Food Microbiology 46 (2015) 635–642

Contents lists available at ScienceDirect

Food Microbiology

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

# Study of the inactivation of spoilage microorganisms in apple juice by pulsed light and ultrasound

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ARTICLE INFO

*Article history:*
Received 13 November 2013
Received in revised form
8 April 2014
Accepted 17 June 2014
Available online 1 July 2014

*Keywords:*
Ultrasound
Pulsed light
Hurdle technology
Fruit juices
Microbiological stability

ABSTRACT

The aim of this study was to evaluate the effect of ultrasound (US) (600 W, 20 kHz and 95.2 μm wave amplitude; 10 or 30 min at 20, 30 or 44 ± 1 °C) and pulsed light (PL) (Xenon lamp; 3 pulses/s; 0.1 m distance; 2.4 J/cm$^2$–71.6 J/cm$^2$; initial temperature 2, 30, 44 ± 1 °C) on the inactivation of *Alicyclobacillus acidoterrestris* ATCC 49025 spores and *Saccharomyces cerevisiae* KE162 inoculated in commercial (pH: 3.5; 12.5 °Brix) and natural squeezed (pH: 3.4; 11.8 °Brix) apple juices. Inactivation depended on treatment time, temperature, microorganism and matrix. Combination of these technologies led up to 3.0 log cycles of spore reduction in commercial apple juice and 2.0 log cycles in natural juice; while for *S. cerevisiae*, 6.4 and 5.8 log cycles of reduction were achieved in commercial and natural apple juices, respectively. In natural apple juice, the combination of US + 60 s PL at the highest temperature build-up (56 ± 1 °C) was the most effective treatment for both strains. In commercial apple juice, US did not contribute to further inactivation of spores, but significantly reduced yeast population. Certain combinations of US + PL kept on good microbial stability under refrigerated conditions for 15 days.



## 1. Introduction

Consumers are increasingly aware of health benefits and risks associated with food consumption and tend to opt for healthy foods that have been subjected to less extreme treatments (less heat and chill damage), with lower levels of salts, fats, acids, and sugars and/or the complete or the partial removal of chemically synthesized additives. Thermal processing ensures the safety and shelf life of fruit juices, but can result in the loss of this claimed sensory and nutritional quality. In the last decade, emerging preservation procedures have been included as hurdles in combined preservation systems to ensure food safety and to retain or improve food quality (Alzamora et al., 2011). This type of processing techniques for food preservation reduce microbial load and at the same time, allow better retention of product flavour, texture, colour and nutrient content than comparable conventional treatments. There is a wide range of novel alternative physical agents, intensely investigated in the last 25 years, which can cause inactivation of microorganisms at ambient or sublethal temperatures (*i.e.*, high hydrostatic pressure, pulsed electric fields (PEF), ultrasound (US), pulsed light (PL), and ultraviolet light (UV), among others). The choice of non-thermal hurdles involved in the combined processes depends on the target within the microbial cells (e.g. cell membrane, DNA or enzymes system) or the extrinsic environment surrounding them (e.g. pH, temperature or water activity).

Because of its acidity, fruit juices were thought to be spoiled by yeasts, predominantly *Saccharomyces* spp. (Pontius et al., 1997; Martinez et al., 2000); moulds like *Aspergillus ochraceus*, and lactic acid bacteria like *Lactobacillus* and *Leuconostoc* spp. (Stratford et al., 2000) However, *Alicyclobacillus acidoterrestris* is a thermoacidophilic, heat-resistant bacteria that is capable of surviving the pasteurization procedures normally applied to fruit juices (Bahçeci et al., 2005). Therefore, the endospores can germinate and increase in products to cell concentrations high enough to produce taint compounds (Smit et al., 2010). Few scientific studies focus on the effects of emerging preservation technologies to inactivate *A. acidoterrestris*. Baysal et al. (2013) examined the efficiency of UV-C (0.5 J/cm$^2$) at constant depth (0.15 cm), as an alternative to thermal treatment, on the inactivation of *A. acidoterrestris* spores in fruit juices. They reported significant population reductions in white grape (5.5 log-cycles) and apple (2.1 log-cycles) juices.

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http://dx.doi.org/10.1016/j.fm.2014.06.017
0740-0020/

Among physical hurdles, US (*i.e.* intensities higher than 1 W/cm$^2$; frequencies between 18 and 100 kHz) has attracted considerable interest in food preservation applications (Knorr et al., 2004; Alzamora et al., 2011). The lethal effect of US has been attributed to the cavitation phenomenon, in which microbubbles of gas and/or vapour formed within a liquid, undergo violent collapse during the compression cycle of the wave (Guerrero et al., 2001, 2005). The mechanism of microbial inactivation is mainly due to the thinning of cell membranes, localized heating and production of free radicals (Butz and Tauscher, 2002; Alzamora et al., 2011). The use of ultrasonic waves as a unique preservation factor is unable to effectively kill all the microorganisms since the high levels needed of US could adversely modify nutritional and sensory properties of the food. This limitation has suggested that US could be more effective when used in combination with other techniques such as heat (Guerrero et al., 2001; Bermúdez-Aguirre and Barbosa-Cánovas, 2008), pressure (Raso et al., 1998), natural antimicrobials (Guerrero et al., 2005; Ferrante et al., 2007), UV light (Lopez-Malo et al., 2005a) for inactivation of some pathogenic and spoilage microorganisms.

Implementation of PL to inactivate microorganisms has gained interest due to the short-time treatments involved (Krishnamurthy et al., 2010). This technology uses short time pulses (100–400 μs) of an intense broad spectrum between 100 and 1100 nm with 54% of emitted energy in the ultraviolet range (Gómez-López et al., 2007; Oms-Oliu et al., 2010). Exposure to PL causes the formation of pyrimidine dimmers which impairs the process of cell replication (photochemical mechanism) (Gómez-López et al., 2007). Moreover, membrane disruption was also reported (Wekhof, 2000; Ferrario et al., 2013a) as a result of a momentous overheating. This phenomenon is attributed to a difference in UV light absorption between the microorganism and its surrounding environment (photothermal effect). Besides, structural damages in microbial cells like cytoplasmic membrane shrinkage were also reported (photophysical effect) by Krishnamurthy et al. (2010). It is possible for these mechanisms to coexist and, the relative importance of each one would depend on the fluence and target microorganism (Gómez-López et al., 2007). PL application in fruit juices is very promising as some studies reflect its effectiveness as a decontamination technique. Among them, Sauer and Moraru (2009) studied the inactivation of *Escherichia coli* in apple juice (pH: 4.0; 11.3 °Brix) and reported 2.7 log reductions (12.6 J/cm$^2$, 1.3 mm layer liquid thickness) under static conditions. In addition, they observed up to 7.3 log reductions (8.8 J/cm$^2$) when generated turbulence in the liquid substrate during the PL treatment (3000 rpm, orbital shaker).

Certain combinations of PL with other technologies like PEF, manothermosonication, UV-C and heat have already been reported for microbial inactivation (Palgan et al., 2011; Muñoz et al., 2011, 2012a, 2012b; Marquenie et al., 2003). In particular, Muñoz et al. (2011, 2012a) assayed combinations of PL and thermosonication (TS) in a continuous flow system to inactivate *E. coli* in orange and apple juices. They observed that all the different combinations tested showed an additive effect. Combinations of PL with mild temperatures were also reported. Marquenie et al. (2003) found that the combination of PL with thermal treatment at 40 °C in culture media had a significant higher fungal inactivation than single treatments. (In addition, Krishnamurthy et al. (2007) suggested that the heat produced during the PL process itself could have been responsible for higher *Staphylococcus aureus* inactivation in milk treated with PL in a continuous flow.

The combination of US and PL may improve food microbial stability due to their different inactivation mechanisms. The purpose of this study was to analyse the response of *Saccharomyces cerevisiae* cells and *A. acidoterrestris* spores in apple juice as affected by combinations of US and PL with different temperature build-up generated from the PL process itself. Storage studies were also carried out for some US-PL combinations to assess the evolution of treated microorganisms under refrigerated conditions.

## 2. Materials and methods

### 2.1. Strains and preparation of inocula

Experiments were performed using *S. cerevisiae* KE162 and *A. acidoterrestris* ATCC 49025 spores. Initial yeast inoculum was prepared by transferring a loopful of Potato Dextrose Agar (PDA; Britania, Buenos Aires, Argentina) slant stock culture to a 20 mL Erlenmeyer-flask of Sabouraud Dextrose Broth (Britania, Buenos Aires, Argentina). It was incubated at 27 ± 1 °C under agitation for 24 h until it reached stationary phase. Yeast inocula was harvested by centrifugation (5000 rpm, 5 min) (Labnet International Inc., Edison, NJ, USA), washed twice with saline and re-suspended in peptone water to give a cell density of $10^7$–$10^8$ CFU/mL. For bacterial culture, the initial inoculum was prepared by transferring a loopful of a fresh stock culture maintained in *Bacillus acidoterrestris* medium (BAM) to an Erlenmeyer-flask containing 20 mL of BAM Broth and subsequently incubated at 43 ± 1 °C for 24 h. The production of spores was performed by sowing the inoculum onto bottles containing *A. acidoterrestris* medium (AAM) and incubating 1 week at 43 ± 1 °C. Spores were removed by the procedure explained by Silva and Gibbs (2001), and maintained at −18 ± 1 °C until use. The BAM used in this study was prepared according to Silva and Gibbs (2001).

### 2.2. Apple juice samples

Commercial (CEPITA, Coca- Cola, Argentina) (pH: 3.5 ± 0.1; 12.5 ± 0.1 °Brix; $A_{660\ nm}$: 0.063 ± 0.003) and natural squeezed apple juice (*Pyrus malus* L, var Granny Smith, pH: 3.4 ± 0.1; 11.8 ± 0.6 °Brix; $A_{660\ nm}$: 0.071 ± 0.005) were used in this study. Commercial apple juice characterized by a high penetration of light was used in order to compare it with a matrix with dissolved particles. Natural juice was aseptically obtained from fruits that were rinsed with 0.02% sodium hypochlorite and sterile water to eliminate surface microbial load and gently dried with a sterile cloth. Juice was obtained under aseptic conditions in a 90% ethanol sanitized and 10 min UV-C exposed household juicer (Bluesky, Ningbo, China), centrifuged in order to reduce pulp amounts (5000 rpm, 10 min) (Eppendorf, model 5804 R, Hamburg, Germany) and collected for subsequent processing. Juice turbidity was measured by centrifuging samples (1500 rpm, 10 min), and measuring the supernant absorbance at 660 nm (Rivas et al., 2006). Measurements were performed in triplicate. For these studies, uninoculated juice samples were used.

### 2.3. Ultrasonic treatment

Treatments were carried out in a 150 mL-double wall cylindrical vessel (diameter 6.3 cm; height: 7.6 cm) connected to a thermostatically controlled water bath (HAAKE, Model Rotovisco RV12, Germany), to attain 20, 30 or 44 ± 1 °C in the vessel. Ninety-five (95 mL) of juice were poured into the vessel. Ultrasound (Vibracell®, net power output: 600 W, Sonic Materials Inc., Newtown, CT, USA) at 20 kHz and 95.2 (80%) μm of wave amplitude was applied to the medium with an immersed 13 mm diameter probe. The equipment has automatic amplitude compensation to ensure uniform probe amplitude regardless of the varying loading conditions and line voltage fluctuations. The probe was previously calibrated following the steps of the manufacturer. After three minutes of sonication, the desired temperature was reached, and it was maintained constant at the predetermined temperature value

±0.5 °C throughout the experiment. When the desired temperature was achieved, 5 mL-microbial suspension was inoculated into the vessel (~2 × $10^6$ CFU $mL^{-1}$) and treated up to 30 min. Due to bubbles generated by the cavitation process, the systems were always highly mixed from the start of the experiments. Temperature of samples was continuously monitored by a thermocouple attached to the US device. For kinetic studies, samples were taken at preset intervals for their subsequent analysis. Ten (10) or 30 min sonicated juice samples were immediately PL processed (US + PL combined treatments) or not (single US treatments). Experiments were performed in triplicate.

### 2.4. Pulsed light processing

PL treatments were performed with a RS-3000B Steripulse-XL system (Xenon Corporation, Wilmington, MA, USA), which produce polychromatic radiation in the wavelength range from 200 to 1100 nm. The PL device consisted of an RC-747 power/control module, a treatment chamber that housed a xenon flash lamp (non toxic, mercury free) and the air cooling system attached to the lamp housing to avoid lamp overheating during operation (Ferrario et al., 2013b). It generated high intensity pulsed light at a pulse rate of 3 pulses per second and a pulse width of 360 μs. According to the specifications supplied by the manufacturer, each pulse delivered 1.27 J/$cm^2$ for an input of 3800 V at 1.9 cm below the quartz window surface of the lamp. The different PL doses were obtained by altering the number of applied pulses. Fluence measurements were taken by a pyroelectric head model ED500 (Gentec Electro-Optics, Québec, Canada) connected to an oscilloscope model TDS 2014 (Tektronix, Beaverton, USA), with an aperture cover of 20.3 $cm^2$ (Gómez et al., 2012). Experiments were performed in triplicate.

For each PL treatment, 4.9 mL of inoculated juice previously US treated or not (initial temperature, $T_{PLi}$ = 2, 30 and 44 ± 1 °C) were poured into a 100 mm diameter Petri dish to ensure that the entire dish surface was covered with sample to a depth of 1 × $10^{-3}$ m. Samples were exposed to PL treatment for 2–60 s at a distance of 0.1 m from the quartz window, corresponding to applied fluencies between 2.4 J/$cm^2$ and 71.6 J/$cm^2$ with three different final temperatures ($T_{PLf}$) due to the heat build-up during PL treatment and the matrix. To process juice at the lowest temperature, juice sample (5 mL) contained in the 100 mm Petri dish was placed into a 150 mm one, filled with ice flakes before PL treatment. Therefore, juice temperature immediately decreased to 2–3 ± 1 °C, reaching after PL processing a $T_{PLf}$ of 12 ± 1 °C.

In the case of US + PL combined treatments, 5 mL of sonicated juice were poured into the Petri dish and exposed to selected doses corresponding to 20 s or 60 s (23.9 J/$cm^2$ or 71.6 J/$cm^2$; $T_{PLi}$ = 2, 30 or 44 ± 1 °C). Inoculated untreated samples were used as controls. Temperature evolution of juices during PL treatment was monitored using a T-type thermocouple connected to a data logger DigiSense model 69202-30 (Barnant Company Division, Barrington, NH, USA). Experiments were performed in triplicate.

### 2.5. Microbial enumeration

After single US, PL or combined US + PL treatments and before plating, *A. acidoterrestris* spores were shock-heated for 10 min at 80 ± 1 °C, in order to stimulate spores germination and to inactivate vegetative organisms (Vercammen et al., 2012; Djas et al., 2011; Podolak et al., 2009). Peptone water (0.1% w/v) tenfold dilution aliquots were surface plated by duplicate onto PDA for *S. cerevisiae* or BAM for *A. acidoterrestris* using a spiral plater (Autoplate 4000, Spiral Biotech, USA). When treatment resulted in low counts (longer treatment times), up to 3-mL of fruit juice was directly pour plated into each Petri dish. Plates were incubated for 72 h at 43 ± 1 °C (bacteria) and 27 ± 1 °C (yeast), respectively. A counting grid was used for enumeration of colonies in the case of spiral plating. Survival curves were generated from experimental data by plotting log $N/N_0$ (where $N$ is the number of CFU/mL at a given time and $N_0$ the initial number of CFU/mL) versus time of treatment. Non-viable cells were expressed as (log $N_0$−log$N$).

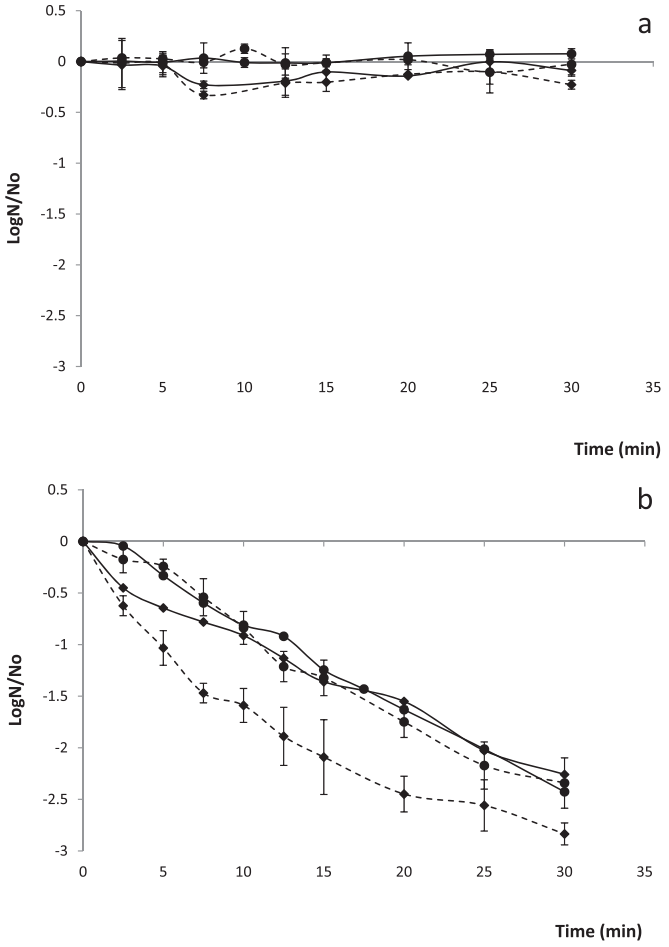


**Fig. 1.** Survival curves of *A. acidoterrestris* spores (a) and *S. cerevisiae* cells (b) treated 30 min with US at 30 ± 1 °C (●) or 44 ± 1 °C (◆) in commercial (╍) and natural squeezed (—) apple juice; standard deviation (I).

### 2.6. Storage studies

The evolution of surviving cells along juice storage was evaluated in control and in US + PL treated samples. Samples were dispensed in 10 mL flasks in a biological safety cabinet (Nuaire Inc., Plymouth, USA) and stored in the dark at 5 ± 1 °C for 15 days. During storage, flasks were taken at preset time intervals for the analysis of survivors in triplicate.

### 2.7. Statistical analysis

Statistical analyses were carried out using InfoStat 2009 (InfoStat Group, FCA-UNC, Córdoba, Argentina). A two-way analysis of variance (ANOVA) was applied to evaluate the influence of treatment time (single US or PL and combined US + PL) and temperature. Significance level was set at $p < 0.05$. Homoscedasticity and normality assumptions were verified and outliers were removed from data set in case it was appropriated. Interaction contrasts were evaluated by the Tukey Test.

## 3. Results and discussion

### 3.1. Ultrasonic effectiveness

Fig. 1 shows the survival curves of *A. acidoterrestris* spores and *S. cerevisiae* cells treated with 30 min US at 30 ± 1 °C or 44 ± 1 °C in commercial and natural fresh squeezed apple juices. US treatment resulted unable to achieve inactivation of *A. acidoterrestris* spores, as no reductions were observed after 30 min of sonication (Fig. 1a). There are very few studies which investigated spore inactivation by single US. Among these, Djas et al. (2011) reported less than a 0.12-log reduction of *A. acidoterrestris* spores after the application of single US (10 min, 330 W) in apple juice concentrate. However, a vast amount of scientific literature has been published in the last decade about spore inactivation by the combination of ultrasound and other technologies (Sagong et al., 2013; López-Malo et al., 2005b; Coronel et al., 2011). Inactivation curves of *S. cerevisiae* resulted linear for commercial apple juice at both $T_{PLi}$, and for natural apple juice only at the lowest $T_{PLi}$ (Fig. 1b). The inactivation *of S. cerevisiae* by US described by a linear behaviour in single US treatments was also reported by Guerrero et al. (2001) in model system in a temperature range from 35 ± 1 °C to 55 ± 1 °C. A non-linear behaviour was observed for *S. cerevisiae* in natural apple juice treated at $T_{PLi}$ 44 ± 1 °C (Fig. 1b). A moderate 2.5-log decrease was observed in *S. cerevisiae* cells after 30 min of sonication at 30 ± 1 °C for both juices and at 44 ± 1 °C for commercial apple juice only (Fig. 1b). On the other hand, a slight higher inactivation of a 2.8-log reduction was obtained in natural apple juice treated with US at 44 °C (Fig. 1b). Char et al. (2010) obtained a 1-log reduction for *S. cerevisiae* in orange juice (pH 3.4, 10 °Brix) after 20 min of sonication (20 kHz, 80% amplitude, 500 mL) at 40 ± 1 °C. While in this study (US: 20 kHz, 80% amplitude, 100 mL) 1.5 and 2.4 log cycles of reduction were obtained for the same treatment time and 44 °C in commercial apple juice and natural squeezed apple juice, respectively. The use of a smaller container which improves the sonication process (Guerrero et al., 2001) and a different matrix could be responsible for the greater inactivation observed in this study under the same US conditions.

### 3.2. PL effectiveness

#### 3.2.1. Juice temperature

Fig. 2 shows the temperature evolution during PL treatment in apple juice for different initial temperatures. During PL treatments, juice temperature increased with time as a consequence of light absorption. On average, the temperature of samples treated during 60 s increased between 10 and 14 ± 1 °C, depending on the initial temperature and the type of juice. Natural squeezed apple juice exhibited higher increase in temperature than commercial apple juice for all assayed conditions, probably due to its higher absorbance in the UV- infrared (IR) range (Ferrario et al., 2013b). Systems with $T_{PLi}$ 2 °C and 30 °C showed a linear temperature increase profile with scarce differences between both matrixes. Meanwhile, notorious differences were observed for the condition of $T_{PLi}$ 44 °C where natural squeezed apple juice reached $T_{PLf}$ 56 °C at 20 s PL treatment, whilst for commercial apple juice, 60 s of PL exposure were needed to reach that final temperature. Nevertheless for this condition, both matrixes showed high increase in temperature up to the first 20 s of PL treatment, followed by a second period with a lower increase rate.

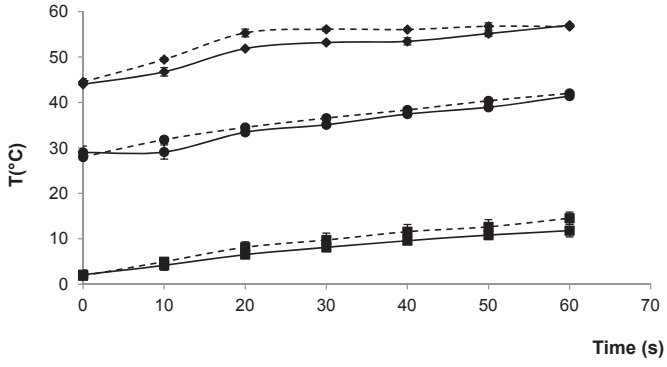


**Fig. 2.** Temperature profiles of commercial (▪) or natural squeezed (—) apple juice at $T_{PLi}$ 2 ± 1 °C (■), 30 ± 1 °C (●) or 44 ± 1 °C (◆), during treatment with PL at 10 cm from the lamp, T: juice temperature at a given time of treatment. Standard deviation (I).

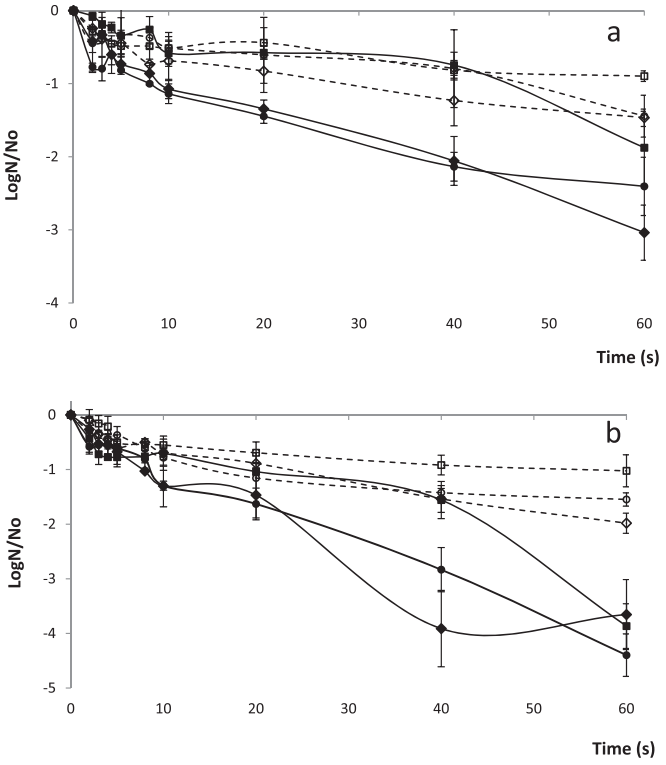


**Fig. 3.** Inactivation curves of *A. acidoterrestris* spores (a) and *S. cerevisiae* cells (b) in commercial (solid symbols and line) and natural squeezed (open symbols and dashed line) apple juice, treated with PL ($T_{PLi}$: 2 ± 1 °C (■; □), 30 ± 1 °C (●; ○), or 44 ± 1 °C (◆; ◊)); standard deviation (I).

#### 3.2.2. PL inactivation

Survival curves of *A. acidoterrestris* spores and *S. cerevisiae* cells in commercial and natural apple juices processed by PL are shown in Fig. 3. Inactivation curves of both strains exhibited a notorious upward concavity in natural apple juice, while in commercial apple juice survival curves of *S. cerevisiae* cells resulted in a sigmoidal shape. PL exposure in commercial apple juice was highly effective obtaining up to 3.0 and 4.4 log reductions for *A. acidoterrestris* spores (Fig. 3a) and *S. cerevisiae* cells (Fig. 3b), respectively. On the other hand, in natural squeezed juice, only 1.5 and 2 log-cycles of reduction were obtained for *A. acidoterrestris* spores (Fig. 3a) and *S. cerevisiae* cells (Fig. 3b), respectively. This behaviour could be attributed to differences in juice absorptivity, as commercial apple juice exhibited less absorption in the lethal UV region (Ferrario et al., 2013b). Besides, the presence of pulp particles in the natural squeezed apple juice may contribute to decrease transmission of

light due to reflection and scattering and hence affecting the effectiveness of the PL treatment (Gómez-López et al., 2007). In natural apple juice, a pronounced decrease in the number of survivors occurred during the first 10 s of PL treatment (fluence ≤ 12 J/cm$^2$), followed by a lower inactivation rate, which led to the presence of tail in these survival curves (Fig. 3). This behaviour has been explained in several different ways, like lack of homogeneous population (Xiong et al., 1999), multi-hit phenomena (Yousef and Marth, 1988), presence of soluble solids (Koutchma, 2009), different abilities of cells to repair DNA mutations (Wekhof, 2000), sample topography, and shading effect that may have been originated by the edge of the Petri dishes used in the experiment (Gómez-López et al., 2007; Yaun et al., 2003, 2004).

Higher levels of inactivation were reached for the systems with higher temperature build-up compared to the lowest one, for both strains and matrixes. The most notorious difference was obtained at 40 s (47.76 J/cm$^2$) PL exposure. At this dose, in commercial apple juice, 0.74 or 2.05 log cycles of reduction for *A. acidoterrestris* spores were obtained, depending on whether the $T_{PLi}$ had been 2 ± 1 °C or 44 ± 1 °C and hence their heat build-up history (Figs. 2 and 3a). Inactivation of *A. acidoterrestris* spores by PL was already reported by Chaine et al. (2012) who obtained almost 4 log cycles of reduction in 65 °Brix sugar syrup at 1.5 J/cm$^2$ in a continuous flow. Furthermore, at 40 s (47.76 J/cm$^2$) of PL exposure, 1.56 or 3.91 log reductions were obtained for *S. cerevisiae* when the $T_{PLi}$ was 2 ± 1 °C or 44 ± 1 °C, respectively (Figs. 2 and 3b). Under the same conditions, 0.80 and 1.23 log-cycles were obtained for *A. acidoterrestris* spores in natural squeezed apple juice (Figs. 2 and 3a) whilst similar reductions were obtained for *S. cerevisiae* in the same matrix (0.91 and 1.53 log cycles) (Figs. 2 and 3b). A slight difference in *A. acidoterrestris* spores inactivation was also observed at 60 s (71.6 J/cm$^2$) PL exposure in commercial apple juice, where 1.87 and 3.03 log cycles of reduction were observed for $T_{PLi}$ 2 ± 1 °C and 44 ± 1 °C, respectively (Fig. 3a). These results may support the photothermal hypothesis as one of the possible inactivation mechanisms of PL. Our results are in concordance with Krishnamurthy et al. (2007) who studied the inactivation of *S. aureus* in milk treated with PL in a continuous flow. They reported that the temperature build-up of milk caused by the IR portion of PL may have played a major role in enhancing the inactivation caused by the PL exposure. They emphasized that the temperature increase could not have been responsible for inactivating *S. aureus* as the highest temperature recorded was a non-lethal value of 38 °C. Moreover, Gómez-López et al. (2005) suggested that both the photochemical and photothermal effect were responsible for the inactivation of several microorganisms exposed to PL, as the number of survivors did not fit the equations for each phenomenon separately, but it fitted the equation which involved both factors. Cheigh et al. (2012) also proposed a multitarget inactivation mechanism by PL as the shape of *Listeria monocytogenes* and *E. coli* UV-C treated cells were similar to control cells. Transmission electron microphotographs revealed that PL treated cells showed destruction of cell wall and cytoplasmic membrane; and rupture of the internal organization.

### 3.3. Microbial inactivation by combined US and PL treatments

The survival curves of *A. acidoterrestris* spores and *S. cerevisiae* cells inoculated in commercial or natural squeezed apple juice and treated with PL or with prior application of 30 min US are shown in Fig. 4. When US was applied prior to PL treatment, no differences in the shape of the survival curves or in the level of inactivation achieved were detected for *A. acidoterrestris* spores in both matrixes (Fig. 4a), supporting the fact that US did not contribute to the spore inactivation. On the other hand, the shape of the PL

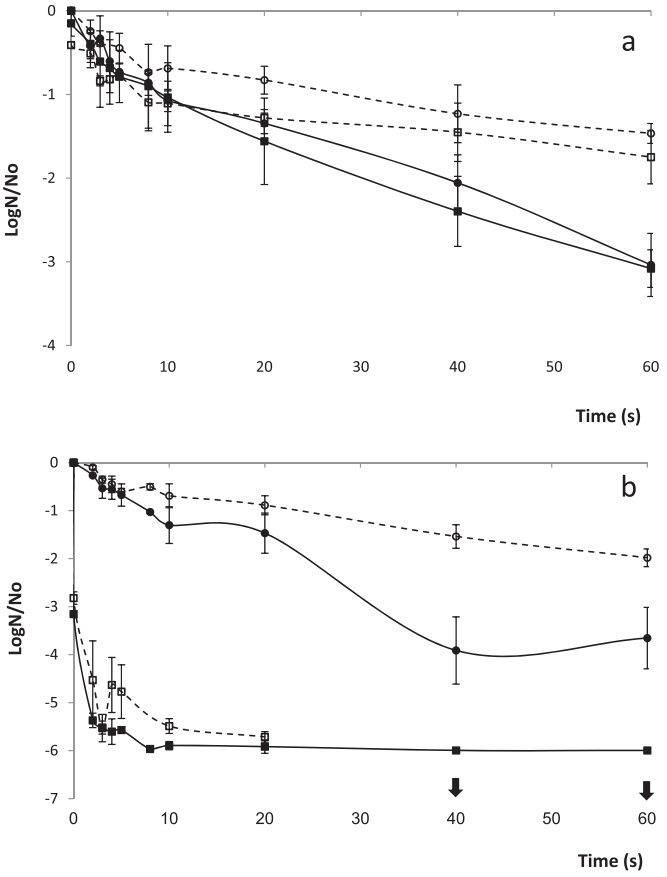


**Fig. 4.** Inactivation curves of *A. acidoterrestris* spores (a) and *S. cerevisiae* cells (b) in commercial (solid symbols and line) and natural squeezed (open symbols and dashed line) treated with 60 s PL ($T_{PLi}$: 44 ± 1 °C)(●; ○) or with 60 s PL prior 30 min US treatment (■; □).

inactivation curve resulted very different from the survival curve of single PL for *S. cerevisiae* in both matrixes, thus suggesting the existence of a combined effect between US and PL in the yeast inactivation (Fig. 4b). The application of single US (30 min, 44 ± 1 °C) resulted in approximately 2.5 log cycles of yeast reduction for both matrixes and, 2.0 and 3.7 log reductions for single PL (60 s, 71.4 J/cm$^2$, $T_{PLi}$ 44 ± 1 °C) in natural squeezed and commercial apple juices, respectively. An improved decrease in population was obtained (up to 6 log-cycles) applying PL with prior US treatment at 10 s (fluence 12 J/cm$^2$), for both juices. The shape of *S. cerevisiae* inactivation curves treated by PL exhibited upward concavity and were characterized by a more pronounced decrease during the first 10 s of treatment and then, the number of survivors decreased slowly as treatment time increased. Upward concavity, which indicates that the process became less effective for higher doses, led to the presence of tail in several survival curves. The application of US prior to PL increased the inactivation rate at low PL doses (Fig. 4b).

Therefore, the high inactivation observed at the beginning of PL treatment was emphasized by the application of prior US treatment. No survivors were detected at the end of the treatment in commercial apple juice, whereas in the natural squeezed one, the survival curves exhibited a pronounced tail (Fig. 4b).

The inactivation degree obtained by single or combined US + PL at selected doses and different temperature build-up and the corresponding significant differences among treatments obtained by the Tukey Test are shown in Fig. 5. Treatment*temperature

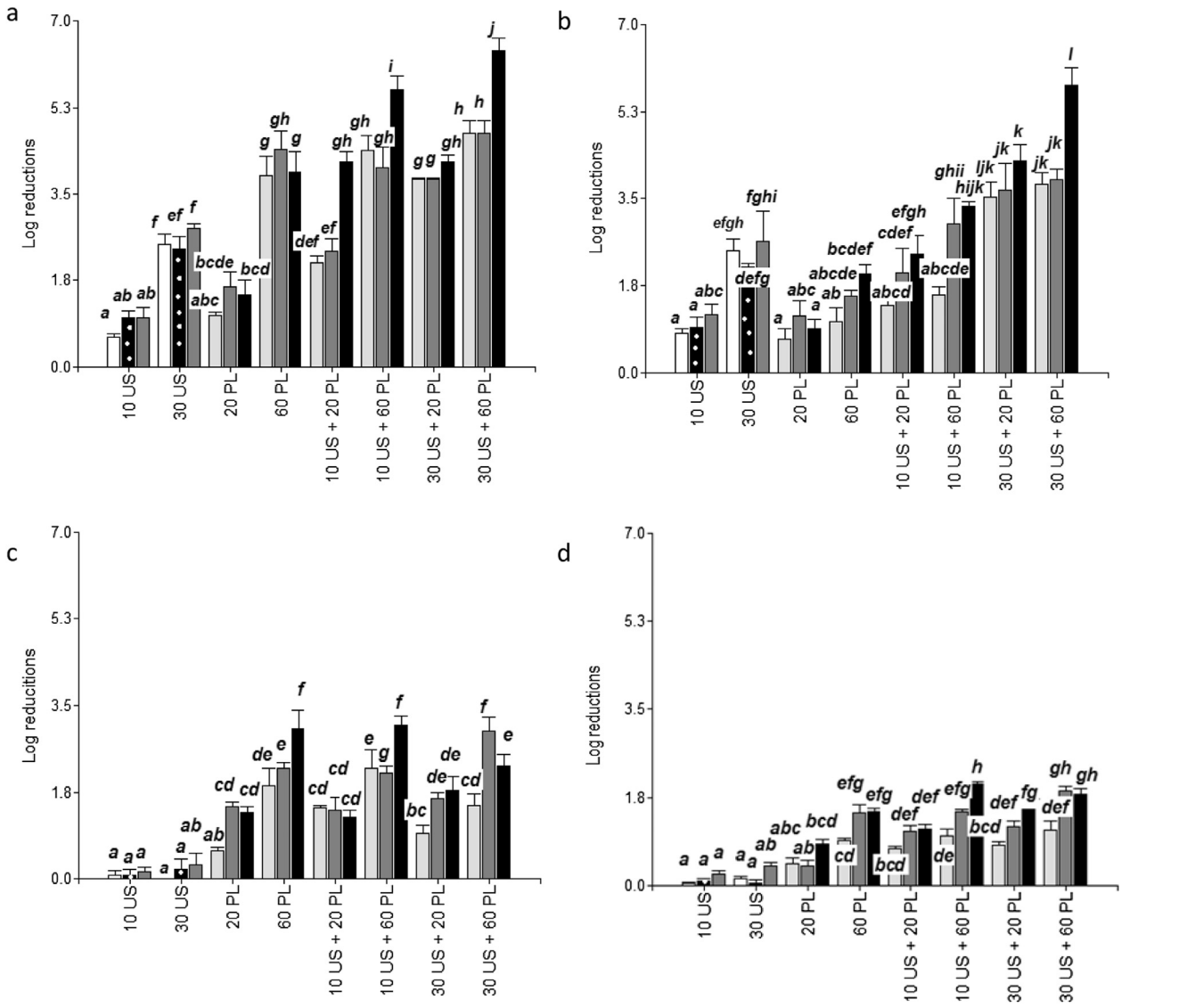


**Fig. 5.** Log- reductions and significant differences among treatments obtained by ANOVA and the Tukey Test of *S. cerevisiae* cells in commercial (a) or natural squeezed apple juice (b) and of *A. acidoterrestris* spores in commercial (c) or natural squeezed apple juice (d) treated with single US or PL or their combinations. Temperature of single US: 20 ± 1 °C (□), 30 ± 1 °C (▦) or 44 ± 1 °C (▩); $T_{PLi}$ for single or combined PL: 2 ± 1 °C (○), 30 ± 1 °C (▒) or 44 ± 1 °C (■) In the labels, number means the time of treatment in min (US) or seconds (PL). Different letters above the bars represent significant differences ($p < 0.05$) among treatments according to the Tukey Test.

interactions were statistically significant variables for predicting log $N/N_0$ with highly *F*-values (*p*-value < 0.001). The *R*-squared statistic indicates that the model explained the observed variability in a range of 92–96% (data non shown). For *S. cerevisiae*, in natural squeezed apple juice, no differences were observed between single 20 s (23.9 J/cm$^2$) or 60 s (71.6 J/cm$^2$) PL (Fig. 5a, b). The combination US + PL treatment significantly increased the observed inactivation respect to single treatments. For example, the combination 10 min US + PL resulted more effective than single PL at $T_{PLi}$ 44 ± 1 °C for both matrixes (Fig. 5a, b). In addition, the combination 30 min US + 20 s and 60 s PL was more efficient than applying single US or PL for both matrixes. Temperature build-up of 56 ± 1 °C enhanced the effectiveness of the combination 10 min US + 20 s and 60 s PL and 30 min US + 60 s PL compared to the lowest temperature increase (Fig. 5a, b).

For both matrixes, the combined treatment 30 min US + 60 s PL at $T_{PLi}$ 44 °C was the most effective one inactivating *S. cerevisiae*, as 6.4 and 5.8 log reductions were obtained for commercial and natural squeezed juice, respectively (Fig. 5a, b). Overall, the combinations of these non-thermal agents showed additive effects. Muñoz et al. (2012b) studied the combination of PL with subsequent US for inactivating *E. coli* and *Listeria innocua* inoculated in buffer solution. They reported that the consecutive application of US contributed to an increase in the inactivation observed by single PL for *L. innocua* but not for *E. coli.* Muñoz et al. (2011, 2012a) also studied the combination of PL and TS in the inactivation of *E. coli* in orange and apple juices in a continuous flow. In agreement with our results, they found that the combined treatments showed an additive effect.

On the other hand, the combination with US treatment did not improve the observed inactivation for *A. acidoterrestris* spores when single PL treatments were applied (Fig. 5c, d). Djas et al. (2011) studied the inactivation of *A. acidoterrestris* spores in apple juice concentrate by the combination of US and PEF. In contrast with our findings, they reported that the highest efficiency of inactivation was obtained when US was applied before PEF

treatment. They attributed this phenomenon to the possible disaggregation of clusters during the sonication, facilitating the inactivation by PEF. Alternatively, damaged spores could be transformed to vegetative cells during the US treatment which could be easily inactivated by PEF. Nevertheless, the temperature build-up enhanced the effectiveness of single 60 s (71.6 J/cm$^2$) PL, 10 min US + 60 s PL and 30 min US + PL in both matrixes (Fig. 5c, d). Therefore, the highest temperature generated from the PL processing itself, enhanced the disinfection process for single PL or certain combinations of US + PL for both strains assayed compared to the lowest temperature build-up.

### 3.4. Storage studies

The evolution of surviving cells along apple juice storage under refrigerated conditions in control and in single PL or US + PL treated samples is shown in Fig. 6. The level of inactivation of *A. acidoterrestris* spores achieved by 60 s (71.6 J/cm$^2$; $T_{PLi}$ 44 ± 1 °C) PL exposure was maintained up to 15 days of storage. Even the untreated population (control system) remained constant, due to the very low temperature conditions for this thermo-acidophilic strain (Fig. 6a). *S. cerevisiae* control cells were able to grow up to 2.6 log-cycles in refrigerated conditions from the third day of storage. The application of 60 s- PL (71.6 J/cm$^2$; $T_{PLi}$ 44 °C), which decreased the yeast load by 1.7 log cycles, prevented from recovering of treated cells for 7 days of storage. After that, treated cells began to grow at the same rate as the untreated ones. After 15 days of storage; PL exposed cells recovered by 2.4 log cycles, although the control load remained higher than the treated one (Fig. 6b). The combination of 10 min US + 20 s PL led to a 3.6-log reduction and after 15 days of storage, the population was able to recover only 1.1 log-cycles. The difference between the control and the treated samples was of almost 5 log cycles of reduction (Fig. 6b).

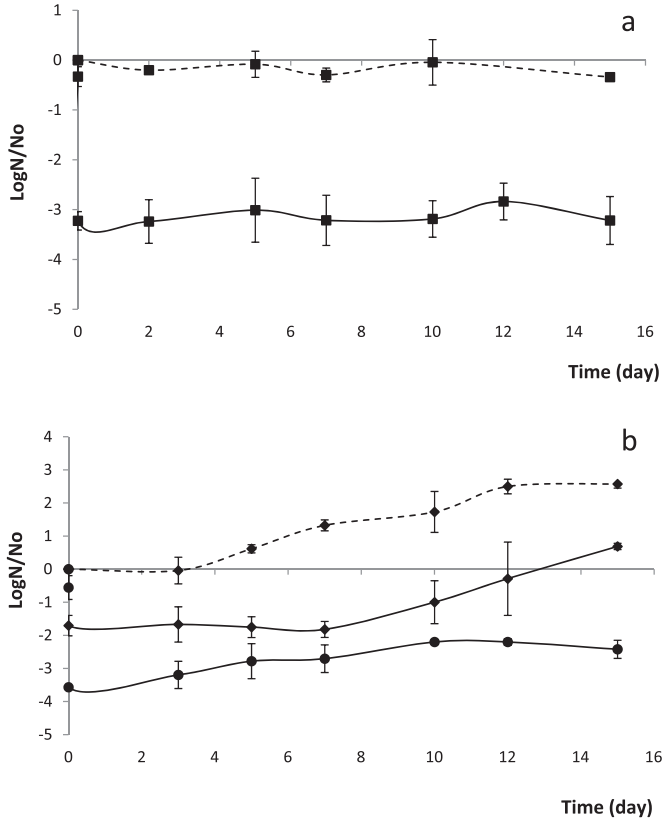


**Fig. 6.** Storage of *A. acidoterrestris* spores (a) and *S. cerevisiae* cells (b) in commercial apple juice at 5 ± 1 °C. Control (dotted line) and treated (solid line) 60 s PL (■), 20 s PL (◆) or 10 min US/20 s PL (●) at $T_{PLi}$ 44 ± 1 °C; standard deviation (I).

## 4. Conclusions

The present work demonstrates the potential of moderate temperature build-up generated from the PL processing itself to enhance the inactivation effectiveness. In addition, the use of a US pretreatment turned out to be a promising alternative for inactivating *S. cerevisiae* KE162 cells and *A. acidoterrestris* ATCC 49025 spores in apple juice. Nonetheless, this work contributed to address some limitations. In particular, the application of 30 min of US followed by 60 s of PL with final temperature in the sample of 56 °C was the most effective treatment, reaching 6.4 and 5.8 log reductions of *S. cerevisiae* in commercial and natural squeezed apple juice, respectively. However, in the case of *A. acidoterrestris*, single PL treatment provoked 3.8 log reductions in clear apple juice and poor inactivation in natural squeezed apple juice. Nonetheless, all assayed combinations of US an PL were less effective than the single PL treatment.

Storage studies revealed that the level of inactivation reached by the application of US and PL was extended throughout storage compared to fresh, untreated juice. Further studies with other microorganisms of concern would be necessary to assess the eventual existence of sub-lethal cell damages induced by the combination of the imposed stresses. This would help a better understanding of the microbial response to the proposed combined treatments.

## Acknowledgements

The authors would like to acknowledge the financial support from Universidad de Buenos Aires (N° 20020100100231; R CS 2657/11), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (2012-289 Project) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) of Argentina and from Banco Interamericano de Desarrollo (BID) (2008-0197 and 2011-0288 Projects).

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