2. Antimicrobial assays

2.2.1. Antimicrobial agents

Pure essential oils (EOs) previously extracted by Ambrosio et al. (2017) from leaves and branches of *Cordia verbenacea*, *Corymbia citriodora*, *Cymbopogon winterianus*, *Eucalyptus camaldulensis*, *Eucalyptus staigeriana*, *Eucalyptus urograndis*, *Lippia sidoides*, *Melaleuca alternifolia*, *Melaleuca leucadendron*, *Pimenta pseudochariophyllus* and *Thymus vulgaris* were investigated. Working concentrations of all EOs were prepared before each assay in ultrapure water with 0.15% (w/v) bacteriological agar (Kasvi) as stabilizing agent (Remmal et al., 1993). Peracetic acid (39% v/v) and sodium hypochlorite (Sigma-Aldrich, Brazil) were also evaluated as examples of industrial disinfectants, being diluted in ultrapure water.

2.2.2. Minimal bactericidal concentration (MBC)

The MBC of disinfectants against planktonic cells of *L. monocytogenes* strains was assessed as the lowest concentration at which no viable cells was detected under experimental conditions. A method previously optimized was followed (Vázquez-Sánchez et al., 2017). Planktonic cells were exposed to final concentrations of 0.010%, 0.025%, 0.050%, 0.075%, 0.100%, 0.500%, 1%, 2%, 3% and 4% (v/v) of each disinfectant, being tested in triplicate in two independent experiments. Positive controls (inoculums treated with sterile distilled water), negative controls (disinfectants applied to wells containing only TSB) and blanks (with only TSB) were also included.

2.2.3. Logarithmic reduction of viable biofilm cells (LR)

2.2.3.1. Single treatments. The efficacy of peracetic acid, sodium hypochlorite and the three EOs with the highest efficacy against planktonic cells against *L. monocytogenes* biofilms was determined in terms of logarithmic reduction of viable biofilm cells (LR). Biofilms formed on stainless steel and polystyrene surfaces for 24 h at 25 °C were exposed for 30 min to single applications of each disinfectant, following the methodology described in Vázquez-Sánchez et al. (2017). Doses of 0.10%, 0.50%, 1%, 1.25%, 1.5%, 1.75%, 2%, 2.25%, 2.5%, 2.75% and 3% (v/v) of each disinfectant were tested in triplicate in two independent assays, including also positive controls (i.e. biofilms exposed to sterile ultrapure water). The LR was calculated as follows:

$$LR = \log(VC) - \log(SC)$$

where VC is the number of viable cells in non-treated biofilms and SC the number of surviving viable cells in disinfected biofilms.

2.2.3.2. Combined treatments. The LR caused by binary combinations of selected EOs, as well as combinations of these EOs with peracetic acid, on 24-h-old *L. monocytogenes* biofilms was also determined. Second order rotatable factorial designs (Box et al., 1989), with the concentrations of disinfectant as independent variables (Table 1), were used. Experimental conditions were similar to that used in the single treatments (i.e. 30 min of exposure to 0.5 mL of the blend of disinfectants and 10 min of neutralization). After calculation of the LR values, models were obtained by means of a least-squares method.

2.3. Chemical composition of the selected essential oils

The chemical composition of the EOs with the highest efficacy against *L. monocytogenes* biofilms and planktonic cells was determined by gas chromatography/mass spectrometry (GC/MS) analysis, using a GC/MS QP2010 Plus (Shimadzu, Japan) equipped with a capillary column Rtx–5MS (30 m × 0.25 mm ID × 0.24 film thickness, 5% diphenyl and 95% dimethyl-polysiloxane; RESTEK, USA). The oven temperature was held at 50 °C for 1.5 min, then raised to 200 °C at 4 °C/min and finally to 240 °C at 10 °C/min, being kept this temperature for 7 min. The temperature of injector was 240 °C and 220 °C for the interface. Aliquots of 1 μL of samples diluted in *n*-hexane were injected in the "split" mode at a ratio of 1:200. A constant flow rate of 1.2 mL/min of helium was used. The mass detector operated in scan mode in the range of 40–500 *m/z*. The components of EOs were identified by comparing their Linear Retention Index (LRI) and their mass spectra with those of Adams (2007), NIST 07 or Wiley 8 libraries.

2.4. Statistical analysis

Statistics were performed in IBM SPSS 19.0. A one-way ANOVA determined the data significance. Homogeneity of variances was

Table 1
Experimental domain and codification of independent variables in factorial designs.

Coded values[a]		Natural values (% v/v)	
Disinfectant 1	Disinfectant 2	Disinfectant 1	Disinfectant 2
1	1	1.25	1.25
1	−1	1.25	0.25
−1	1	0.25	1.25
−1	−1	0.25	0.25
1.41	0	1.46	0.75
−1.41	0	0.04	0.75
0	1.41	0.75	1.46
0	−1.41	0.75	0.04
0	0	0.75	0.75

[a] Five replicates were used in the center of the domain, instead of three.