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ORIGINAL PAPER

Use of High-Intensity Ultrasound and UV-C Light to Inactivate Some Microorganisms in Fruit Juices

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Abstract Novel technologies that involve non-thermal processes have been investigated in the last two decades as full or partial alternatives to conventional heat treatment. The main objective of this study was to evaluate the survival of single or strain cocktail of *Escherichia coli, Saccharomyces cerevisiae*, and a yeast cocktail in orange (pH 3.5; 9° Brix) and/or apple (pH 3.1; 12° Brix) juices and in 0.1% *w/w* peptone water processed by two non-thermal techniques: high-intensity ultrasound (USc) and/or short-wave ultraviolet radiation (UV-C). USc treatments (20 kHz, 95 μm-wave amplitude) were performed using a stainless steel continuous flow cell with a 13-mm probe (0.2 L/min; 40°C). The UV-C device consisted of a 90-cm long UV-C-lamp (100 W) placed inside a glass tube leaving an annular flow space (0.2 L/min; 40°C). Inoculated systems were recirculated through simultaneous or consecutive USc and UV-C devices and samples were taken at preset time intervals. Microbial populations were monitored by plate count technique. In peptone water and apple juice, UV-C radiation provoked higher *E. coli* ATCC 35218 inactivation than USc treatment. *E. coli* ATCC 35218 and its cocktail were more sensitive than *S. cerevisiae* KE162 and the cocktail of yeasts. UV-C efficiency was highly dependent on media nature. The poor single effect of UV-C light in orange juice was enhanced by the combination with USc. Combined treatment was more effective in simultaneous rather than in a series of USc−UV-C arrangement.

Keywords High-intensity ultrasound · UV-C light · Fruit juices

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Introduction

Thermal treatment constitutes the most extensively available method for the inactivation of microorganisms in fruit juices to achieve the required 5-log reduction in number of the most resistant pathogens (FDA 2000), but causes side effects on their flavor and nutritional quality. Minimal processing of fruits may include many novel technologies aimed to minimize these changes and to improve shelf life (Alzamora et al. 2000).

Short-wave ultraviolet light (UV-C) is a radiation in the range 200–280 nm in the UV spectrum, which mainly breaks down DNA molecules resulting in germicidal effect on bacteria, virus, protozoa, fungi, and algae (Shama 1999; Unluturk et al. 2008). Numerous studies have examined its effect in the disinfection of water (Litved and Cripps 1999; Sutton et al. 2000). Since FDA has approved the use of UV-C light as a novel technology for pasteurization of fruit juices (US FDA 2000, 2004), in recent years this technology has been focused to the treatment of liquid foods and beverages (Unluturk et al. 2008; Koutchma 2009; Oteiza et al. 2009).

It does not produce byproducts or generate chemical residues that could change the sensory characteristics in the final product (Guerrero-Beltrán and Barbosa-Cánovas 2004). Another advantage is that it does not deliver residual radioactivity as ionizing radiation. Furthermore, it is a cold

and dry process requiring very low maintenance (Morgan 1989). However, UV-C radiation has a limited penetration depth (Bintsis et al. 2000). Therefore, it is necessary to apply UV-C to a thin surface. Foods vary enormously in their sensitivity to UV light. It is usually stated that many nutrients (like vitamins, carotenes, tryptophan, unsaturated fatty acid residues in oils, solid fats, etc.) and pigments are "light sensitive" (Koutchma 2009). For this reason, the intensity of this treatment should be minimized to prevent quality loss as undesired effect, achieving this by combining with other techniques (Marquenie et al. 2003).

High-intensity ultrasound could be used as an additional factor for the development of minimally processed juices. The lethal effect of ultrasound has been attributed to the cavitation phenomenon (Rhaman 1999), which releases large amounts of energy, generating temperatures of ≈4,700°C and shock waves with pressures of several atmospheres. Ultrasound alone is not very effective in killing microorganisms in food since longer treatment times are required; however, in combination with other emerging or traditional techniques (high pressure, natural, and synthetic antimicrobials; moderate temperature) has demonstrated to enhance its inhibitory effect on a vast variety of microorganisms (Pagán et al. 1999; Guerrero et al. 2001, 2005; Ferrante et al. 2007).

The aim of this research was to study the response of *E. coli* ATCC 35218 and *S.cerevisiae* to the single and/or combined effect of USc and UV-C light in peptone water, orange juice, and/or apple juice. Combined treatments were performed using two configurations: simultaneous or serial applications of UV-C and USc. Additionally, the responses of strain mixtures and single cultures were studied and compared.

Materials and Methods

Tested Cultures and Preparation

The following strains were assessed in this study: *E. coli* ATCC 35218 and a cocktail of four *E. coli* strains (ATCC 35218, ATCC 8738, ATCC 11229, and ATCC 25922); *S. cerevisiae* KE 162 and a cocktail of five yeasts (*S. cerevisiae* KE 162; *Candida parapsilosis* ATCC 22019; *Zygosaccharomyces bailii* NRRL 7256; *Zygosaccharomyces rouxii* ATCC 52519; and *Pichia anomala* NRRL 3668).

Initial *E. coli* inoculum was prepared by transferring a loopful of a Trypticase Soy Agar plus 0.6% *w*/*w* Yeast Extract (TSAYE) stock culture slant to a 20-mL Erlenmeyer-flask of Trypticase Soy Broth supplemented with 0.6% *w*/*w* yeast extract (*w*/*w*). It was incubated at 37°C (±1°C) under agitation until it reached the stationary phase (≈24 h).

A similar procedure was repeated for yeast cultures. Potato Dextrose Agar (PDA; pH 5.6) stock cultures were maintained at 4°C for a maximum of 2 months. The initial inoculum was prepared by transferring a loopful to an Erlenmeyer-flask containing 20 mL of Potato Dextrose Broth. The organism was incubated at 27°C (±1°C) until it reached the stationary phase (≈36 h).

For mixed cultures, equal aliquots of each individual strain in the stationary phase were aseptically combined to produce a cocktail of four strains of *E.coli* or five strains of yeasts.

For each experiment, 5-mL inoculum was added to peptone water or fruit juices in order to obtain initial levels of ~10^7CFU/mL for bacteria and ~10^6CFU/mL for yeasts, respectively. All microbiological media were purchased from Laboratorios Britania S.A. (Buenos Aires, Argentina).

Preparation of Samples

Peptone water (0.1% *w*/*w*) was used due to its transparency and colorless to evaluate the UV-C and/or ultrasonic treatments without considering juice characteristics effect. Commercial clear pasteurized apple juice without additives and/or preservatives (pH 3.1±0.2; 12° Brix) was purchased from a local market (Buenos Aires, Argentina). Orange juice (pH 3.4±0.2; 10 ºBrix) was aseptically obtained as follows: Valencia oranges (pH ~3.4±0.2; 10 °Brix) were purchased from a local market and washed in chlorinated water to reduce external flora. The fruit were squeezed under aseptic conditions in a class II security cabinet (Nuaire Inc., Plymouth, Minn.). The juice was centrifuged to reduce pulp amounts, dispensed into 250-mL sterile dark flasks and stored at 18°C until use (for a maximum of 2 months).

USc and UV-C Treatments

Ultrasonic treatment (USc): A stainless steel continuous flow ultrasonic cell (horn: 40-mm diameter, 120-mm long) screwed onto a 13-mm diameter probe connected to an ultrasonic processor (VCX 600 Vibra cell, Sonics & Materials, USA) was used. For a greater intensity of cell disruption, 250 mL of each system were fed in the reverse flow mode and sonicated at 20 kHz and 95.2 μm (80%) of wave amplitude. Systems were recirculated at 0.2 L/min using a peristaltic pump (CPX-400, Cole Parmer, Illinois, USA) and collected in a 600-mL double-wall cylindrical vessel connected to a thermostatically controlled water bath (to attain 40±1°C). A degasification glass device was included in the circuit, before the USc device, in order to dump pump pulses through the ultrasonic probe. All pieces of the USc equipment were steam sterilized before each experience. When the desired temperature (40±1°C) was

reached, 5 mL of single or mixed inoculum were added to the vessel containing sterile peptone water or juice and USc treatment proceeded.

Ultraviolet light treatment (UV-C): Previous to each experience, the UV-C reactor was sanitized by recirculating sterile water during 5 min with the turned on lamp. Subsequently, water was discharged and the lamp was turned off. Five hundred (500) milliliters of inoculated systems were recirculated with a peristaltic pump (0.2 L/min; 40°C) through the UV-C device which consisted of a 0.87-m long glass tube with a UV-C lamp (TUV-100 W, 253.7 nm, Philips, Holland) leaving a annular flow space (outer diameter=0.031 m; inner diameter=0.024 m; volume=0.22 L). When UV-C lamp was stabilized (2–3 min), 5 mL of single or mixed inoculum were added to the vessel containing peptone water or juice, which were recirculated through the USc device. The UV-C dose emitted from the lamps was determined by using the technique designed to only measure germicidal radiation proposed by Rhan (1997). A 0.6 M iodide/0.1 M iodate solution in 0.01 M borate buffer (pH 9.25) was used as a chemical actinometer. Irradiation of this solution resulted in the linear formation of triiodide, which was quantified by measuring its absorbance at 352 nm (UV-visible spectrophotometer, Agilent technologies, M8453, California, USA) for each dose of irradiation. The number of einsteins of photons absorbed by the irradiated sample were calculated from the moles of generated triiodide (Rhan 1997). The UV-C radiation doses varied between 0 and 18.7 kJ/m^2 and were obtained by altering the exposure time up to 20 min.

Combined USc and UV-C treatment: They were performed using two different configurations of processes in order to detect interaction effects, if any. First, it was tried consecutive treatments involving USc (10 min) followed by UV-C (10 min) (USc−UV-C). The second configuration consisted of the simultaneous application of both technologies during 20 minutes (USc + UV-C). Figure 1 schematizes this arrangement. Five hundred (500) milliliters of inoculated orange juice were recirculated (0.2 L/min; 40°C) through the two arrangements described below.

Enumeration of Surviving Cells

During each treatment samples were taken from the 600 mL vessel at selected intervals and analyzed for enumeration of survivors. Proper dilutions were made and 0.1 mL of sample suspension was surface plated in duplicate using TSAYE (*E. coli*) or PDA (yeasts). Plates were incubated at 37°C for 24 h (*E. coli*) or 27°C for 48 h (yeasts). Trials were replicated at least three times and the average survival fraction was reported. Survival curves were generated from experimental data by plotting Log N/N_0 (where N is the number of colony forming units per milliliter at a given time and N_0 the initial number of number of colony forming units per milliliter) versus time of treatment.

Statistical Analyses

Analysis of variance (ANOVA) was performed to detect differences and interactions among systems (different liquid media), treatments (UV-C, USc, and combinations) and/or microorganism responses using the general linear model

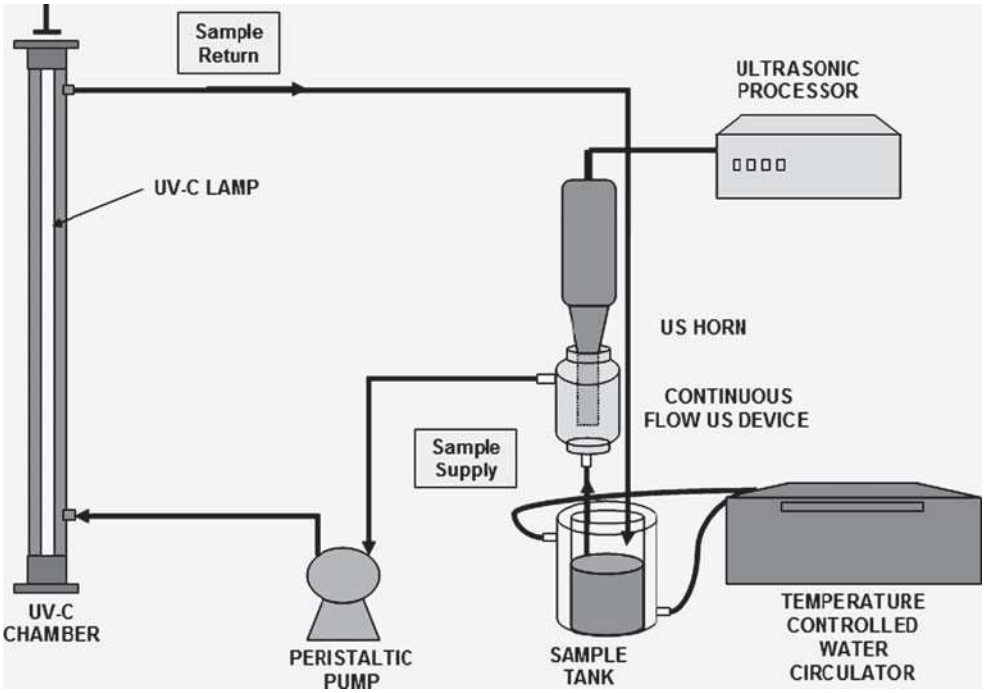

Fig. 1 Diagram showing the ultrasonication and ultraviolet light apparatus used in this study to work in simultaneous conditions USc + UV-C

procedure. The variability of each descriptor was analyzed; treatment, microorganism, and system were considered as categorical factors, and processing time was considered as a quantitative factor. Multiple mean comparisons among treatments were analyzed using the Bonferroni test at 95% confidence level. Statistical procedures were carried out using the STATGRAPHICS PLUS for Windows 3.0 ® Package (Statistical Graphics Corp., Washington, USA). Internal validation of the model was carried out through the comparison between the observed and the predicted values, residual analysis and the evaluation of the adjusted coefficient of determination (R_{adj}^2).

Results and Discussion

The USc treatment provoked up to 2.2 log reduction of *E. coli* ATCC 35218, and the inactivation appeared to follow a first-order kinetics throughout the assayed sonication time (Fig. 2a). The use of high-intensity ultrasound treatment in a continuous flow mode, with major application in industrial needs, generated inactivation curves similar to those obtained under batch processes. Guerrero et al. (2001) also observed that *S. cerevisiae* inactivation in culture media treated by ultrasound alone (600 W; 20 kHz; 30–55°C; 91.2 μm) in batch arrangement, generally followed a first-order kinetics during the most part of the process, while combination with other stress factors showed non-linear survival curves (Guerrero et al. 2005). Ferrante et al. (2007) studied *L. monocytogenes* survival in batch sonicated (600 W, 20 kHz, 91.2 μm wave amplitude; 45°C) squeezed orange juice (pH 3.5) containing vanillin (0 to 1,500 ppm). Survival curve in batch sonicated orange juice without antimicrobial was linear but when vanillin was added, the correspondent survival curves were completely sigmoid or semi-sigmoid.

When considering UV-C treatment, there were notorious differences in survival curves of *E. coli* ATCC 35218

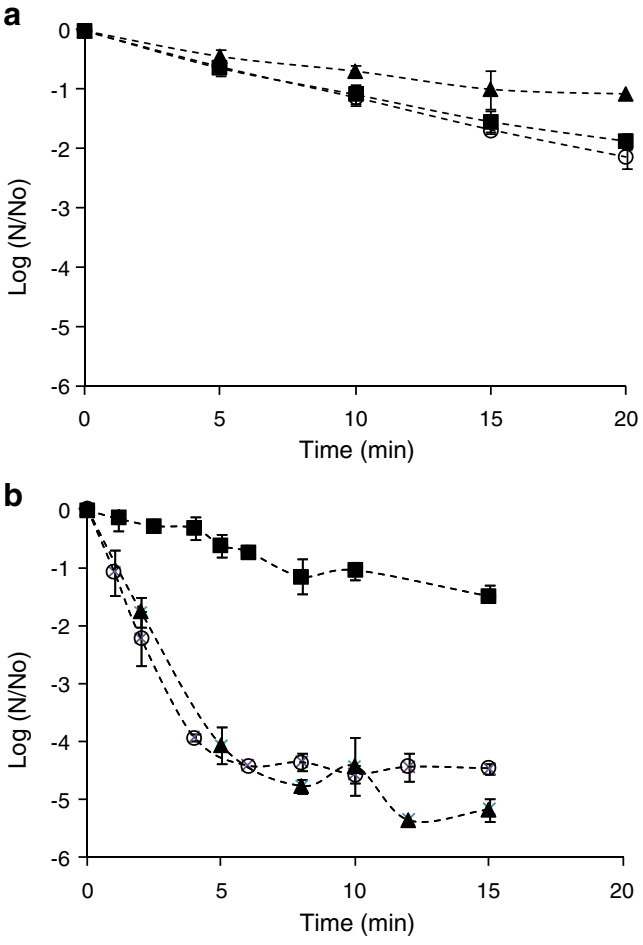

Fig. 2 Survival curves of *E. coli* ATCC 35218 as affected by USc (**a**) or UV-C (**b**) in peptone water (*empty circle*), apple juice (*filled triangle*), and orange juice (*filled square*), standard deviation (*vertical line*)

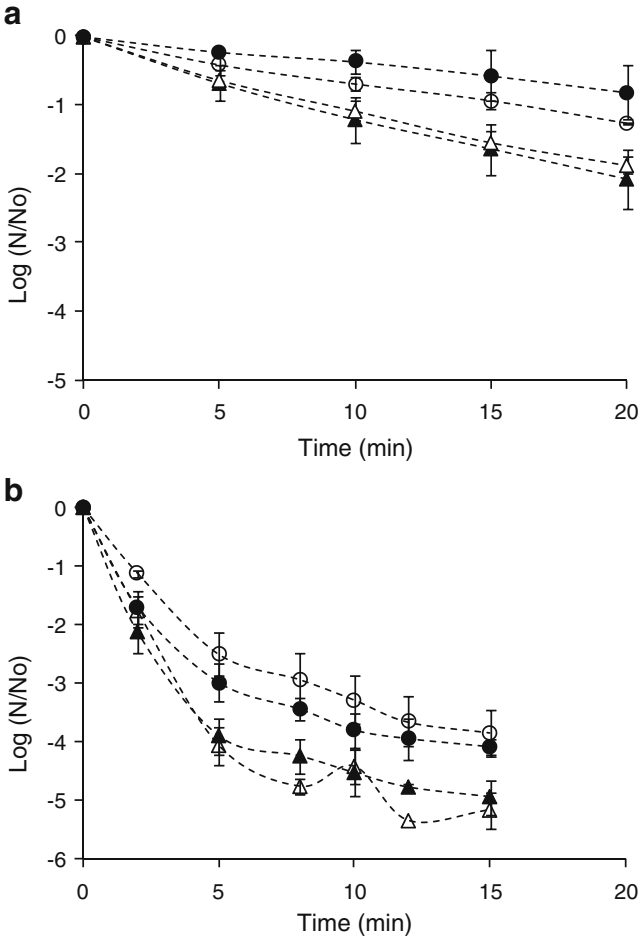

Fig. 3 Survival of *S. cerevisiae* KE162 (*empty circle*), cocktail of yeasts (*filled circle*), *E. coli* ATCC 35218 (*empty triangle*), and cocktail of *E. coli* (*filled triangle*) as affected by USc treatment in orange juice (**a**) or UV-C treatment in apple juice (**b**)

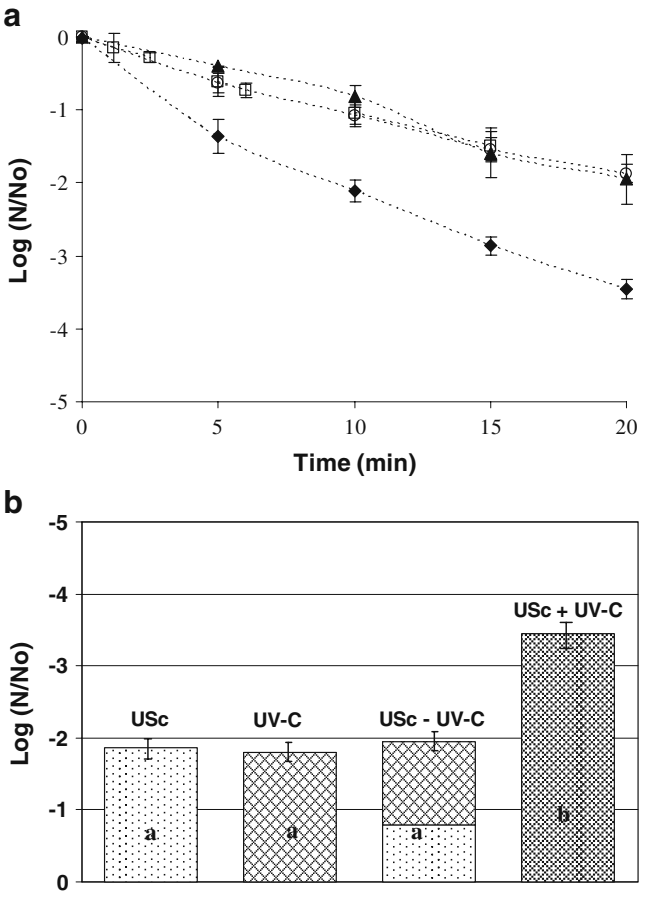

Fig. 4 **a** Semilogarithmic survival curves of *E. coli* ATCC 35218 in orange juice as affected by USc (*empty circle*), UV-C (*empty square*), consecutive USc − UV-C (*filled triangle*) or simultaneous USc + UV-C (*filled diamond*). **b** *E. coli* ATCC 35218 log reduction in orange juice achieved after 20-min single or combined treatments: USc, UV-C, USc − UV-C, and USc + UV-C. USc − UV-C implies that USc was the first treatment (10 min), followed by the UV-C treatment (10 min). *Different letters inside the bars* represent significant differences ($p<0.05$) among treatments according to the 95% Bonferroni's multiple comparison procedure

compared with USc treatment (Fig. 2b). A considerable level of inactivation of *E. coli* ATCC 35218 (≈4.5 log cycle reduction) was obtained in apple juice and in peptone water after 5 min of UV-C treatment. However, there was a markedly lower efficiency of UV-C disinfection in orange juice, probably due to the presence of colored compounds and pulp particles which caused poor UV-C light transmission (Shama 1999). The use of this technology to reduce microbial populations led to the formation of curves with upward concavity and pronounced tailing effect, especially in peptone water and apple juice. Other researchers have also reported that UV-energy inactivates microorganisms generating curves with a notorious tail. Yaun et al. (2004) studied the inhibition of some pathogens by ultraviolet light on the surface of fresh tomatoes, lettuce, and delicious apples. Schenk et al. (2008) studied the inactivation by UV-C of some microorganisms inoculated onto pear slices with and without peel. All observed survival curves with upward concavity; almost biphasic and with a well-defined tail. The tail in UV-C inactivation curves has been explained in several different ways. One explanation is the multiple hit phenomena described by Yousef and Marth (1988), which stated that survival curve was accounted for on the basis of multiple UV hits on a single cell or single UV hits on multiple cells. Other potential causes of tailing have been mentioned, like experimental bias, aggregation of microorganisms and resistant subpopulation as well as non-homogeneity of UV treatment and presence of soluble solids (Koutchma 2009).

Figure 3 shows the inactivation curves of *S. cerevisiae* KE162; *E. coli* ATCC 35218 and the cocktails in orange juice treated by USc (Fig. 3a) and in apple juice treated by UV-C (Fig. 3b). For the period of time assayed, the inactivation response to ultrasound, as it was previously commented, appeared to be closer to a first-order kinetics while under UV-C treatment survival curves exhibited a notorious upward concavity. The cocktails of strains showed survival curves similar to the ones of individual strains for both sonication in orange juice and UV-C

Table 1 Summary of analysis of variance of response (survivor fraction) of various microorganisms (*E. coli*, *S. cerevisiae*, and cocktails of *E. coli* or yeasts) recovered on different systems (orange juice, apple juice and/or model) processed by emerging technologies (USc, UV-C, and USc followed by UV-C and/or simultaneous USc and UV-C)

Source	Sum of squares	DF	Mean square	*F* value	*P* value
Model	154.1470	19	8.1130	567.10	0.0000
Microorganism	0.2180	3	0.0727	5.08	0.0044
Time	2.3784	1	2.3784	166.25	0.0000
System	5.3314	2	2.6657	186.33	0.0000
Time*treatment	0.5254	3	0.1751	12.24	0.0000
Time*system	0.3526	2	0.1763	12.32	0.0001
Time*microorganism	0.5045	3	0.1682	11.75	0.0000
Treatment*system	23.084	5	4.6168	322.72	0.0000
Residual	0.5866	41	0.0143		
R^2	99.62				
R^2adj.	99.45				

treatment in apple juice but the inactivation curves were dependent on the type of microorganism and treatment. This behavior was also observed by Schenk et al. (2008) in pear slices inoculated with *Z. bailii; a* yeast cocktail; *E. coli* and its cocktail treated with UV-C light during 15 min. As it can be seen in Fig. 3, *E. coli* ATCC 35218 and its cocktail were more sensitive to these treatments than *S. cerevisiae* KE162 or the yeast cocktail, reaching higher lethality in the same process time.

The single effect of UV-C light on the inactivation of *E. coli* ATCC 35218 in orange juice was enhanced by the combination with USc (Fig. 4). An interesting result was that the way the combined treatment was performed notoriously changed the inactivation curve. Simultaneous USc + UV-C treatment significantly increased the observed inactivation with respect to single treatments, showing an additive effect. On the other hand, the serial application of USc followed by UV-C did not contribute to increase the efficiency of single processes since their inactivation curves were almost overlapped (Fig. 4). Lopez-Malo et al. (2005) studied the survival of *S. cerevisiae* and *L. monocytogenes* in apple juice (pH 3.5; 12° B) and in a model system (buffer phosphate; pH 3.5) processed by single and combined treatments: UV-C (100 W) and ultrasound (US) (20 kHz, 95 μm-wave amplitude) in a similar apparatus like the one used in this study but in batch instead of continuous flow arrangement. They observed that the use of UV-C light combined with high-intensity ultrasound was effective for enhancing individual inactivation effects on *L. monocytogenes* and *S. cerevisiae* in apple juice.

Statistical analysis of overall data was also performed. ANOVA for Log N/N_0 is summarized in Table 1. Microorganism, time, system, and time*treatment, time*system, time*microorganism, and treatment*system interactions were statistically significant variables for predicting Log N/N_0 with highly significant F values (P value <0.000001).The R^2 statistic indicates that the proposed model as fitted explains 99.62% of the observed variability in the number of survivors (Log N/N_0). System (peptone water or fruit juices) was the most significant single term followed by time of treatment and microorganism (*E. coli* ATCC 35218; *S. cerevisiae* KE162 or cocktails) while the most significant interaction was between treatment and system ratifying that the Log number of survivors varied within treatments depending on the treated media (system) as was previously commented.

Multiple comparisons between treatment means were performed (data not shown). Two homogeneous groups were identified using the Bonferroni procedure at 95% confidence level between 20 minute treatments for *E.coli* ATCC 35218 response (Log N/N_0) in orange juice. Simultaneous treatment (USc + UV-C) mean was significantly different from those corresponding to single USc or UV-C treatments and the serial treatment (USc − UV-C) (Fig. 4b).

Conclusions

This work contributed to address some limitations of novel technologies when applied to food systems. The application of high-intensity ultrasound in a continuous flow mode had low efficacy for all studied microorganisms inoculated in peptone water, orange, or apple juices. UV-C radiation was effective inactivating *E. coli* ATCC 35218, an *E. coli* cocktail, *S. cerevisiae* KE162, and a yeast cocktail in peptone water and clarified apple juice but had poor inactivation effect in the squeezed orange juice. It is well known that UV-C light is a valuable technology for microbial inactivation in water and fruit juices, but its efficiency is limited to clear, non-opaque media. The combination of UV-C with USc turned out to be a promising approach to lead with this kind of troubles that frequently appear when working with real food systems as in the case of the orange juice studied in this work. Further investigation is needed to elucidate the way combined treatments may be performed to increase their efficiency and in order to evaluate the efficacy of the combined treatment in other media or with other microorganisms of relevance.

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