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Impact of a combined processing technology involving ultrasound and pulsed light on structural and physiological changes of *Saccharomyces cerevisiae* KE 162 in apple juice

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ABSTRACT

This study analyzed the effect of single ultrasound (US) (600 W, 20 kHz and 95.2 μm wave amplitude, 10 or 30 min at 20 or 44 ± 1 °C), or combined with pulsed light technology (PL) with controlled heat build-up (Xenon lamp, 3 pulses/s, 71.6 J/cm^2, temperature ranges: 2–20 ± 1 °C and 44–56 ± 1 °C) on the inactivation of *Saccharomyces cerevisiae* KE 162 cells in commercial (pH: 3.5 ± 0.1; 12.5 ± 0.1 °Brix) and freshly pressed (pH: 3.4 ± 0.1; 12.6 ± 0.1 °Brix) apple juices. Structural damages were analyzed by transmission electronic microscopy (TEM) and induced damage by flow cytometry (FC). Cells were labeled with fluorescein diacetate (FDA) and propidium iodide (PI) for monitoring membrane integrity and esterase activity. US+PL treatment at the highest heat build-up led up to 6.4 and 5.8 log-cycles of yeast reduction in commercial and freshly apple juices, respectively. TEM images of treated cells revealed severe damage, encompassing loss and coagulated inner content and cell debris. In addition, FC revealed a shift of yeasts cells with esterase activity and intact membrane to cells with permeabilized membrane. This effect was more notorious after single 30-min US and all combined US+PL treatments, as 91.6–99.0% of treated cells showed compromised membrane. Additionally, heat build-up enhanced this shift when applying 10 min US (20 °C) in both juices.

1. Introduction

Fruit juices are predominantly spoiled by yeasts, which undergo alcoholic fermentation, being *Saccharomyces* spp. the most frequent strain because of its ethanol tolerant condition (Martinez et al., 2000). A wide range of novel alternative technologies has been intensely investigated in the last decades with the purpose of replacing thermal treatment while avoiding deleterious effects on product quality. Many emerging technologies are currently in broad development, such as high hydrostatic pressure, pulsed electric fields, ultrasound, ozone, pulsed light, and ultraviolet light, among others (Guerrero et al., 2016).

Nowadays, US has attracted increasing interest in food preservation applications as it has become much more commonplace and costs for its use have been reduced (Chemat et al., 2011). The lethal effect of US has been attributed to the cavitation phenomenon. Pressure waves created by mechanical vibrations of low frequency form millions of microscopic bubbles or cavities containing gas and vapor, which expand during the negative pressure excursion and implode violently during the positive excursion with the release of large amounts of energy (Mason et al., 2005). US appears to weaken microbial membranes through cavitation induced by ultrasonic shock waves (Guerrero et al., 2001, 2005; Butz and Tauscher, 2002), thereby microorganisms would become more vulnerable to additional stresses (Piyasena et al., 2003; Wong et al., 2012; Pagan et al., 1999). In particular, bacterial spores and most enzymes remain difficult to inactivate with this technology, thus the use of US as a single hurdle has some limitations (Sala et al., 1995; Piyasena et al., 2003).

Likewise, PL is another emerging technology which has been recently proposed for food preservation purposes. It is based on the application of short intense pulses (100–400 μs) of broad spectrum between 100 and 1100 nm with 54% of emitted energy in the UV range (Gómez-López et al., 2007; Oms-Oliu et al., 2010). The germicidal action of PL has been attributed to three different

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mechanisms which may coexist: the photochemical effect (Gómez-López et al., 2007), the photothermal effect (Wekhof, 2000; Ferrario et al., 2013a) and the photophysical effect Krishnamurthy et al. (2010). The relative importance of each mechanism would depend on the fluence imparted to the food and target microorganism (Gómez-López et al., 2007).

Therefore, many of these emerging technologies used alone are insufficient to inactivate many microbial species or require excessively long treatment times (FDA, 2000), due to lack of efficacy in some circumstances (Søltoft-Jensen and Hansen, 2005). Thus, many recent developments based on the hurdle concept, have intended to combine some emerging technologies with each other or with other traditional hurdles to achieve higher inactivation levels. The attack on various cellular targets would have a synergistic effect by making microbial cells implement every possible repair mechanism simultaneously (Leistner, 2000). Nevertheless, the original hurdle concept has been questioned arguing that although agents targeting different sites could inflict sublethal damage in several parts of the cell, they would fail to inactivate those (Ross et al., 2003). Whereas, the use of treatments with the same target would improve the chances of causing irreversible damage. . Overall, the prime cause for cell death provoked by many nonthermal agents is still not clearly defined, and this uncertainty hinders an intelligent selection of hurdles. Until now, most of binary or ternary nonthermal combinations reported in literature have shown additive rather than synergistic inactivation effect (Guerrero et al., 2016).

The commercial success of a given novel processing is based on the need to summarize scientific knowledge, which encompasses the use of engineering principles to address microbial and chemical safety challenges that contribute to elucidate some aspects related to food safety. Accordingly, the use of flow cytometry (FC) allows exploring changes in the physiological state of microorganisms from analyzing large populations of cells in a very short time (Comas-Riu and Rius, 2009). This goal can be achieved by adding fluorescent dyes, which put on evidence possible functional and structural changes that are aimed at specific cellular targets suspected to be involved in cellular injury or compromised metabolic activity induced by a given process (Ananta et al., 2005).

The aim of the present work was to analyze the physiological status of *S. cerevisiae* cells subjected to single or combined US and PL treatments in apple juice by FC, under dual staining with fluorochromes. Additionally, cell structure changes were evaluated by transmission electron microscopy observations.

2. Materials and methods

2.1. Inoculum preparation

Experiments were performed using *Saccharomyces cerevisiae* KE 162. 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 until it reached stationary phase (24 h) and was harvested by centrifugation (1475 g, 5 min) (Labnet International Inc., Edison, NJ, USA), washed twice with saline and resuspended in peptone water to achieve a cell density of 10^7–10^8 CFU/mL.

2.2. Apple juice

Two types of juices were used with the aim of evaluating the influence of suspended particles, particle size and light penetration on treatment efficacy. Commercial clarified apple juice without any additives (CAJ; CEPITA, Coca- Cola, Argentina) and freshly pressed apple juice (NAJ; *Pyrus malus* L, var. Granny Smith) were used in this study. NAJ 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. . NAJ was obtained by fruit pressing using a household juicer (Bluesky, Ningbo, China) which was previously sanitized with 90% ethanol and subsequently exposed to ultraviolet light during 10 min. Juice was immediately centrifuged in order to reduce pulp amounts (5000 rpm, 10 min) (Eppendorf, model 5804 R, Hamburg, Germany) and collected for subsequent processing.

2.3. Juice characterization

For these studies, uninoculated juice samples were used. The pH of juices was determined by a pH meter (PerpHect pH/ISE, 310 model, Orion Research Inc., Beverly, USA), while the soluble solid concentration (° Brix) was determined by a refractometer (PR-101 Palette, ATAGO Co. LTD, Japan). Juice turbidity was measured by a turbidimeter (LaMotte, Maryland, USA) using a formazin pattern of 100 NTU. Particle size of apple juices in the range from 0.6 nm to 6 μm was determined in triplicate by dynamic light scattering (DLS) at 20 °C in a Zetasizer Nano-Zs (Malvern, Worcestershire, UK) provided with a He-Ne laser (633 nm) and a digital correlator (Model ZEN3600), according to Ferrario et al. (2015b). All measurements were performed in triplicate.

2.4. Single and combined US and PL treatments

2.4.1. Ultrasonic treatment

Treatments were carried out in a 150 mL-double wall cylindrical vessel (diameter 6.3 cm; height: 7.6 cm) containing 95 mL of juice and connected to a thermostatically controlled water bath (HAAKE, Model Rotovisco RV12, Germany) to attain 20 or 44 ± 1 °C. . Ultrasound (Vibracell®, Sonic Materials Inc., Newtown, USA) was applied (20 kHz, 600 W, 95.2 (80%) μm of wave amplitude) to the medium with an immersed 13 mm diameter probe, which was previously calibrated according to the manufacturer's instructions. After three minutes of sonication, the desired temperature was reached, and maintained constant (±0.5 °C) throughout the experiment. When the desired temperature was achieved, 5 mL-microbial suspension was added to the juice (final microbial concentration ~ 2×10^6 CFU mL^{-1}), which was subsequently US-treated during 10 or 30 min. Systems were highly mixed from the start of the experiments due to bubbles generated by the cavitation process. Temperature of samples was continuously monitored by a thermocouple attached to the US device. Sonicated juice samples were immediately PL processed (US + PL combined treatments) or not (single US treatments). Treatments were run in triplicated independent trials for each condition.

2.4.2. Pulsed light processing

PL treatments were performed with a RS-3000B Steripulse-XL system (Xenon Corporation, Wilmington, MA, USA), which produces 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 (nontoxic, mercury free) and an air cooling system attached to the lamp housing to avoid lamp overheating during operation (Ferrario et al., 2013b). It generated high intensity PL at a pulse rate of 3 pulses per second and a pulse width of 360 ìs. 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. Treatments were run in triplicated independent trials for each condition.

For each PL treatment, 5 mL of inoculated juice previously sonicated or not (initial temperature, T_{PLi}: 2 ± 1 °C 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. For PL processing at the lowest temperature range, the Petri dish was placed, before PL treatment, into a bigger one filled with ice flakes. Therefore, juice temperature immediately decreased to 2 ± 1 °C, reaching after PL processing a final temperature value, T_{PLf} of 20 ± 1 °C. Juice samples were exposed to PL for 60 s (energy dose: 71.6 J/cm^2, 1.1 × 10^3 J/mL) at a distance of 0.1 m from the quartz window with two different final temperatures (T_{PLf}) due to the heat build-up produced during PL treatment (20 ± 1 °C and 56 ± 1 °C). Temperature evolution of juice samples during PL treatment was monitored using a T-type thermocouple connected to a data logger Digi-Sense model 69202-30 (Barnant Company Division, Barrington, USA) and the temperature profiles were previously published (Ferrario et al., 2013b). Treatments were performed in triplicated independent trials for each condition.

2.4.3. Electrical energy per order (E_{EO}) estimation

E_{Eo}, an electrical figure-of-merit defined as the electric energy in kilowatt hours [kWh] required to reduce microbial load by one order of magnitude in 1 m^3 of contaminated sample, was calculated to determine the involved energy delivered by the treatments and thus, their efficiency. The E_{Eo} values were estimated according to the equations proposed by Bolton et al. (2001) for batch operation.

2.5. Microbial enumeration

Peptone water (0.1% w/v) tenfold dilution aliquots were surface plated by duplicate onto PDA 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 27 ± 1 °C. A counting grid was used for the 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).

2.6. Staining procedure and FC analysis

Use of double staining with FDA (Calbiochem, Darmstadt, Germany) and PI (Sigma Aldrich, St Louis, MO, USA) was used for FC analysis (Schenk et al., 2011; Ferrario et al., 2013a). FDA is a non-fluorescent precursor that readily diffuses across membranes, and it is used for the evaluation of enzymatic activity and membrane integrity. In the intracellular compartment of metabolically active cells, FDA undergoes hydrolysis by enzymes with esterase activity into fluorescein (F), a polar fluorescent green compound that is unable to diffuse out of the cell. Thus, only cells with intact membranes will remain fluorescent due to the presence of internal F. In contrast, PI is a nucleotide-binding probe, widely used for the assessment of membrane integrity, supposed to enter only cells with damaged membranes. Following loss of membrane integrity, PI diffuses into the cell and stains the DNA giving red fluorescence (Schenk et al., 2011).

Non-irradiated stained cells and cells treated at 85 °C for 15 min and subsequently stained with FDA or PI, respectively, were used as controls to define negative and positive histogram regions. Moreover, a non-stained and non-treated control was employed for determining the auto fluorescence of cells. Control and treated cells were initially incubated with 0.5 μL FDA (5 mg/ml acetone) at 37 °C for 30 min. After labeling, cells were centrifuged twice (8000 rpm, 5 min) and resuspended in 1 mL phospate buffered saline solution (PBS buffer, pH 7.0). This step was followed by addition of 0.5 μL PI (1 mg/ml sterile water) and by incubation at room temperature for 10 min in the dark. Following incubation with PI, samples were placed on ice in the dark until analysis (maximum elapsed time: 1 h).

Analysis of microbial cells was performed on a flow cytometer (BD FACSAria II, New Jersey, USA). Scatter and fluorescence signals of individual cells passing through the laser zone were collected as logarithmic signals. Green fluorescence of cells stained with FDA was collected in the FL1 channel (525 ± 15 nm), whereas red fluorescence of cells labeled with PI was collected in the FL2 Channel for (620 ± 15 nm). Flow rate and cell concentration of samples were adjusted to keep acquisition at 200 microbial cells per second. A total of 20.000 events were registered per sample. Treatments were performed in triplicated independent trials for each condition. For cell sorting assay, cells corresponding to quadrant 4 (F^-/PI^+) in commercial apple juice treated single 30 min US (T_{US}: 20 or 44 ± 1 °C) or combined with PL (T_{PLi}: 2 and 44 ± 1 °C) were sorted at a threshold rate of 2000 events/s until an acquisition of 100.000 events. Obtained aliquots were then cultured in Sabouraud dextrose agar for 72 h to analyze possible recovery.

2.7. Data analysis

Dot plots representing forward scatter light (FSC) versus side scatter light (SSC) were obtained during measurements. FSC is measured in the plane of the beam and gives relative information on cell size. SSC is measured at 90° to the beam and can provide information on cell granularity. Dot plot analysis of FL1 vs. FL2 was applied to determine fluorescence properties of the population. F^+ PI^- cells (gate I) represent active esterase and intact membrane, while F^+ PI^+ cells (gate II) encompasses active esterase activity with compromised membrane. In addition, F^- PI^+ cells (gate IV), corresponds to undetectable esterase activity with compromised membrane. Residual esterase activity following treatments was calculated using Eq. (1), in which the post treatment enzyme activity of the population was set in relation to the activity of untreated cells. Only cells solely labeled by F (in gate 1) were considered in the esterase activity (*%EA*) of the whole population.

$$\%EA = \left(\frac{\#1_T}{\#1_{Ctrl}}\right) \times 100 \quad (1)$$

where *%EA* is the percentage of residual enzymatic activity in response to a particular treatment; $\#1_T$ is the percentage of population in gate 1 following single US, single PL or combined US + PL treatments, and $\#1_{Ctrl}$ is the percentage of population in gate 1 prior to the treatments. The increase in permeabilized cells (PI uptake) was calculated as the difference between Log No_T and Log N_T, (log No_T-log N_T), where No_T is the number of cells that had not taken up PI (i.e., cells that did not show red fluorescence) before treatment and N_T is the number of cells that had not incorporated PI (gate 4) following treatment.

2.8. Transmission electron microscopy

Treated samples were centrifuged at 1475 g for 5 min and the supernatant was discarded. The yeast cells were resuspended in 2 mL of glutaraldehyde (2.5 g/100 mL) in 0.1 M phosphate buffer,

pH 7.1 for 2 h at 4 °C; washed with 0.1 M phosphate buffer and post-fixed with $KMnO_4$ (2 g/100 mL) in 0.1 M phosphate buffer for 17 h at 4 °C; washed well with distilled water, dehydrated with alcohol series and embedded in Epon 812 (Shell Chemical Company, USA), during 48 h (Bolondi et al., 1995). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a microscope TEM Philips EM 301 (Buenos Aires, Argentina). Non-treated cells were fixed with a similar procedure and served as a control.

2.9. Statistical analysis

An analysis of variance (ANOVA) was used to evaluate the influence of treatment (single US and combined US + PL) and temperature. Significance level was set at $p < 0.05$. Homocedasticity and normality assumptions were verified and outliers were removed from data set when corresponded. Interaction contrasts were evaluated by the Tukey Test. InfoStat 2009 (InfoStat Group, FCA-UNC, Córdoba, Argentina) software was used to perform the analyses. The software WinMDI 2.8 was used to analyze flow cytometric data.

3. Results and discussion

3.1. Juice characterization

CAJ (pH: 3.5 ± 0.1, 12.5 ± 0.1 °Brix) was characterized by low particle size (1.37 ± 0.15 nm) and few suspended particles (turbidity: 1.18 ± 0.06 NTU); whilst NAJ (pH: 3.4 ± 0.1; 12.6 ± 0.1 °Brix) showed high particle size (1.37 ± 0.15 nm) with many suspended particles (turbidity: 45.7 ± 2.2 NTU). Therefore, CAJ exhibited higher penetration of light ($A_{254\ nm}$: 0.031 ± 0.001) compared to NAJ ($A_{254\ nm}$: 0.070 ± 0.007).

3.2. Microbial inactivation by combined US and PL treatments

The inactivation of *S. cerevisiae* KE 162 cells inoculated in both juices treated with single or combined US+PL at different temperature, and the corresponding significant differences among treatments obtained by the Tukey Test are shown in Fig. 1. The adjusted R-squared statistic indicated that the model explained the observed variability between 82 and 99% (data not shown). *S. cerevisiae* KE 162 was reduced by 1.2 or 2.6 log cycles after exposure to 10-min or 30-min single US treatments, respectively. Regarding the single PL treatment, lower inactivation, ranging between 1.0 and 2.0 log reductions, was observed in NAJ; while 3.9 log reductions were achieved in CAJ (Fig. 1a and b). Additionally, a significant increase in yeast inactivation by single PL was observed in NAJ, probably attributed to the heat build-up produced by PL processing itself (Fig. 1b). Otherwise, the use of the combined treatments, 10-min and 30-min US + PL, increased the inactivation achieved by single treatments at both temperatures, thus obtaining up to 5.8 and 6.4 log reduction cycles in NAJ and CAJ, respectively (Fig. 1a and b). Exceptionally, 10-min US+PL treatment in CAJ exhibited significant differences compared to both single treatments only at the highest heat build-up. Overall, these treatment combinations exhibited additive effects on *S. cerevisiae* KE 162 inactivation. In agreement with these results, Muñoz et al. (2011, 2012) found that combinations of PL (4.0–5.1 J/cm^2, 35 °C) and thermosonication (24 kHz, 100 mm, 400 W, 40–50 °C, 8–14 mL/min) in continuous flow mode, showed additive effect for inactivating *E. coli* in orange and apple juices (pH: 3.6, 12 °Brix). Moreover, in a previous study, Ferrario et al. (2015a) reported that different combinations of US (600 W, 20 kHz, 10 or 30 min) and PL (23.9 J/cm^2 or 71.6 J/cm^2) in batch mode with different temperature rise, showed additive effect for inactivating *S. cerevisiae* cells in commercial (pH: 3.5; 12.5 °Brix) and freshly pressed (pH: 3.4; 11.8 °Brix) apple juices.

Higher inactivation levels were achieved in treatments with higher temperature rise. In particular, an increase in final temperature improved the effectiveness of *S. cerevisiae* KE 162 inactivation achieved by single PL in NAJ, 10-min US+PL in CAJ and 30-min US+PL in both juices. These results may support the photothermal hypothesis as one of the possible inactivation mechanisms produced by PL (Gómez-López et al., 2007). On the same fashion, in a previous study (Ferrario et al., 2015a) the effect of PL on the inactivation of *Alicyclobacillus acidoterrestris* ATCC 40925 spores and *S. cerevisiae* KE 162 cells, inoculated in the same apple juices used in the present study, was investigated. Juice samples were exposed to PL (batch mode, 2.4 J/cm^2- 71.6 J/cm^2) with three different final temperatures between 20 and 56 °C due to the heat build-up provoked during PL processing Higher inactivation was achieved in systems with higher final temperature (44 and 56 °C) compared to the lowest one (20 °C), for both strains and matrixes. The most notorious difference was obtained at 40 s of PL exposure

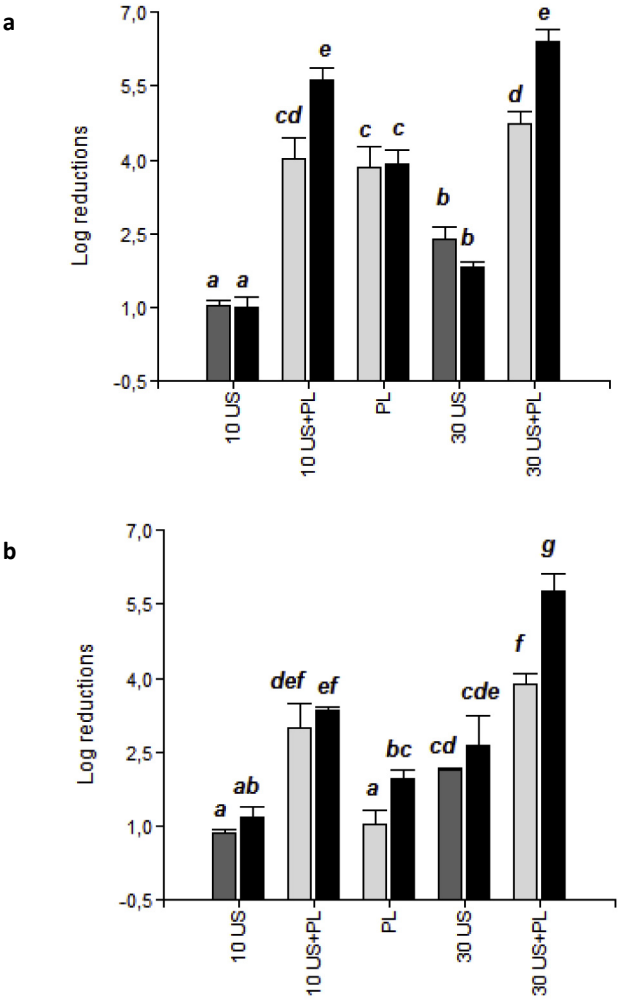

Fig. 1. Log-reductions and significant differences among treatments obtained by ANOVA and the Tukey Test of *S. cerevisiae* KE 162 cells in commercial apple (a) or freshly pressed apple juices (b) treated with single or combined US and PL. Temperature of single US: 20 °C (■), 44 °C (■); PL initial temperature, T_{PLi}: 2–20 °C (■) or 44–56 °C (■). In the labels, number means the treatment time in min for US. Different letters above the bars represent significant differences ($p < 0.05$) among treatments according to the Tukey Test. Standard deviation (I).

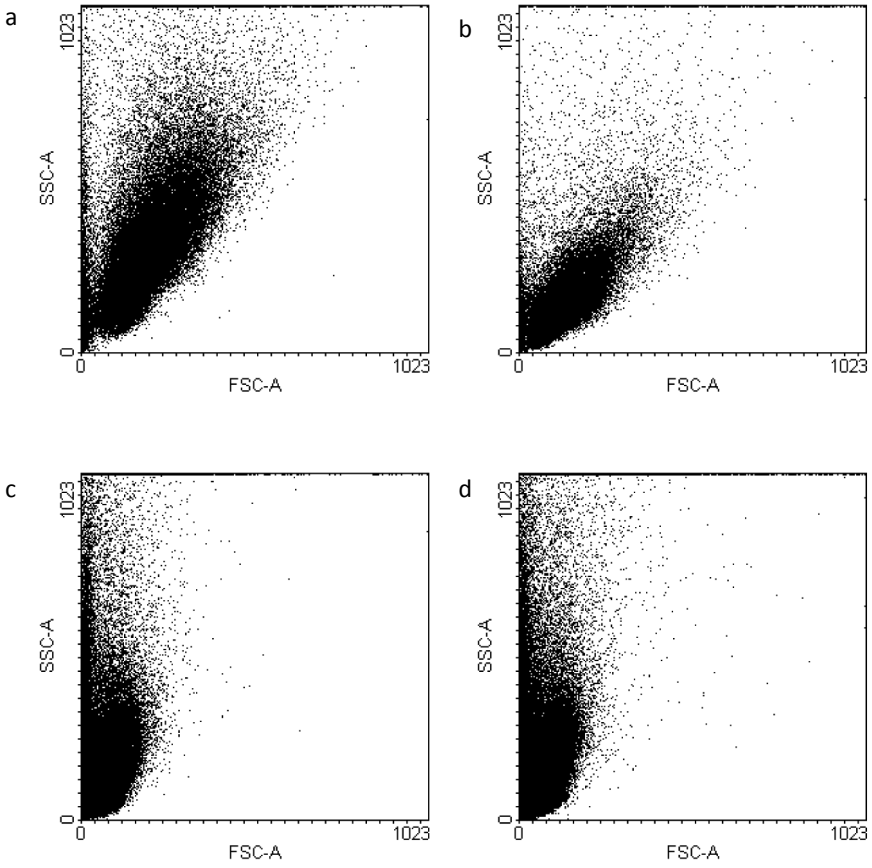

Fig. 2. Dot plot representing forward scatter light (FSC) versus side scatter light (SSC) of *S. cerevisiae* KE 162 cells in commercial apple juice. a) untreated cells; b) 60 s PL treated cells; c) 30-min US treated cells (T_{US}: 44 °C); d) 30-min US + PL. T_{US}: 44 °C/T_{PL}: 44–56 °C.

(47.8 J/cm^2). At this dose, it was reported, 0.7 or 2.1 log-reductions for *A. acidoterrestris* ATCC 49025 spores, and 1.56 or 3.91 log-reductions for *S. cerevisiae* KE 162 in commercial apple juice, depending on whether final sample temperature had been 20 or 56 °C. Moreover, Krishnamurthy et al. (2008) also reported that temperature build-up caused by the IR portion of PL (0.98 W/cm^2, continuous mode) in continuous flow enhanced the inactivation of *Staphylococcus aureus* ATCC 25923 in milk. They suggested that temperature increase could not have been responsible for inactivating *S. aureus* as the highest temperature recorded was a non-lethal value of 38 °C.

The $\boldsymbol{E_{EO}}$ parameter, which allows to estimate treatment decontamination efficacy, was 6.4×10^5–1.1×10^6 or 9.8×10^5–3.8×10^6 kW h/m^3/order for single US and PL treatments, respectively. While, combined 10-min and 30-min US + PL exhibited $\boldsymbol{E_{EO}}$ values in the same range than single treatments (1.6×10^6–4.9×10^6 kW h/m^3/order). Literature focused on $\boldsymbol{E_{EO}}$ estimation is available mainly for measurements of the cost effectiveness of UV-based AOP (Advanced Oxidation Process) systems (Li et al., 2008; Behnajady et al., 2009). Unfortunately, there is little information regarding E_{EO} estimation for reducing microbial load in juices or other food matrixes processed by PL or UV-C light for comparison purposes. In particular, Ferrario and Guerrero (2016) evaluated PL decontamination efficiency of *Escherichia coli* ATCC 35218, *Salmonella* Enteritidis MA44 and *Saccharomyces cerevisiae* KE 162 inoculated in commercial clarified apple juice (pH: 3.5, 11.1 °Brix) and centrifuged freshly pressed apple juice (pH: 3.5, 12.6 °Brix) treated by single US (600 W, 20 kHz, 95.2 μm of wave amplitude, 30 min, 25 °C) or PL (0–71.6 J/cm^2, T < 12 °C) treatments in batch mode operation. In agreement with the results shown in the present work, similar $\boldsymbol{E_{EO}}$ values were obtained when applying single US ($\boldsymbol{E_{EO}}$: 4.4×10^5–1.1×10^6 kW h/m^3/order) and single PL (6.2×10^6–3.8×10^7 kW h/m^3/order) treatments.

3.3. Flow cytometry study

Dot plots corresponding to *S. cerevisiae* KE 162 control cells subjected to single PL (T_{PL}: 44–56 °C), single 30 min US (T_{US}: 44 °C) or combined 30 min US+PL (T_{US}: 44 °C/T_{PL}: 44–56 °C) in CAJ are shown in Fig. 2. Untreated *S. cerevisiae* KE 162 cells (Fig. 2a) produced unimodal clustering with respect to SSC and FSC signals, extending virtually from small to high sizes and granulometries, but more concentrated in the range from smaller to intermediate values. This could be attributed, at least partially, to different orientation of the elongated yeast cells as they travel through the laser beam generating different granulometry and size (Schenk et al., 2011). Single PL produced a more uniform distribution with respect to FSC and SSC of *S. cerevisiae* KE 162 cells in apple juice (Fig. 2b). Higher reductions in both signals were observed after single US and combined US+PL treatments (Fig. 2c and d), indicating that cells became more symmetric in shape, with smaller size and lower granularity. These results are in agreement with Ferrario et al. (2013a), who observed a reduction in FSC and SSC signals of *S. cerevisiae* KE 162 cells exposed to PL (batch mode, 2.4–71.6 J/cm^2, initial T_{PL}: 2 °C) in peptone water and commercial apple juice. All treatments assayed in NAJ shown similar light

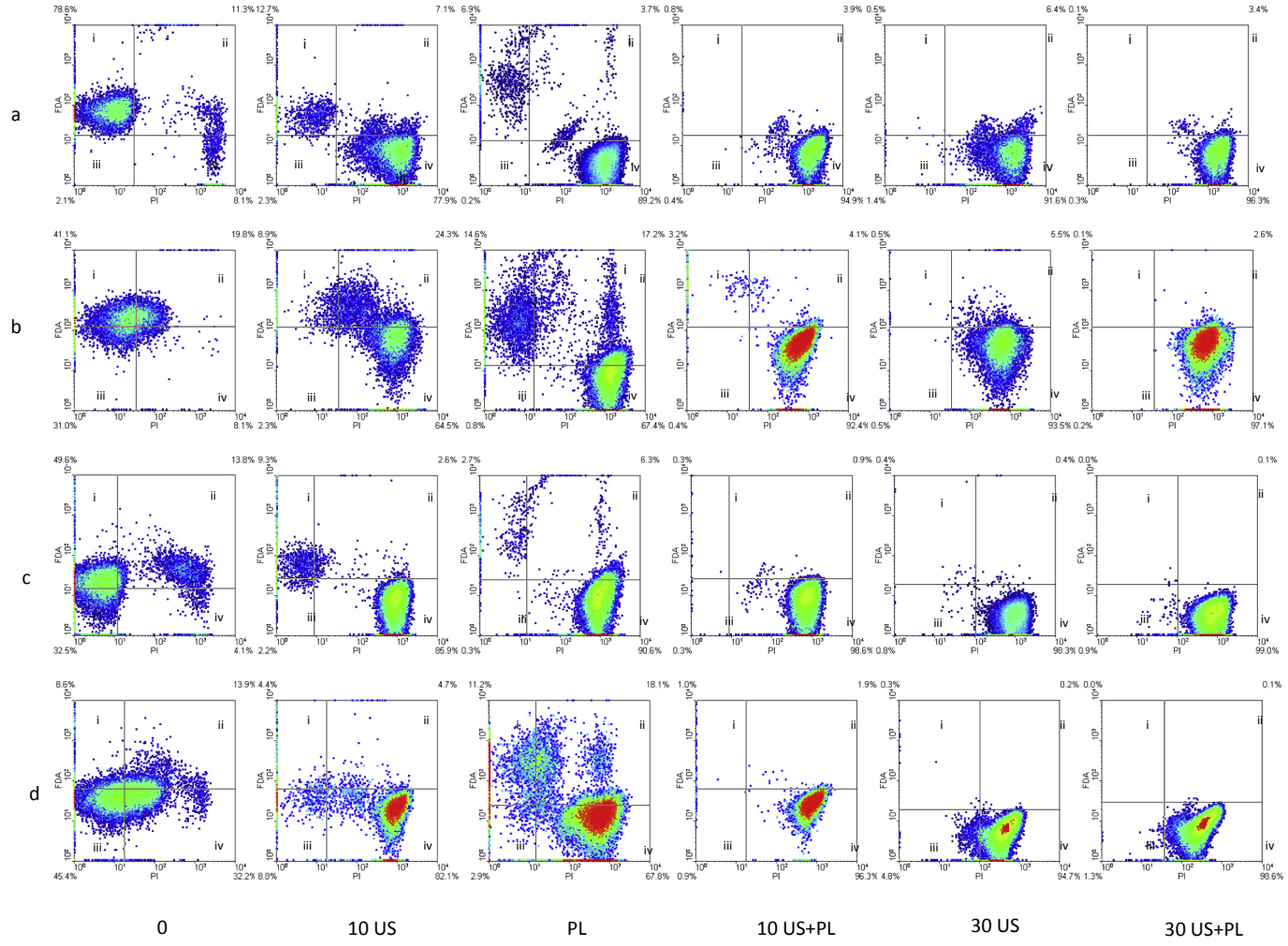

Fig. 3. Fluorescence density plots of *S. cerevisiae* KE 162 in response to staining with FDA and PI after single US (10 and 30 min), single PL (71.6 J/cm^2) and 10-min or 30-min US+PL treatments. T_{US}: 20 °C; T_{US}: 20 °C/T_{PL}: 2–20 °C in commercial apple (a) and freshly pressed apple (b) juices; T_{US} 44 °C, T_{US}: 44 °C-T_{PL}: 44–56 °C in CAJ (c) and NAJ (d). The percentage of microbial populations which falls in each gate are displayed in the four edges of each plot.

scattering behaviour compared to CAJ (data not shown).

Fig. 3 shows dual-parameter density plots of green fluorescence (y-axis) and red fluorescence (x-axis), monitoring ability of *S. cerevisiae* KE 162 cells in CAJ and NAJ to accumulate and retain F as an indicator of membrane integrity and enzyme activity, and uptake of PI to assess membrane damage after exposure to single or combined US and PL. The percentage of microbial populations which falls in each gate can be found in the four edges of each plot in Fig. 3.

Exposure of *S. cerevisiae* cells to single or combined treatments led to a shift of cells from gate 1 (cells with esterase activity and intact membrane) to gate 4 (cells with permeabilized membrane), being the shift almost complete as 91.6–98.3% (Fig. 3a–d) and 92.4–99.0% cells were found in gate 4 for single 30-min US and combined 10-min and 30-min US+PL treatments, respectively. Otherwise, single 10-min US produced a less pronounced shift with 64.5–85.9% of cells found in gate 4, depending on the type of juice and PL temperature range (Fig. 3a–d). Besides, single PL produced a shift to gate 4 of ~90% and ~67% cells in CAJ and NAJ, respectively at both temperatures. The less proportion of stained F^-PI^+ cells observed for NAJ is in concordance with the lower yeast inactivation observed by plate count technique (Fig. 1a and b).

An important fraction of double stained F^+PI^+ cells (~18%) was found in NAJ after single PL at both temperature ranges, which indicates that cytoplasmic membrane integrity was affected by the treatment, but cells retained esterase activity by hydrolyzing the FDA fluorochrome. Presence of this sublethal injured subpopulation could seriously influence shelf life of PL-treated foods (Zhao et al., 2011) since cells are metabolically active but cannot be detected by conventional culture methods (Ueckert et al., 1995; Schenk et al., 2011). In a previous study, the induced damage of *S. cerevisiae* KE 162 cells in peptone water (pH 3.5 and 5.6) and apple juice (pH 3.5) processed by different doses of PL (batch mode, dose range: 2.4–71.6 J/cm^2, T < 20 °C) was analyzed by FC (Ferrario et al., 2013a). In agreement with the actual results, most of the population was located at F^-PI^+ gate at the highest PL dose (71.6 J/cm^2), probably indicating that PL treatment provoked rupture of cytoplasm membrane and progressive loss of esterase activity. However, at low doses (2.4–6.0 J/cm^2), the presence of double-stained *S. cerevisiae* KE 162 cells in apple juice was observed (Ferrario et al., 2013a). The existence of sublethally injured *E. coli* DH5-á and *L. innocua* 11288 cells with compromised membrane was also observed by Pataro et al. (2011) after PL treatment (continuous flow mode; T < 20 °C, 1.8–5.1 J/cm^2) in apple (pH: 3.5) and orange (pH: 3.8) juices by culturing in selective and non-selective agar. They also found that the proportion of this

subpopulation depended on the delivered energy dose and the type of juice.

The application of PL after 10-min US treatment, considerably increased the $F^{-}PI^{+}$ population for both juices and temperature ranges. These observed changes suggest that all treatments destroyed membrane integrity, allowing PI to easily penetrate cells, causing accumulation of red fluorescence due to its binding to DNA, as well as releasing of F. Several authors have evaluated the induced damage of cells after exposure to different emerging physical agents including US (Ananta et al., 2005; Joyce et al., 2010; Wu et al., 2012), UV-C (Schenk et al., 2011), PEF (Zhao et al., 2011), PL (Ferrario et al., 2013a), among others by FC in order to provide a better understanding of the mechanisms involved in cell inactivation. In particular, our results are in agreement with Joyce et al. (2010) who reported 76.5 and 90.9% of dead cells after 10 and 15 min sonication of an *E. coli B* suspension in phosphate buffer saline (200 mL, 20 kHz, 25 °C), respectively. Moreover, Wu et al. (2012) studied integrity and viability of a *Microcystis aeruginosa* CCAP 1450/15 suspension treated with 30 min US (200 mL, 20 kHz, 25 °C) by FC. They found that 85% of the microbial counts were in the " dead gate". They attributed cell viability loss to the mechanical and almost instantaneous effect of US which breaks down cell membranes. In contrast with our findings, Ananta et al. (2005) studied membrane rupture by FC of a suspension of *E. coli* K12 DH 5a and *Lactobacillus rhamnosus* GG cells in phosphate buffer saline after 18 or 20 min of sonication, respectively (20 kHz, 10 mL, T < 20 °C). They reported that only 11% *L. rhamnosus* cells exhibited seriously compromised cytoplasmic membrane integrity, while there were not *E. coli* cells solely stained with PI.

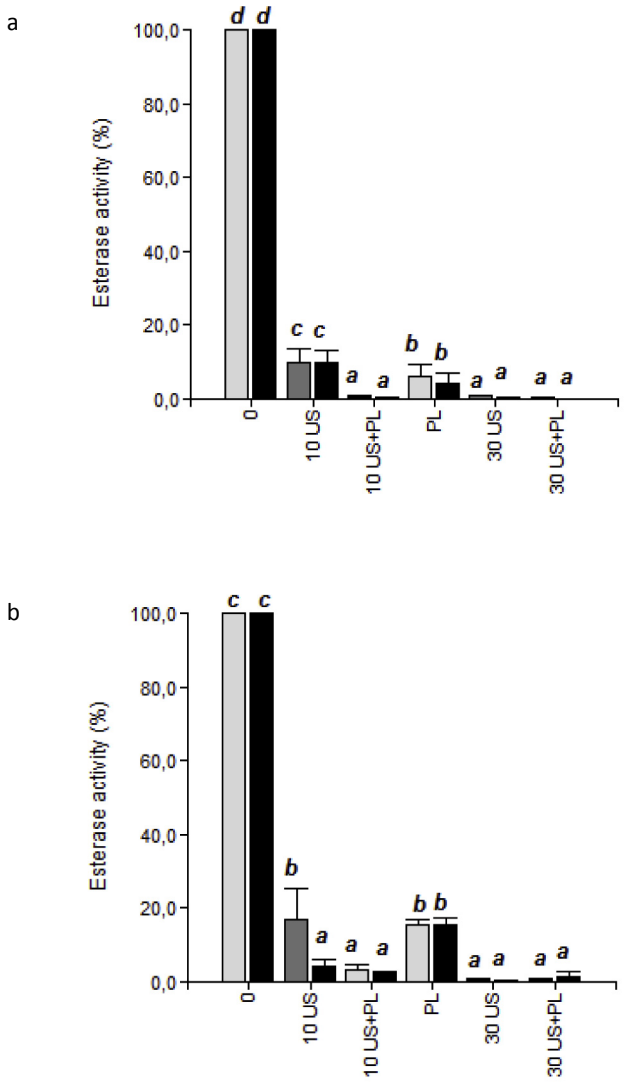

Fig. 4. Relative changes in esterase activity, *(%EA)* of *S. cerevisiae* KE 162 control and treated cells in commercial apple (a) and freshly pressed apple juices (b). 10 and 30 min- US, T_{US}: 20 °C (▒) or T_{US} 44 °C (■); 60 s PL, T_{PL}: 2–20 °C (▒) or T_{PL}: 44–56 °C (■); US + PL, T_{US}: 20 °C/T_{PL}: 2–20 °C (▒) or T_{US} 44 °C/T_{PL}: 44–56 °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. Standard deviation (I).

After 10 min US at 20 °C, 77.9 and 64.5% of cells were found in gate 4 in CAJ and NAJ; whereas a higher fraction of cells in the range from 82.1 to 85.9% was found when temperature of US treatment was set at 44 °C (Fig. 3a–d). Thus,, temperature increase enhanced the shift from gate 1 to 4 in these juices. In the case of NAJ sonicated during 10 min at 20 °C, a significant proportion of double stained cells (24.3%) was found. In agreement with these results, Ananta et al. (2005) observed a notorious fraction of double stained *E. coli* K12 DH 5α cells (16%) after sonication (18 min, 20 kHz, 10 mL, T < 20 °C). They attributed this phenomenon to an intermediate state of membrane damage, which may allow PI to penetrate the cell and avoid release of F, unless membrane degradation proceeds to a more compromised condition.

The lower percentage of cells found in gate 4 for the 10 min US condition (Fig. 3a–d) positively correlates with the lower inactivation obtained by the plate count method (Fig. 1). In addition, there were no differences in the percentage of $F^{-}PI^{+}$ subpopulation observed between single 30-min US (91.0–98.3%) and combined 10-min and 30-min US+PL (92.4–99.0%) treatments at both temperature ranges of PL treatment, and for both juices (Fig. 3a–d). Nevertheless, these treatments exhibited differences in *S. cerevisiae* KE 162 inactivation achieved by the plate count method (Fig. 1). Cell sorting of the subpopulation found in gate 4 corresponding to 30-min US+PL treatment at both temperatures, was not able to recover. In contrast, after plating the subpopulation found in gate 4, corresponding to 30-min sonication, 2×10^2 CFU/mL and 1×10^2 CFU/mL were able to recover at T_{US}: 20 and 44 °C, respectively. These results suggest that although membrane permeabilization occurred after single US exposure, would not the only responsible factor for yeast inactivation.

The relative changes in esterase activity obtained after juice exposure to single US or combined US+PL treatments at different temperature ranges, and the corresponding significant differences among treatments obtained by the Tukey Test are shown in Fig. 4. *Treatment* × *temperature* interactions were statistically significant with high F-partial values (p-value< 0.001) in the case of NAJ, while only *treatment* parameter resulted significant (p-value< 0.001) in the case of CAJ. The R-squared statistic explained the observed variability by 99% (data not shown). Esterase activity was reduced to 10–16% after exposure to 10-min US at both temperatures in CAJ, and only at 20 °C in NAJ (Fig. 4). Single PL reduced esterase activity to ~15% in NAJ and 4–6% in CAJ at both temperatures. Less than 2% of esterase activity was found after 10 and 30-min US + PL and single 30-min US for both matrixes and temperature profiles (Fig. 4).

Fig. 5 illustrates the permeabilized *S. cerevisiae* KE 162 cells in CAJ (a) and NAJ (b), determined by PI uptake according to different single and combined treatments along with the corresponding significant differences among treatments obtained by the Tukey Test. *Treatment* × *temperature* interactions were statistically significant parameters with high F-partial values (p-value< 0.001) and R^2_{adj} values from 0.94 to 0.96 (data not shown). All treatments induced membrane permeabilization. Non-significant differences

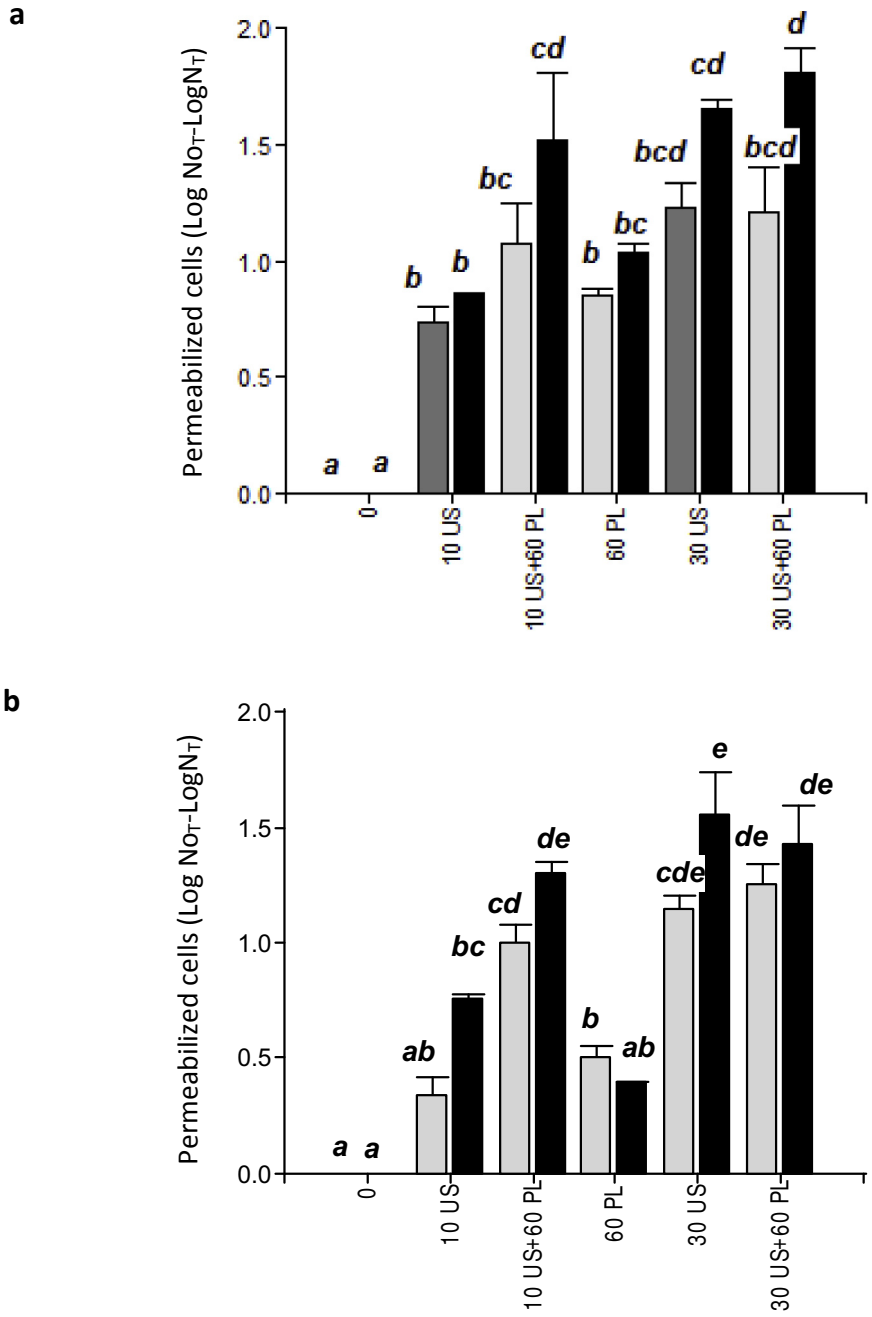

Fig. 5. Permeabilized *S. cerevisiae* KE 162 cells determined by PI uptake (Log No$_T$-LogN$_T$) as a function of treatment in commercial apple (a) and freshly pressed apple juices (b). 10 and 30 min- US, T_{US}: 20 °C (▒) or T_{US} 44 °C (■); 60 s PL, T_{PL}: 2–20 °C (▒) or T_{PL}: 44–56 °C (■); US + PL, T_{US}: 20 °C/T_{PL}: 2–20 °C (▒) or T_{US} 44 °C/T_{PL}: 44–56 °C (■). In the labels, number means time of treatment in min (US) or seconds (PL). Standard deviation (I).

were observed between single 10-min US and single PL treatments at both temperatures and matrices, as up to 0.8–0.9 or 0.5–1.0 log increases in membrane permeabilization were obtained, respectively (Fig. 5a and b). However, single 30-min US provoked, higher *S. cerevisiae* KE 162 membrane permeabilization (1.5–1.6 log) than single 10-min US (0.8–0.9 log) in NAJ, and only at the highest temperature in CAJ (Fig. 5a and b). These observations are in agreement with the higher inactivation observed by the plate count method for 30-min US treatment (Fig. 1). Furthermore, temperature increase enhanced *S. cerevisiae* KE 162 membrane damage in NAJ subjected to single 10-min US (Fig. 5b). The combined 10-min US+PL treatment, which provoked up to 1.3 log increase in membrane permeabilization, was significantly higher compared to single 10-min US at both temperatures in NAJ, and only at the highest temperature range in CAJ (Fig. 5a and b).

Increase in permeabilized cells versus log reductions achieved after exposure to single or combined treatments is displayed in Fig. 6. This plot led to analyze the relationship between the decrease in *S. cerevisiae* KE 162 cells viable population (determined by the plate count method) and membrane permeabilization (corresponding to cells that had incorporated PI). In accordance with Aronsson et al. (2005), Ananta et al. (2005) and our previous studies (Ferrario et al., 2013a), reported a higher number of inactivated cells in relation to the corresponding

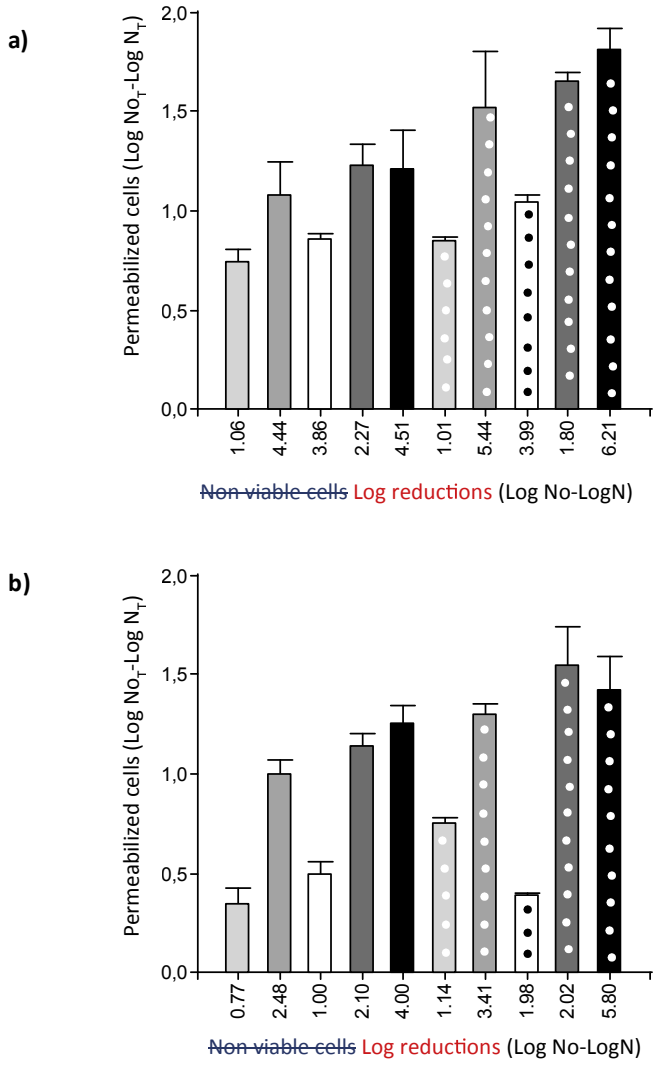

Fig. 6. Permeabilized *S. cerevisiae* KE 162 cells determined by PI uptake as a function of log reductions determined by CFU method in commercial apple juice (a) and freshly pressed apple juice (b). 10 min US (▫), 10 min + PL (▪), PL (□), 30 min US (■), 30 min + PL (■); T_{US}: 20 °C/T_{PL}: 2–20 °C (solid bar) and T_{US} 44 °C, T_{US} 44 °C/T_{PL}: 44–56 °C (dotted bar). In the labels, number means the time of treatment in min (US) or seconds (PL). Standard deviation (I).

permeabilized cells. For example, 5.7 and 6.2 log reductions were obtained by the plate count method after the combined treatment 30-min US + PL at the highest temperature assayed in CAJ and NAJ, respectively. Nevertheless, only 1.8 and 1.4 log of permeabilized cells were detected by FC. Moreover, single 30-min US treatment carried out at 44 °C provoked only 2 log reductions of *S. cerevisae* KE 162; but exhibited a membrane permeabilization degree similar to those systems which had reached more than 5 log-reductions. Aronsson et al. (2005) attributed this phenomenon to a possible reversible permeabilization, which may have not been disclosed by PI uptake. Otherwise, Ananta et al. (2005) obtained a very low percentage of F^-PI^+ stained cells after 20 min of sonication in relation to cell inactivation determined by plate count method, and suggested that cell death caused by sonication could even occur without any severe membrane damage. Therefore, these results strongly suggest the occurrence of other inactivation mechanisms, which along with membrane permeabilization, may explain yeast inactivation.

3.4. Transmission electron microscopy

Ultrastructural studies corresponding to some selected treatments were carried out to analyze possible structural damage caused in yeast cells, conferring a better understanding about the microbial inactivation mechanism. Untreated (control) and treated *S. cerevisiae* KE 162 cells in CAJ processed with single 10-min US (T_{US}: 44 °C) and PL (T_{PL}: 44–56 °C) treatments and the correspondent combined treatment are exhibited in Fig. 7. Untreated cells (Fig. 7a–c) showed the typical ellipsoidal shape, while cell structures; such as walls, organelles and membranes, appeared intact with high electronic density, with a continuous smooth cell wall. Exposure to single US (Fig. 7d–f) resulted in puncturing of cell walls and discontinuity of cytoplasmic membrane with or without leakage of content (Fig. 7d and e), and complete disorganized lumen (Fig. 7f). Likewise, PL treatment (Fig. 7g–i) provoked more rounded and less densely stained cells (Fig. 7h), plasmalemma shrinking (7 g) with coagulated lumen (Fig. 7g–i) or even vacuolated (Fig. 7g) Cytoplasmic membrane shrinkage could have resulted in loss of membrane semi-permeability; and hence, the osmotic equilibrium may have been disturbed. Whereas, the observed cytoplasmic membrane damage, which encompasses change of cell shape, vacuolization and cell membrane distortion, may have occurred due to the disturbance caused by intermittent high intensity pulses, which leads to the leakage of cellular content from the cytoplasm and eventually to cell death (Krishnamurthy et al., 2008). Moreover, the observed coagulated inner content may be attributed to the photothermal effect which could had cause a localized heating of the cell content and its consequent vaporization (Krishnamurthy et al., 2008).

In contrast to sonicated cells, no efflux of intracellular material was detected in cells subjected to single PL. When cells were exposed to combined US+PL treatment (Fig. 7j–l), more severe damage was observed, including more rounded cell shape and swollen walls (Fig. 7j); complete coagulated lumen (Fig. 7j,l); dispersed intracellular material without visible plasma membrane and cell wall, commonly denominated “ghost cells” (Fig. 7j), vacuolated inner content (Fig. 7k); broken cell wall with or without efflux of intracellular material (Fig. 7k and l). Therefore, several additional targets to membrane damage were observed.

Previous studies (Ferrario et al., 2013a) revealed that *S. cerevisiae* KE 162 cells in peptone water (pH: 3.5) and apple juice (pH: 3.5, 9.5° Brix) subjected to PL treatment (batch mode operation, 0–71.4 J/cm^2) exhibited ultrastructural changes, which were more severe as PL dose increased. In agreement, PL treated cells exhibited raised and expanded vacuoles, less differentiated cytoplasm with more rounded unnatural shape, puncture sites at the cell surface, discontinuity of plasma membrane with leakage of content and swollen walls. Accordingly, Takeshita et al. (2003) reported rounded cell shape, plasma membrane distortion and increase in vacuole size after PL exposure (1.4–2.1 J/cm^2, batch mode operation) of *S. cerevisiae* IFO2347 cells in model media. Moreover, Krishnamurthy et al. (2008) observed cell wall damage and cell content leakage of *S. aureus* ATCC 25923 in model solution treated with PL (5 s, 4.95 J/cm^2, 12 mL, batch mode operation). They also reported plasmalemma shrinking and collapse of internal structures In addition, TEMs observations corresponding to US conditions are in agreement with previous studies achieved by Guerrero et al. (2005), who analyzed the response of *S. cerevisiae* KE 162 to 20 min US treatment in culture media (100 mL, 45 °C, 20 kHz) and reported puncturing of cell walls and discontinuity of plasmalemma with leakage of content, wall rupture or fragmentation, and disruption of organelles.

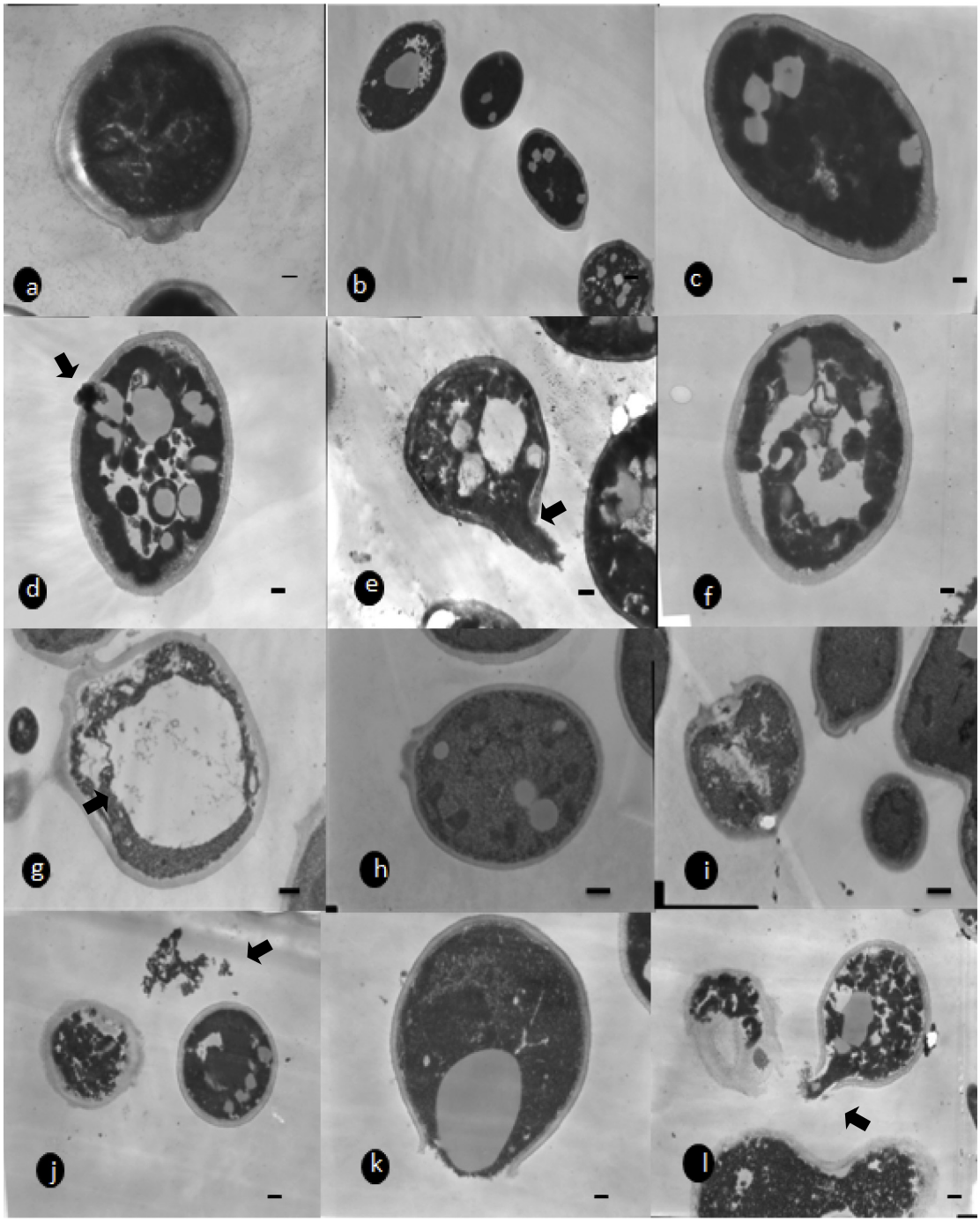

Fig. 7. TEM images of *S. cerevisiae* KE 162 cells. a,b,c: untreated cells; d–f: 10-min US (T_{US}: 44 °C) treated cells; g–i: PL (T_{PL}: 44–56 °C); j–l: 10-min US + PL (T_{US}: 44 °C, T_{PL}: 44–56 °C) treated cells in commercial apple juice. Scale: a, d, f, k: 200 nm; c, e, l: 270 nm; j: 350 nm; g: 500 nm; b: 590 nm; h,i: 600 nm. a,c) General aspect of intact cells. b) Group of intact cells with similar general aspect. d) Yeast cell showing complete disorganized inner content with single puncture site (arrow). e) Single puncture site at the cell surface with efflux of inner material (arrow). f) Discomposed inner structure and cytoplasmic membrane, but cell wall not broken. g) expanded vacuole (arrow). h) Detail of rounded damaged cell wall with intracellular content undistinguishable. i) Group of cells exhibiting more rounded shape, disorganized and coagulated inner content. j) Group of rounded cells showing complete disorganized lumen and residues of inner content without visible plasma membrane and cell wall ("ghost cells") (arrow). k) Broken cell wall and cellular membrane (arrow) and expanded vacuole. l) Broken cell wall and plasmalemma and inner material radiating from the cell (arrow), vacuolated and disrupted inner content.

Many distinct structural changes in yeast cells were visualized after exposure to single or combined US and PL treatments, evidencing various targets other than membranes. Results revealed by TEM images may confirm the observed discrepancy between the reductions achieved by plate count technique and the permeabilized cells observed by FC (Fig. 6). A great proportion of cells appeared with apparently intact wall and membrane, but with coagulated inner content, cell shape distortion, and disruption of inner material, strongly suggesting its contribution to microbial inactivation. It is also important to highlight that TEM observations, which revealed that treated cells exhibited more rounded shape and total or partial loss of inner content, were well correlated with the reduction in cell size and granularity observed by FSC and SSC signals (Fig. 2).

4. Conclusions

The present study revealed that the application of moderate heat build-up generated from the PL processing itself, improved the inactivation effectiveness. In addition, the use of pretreatment involving US turned out to be a promising alternative for inactivating *S. cerevisiae* KE 162 in apple juice. Greater inactivation was obtained in clear apple juice compared to the freshly pressed one, due to its lower absorbance and turbidity. FC and TEM techniques contributed to a better understanding of the inactivation mechanism of *S. cerevisiae* KE 162 cells in apple juice subjected to single PL with different heat build-up or combined with US. In one hand, FC allowed quantification of double stained cells in certain treatments which were unable to be detected by the plate count method. On the other hand, FC revealed almost complete yeast membrane permeabilization and loss of metabolic activity, despite the fact that a large proportion of viable cells were detected by the traditional plate count technique. TEM images revealed other structural changes like altered cell shape, vacuolization, coagulation of inner content and loss of cell wall; which along with membrane permeabilization, would be involved in the inactivation process. The additional structural changes revealed by TEM would explain the discrepancy between the reductions achieved by the plate count technique and the permeabilized cells observed by FC. Further studies with other microorganisms of concern would be necessary for a better understanding of microbial response to the proposed combined treatments.

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