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Study of pulsed light inactivation and growth dynamics during storage of *Escherichia coli* ATCC 35218, *Listeria innocua* ATCC 33090, *Salmonella* Enteritidis MA44 and *Saccharomyces cerevisiae* KE162 and native flora in apple, orange and strawberry juices

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Summary The response of some inoculated strains and native flora to PL treatment (Xenon lamp, 3 pulses s^{-1}, 10 cm distance from the lamp, 71.6 J cm^{-2}) in apple, orange and strawberry fresh juices with different absorbance, turbidity and particle size was investigated. Microbial growth dynamics during 12-day storage (5 °C) of PL-treated juices was also evaluated. PL treatments provoked 0.3–2.6 log reductions for inoculated microorganisms and 0.1–0.7 for native flora. High turbidity and particles with high UV absorbance seemed to play a major role in the PL efficiency compared to particle size. Cold storage of PL-processed juices provoked an increase in *Salmonella* Enteritidis and *Listeria innocua* inactivation, achieving 5.0–8.0 log reductions, while no recovery of *Escherichia coli* and retardation for yeast growth was observed, compared to untreated samples. This study gives valuable information regarding the influence of juice variables on PL effectiveness and emphasises the beneficial effect of a postcold storage on microbial safety of PL-treated juices.

Keywords Food safety, fruit juices, physical preservation methods, pulsed light, shelf life.

Introduction

Fruit juices are susceptible to spoilage thus having a limited shelf life. Yeasts and moulds are considered the main spoilage agents due to the low pH of most fruits (Raybaudi-Massilia *et al.*, 2009). Several yeast species may initiate fermentation, but are soon overgrown by more ethanol-tolerant strains like *Saccharomyces cerevisiae*. Moreover, pathogens such as *Listeria monocytogenes*, *Escherichia coli* and *Salmonella* Enteritidis may form part of juice microflora, posing a potential safety problem (Vojdani *et al.*, 2008). The US Food and Drug Administration (FDA) issued a final rule requiring fruit and vegetable juice producers to implement a 5-log pathogen reduction process (FDA, 2004). To fulfil with these requirements, fruit juices are nowadays processed by thermal procedures. Nevertheless, pasteurisation cause significant damage on organoleptic, nutritional and physicochemical properties of fluid foods (Elmnasser *et al.*, 2008). The new approaches mostly involve nonthermal fruit juice preservation technologies that offer complete or partial alternatives to heat treatments. They include, among other physical procedures, the application of pulsed electric fields (PEF) in combination with PL (Caminiti *et al.*, 2011), PL combined with thermosonication (TS) (Muñoz *et al.*, 2012), pulsed ultraviolet light (UV-C) (Orlowska *et al.*, 2013), high-intensity ultrasound (US) (Char *et al.*, 2010) and high-hydrostatic pressure (HPP) (Guerrero-Beltran *et al.*, 2011). Some of these methods have already been commercialised. Some are still in the research or pilot scale.

Pulsed light (PL) is one of the emerging preservation technologies that are being studied as a viable alternative to conventional processes. It involves the use of

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intense and short-duration (1 μs–0.1 s) pulses of broad spectrum light of wavelength ranging from UV to near infrared (200–1100 nm). Power is magnified by storing electricity in a capacitor over relatively long times (fractions of a second) and releasing it in a short time (millionths of thousandths of a second) (Gómez-López *et al*., 2007). It has, comparatively to continuous UV light, higher penetration depth and emission power (Krishnamurthy *et al*., 2007). Its use has been approved by the FDA (2004) for the decontamination of food and food surfaces. PL is one of the most promising nonthermal decontamination technologies for food products due to the significant microbial reduction in very short treatments time, the limited energy cost, the low environmental impact, the lack of residual compounds and its great flexibility (Oms-Oliu *et al*., 2010). Its efficacy has been mainly attributed to microbial DNA damages by thymine dimmer formation (photochemical effect) and/or to localised overheating of microbial cells (photothermal effect) and/or to structural damage caused by the pulsing effect (photophysical effect) (Wekhof, 2000; Krishnamurthy *et al*., 2008). It is possible that all these mechanisms coexist, and the relative importance of each one would depend on the fluence imparted to the food and target microorganism (Gómez-López *et al*., 2007). PL has shown to inactivate vegetative cells and spores in a variety of food products such as milk, corn meal, lettuce, white cabbage, carrots, cut apple, infant food, sugar syrup, meat and fruit juices, among others (Jun *et al*., 2003; Choi *et al*., 2010; Izquier & Gómez-López, 2011; Palgan *et al*., 2011a; Gómez *et al*., 2012; Ganan *et al*., 2013; Ferrario *et al*., 2013a). Despite the fact that there is a vast scientific literature focused on the inactivation of relevant pathogens or spoilage microorganisms exposed to PL, very few studies investigate the treated microbial load evolution over time (Uesugi & Moraru, 2009; Palgan *et al*., 2011a; Gómez *et al*., 2012). The relevance of studying this issue lies in the fact that PL-treated surviving cells could be sublethally damaged and are capable to recover and return to their normal function (Pataro *et al*., 2011; Ferrario *et al*., 2013b). In fact, Lasagabaster & Martínez de Marañón (2014) investigated the impact of post-PL treatment temperature conditions on *L. innocua*. They suggested that PL induced sublethal damages that could make *L. innocua* cells more sensitive to the subsequent refrigeration temperature.

Some authors reported that PL was successful in extending the product's shelf life. Gómez *et al*. (2012) determined that PL treatment extended fresh cut apple discs shelf life by 7 days. According to Gómez-López *et al*. (2005a), iceberg lettuce treated with PL only had a 3-day shelf life exceeding after this period of time the microbial recommended counts.

The purpose of the present work was to evaluate the effect of PL treatment on the response of some relevant microorganisms inoculated in fruit juices as well as its native flora. The effect of a postexposure of PL-treated samples to low-temperature conditions was studied in order to determine whether cold storage contributed to improve PL inactivation. In addition, it was also explored how differences in fruit juices absorbance, turbidity and particle size influenced PL effectiveness.

Materials and methods

Strains and preparation of inocula

Experiments were performed using *E. coli* ATCC 35218, *L. innocua* ATCC 33090, *S.* Enteritidis MA44 and *S. cerevisiae* KE162 (all strains were generously provided by Medica-Tec SRL, Buenos Aires, Argentina). *L. innocua* ATCC 33090 was used as a nonpathogenic indicator for *L. monocytogenes* because of its high-phenotypic similarity (Fairchild & Foegeding, 1993). All bacterial inocula were prepared by transferring a loopful of Trypticase Soy Agar plus 0.6 g/100 g Yeast Extract (TSAYE) slant stock culture to a 20-mL Erlenmeyer flask of Trypticase Soy Broth supplemented with 0.6 g/100 g Yeast Extract (TSBYE). They were incubated at 37 °C under agitation for 18 h until they reached stationary phase. A similar procedure was repeated for the yeast culture, where the initial inoculum was prepared by transferring a loopful of a fresh stock culture maintained in Potato Dextrose Agar (PDA) to an Erlenmeyer flask containing 20 mL of Sabouraud Dextrose Broth. Incubation was performed at 27 °C for 24 h. All inocula were harvested by centrifugation (5000 r.p.m., 5 min) (Labnet International Inc., Edison, NJ, USA), washed twice with saline solution and re-suspended in peptone water (0.1% w/v) to give a cell density of 10^7–10^9 CFU mL^{-1}. For PL treatment, inoculated juice samples were independently prepared. Six tuplicates of 100 μL of microbial suspension were added to 4.9 mL fruit juice prior to the PL treatment achieving a final microbial concentration of 10^6–10^8 CFU mL^{-1}, depending on the considered micro-organism. Three replicates were PL processed and three were analysed without any treatment (control samples). All microbiological procedures were performed in a Class II security cabinet (Nuaire Inc., Plymouth, MN, USA). All microbiological media used in this study were purchased from Biokar (Biokar Diagnostics, Beauvais, France).

Fruit juices

Natural squeezed juices of orange (*Citrus sinensis*, var. Valencia, pH: 4.3 ± 0.2, 10.1 ± 1.4 Brix), apple (*Pyrus malus* L, var Granny Smith, pH: 3.4 ± 0.2, 12.6 ± 0.1 Brix) and strawberry (*Fragaria ananassa* var. Duch, pH: 3.8 ± 0.3, 8.8 ± 1.4 Brix) were used in this study.

These juices were selected based on the results of a previous work which showed differences among these fruit juices in microbial inactivation during PL exposure with doses ranging from 2.4 J cm^{-2} to 71.6 J cm^{-2} (Ferrario *et al.*, 2013a). In this study, the inactivation curves were characterised by different predictive models, which revealed the existence of PL-resistant subpopulations, which might have variable growth dynamics during storage.

Before juice processing by PL, fruits were washed with water, dipped in 0.02/100 mL sodium hypochlorite and sterile water to eliminate surface microbial load and gently dried with a sterile cloth. Juices were obtained using a household juicer (Bluesky, Ningbo, China), immediately centrifuged to reduce pulp amounts (2500–6000 r.p.m., 10 min) (Eppendorf, model 5804 R, Hamburg, Germany) and collected in a sterile flask for their subsequent processing. The overall preparation of juice samples was made inside the Class II security cabinet previously mentioned. All cutting boards, tools, holding vessels and the juicer parts were sanitised before use in the same way as described for fruits and exposed to UV-C light during 10 min.

For native flora assay, fruit juices were obtained as described above but sample processing was not performed in aseptic conditions to increase the initial level of native flora.

Pulsed light treatment

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 (nontoxic, mercury free) and an air-cooling system attached to the lamp housing to avoid lamp overheating during operation (Ferrario *et al.*, 2013a). It generated high-intensity pulsed light at a pulse rate of 3 pulses s^{-1} (pulse magnitude with a peak of ~18 kV) 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. Fluence measurements were taken in triplicate by a pyroelectric head model ED500 (Gentec Electro-Optics, Québec, Canada) connected to an oscilloscope model TDS 2014 (Tektronix, Beaverton, OR, USA), with an aperture cover of 20.3 cm^2. For each PL treatment, 4.9 mL of refrigerated juice (~4 °C) was poured into a 100 mm (external diameter) Petri dish to ensure that the entire plate surface was covered with sample to a depth of 1 mm and was immediately inoculated as described above. The Petri dish containing the inoculated juice sample was placed inside a 150 mm (external diameter) Petri dish filled with ice flakes to minimise juice temperature increase, and altogether were put below the lamp (10 cm distance) and over the centre line of a stainless steel shelf inside the PL unit. The sample was exposed to PL during 60 s, corresponding to a dose of 71.6 J cm^{-2}. The selected sample location and dose were optimised in previous studies, being those that allowed minimal variations in PL dose according to fluence measurements, minimal sample heating and significant microbial reductions (Gómez *et al.*, 2012; Ferrario *et al.*, 2013a). Inoculated and PL unprocessed juice samples were used as controls for challenge test studies during refrigerated storage. Uninoculated and PL unprocessed samples were used as controls for native flora studies during storage. Experiments were run in triplicate for each condition.

A separate study was conducted to monitor the temperature evolution during PL treatment of uninoculated juice samples using a T-type thermocouple whose tip was placed at the centre of the Petri dish containing the juice sample. The thermocouple was connected to a data logger Digi-Sense model 69202-30 (Barnant Company Division, Barrington, IL, USA), and measurements were taken in triplicate (Ferrario *et al.*, 2013a).

Particle size, absorbance and turbidity measurements

For these studies, uninoculated juice samples were used. Particle size of each fruit juice 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). Measurements were taken at a fixed scattering angle of 173°, with a measurement range according to the manufacturer. The relationship between the particle size and the diffusion coefficient is defined by the Stokes–Einstein equation ($d(H) = (k.T)/(3.\pi.\eta.D)$) (Malvern Instruments, 2004), where, $d(H)$: hydrodynamic diameter (m), D: translational diffusion coefficient ($m^2\ s^{-1}$), k: Boltzmann's constant (1.38×10^{-23} N m K^{-1}), T: absolute temperature (K), η: solvent viscosity (N s m^{-2}). The intensity distribution obtained was converted to volume distribution, using the Mie theory (Malvern Instruments, 2004). Fruit juice particle size in the range from 0.1 to 1000 μm was measured in triplicate by static light scattering (SLS) using a Mastersizer 2000 (Malvern) device equipped with a Hydro 2000MU as dispersion unit (Malvern). The pump speed was settled at 1800 r.p.m.

For both particle size ranges, the refractive index (RI) of juices (1.35) and their absorption parameter (0.1) were used, according to the specifications provided by the manufacturer for coloured samples (Malvern Instruments, 2004). Absorbance of 0.1% v/v dilution of each sample was measured at 254 nm in 1 cm-path

quartz cuvettes using an UV-VIS spectrophotometer (V-630; Jasco, Tokyo, Japan) to determine the transparency of different juices at the most germicidal portion of the UV range. In addition, turbidity was determined by centrifuging samples (1500 r.p.m., 10 min), and measuring the supernatant absorbance at 660 nm (Rivas *et al*., 2006). Absorbance and turbidity measurements were performed in triplicate.

Storage studies

For each condition, treated and unprocessed (control) inoculated PL juice samples were aseptically pooled into one sterile vessel, and subsequently fractioned into 20-mL aliquots dispensed into caramel flasks which were immediately stored in the dark at 5 ± 1 °C for 12 days. Throughout storage, two flasks were taken at preset time intervals (24–72 h) for analysis of survivors. Peptone water (0.1% ten-fold dilution aliquots) were surface plated by duplicate onto TSAYE for *E. coli*, *S.* Enteritidis and *L. innocua* or, PDA for *S. cerevisiae,* using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA, USA). A counting grid was used for enumeration of colonies. When treatment resulted in low counts ($<10^3$ CFU mL^{-1}), up to 3 mL of fruit juice was directly pour plated into each Petri dish. Plates were incubated for 72 h at 37 ± 1 °C in the case of bacteria and at 27 ± 1 °C in the case of yeasts. Experiments were run in triplicate. Plots of log N/N_0 (where N is the number of colony forming units per juice millilitre (CFU mL^{-1}) at a given time and N_0 the initial number of CFU mL^{-1} vs. treatment time were obtained.

Native flora study

Uninoculated and kept overnight (to increase native microflora level) juice samples of orange, apple and strawberry juices were used for this study. To collect enough volume sample, four independent samples treated or not (control) by PL were pooled into 20-mL caramel flasks and stored under refrigeration conditions (5 ± 1 °C) for 10 days. Throughout storage, two flasks were taken at each preset time interval (24–72 h) for analysis in duplicate of total mesophilic aerobic and, yeast and moulds survivors. Total mesophilic aerobic populations were determined on Plate Count Agar (PCA) plates incubated for 72 h at 37 ± 1 °C, whereas yeasts and moulds populations were cultured on Chloramphenicol Glucose Agar (CGA) plates and incubated at 25 ± 1 °C for 5 days. Plots of log N vs. treatment time were obtained.

Statistical analysis

Statistical analyses were carried out using InfoStat 2009 (InfoStat Group, FCA-UNC, Córdoba, Argentina). Multivariate analysis of variance (MANOVA) was applied to detect differences in absorbance at 254 nm, turbidity and particle size of juices. A two-way analysis of variance (ANOVA), from triplicates, was used to evaluate the influence of juice and strain after 60 s PL and by 12 days of refrigerated storage. In case of finding significant differences, Tukey's test was performed Partial correlation analyses, with a significance level at $P < 0.05$, were performed to find associations between the *log reductions* and the juice parameters. For all statistical analyses, significance level was set at $P < 0.05$. Principal component analysis (PCA) was applied to illustrate the relationship among tested systems and absorbance at 254 nm, turbidity, particle size and the log reductions achieved after PL treatment. The cophenetic correlation coefficient (*CCC*) was obtained as a measure of how faithfully the analysis preserved the original euclidean distances among data points. An adequate PCA corresponded to a *CCC* value close to 1.0.

Results and discussion

Absorbance and turbidity and particle size measurements

Table S1 shows the absorbance at 254 nm ($A_{254\ nm}$), particle size and turbidity of the juices. Strawberry juice exhibited the highest absorptivity and turbidity, followed by orange and apple juices. Turbidity is a measure of the quantity of particulates in a solution. Thus, systems with higher turbidity will show lower UV transmission, diminishing the efficiency of the PL pasteurisation process (Koutchma *et al*., 2009). Besides, contact between photons and the target cells should occur. Therefore, the presence of absorbing particles in the UV range, which will be reflected on the absorptivity value, is detrimental for the disinfection process efficacy (Gómez-López *et al*., 2007). Accordingly, systems with higher turbidity and $A_{254\ nm}$ would exhibit lower PL inactivation. Particle size distribution of juices, exhibited in Fig. S1, resulted multimodal. Similar distributions from 59 to 6439 nm and 21.4 to 6439 nm were obtained for strawberry and apple juices, respectively, showing peaks at 712 and 5560 nm. An additional peak was found for apple juice at 51 nm. Regarding to orange juice, particle size was considerably higher having two peaks at 0.9 and 677.7 μm. Betoret *et al*. (2009) reported similar particle size for centrifuged fresh orange juice (var. Salustiana, pH: 3.5, 12.7 Brix) with a peak at 552.4 μm, whilst Corredig *et al*. (2001) observed a distribution with a peak in 1000 nm in commercial orange juice (pH: 4.0, 12 Brix). Other juice variables, such as pH and Brix, did not appear to play a major role in the inactivation efficacy as strawberry juice showed lower values of these parameters than orange juice; however,

no significant differences in the inactivation level were determined between both juices (Fig. S2). In agreement with these results, Murakami *et al.* (2006) and Koutchma *et al.* (2004) showed that solutions with different soluble solids content (Brix) did not affect inactivation rates during continuous UV-C treatment.

Effects of PL treatment on inoculated micro-organisms

The initial sample temperature averaged ~2 ± 1 °C, according to the adopted process design. During PL treatments juice temperature increased with time, however, it was always below 20 °C. On average, juice temperature increased between 8.0 and 16.8 °C after PL exposure, depending on the matrix. Strawberry juice exhibited the highest temperature increase (16.8 °C), followed by apple (12.7 °C) and orange juices (8.0 °C) (data not shown). In a previous study, the absorbance spectra of the juices in the range from 200 to 1100 nm were examined (Ferrario *et al.*, 2013a). All juices absorbed mainly in the UV range, with negligible absorption in the visible or near infrared spectra. Strawberry juice exhibited the highest absorptivity in the UV range, followed by orange (with an absorption peak in 263 nm) and natural apple juices. Therefore, the different temperature increase of juices could be attributed to differences in their absorptivities in the UV range.

The inactivation degree obtained by PL for different micro-organisms inoculated in natural squeezed apple, strawberry or orange juices are illustrated in Fig. S2. Inactivation in orange and strawberry juices ranged between 0.3 and 0.8 log cycles of reduction for all evaluated strains. Otherwise, 1.6, 2.1 and 2.4 log reductions were achieved for *L. innocua, E. coli*, and *S.* Enteritidis, respectively, in apple juice. *S. cerevisiae* was the most resistant strain to PL treatment of apple juice as only 1.0 log cycle reduction was obtained. Therefore, PL treatment was more effective in apple juice, which was the system with lower turbidity and absorptivity at the most germicidal portion of the UV range (Table S1).

In agreement with these results, Koutchma *et al.* (2004) examined the effect of turbidity (1400 and 2400 NTU) on the inactivation rate of *E. coli* K12 exposed to UV in a continuous flow device (flow rate: 57 or 166 mL s^{-1}) in commercial apple cider. They reported a faster inactivation in the less turbid system at the higher flow rate. Regarding to the liquid absorptivity, Sauer & Moraru (2009) studied the inactivation of *E. coli* 25922 in Butterfield's phosphate buffer (BPB), Trypticase soy broth (TSB), 45 μm-microfiltered apple juice and cider treated with PL under static conditions (13.1 J cm^{-2}). They observed the highest inactivation (8.5 log reductions) in BPB, which showed no significant absorbance in the UV region. Reductions of 2.6 and 2.3 were obtained for apple juice and cider, respectively, and they both showed high absorbance of light in the range from 200 to 400 nm. A decrease of 3.2 log cycles of *E. coli* population was observed in TSB, which absorbed a small amount of light at wavelengths higher than 300 nm. It is important to highlight that in this study, fresh apple juice was assessed. In a previous work, Ferrario *et al.* (2013a) demonstrated that commercial apple juice (pH: 3.5, 12.5 Brix), which possessed lower absorbance in the UV range than fresh apple juice, exhibited greater PL inactivation in accordance with the results reported by other authors (Sauer & Moraru, 2009; Palgan *et al.*, 2011a). Moreover, Chaine *et al.* (2012) also observed a lower inactivation of *B. subtilis* spores in sugar syrup (65 Brix, 3.0 log reductions) than in distilled water (4.6 log reductions) after exposure to PL (1.8 J cm^{-2}) under static conditions. They suggested that these differences could be attributed to differences in the light transmission in the UV-C region as the absorption coefficient of clear syrup at 254 nm resulted 200-fold higher than that corresponding to distilled water.

The analysis of variance (ANOVA) determined that the terms *matrix*strain*, *strain* and *juice* were statistically significant for predicting log N/No with highly F-values (P-value <0.001, data not shown) which indicated that the sensitivity of each strain to PL treatment depended on the considered fruit juice. The obtained adjusted R-squared statistic (R^2 adj) revealed that 90% of the variability in the inactivation observed was explained by the variables juice and strain. *S.* Enteritidis was the most sensitive strain to PL treatment in apple juice, while no differences were observed between *E. coli* and *L. innocua*. Moreover, the degree of inactivation achieved by *L. innocua* was not significantly different from *S. cerevisiae* (Fig. S3). These results are in agreement with Gómez-López *et al.* (2005b) who did not find a sensitivity pattern to PL among an extensive variety of microorganisms. In contrast with our findings, Anderson *et al.* (2000) reported that Gram-negative bacteria were more sensitive than Gram-positive ones. Moreover, Palgan *et al.* (2011a) reported that *E. coli* was more sensitive to PL than *L. innocua* probably due to structural and compositional differences in the cell wall and membranes.

Growth dynamics of surviving micro-organisms during cold storage of fruit juices inoculated microorganisms

Storage studies were carried out to analyse the possibility of microorganism recovery in fruit juices processed by the exposure to PL treatment (71.6 J cm^{-2}). The survival curves of *E. coli*, *S.* Enteritidis, *L. innocua* and *S. cerevisiae* in PL treated apple juice throughout the cold storage are shown in Fig. S3. Log reductions of *S. cerevisiae* achieved after PL treatment

were maintained during 9 days, while 1.1 log cycles were recovered by the end of storage (Fig. S3i). PL treatment retarded yeast recovery in apple juice as control samples began to grow 3 days earlier (Fig. S3i). Reductions of 2.0 and 2.5 log cycles were obtained for *L. innocua* (Fig. S3ii) and *S.* Enteritidis (Fig. S3iii) populations in apple juice after PL exposure, respectively. However, an important decay of both PL treated populations was observed during cold storage, reaching 7.8 log reductions for *L. innocua,* while no colonies were detected in the case of *S.* Enteritidis. *L. innocua* and *S.* Enteritidis populations decreased in apple juice without PL treatment (control samples), as 2.9 and 3.5 log reductions were obtained, respectively, after 12 days of storage (Fig. S3ii, iii). This behaviour could be attributed to the low pH and temperature conditions. On the contrary, PL-treated cells which would probably be more sensitised to the low-pH environment, exhibited a higher decrease in population towards the end of storage. Thus, the combination of PL with postcold storage (5 °C) had a synergistic effect on the inactivation of *S.* Enteritidis and *L. innocua* in apple juice. These results suggest that PL treatment might damage proteins or enzymes involved in the mechanisms that allow these strains to grow at low temperatures and control the proton gradient across membrane. Recent studies obtained by flow cytometry reported the existence of sublethally damaged *S. cerevisiae* cells in peptone water (pH 3.5) treated with PL (static condition, 0.1 m distance from the lamp, 2.1–71.6 J cm^{-2}) which were stressed and lost their ability to grow in agar but still showed metabolic activity (Ferrario *et al.*, 2013b). Moreover, sublethal damage has already been reported by Pataro *et al.* (2011) by culturing in selective and nonselective agar. They observed that *E. coli* and *L. innocua* cells exhibited compromised membrane as a consequence of PL exposure (continuous flow, 500 mL volume, 1.8–5.1 J cm^{-2}) in commercial orange (pH: 3.8, 11.1 Brix) and apple (pH 3.5, 10.9 Brix) juices. In agreement with our previous findings, the proportion of this subpopulation depended on the type of matrix, being higher in the apple juice sample, which exhibited lower absorptivity.

With regard to *E. coli* population, PL treatment provoked a slight decrease of 1.3 log reductions in apple juice remaining almost constant, without further recovery, during storage (Fig. S3d).

Figure S4 shows the growth dynamics of the surviving microbial populations in PL-processed orange juice during refrigerated storage. *S. cerevisiae* cells that were not exposed to PL treatment (control) were able to grow 0.7 log cycles towards the end of storage. *S. cerevisiae* population treated with PL remained constant in orange juice until the ninth day and then they started to grow up to 0.9 log cycles by the end of storage (Fig. S4i). In addition, *L. innocua* cells treated with PL decreased 1 log and continued to decline during storage, reaching 3.9 log cycles whereas control cell counts remained constant (Fig. S4ii). This could indicate that although *L. innocua* cells seemed to be resistant to PL treatment, they may exhibit certain level of damage, which combined with a low pH and temperature environment, would ban their recovery during cold storage. Cold storage of juice samples inoculated with *S.* Enteritidis and *E. coli* and PL treated added only 0.7 log reductions to the inactivation achieved by PL exposure (0.5 log reductions Fig. S4iii, iv). The corresponding populations in control orange juice remained roughly constant throughout the whole storage.

Survival curves corresponding to microbial populations in PL processed strawberry juice during refrigerated storage are shown in Fig. S5. Despite the fact that PL treatment was ineffective inactivating all inoculated microorganisms in strawberry, the refrigerated storage prevented from yeast recovery (Fig. S5i). In addition, *L. innocua* population was reduced up to 6 log cycles in stored strawberry juice within the second day of cold storage maintaining this value towards the end of storage. Whilst control juice exhibited a less pronounced decrease of *L. innocua* population reaching 4 log reductions by 7 days of storage (Fig. S5ii). Otherwise, scarce reductions between 0.9 and 1.3 log cycles were observed for treated *S.* Enteritidis and *E. coli*, while control samples were reduced in a range from 0.6 to 0.2, respectively (Fig. S5iii, iv).

An ANOVA was conducted to detect differences between control and PL-treated samples stored 12 days at 5 ± 1 °C in all the assayed juices. The terms *treatment*strain*, *treatment* and *strain* resulted significant for predicting the log reductions with high F values ($P < 0.0001$), while the R^2_{adj} ranged from 0.98 to 1.00 (data not shown). Overall, PL-treated samples showed lower log reductions than the unprocessed ones (Fig. S5i–iii). In particular, an important decrease in PL-treated population along storage was only observed for *L. innocua* in the three examined juices. Therefore, this bacteria resulted the most sensitive, among all evaluated strains, to an environment of high acidity and low temperature after PL exposure.

Most previous studies in the literature had focused on the inactivation of microorganisms by PL, but they did not evaluate the behaviour of micro-organisms after PL treatment, thus being this information scarce. However, different patterns of microbial response in PL-treated fruit products have been identified by different authors facing complications regarding microbial stability of PL-treated products during cold storage. These patterns include scarce or no inactivation followed by slower growth rate than untreated samples, or no growth of surviving population. In concor-

dance with our findings, Palgan *et al*. (2011a) studied *E. coli* and *L. innocua* inactivation in apple and orange juices treated in a static PL device (0.25 m distance from the lamp 1 mm sample depth 7–28 J cm^{-2}). They reported that neither *E. coli* nor *L. innocua* PL-treated cells recovered after 48 h of storage under cold conditions. Besides, Gómez *et al*. (2012) reported no increase in *E. coli* population in fresh cut apple discs treated with PL (71.6 J cm^{-2}, 0.1 m distance from the lamp) after 7 days of cold storage. They also found that *L. innocua* population continued decreasing along storage, reaching 2.5 log cycles reduction after 7 days. In addition, they reported that PL-treated *S. cerevisiae* was able to recover 0.4 log cycles after the third day of storage at 5 °C. Whist, in this study, *S. cerevisiae* population remained the level of reductions achieved after PL exposure (1.3 log reductions) after 9 days of storage at 5 °C and was able to recover 1.1 log cycles by 12 days of storage. Lasagabaster & Martínez de Marañón (2014) studied the effect of temperature culturing conditions (4 or 37 °C) of *L. innocua* treated with static PL (0–0.421 J cm^{-2}, 11 cm distance from the lamp) in BHI agar. They observed a higher inactivation rate at 4 °C compared to 37 °C, and in agreement with our results, suggested that PL induced sublethal damage that could make *L. innocua* cells more sensitive to the subsequent refrigeration conditions. Moreover, Garcia-Graells *et al*. (1998) observed a significant decrease upon storage at 8 °C of *E. coli* pressured treated (300–500 MPa, 15 min at 20 °C) in apple, orange and melon juices. These authors reported that the subsequent decline during storage was considerable compared to the inactivation achieved by pressurisation. They attributed this behaviour to a sublethal injury provoked by pressure treatment, resulting in a reduced resistance to low pH.

According to partial correlation analysis, a negative correlation ($P < 0.0001$) was obtained between the *log reductions* and $A_{254\ nm}$ (−0.90), *turbidity* (−0.89) and *particle size* (−0.87), thus indicating that at higher parameters values, lower inactivation was achieved. A PCA was performed in order to find how the different samples assayed were spatial distributed with respect to the *log reductions,* $A_{254\ nm}$, *turbidity* and *particle size*.

Figure S6 exhibits a bi-plot of principal components 1 and 2 (PC_1 and PC_2). The *CCC* obtained was 0.99, indicating that an accurate reduction in the number of variables employed was achieved with the analysis. The first two principal components, PC_1 and PC_2, explained 63.2 and 34.2% of variance, respectively. The PC_1 separated the $A_{254\ nm}$, which was positively associated, from the *log reductions* (negatively associated). On the other hand, PC_2 was associated positively with *particle size* and negatively with *turbidity*. Apple juice showed the lowest $A_{254\ nm}$ and *turbidity*, and similar *particle size* than strawberry juice, being both juice parameters lower than orange juice. Apple juice exhibited the highest inactivation for all strains, as expected due to the low juice parameter values obtained. Strawberry juice showed the lowest inactivation which could be attributed to the highest $A_{254\ nm}$ and *turbidity* values, as *particle size* resulted similar to that corresponding to apple juice. This behaviour could be attributed to the presence of anthocyanins. These compounds are present in berries (Jakobek *et al*., 2007) and have two absorption peaks in the UV region at 290 and 310 nm (Burger & Edwards, 1996). Many authors suggested that anthocyanin content is supposed to reduce DNA damage from UV-B and UV-C radiation. Burger & Edwards (1996) observed less damage of mature leaves of red Coleus varieties with epidermal anthocyanins than no accumulating varieties, exposed to UV-B and UV-C radiation. Moreover, Stapleton & Walbot (1994) demonstrated that the DNA in the *Zea mays* plants that contained flavonoids (primarily anthocyanins) was protected from damage caused by UV radiation compared to plants that were genetically deficient in this compound.

In addition, orange juice exhibited lower *log reductions* compared to apple juice but higher compared to strawberry juice. Although orange juice exhibited lower $A_{254\ nm}$ and *turbidity* values than strawberry juice, it showed considerably higher *particle size* values. Therefore, $A_{254\ nm}$ and *turbidity* were more significant variables than *particle size* explaining PL disinfection efficiency.

Native flora

PL treatment (71.6 J cm^{-2}) scarcely reduced initial mesophilic aerobe populations (0.1–0.7 log reductions) and mould and yeast populations (0.5–0.6 log reductions) in all assayed fruit juices, while its evolution during 10-day cold storage (5 °C) depended on the considered juice (data not shown).

Native flora in PL-treated apple juice samples showed a biphasic grow dynamics remaining without significant variation from its initial value (mesophilic aerobes and yeasts and moulds: ~3.3 log cycles) until the third day of storage with a subsequent proliferation. Nevertheless, the recommended limit for fruit juices, which is $<10^7$ CFU mL^{-1} for the aerobic colony count according to the Health Protection Agency (2009), was not exceeded until the eighth day of cold storage (mesophilic aerobes: 5.1 log cycles and yeasts and moulds: 6.1 log cycles). Unprocessed apple juice indigenous microbiota showed similar growth dynamics profile to PL-treated apple juice, exceeding always its value by 1–1.5 log cycles.

Pl treatment was ineffective reducing indigenous microbiota in orange juice (mesophilic aerobes: 3.4 log

cycles; yeasts and moulds: 3.1 log cycles), and no differences in its growth dynamics were observed during cold storage between PL-treated and unprocessed orange juice samples. In contrast with the other systems assayed, mesophilic aerobes and moulds and yeasts counts began to grow immediately, reaching the recommended limit of the Health Protection Agency (2009) near by day two of storage. A slight higher increase of 0.9 log cycles was observed for moulds and yeasts in the control juice compared to the PL-processed juice by the second day of storage.

The mesophilic aerobe and yeasts and moulds population in strawberry juice were 3.1 and 3.7 log cycles, respectively, after PL exposure. PL treatment only reduced the mesophilic aerobe counts in 0.9 and log cycles, while no inactivation was observed for yeasts and moulds. The recommended limit for fruit juices (Health Protection Agency, 2009) was not exceeded throughout the whole storage for strawberry juice, and no differences in the evolution of treated and control samples were recorded, suggesting that cold storage but not PL retarded native flora's growth dynamics. By the end of storage, counts corresponding to PL-treated samples reached ~5.5 log cycles. Strawberry juice resulted more stable than apple juice in which higher inactivation of inoculated microorganisms was observed. Nevertheless, it is important to highlight that each juice contains a particular microflora, which might have different PL sensitivity (Barth *et al.*, 2010). The type of spoilage microflora present is influenced by differences in physical properties and biochemical characteristics of the fruit, from which juices are obtained,

According to our results, the application of PL in combination with cold storage (5 °C) is maintained the mesophilic aerobes counts under the recommended limit (Health Protection Agency, 2009) during 8 and 10 days for apple and strawberry juices, respectively. To achieve a further extension of shelf life, combination with other nonthermal technologies is required. Successful combinations of PL with other emergent hurdles were reported with additive or synergistic effects on various microbial species in different foods and model systems and over a wide range of experimental conditions. However, very few studies documented native flora evolution of PL-treated samples during storage, being the obtained results highly variable, without any possibility of establishing a common pattern. Palgan *et al.* (2011b) studied shelf life on cold storage of a blend of apple and cranberry juice treated with a combination of PL (continuous flow, 360 μs, 3 Hz, 1.213 J cm^{-2} $pulse^{-1}$) and pulsed electric fields (PEF, 34 kV cm^{-1}, 18 Hz, 93 μs). They reported that although no counts were observed after PL treatment for mesophilic aerobes and moulds and yeasts, an increase of two log cycles was obtained for both groups after 14 days of storage at 4 °C. Besides, Gómez *et al.* (2012) reported no effect of PL (71.6 J cm^{-2}, 0.1 m from the lamp) on mesophilic aerobes population in fresh cut apple discs at the onset of storage. In contrast with our findings, treated mesophiles remained constant after 7 days of storage at 5 °C, while untreated samples showed an increase of two log cycles. Regarding to moulds and yeasts, they observed 1 log reduction after PL exposure and 1.6 log reductions by the end of the storage. Aguiló-Aguayó *et al.* (2013) reported 1 log reduction of aerobic mesophilic bacteria and moulds and yeasts in whole tomato fruit after exposure to PL (5.4 J cm^{-2}). They found that mesophilic aerobe counts remained constant by 3 days of storage at 20 °C, while moulds and yeasts increased 1 log cycle.

Conclusions

This study provided valuable quantitative information about the inactivation of some target microorganisms and native flora of fresh-squeezed fruit juices treated with PL under static conditions. In particular, the combination of PL with cold storage had a synergistic effect inactivating *L. innocua* in all the tested juices and *S.* Enteritidis in apple juice, achieving 5.0–8.0 log reductions. This could be attributed to PL treatment which may damage cells to such extension that sensitises them to other environmental stress factors like high acidity and low temperature. The presence of particles with high absorbance in the UV region or high turbidity of juices could play a major role in diminishing PL efficiency rather than particle size. Despite the initial scarce or moderate inactivation obtained after PL exposure, all strains were not able to recover and proliferate during post-treatment storage at low temperature and high acidity conditions. Although *S. cerevisiae* was able to recover in PL-treated apple and orange juices, it faced more difficulties to grow than in unprocessed juices. Due to the decay or growth retardation observed during cold storage, PL treatment coupled with subsequent low-temperature storage conditions can be applied to provide microbial safety in fruit juices. Nonetheless, this work contributed to address some limitations as PL was ineffective reducing the indigenous microbiota of apple, orange and strawberry juices during 10 days of cold storage. Thus, the combination with other factors is required to extend their shelf life. In addition, further studies encompassing larger volumes of treated juice in a continuous flow PL device are being conducted to assess the effects of PL treatments on juice properties besides microbial safety and spoilage for commercial purposes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Absorbance at 254 nm ($A_{254\ nm}$) and turbidity values of fruit juices.

Figure S1. Particle size distribution of (a) apple juice (▬), strawberry juice (- - - -), (b) orange juice. Standard deviation (I).

Figure S2. Log reductions of *E. coli* ATCC 35218 (□), *L. innocua* ATCC 33190 (■), *S. cerevisiae* KE162 (■) and *S.* Enteritidis MA44 (■) in natural-squeezed apple, strawberry and orange juices processed with 60 s PL.

Figure S3. Evolution of *S. cerevisiae* KE162 (i,●), *L. innocua* ATCC 33190 (ii,■), *S.* Enteritidis MA44 (iii,♦) and *E. coli* ATCC 35218 (iv,▲) in PL processed natural squeezed apple juice stored at 5 ± 1 °C.

Figure S4. Evolution of *S. cerevisiae* KE162 (i,●), *L. innocua* ATCC 33190 (ii,■), *S.* Enteritidis MA44 (iii,♦) and *E. coli* ATCC 35218 (iv,▲) in PL processed natural squeezed orange juice stored at 5 ± 1 °C.

Figure S5. Evolution of *S. cerevisiae* KE162 (i,●), *L. innocua* ATCC 33190 (ii,■), *S.* Enteritidis MA44 (iii,♦) and *E. coli* ATCC 35218 (iv,▲) in PL processed natural squeezed strawberry juice stored at 5 ± 1 °C.

Figure S6. Principal component analysis (PCA) biplots of *E. coli* (*E.c*), *L. innocua* (*L.i*), *S. cerevisiae* (*S.c*) and *S.* Enteritidis (*S.*E) log reductions, absorbance at 254 nm ($A_{254\ nm}$), particle size and turbidity of natural squeezed apple (App), strawberry (Str) and orange (Or) 60 s PL-processed juices.