Control of autochthonous spoilage lactic acid bacteria in apple and orange juices by sensorially accepted doses of *Citrus* spp. essential oils combined with mild heat treatments

Running title: *Citrus* oil and heat to preserve juices

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Abstract

This study assessed the compromised acceptance threshold (CAT) and rejection threshold (RT) of *Citrus lemon* (CLEO) and *Citrus reticulata* essential oil (CREO) in apple and orange juices. The efficacy of CLEO and CREO concentrations below the RT were evaluated alone and combined with mild heat treatment (MHT) (54 ºC, up to 12 min) to inactivate the autochthonous spoilage bacteria *Lactobacillus brevis*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* in apple and orange juices. The CAT of CLEO and CREO varied from 0.15 to 0.17 μL/mL in orange and apple juices. The RT of CLEO was approximately 0.58 μL/mL in apple and orange juices, and the RT of CREO was 0.68 μL/mL in both juices. When CLEO and CREO were assayed alone, the highest concentration (0.50 μL/mL) decreased counts of all strains approximately 2 $\log_{10}$ CFU/mL after 12 min of exposure to 54 ºC. All concentrations of CLEO or CREO in combination with MHT acted synergistically against *L. brevis*, *L. plantarum* and *L. mesenteroides.* Decreases in counts varied with the strain, CLEO and CREO concentrations, juice type and exposure time to the combined treatment. CREO was more effective than CLEO in combination with MHT against the strains in apple and orange juices. Effective combinations of CLEO or CREO with MHT to control the autochthonous spoilage bacteria did not compromise the quality parameters (°Brix, pH and titratable acidity) that characterize unsweetened juices. These results indicate CLEO or CREO at concentrations below the sensory RT in combination with MHT as a feasible technology to control autochthonous spoilage bacteria in fresh fruit juices.

Keywords: Essential oils, heat treatments, sensory threshold, lactic acid bacteria, fruit beverages.

Practical Application

The present study provides novel information concerning the efficacy of sensorially accepted doses of CLEO and CREO combined with MHT against autochthonous spoilers in fruit juice. The valuable synergistic effects that can be observed when combining CLEO and CREO with MHT reveal a feasible preservation technology and alternative to traditional treatments that are successful because they help reduce treatment intensity, thereby avoiding adverse effects on the sensory, physicochemical and nutritional properties of these products.

Introduction

Fresh fruit juices are appreciated and consumed worldwide because of their refreshing properties, nutritional value and health-promoting components (Singh et al., 2015). However, the contamination of fruit juices with lactic acid bacteria naturally present in the fruit may result in loss of nutrients and undesirable sensory alterations (Snyder & Worobo, 2018; Guerrouja, Sánchez-Rubiob, Taboada-Rodríguezc, Cava-Rodac, & Marín-Iniestab, 2016).

Lactobacillus spp. and *Leuconostoc* spp. are common genera of the lactic acid bacteria population found in raw fruits (Di Cagno et al., 2011); therefore, they are easily transferred to juices (Jay & Anderson, 2001). Species of these genera are well-known spoilage bacteria in raw juices because they produce metabolic end-products such as lactic acid, diacetyl, CO_2, ethanol, and acetic or formic acid that generate off-flavor and off-odor (Basak, Ramaswamya, & Piette, 2002; Jay & Anderson, 2001). Spoilage microorganisms are classically inactivated in fruit juices by high temperatures (72 to 82 °C for 0.3 to 15 s) or antimicrobial agents (e.g., benzoic and sorbic acids and sulfur dioxide) (Vally, Misso, & Madan, 2009). However, high temperatures destroy heat-sensitive nutrients such as vitamins compromising fruit juice freshness (Hu, Zhou, Xu, Zhang, & Liao, 2013).

The use of essential oils (EOs) obtained from *Citrus lemon* (CLEO) and *Citrus reticulata* (CREO) has been considered to preserve fruit juices because of their activity against

juice-related bacteria (Espina, Somolinos, Lorán, Conchello, García-Gonzalo, & Pagán, 2011; Espina, Somolinos, Ait Ouazzou, Condón, García-Gonzalo, & Pagán, 2012; Espina, García-Gonzalo, & Pagán, 2014a). Both CLEO and CREO are generally recognized as safe for use in foods and beverages (U.S. Code of Federal Regulations 8008-56-8 for CLEO and 8016-85-1 for CREO). However, the strong flavor and taste characteristic of most EOs have limited the use of EOs as preservatives in fruit juices. The effective concentrations of EOs against spoilage and pathogenic microorganisms typically exceed the sensory rejection threshold (RT) (Almeida, Barbosa, Tavares, Barbosa Filho, Magnani, & De Souza, 2018).

The combined use of *Citrus* spp. EOs with mild heat treatment (MHT) may be an alternative approach for use as antimicrobials in fruit juices (Calo, Crandall, O'Bryan, & Ricke, 2015; Espina et al., 2012). The efficacy of *C. sinensis* EO and CLEO in combination with MHT against the pathogen *Escherichia coli* O157:H7 in apple (Espina et al., 2012) or orange (Espina et al., 2014a) juice was previously reported. However, the concentrations evaluated in those studies, alone or in combination with MHT, did not consider the sensory threshold of the tested EOs in the juices. No previous studies assessed whether concentrations sensorially accepted for CLEO or CREO are effective, alone or in combination with MHT, to inactivate the autochthonous spoilage bacteria in juices.

Therefore, this study was performed to i) determine the sensory thresholds of CLEO and CREO in apple and orange juices, ii) isolate and identify spoilage lactic acid bacteria from the autochthonous microflora and iii) assess the efficacy of CLEO and CREO concentrations below the sensory threshold rejection, alone and combined with MHT, to inactive the selected autochthonous spoilage bacteria.

Materials and methods

EOs and chemical composition

CLEO and CREO extracted through steam distillation were purchased from Indulleida S.A. (Lleida, Spain). The constituents of CLEO and CREO were identified using gas chromatography coupled to mass spectrometry (CG-MS) using a chromatography model CGMS-QP2010 (Ultra Shimadzu, Kyoto, Japan). GC–MS analysis was performed under the following conditions: an RTX-5MS capillary column (30 m × 0.25 mm x 0.25 μm); program temperature 60-240 °C (3 °C/min); injector temperature 250 °C; detector temperature 220 °C; carrier gas helium adjusted to 0.99 mL/min speed; ionizing energy 70 eV; and mass range (m/z) 40-500. Samples were co-injected with the homologous series of n-alkanes (C8-C20). Each component was identified by comparing its mass spectra with the NIST/EPA/NIH Mass Spectral Database (National Institute of Standards Technology, Norwalk, CT) and FFNSC1.3 (Flavour and Fragrance Natural and Synthetic Compounds). The retention index (RI) of each constituent was determined by the Kovats method by co-injection of the homologous series of n-alkanes (C8-C20) (Adams, 2001). The EO constituents were quantified after normalizing the areas of each detected constituent and expressed as a percentage of area (%).

Preparation of juices

Apple (*Pyrus malus*) and orange (*Citrus sinensis*) fruits were purchased from a local wholesale distributor (João Pessoa, Brazil). To minimize variation among the fruits used to produce the juices, the fruits were selected for uniformity in size, form, color, and appearance and for absence of mechanical injuries and visible signs of infection. To prepare the juices, the fruits were surface disinfected by a 5 min immersion in a sodium hypochlorite solution (0.15 μL/mL, pH 7.2), washed with sterile distilled water and dried for 30 min in a biosafety cabinet. Apple juice was prepared by mixing 100 g of apple pulp (aseptically peeled) with distilled water (1:2 w/v) using a domestic blender (for 3 min), and orange juice was extracted from ripe fruit

using a domestic squeezer. The juices were stored in 50 mL aliquots at -20 °C, and when required, an aliquot was thawed under refrigeration (4 ± 0.5 °C) and used for subsequent assays.

Isolation and identification of the autochthonous isolates

To isolate the autochthonous spoilage lactic acid bacteria, 25 mL of each fresh juice prepared as described was dispensed into 225 mL of sterile saline solution (NaCl 0.85 g/100 mL), homogenized (3 min) at room temperature and serially diluted (10^{-1} to 10^{-5}). Subsequently, 100 µL of each dilution was inoculated onto de Man, Rogosa and Sharpe (MRS) agar (HiMedia), MRS agar supplemented with cysteine hydrochloride (0.05 g/100 mL) and M17 agar (HiMedia) to isolate species belonging to the genera *Lactococcus* (30 ºC), *Lactobacillus* (37 ºC), and *Bifidobacterium* (37 - 41 ºC), respectively. The plates were incubated anaerobically using an Anaerobic System AnaeroGen, (ASA; Oxoid) for 48 - 72 h (Garcia et al., 2016).

At least five autochthonous colonies with different morphologies were isolated in each specific medium at the proper temperature for each genus and maintained under refrigeration (4 ± 0.5 °C). Each isolate was subjected to analyses of Gram staining, morphology, catalase production and motility using standard procedures (APHA, 2015). Isolates presumptively identified as lactic acid bacteria (nonmotile, catalase negative, Gram-positive cocci or rods) were stored at -20 °C in MRS broth containing glycerol (15% v/v) for further studies.

The isolates were identified at the species level as previously described (Guo, Kim, Nam, Park, & Kim, 2010). Genomic DNA was extracted using a Genomic DNA extraction kit (Promega Corporation, Wisconsin, USA) according to the manufacturer's instructions. To amplify the 16S rRNA gene sequences, the following primers were used: 27F, 5-AGAGTTTGATCCTGGCTCAG-3, and 1492R, 5-GGTTACCTTGTTACGACTT-3. PCRs were conducted in a volume of 50 µL under the following conditions: initial activation at 94 °C for 2 min, denaturation at 94 °C for 30 s; annealing at 55 °C for 1 min, extension at 72 °C for 1

152 min, and a final cycle at 72 °C for 10 min. PCR products were purified using a DNA purification
153 kit (Invitrogen, Germany) and sequenced using the 27F and 1492R primers in a sequencing
154 reaction using an ABI PrismTM BigDyeTM terminator cycle sequencing reaction kit (Applied
155 Biosystems, USA). The resulting 1465 bp sequences were analyzed using the Pregap4 and Gap4
156 tools in the STADEN 1.6 software. Partial 16S rRNA sequences were compared to those
157 available in the National Center for Biotechnology Information (NCBI) GenBank database
158 using the Local Alignment Search Tool (BLAST) (Guo et al., 2010). Only query sequences
159 with similarity >97% were considered for bacterial identification.
160
161 *Test strains and inoculum preparation*
162 Stock cultures were maintained in cryovials with MRS broth containing glycerol (15 g/100
163 mL) at -80 °C. Each inoculum was obtained by preparing suspensions in sterile saline solution
164 (NaCl 0.85 g/100 mL) from overnight cultures grown anaerobically (ASA) in MRS broth at 37 °C
165 for 18 h to reach stationary growth phase (time determined considering the data of growth behavior
166 assays of the test strains). Cells were harvested using centrifugation (4500 *g* x 15 min, 4 °C),
167 washed twice and resuspended in sterile saline solution to obtain standard cell suspensions with
168 an optical density (OD) at 625 nm (OD_{625}) of 0.8. These suspensions provided viable counts of
169 approximately 8 $\log_{10}$ colony forming units per milliliter (CFU/mL) for all strains (Leite et al.,
170 2016). A final concentration of 7 $\log_{10}$ CFU/mL of the test strains was used in the juices to provide
171 a number of viable cells suitable for measuring the $\log_{10}$ reduction during the treatments.
172
173 *Minimum inhibitory concentration (MIC) of CLEO and CREO*
174 The MIC of CLEO and CREO against *L. brevis*, *L. plantarum* and *L. mesenteroides* was
175 determined using a microdilution in broth assay (CLSI, 2015) with minor modifications. The
176 stock emulsions (32 μL/mL; pH 5.6 ± 0.1) of CLEO and CREO were prepared by directly

adding EOs in MRS broth (HiMedia) containing Tween 80 (1%, v/v; Sigma–Aldrich, USA) as an emulsifier, followed by vigorous shaking using a vortex for 5 min. Using this method, the prepared emulsions presented droplet sizes characteristic of macroemulsions (Kale & Deore, 2017; Friedman, Henika, & Mandrell, 2002). At the assayed concentration (1%, v/v), Tween 80 did not present inhibitory effects against the test strains used in the study. Two-fold serial dilutions from the stock emulsion were added to the wells of a 96-well microtiter plate to provide final concentrations of CLEO or CREO in a range of 16 to 0.13 μL/mL. Then, 50 μL of the bacterial suspension prepared in MRS broth was added to each well (resulting in final viable counts of approximately 7 log CFU/mL). Each microplate included positive (inoculated) and negative (non inoculated) controls. The microtiter plates were covered with a lid and incubated anaerobically (ASA) at 37 °C for 24 h. The MIC of CLEO or CREO was confirmed as the lowest concentration capable of inhibiting visible bacterial growth (Sousa Guedes et al., 2016). The MIC was defined as the highest concentration to be tested in assays for the determination of sensory thresholds.

Compromised acceptance threshold (CAT) and rejection threshold (RT) of CLEO and CREO in fruit juices

All sensory analyses were performed after approval from the Committee on Ethical Research Involving Humans Beings (Federal University of Paraíba, protocol 1.125.993/2015). The rejection threshold (RT) of the CLEO and CREO in the fruit juices and the compromised acceptance threshold (CAT), which indicates the transition point between sensory acceptance and rejection, were assessed using a previously proposed and validated methodology (Lima Filho et al., 2015; 2017). For this, 50 untrained panelists (18 to 58 years old) were preselected according to their interest and frequency of fruit juice consumption. Sensory tests comprised five sessions of acceptance tests (Stone et al., 2012) performed in individual booths under

controlled temperature and light. In each session, the panelists received two samples, one of which was the control sample (fruit juice without CLEO or CREO) and the other was a stimulus sample (fruit juice containing CLEO or CREO at 2, 1, 0.50, 0.25, 0.13 μL/mL). Between sessions, the stimulus sample was presented in ascending order of CLEO or CREO concentration, and the position of the stimulus sample within each pair was randomized (Lima Filho et al., 2017). Approximately 30 mL of each juice (with or without CLEO or CREO) were served in white disposable 50-mL cups encoded with a random three-digit number to avoid panelists to know the sample referred as control or stimulus. Panelists judged the samples using a hedonic scale of nine points ranging from 1 (dislike extremely) to 9 (like extremely). A 5 min interval was observed before offering a new pair of fruit juice samples. Between sessions, panelists were invited to use low-salt biscuits and rinse their mouths with drinking water to cleanse their palates. Panelists were allowed to freely select any of the nine points of the hedonic scale that best reflected their judgments.

For the statistical analysis of the data as well as CAT determination in each session, the *t*-test for paired samples was used to compare the hedonic scores of control and stimulus samples. The obtained *t* values (Y1-axis) were graphically evaluated as a function of the EOs concentrations (X-axis). The point that resulted in significant differences ($P \leq 0.05$) between the control and stimulus samples was represented in the graph by a dotted line (tabulated *t* value). To assess the RT of the CLEO or CREO in fruit juices, a second Y axis (Y2-axis) representing the average hedonic score of the stimulus sample was inserted into the graph. The transition point between the sensory acceptance and rejection of the fruit juices was represented on the graph by a dashed line referring to the hedonic score 5 (category “indifferent”) (Della Lucia et al., 2014). To determine the CAT and RT values, the regression models were adjusted to the points of the graph (Y1-axis points= CAT; Y2-axis points= RT). From the model equation, the CAT was calculated considering where the calculated *t* value became equal to the

standard *t* value ($P = 0.05$) (Y1 = tabulated *t* value); the RT was calculated considering the point where the average hedonic score for CLEO or CREO concentration became equal to "indifferent" in the hedonic scale (Y2=5). The validity of the models generated was determined from the significance of the regression coefficients (SSregression/SStotal).

Inactivation by CLEO or CREO in fruit juices

To assess the inactivation of each test strain in apple or orange juice using CLEO or CREO alone or in combination with MHT, the juices were centrifuged (12,500 x *g*, 15 min, 4 °C) to separate the pulp from the remaining liquid. The supernatants were filtered using a triple-cheesecloth layer and sterilized by autoclaving (121 °C, 1.1 atm, for 15 min). The effects of concentrations of CLEO or CREO below the RT on the viable counts of each test strain in the apple and orange juices were assessed for 0, 2, 4, 6, 8, 10 and 12 min. Initially, an aliquot of 1 mL of the bacterial suspension (8 $\log_{10}$ CFU/mL) was inoculated in 9 mL of juices containing CLEO or CREO at the desired final concentrations prepared as described above. At intervals of 0 (just after homogenization), 2, 4, 6, 8, 10 and 12 min post incubation at room temperature (25 ± 1 °C), an aliquot of 100 µL of each mixture was serially diluted in sterile saline solution (NaCl 0.85 g/100 mL). Subsequently, 20 µL aliquots of each dilution were inoculated onto MRS agar using the microdrop technique (Herigstad, Hamilton, & Heersink, 2001). Control systems without CLEO or CREO were assayed similarly. The plates were incubated anaerobically (ASA) at 37 °C for 24 h, and the results were expressed as $\log_{10}$ CFU/mL.

Inactivation by CLEO or CREO in combination with MHT in fruit juices

The effects of CLEO or CREO concentrations below the RT in combination with MHT (54 °C) on the viable counts of each test strain in apple and orange juices were assessed after 0,

2, 4, 6, 8, 10 and 12 min of exposure. Initially, aliquots of 9 mL of fruit juice containing CLEO or CREO at different concentrations were placed in a shaking bath with the thermostat set at 54 °C. Once the core point of each fruit juice sample reached 54 ºC, an aliquot of 1 mL of bacterial suspension (8 $\log_{10}$ CFU/mL) was added to the flasks. After 0, 2, 4, 6, 8, 10 or 12 min at 54 °C, an aliquot of 100 µL of each fruit juice was serially diluted in sterile saline solution (NaCl 0.85 g/100 mL), and subsequently, aliquots of 20 µL of each dilution were inoculated onto MRS agar as previously described (Herigstad et al., 2001). Systems without CLEO or CREO were assayed similarly to evaluate the effects of MHT alone. The plates were incubated anaerobically (ASA) at 37 °C for 24 h, and the results were expressed as $\log_{10}$ CFU/mL.

To determine the occurrence of synergism between CLEO or CREO and MHT, the results obtained in combined applications were compared to the corresponding theoretical results, which described the sum of the inactivation caused by CLEO, CREO or MHT acting individually (additive effect). The enhanced effects of CLEO or CLEO and MHT acting simultaneously were considered synergistic effects (Arroyo, Cebrián, Pagán, & Condón, et al., 2012).

Modeling the survival curves of test strains in juices treated with CLEO or CREO in combination with MHT

The obtained survival curves of each test strain in orange or apple juice treated with CLEO or CREO in combination with MHT were fitted to the following the Equation 1 (Mafart, Couvert, Gaillard, & Leguerinel, 2002):

$$\log \frac{N_t}{N_0} = \left(\frac{t}{\delta}\right)^p \qquad \text{(Eq. 1)}$$

where t is the treatment time (min); N_t and N_0 are the population densities (CFU/mL) at time t and time zero, respectively; and δ and p are two characteristic parameters of the equation. The

277 δ value is the time to the first decimal reduction (the time necessary to inactivate the first $\log_{10}$
278 cycle of the microbial population). The *p* value is a shape parameter dependent on the profile
279 of the survival curve: $p<1$ for concave upwards survival curves, $p=1$ for linear survival curves,
280 and $p>1$ for concave downwards survival curves. Once the profile of the survival curves was
281 described by Eq. 1, the time needed to achieve a 3-log reduction (3δ value) was estimated as a
282 function of the CLEO or CREO concentration and juice type evaluated.
283
284 *Physicochemical parameters of fruit juices*
285 To assess whether the sensorially accepted concentrations of CLEO or CREO used in
286 combination with the MHT affected the physicochemical parameters of apple and orange juices,
287 samples subjected or not to combined treatments were analyzed for soluble solids content
288 (°Brix), pH and titratable acidity (TA) (CLEO or CREO and MHT) using standard procedures
289 (AOAC, 2016). °Brix was determined using a digital refractometer (model HI 96801, Hanna
290 Instruments, São Paulo, Brazil) (No. 932.12). pH values were determined using a digital
291 potentiometer (model Q400AS, Quimis, São Paulo, Brazil) (No. 981.12). TA was determined
292 using phenolphthalein as an indicator with 0.1 N NaOH, and the results were expressed in g per
293 100 mL of citric acid equivalents (No. 942.15).
294
295 *Statistical analysis and reproducibility*
296 The assays were performed in triplicate in three independent experiments. For the MIC
297 assays, the results were expressed as modal values because the MIC values were the same in
298 all repetitions. For the assays of $\log_{10}$ reduction and physicochemical parameters, statistical
299 analyses were performed to determine significant differences ($P \leq 0.05$) using ANOVA
300 followed by post hoc Tukey's test or Student's t-test. The error bars in the figures indicate the
301 mean ± standard deviations from the data obtained from independent experiments. For kinetic

analysis of the data, the least-squares criterion by the GraphPad PRISM (GraphPad Software, Inc., San Diego, CA) was used.

Results and discussion

Selection of autochthonous lactic acid bacteria test strains

A total of 40 isolates of lactic acid bacteria, comprising 20 isolates from each type of fruit juice, were selected for identification. Species belonging to the *Lactobacillus* genus were predominant (32/40 isolates) in both juices. The following *Lactobacillus* species were identified in both juices: *L. plantarum* (n=8 in apple juice; n=5 in orange juice), *L. brevis* (n=3 in apple juice; n=7 in orange juice) and *L. fermentum* (n=2 in apple juice; n=7 orange juice). *Leuconostoc mesenteroides* (n=8) was identified only in orange juice.

One strain of *L. plantarum* isolated from apple juice and one strain of *L. brevis* and one strain of *L. mesenteroides* isolated from orange juice were selected as target organisms for this study considering that these species commonly act as juice spoilage bacteria (Espirito-Santo, Carlin, & Renard, 2015; Campos & Cristianini, 2007; Elez-Martínez, Escolà-Hernández, Soliva-Fortuny, & Martín-Belloso, 2005; Basak et al., 2002).

Chemical composition of CLEO and CREO

A total of 23 and 18 constituents were identified in CLEO and CREO used in this study, respectively (Table 1). The constituents detected at the highest amounts in CLEO were limonene (66.47%), β-pinene (11.71%), γ-terpinene (9.29%) and sabinene (2.00%). Other constituents, such as α-pinene (1.91%), myrcene (1.71%) and geranial (1.35%), were detected in minor amounts. The majority constituent in the CREO was also limonene (89.38%), followed by myrcene (2.05%). Previous studies also reported limonene (53.57–84.73%), β-pinene (8.23–12.74%) and γ-terpinene (3.38–9.66%) as the predominant constituents in CLEO (Espina et al.,

2011; AL-Jabri & Hossain, 2018). Similarly, limonene (60.74–80.2%) and myrcene (6.7–7.43%) have been described as the majority constituents in CREO (Tao, Jia, & Zhou, 2014; Fouad & Camara, 2017). Differences in the amounts of limonene detected in CLEO and CREO could be explained by the influence of environmental conditions (e.g., altitude, temperature, rainfall and geographical distribution) on the plant source (AL-Jabri & Hossain, 2018). These findings reinforce the importance of determining the chemical characterization of EOs each time a new study is carried out because this characterization may help to determine the differences among the antimicrobial activities of EOs obtained from the same plant species (Espina et al., 2012).

MIC values of CLEO and CREO

The MICs of both CLEO and CREO against *L. brevis*, *L. plantarum* and *L. mesenteroides* were 2 μL/mL. The antimicrobial activities of CLEO and CREO has been primarily related to the high amounts of limonene in the composition of these EOs. An earlier study reported strong antimicrobial efficacy of limonene (MIC 1 μL/mL) against *L. brevis* DSMZ 20054 and *L. plantarum* DSMZ 2601 (Bevilacqua, Corbo, & Sinigaglia, 2010). The hydrophobic characteristics of limonene and other compounds, such as γ-terpinene, terpinolene, linalool and limonene, found in CLEO and CREO could perturb the bacterial cell membrane, increasing its permeability and causing leakage of cellular components (Prashar, Hili, Veness, & Evans, 2003; Tao et al., 2014). Synergistic interactions resulting from disturbing effects of limonene on the bacterial cell membrane that could facilitate the uptake of other constituents present in smaller amounts in CLEO and CREO (e.g., linalool, octanal and β-ocimene) may also contribute to the antimicrobial activities of these EOs (Espina et al., 2011).

CAT and RT of CLEO and CREO in fruit juices

CLEO and CREO were evaluated in concentrations of 0.13, 0.25, 0.50, 1.0 and 2.0 μL/mL to determine the CAT and RT in apple and orange juices. Fig. 1 shows the calculated *t* values (Y1-axis) and the hedonic score (Y2-axis) as a function of CLEO and CREO concentrations in the stimulus fruit juice samples (X-axis). The obtained linear models showed significant regression coefficients ($P \leq 0.05$).

In the orange juice, the CAT of CLEO was 0.17, and the CAT of CREO was 0.15 μL/mL. In the same juice, the RT of CLEO and CREO was 0.58 and 0.68 μL/mL, respectively. In the apple juice, the CAT of CLEO and CREO was 0.15 and 0.16 μL/mL, respectively, while the RT was 0.59 for CLEO and 0.68 μL/mL for CREO. Consequently, apple and orange juices at concentrations of 0.13, 0.25 and 0.50 μL/mL CLEO or CREO were considered acceptable by panelists (≥ CAT < RT). Analyses of acceptance as a function of the CLEO or CREO concentration based on the angular coefficient of CLEO and CREO in apple juice or orange juice showed that the increase in CLEO or CREO concentration decreased the juice acceptance (Table 2).

The hedonic scores of juices added of CLEO or CREO concentrations referring to their CAT values were “like moderately” (hedonic score 7) or “like very much” (hedonic score 8). Otherwise, the hedonic scores of juices added of CLEO or CREO concentrations corresponding to their RT values were “dislike very much” (hedonic score 2) or “dislike extremely" (hedonic score 1).

A previous study reported the overall acceptance of CLEO at potentially antimicrobial concentrations (20 to 200 μL/L) in three distinct matrices (tomato juice, vegetable soup and poultry burgers) (Espina, García-Gonzalo, & Pagán, 2014b). According to these researchers, only the lowest assayed concentration (20 μL/L) of CLEO had acceptance in tomato juice, while higher CLEO concentrations were accepted in vegetable soup (200 μL/L) and poultry burgers (100 μL/L). These results show that the determination of CLEO and CREO sensory thresholds

in the matrix proposed for incorporation results in a successful experimental approach to explore their application because concentrations that exceed the RT, even though they are effective against target bacteria (for example, based on MIC values), would not be applicable.

Inactivation of the test spoilage bacteria by CLEO or CREO in combination with MHT in fruit juices

No decreases ($P > 0.05$) were observed in the counts of *L. brevis*, *L. plantarum* and *L. mesenteroides* after 12 min of exposure to 0.13 μL/mL CLEO or CREO in apple and orange juices (Fig. 2-3). Counts of *L. brevis*, *L. plantarum* and *L. mesenteroides* decreased ($P \leq 0.05$) by approximately 1 $\log_{10}$ CFU/mL after 12 min of exposure to 0.25 μL/mL CLEO in apple and orange juices (Fig. 2). The same concentration of CREO (0.25 μL/mL) decreased ($P \leq 0.05$) 1.6 $\log_{10}$ and 1.1 $\log_{10}$ CFU/mL in the counts of *L. mesenteroides* and *L. plantarum*, respectively, in both juices (Fig. 3), but it did not decrease ($P > 0.05$) the counts of *L. brevis* in apple (Fig. 3A1) or orange (Fig. 3A2) juice after 12 min of exposure.

Interestingly, 0.50 μL/mL CLEO decreased ($P \leq 0.05$) 3.5 $\log_{10}$ CFU/mL in the counts of *L. plantarum* in apple juice and only decreased 1.1 $\log_{10}$ CFU/mL in the counts of *L. brevis* and *L. mesenteroides* in the same juice after 12 min of exposure (Fig. 2A1-C1). In orange juice, 0.50 μL/mL CLEO caused decreases ($P \leq 0.05$) of 1.7 $\log_{10}$ CFU/mL in the counts of *L. plantarum*, 1.4 $\log_{10}$ CFU/mL in the counts of *L. brevis* and 1.1 $\log_{10}$ CFU/mL in the counts of *L. mesenteroides* after 12 min of exposure (Fig. 2A2-C2). In apple juice, 0.50 μL/mL CREO decreased the counts of *L. plantarum* and *L. mesenteroides* ($P \leq 0.05$) by 1.5 $\log_{10}$ CFU/mL (Fig. 3B1-C1) and the counts of *L. brevis* by 1 $\log_{10}$ CFU/mL (Fig. 3A1). Comparatively, 0.50 μL/mL CREO decreased ($P \leq 0.05$) the counts of *L. mesenteroides* by approximately 2 $\log_{10}$ CFU/mL and the counts of *L. brevis* and *L. plantarum* by 1.2 $\log_{10}$ CFU/mL in orange juice after 12 min of exposure (Fig. 3A2-C2).

In combination with MHT, the decrease in the counts of *L. brevis, L. plantarum* and *L. mesenteroides* varied with the concentration of CLEO or CREO evaluated and the time of exposure. A decrease ($P \leq 0.05$) of approximately 5 $\log_{10}$ CFU/mL was observed in the counts of *L. brevis* after 12 min of exposure to 0.50 μL/mL CLEO or CREO in combination with MHT in both apple and orange juices (Fig. 4). Similar results were observed after 8 min of exposure to 0.50 μL/mL CREO and MHT in orange juice ($P \leq 0.05$) (Fig. 4D).

As observed in Fig. 4, the level of inactivation of *L. brevis* caused by the experimental combined treatment was higher than that theoretically estimated by the sum of the lethality of both hurdles acting individually (additive effect), indicating the occurrence of a remarkable synergistic effect. The magnitude of the synergism was greater when using CREO in combination with MHT. The use of CREO caused more than 2 extra $\log_{10}$ cycles of inactivation after 4 and 6 min of treatment in apple and orange juices, respectively. Similar results were observed for all concentrations assayed (0.13, 0.25 and 0.50 μL/mL) of CLEO or CREO in combination with MHT against *L. plantarum* and *L. mesenteroides* (data not shown). The synergism observed against the test strains was probably a result of an initial sublethal damage in the cell envelopes caused by MHT, which would help the hydrophobic CREO or CLEO constituents (e.g., limonene, β-pinene and myrcene) cross the bacterial membranes and act directly in the cell (Mañas & Pagán, 2005; Espina et al., 2011; Espina et al., 2012).

To assess the resistance of each strain to the combined process, the survival curves were fitted using Eq. 1 (Table 3), which described the survival curve profile obtained after 12 min of exposure to the combined processes, independent of the spoilage bacteria, CLEO or CREO concentration and juice type (apple and orange). The results demonstrate that the δ values decreased when the EO concentration increased and that p values were less than 1 and varied as a function of the strain and treatment conditions applied. The estimated parameters with their

95% confidence limits are listed in Table 3. The root mean square error (*RMSE*) and determination coefficient (R^2) values indicated the goodness of fit.

Based on the estimated parameters obtained by Equation 1, the time to inactivate 99.9% (3δ value) of the microbial population was estimated. Fig. 5 shows the relationship between the $\log_{10}$ of 3δ values and the concentration of CLEO (Fig. 5A-B) or CREO (Fig. 5C-D) for *L. brevis*, *L. plantarum* and *L. mesenteroides* inactivation under the combined treatment in apple (Fig. 5A-C) and orange (Fig. 5B-D) juices. As observed in Fig. 5, the time to cause 3-log reduction (3δ) in *L. brevis*, *L. plantarum* and *L. mesenteroides* counts decreased ($P \leq 0.05$) when CLEO or CREO concentrations in combination with MHT increased, independent of the exposure time. An earlier study reported that the inactivation rate of *E. coli* O157:H7 in orange juice increased with the increase in *C. sinensis* EO concentration in combination with MHT (54-60 °C) as well as with the time of exposure to the combined treatment (Espina et al., 2014a).

The efficacy of CLEO or CREO concentrations combined with MHT varied between the apple and orange juices. *L. brevis*, *L. plantarum* and *L. mesenteroides* showed high sensitivity ($P \leq 0.05$) to the same CLEO concentrations combined with MHT in orange juice (Fig. 5A-B). These results could be associated with the distinct composition of the juices because the constituents of the food matrices play an important role in bacterial protection against heat (Espina et al., 2014a). Similarly, food matrix components may influence the antimicrobial efficacy of EOs (Gutierrez, Barry-Ryan, & Bourke, 2009).

CREO in combination with MHT showed higher efficacy ($P \leq 0.05$) in inhibiting *L. brevis*, *L. plantarum* and *L. mesenteroides* (Fig. 5C-D) than that of CLEO (Fig. 5A-B) in both apple and orange juices. The higher amounts of oxygenated monoterpenes (e.g., octanal, linalool, decanal and α-sinensal) ($P \leq 0.05$) in CREO (7.02%) than in CLEO (4.2%) probably influenced the antibacterial effects of CREO because both EOs had limonene as the major constituent. The stronger antimicrobial activity exerted by CREO against *Pseudomonas*

aeruginosa ATCC 10145 than that exerted by CLEO has already been reported and was putatively associated with the higher proportion of oxygenated monoterpenes in CREO than in CLEO (Espina et al., 2011).

L. mesenteroides was the most susceptible ($P \leq 0.05$) to CREO and MHT in apple and orange juices (Fig. 5C-D) and the most resistant to CLEO and MHT in orange juice (Fig. 5B). Otherwise, *L. brevis* was the most susceptible ($P \leq 0.05$) to CLEO and MHT in orange juice (Fig. 5B) and the most resistant to CREO and MHT in both juices (Fig. 5C-D). The distinct susceptibility of spoilage microorganisms such as *Saccharomyces cerevisiae* (Tyagi, Gottardi, Malik, & Guerzoni, 2013), *Zygosaccharomyces rouxii* and *Z. bailii* to EOs in fruit juices has been reported (Tyagi et al., 2013; Karaman, Sagdic, & Yilmaz, 2016). However, no previous studies have focused on the efficacy of CREO and CLEO applied alone or combined with MHT against autochthonous spoilage bacteria in apple and orange juices.

Apple and orange juices treated with 0.13, 0.25 and 0.50 μL/mL CLEO or CREO and MHT for 12 min presented °Brix in a range of 10.56 to 10.93 (Table 4). The pH of apple and orange juices varied from 3.91 to 4.06, while TA ranged from 0.15 to 1.73 (Table 4). No differences were observed between the physicochemical characteristics of treated and control juices (Table 4). These results are important because they show that sensorially accepted concentrations of CREO or CREO (0.13, 0.25 or 0.50 μL/mL) in combination with MHT (54 °C; 12 min) did not compromise the quality aspects of orange and apple juices that characterize unsweetened fruit juices (Brazilian Legislation, 2016).

Conclusion

The application of doses below the RT of CLEO and CREO was not effective in reducing the counts of *L. brevis*, *L. plantarum* and *L. mesenteroides* in apple and orange juices. However, doses below the RT of CLEO and CREO when applied in combination with MHT

were effective in reducing the counts of *L. brevis*, *L. plantarum* and *L. mesenteroides* in the juices up to 5 $\log_{10}$ CFU/mL. The antibacterial efficacy of the combined treatments varied among the strains, EOs and juice type. The magnitude of the synergism was greater when MHT was combined with CREO than with CLEO. These results indicate that the doses of *Citrus* spp. EOs, primarily CREO, below the RT in combination with MHT could serve as an alternative to control autochthonous spoilage bacteria in apple and orange juices. These findings clearly indicate that the determination of sensory limits is critical to determine the potential application of *Citrus* spp. EOs as preservatives in fruit juices.

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Author Contributions

GTSP, RP and MM designed the research; GTSP and RC conducted the experiments; and GTSP, RP, DB, RC, ELS and analyzed the data and performed the statistical analysis.

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640 **Table 1.** Constituents identified by CG-MS in the essential oil from *Citrus lemon* (CLEO) and

641 *Citrus reticulata* (CREO).

Constituents	RI[a]	Percentage area (%)**		Identification[b]
		CLEO	CREO	
2-Hexanone, 3,3-dimethyl	787	0.46	0.45	RI, MS
4-Butoxy-2-butanone	603	0.36	0.35	RI, MS
α-Thujene	927	0.43		RI, MS
α-Pinene	936	1.91	0.58	RI, MS, PC
Cyclopentane, 1-acetyl-1,2-epoxy	947	0.21	0.21	RI, MS
Sabinene	975	2.00	0.70	RI, MS
β-Pinene	981	11.71		RI, MS, PC
Myrcene	990	1.71	2.05	RI, MS
Octanal	1006		0.13	RI, MS
α-Terpinene	1020	0.19		RI, MS
p-Cymene	1028	0.19		RI, MS, PC
Limonene	1033	66.47	89.38	RI, MS, PC
γ-Terpinene	1061	9.29	0.27	RI, MS
Linalool	1102	0.10	0.26	RI, MS, PC
(E)-β-Ocimene	1048	0.10		RI, MS
Terpinolene	1089	0.38		RI, MS
Decanal	1135		0.30	RI, MS
(R)-6-Octenal, 3,7-dimethyl	1155	0.14		RI, MS
(Z)-2,6-Octadienal, 3,7-dimethyl	1206	0.78		RI, MS
Nery acetate	1263	0.53		RI, MS
Geranial	1272	1.35		RI, MS
(E)-Caryophyllene	1265	0.23		RI, MS
Geranyl acetate	1285	0.37		RI, MS
β-Bisabolene	1368	0.69		RI, MS
α-*cis*-Bergamotene	1418	0.41		RI, MS
Butylated hydroxytoluene	1495		0.15	RI, MS
α-Sinensal	1700		0.12	RI, MS
Methyl tetradecanoate	1722		0.27	RI, MS
Hexadecanoic acid, methyl ester	1962		1.40	RI, MS
(E)-9-Octadecenoic acid, methyl ester	2126		1.05	RI, MS
Octadecanoic acid, methyl ester	2169		0.89	RI, MS
(Z,Z)-9,12-Octadecadienoic Acid, methyl ester	2189		0.44	RI, MS

642 [a] Retention index relative to n-alkanes;
643 [b] RI: Identification by Kovats index (Adams, 2001); MS: Identification by NIST/EPA/NIH; PC: Identification by
644 authentic standards analyzed by mass spectrometry.

Table 2. Adjusted models of compromised acceptance threshold (CAT) and rejection threshold (RT) determination of *Citrus lemon* (CLEO) or *Citrus reticulata* (CREO) essential oil in apple and orange juices and their respective coefficients of determination.

Essential oil	Juice	Equation	Model	r^2
CLEO	Apple	2	Y1 = 81.761x – 10.183	0.95
		3	Y2 = -3.643x + 7.1641	0.79
	Orange	4	Y1 = 102.19x – 15.63	0.93
		5	Y2 = -3.6078x + 7.0776	0.78
CREO	Apple	6	Y1 = 57.821x – 7.1036	0.99
		7	Y2 = -3.9289x + 7.655	0.80
	Orange	8	Y1 = 59.593x – 6.5039	0.99
		9	Y2 = -3.9221x + 7.6772	0.81

Y1: calculated t values; Y2: mean hedonic score; X: concentration of *Citrus lemon* or *Citrus reticulata* essential oil; r^2: coefficient of determination.

688 **Table 3.** p and δ values estimated from the fitting of Equation 1 to experimental data in assays
689 with *Lactobacillus brevis*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* in apple
690 and orange juices treated with MHT (54 ºC for 12 min) and essential oils from *Citrus lemon*
691 (CLEO) and *Citrus reticulata* (CREO).

Juice	Strain	EO*	Concentration (µL/mL)	p**	δ***	*RMSE*	R^2
Apple	*L. brevis*	CLEO	0.50	0.38 ± 0.12	0.19 ± 0.19	0.39	0.94
			0.25	0.39 ± 0.15	0.27 ± 0.38	0.43	0.92
			0.13	0.43 ± 0.14	0.43 ± 0.42	0.38	0.93
		CREO	0.50	0.54 ± 0.08	0.71 ± 0.32	0.19	0.98
			0.25	0.61 ± 0.11	1.18 ± 0.55	0.22	0.97
			0.13	0.68 ± 0.16	1.89 ± 0.93	0.25	0.96
	L. mesenteroides	CREO	0.50	0.57 ± 0.09	0.26 ± 0.11	0.24	0.97
			0.25	0.51 ± 0.15	0.39 ± 0.36	0.42	0.94
			0.13	0.38 ± 0.11	0.40 ± 0.33	0.17	0.98
		CLEO	0.50	0.42 ± 0.07	0.38 ± 0.22	0.17	0.98
			0.25	0.46 ± 0.05	0.72 ± 0.26	0.10	0.99
			0.13	0.48 ± 0.08	1.19 ± 0.53	0.14	0.98
	L. plantarum	CREO	0.50	0.50 ± 0.10	0.39 ± 0.26	0.33	0.96
			0.25	0.48 ± 0.06	0.43 ± 0.18	0.17	0.99
			0.13	0.48 ± 0.07	0.63 ± 0.29	0.17	0.98
		CLEO	0.50	0.47 ± 0.03	0.55 ± 0.12	0.08	1.00
			0.25	0.52 ± 0.03	0.99 ± 0.17	0.07	1.00
			0.13	0.64 ± 0.03	2.00 ± 0.18	0.04	1.00
Orange	*L. brevis*	CREO	0.50	0.82 ± 0.17	1.11 ± 0.47	0.23	0.98
			0.25	0.79 ± 0.19	1.40 ± 0.71	0.30	0.97
			0.13	0.71 ± 0.16	1.43 ± 0.76	0.31	0.96
		CLEO	0.50	0.50 ± 0.08	0.53 ± 0.27	0.26	0.96
			0.25	0.59 ± 0.07	0.86 ± 0.28	0.16	0.99
			0.13	0.76 ± 0.13	1.49 ± 0.56	0.24	0.98
	L. mesenteroides	CREO	0.50	0.31 ± 0.17	0.23 ± 0.31	0.96	0.91
			0.25	0.43 ± 0.12	0.51 ± 0.41	0.39	0.94
			0.13	0.34 ± 0.06	0.62 ± 0.28	0.30	0.95
		CLEO	0.50	0.57 ± 0.09	1.08 ± 0.47	0.18	0.98
			0.25	0.66 ± 0.11	1.93 ± 0.64	0.16	0.98
			0.13	0.67 ± 0.09	2.93 ± 0.67	0.10	0.99
	L. plantarum	CREO	0.50	0.25 ± 0.05	0.03 ± 0.03	0.31	0.96
			0.25	0.37 ± 0.08	0.23 ± 0.16	0.29	0.95
			0.13	0.48 ± 0.13	0.78 ± 0.38	0.38	0.94
		CLEO	0.50	0.50 ± 0.05	0.69 ± 0.19	0.18	0.96
			0.25	0.51 ± 0.04	0.83 ± 0.18	0.08	1.00
			0.13	0.52 ± 0.08	1.15 ± 0.46	0.15	0.98

692 *EO: essential oil; **p: shape parameter dependent on the profile of the survival curve; ***δ time to the first
693 decimal reduction; *RMSE*: root mean square error; R^2: determination coefficient.

Table 4. Physicochemical parameters (average ± standard deviation; n = 6) of apple and orange juices treated with *Citrus lemon* (CLEO) or *Citrus reticulata* (CREO) essential oil and MHT (54 °C; 12 min).

Juices	EO (μL/mL)		Physicochemical parameters (storage time interval)		
			Total soluble solids (°Brix)	pH	Titratable acidity (g/100 g)
Apple	CLEO	0.13	10.82 ±0.11^{Aa}	3.92 ±0.02^{Aa}	0.15 ±0.03^{Aa*}
		0.25	10.83 ±0.09^{Aa}	3.93 ±0.02^{Aa}	0.16 ±0.06^{Aa*}
		0.50	10.81 ±0.13^{Aa}	3.92 ±0.05^{Aa}	0.16 ±0.07^{Aa*}
		Control	10.82 ±0.10^{Aa}	3.94 ±0.02 ^{Aa}	0.17 ±0.06^{Aa*}
Orange	CLEO	0.13	10.56 ±0.13^{Aa}	4.03 ±0.04^{Aa}	1.58 ±0.07^{Aa**}
		0.25	10.59 ±0.11^{Aa}	4.05 ±0.01^{Aa}	1.64 ±0.04^{Aa**}
		0.50	10.62 ±0.09^{Aa}	4.05 ±0.03^{Aa}	1.63 ±0.09^{Aa**}
		Control	10.61 ±0.12^{Aa}	4.06 ±0.03^{Aa}	1.68 ±0.03^{Aa**}
Apple	CREO	0.13	10.92 ±0.10^{Aa}	3.91 ±0.03^{Aa}	0.16 ±0.04^{Aa*}
		0.25	10.90 ±0.08^{Aa}	3.92 ±0.03^{Aa}	0.15 ±0.07^{Aa*}
		0.50	10.92 ±0.12^{Aa}	3.91 ±0.04^{Aa}	0.16 ±0.05^{Aa*}
		Control	10.93 ±0.11^{Aa}	3.95 ±0.01 ^{Aa}	0.16 ±0.08^{Aa*}
Orange	CREO	0.13	10.89 ±0.08^{Aa}	4.04 ±0.03^{Aa}	1.73 ±0.06^{Aa**}
		0.25	10.90 ±0.10^{Aa}	4.04 ±0.02^{Aa}	1.66 ±0.03^{Aa**}
		0.50	10.93 ±0.09^{Aa}	4.02 ±0.03^{Aa}	1.73 ±0.05^{Aa**}
		Control	10.92 ±0.11^{Aa}	4.03 ±0.04^{Aa}	1.69 ±0.02^{Aa**}

EO: essential oil; *Total acidity expressed as malic acid (g /100 g) for apple juice; **Relation of solids soluble in brix/ acidity (g/100 g) of citric acid anhydrous for orange juice; Control: fruit juice not subjected to combined treatment (CLEO or CREO and MHT); Different superscript capital letters in the same row indicate significant difference ($P \leq 0.05$), based on Student's t-test; Different superscript small letters in the same column indicate significant difference ($P \leq 0.05$), based on a Tukey test.

Figure captions

Fig. 1. Calculated *t* values (Y1 - axis) and mean hedonic scores (Y2 - axis) in the function of *Citrus lemon* (A, B) or *Citrus reticulata* (C, D) essential oil concentration (X - axis) for apple (A, C) and orange (B, D) juices. The black dashed line represents the tabulated *t* (t_{tab} = 2.01), and the black circle (○) represents the compromised acceptance threshold. The gray dashed line represents a mean hedonic score of 5 and the gray circle (○) the rejection threshold.

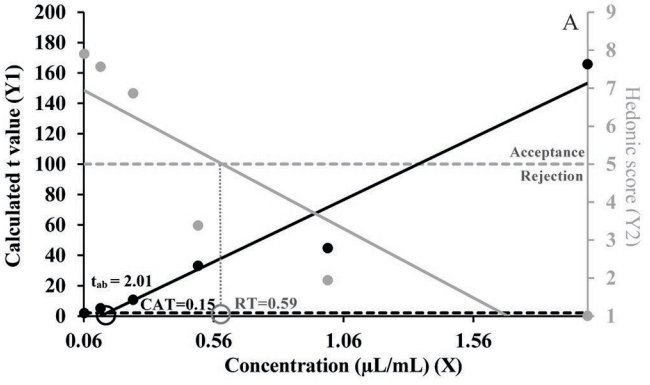
Fig. 2. Log_{10} cycles of inactivation of *Lactobacillus. brevis* (A), *Lactobacillus plantarum* (B) and *Leuconostoc mesenteroides* (C) in apple (A1-C1) and orange (A2-C2) juices at room temperature as a function of exposure time and *Citrus lemon* essential oil concentration: (●) control: 0 μL/mL; (■) 0.13 μL/mL; (▲) 0.25 μL/mL; (▼) 0.50 μL/mL. Data represent the means ± standard deviations (error bars) of at least three independent experiments.

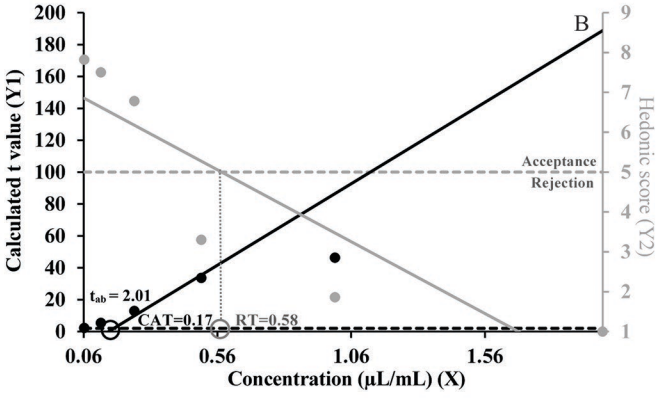
Fig. 3. Log_{10} cycles of inactivation of *Lactobacillus brevis* (A), *Lactobacillus plantarum* (B) and *Leuconostoc mesenteroides* (C) in apple juice (A1-C1) and orange juice (A2-C2) at room temperature as a function of exposure time and *Citrus reticulata* essential oil concentration: (●) control: 0 μL/mL; (■) 0.13 μL/mL; (▲) 0.25 μL/mL; and (▼) 0.50 μL/mL. Data represent the means ± standard deviations (error bars) of at least three independent experiments.

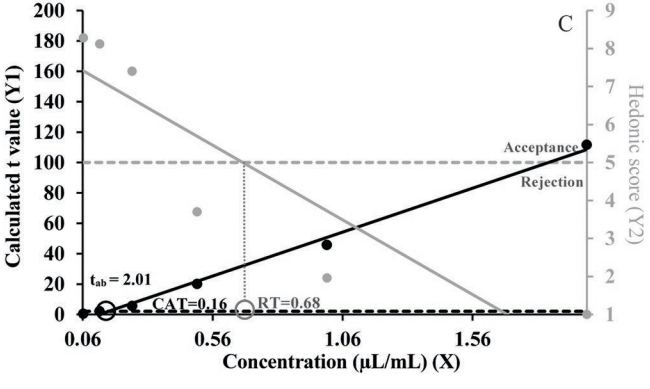
Fig. 4. Survival fraction of *Lactobacillus brevis* after treatment: (●) heated at 54 ºC; (■) *Citrus lemon* (A, B) or *Citrus reticulata* (C, D) essential oil applied at 0.50 μL/mL; and (▲) combined treatment (heat treatment (54 ºC) in the presence of the EO (0.50 μL/mL) in apple (A, C) and orange (B, D) juices. The figure includes the theoretical inactivation curves obtained by considering the lethality caused by the heat and the EO treatment acting separately (additive effect) (□) and the fitting of

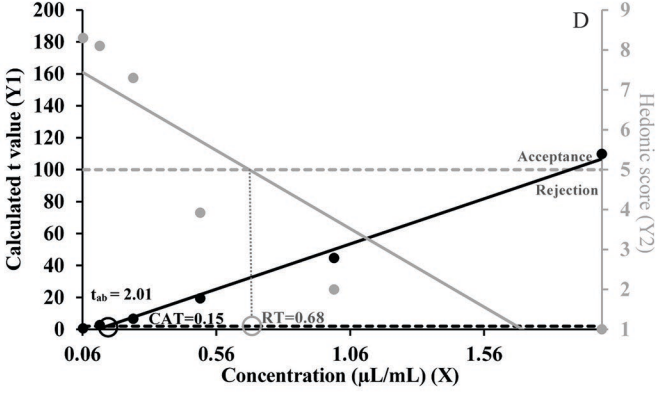
Equation 1 to the survival curves obtained after the combined treatments. Data represent the mean ± standard error of the mean (error bars) of at least three independent experiments.

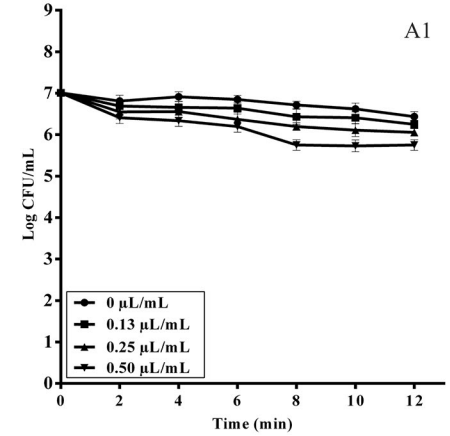
Fig. 5. Relationship between the EO concentration and the log of 3δ values of *Lactobacillus brevis* (●), *Lactobacillus plantarum* (■) and *Leuconostoc mesenteroides* (▲) in apple (A, C) and orange (B, D) juices and heat treated at 54 ºC in the presence of *Citrus lemon* (A, B) and *Citrus reticulata* (C, D) essential oils.

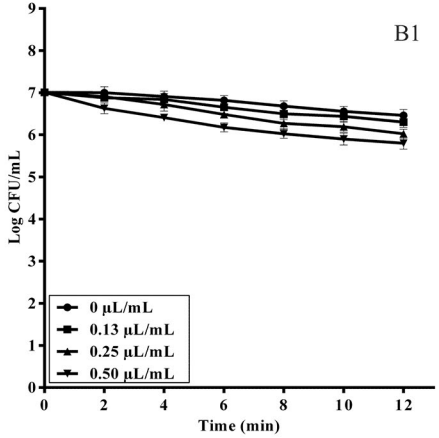

A
Calculated t value (Y1)
Hedonic score (Y2)
Concentration (µL/mL) (X)
Acceptance
Rejection
t_{ab} = 2.01
CAT=0.15
RT=0.59
0.06
0.56
1.06
1.56

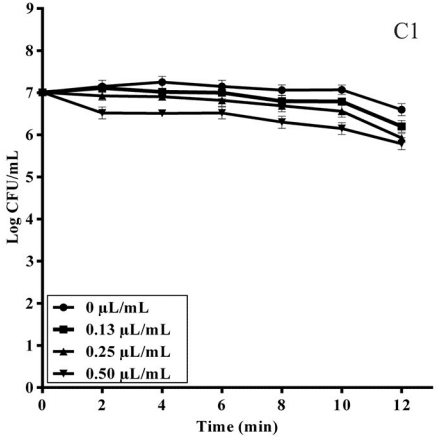

B
Calculated t value (Y1)
Hedonic score (Y2)
Concentration (µL/mL) (X)
Acceptance
Rejection
t_{ab} = 2.01
CAT=0.17
RT=0.58
0.06
0.56
1.06
1.56

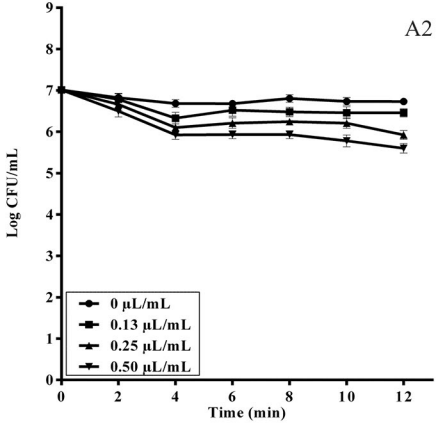

C
Calculated t value (Y1)
Hedonic score (Y2)
Concentration (µL/mL) (X)
Acceptance
Rejection
t_{ab} = 2.01
CAT=0.16
RT=0.68
0.06
0.56
1.06
1.56

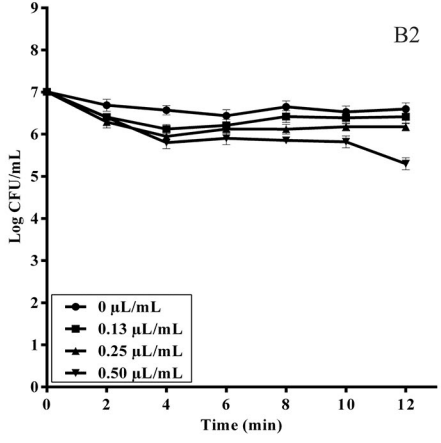

D
Calculated t value (Y1)
Hedonic score (Y2)
Concentration (µL/mL) (X)
Acceptance
Rejection
t_{ab} = 2.01
CAT=0.15
RT=0.68
0.06
0.56
1.06
1.56

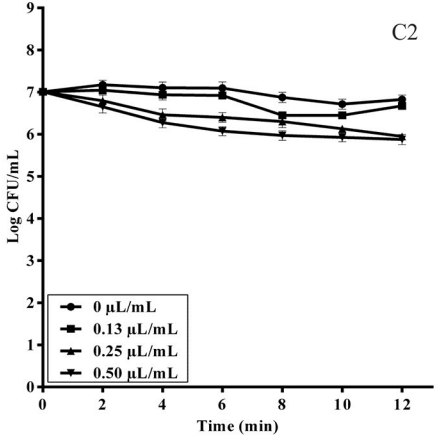

A1
Log CFU/mL
Time (min)
0 μL/mL
0.13 μL/mL
0.25 μL/mL
0.50 μL/mL

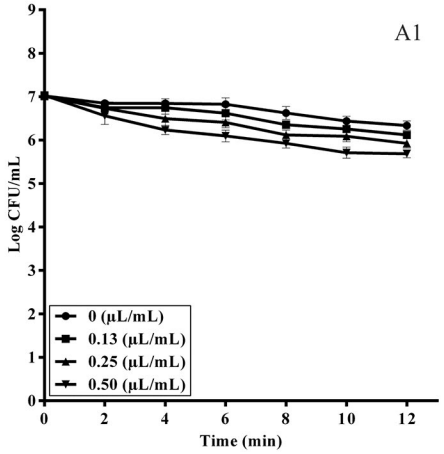

B1
Log CFU/mL
Time (min)
0 μL/mL
0.13 μL/mL
0.25 μL/mL
0.50 μL/mL

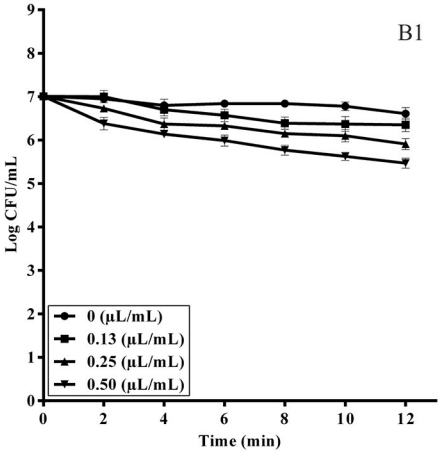

C1
Log CFU/mL
Time (min)
0 μL/mL
0.13 μL/mL
0.25 μL/mL
0.50 μL/mL

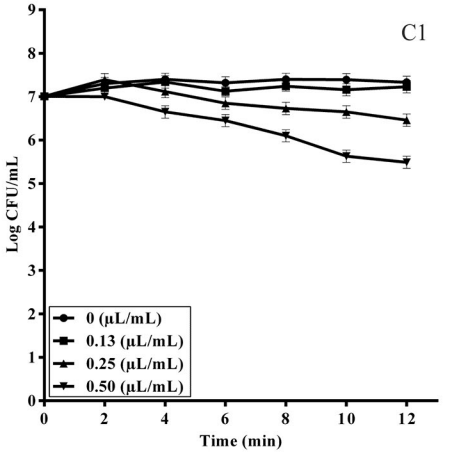

A2
Log CFU/mL
Time (min)
0 μL/mL
0.13 μL/mL
0.25 μL/mL
0.50 μL/mL

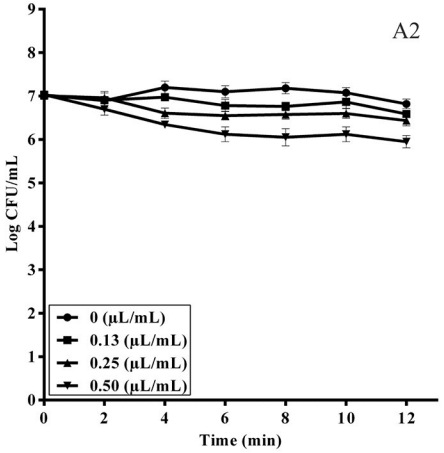

B2
Log CFU/mL
Time (min)
0 μL/mL
0.13 μL/mL
0.25 μL/mL
0.50 μL/mL

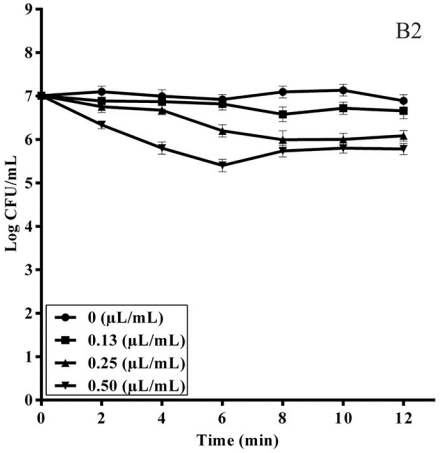

C2
Log CFU/mL
Time (min)
0 μL/mL
0.13 μL/mL
0.25 μL/mL
0.50 μL/mL

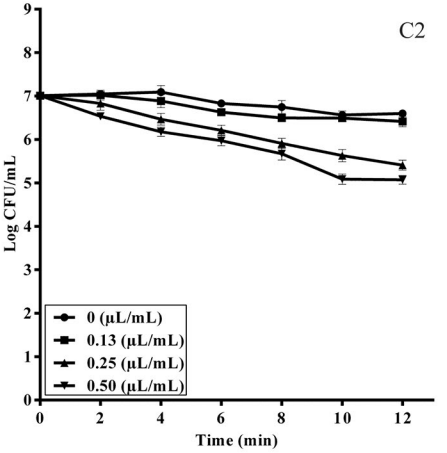

A1
Log CFU/mL
Time (min)
0 (μL/mL)
0.13 (μL/mL)
0.25 (μL/mL)
0.50 (μL/mL)

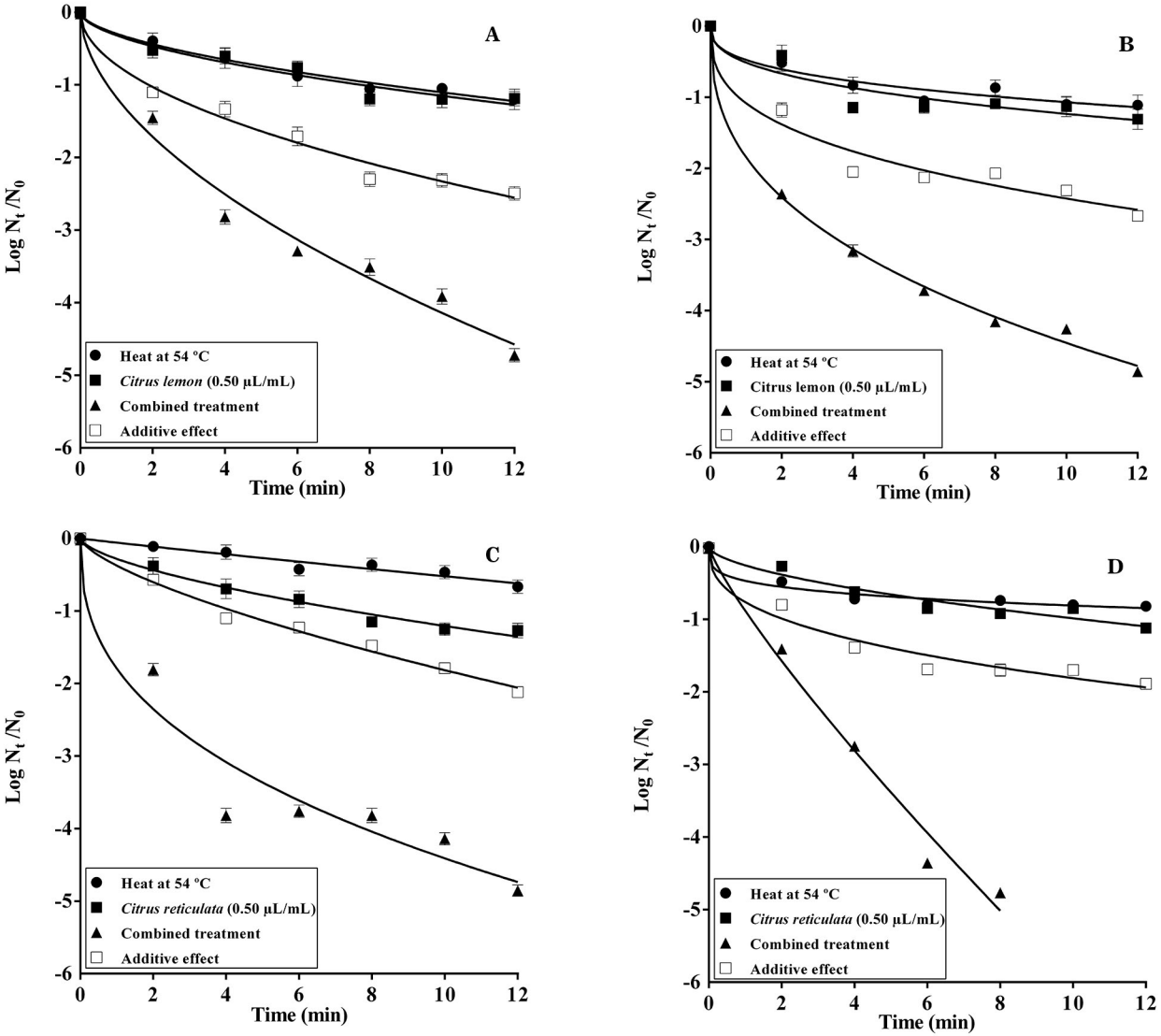

B1
Log CFU/mL
Time (min)
0 (μL/mL)
0.13 (μL/mL)
0.25 (μL/mL)
0.50 (μL/mL)

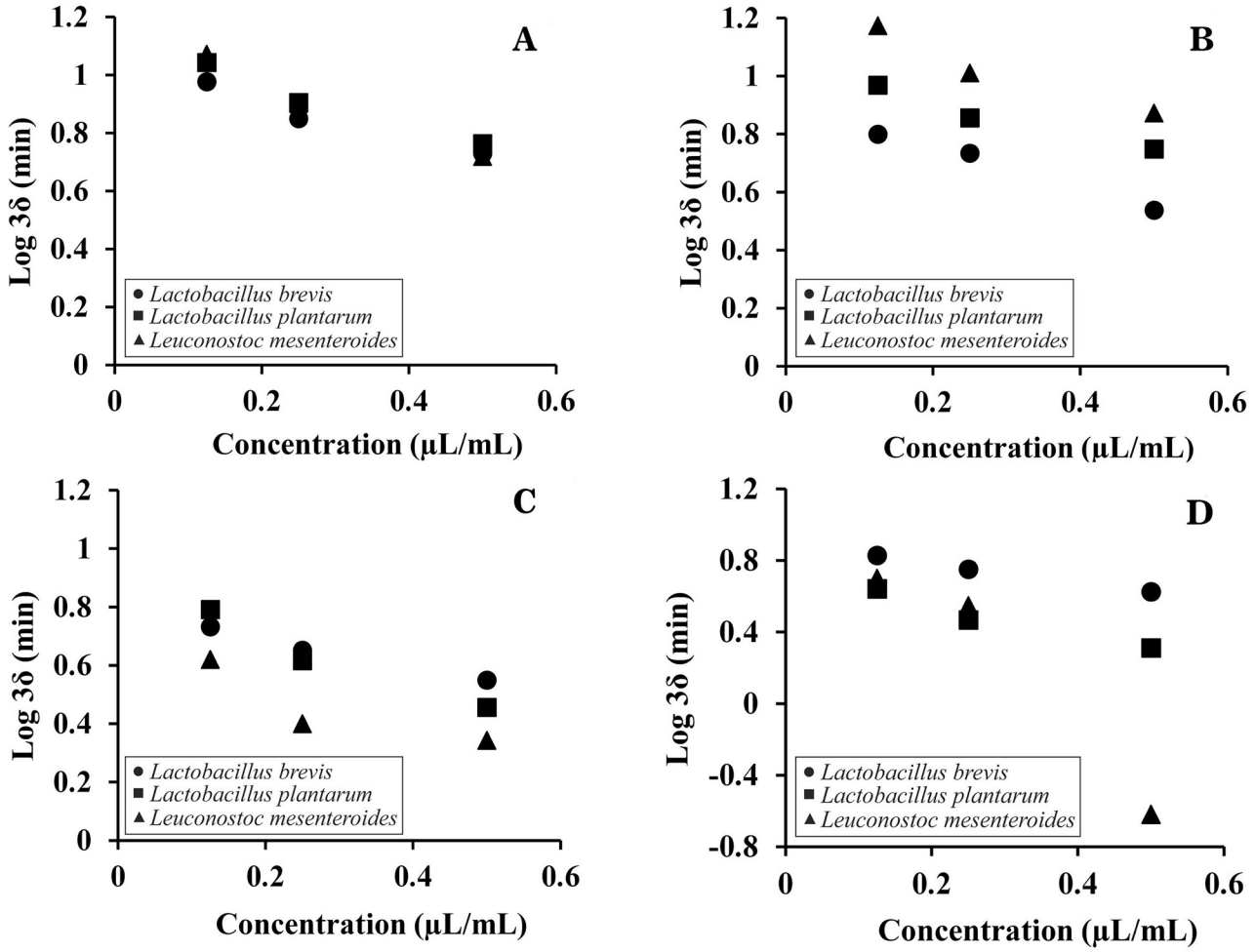

C1
Log CFU/mL
Time (min)
0 (μL/mL)
0.13 (μL/mL)
0.25 (μL/mL)
0.50 (μL/mL)


A2
Log CFU/mL
Time (min)
0 (μL/mL)
0.13 (μL/mL)
0.25 (μL/mL)
0.50 (μL/mL)


B2
Log CFU/mL
Time (min)
0 (μL/mL)
0.13 (μL/mL)
0.25 (μL/mL)
0.50 (μL/mL)


C2
Log CFU/mL
Time (min)
0 (μL/mL)
0.13 (μL/mL)
0.25 (μL/mL)
0.50 (μL/mL)


A
Log N_t/N_0
Time (min)
Heat at 54 °C
Citrus lemon (0.50 µL/mL)
Combined treatment
Additive effect
B
Log N_t/N_0
Time (min)
Heat at 54 °C
Citrus lemon (0.50 µL/mL)
Combined treatment
Additive effect
C
Log N_t/N_0
Time (min)
Heat at 54 °C
Citrus reticulata (0.50 µL/mL)
Combined treatment
Additive effect
D
Log N_t/N_0
Time (min)
Heat at 54 °C
Citrus reticulata (0.50 µL/mL)
Combined treatment
Additive effect


A
B
C
D
Log 3δ (min)
Concentration (µL/mL)
Lactobacillus brevis
Lactobacillus plantarum
Leuconostoc mesenteroides
1.2
1
0.8
0.6
0.4
0.2
0
-0.4
-0.8
0.2
0.4
0.6