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Bacterial inactivation in fruit juices using a continuous flow Pulsed Light (PL) system

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ABSTRACT

In this work, the susceptibility to pulsed light (PL) treatments of both a Gram-positive (*L. innocua 11288*) and a Gram-negative (*E. coli DH5-*α) bacteria inoculated in apple (pH = 3.49, absorption coefficient 13.9 cm^{-1}) and orange juices (pH = 3.78, absorption coefficient 52.4 cm^{-1}) was investigated in a range of energy dosages from 1.8 to 5.5 J/cm^2. A laboratory scale continuous flow PL system was set up for the experiments, using a xenon flash-lamp emitting high intensity light in the range of 100–1100 nm. The flashes lasted 360 µs at a constant frequency of 3 Hz.

The results highlighted how the lethal effect of pulsed light depended on the energy dose supplied, the absorption properties of liquid food as well as the bacterial strain examined. The higher the quantity of the energy delivered to the juice stream, the greater the inactivation level. However, the absorbance of the inoculated juice strongly influenced the dose deliver and, therefore, the efficiency of the PL treatment. Among the bacteria tested, *E. coli* cells showed a greater susceptibility to the PL treatment than *L. innocua* cells in both apple and orange juices. Following treatment at 4 J/cm^2, microbial reductions in apple and orange juices were, respectively, 4.00 and 2.90 Log-cycles for *E. coli* and 2.98 and 0.93 Log-cycles for *L. innocua*.

Sublethally injured cells were also detected for both bacterial strains, thus confirming that membrane damage is an important event in bacterial inactivation by PL.

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1. Introduction

Consumer demand for freshly squeezed fruit juices has increased over recent years, even though such products are susceptible to spoilage and thus have a limited shelf-life (Jordan, Pascual, Bracey, & Mackey, 2001). Although most pathogens do not grow in fruit juices due to their low pH, they can survive and adapt to the acid environment, increasing their tolerance to unfavorable growth conditions (Mazzotta, 2001). In fact, outbreaks involving *E. coli* O157:H7 and different serotypes of *Salmonella* occurred in unpasteurized orange and apple juices as well as cider, causing major health concerns worldwide (Char, Guerrero, & Alzamora, 2009). Even if *Listeria monocytogenes* is not known to have caused outbreaks through the consumption of fruit juices, it has been isolated in unpasteurized apple juice (Sado, Jinneman, Busby, Sorg, & Omiecinski, 1998).

Consequently, in order to provide more effective food preservation measures, the US Food and Drug Administration (US FDA, 2004) published a definitive regulation to increase the food safety of fruit and vegetable juices, requiring juice producers to implement a 5-Log_{10} pathogen reduction process.

In order to achieve this objective, food processors have recently shown increasing interest in replacing traditional preservation technologies with milder and more environmentally friendly alternatives.

Pulsed light (PL) is considered, among others, to be one of the most promising non-thermal decontamination technologies for food products. This technology, which can be considered an alternative to continuous UV light treatment for rapid and effective microbial inactivation in solid and liquid foods (Oms-Oliu, Martín-Belloso, & Soliva-Fortuny, 2010), involves a wide broad-spectrum light in the wavelength range of 100–1100 nm, with approximately 54% of energy emitted in the ultraviolet range. During the PL treatment, electrical energy cyclically accumulated in a high power capacitor is released over an inert gas (e.g. xenon) lamp as intermittent intense short pulses of light, which typically last for a few hundred microseconds, to considerably magnify the power (Krishnamurthy, Tewari, Irudayaraj, & Demirci, 2008).

A significant number of publications on the subject have documented the ability of PL to inactivate bacterial species (both as vegetative cells as well as spores), yeast, fungi and viruses either spread on agar surfaces or suspended in aqueous solutions (Anderson, Rowan, MacGregor, Fouracre, & Farish, 2000; Elmnasser et al., 2007; Oms-Oliu et al., 2010; Sharifi-Yazdi & Darghahi, 2006).

The most important factor determining the inactivation efficacy of PL is the energy dose (or fluence) incident on the sample. Additionally, the composition of the emitted light spectrum, the distance of the sample from the light source, the thickness, color, opacity, viscosity and product

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flow conditions of the liquid samples as well as the presence of particulate material, the inoculum size, the geometry of the treatment cell and the number of lamps are critical parameters that should be optimized in order to obtain the maximum effectiveness of the treatment along with minimum product alteration (Choi, Cheigh, Jeong, Shin, & Chung, 2010; Gómez-López, Ragaert, Debevere, & Devlieghere, 2007; Krishnamurthy, Demirci, & Irudayaraj, 2007).

The inactivation mechanism of PL is still not fully understood. However, it appears that the mode of the action of the PL process is attributed to the effects of the high peak power and the UV component of the broad spectrum of the flash (Oms-Oliu et al., 2010; Rajkovic, Smigic, & Devlieghere, 2010). Consequently, the lethal action of PL might be due to the coexistence of different mechanisms. Even though the efficacy of PL in microbial reduction has been traditionally attributed to microbial DNA damages by thymine dimer formation (photochemical effect) (Wang, MacGregor, Anderson, & Woolsey, 2005) and/or (to a lesser extent) to localized overheating of microbial cells (photothermal effect) (Hiramoto, 1984; Wekhof, 2000; Wekhof, Trompeter, & Franken, 2001), structural damage caused by the pulsing effect (photophysical effect) has been also detected (Krishnamurthy et al., 2008; Takeshita et al., 2003; Wuytack et al., 2003). It is therefore possible that the relative importance of each mechanism depends on the fluence, the type of microorganism as well as the food absorption characteristics.

There is not a great deal of information on the occurrence of sub-lethal damage to bacterial cells following exposure to PL as well as the influence of treatment conditions on the extent of damage (Rajkovic et al., 2009; Van Houteghem et al., 2008; Woodling & Moraru, 2005; Wuytack et al., 2003). Nevertheless, this is a critical aspect with regard to food safety as, under suitable conditions, sublethally injured cells can recover and return to normal function (Wu, 2008). In addition, more detailed information on this subject will allow to determine the most suitable conditions for PL processing as well as have a more comprehensive understanding on the microbial inactivation mechanisms of PL.

Current literature on food applications is relatively scarce, mainly dealing with the PL decontamination of solid and semisolid products such as vegetables (Gómez-López, Devlieghere, Bonduelle, & Debevere, 2005b), fruit (Marquenie et al., 2003), food powers and seeds (Fine & Gervais, 2004; Jun, Irudayaraj, Demirci, & Geiser, 2003; Sharma & Demirci, 2003), dairy products (Dunn et al., 1991), fish (Ozer & Demirci, 2006), honey (Hillegas & Demirci, 2003) and infant powder milk (Choi et al., 2010).

In addition, there is extremely limited data available on the application of PL for the decontamination of liquid foods other than clear water, such as fruit juices (Sauer & Moraru, 2009), infant foods (Choi et al., 2010) and milk (Krishnamurthy et al., 2007). Furthermore, to date, only one paper deals with the processing of a liquid food in a continuous flow PL unit (Krishnamurthy et al., 2007).

For these reasons, more detailed research is needed to demonstrate the suitability of the technology for the continuous flow treatment of non-clear liquid foods such as fruit juices, since food transparency, penetration depth, color, viscosity, opaqueness, soluble solids content and composition may have a negative impact on the penetration of UV light through the liquid.

This experimental study deals with microbial inactivation by PL in a continuous flow system. It aims at investigating the lethal and sub-lethal effects of PL treatments depending on the energy dose and absorption properties of two different fruit juices inoculated with a Gram-positive and a Gram-negative bacterial strain.

2. Material and methods

2.1. Microorganisms and growth conditions

Cells of the Gram positive *Listeria innocua* 11288 and the Gram negative *Escherichia coli* DH5-α were used throughout this study, with both strains being maintained on slants of Tryptone Soya Agar (TSA) (Oxoid, LTD., Basingstoke, Hampshire, England) and stored at 4 °C.

Cultures of each microorganism were prepared by transferring one colony of the organisms from the agar slants to 50 mL of sterile Tryptic Soya Broth (TSB) (Oxoid). The microbial suspensions were then incubated at 37 °C for 24 h without shaking for *L. innocua* or under agitation for *E. coli* in order to obtain cells in the early stationary phase. The final concentration reached by the microorganisms in the growth media was approximately 10^9 colony forming unit (CFU)/mL.

2.2. PL apparatus

Microbial inactivation experiments were carried out in a laboratory scale continuous flow PL unit which is illustrated in Fig. 1. PL

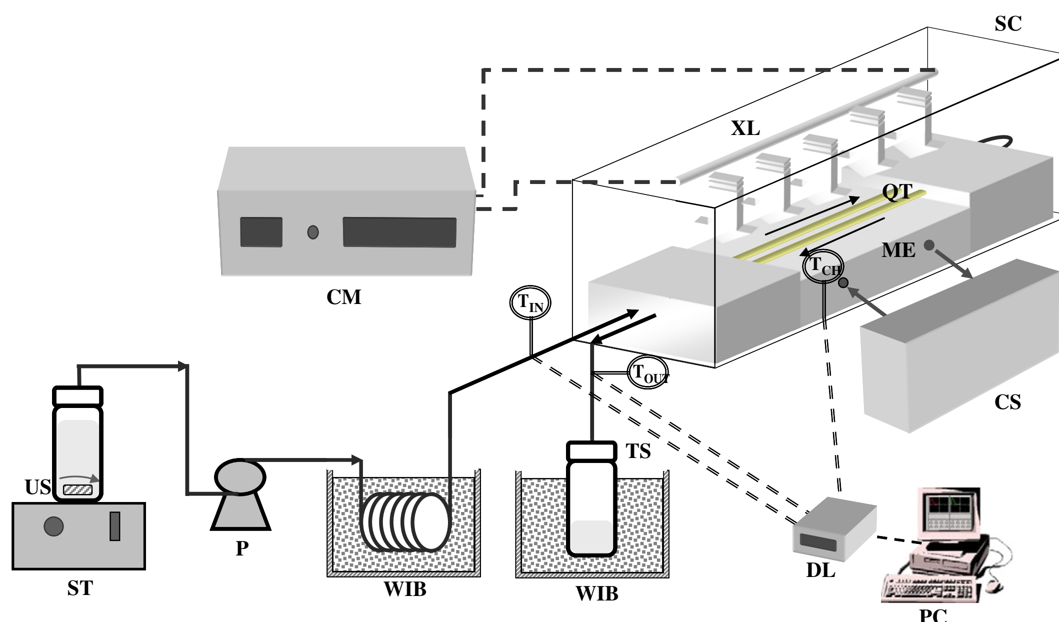

Fig. 1. Schematic diagram of the continuous flow PL system. ST, stirrer; US, untreated sample; P, pump; WIB, water–ice bath; SC, sterilization chamber; XL, xenon lamp; QT, quartz tubes; ME, metal enclosure; CS, cooling system; CM, power/control module; T_{in}, T_{out}, $T_{ch,}$ thermocouples; TS, treated sample; DL, data logger; PC, computer.

was generated by SteriPulse®-XL 3000 Pulsed light Sterilization System (Xenon Corp., Wilmington, Mass., USA) which consisted of a power/control module, a linear Xenon flash lamp and a lamp housing module. The sterilization system generated 3 pulses/s (pulse width 360 µs) of polychromatic light in the wavelength range of 100 to 1100 nm with, approximately, 1.21 J/cm^2/pulse at 1.9 cm from the quartz window surface for an input voltage of 3800 V as set out in the manufacturer's specifications. The distance between the quartz window and the central axis of the lamp was 5.8 cm. A factory supplied photo electric detector module (LiteMark-XL) mounted on the lamp housing, was used to check that the optical emissions from the source were consistent throughout the duration of the experiments. A forced air system with filter was used to remove ozone and heat from both the housing lamp and treatment zone.

As shown in Fig. 1, a peristaltic pump (model No. 77200–60, Masterflex® L/S ®, Cole-Parmer Instrument Company Ltd, London, U.K.) equipped with food grade tygon tube was used to pump the pre-stirred inoculated fruit juice through two consecutive quartz tubes (1 mm i.d., 0.5 mm wall thickness,) placed 1.9 cm below the quartz window. In order to maximize the energy absorption, the central axes of the quartz tubes were aligned with that of the Xenon lamp. Furthermore, only 20 cm of the tubes length was exposed to the light source, while the rest of the tubing, including flexible connections tubing between the two quarts tubes, was covered with aluminum foil to block UV-light exposure. The quartz tubes were kept in position by placing them on the grooved surface of a hollow metal enclosure. The latter was filled with a recirculating water–ethylene glycol solution from an external refrigerated bath (Model No. Viscotherm VT 100, Physica Messtechnik GmbH, Ostfildern, Germany) kept at −10 °C. This system contributed to both reducing the heating of the product during the pulse treatment as well as cooling the air in the sterilization chamber.

Stainless steel coiled tubes submerged in an ice–water bath were used to cool the juice product both before entering and immediately after leaving the chamber.

2.3. PL treatment

PL treatments were carried out by suspending microorganisms in reconstituted commercial apple and orange juices (Batchelors, Dublin, Ireland) whose physical and optical properties are reported in detail in Table 1. The juices were reconstituted from the concentrates using a water:juice ratio of 6.8:1 and 6.2:1 for apple and orange juices, respectively. A volume of 500 mL of each juice was inoculated with a pure culture suspension of either *E. coli* or *.L. innocua* in order to obtain an initial microbial load of approximately 10^6 CFU/mL. The inocula were prepared at least 1 h before treatment in order to allow the cells to adapt to the new environment. During this period of time no change in cell viability was detected (data not shown).

Prior to each experiment being carried out, the hydraulic line was first washed with distilled water, then sanitized by pumping a 5% (v/v) Milton solution and afterwards rinsed thoroughly with sterile distilled water. After the sanitization step, a sample of the water was taken to check the sterility of the system. Then, the inoculated juice was pumped through the system, with treatments at different PL energy dosages being carried out by changing the flow rate and, therefore, the residence time of the juice inside the quartz tubes (Table 2). Once the distilled water had been fully displaced and the outlet temperature of the product as well as the light parameters were steady, samples of the treated product were collected in sterile vials wrapped in aluminum foil, as a precaution to prevent photo-reactivation. The samples were, then, immediately placed in ice until the microbiological analyses. In all the experiments the inlet temperature of the juice to the sterilization chamber (T_{IN}) was set at 10 °C ± 1 °C, while the initial temperature of the air inside the chamber (T_{CH}) was approximately 18 ± 1 °C.

Table 1
Physical properties of fruit juices.

	pH (−)	Brix (−)	Absorption coefficient (cm^{-1})
Apple juice	3.49 ± 0.08	10.9 ± 0.2	13.9 ± 0.9
Orange juice	3.78 ± 0.6	11.1 ± 0.5	52.4 ± 1.2

Table 2
Processing conditions.

Flow rate (ml/min)	t_r[a] (s)	n[b] (−)	W_T[c] (J/cm^2)
38.4	0.49	1.5	1.8
27.0	0.70	2.1	2.5
20.8	0.91	2.7	3.3
17.0	1.11	3.3	4.0
13.4[d]	1.41	4.2	5.1
12.5[e]	1.51	4.5	5.5

[a] Residence time calculated from the volume of the quartz tubes (0.314 ml) and the flow rate.
[b] Number of pulses calculated from t_r and pulse repetition frequency (3 Hz).
[c] Energy dose calculated from the energy dose delivered for each pulse (1.21 J/cm^2) and n.
[d] Apple juice only.
[e] Orange juice only.

2.4. Temperature measurement

The inlet (T_{IN}) and outlet (T_{OUT}) temperatures of the fruit juice as well as the temperature of the air surrounding the quartz tubes inside the chamber (T_{CH}) were monitored by T-type thermocouples and recorded at 1 s intervals using a data logger (Mod. SQ2040-2F16, Grant Instruments Ltd, Cambridge, UK). Steady state values were then obtained and plotted against the energy dose delivered to the product stream. For each set of conditions investigated (Table 2), triplicate measurements were carried out.

2.5. Analytical measurements

The absorption coefficients of the tested fruit juices were determined according to the method of Koutchma, Keller, Parisi, and Chirtel (2004). Absorbance of the samples placed in fused quartz cuvettes with path lengths of 0.1, 0.2, 0.5 and 1.0 cm was measured at 254 nm using V-670 UV–vis spectrophotometer (Jasco Instruments, USA). The absorption coefficients of each juice were determined from the slope of the linear plot of the absorbance vs. path length.

°Brix evaluations, as the soluble solid content, were carried out at 25 °C using a hand held refractometer (0–50% Sugar Refractometer, Bellingham & Stanley Ltd., Tunbridge Wells, UK). The pH was measured at 25 °C using a pH meter (Model No. 9450, Unicam Ltd., Cambridge, UK).

All the measurements were carried out in triplicate.

2.6. Enumeration of viable and injured cells

The enumeration of viable and injured cells of both *E. coli* and *L. innocua* cells before and after PL treatment was measured by a colony count on agar medium plates. Control and PL treated samples were serially diluted in 0.1% (w/v) sterile peptone water. The non-selective agar medium used for enumeration of viable cells of both strains was Tryptone Soya Agar (TSA) (Oxoid, LTD., Basingstoke, Hampshire, England). Two selective agars were used in order to determine the number of injured bacterial cells: Eosin Methylene Blue agar (EMB) (Oxoid) was used to count *E. coli* and *Listeria* Selective Agar (LSA) (Oxoid) added with *Listeria* selective supplement (Oxoid) was used to count *L. innocua*.

From selected dilutions, 0.1 mL samples were spread in duplicates on the surface of non-selective and selective agar media plates. The plates were incubated at 37 °C for 24 h (*E. coli*) or 48 h (*L. innocua*) to

detect the viable cells. Plates containing selective media were incubated for 24 h more than those containing non selective media. Longer incubation times did not increase the microbial counts. The number of sublethally injured bacterial cells was estimated by the difference between the counts obtained from the non-selective (TSA) and selective agars (EMB or LSA).

The results are means on data from at least three experiments, with the standard deviations being indicated by error bars.

3. Results and discussion

3.1. Sample temperature

A well documented side effect of PL treatments is sample heating caused by the absorption of light by the food or by lamp heating. This aspect has been reported as the most important limiting factor of PL for practical applications (Elmnasser et al., 2007). In most studies, the use of high fluencies or long treatment times necessary to obtain the desired level of microbial inactivation resulted in a greater than desirable temperature increase causing a serious impairing of the food quality attributes. For example, when studying the inactivation of *A. niger* spores on corn meal, Jun et al. (2003) found that under some of the experimental conditions used, sample temperatures rose to120 °C. This effect, was likely to have been due to the large amount of heat generated by the lamps, even though an air cooling system was present. The generation of excessive heating also limited the treatment of alfalfa seeds (Sharma & Demirci, 2003), grated carrots (Gómez-López et al., 2005b), and raw salmon fillets (Ozer & Demirci, 2006). It was also reported to occur in honey (Hillegas & Demirci, 2003) and agar (Gómez-López, Devlieghere, Bonduelle, & Debevere, 2005a). In food powders (black pepper and wheat flour), the thermal effect of PL resulted in undesirable product color alterations occurring prior to microbial inactivation being completed (Fine & Gervais, 2004). To date, only Krishnamurthy et al. (2007) carried out measurements of the temperature increase during the PL treatment of milk in a continuous flow system. Milk temperature increased up to 38 °C, depending on the residence time as well as the distance of the product from the light source. This temperature increase caused a fouling effect as well as possible changes in milk quality.

The results of all these studies suggest that without an efficient cooling system incorporated in the equipment, PL treatments for long treatment times can seriously compromise food quality due to an excessive temperature increase (Elmnasser et al., 2007; Rajkovic et al., 2010). Furthermore, the excessive temperature rise may also introduce difficulties in separating the lethal effects induced by thermal energy from those related to PL energy.

In this study, the PL device was equipped with an external water–ethylene glycol cooling system described in Section 2.2, in addition to the manufacturer blower kit required for the dissipation of the heat generated by the lamp inside the lamp housing.

The results showed that, despite the differences in optical properties, the two juices showed a similar temperature rise during the PL process (data not shown). Therefore, only the results relating to the PL treatments of orange juice are presented.

Fig. 2 shows the typical profiles of the temperature increase of orange juice obtained with and without cooling as a function of the running time of the PL sterilization system. In both cases, the residence time of the product inside the quartz tubes was 0.91 s and the total energy dose delivered was 3.3 J/cm^2.

The results show that the temperature rise of the juice increased with increasing running time. However, the presence of a cooling system limited the heating rate and the final temperature of the samples. In addition, whereas after about 300 s of running time, the heat generated by the light absorption as well as lamp heating was equilibrated by that removed by the cooling system, when the same experiment was carried out without cooling, even after 1000 s of running time no steady state condition was reached. Similar time–temperature profiles were obtained when different processing conditions were used (Table 2) (data not shown).

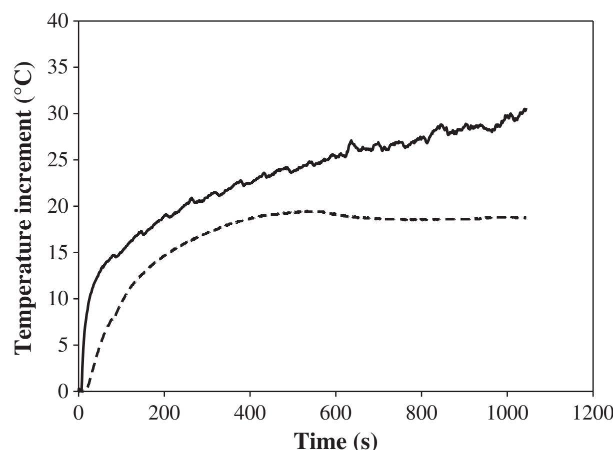

Fig. 2. Typical profiles of the temperature increment in orange juice obtained with (dashed lines) and without (solid line) cooling as a function of the running time of the PL sterilization system. Energy dose delivered 3.3 J/cm^2.

The steady state profiles of the temperature of both the air inside the sterilization chamber and the juice during the different processing conditions in presence of the cooling system are shown in Fig. 3. The results show that both temperatures increased gradually as the energy dose increased. However, although the use of the fan ensured that the air inside the chamber was continuously removed and replaced by external fresh air, the heating of the air immediately surrounding the quartz tubes was always greater than that of the juice flowing inside them, with this probably being due to the cooling effect provided by the metal box which was in direct contact with the quartz tubes. For example, increasing the fluence delivered to the sample from 1.8 to 5.5 J/cm^2 the temperature increase of the air inside the chamber changed from 32 to 37 °C, while that of the juice increased from 18 to 24 °C. However, it can be noted that, as the inlet temperature of the juice was kept at a constant value of approximately 10 °C, the corresponding outlet temperature of the juice was between 28 and 34 °C, thus never reaching values that would have any impact on the lethality of the microorganisms tested.

Due to the advantage presented by the use of the cooling system, all the microbial inactivation experiments described in this work were

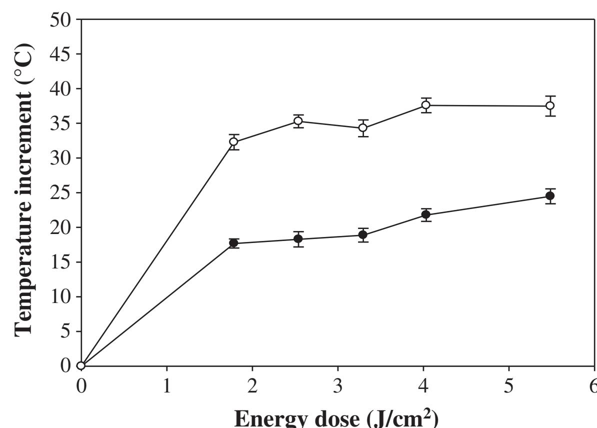

Fig. 3. Steady state profiles of the temperature increase of the fruit juice (●) and the air surrounding the quartz tubes inside the chamber (○) as a function of the energy dose in the presence of the cooling system.

carried out with the additional cooling system with recirculating water/ethylene glycol.

3.2. Bacterial inactivation in apple and orange juices

The effectiveness of the PL in microbial inactivation is a direct function of the energy dose absorbed by microorganisms that, in turn, is affected by the light transmittance through the liquid food being treated. While liquids such as high purity water as well as drinking water, have a high degree of transparency to a broad range of wavelengths including visible and UV light, most liquid foods such as fruit juices may exhibit a more limited transparency due to their absorption properties. This could drastically reduce the penetration of the light pulses into the liquid and, therefore, decrease the efficiency of the treatment. Furthermore, it should be noted that the PL sensitivity of the various groups of microorganisms may be diverse since each organism has a different requirement in terms of lethal dose.

In this work, the susceptibility to PL treatment of a Gram-positive (*L. innocua 11288*) and a Gram-negative (*E. coli DH5*-α) bacteria inoculated in fruit juices with different absorption characteristics was investigated. Fig. 4 shows the inactivation curves of *L. innocua* and *E. coli* in both apple and orange juices as a function of the energy dose received. The shape of the inactivation curves and the lethality of the PL treatment depended on the energy dose, the absorption characteristics of the inoculated liquid as well as the type of microorganism examined. The lethal effect of PL intensified upon increasing energy delivered to the juice. However, according to the findings of other authors (Anderson et al., 2000; Fine & Gervais, 2004; MacGregor et al., 1998; Marquenie et al., 2003; Rowan et al., 1999), the inactivation curves showed a slight initial shoulder due to an injury phase up to a certain energy level being reached. Beyond this energy, survivor numbers rapidly declined. Furthermore, it is worth highlighting that, regardless of the microorganisms examined, the end of the curve could present or not a tailing behavior. In current literature, there are several explanations to the cause of tailing, which include the lack of homogeneous population, multi-hit phenomena, the presence of suspended solids, aggregation of microorganisms, as well as the ineffective exposure of microbial cells to light pulses by the edge of the Petri dishes used in some experiments (Gómez-López et al., 2007; Marquenie et al., 2003; Shama, 1999). In this experiment, the occurrence of the tailing phase could be mainly attributed to shading effects due to insufficient light penetration in the depth of lower transmittance juices. In fact, as shown in Table 1, the absorption coefficient of the tested juices varied considerably, being that the absorptivity of apple juice is significantly lower than that of orange juice. Similar differences in absorption properties between apple and orange juices were also reported by Koutchma et al. (2004) and Koutchma (2009). Consequently, the PL resistance of both *E. coli* and *L. innocua* was greater in orange juice than in apple juice. For each bacterial strain examined, due to the differences in light transparency between the two juices, after an initial plateau, the microbial inactivation rapidly decreased when bacterial cells were suspended in apple juice. However, when they were inoculated in orange juice, there was a remarkable reduction of the inactivation rate. In addition, when *L. innocua* was inoculated in orange juice no more than 1 Log_{10} reduction was obtained regardless of the energy dose delivered to the bacterial cells.

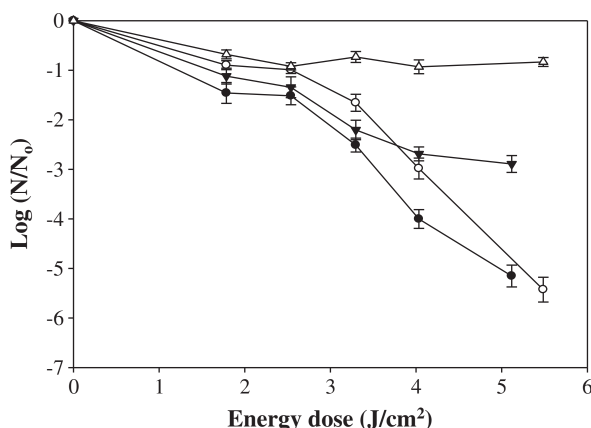

Fig. 4. Inactivation curves of *E. coli DH5*-α in apple (●) and orange juice (▼) and of *L. innocua 11288* in apple (○) and orange juice (Δ) as a function of the energy dose.

Although only few data have been published on the use of PL in treating liquid food, similar results were also found by other authors. Sauer and Moraru (2009) studied the effectiveness of PL treatment for the inactivation of *E. coli* in liquids with different levels of clarity such as butterfield's phosphate buffer (BPB), tryptic soy broth (TSB), apple juice and apple cider. They observed that susceptibility to PL treatment decreased moving from BPB to TSB and to apple juice and apple cider. Choi et al. (2010) studied the inactivation of *L. monocytogenes* in a light colored thin infant beverage as well as a dark colored viscous infant meal, finding that the inactivation in the latter was effective but significantly lower than in the infant beverage. Therefore, they concluded that the factors affecting the inactivation of microorganisms in liquid food include its color, viscosity and opaqueness.

Further studies should be carried out in order to have a more comprehensive understanding of the role played by the physical and optical properties of the liquid foods on the effectiveness of PL treatment.

Finally, the results presented in this study also highlighted that sensitivity to light pulses varies for different species of microorganisms. As shown in Fig. 4, in each inoculated juice and at a fixed amount of energy dose delivered, *E. coli* cells displayed a greater susceptibility to light pulses than *L. innocua*. For example, when an energy dose of 4 J/cm^2 was delivered, microbial reduction in apple and orange juices were, respectively, 4.00 and 2.90 Log-cycles for *E. coli* and 2.98 and 0.93 Log-cycles for *L. innocua*. Although no clear pattern can be established regarding the differences in PL sensitivity of the different microorganisms investigated (Gómez-López et al., 2005a), it has been generally observed that Gram-positive bacteria are more resistant than Gram-negative organisms (Anderson et al., 2000; MacGregor et al., 1998; Rowan et al., 1999; Sharifi-Yazdi & Darghahi, 2006).

Considering that the mechanism of microbial inactivation by PL seems to be also related to the effects on the cell envelopes, it is likely that the difference in susceptibility to light pulses was caused by the differences in structure and composition of the cell wall of Gram-positive and Gram-negative bacteria (Sharifi-Yazdi & Darghahi, 2006). Furthermore, it is also worth considering that the natural habitat of many Gram-positive bacteria types is on material surfaces and consequentially these are more likely to be exposed to solar UV-radiation. The UV-sensitive Gram-negative entero-pathogens are essentially restricted to the totally dark confines of animal/human alimentary tracts and consequentially, may have evolved very comparatively little UV-radiation resistance (Anderson et al., 2000). However, further research is necessary in order to determine the inactivation mechanisms of Gram-positive and Gram-negative bacteria by PL.

3.3. Sublethal injury after PL treatment

The inactivation mechanism for PL has mainly been attributed to the structural changes of microbial DNA (Wang et al., 2005), and, to a lesser extent, to the local overheating of microbial cells (Hiramoto, 1984; Wekhof, 2000; Wekhof et al., 2001). However, microscopic observation, such as those reported by Krishnamurthy et al. (2008) on the microbial cells of *S. aureus*, clearly indicated the presence of damage to the cellular structure such as cell wall damage, cytoplasmic membrane shrinkage, cellular content leakage, and mesosome disintegration occurring during PL treatment due to the pulsing effect. These results are in agreement with those previously published by Takeshita et al. (2003), who compared the inactivation of *S. cerevisiae* by continuous-wave UV light and broad-

spectrum flash lights. These authors observed that the DNA damage induced in yeast cells was essentially the same for both methods. However, the increased concentration of eluted protein and structural change in the cells exposed to flashes were observed only in the case of PL.

Other techniques can also be used to confirm whether structural cell damage may occur after PL treatment. Using a selective medium plating to observe the sub-lethal injury is a further measure of cell membrane integrity and functionality (Mackey, 2000). Nevertheless, there is no or very little information on sub-lethal damage to bacterial cells induced by PL treatment. Wuytack et al. (2003) determined the occurrence of sub-lethal injury in PL treated cultures of *S. typhimurium* when selective media such as violet red bile glucose agar (VRBG) and Tryptone Soya Agar (TSA) supplemented with 3% NaCl were used, while no sub-lethal injury occurred when TSA (pH 5.5) was used. Sub-lethal injury was also observed in *L. innocua* cells inoculated on stainless-steel surface coupons treated with a different number of intense light pulses (Woodling & Moraru, 2005), and in *L. monocytogenes* inoculated in an aqueous solution (0.85% NaCl and 0.1% peptone) (Rajkovic et al., 2009; Van Houteghem et al., 2008). However, to date, no publications exist on the occurrence of injury cells inoculated in liquid food after exposure to light pulses.

In this study, in order to estimate the degree of sublethal injury caused by PL treatment, treated and untreated samples were recovered on non-selective agar (TSA) which allows cells to repair sub-lethal damage and recover, and two selective agars (LSA for *Listeria* and EMB for *E. coli*), in which damaged survivors are unable to repair their damage and eventually die.

The results reported in Figs. 5 and 6 show that sub-lethal injury can be observed following PL treatment depending on the energy dose supplied, the types of microorganisms investigated and, mostly, on the absorption characteristics of the inoculated fruit juice.

As shown in Figs. 5a and 6a, when bacterial cells were inoculated in apple juice the proportion of sublethally injured cells increased when increasing the energy dose delivered. No appreciable differences in the proportion of sub-lethal damage induced by light pulses could be detected between the two microbial strains investigated. The higher proportion of sub-lethally injured cells, about 90% of survivors for *L. innocua* and slightly more for *E. coli*, was observed when the microbial cells were PL treated at 4 J/cm^2.

In contrast, the absence or a much lower proportion of sublethally injured cells was detected when the bacterial cells were inoculated in orange juice (Figs. 5b and 6b). In particular, the proportion of sub-lethally injured cells of *E. coli* increased with the increasing energy dose up to a maximum of less than 90%, and tended to decrease slightly as the energy dosage increased further to 5.1 J/cm^2. However, in the case of *L. innocua,* negligible sub-lethal injury was detected at all the energy doses investigated. This result would suggest that, due the very low light transparency of orange juice (Table 1), only the microorganisms located on the outermost layer of the juice, close the surface of the quartz tubes were reached by a sufficient dose of irradiation and, therefore, were inactivated or experienced sublethal injury. In the depth of the liquid, due the strong light attenuation effect, the energy that ultimately reached the bacterial cells may have been insufficient and therefore no appreciable lethal or sub-lethal

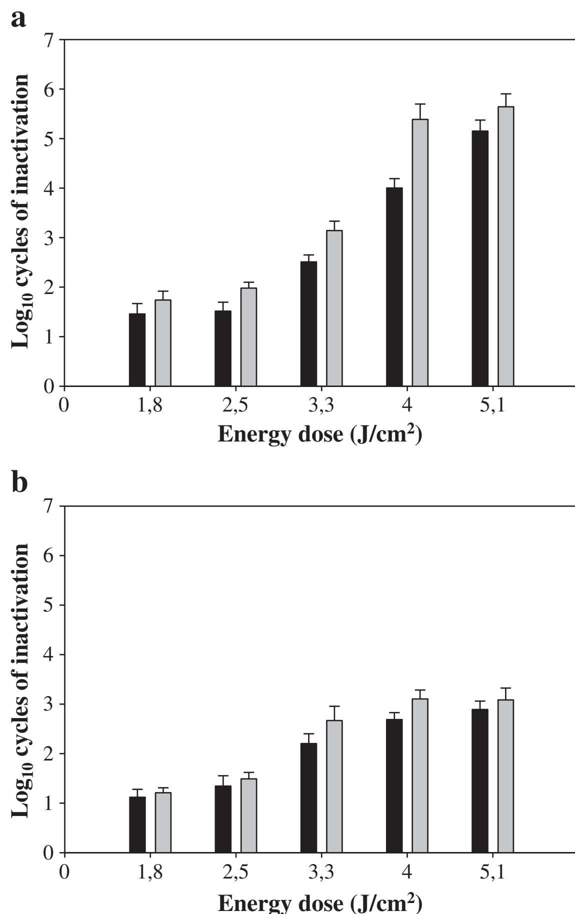

Fig. 5. Log_{10}-cycles of inactivation of *E. coli DH5-α* cells in apple (a) and orange (b) juice after PL treatments at different energy doses and recovered in the nonselective (TSA) (black bars) and selective (EMB) (gray bars).

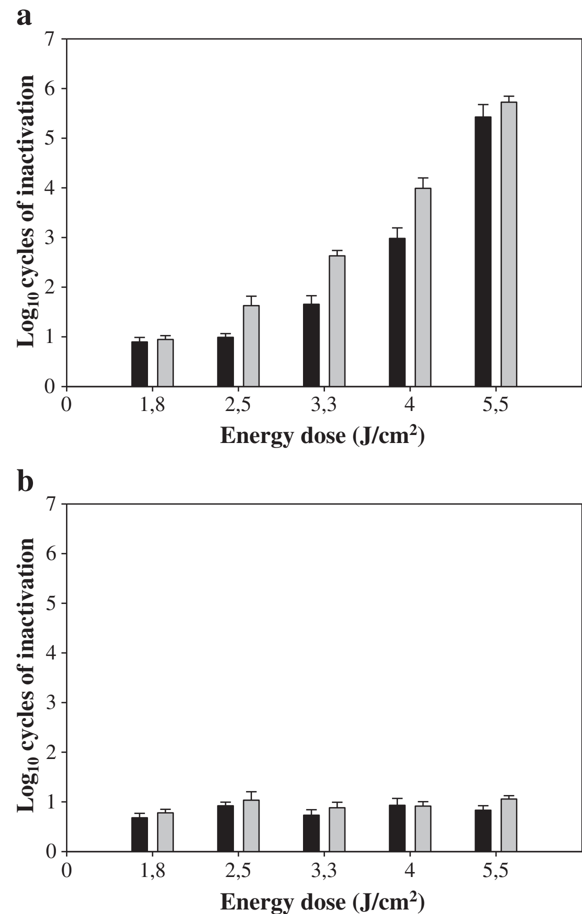

Fig. 6. Log_{10}-cycles of inactivation of *L. innocua 11288* cells in apple (a) and orange (b) juice after PL treatments at different energy doses and recovered in the nonselective (TSA) (black bars) and the selective (LSA) (gray bars).

damage was detected, especially in the bacterial strain that showed greater PL resistance.

Thus, the results of this study would confirm that membrane damage is an important event in bacterial inactivation by PL with it depending on the actual energy dose absorbed by microorganisms as well as their sensitivity to light pulses.

4. Conclusion

This study explored the possibility to use a continuous flow PL system for microbial inactivation of *E. coli* and *L. innocua* inoculated in fruit juices with different optical properties.

The results highlighted that the temperature of both the juice and air inside the sterilization chamber may increase significantly during processing, unless an efficient cooling system is incorporated into the equipment. Microbial inactivation experiments demonstrated that PL treatment can be successfully applied to obtain high levels of destruction with respect to the selected foodborne pathogens, even though the treatment efficiency strongly depended on the energy dose that is actually absorbed by the microorganisms, that in turn, is affected by the absorption properties of the liquid food.

An appropriate design of the PL equipment, including the reactor and the cooling system, could make it possible to minimize the temperature build-up during the pulse treatment as well as improve the treatment homogeneity by ensuring that all the fluid elements receive the same energy dose.

The results of this work also highlighted the occurrence of sublethally injured cells after PL treatment. This fact, along with the different susceptibility to light pulses of bacterial cells with different structures and cell wall compositions, is confirmation that membrane damage is an important event in the inactivation mechanisms of bacteria by PL.

However, more research is needed for a more comprehensive understanding of how the fluence, the type of microbial strain as well as the physical and optical properties of the liquid foods may affect the occurrence of the lethal and sub-lethal effects induced by pulsed light treatments.

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