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Nanoemulsions of *Mentha piperita* L. essential oil in combination with mild heat, pulsed electric fields (PEF) and high hydrostatic pressure (HHP) as an alternative to inactivate *Escherichia coli* O157:H7 in fruit juices

Running title: Nanoemulsions of peppermint oil in combined treatments.

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Abstract

This study was performed in order to obtain and characterize nanoemulsions of *Mentha piperita* L. essential oil (n-MPEO) to assess its efficacy in combination with mild heat (MHT) (50, 52, 54ºC; 10 min), pulsed electric fields (PEF) (20, 25, 30 kV/cm; 150 μs), and high hydrostatic pressure (HHP) (150, 200, 300 MPa; 15 min) treatments in causing a 5- $\log_{10}$ reduction in survival counts (from 7 to < 2 $\log_{10}$ CFU/mL) of *Escherichia coli* O157:H7 in guava and mango juices. The droplets of n-MPEO were < 200 nm and showed good stability for 4 months at 4 ºC. The n-MPEO at 5.0 μL/mL displayed a more efficacious long-term antimicrobial activity than suspensions of MPEO (s-MPEO). Combined treatments of s-MPEO or n-MPEO and MHT, PEF, or HHP acted synergistically against *E. coli*. Nevertheless, combined treatments with n-MPEO showed the same or even higher efficacy than those with s-MPEO (up to 1- additional $\log_{10}$ reduction). Thus, by using nanoemulsions, lower doses of antimicrobial compounds (up to 4 times) or milder MHT (up to 4ºC), PEF (up to 5 kV/cm) and HHP (up to 100 MPa) treatments can be applied, while still guaranteeing the microbial safety of tropical fruit juices.

Industrial relevance

The exploration of a series of different delivery systems of antimicrobial compounds in food products improves their antimicrobial efficacy and aids in the establishment of successful combined treatments for food preservation. The preparation of nanoemulsions of MPEO not only enhances the compound's stability, but also its antimicrobial efficacy (20% reduction in treatment time as compared with not emulsified MPEO). Valuable synergistic effects that can be observed when combining n-MPEO with MHT, PEF, or HHP (up to 2.5- additional $\log_{10}$ reductions) reveal alternatives to traditional treatments

that are successful because they help reduce treatment intensity, thereby helping to avoid adverse effects on juice quality without compromising food safety.

Keywords: natural antimicrobials; essential oils; nanoemulsions; emerging technologies; food preservation; tropical fruit juices

1. Introduction

The consumption of tropical fruit juices has been increasing worldwide, mainly because consumers are demanding healthy, fresh products that associate nutritional value with convenience (Neves, Trombin, Lopes, Kalaki, & Milan, 2011; Pereira et al., 2015; Petruzzi et al., 2017). Despite the intrinsic low pH of fresh fruit juices, they have been increasingly linked with food outbreaks (Callejón et al., 2015). *Escherichia coli* O157:H7 has been cited as a pathogen frequently involved in fruit juice outbreaks worldwide (CDCP, 2016; Choi et al., 2012; EFSA, 2015). It can survive in acidic foods and beverages such as fruit juices because of its acid adaptability and its tolerance to certain organic acids (Lee, Kim, & Kang, 2015). The US Food and Drug Administration has identified *E. coli* O157:H7 as the most suitable pathogen for the evaluation of safety in fruit juices, and requires procedures that are capable of reducing at least 5 $\log_{10}$ cycles in counts of this pathogen in the final product (FDA, 2001).

The use of plant essential oils (EOs) has been suggested as a technology that has promising potential for fruit juice preservation, particularly in view of consumers' increased wariness of chemical preservatives (e.g., sodium benzoate and potassium sorbate) (Basak, 2018; Seow, Yeo, Chung, & Yuket, 2014). Moreover, the high temperatures (72 to 82 °C for 0.3 to 15 seconds), usually applied to preserve fruit juices (FDA, 2004) cause undesirable changes in several quality parameters such as flavor and color. High temperatures also destroy

heat-sensitive nutritional components (e.g. vitamins), thereby compromising product freshness (Hu, Zhou, Xu, Zhang, & Liaoet, 2013).

The main obstacle impeding the use of EOs in fruit juices is their strong flavor (Macwan, Dabhi, Aparnathi, & Prajapati, 2016; Souza, Almeida, & Sousa Guedes, 2016). To attain the desired antimicrobial effects, the required EO concentration may cause undesired sensory changes that reach the rejection threshold of consumers (Hyldgaard, Mygind, & Meyeret, 2012). However, by combining EOs with traditional technologies, such as mild heat treatment (MHT), or emerging technologies, such as pulsed electric fields (PEF) or high hydrostatic pressure (HHP), antimicrobial efficacy can be achieved at lower EO concentrations: this is thus a recommendable strategy for the prevention of undesirable taste effects (Tyagi, A., Gottardi, D., Malik, A. & Guerzoni, M. E., 2013; Cherrat, Espina, Bakkali, Pagán, & Laglaoui, 2014). MHT can enhance the antimicrobial efficacy of EOs because it exerts an influence on the vapor phase formation of their volatiles, and thereby improves their solubilization in the cell membrane (Burt, 2004). Otherwise, PEF and HHP induce structural changes in the cell membrane via the formation of pores which, in turn, favor the diffusion of the EOs into the cell, resulting in damage to vital cell functions (Baptista, Rocha, Cunha, Saraiva, & Almeida, 2016; Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Bellosoet, 2008).

It has been suggested, however, that the high reactivity and hydrophobicity of EOs represents a challenge to their direct incorporation in food and beverage products (Donsì & Ferrari, 2016). Therefore, new delivery systems designed to encapsulate and release EOs in food products, such as emulsion-based delivery systems based on emulsion phase inversion (EPI), could improve the dispersion of EOs into food products, minimize the phase separation, and thereby enhance their antimicrobial properties. Moreover, this methodology could be of great interest due to ease of implementation, lower equipment and operation costs, and higher energy efficiency (Komaiko & McClements, 2016). In this regard, Pagán, Berdejo, Espina,

García-Gonzalo, and Pagán (2018) have assayed the antimicrobial effect of EPI nanoemulsions of citral in combination with heat or PEF in laboratory media, obtaining unexpected results: while nanoemulsions of citral were more effective when inhibiting or inactivating *E. coli* O157:H7 than citral used in form of suspensions, nanoemulsions did not offer any advantage when combined with mild heat or PEF.

Mentha piperita L. essential oil (MPEO) possesses strong activity against fruit-related pathogens and spoilers (Sousa Guedes et al., 2016). An earlier study reported that it shows good efficacy when applied in juices (Sousa Guedes et al., 2016); however, the doses required were relatively high, which might affect the juices' sensory characteristics. No previous studies have explored the potential of applying reduced doses of MPEO in the form of nanoemulsions combined with MHT, PEF, and HHP to preserve juices.

Therefore, the aims of this study were i) to obtain and characterize EPI nanoemulsions of MPEO (n-MPEO) in terms of stability and reproducibility and, ii) to assess the efficacy of MPEO as a function of the MPEO preparation procedure: n-MPEO *vs*. the use of a simple vigorous shaking method by vortex agitation (suspension of MPEO; s-MPEO), each applied as a single hurdle or in combination with MHT, PEF and HHP, to cause ≥ 5- $\log_{10}$ reduction in survival counts of *E. coli* O157:H7 in guava and mango juices.

2. Materials and methods

2.1. Microorganism and growth conditions

Escherichia coli O157:H7 (VTEC - phage type 34) isolated from cattle by Dr. P. A. Chapman (Chapman et al., 1993) was used as test strain in the present study. The stock culture was maintained at − 80 °C in cryovials. Broth subcultures were prepared by inoculating one single colony from a plate into a test tube containing 5 mL of sterile tryptic soy broth (Biolife, Milan, Italy) with 0.6% yeast extract added (Biolife) (TSBYE). After

inoculation, the tubes were incubated under agitation (130 rpm; Selecta, mod. Rotabit, Barcelona, Spain) at 37 °C for 18 h to reach stationary growth phase. Thus, cells were harvested through centrifugation (4500 g x 15 min, 4 °C), washed twice with sterile saline solution (0.85 % NaCl), and re-suspended in TSBYE to obtain standard cell suspensions with an optical density (OD) at 625 nm (OD_{625}) of 0.09 that provided viable counts of approximately 8 $\log_{10}$ colony-forming units per milliliter (CFU/mL) (Leite et al., 2016). Prior to experiments, cells were re-centrifuged and re-suspended at a concentration of 7 $\log_{10}$ CFU/mL in fruit juices with or without MPEO. This initial bacterial concentration was chosen in order to provide a number of viable cells that allow to measure ≥ 5- $\log_{10}$ reduction of the test pathogen under the assayed conditions.

2.2. Fruit juices

The guava (*Psidium guajava* L.) and mango (*Mangifera indica* L.) fruits were purchased from Fruits CMR, S.A. (Barcelona, Spain) in the commercial maturation stage and selected for similar shape and uniform color, with absence of mechanical damages and no visible signs of infection. The fruits were surface-disinfected through immersion in a sodium hypochlorite solution (150 ppm, pH 7.2 adjusted using 1 M NaOH) for 5 min, washed with sterile distilled water, and dried for 30 min in a biosafety cabinet. Subsequently, the fruits were aseptically peeled, cut into small pieces and crushed using a food processor (Robot-Coupe, Blixer 6 V.V., Burgundy, France). The fruit pulps were sealed and stored at − 18 °C in 100 g-polypropylene bags. When required for assays, the fruit juices were prepared by mixing 100 g of the fruit pulp with distilled water (1:1 ratio) using a domestic blender (for 3 min). The obtained juices were centrifuged (12,500 g x 15 min, 4 °C) to separate the pulp from the remaining liquid. The supernatants were sterilized by autoclaving (121 °C, for 15 min) and

used for subsequent assays (Sousa Guedes et al., 2016). The final pH of guava and mango juices used in these experiments was 3.9 and 4.5, respectively.

2.3. MPEO, direct addition procedure and preparation of nanoemulsions

The MPEO was purchased from Ferquima Ind. Com. Ltd. (São Paulo, Brazil). According to the company, the MPEO was extracted through steam distillation (batch 187; density at 20 °C, 0.900; refractive index at 20 °C, 1.460; 40.41% of menthol, 19.78% of isomenthone, 8.68% of neomenthol, 6.72 of menthyl acetate and 6.53% of eucalyptol). A vigorous shaking method (Friedman, Henika, & Mandrellet, 2002) was used to prepare MPEO suspensions (s-MPEO) in guava and mango juices.

Nanoemulsions of MPEO (n-MPEO) were prepared by applying the catastrophic phase inversion method (Zhang, Zhang, Fang, & Liu, 2017), also known as the emulsion phase inversion (EPI) method. The n-MPEO were prepared from a mixture of oily phase by slowly adding aqueous phase with gentle magnetic agitation (1000 rpm). The aqueous phase was prepared by mixing 1.5 mL of ethanol (Sigma–Aldrich, USA) with 40.5 mL of sterile distilled water (Pagán, Berdejo, Espina, García-Gonzalo, & Pagán, 2018). The oily phase was prepared by mixing 3 mL of Tween 80 (Panreac, Barcelona, Spain) with 5 mL of MPEO. The addition rate of aqueous phase was kept constant at approximately 1.0 mL/min by using a burette. A water-in-oil (W/O) emulsion with a high oil-to-water ratio was formed; increasing amounts of water were subsequently added to the system with continuous stirring. The amount of water added to a W/O emulsion was progressively increased until a phase inversion occurred and an oil-in-water (O/W) emulsion was formed. Final concentration of MPEO in the nanoemulsion was 90 g/L.

2.4. Characterization, stability and reproducibility of n-MPEO

The nanoemulsions (n-MPEO) were characterized for droplet size and size distribution (polydispersity index-PDI) using a particle size analyzer (Brookhaven, 90 Plus, New York, USA). Droplet size was analyzed with the dynamic light scattering (DLS) technique. Prior to the experiments, the n-MPEO were diluted with water to eliminate multiple scattering effects. Emulsion droplet size was estimated by an average of three measurements and is presented as the mean diameter of volume distribution. Droplet size was evaluated after fresh preparation, and then after 1 and 4 months of storage at 4 ºC. The reproducibility of the protocol for preparing the n-MPEO and their stability during 30 days was also evaluated by comparing the inactivation curves of *E. coli* O157:H7 (obtained weekly) in the presence of n-MPEO. Cells from stationary-phase cultures were added at final concentrations of 7 $\log_{10}$ CFU/mL in mango juice with 5 µL/mL of n-MPEO. The different systems were gently hand-shaken for 30 s and maintained at room temperature (23 ± 2 ºC). Samples were taken at preset intervals and survivors were enumerated as described below.

2.5. Evaluation of the antimicrobial activity of s-MPEO and n-MPEO

MPEO (s-MPEO and n-MPEO) were evaluated to determine the minimum inhibitory concentration (MIC), and to obtain survival curves of *E. coli* O157:H7. The MIC values were determined using a microdilution in broth assay (CLSI, 2015). Approximately 50-µL aliquots of TSAYE with different concentrations of s-MPEO or n-MPEO (0.312 - 20 µL/mL) were dispensed into each well of a 96-well microplate. Subsequently, 50 µL aliquots of bacterial suspension were added to each well (final viable counts of 6 $\log_{10}$ CFU/mL). Negative control (growth media non-inoculated with the test pathogen), positive control (growth media inoculated with the test pathogen), and diluent control (the amount of ethanol corresponding to the maximum n-MPEO concentration assayed − 20 µL/mL) were also prepared. The microplate was loosely wrapped with cling wrap to prevent bacterial dehydration and s-

MPEO or n-MPEO volatilization, and incubated at 37 °C for 24 h. The MIC was confirmed as the lowest s-MPEO or n-MPEO concentration capable of inhibiting visible bacterial growth (Carvalho et al., 2015).

To assess the inactivating effect of n-MPEO and s-MPEO against *E. coli* O157:H7 in mango and guava juices, cells from stationary-phase cultures were added at final concentrations of 7 $\log_{10}$ CFU/mL in guava and mango juices with n-MPEO and s-MPEO MIC values (5 µL/mL). The different systems were gently hand-shaken for 30 s and maintained at room temperature (23 ± 2 ºC). At intervals of 0 (just after homogenization), 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min of treatment, samples were taken and survivors were enumerated.

2.6. Application of combined preservation processes: MHT, PEF or HPP and MPEO

2.6.1 MHT and MPEO

Cell inactivation of *E. coli* O157:H7 caused by MPEO in combination with MHT (50, 52 and 54 °C) after 10 min of exposure was assessed. Exposure time intervals and temperature were selected based on results of previous studies featuring the combination of EOs and MHT to preserve juices (Espina et al., 2011; 2012). The concentrations 0.16, 0.31, and 0.63 µL/mL of MPEO selected for this study lay below the rejection threshold in sensory tests (unpublished results). Aliquots of 5 mL of each cell suspension prepared in guava and mango juices with and without MPEO were placed in a shaking bath thermostated at 50, 52 and 54 ± 0.2 °C (140 rpm; model 1620; Bunsen, mod. BTG, Madrid, Spain). The MPEO was added once the treatment media was tempered, prior to microbial inoculation. After treatment, cell suspensions were extracted, immediately placed on ice, and survivors were evaluated.

2.6.2. PEF treatments and MPEO

PEF treatments were carried out using ScandiNova equipment (Modulator PG, ScandiNova, Uppsala, Sweden) described by Saldaña, Puértolas, Condón, Álvarez, and Raso (2010). An aliquot of 0.5 mL of the microbial suspensions prepared in guava and mango juices (electrical conductivity of 1.9 and 1.2 mS/cm, respectively) with and without s-MPEO or n-MPEO (0.16, 0.31 and 0.63 µL/mL) was placed into the treatment chamber with a sterile syringe. Exponential waveform pulses at an electrical field strength of 20, 25 and 30 kV/cm and a pulse repetition rate of 1 Hz were used in this study. The specific energy input of each pulse was 2.3 kJ/kg, 3.6 kJ/kg and 5.1 kJ/kg in guava juice, and 1.4 kJ/kg, 2.3 kJ/kg and kJ/kg in mango juice, respectively. Cell suspensions were treated for 50 pulses (pulse width 3 µs). Experiments began at room temperature (23 ± 2 ºC), and in all experiments the temperature of the samples after the application of 50 pulses was lower than 35 ºC. After treatment, samples were taken, and survivors were evaluated.

2.6.3. HHP treatments and MPEO

HHP treatments were carried out following the procedure described by Espina, García-Gonzalo, Laglaoui, Mackey, and Pagán (2013). Cell suspensions (1 mL each) prepared in guava and mango juices with and without s-MPEO or n-MPEO (0.16, 0.31 and 0.63 µL/mL) were placed in sterile plastic pouches that were heat-sealed and kept on ice before pressurization. Cell suspensions were pressure-treated in a 300-mL pressure vessel (model S-FL-085-9-W; Stansted Fluid Power, Stansted, United Kingdom) at room temperature (23 ± 2 °C). The pressure-transmitting fluid was monopropylene glycol-water (30:70). Cell suspensions were exposed to pressures of 150, 200, and 300 MPa for 15 min. The come-up time to 300 MPa was less than 10 s and the maximum temperature reached during pressurization was 30 °C. After decompression, the pouches were removed from the unit and placed on ice until survivors were evaluated.

2.7. Counts of viable cells

After treatment, samples were adequately diluted in 0.1% w/v peptone water (Biolife). Subsequently, 20 µL-aliquots of each dilution were inoculated onto Tryptic Soy Agar (Biolife) with 0.6% Yeast Extract added (Biolife) (TSAYE) using the microdrop technique (Herigstad, Hamilton, & Heersinket, 2001). Inoculated control samples without s-MPEO or n-MPEO were similarly assayed. The plates were incubated at 37 °C for 24 h. Previous experiments showed that longer incubation times did not influence the surviving cell counts. The results were expressed as $\log_{10}$ CFU/mL. The detection limit was 2 $\log_{10}$ CFU/mL for all experiments and strains tested.

2.8. Statistical analysis

Statistical analyses were performed to determine significant differences ($p \leq 0.05$) using ANOVA, followed by post-hoc Tukey's test or Student's t-test. For MIC determination assays, the results are expressed as modal values because the MIC values were the same in all repetitions. The error bars in the figures indicate the mean ± standard deviation from the data obtained from at least three independent experiments. All statistical analyses were performed using Sigma Stat 3.5 computer software (Jandel Scientific Software, San Jose, California).

3. Results

3.1 Characterization, stability and reproducibility of n-MPEO

In the nanoemulsions (n-MPEO), droplet size remained below 200 nm during all 4 months of storage, and the PDI values were normally ≤ 0.32 (Table 1). No significant differences ($p \leq 0.05$) were observed between the inactivation of *E. coli* O157:H7 in mango juice induced by the fresh preparations of n-MPEO (5 µL/mL) and by n-MPEO stored at 4 ºC

up to 4 months (Fig. 1), which indicates that the EPI method assayed allows the obtainment of reproducible and stable nanoemulsions of MPEO.

3.2. Evaluation of the antimicrobial activity of s-MPEO and n-MPEO

The MIC value of n-MPEO or s-MPEO against *E. coli* O157:H7 was 5.0 µL/mL. This value was used in an experiment conducted to compare the effect of the addition of n-MPEO and s-MPEO in the inactivation of 5 $\log_{10}$ of *E. coli* O157:H7 in mango and guava juices. Although the MIC value was similar for n-MPEOs and s-MPEOs, the study of cell inactivation indicated that the n-MPEOs showed a more efficacious long term antimicrobial activity compared to the s-MPEOs (Fig. 2): while the inactivation of 5-$\log_{10}$ of *E. coli* O157:H7 in guava and mango juices using 5.0 µL/mL of s-MPEO required 90 and 100 min respectively, in the presence of n-MPEO it was achieved after 70 and 90 min, respectively (20 min reduction).

3.3. Application of combined preservation processes: MHT, PEF, or HPP

Figures 3, 4 and 5 show the $\log_{10}$ cycles of inactivation of *E. coli* O157:H7 in guava and mango juices after different combined preservation treatments based on the simultaneous application of MHT (Fig. 3), PEF (Fig. 4), or HHP (Fig. 5) and s- or n-MPEO at three different concentrations (0.16, 0.31 and 0.63 µL/mL). As a control, the figures include the degree of inactivation achieved by MHT, PEF, or HHP acting as single hurdles (0). Due to the insignificant inactivation caused by s- or n-MPEO applied directly at the highest concentration tested (0.63 µL/mL) (data not shown), the comparison of the degree of inactivation achieved by MHT, PEF, or HHP acting as single hurdles and in combination with s- or n-MPEO allowed us to ascertain lethal synergistic effects associated with a higher degree of inactivation ($p \leq 0.05$) achieved as a result of the combined treatment.

MHT at 54 ºC for 10 min caused a 2.7 $\log_{10}$ and 2.3 $\log_{10}$ reduction of *E. coli* O157:H7 in guava (Fig. 3C) and mango (Fig. 3F) juices, respectively. The higher degree of inactivation observed when combining MHT at 54ºC with s- or n-MPEO in comparison with MHT acting as a single hurdle ($p \leq 0.05$) indicates synergism between the hurdles at any MPEO concentration tested. MHT at 54 ºC combined with 0.16, 0.31, and 0.63 µL/mL of s- and n-MPEO caused a 5 $\log_{10}$ reduction in survival counts of *E. coli* O157:H7 in guava juice (Fig. 5C). In mango juice (Fig. 5F), MHT at 54 ºC combined with 0.31 and 0.63 µL/mL of s- and n-MPEO also caused a 5 $\log_{10}$ reduction in survival counts of *E. coli* O157:H7. However, the combination of MHT at 54 ºC with 0.16 µL/mL of s-MPEO caused an approximately 4 $\log_{10}$ reduction of *E. coli* O157:H7. In order to ensure the desired 5 $\log_{10}$ reduction at 54ºC at the lowest MPEO concentration, it was necessary to use MPEO in the form of nanoemulsion.

In both juices, milder heat treatments at 50 (Fig. 3A and 3D) and 52 °C (Fig. 3B and 3E) for 10 min caused < 1 $\log_{10}$ reduction in survival counts of *E. coli* O157:H7. The degree of inactivation when combining MHT at 50 or 52 ºC with n-MPEO was higher at any concentration tested ($p \leq 0.05$), with the exception of 0.16 µL/mL n-MPEO and 50 ºC in mango juice (Fig. 3D). Overall, the inactivation achieved with n-MPEO instead of s-MPEO was greater, showing significant differences ($p \leq 0.05$) when applying 0.31 and 0.63 µL/mL of s- and n-MPEO in guava and mango juices. Despite the synergistic lethal effects observed at all tested concentrations, combined treatments at temperatures lower than 54ºC did not achieve a 5 $\log_{10}$ reduction of *E. coli* O157:H7.

Results obtained with PEF treatments (Fig. 4) acting as a single agent show an inactivation of 2.3 – 4.2 $\log_{10}$ cycles of *E. coli* O157:H7 as a function of treatment intensity (150 µs at 20, 25 or 30 kV/cm) and of the juice assayed. The combination of PEF and MPEO achieved a 5- $\log_{10}$ reduction of *E. coli* O157:H7 at the highest electrical field strength tested (30 kV/cm) (Fig. 4C and 4F). Nevertheless, that degree of inactivation was achieved by both

s- or n-MPEO in guava juice (Fig. 4C) at all tested MPEO concentrations, but only at the maximum concentrations tested (0.31 and 0.63 μL/mL) in mango juice (Fig. 4F). In the latter, only the use of MPEO in the form of nanoemulsion at the lowest concentration (0.16 μL/mL) allowed us to achieve the desired 5 $\log_{10}$ reduction ($p \leq 0.05$).

Reduction of the electrical field strength to 25 kV/cm also achieved a 5 $\log_{10}$ reduction when combined with n-MPEO in all tested concentrations, and with s-MPEO at the highest concentration (0.63 μL/mL) tested in guava juice (Fig. 4B), and when combining with s- or n-MPEO at 0.63 μL/mL and with n-MPEO at 0.31 μL/mL in mango juice (Fig. 4E). Synergism between the hurdles was clearly noticeable in guava and mango juice at the highest concentration of n-MPEO (0.63 μL/mL) tested ($p \leq 0.05$).

At the lowest electrical field strength (20 kV/cm) (Fig. 4A and 4D), the synergistic effect when combining with MPEO was remarkable, especially with MPEO in the form of nanoemulsion ($p \leq 0.05$) and with s-MPEO at the highest concentrations tested: 0.63 μL/mL in guava juice, and 0.31 and 0.63 μL/mL in mango juice ($p \leq 0.05$). The combined use of PEF at 20 kV/cm and 0.16 and 0.31 μL/mL of s-MPEO caused a degree of inactivation similar to that achieved by PEF treatment acting as a single hurdle (3 $\log_{10}$ reduction approx. in survival counts of *E. coli* O157:H7); however, the combination of PEF and 0.16 and 0.31 μL/mL of n-MPEO helped to achieve 1 extra $\log_{10}$ reduction ($p \leq 0.05$) (around 4 $\log_{10}$ reduction in both juices). The synergism was at its maximum at the highest tested concentration (0.63 μL/mL) of n-MPEO, inducing the inactivation of more than 2 extra $\log_{10}$ cycles in guava (Fig. 4A) and mango juices (Fig. 4D) ($p \leq 0.05$) .

Regarding HHP treatments, the results show that 300 MPa for 15 min applied as a single hurdle caused a 5 $\log_{10}$ reduction of *E. coli* O157:H7 in guava juice (Fig. 5C) and a 4.2 $\log_{10}$ reduction in mango juice (Fig. 5F). The 5 $\log_{10}$ reduction in mango juice was achieved

after combining with s-MPEO or n-MPEO at 0.31 and 0.63 µL/mL, or only with n-MPEO at the lowest concentration (0.16 µL/mL) tested ($p \leq 0.05$).

HHP at 200 MPa for 15 min caused a 3.8 $\log_{10}$ reduction of *E. coli* O157:H7 in guava juice (Fig. 5B), and 3.4 $\log_{10}$ in mango juice (Fig. 5E). Again, a synergistic lethal effect was observed when combining with MPEO, especially in the form of nanoemulsion ($p \leq 0.05$). The combination of HHP and n-MPEO at 0.31 and 0.63 µL/mL caused the inactivation of 5 $\log_{10}$ cycles of *E. coli* O157:H7 in both juices. The use of s-MPEO only accomplished the objective of 5 $\log_{10}$ reduction at the highest concentration (0.63 µL/mL) tested in both juices, and at 0.31 µL/mL in guava juice.

Finally, the application of 150 Mpa of pressure for 15 min was not intense enough to cause a significant microbial inactivation (1 $\log_{10}$ reduction approx.) (Fig 5A and 5D). A synergistic lethal effect was only observed when combining with n-MPEO at 0.31 µL/mL and with s- or n-MPEO at the highest concentration (0.63 µL/mL) tested ($p \leq 0.05$), achieving less than 1 extra $\log_{10}$ cell cycle when combining with s-MPEO, and more than 2 extra $\log_{10}$ cell cycles with n-MPEO in guava (Fig. 5A) and mango juice (Fig. 5D). Nevertheless, no combined treatment at 150 MPa allowed us to achieve a 5 $\log_{10}$ reduction of *E. coli* O157:H7 in guava and mango juice.

4. Discussion

Particles with a diameter of < 200 nm, organized into two immiscible phases (a dispersed and a continuous phase) as in this study, are characterized as nanoemulsions (McClements, 2012). The polydispersity index (PDI) describes the variation in size of a given emulsion, ranging from 0 to 1 (Ragelle et al., 2012). A low PDI stands for a relatively narrow distribution of EO droplets in aqueous systems, indicating a more stable emulsion state (Basak & Guha, 2017a; Topuz et al., 2016; Kang & Song, 2018). In the present study, PDI

values were generally ≤ 0.32, indicating that all of the MPEO nanoemulsions remained within a relatively narrow range of size distribution with a good stability.

The similar survival curves of *E. coli* O157:H7 obtained in mango juice ($p \leq 0.05$) when applying either freshly prepared n-MPEO or n-MPEO stored up to 4 months at 4 ºC (Fig. 1) indicated that the EPI method assayed allowed us to obtain reproducible and stable nanoemulsions. To the best of our knowledge, this is the first study to have used MPEO nanoemulsions prepared via EPI method. Earlier studies reported good stability of nanoemulsions of individual constituents of essential oils such as D-limonene (up to 90 days) (Zhang, Vriesekoop, Yuan, & Liang, 2014) and citral (up to 120 days) (Pagán, Berdejo, Espina, García-Gonzalo, & Pagán, 2018) using the same method.

In the present study, no differences were observed between MIC values of s-MPEO and n-MPEO, thereby indicating that the preparation method for the nanoemulsions neither compromised nor improved the bacteriostatic activity of the EO. Earlier studies have reported that the use of D-limonene in nanoemulsions enhanced that compound's bacteriostatic activity against *L. delbrueckii*, *S. cerevisiae* and *E. coli* (Donsì, Annunziata, Sessa, and Ferrari, 2011) as well as that of citral when used against *E. coli* O157:H7 (Pagán, Berdejo, Espina, García-Gonzalo, & Pagán, 2018).

Regarding bactericidal activity, the time required to inactivate 5 $\log_{10}$ cells cycles of *E. coli* O157:H7 in mango and guava juices was shorter in assays with n-MPEO than with s-MPEO at the same concentration (5 µL/mL) (Fig. 2A and 2B). This would indicate that the preparation of EPI nanoemulsions of MPEO is a procedure that is not only capable of improving this essential oil's stability, but also its antimicrobial activity. Similar results were reported for nanoemulsions and suspensions of a peppermint essential oil (PEO) blended with virgin coconut oil at MIC value (5 µL/mL) against *L. monocytogenes* and *S. aureus* in lab media (Liang et al., 2012) and for nanoemulsions of citral against *E coli* O157:H7 (Pagán,

Berdejo, Espina, García-Gonzalo, & Pagán, 2018). It is reported that the use of nanoemulsions improves the miscibility of the hydrophobic constituents of EOs, thereby increasing their constituents' chemical and physical stability (Oliveira et al., 2017): this might likewise facilitate long-term antimicrobial activity. According to Wu, Lin, and Zhang (2014), emulsions with droplets in the nano range can efficiently permeate the porin proteins of the outer membrane of the bacterium, thus effectively delivering EOs and leading to a higher antimicrobial efficacy, as observed here.

The efficacy of n-MPEO or s-MPEO (5 μL/mL) against *E. coli* O157:H7 varied with the juice assayed. In guava juice, s-MPEO inactivated the required 5 $\log_{10}$ cell cycles after 90 min of exposure, and in mango juice this inactivation occurred after 100 min of exposure. The same occurred when using n-MPEO. It was necessary to apply 70 min of exposure in order to inactivate 5 $\log_{10}$ cycles in guava juice and 80 min in mango juice, thus reinforcing the effects of the food matrix − mainly in relation to pH, which was lower in guava juice (3.9) than in mango juice (4.5) − on the antibacterial action of MPEO. The hydrophobicity of EOs typically increases at lower pH values, enabling them to dissolve more readily into the cell membrane lipids of the target bacteria (Schelz, Molnar, & Hohmannet, 2006).

Although MPEO achieved the inactivation of 5 $\log_{10}$ cycles of *E. coli* O157:H7 in guava and mango juice, the treatment required more than 1 h of incubation at 37 ºC and doses (5 μL/mL) lying well above the sensory threshold for this EO (0.63 μL/mL) in both juices (data not shown). Therefore, in this study the use of MPEO at greatly reduced concentrations (≤0.63 μL/mL) in combination with MHT, or with emerging technologies such us PEF or HHP, has been proposed for the efficacious preservation of guava and mango juice.

Researchers have demonstrated remarkable synergistic lethal effects when combining MHT, PEF, or HHP with EOs at very low EO doses (Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Bellosoet, 2008; Espina et al., 2012; Espina, García-Gonzalo, Laglaoui, Mackey, &

Pagán, 2013). However, some studies have pointed out the lack of stability of these hydrophobic compounds and the lack of treatment homogeneity when EOs are added to aqueous systems, and have suggested that it is preferable to use nanoemulsions (Basak & Guha, 2017b; Donsì & Ferrari, 2016; Donsì, Annunziata, Sessa, & Ferrari, 2011).

As mentioned before, EO nanoemulsions, resulting from different preparation methods, have previously been reported to be effective against microorganisms (Liang et al., 2012; Zhang, Vriesekoop, Yuan, & Liang, 2014; Zhang, Zhang, Fang, & Liu, 2017; Ziani, Chang, McLandsborough, & McClements 2011). However, few studies have evaluated their behavior when combined with other technologies such as MHT, PEF, or HHP.

Our results show the occurrence of synergistic lethal effects achieved through the application of MHT, PEF, or HHP in combination with both s-MPEO and n-MPEO. The magnitude of the synergism depends on the technology, on the form of MPEO (in suspension or nanoemulsion), and on the concentration applied.

Overall, combined treatments with MHT, PEF, or HHP and n-MPEO showed the same or even greater lethality against *E. coli* O157:H7 than those with s-MPEO: thus, under these circumstances, the use of nanoemulsions also seemed to facilitate the access of EOs to the cells to cause their inactivation. Thus, the use of nanoemulsions allowed us to propose lower doses of antimicrobial or milder MHT, PEF or HHP treatments to achieve a 5 $\log_{10}$ reduction in survival counts of *E. coli* O157:H7 in guava and mango juices. For instance, while the inactivation of 5 $\log_{10}$ cycles of *E. coli* O157:H7 by PEF (35 kV/cm-150 μs) and s-MPEO in mango juice required 0.31 μL/mL (Fig. 4F), the same lethal effect was achieved with a lower concentration (0.16 μL/mL) of n-MPEO ($p \leq 0.05$). In guava juice, similar results were obtained at 30 kV/cm (Fig. 4B): it was possible to maintain the lethal effect with 0.31 μL/mL of s-MPEO or 0.16 μL/mL of n-MPEO ($p \leq 0.05$). Moreover, the use of nanoemulsions in guava juice also allowed us to reduce the intensity of the PEF treatment from 35 to 30 kV/cm

in combination with 0.31 μL/mL of n-MPEO ($p \leq 0.05$) (Fig. 4B and 4C), while still achieving the desired safety objective (a 5 $\log_{10}$ reduction of *E. coli* O157:H7).

The differences in pH or conductivity (in the case of PEF treatment) among juices did not affect the efficacy of MHT, PEF, or HHP acting as single agents against *E. coli* O157:H7, since no differences were observed ($p \leq 0.05$) among the number of survivors obtained after the application of each technology in both juices, with the exception of HHP at 300 MPa. However, combined treatments, especially PEF or HHP in conjunction with MPEO, achieved a generally greater degree of inactivation in guava juice (pH 3.9) than in mango juice (pH 4.5). This rather negligible variation in pH did not seem to influence the physical technologies' inactivation mechanism, but the lower pH of guava juice might be responsible for a greater hydrophobicity of the EOs, thereby improving their antimicrobial action (Schelz, Molnar, and Hohmannet, 2006), and, thus, their effectivity in combined treatments.

Moreover, the examination of the occurrence of sublethal injury after MHT, PEF, or HHP by using the selective recovery medium technique (data not shown) revealed a slightly higher percentage of sublethally injured cells in the outer and cytoplasmic membranes of *E. coli* 0157:H7 when suspended in guava juice than in mango juice, as a function of treatment intensity. According to previous reports (Burt, 2004; Espina et al., 2011; Hyldgaard, Mygind, & Meyeret, 2012), the occurrence of sublethal injuries at the cell envelope level might facilitate the access of EO constituents to the cell targets: thus, the greater sensitivity of sublethally injured cells to the EOs' action might also facilitate a more effective synergism among the hurdles observed in guava than in mango juice. Thus, the achievement of the safety objective was reached in guava juice at lower HHP and PEF treatment intensities than in mango juice, and also at a lower MPEO concentration in combination with MHT or PEF. Pagán, Berdejo, Espina, García-Gonzalo, and Pagán (2018) observed that the use of nanoemulsions of citral (n-citral; prepared by EPI method) did not affect the slight synergism

observed when citral and PEF were combined (150 µs, 30 kV/cm) to inactivate *E. coli* O157:H7 Sakai in lab media (pH 7.0 and 4.0). In contrast, when combined with heat (53 °C), they observed a greater synergistic effect of s-citral rather than of n-citral, either in lab media (pH 7.0 and 4.0) or in apple juice. According to the authors, in the case of citral, the vigorous agitation applied would have been sufficient to disperse it correctly and favour its antimicrobial activity during the short duration of the combined treatment. Our results with MPEO showed the opposite behavior. The use of the EPI method to prepare nanoemulsions of a complex mixture of constituents, such as MPEO, does not impair but maintains or even reinforces their antimicrobial effectivity in combination with MHT, PEF or HHP. These results show that the behavior of nanoemulsions of different EOs or EO constituents might vary as a function of their chemical characteristics, of their use as a single hurdle or in combined treatments, and of the food matrix. Therefore, further research is needed in order to assess which is the best EO nanoemulsion preparation procedure for food preservation.

To summarize: the results obtained in this study contribute to scientific knowledge regarding the effect of combined treatments of MHT, PEF, or HHP and n-MPEO with the purpose of ensuring the microbiological safety of fruit juices and reducing the risk of food-borne illness.

5. Conclusion

The use of MPEO nanoemulsions prepared by EPI method displayed good physical stability and antimicrobial activity. MPEO was generally more effective in guava than in mango juice. Both s-MPEO and n-MPEO acted synergistically in combination with MHT, PEF and HHP against *E. coli* O157:H7. The efficacy of s-MPEO and n-MPEO in reducing 5-$\log_{10}$ of *E. coli* O157:H7 when combined with MHT, PEF, or HHP varied with the tested concentrations, treatment intensity, and the food matrix. Nevertheless, in guava and mango

juices, combined treatments with MPEO in the form of nanoemulsion showed the same or even higher efficacy than those with s-MPEO, thus suggesting that the use of n-MPEO in combination with mild heat and with the emerging technologies tested herein can be regarded as a promising method to ensure microbial safety in fruit juices.

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Table 1. Droplet size and polydispersity index (PDI) of nanoemulsions of *Mentha piperita* L. essential oil stored at 4 ºC. Data represent the mean ± standard deviation of the mean of at least three independent experiments.

Storage time (months)	Droplet size (nm)	PDI
0	134 ± 1^{a}	0.322 ± 0.009^{a}
1	191 ± 8^{b}	0.299 ± 0.005^{a}
4	164 ± 2^{c}	0.243 ± 0.017^{b}

Different superscript letters in the same column indicate significant difference ($p \leq 0.05$), based on student t-test.

Figure captions

Fig. 1. Log_{10} cycles of inactivation of *E. coli* O157:H7 after exposure to n-MPEO at 5 µL/mL for 80 min in mango juice at 35 °C. Bars correspond to freshly prepared nanoemulsions (black bars), and after 1st week (dark grey bars), 2nd week (light grey bars), 3th week (dotted bars), and 4th month (white bars) of storage at 4 ºC. The error bars in the figures indicate the standard deviations of the means for data obtained from three independent experiments. The dotted line represents the detection limit.

Fig. 2. Survival curves of *E. coli* O157:H7 after exposure to s-MPEO (○) or n-MPEO (●) at 5 µL/mL for 100 min in guava (A) and mango (B) juices at 35 °C. The error bars in the figures indicate the standard deviations of the means for data obtained from three independent experiments. The dotted line represents the detection limit.

Fig. 3. Log_{10} cycles of inactivation of *E. coli* O157: H7 after a heat treatment for 10 min at 50 °C (A; D), 52 °C (B; E) and 54 °C (C; F) applied as a single agent (0.0; grey bars) or in combination with different concentrations (0.13, 0.31 and 0.63 µL/mL) of *Mentha piperita* L. essential oil in the form of a nanoemulsion (white bars) or a suspension (black bars) in guava (A; B; C) and mango (D; E; F) juices. The error bars in the figures indicate the standard deviations of the means for data obtained from three independent experiments.

Fig. 4. Log_{10} cycles of inactivation of *E. coli* O157: H7 after a pulsed electric field treatment for 150 µs at 20 kV/cm (A; D), 25 kV/cm (B; E) and 30 kV/cm (C; F) applied as a single agent (0.0; grey bars) or in combination with different concentrations (0.13, 0.31 and 0.63 µL/mL) of *Mentha piperita* L. essential oil in the form of a nanoemulsion (white bars) or a suspension (black bars) in guava (A; B; C) and mango (D; E; F) juices. The error bars in the

figures indicate the standard deviations of the means for data obtained from three independent experiments.

Fig. 5. Log_{10} cycles of inactivation of *E. coli* O157: H7 after a high hydrostatic pressure treatment for 15 min at 150 MPa (A; D), 200 MPa (B; E) and 300 MPa (C; F) applied as a single agent (0.0; grey bars) or in combination with different concentrations (0.13, 0.31 and 0.63 μL/mL) of *Mentha piperita* L. essential oil in the form of a nanoemulsion (white bars) or a suspension (black bars) in guava (A; B; C) and mango (D; E; F) juices. The error bars in the figures indicate the standard deviations of the means for data obtained from three independent experiments.

Research highlights

M. piperita EO nanoemulsions showed a greater lethality against *E. coli* O157:H7 than the not emulsified EO

M. piperita EO offers the potential to improve heat, PEF and HHP lethality

Valuable synergistic effects were observed between heat/PEF/HHP treatments and *M. piperita* EO

Combined processes (heat/PEF/HHP and *M. piperita* EO) inactivated 5 $\log_{10}$ cycles of *E. coli* O157:H7 in tropical juices

The use of nanoemulsions may allow reducing treatment intensity and EO doses

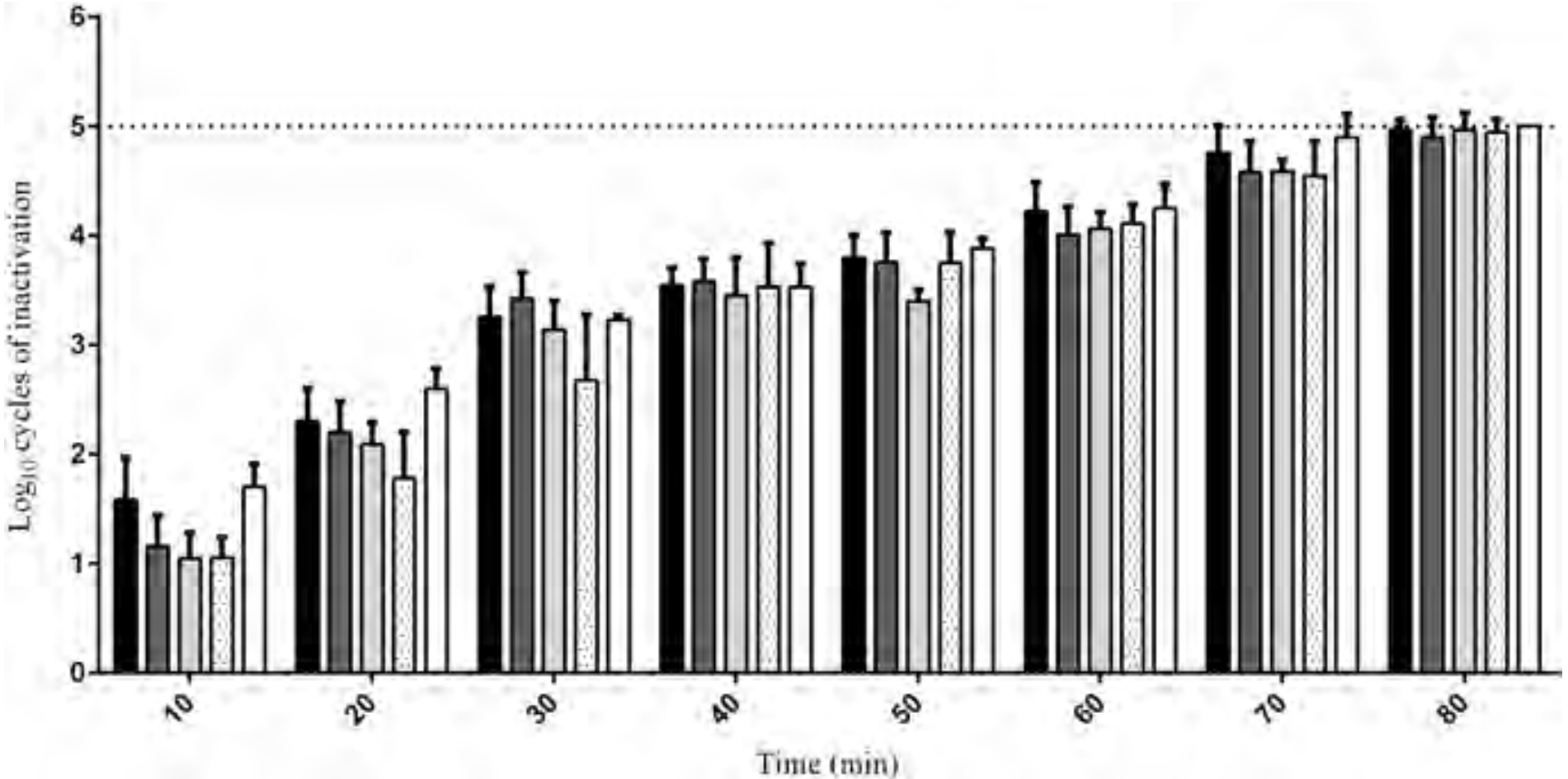
Log10 cycles of inactivation
0
1
2
3
4
5
6
10
20
30
40
50
60
70
80
Time (min)

Figure 1

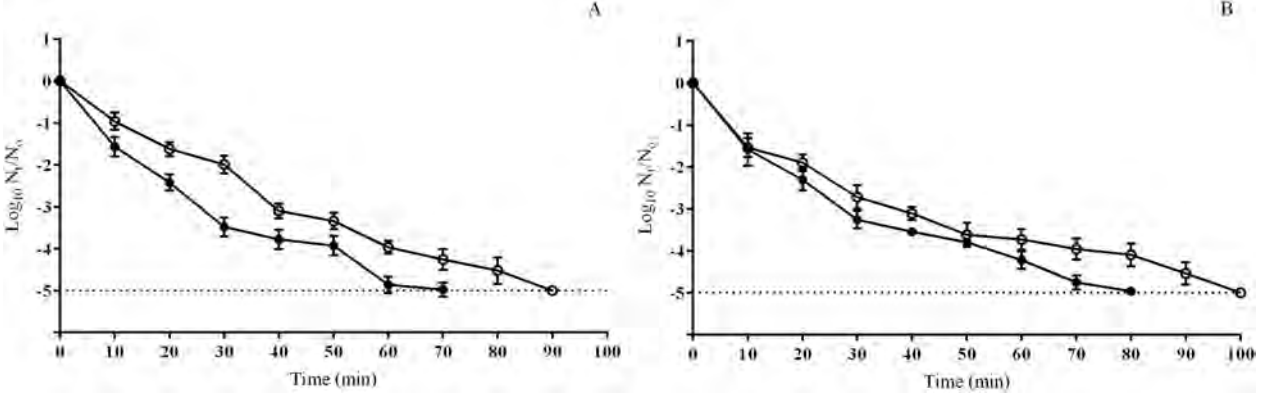

Figure 2

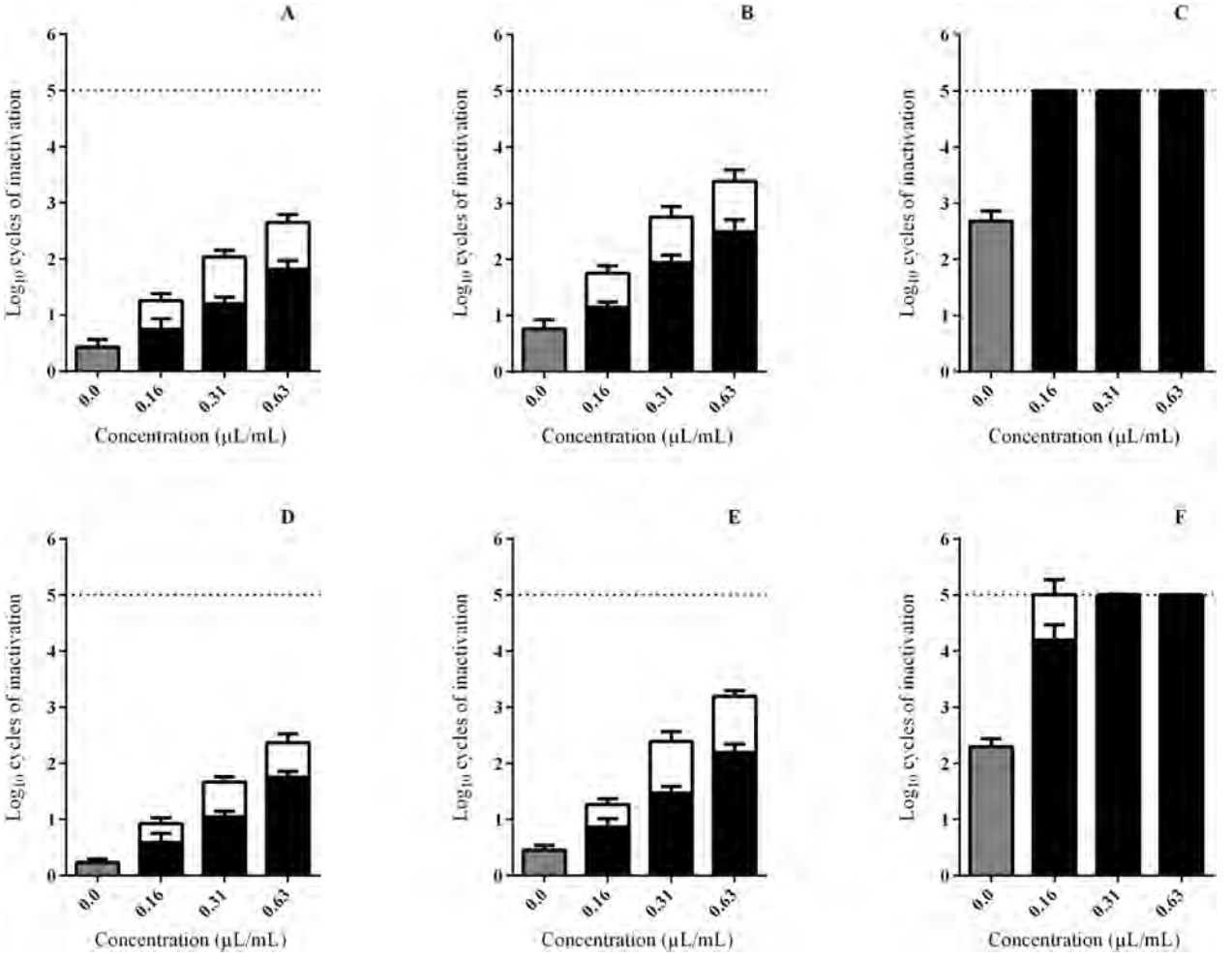
A
B
C
D
E
F
Log10 cycles of inactivation
0
1
2
3
4
5
6
0.0
0.16
0.31
0.63
Concentration (µL/mL)

Figure 3

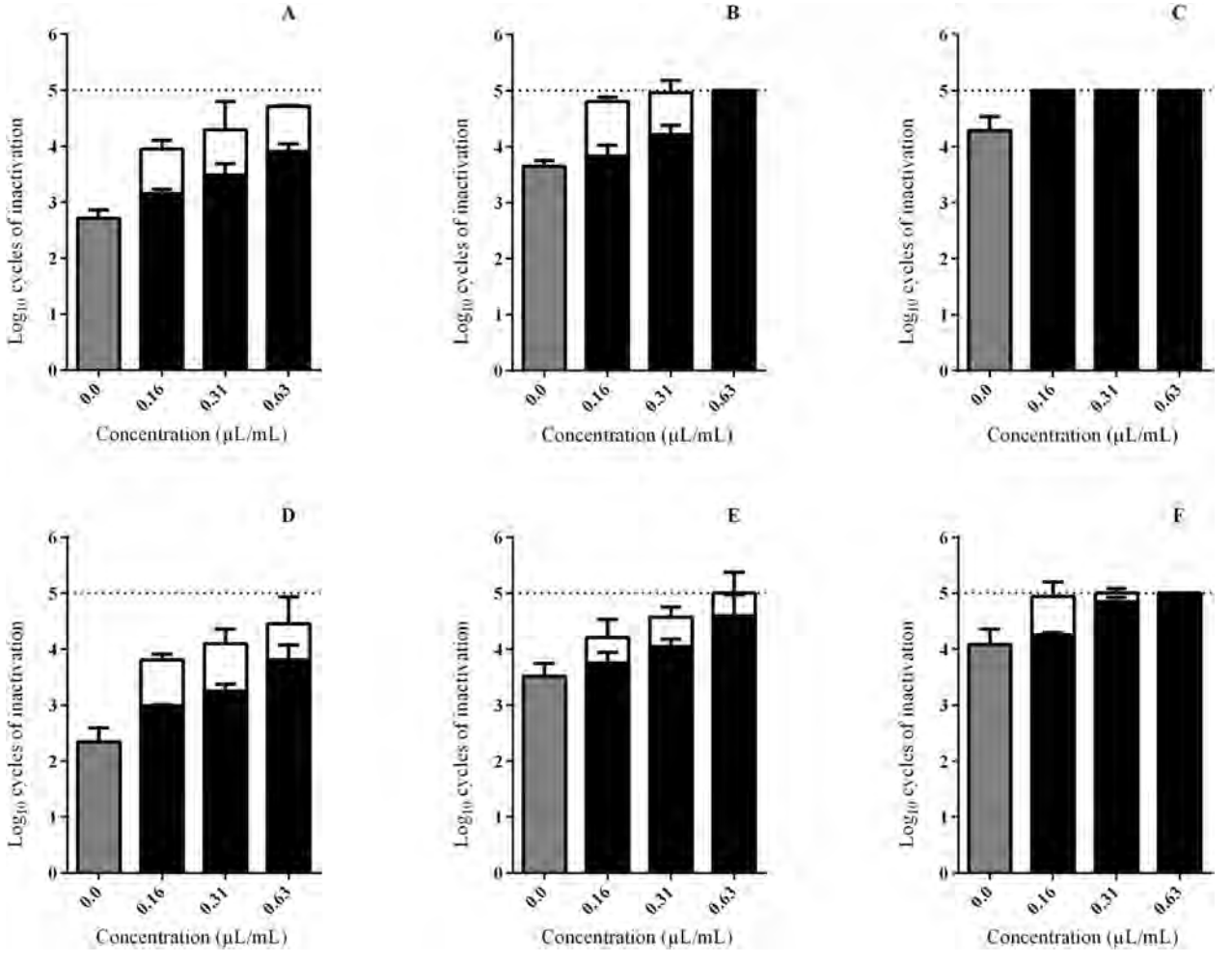
A
B
C
D
E
F
Log10 cycles of inactivation
0
1
2
3
4
5
6
0.0
0.16
0.31
0.63
Concentration (µL/mL)

Figure 4

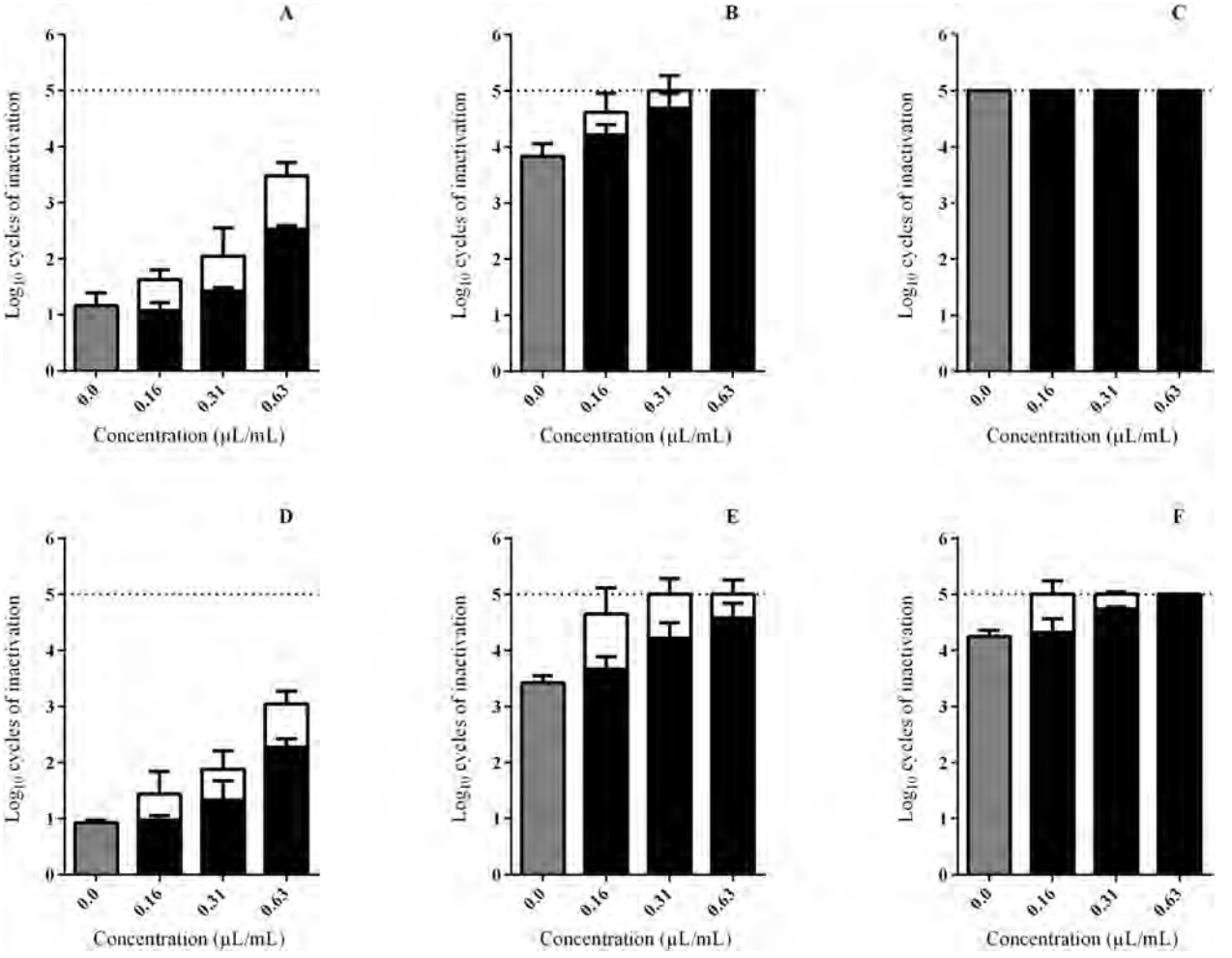
A
B
C
D
E
F
Log10 cycles of inactivation
0
1
2
3
4
5
6
0.0
0.16
0.31
0.63
Concentration (µL/mL)

Figure 5