TITLE:

Inactivation of spoiling microorganisms in apple juice by a combination of essential oils' constituents and physical treatments

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Abstract

A combination of different hurdles, such as mild heat (54 °C for 10 min) or pulsed electric field (PEF) (25 pulses; 25 kV/cm; 3.35 kJ/cm per pulse) treatments and essential oils (EOs) constituents (carvacrol, citral and (+)-limonene), to reduce spoiling bacteria and yeasts in apple juice was evaluated. For this purpose, the heat and PEF resistances of five strains of *Leuconostoc* spp. and five *Saccharomyces* spp. strains were assayed, achieving different inactivation levels for each treatment and strain. For instance, *L. fallax* 74, the most heat-resistant strain, was the second-most sensitive strain to PEF. The most resistant strains were exposed to combined processes of heat or PEF and 0.2 µL/mL EOs constituents. The combination of heat and EOs constituents proved to be synergistic against both microorganisms in apple juice. The most effective was the combination of mild heat and carvacrol, which caused the inactivation of 99% of *L. fallax* 74 and 99.99% of *S. cerevisiae* CECT 1172 cells. Therefore, this study shows the great potential of carvacrol, citral and (+)-limonene in combined treatments with mild heat to achieve a higher degree of inactivation of spoiling microorganisms in apple juice, and thus, to extend its shelf life.

Keywords

Yeasts, Lactic acid bacteria, Non-thermal preservation technologies, Thermal processing, Fruit juices

Abbreviated short title: Synergistic combined processes to preserve apple juice

INTRODUCTION

Fruit juices are popular drinks due to their sensory and nutritional qualities. They are low in sodium, cholesterol and fat but rich in vitamin C, polyphenols and antioxidants (Lee et al., 2003; Leontowicz et al., 2003). In addition, the regular moderate intake of fruit juice is included in dietary recommendations for healthy eating (Reedy and Krebs-Smith, 2008).

The spoilage of fruit and vegetable juices is primarily due to the proliferation of their natural acid-tolerant and osmophilic microbiota (Tahiri et al., 2006). Fruit juices are generally rich in simple carbohydrates and complex nitrogen sources, and hence are ideal substrates for yeasts, which produce metabolites that negatively affect sensory quality (Patil et al., 2011; Zook et al., 1999). Many authors have reported that juice concentrates, fruit pulps, packed fruit juices and soft drinks are particularly prone to fermentative spoilage by *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and, to a lesser extent, by *Saccharomyces pastorianus* (Arias et al., 2002; Las Heras-Vazquez et al., 2003; Patil, 2001; Patrignani et al., 2009; Sancho et al., 2000;). Moreover, lactic acid bacteria (LAB) have also been implicated in the microbiological spoilage of fruit juices, especially *Lactobacillus* spp. and *Leuconostoc* spp., which cause undesirable butter mill and fermented flavors due to diacetyl and organic acid production, as well as swelling of packages as a result of the production of carbon dioxide (Tajchakavit et al., 1998).

Heat treatment is still the most widely used method for bacterial inactivation in fruit and vegetable juices (De Massaguer et al. 2014; FDA, 2001). However, the thermal pasteurization of apple juice can lead to temperature-dependent reactions with undesirable effects in juices, such as non-enzymatic browning, production of off-

flavors, decreased fresh juice flavor (Aguilar-Rosas et al., 2007; Basak and Ramaswamy, 1996) and reduction of vitamin content (Braddock, 1999; Vikram et al., 2005). Consequently, several alternatives–such as the use of emerging technologies (high hydrostatic pressures, pulsed electric fields, ultraviolet light, etc.), new chemical preservatives, and the development of more effective combined treatments−have been proposed (Guerrero-Beltrán et al., 2009; Mañas and Pagán, 2005).

The use of pulsed electric fields (PEF) has been proposed to inactivate pathogenic and spoiling microbial populations, while avoiding the negative effects of heat treatments (Somolinos et al., 2010a; Timmermans et al., 2014). However, their effectiveness against some pathogenic bacteria, such as *Escherichia coli* 0157:H7 under acid conditions, is limited (Ait-Ouazzou et al., 2013; Iu et al., 2001).

Regarding the use of chemical preservatives, consumer demand has shown a preference for naturally occurring substances, such as plan essential oils (EOs) or their constituents (Burt, 2004). Among the latest, carvacrol, citral and (+)-limonene are common EOs constituents that exist at significant concentrations in many aromatic plants (Ait-Ouazzou et al., 2011; Burt, 2004). As Generally Recognized as Safe (GRAS) food additives, they are used as flavoring agents in the food industry (Dorman and Deans, 2000; Fenaroli and Burdock, 2002), as well as antimicrobials or preservatives in a variety of sanitary, pharmaceutical or make-up products (Bakkali et al., 2008). Nevertheless, the concentration of these compounds needed to achieve a significant antimicrobial effect in food could lead to undesirable organoleptic changes (Burt, 2004).

As a result, and based on the hurdle theory proposed by Leistner and Gorris (1995), many researchers have assessed the simultaneous use of traditional heat or emerging PEF with natural antimicrobials to keep food safety and quality, as well as to

extend food shelf life while lowering treatment temperatures and/or antimicrobial doses, or increasing PEF effectiveness (Burt, 2004; Corbo et al., 2009; Periago et al., 2001; Raybaudi-Massilia et al., 2009). Recently, our research group has observed outstanding synergistic effects when exploring the simultaneous action of mild heat and EOs, or their constituents against pathogenic bacteria in apple juice (Ait-Ouazzou et al., 2011; 2013; Espina et al., 2010; 2012; Somolinos et al., 2010b). Among EOs constituents, carvacrol, citral and (+)-limonene have been demonstrated to be the most effective antimicrobials, whether acting alone or in combined treatments (Ait-Ouazzou et al., 2011; Somolinos et al., 2010a). The use of EOs constituents instead of EOs has been suggested in order to avoid the interference of the multiple aromatic compounds present in EOs extracts that might affect the organoleptic properties of food. On the other hand, the effectiveness of the combination of these substances and PEF has shown to be dependent on the microorganism investigated (Ait-Ouazzou et al., 2011; Arroyo et al., 2010), thus not allowing extrapolation of conclusions to other microorganisms.

Despite the intensive research carried out on foodborne pathogens, to the best of our knowledge, scarce information is available on the effectiveness of the combination of mild heat or PEF and these EOs constituents to inactivate the most common spoiling microorganisms, yeasts and LAB in single-strength apple juice.

Therefore, the objectives of this work were to evaluate: a) the resistance to mild heat and PEF treatments of *Saccharomyces* spp. and *Leuconostoc* spp. suspended in apple juice; and b) the effectiveness of a combined process, based on the simultaneous use of three EOs constituents (carvacrol, citral and (+)-limonene) and mild heat or PEF treatments against the most resistant yeast and LAB strains, suspended in apple juice.

MATERIALS AND METHODS

Microorganisms and growth conditions

The strains of *Saccharomyces* spp. (*S. cerevisiae* CECT 1170, *S. cerevisiae* CECT 1172, *S. cerevisiae* CECT 1996, *S. cerevisiae* CECT 11034, *S. bayanus* CECT 11185) and *L. mesenteroides* subsp. *mesenteroides* CECT 394 used in this investigation were supplied by the Spanish Type Culture Collection. *Leuconostoc* spp. 75, *L. mesenteroides* subsp. *mesenteroides* 67, *L. fallax* 74 and *L. lactis* 88 were naturally occurring strains in food that were isolated and identified by Dr. Arvizu (Universidad Autónoma de Querétaro, Mexico). The cultures were maintained in cryovials at −80 °C.

Microbial subcultures were prepared by inoculating, with one single colony from a plate a test tube containing 5 mL of MRS (De Man, Rogosa and Sharpe) broth (Oxoid, Basingstoke, Hampshire, England) for *Leuconostoc*, or Sabouraud broth (Biolife, Milan, Italy) for *Saccharomyces*. After inoculation, the tubes were incubated for 24 h at 30 °C. 250-mL Erlenmeyer flasks containing 50 mL of MRS broth or Sabouraud broth were inoculated with 100 µL of these subcultures. These flasks were incubated under agitation (130 rpm) (Selecta, mod. Rotabit, Barcelona, Spain) at 30 °C until the stationary growth phase was reached (24 h for *Leuconostoc* and 48 h for *Saccharomyces* spp. strains).

Evaluation of microbial inactivation by EOs constituents

Carvacrol (98%), citral (95%) and (+)-limonene (97% purum) were purchased from Sigma-Aldrich (Steinheim, Germany). A vigorous shaking method by vortex (Genius 3, Ika, Königswinter, Germany) agitation was used to prepare suspensions in apple juice following the previously described procedure (Friedman et al., 2002). A commercial

clear shelf-stable apple juice (Don Simón, Murcia, Spain) was purchased from a local supermarket (pH 3.58, conductivity 1.85 mS/cm).

Cells from stationary-phase cultures were added at approximately 2×10^6 CFU/mL to apple juice with 0.2 µL/mL of carvacrol, citral or (+)-limonene. The apple juice's pH was not modified as a consequence of adding EOs constituents. Before treatment, microorganisms were washed once with sodium chloride solution 0.85% w/v, centrifuged and resuspended in apple juice, where they were left for 20 min at 4 °C, so that the cells were adapted to the treatment media. Carvacrol, citral and (+)-limonene treatments were carried out at 20 °C for 10 min. Samples were taken, and the survivors were enumerated. Previous experiments showed that untreated cells of the strains used in this study at concentrations of 2×10^6 CFU/mL were insensitive to incubation for 10 min at 20 °C in apple juice.

Measurement of cell inactivation by heat treatments and EOs constituents

Survival data for heat and combined treatments were obtained in an incubator (FX Incubator, Ref ZE/FX, from ZEULAB, Zaragoza, Spain) at 54 °C with a thermocouple (Ahlborn, mod. Almemo 2450, Holzkirchen, Germany) to monitor the temperature during the heat treatment. Once the temperature had stabilized, 50 µL of an adequately diluted cell suspension were added into a sterile tube containing 450 µL of the treatment media. The initial bacterial concentration was approximately 2×10^6 CFU/mL. The treatment media included apple juice and apple juice with carvacrol, citral or (+)-limonene (0.2 µL/mL). After 10 min, samples were taken and the survivors were enumerated.

Measurement of cell inactivation by PEF treatments and EOs constituents

The survival data for the PEF treatments and combined treatments were collected using equipment that delivered exponential-decay pulses, as previously described (García et al., 2005), which were provided with a parallel-electrode treatment chamber, with a distance of 0.25 cm between electrodes and an area of 2.01 cm^2.

Before treatment, the microorganisms were likewise centrifuged and resuspended at a final concentration of approximately 2×10^6 CFU/mL in apple juice, as well as in this treatment media with carvacrol, citral or (+)-limonene added to a final concentration of 0.2 µL/mL. Next, 0.5 mL of the samples were placed into the treatment chamber with a sterile syringe. Exponential waveform pulses at electrical field strengths of 25 kV/cm, corresponding to a specific energy of 3.35 kJ/kg per pulse, and a pulse repetition rate of 1 Hz were used in this study. The experiments started at room temperature. In all the experiments, the temperature of the samples after the application of 25 pulses was lower than 35 °C. After treatment, samples were taken and the survivors were enumerated.

Counts of viable cells and detection of sublethal injury

After the treatments, the samples were adequately diluted in 0.1% w/v peptone water (Biolife). Next, 0.1 mL samples were pour-plated onto MRS agar (Oxoid), for *Leuconostoc*, or Potato Dextrose Agar (PDA; Oxoid), for *Saccharomyces* spp. In order to determine the microbial cell injuries in the cytoplasmic membrane, treated samples were also plated onto agar containing the maximum concentrations of sodium chloride that caused no reduction in the colony counts of untreated cells for each of the strains used in this study: 5% for *Leuconostoc* spp. 75, *L. lactis* 88 and *L. mesenteroides* CECT 394; 6% for *L. mesenteroides* 67, *L. fallax* 74, *S. cerevisiae* CECT 1996 and *S. cerevisiae* CECT 1172; and 7% for *S. cerevisiae* CECT 11034, *S. cerevisiae* CECT

1170 and *S. bayanus* CECT 11185. The extent of the sublethal injury in a population of treated cells was expressed as the difference between the log_{10} count (CFU) on a non-selective medium (MRS agar or PDA) and the log_{10} count on a selective medium (MRS agar-SC or PDA-SC). According to this representation, "two log_{10} of injury" means a 2 log_{10} difference in the counts of selective and non-selective media or that 99% of survivors were sublethally injured. As indicated in the figures, the detection limit corresponds to the inactivation of four log_{10} cycles (99.99% of the initial population).

Data analysis

Inactivation was expressed in terms of the extent of reduction, in log_{10} counts, after every treatment. The error bars in the figures indicate the mean ± standard deviations from the data obtained from at least three independent experiments. ANOVA and t-tests were performed with GraphPad PRISM® (GraphPad Software, Inc., San Diego, USA) and differences were considered significant if $p \leq 0.05$

RESULTS

Inactivation of *Leuconostoc* spp. by heat and PEF treatments

Under the treatment conditions assayed, a great variation in resistance was observed as a function of the strain. Figure 1 shows the inactivation of five strains of *Leuconostoc* spp. in apple juice after heat (54 °C for 10 min) (Figure 1a) and PEF (25 pulses at 25 kV/cm) (Figure 1b) treatments, evaluated in non-selective and selective media. *L. mesenteroides* subsp. *mesenteroides* CECT 394 was the most sensitive strain to both treatments (p<0.05). The heat treatments inactivated more than 4 log_{10} cycles of its initial population, while the PEF treatments achieved more than 1 log_{10} cycle of bacterial inactivation, as assessed by the recovery of survivors in a non-selective medium. A comparison of the survival counts in both recovery media showed that 1.5 log_{10} cycles of survivors were sublethally injured at the cytoplasmic membrane. One of the most heat-resistant strain was *L. fallax* 74 (p<0.05), which showed less than 0.5 log_{10} cycles of inactivation. However, the proportion of sublethally injured bacterial cells after heat treatments in this strain was nearly 2 log_{10} cycles of the surviving population. The most PEF-resistant strains (p<0.05) were *Leuconostoc* spp. 75 and *L. lactis* 88, which showed similar resistance (p>0.05), with less than 1 log_{10} cycles of inactivation after PEF treatments.

Inactivation of *Saccharomyces* spp. by heat and PEF treatments

In order to obtain comparable data for *Leuconostoc* spp. and *Saccharomyces* spp., the intensities of the applied treatments were the same for both microbial groups. Figure 2 shows the levels of inactivation for five strains of *Saccharomyces* in apple juice after heat (Figure 2a) and PEF (Figure 2b) treatments, as determined after plating the treated

cells in both non-selective and selective media. A comparison of Figures 1 and 2 demonstrates that the yeasts were more sensitive than bacteria to both heat (p<0.05) and, especially, PEF treatments (p<0.05). The five strains of *Saccharomyces* spp. were even more sensitive to PEF than the most sensitive strain of *Leuconostoc* spp. (*L. mesenteroides* subsp. *mesenteroides* CECT 394) (p<0.05). The two most heat-resistant strains (p<0.05) were *S. cerevisiae* CECT 1172 and *S. cerevisiae* CECT 1170, with similar degrees of inactivation (p>0.05) of around 1–2 $\log_{10}$ cell cycles. Nevertheless, a slightly higher proportion of surviving cells with sublethally injuries at their cytoplasmic membranes was detected in *S. cerevisiae* CECT 1172 than in *S. cerevisiae* CECT 1170. On the other hand, the less heat-resistant strains (p<0.05) were *S. cerevisiae* CECT 11034 and *S. bayanus* CECT 11185 which showed more than 4 $\log_{10}$ cycles of inactivation, exceeding the detection limit. However, *S. bayanus* CECT 11185 was one of the most resistant strains to PEF treatments among the five yeasts strains studied. Notably, the PEF treatments did not cause sublethal injuries at the cytoplasmic membranes of the *Saccharomyces* strains (p>0.05) (Figure 2b), contrarily to what was observed for most of the *Leuconostoc* strains (Figure 1b).

Inactivation of *Leuconostoc* spp. by combined processes

The most resistant strain to the physical treatment, either with heat or PEF treatment, was selected to study the effectiveness of the combined processes of heat or PEF with EOs constituents. Thus, one of the most heat resistant strain (Figure 1a), *L. fallax* 74, was chosen for combining the EOs constituents with heat; and *Leuconostoc* spp. 75, which showed the highest PEF resistance (Figure 1b) for the combined process evaluation including PEF.

Figure 3 shows the inactivation of the selected strains by the treatments acting alone (carvacrol, citral, (+)-limonene and heat (Figure 3a) or PEF (Figure 3b)), and by combining these hurdles. For both strains, the inactivation level after 10 min at room temperature caused by 0.2 µL/mL of any EOs constituent was less than 0.5 $\log_{10}$ cycles, independently of the recovery media used. However, the combination of heat and EOs constituents (Figure 3a) showed a highly synergistic lethal effect ($p<0.05$) against *L. fallax* 74 suspended in apple juice. Compared to the sum of the lethality reached by heat and EOs constituents acting separately, an extra-inactivation of *ca.* 1.5 $\log_{10}$ cycles was observed when applying both technologies simultaneously.

Recovery in selective medium also showed a synergistic effect ($p<0.05$) for the combination of heat and carvacrol or (+)-limonene, with survival counts exceeding our detection limit, as well as an additive effect for heat combined with citral (Figure 3a). Interestingly, the levels of inactivation by the combined process of heat and EOs constituents, as determined in a non-selective medium, were similar ($p>0.05$) to those determined in a selective medium after heat treatments with no added constituents (Figure 3a).

In contrast, the combination of PEF and citral or (+)-limonene (Figure 3b) showed additive effects against *Leuconostoc* spp. 75 in both recovery media. Only the combination of PEF and carvacrol acted synergistically ($p<0.05$), although the additional inactivation achieved was lower than 1 $\log_{10}$ cell cycles.

Inactivation of *Saccharomyces* spp. by combined processes

Based on the higher resistance criterion *S. cerevisiae* CECT 1172 strain was chosen for the study of the effectiveness on the simultaneous application of heat and EOs

constituents, as well as *S. bayanus* CECT 11185 for the combined process of PEF with EOs constituents.

The inactivation of *S. cerevisiae* CECT 1172 by carvacrol, citral or (+)-limonene for 10 min at room temperature was lower than 1 $\log_{10}$ cycle (Figure 4a). Interestingly, the proportion of sublethally injured cells after treatment with these EOs constituents was *ca.* 2 or 3 $\log_{10}$ cycles after carvacrol and (+)-limonene, or citral treatments as determined by the difference in survival counts between the non-selective and selective media (Figure 4a). The survival counts in the non-selective medium showed a synergistic ($p<0.05$) effect when combining heat and (+)-limonene or carvacrol since the inactivation achieved was almost 1 or 2 $\log_{10}$ cycles higher, respectively, than the sum of both hurdles acting separately. In contrast, the combination of heat and citral showed an additive effect rather than a synergistic effect, since simultaneous application of both technologies did not cause any additional inactivation, with regard to their separated application.

On the other hand, the combination of PEF and EOs constituents (Figure 4b) did not increase either inactivation or injury levels ($p>0.05$) of *S. bayanus* CECT 11185, in comparison to inactivation by these treatments separately.

DISCUSSION

The first step in evaluating an alternative to traditional food preservation methods is to assure its ability for inactivating pathogenic microorganisms. Then, the following stage is to study resistance of the most common spoiling microorganisms. Accomplishing both objectives will allow safe food to be offered with a prolonged shelf-life. In the last several years, many studies have confirmed the efficacy of new combined processes against different microorganisms, based on the simultaneous application of EOs or their main constituents and heat (mild temperatures) or new emerging technologies, such as PEF (Ait-Ouazzou et al., 2011; Belletti et al., 2010; Bevilacqua et al., 2013; Char et al., 2009; Espina et al., 2011; Periago et al., 2006). Our research group has assessed the synergism between these technologies or hurdles on food, especially on fruit juices such as apple or orange juice, achieving the inactivation of more than 5 log_{10} cycles of the most resistant pathogen, *E. coli* O157:H7 (Ait-Ouazzou et al., 2011; Espina et al., 2010; 2011; 2012; Somolinos et al., 2010a). These results suggest the potential of this approach for inactivating common spoiling microorganisms in apple juice, such as *Leuconostoc* spp. and *Saccharomyces* spp.

Microorganisms, or even strains of the same microorganism, do not show highly or poorly resistance to all inactivation technologies (Mañas and Pagán, 2005). Therefore, a wide range of different strains should be studied in order to prove the effectiveness of a new method for microbial inactivation in a target microorganism.

Most *Leuconostoc* spp. strains were more resistant than *Saccharomyces* spp. strains, which is in agreement with the general assumption that bacteria are more resistant than yeasts to heat and other physical technologies, including PEF (Jay et al., 2008; Mañas and Pagán, 2005); however, there were exceptions when comparing their

heat resistance, since the yeasts *S. cerevisiae* CECT 1172 and CECT 1170 exhibited higher heat ($p<0.05$) resistances than the bacterium *L. mesenteroides* subsp. *mesenteroides* CECT 394.

On the other hand, the group of strains of *Leuconostoc* spp. tested in this study showed diverse susceptibility to both heat and PEF treatments. In this regard, it was noticeable that *L. fallax* 74, one of the most heat-resistant strains, was the second most PEF-sensitive (Figure 1). The fact that one strain ranks differently when comparing the resistance of a group of strains to heat and to PEF treatments would confirm the different mechanisms of action involved in these two technologies (Mañas and Pagán, 2005). Therefore, a different strain was necessarily selected as the target microorganism for the development of combined processes with each physical technology—heat and PEF. For this study, and in order to be able to compare the results on spoiling microorganisms with previous results on pathogenic microorganisms (Ait-Ouazzou et al., 2011; 2013; Espina et al., 2010; 2011; 2012; Somolinos et al., 2010a), a dose of 0.2 μL/mL of each EOs constituent was assayed in the combined treatments.

In synergistic combinations of two hurdles, the overall inactivating effect was greater than the sum of the inactivation achieved by each hurdle acting alone (Leistner and Gorris, 1995). Synergistic lethal effects were observed when combining mild heat and each EOs constituent against *L. fallax* 74, causing the inactivation of 99% of the cells suspended in apple juice. The synergistic effects were probably due to the occurrence of sublethal injuries in heat- or PEF-treated cells, which would facilitate the interaction of antimicrobials with the cytoplasmic membrane as the primary site of action, or their access into the cytoplasm to reach other key targets, leading to cell death (Prashar et al., 2003). Nevertheless, the combinations of heat and carvacrol or (+)-limonene were more effective than that with citral because 2 additional $\log_{10}$ cycles of

survivors were sublethally injured as a result of the first combination. In these cases, the EOs constituents not only caused the inactivation of the cells that were sublethally injured by heat, but also contributed to increasing the occurrence of injured cells. This sublethally injured population might be sensitive to the acidic environmental conditions of apple juice during subsequent storage, being responsible for its death (Espina et al., 2010; Somolinos et al., 2008). On the contrary, the study of the effectiveness of heat and EOs constituents against *S. cerevisiae* CECT 1172 demonstrated that not all the EOs constituents were similarly active against yeasts because the combination of heat and citral did not show any synergistic or additive effect (p>0.05). The most efficient and effective combination against *S. cerevisiae* CECT 1172 was heat and carvacrol, causing the inactivation of more than 4 $\log_{10}$ cells cycles, exceeding our detection limit.

Despite previous findings about the effectiveness of the combination of PEF and citral against *Cronobacter sakazakii* (Arroyo et al., 2010), none of the combinations of PEF and EOs constituents assayed showed any synergism. These results might be explained by the absence of sublethally injured cells after PEF treatments (Somolinos et al., 2008; Arroyo et al., 2010).

In brief, the most effective combined process to inactivate the most resistant *Leuconostoc* spp. and *Saccharomyces* spp. strains in apple juice, among the six alternative treatments explored, was the combination of mild heat and carvacrol, which caused the inactivation of 2 $\log_{10}$ cycles of *L. fallax* 74, as well as induced sublethal injuries to 2 extra $\log_{10}$ cell cycles, and the inactivation of more than 4 $\log_{10}$ cycles of *S. cerevisiae* CECT 1172. Under the same experimental conditions, Ait-Ouazzou et al. (2013) demonstrated the inactivation of more than 5 $\log_{10}$ cycles of *Escherichia coli* O157:H7 suspended in apple juice. Consequently, this combined process seems to be an interesting alternative to extend the shelf life of a safe food product at mild heat

treatment intensity, which would diminish the undesirable effects of heat on food quality. Further research to optimize the treatment conditions would be required in order to study the influence of the treatment temperature and time on the effectiveness of the combined treatment. Also, a reduction of the carvacrol dose would be required to avoid undesirable changes in the sensory attributes of the juice (Ait-Ouazzou et al., 2013; Burt, 2004). In this regard, Ait-Ouazzou et al. (2013) demonstrated that the reduction of microbial contamination from 2×10^7 CFU/mL to 2×10^4 CFU/mL, mimicking the initial conditions before the hygienization of fruit juices (Stratford et al., 2000), would cause a reduction of the carvacrol dose by 3 times, while maintaining its synergistic effect in combination with heat. The influence of these combined treatments on the sensory attributes of apple juice has been previously described (Espina et al., 2012).

In conclusion, this study shows the great potential of carvacrol, citral and (+)-limonene in combined treatments with mild heat to achieve a higher degree of inactivation of spoiling microorganisms in apple juice, and thus, to extend its shelf life. In contrast, none of the assayed combinations of PEF and EOs constituents showed any promising synergism against the spoiling microorganisms assayed.

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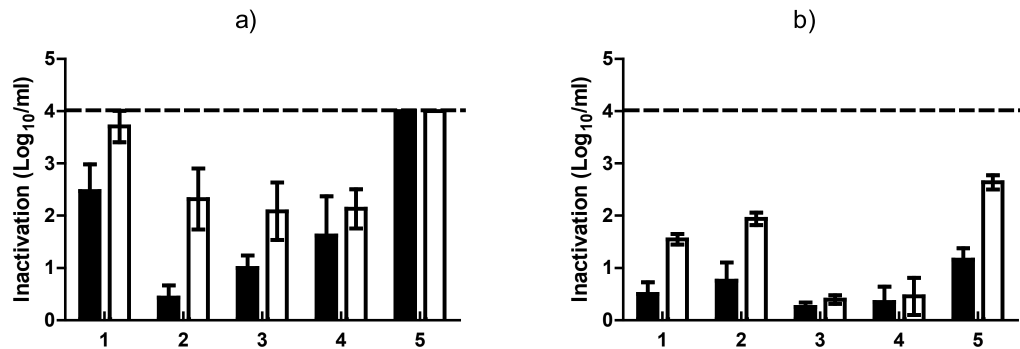

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7 **Fig. 1.** Inactivation of five strains of *Leuconostoc* spp. using heat (a) and PEF (b). Cell
8 suspensions were suspended in apple juice and exposed to the following treatments: 54
9 °C for 10 min (heat) or 25 pulses at 25 kV/cm (PEF). The strains used were *L.*
10 *mesenteroides* 67 (1), *L. fallax* 74 (2), *Leuconostoc* spp. 75 (3), *L. lactis* 88 (4), *L.*
11 *mesenteroides* CECT 394 (5). Treated cells were recovered on the non-selective MRS
12 agar (black bar) and the selective MRS agar-SC (white bar). Data are means±standard
13 deviations (error bars). The dotted line represents the detection limit (four log_{10} cycles).

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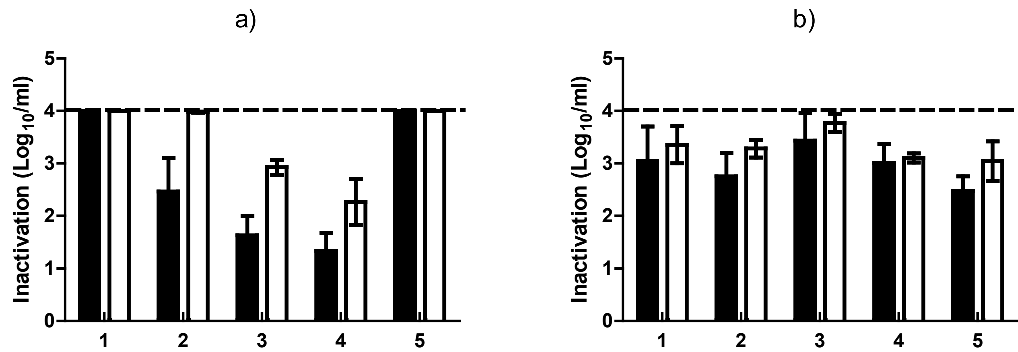

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22 **Fig. 2.** Inactivation of five strains of *Saccharomyces* spp. using heat (a) and PEF (b).
23 Cell suspensions were suspended in apple juice and exposed to the following
24 treatments: 54 °C for 10 min (heat) or 25 pulses at 25 kV/cm (PEF). The strains used
25 were *S. cerevisiae* CECT 11034 (1), *S. cerevisiae* CECT 1996 (2), *S. cerevisiae* CECT
26 1172 (3), *S. cerevisiae* CECT 1170 (4), *S. bayanus* CECT 11185 (5). Treated cells were
27 recovered on the non-selective PDA (black bar) and the selective PDA-SC (white bar).
28 Data are means±standard deviations (error bars). The dotted line represents the
29 detection limit (four log_{10} cycles).

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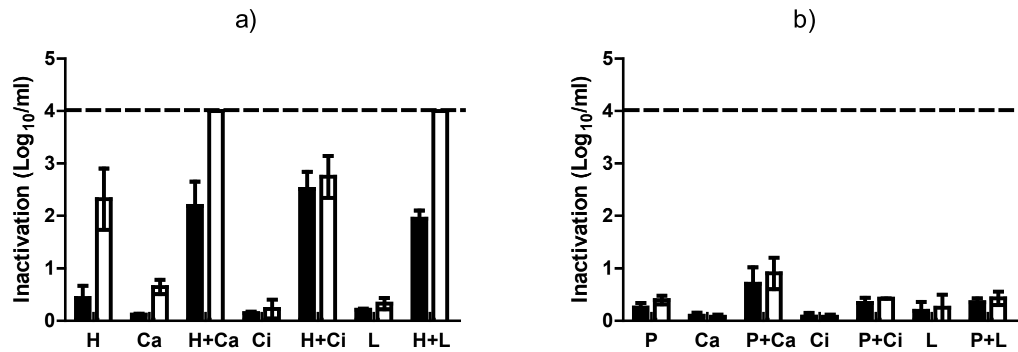

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38 **Fig. 3.** Inactivation of *L. fallax* 74 (a) or *Leuconostoc* spp. 75 (b) by heat (H), PEF (P),
39 EO constituents (carvacrol (Ca), citral (Ci) and (+)-limonene (L)) and combined
40 treatment of heat or PEF with each EO constituent. Cells were suspended in apple juice
41 and exposed to the following treatments: 54 °C for 10 min (heat), 25 pulses at 25 kV/cm
42 (PEF), 0.2 μL/mL of carvacrol, citral or (+)-limonene and combined treatments applied
43 simultaneously. Treated cells were recovered on the non-selective MRS agar (black bar)
44 and the selective MRS agar-SC (white bar). Data are means±standard deviations (error
45 bars). The dotted line represents the detection limit (four log_{10} cycles).

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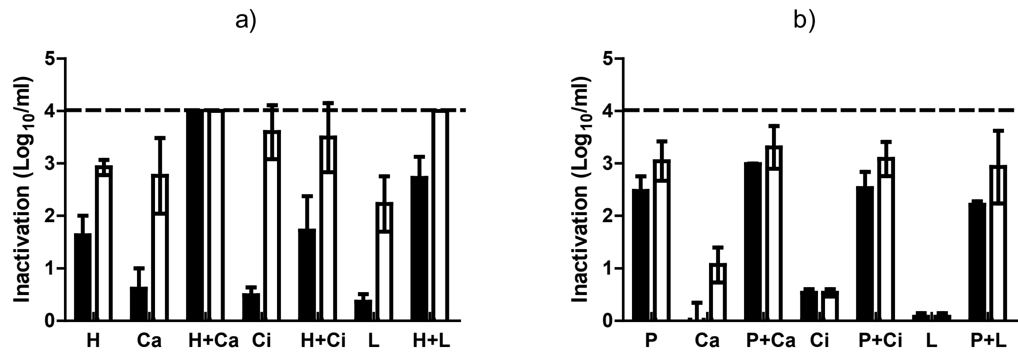

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54 **Fig. 4.** Inactivation of *S. cerevisiae* CECT 1172 (a) or *S. bayanus* CECT 11185 (b) by
55 heat (H), PEF (P), EO constituents (carvacrol (Ca), citral (Ci) and (+)-limonene (L)) and
56 combined treatment of heat or PEF with each EOs constituent. Cells were suspended in
57 apple juice and exposed to the following treatments: 54 °C for 10 min (heat), 25 pulses
58 at 25 kV/cm (PEF), 0.2 µL/mL of carvacrol, citral or (+)-limonene and combined
59 treatments applied simultaneously. Treated cells were recovered on the non-selective
60 PDA (black bar) and the selective PDA-SC (white bar). Data are means±standard
61 deviations (error bars). The dotted line represents the detection limit (four $\log_{10}$ cycles).

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