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Abstract

The decontamination effect of pulsed and continuous power ultrasound, provided at either controlled or uncontrolled temperature regimes, was studied with reference to native microflora and inoculated pathogenic bacteria in wastewater obtained by fresh-cut lamb's lettuce washing. Results showed that decontamination efficacy increased with increasing specific energy and was higher when ultrasound treatment was provided under uncontrolled temperature regime. Continuous ultrasound supplied without temperature control allowed to achieve 3.2 Log reductions of native microflora during 20 min treatment, while 5 Log reductions of inoculated *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enterica* were attained within 5 min of ultrasonication. The heat generated during continuous ultrasound accounted for approximately 58% of the total decontamination effect against *L. monocytogenes,* while it contributed for 100% to *E. coli* and *S. enterica* inactivation.

Industrial relevance

The application of power ultrasound combined with *in situ* generated heat could represent an effective tool for water decontamination and recycling in the fresh-cut industry. In addition, besides safety requirements, this technology would also meet cost-effectiveness criteria and existing standards.

Keywords: Ultrasounds, Wastewater disinfection, Water recycling, *In situ* generated heat, Fresh-cut industry

1. Introduction

Nowadays, water scarcity is a major issue at global level. It has been estimated that in the next 15-20 years the water supply-to-demand gap will be approximately 40%. Tackling the water gap is a challenge for EU research (Horizon 2020). The food sector greatly contributes to water scarcity. It has been estimated that about 20-50% reduction in water consumption in the food sector can be achieved by recycling and reuse of water (Hiddink, Schenkel, Buitelaar, & Rekswinkel, 1999).

The fresh-cut vegetables market has grown considerably in the last few decades in response to an increased demand for fresh-like, healthy and convenient foods. Fresh-cut vegetables production requires intensive use of water to both wash and move vegetables along the production line. In order to secure water supply and protect the environment from the adverse effects of the wastewater discharges (EEC 1991), water recycling in the fresh-cut industry has to be improved. Recycling of water that is intended to re-enter the washing step, implies wastewater disinfection. As well known, a 5 Log reduction of pathogenic bacteria is the generally accepted requirement for safe water disinfection. Wastewater decontamination may be accomplished by means of chemical and physical interventions (Casani, Rouhany, & Knøchel, 2005; Olmez & Kretzschmar, 2009). Among these, sodium hypochlorite is the most used due to its low cost and easy use (Olmez & Kretzschmar, 2009; Gil, Selma, López-Gálvez, & Allende, 2009). However, not only wastewater containing chlorine has a great environmental impact, but also chlorination disinfection by-products are known to represent a potential risk for human health (Itoh, Gordon, Callan, & Bartram, 2011). Consequently, there is great effort to find suitable technologies to allow wastewater recycling (Casani et al., 2005; Olmez & Kretzschmar, 2009; Artés, Gómez, Aguayo, Escalona, Artés-Hernández, 2009). Power ultrasound has been suggested as a technology alternative to chlorination for wastewater decontamination (Neis & Blume, 2002; Piyasena, Mohareb, & McKellar, 2003). Ultrasound frequencies higher than 20 kHz are actually considered safe, non-toxic and environmentally friendly (Kentish & Ashokkumar, 2011). During ultrasound treatment, cavitation phenomena occur into the liquid medium causing a rapidly alternating compression and decompression zones, that are in turn responsible for generating shock waves with associated local very high temperatures and pressures, as well as free radicals and hydrogen peroxide (Leighton, 1994; Mason, Joyce, Phull, & Lorimer, 2003). Ultrasound effectiveness in wastewater decontamination was found to increase with the power input and

exposure time, and to depend on microorganism type (Scherba, Weigel, & O'Brien, 1991; Joyce, Phull, Lorimer, & Mason, 2003; Hulsmans, Joris, Lambert, Rediers, Declerk, Delaedt, Olleveir, & Liers, 2010; Elizaquivel, Sanchez, Selma, & Aznar, 2011; Gao, Lewis, Ashokkumar, & Hemar, 2014). Improved efficiency of ultrasound technology can be obtained by its combination with other biocidal treatments, such as chlorination (Drakopoulou, Terzakis, Fountoulakis, Mantzavinos, & Manios, 2009; Ayyildiz, Sanik, & Ileri, 2011), organic acids (Gómez-López, Gil, Allende, Vanhee, & Selma, 2015), and ultraviolet irradiation (Blume & Neis, 2004; Mason et al., 2003; Naddeo, Land, Belgiorno, & Napoli, 2009; Gómez-López et al. 2015). An increase of microbial sensitivity to ultrasound in combination with temperature increase, experienced with ultrasonic treatment, for wastewater disinfection has been also reported (Madge & Jensen, 2002; Salleh-Mack & Roberts, 2007; Gómez-López, Gil, Allende, Blancke, Schouteten, & Selma, 2014). It has been estimated that the heat generated during ultrasound processing accounted for approximately 52% of the resulting disinfection (Madge & Jensen, 2002).

In contrast with the huge number of studies in the literature dealing with ultrasound decontamination of municipal wastewater and effluents as well as model fluids, very few studies investigated ultrasound effectiveness for water decontamination deriving from fresh-cut vegetable production (Elizaquível et al., 2012; Gómez-López et al., 2014; Gómez-López et al., 2015). It has been demonstrated that power ultrasound was effective in inactivating pathogenic bacteria inoculated in fresh-cut lettuce wash water (Elizaquível et al., 2012), especially in the presence of the residual peroxyacetic acid concentration that can be found in the wash water (Gómez-López et al., 2015). In these studies, ultrasonic treatments were carried out with temperature control, allowing the inactivation effects of ultrasound alone to be evaluated. In another study, Gómez-López et al. (2014) showed that ultrasound disinfection against *Escherichia coli* O157:H7 inoculated in fresh-cut lettuce wash water can be increased by combination with heating. Reductions of 6 Log of this microorganism were actually achieved after 60 and 20 min of ultrasonication with and without temperature control, respectively.

In light of this, there is a lack of knowledge on the efficacy of power ultrasound in combination with *in situ* generated heat against naturally occurring microflora and foodborne pathogens, other than *E. coli*, potentially contaminating fresh-cut vegetable wash water.

In this study the efficacy of power ultrasound in decontaminating wastewater deriving from fresh-cut vegetable washing was investigated. To this aim, wastewater obtained by washing fresh-cut lamb's lettuce was subjected to power ultrasound, provided in pulsed or continuous modality, with or without temperature control. The decontamination efficacy of the treatments was evaluated on both the native microflora and inoculated pathogenic bacteria, i.e. *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enterica*. These microorganisms were chosen due to their natural occurrence in a water environment and because they are generally considered indicators of fecal contamination (Szewzyk, Szewzyk, Manz, & Schleifer, 2000). The final goal was to find the potentiality of combined ultrasound with *in situ* generated heat in the attempt to implement strategies for efficient management of water resource in the fresh-cut industry. To this regard, the decontamination efficacy was related to the ultrasound cavitation and heat contributions.

2. Materials and methods

2.1. Preparation of fresh-cut vegetable wash water

Lamb's lettuce (*Valerianella locusta* Laterr.) was purchased from a local market. Lettuce leaves were placed into a beaker containing tap water at 18 °C ± 2 °C (the vegetable-water ratio was 1:30 w/v). After 1 min of washing, water was separated from the leaves by using a domestic salad spinner.

2.2. Bacterial strains and inoculum preparation

The microorganisms used for inoculum were *Listeria monocytogenes Escherichia coli* and *Salmonella enterica* subsp. *enterica* 9898 DSMZ, obtained from the bacterial culture collection of the Department of Food Science of the University of Udine (Italy). Strains were maintained at -80 °C in Brain Heart Infusion broth (BHI, Oxoid, UK) with 30% sterile glycerol as cryoprotectant until use. Strains were incubated in BHI at 37 °C for 24 h, subsequently cultured in 5 mL of BHI at 37 °C for 24 h, and finally collected by centrifugation at 14170 *g* for 10 min at 4 °C (Beckman, Avanti TM J-25, Palo Alto, CA, USA) and washed three times with Maximum

Recovery Diluent (MRD, Oxoid, UK). The final pellets were suspended in MRD and used as inoculum. A final concentration of approximately 10^6 CFU/mL was obtained for each bacteria suspension.

2.3. Power ultrasound treatment

An ultrasonic processor (Hieschler Ultrasonics GmbH, mod. UP400S, Teltow, Germany) with a titanium horn tip diameter of 22 mm was used. The instrument operated at constant ultrasound amplitude and frequency of 100 μm and 24 kHz, respectively. Aliquots of 200 mL of wash water inoculated or not with *L. monocytogenes*, *E. coli* and *S. enterica* were introduced into 250 mL capacity (110 mm height, 60 mm internal diameter) glass vessels. The tip of the sonicator horn was placed in the centre of the solution, with an immersion depth in the fluid of 10 mm. The ultrasound treatments were performed for increasing lengths of time up to 20 min. During the ultrasonication experiment, the temperature was either controlled using an ice bath, to dissipate the heat generated during treatment, or uncontrolled, leaving the temperature to rise due to heat dissipation. The sonicator operated either in pulsed mode or continuous mode. In the pulsed mode, the pulse duration period of 0.5 s was followed by a pulse interval period of 0.5 s, during which the sonochemical reactor was switched off. Before and after each experiment, the ultrasound probe was disinfected by washing with ethanol followed by through rinsing with sterile water.

2.4. Thermal treatment

The total temperature-time combination received by water during continuous ultrasound under uncontrolled temperature regime was applied to the wastewater in the absence of the ultrasound treatment. To this purpose, aliquots of 200 mL of wash water were introduced into 250 mL capacity glass vessels and heated in a thermostatic water bath (Ika Werke, MST BC, Staufen, Germany) under continuous stirring, by mimicking the same temperature rise produced by the probe during continuous ultrasound treatment under the uncontrolled temperature regime.

2.5. Microbiological analysis

147 Both naturally present and inoculated microorganisms were quantified at different time intervals during the
148 ultrasound and heat treatments. The wastewater samples were diluted 10 fold with MRD (Oxoid, UK). Total
149 viable count of non inoculated water was enumerated by spreading onto plates with Plate Count Agar (PCA,
150 Oxoid, UK) and incubating at 30 °C for 48 h. *L. monocytogenes* and *S. enterica* concentrations were determined
151 by plating on Palcam Agar (PA, Oxoid, UK) and Xylose Lysine Desoxycholate agar (XLD, Oxoid, UK),
152 respectively, at 37 °C for 48 h, while the Coli ID medium (BioMerieux, Mercy L'Etoile, France) was used for
153 *E. coli* concentration determination, followed by incubation at 37 °C for 24 h.
154 Preliminary trials were carried out on the non inoculated wastewater to check for *Salmonella* spp. and *L.*
155 *monocytogenes* presence and enumerate *E. coli*. For *Salmonella* spp., 25 mL of wastewater was diluted with
156 225 mL of Buffered Peptone Water (BPW, Oxoid, UK), homogenised in a Stomacher Lab-Blender 400 (VWR
157 International PBI srl, Milano, Italy) for 2 min and incubated at 37 °C for 24 h. Aliquots of 0.1 mL of BPW
158 were added with 9.9 mL Rappaport Vassiliadis (RV, Oxoid, UK) and incubated at 42-43 °C for 18-24 h.
159 Presence/absence of *Salmonella* spp. was checked by spreading onto XLD agar plates and incubating at 37 °C
160 for 24 h. For *L. monocytogenes*, 25 mL of wastewater were diluted with 225 mL of Fraser Broth (FB, Oxoid,
161 UK), homogenised in a Stomacher for 2 min and incubated at 30 °C for 36-48 h. 1 mL of FB was added with
162 9 mL of FB and incubated at 37 °C for 24-48 h. Presence/absence of *L. monocytogenes* was checked by
163 spreading onto PA plates and incubating at 37 °C for 24-48 h. To evaluate the presence of *E.coli* the Coli ID
164 medium at 37 °C for 24 h was used.
165 In order to investigate whether treatments were responsible for bacteria sub-lethal injury, resuscitation trials
166 were carried out. For each inoculated strain, 10 mL of wastewater was transferred into 10 mL of BHI broth
167 and then incubated at 30 °C for 2h. Afterwards, presence/absence of *L. monocytogenes*, *E. coli* and *S. enterica*
168 was checked by spreading onto PA, Coli ID and XLD agar media, respectively.
169
170 *2.6. Temperature measurement*
171
172 The temperature was recorded as a function of time using a copper-constantan thermocouple probe (Ellab,
173 Denmark), connected to a data-Logger (CHY 502A1, Tersid, Milano, Italy).
174

175 *2.7. Specific power and energy computation*

176

177 The specific power or power density (*P*, W/L) transferred from either the probe or the water bath to the sample
178 was determined calorimetrically by recording the temperature (*T*, K) increase against the time (*t*, s) of
179 ultrasound or heat application (Raso, Manas, Pagan, & Sala, 1999). The following equation (1) was used:

180

181 $$P = dc_p(\partial T / \partial t) \quad (1)$$

182

183 where c_p is the water heat capacity (4.18 J/kg K), and *d* is the sample density (kg/L). The specific energy (kJ/L)
184 was calculated by multiplying the power density value by the duration of the treatment (Hulsmans et al., 2010).

185

186 *2.8. Statistical analysis*

187

188 Results are averages of two measurements carried out on two replicated samples and are reported as means ±
189 SD. Analysis of variance (ANOVA) was performed with significance level set to $p<0.05$ (Statistica for
190 Windows, ver. 5.1, Statsoft Inc. Tulsa, USA, 1997). The Tukey procedure was used to test for differences
191 between means. Linear regression analysis was performed by using Microsoft Excel 2007. The goodness of
192 fitting was evaluated based on visual inspection of residual plots and by the calculation of R^2 and p.

193

194 **3. Results and discussion**

195

196 *3.1. Decontamination efficiency of continuous power ultrasound provided under controlled temperature*
197 *regime*

198

199 Initial total microbial count of wastewater deriving from fresh-cut lamb's lettuce wash water was 4.92 ± 0.15
200 Log CFU/mL. This value was in the same magnitude range of those reported in the literature for wastewater
201 obtained by washing fresh-cut vegetable (Elizaquivel et al., 2011; Gomez-Lopez et al., 2015). As reported by
202 Ignat, Manzocco, Bartolomeoli, Maifreni and Nicoli (2015) for wastewater obtained from lamb's lettuce

203 washed in analogous conditions as those performed in the present study, the microbial count was mainly
204 represented by *Pseudomonas* spp, Enterobacteriaceae and total coliforms. No presence of *L. monocytogenes*,
205 *E. coli* and *S. enterica* cells was detected in wastewater.
206 Wastewater obtained by washing fresh-cut lettuce was subjected to ultrasound treatment for up to 20 min in
207 continuous mode and controlled temperature regime. To avoid temperature increase, the vessel containing the
208 sample was placed into an ice bath to remove the heat generated during the ultrasound process into the fluid.
209 The controlled temperature regime allowed values never exceeding 35 °C to be obtained. The power density
210 transferred from the ultrasound probe into the fluid, quantified calorimetrically using eq. 1, was equal to 270
211 W/L. Accordingly, the specific acoustic energy values ranged between 15 kJ/L and 314 kJ/L, depending on
212 treatment time.
213 Fig. 1 shows the decontamination efficiency of continuous power ultrasound provided under controlled
214 temperature regime against the total microbial count as well as *L. monocytogenes*, *E. coli* and *S. enterica*
215 inoculated in the wastewater obtained by fresh-cut lettuce washing. Following the ultrasound treatments, Log
216 reductions of the total microbial count as well as *L. monocytogenes, E. coli* and *S. enterica* of the wash water
217 increased linearly with exposure time ($p<0.05$). In particular, the rate constants computed from the slopes of
218 the linear regression of the logarithm of microbial counts as a function of ultrasonication time were 0.127,
219 0.09, 0.195 and 0.226 min^{-1} ($0.783<R^2<0.973$) for native microflora, *L. monocytogenes, E. coli* and *S. enterica,*
220 respectively. These differences in rate constants indicate different resistances to ultrasonication among the
221 microorganisms. A total microbial count reduction of approximately 2.8 Log units was obtained after 20 min
222 application of this treatment. Based on the above rate constants, a 5 Log reduction of *L. monocytogenes, E.*
223 *coli* and *S. enterica*, that is the minimum requirement for water disinfection, can be achieved by the application
224 of 56, 26 and 22 min of power ultrasound, respectively. It is noteworthy that these treatments are hardly
225 applicable at the industrial level because time and cost consuming. In our experimental conditions, higher
226 decontamination effects were achieved as compared with those of the literature. Neis and Blume (2002)
227 reported that reductions of 0.9 and 2.9 Log units of fecal streptococci and *E. coli*, respectively, were achieved
228 following 60 min at 400 W/L. Similar Log reductions of total coliforms and fecal streptococci in municipal
229 wastewater subjected to 1500 W/L power density were reported by Drakopoulou et al. (2009). Ayyildiz et al.
230 (2011) found that *E. coli* Log reductions ranged from approximately 0.5 and 1.1 for municipal wastewater

231 processed at 75 to 300 W/L for 10 min. Elizaquivel et al. (2011) reported 2.4 Log reductions of *E. coli* O157:H7
232 inoculated in fresh-cut vegetable wastewater following 30 min ultrasonication at 280 W/L, while 60 min were
233 required to achieve complete inactivation (5 Log reductions). Similarly, Gómez-López et al. (2015) reported
234 that 30 min ultrasound treatment at 280 W/L of wastewaster obtained by lettuce washing allowed 2 Log
235 reductions for *E. coli* and *S. enterica*, and 1 Log reduction for *L. monocytogenes* to be achieved.
236 To actually quantify the effect of power ultrasound, the decimal reduction time D_{US} for the inoculated
237 pathogenic bacteria was calculated using procedures analogous to those employed in thermal death time
238 studies. In particular, D_{US} was defined as the ultrasonication time needed to reduce the number of
239 microorganisms by 90% at a given ultrasound power. D_{US} values of 11.1, 5.1 and 4.4 min were obtained for *L.*
240 *monocytogenes*, *E. coli* and *S. enterica*, respectively. According to the above mentioned definition, the higher
241 the D_{US} value, the less the microorganism susceptibility to the ultrasonication power. Therefore, *S. enterica*
242 resulted to be slightly more susceptible to the ultrasound treatment than *E. coli*, that in turn was more sensitive
243 than *L. monocytogenes*, in agreement with Gómez-López et al. (2015). The greater resistance of *L.*
244 *monocytogenes* to ultrasound treatments can be attributed to its Gram status. As known, the Gram-positive cell
245 wall of microorganisms presents a thicker and more tightly adherent peptidoglycan layer than that of the
246 Gram-negative microorganims (Cummins, 1989). Thus, *L. monocytogenes* would be capable to better
247 withstand extreme pressure and temperature variations due to cavitation.
248
249 *3.2. Decontamination efficiency of continuous and pulsed power ultrasound provided under uncontrolled*
250 *temperature regime*
251
252 In order to study the decontamination potential of combined ultrasound processing with *in situ* generated heat,
253 wastewater obtained by washing fresh-cut lamb's lettuce was subjected to ultrasound treatments under
254 uncontrolled temperature regime. To this purpose, sample temperature was left to rise during the ultrasound
255 process due to heat dissipation. Trials without temperature control were performed in pulsed mode or
256 continuous mode. In the former case, samples were subjected to pulsing at 0.5/0.5 seconds on/off. This
257 modality has been already used to allow to contain the temperature rise during ultrasound process (Madge &
258 Jensen, 2002; Bermúdez-Aguirre & Barbosa-Cánovas, 2012). Fig. 2 shows the time-temperature profiles of

wash water during continuous or pulsed ultrasound without temperature control. As expected, temperature increased during treatments, reaching approximately 90 °C after 15 min of continuous ultrasound, whereas temperature values not exceeding 65 °C were recorded for the pulsed modality. In fact, pulsed ultrasound decreased the temperature rise compared with continuous ultrasound, because the "off" interval period allowed heat to be dissipated (Madge & Jensen, 2002). The power densities transferred into the wastewater sample during the pulsed and continuous power ultrasound processes were of 205 and 572 W/L, respectively. Accordingly, the specific acoustic energy values ranged between 60 and 244 kJ/L, and 32 and 687 kJ/L for the pulsed and continuous ultrasound modalities, respectively.

Fig. 3 shows the effect of pulsed and continuous power ultrasound provided under uncontrolled temperature regime on the total microbial count of the wastewater obtained by fresh-cut lettuce washing. The effect of heat alone, i.e. generated by providing the water sample the same time-temperature combinations received during the continuous ultrasound without temperature control, on the native microflora is also shown. The Log reductions of the total microbial count of wastewater increased linearly with exposure time ($p<0.05$). In particular, the rate constants computed from the slopes of the linear regression of the logarithm of total microbial count *vs* exposure time were 0.109, 0.147 and 0.142 min^{-1} ($0.711<R^2<0.874$) for the pulsed ultrasound, continuous ultrasound and heating, respectively. It can be observed that the rate constants of the pulsed and continuous ultrasound increased with increasing levels of power density (205 and 572 W/L, respectively), in agreement with previous findings (Patil, Bourke, Kelly, Frias, & Cullen, 2009; Gao et al., 2014). Thus, the lowest Log reductions were attained during pulsed ultrasound. In fact, 20 min of this treatment resulted in 2.4 Log reductions of the total bacterial count. According to the classification suggested by Madge and Jensen (2002), this value accounts for a good disinfection efficiency of the pulsed ultrasound. It is noteworthy that the same Log reduction was achieved by applying continuous power ultrasound with temperature control (Fig. 1). It could be argued that the additional thermal effect produced during the pulsed treatment is likely to compensate the lower cavitation effect generated during the continuous ultrasound process at controlled temperature regime. Microorganisms responded similarly to the continuous ultrasound and heating alone (Fig. 3). Twenty min application of both treatments allowed a 3.2 Log reduction of the native microflora to be achieved, thus indicating that the *in situ* generated heat contributed to microbial inactivation, in agreement with previous findings (Madge & Jensen, 2002; Salleh-Mack & Roberts, 2007; Gómez-López et

al., 2015). Overall, data reported here suggest that cavitation may be not the only mechanism of microbial decontamination. Besides physical (i.e. extreme pressure variations and micro-streaming) and chemical (i.e. formation of free radicals and H_2O_2) mechanisms, temperature rise, occurring during ultrasound, plays an important role towards microbial inactivation.

Fig. 4 shows the Log reductions of the total microbial count in the wastewater derived from washing fresh-cut lettuce as a function of the specific energy generated upon the pulsed and continuous power ultrasound processes without temperature control as well as heating alone. As the specific energy brings together transferred power, time of exposure and treated volume (Hulsmans et al., 2010), it was used as a reference parameter to make possible the comparison. It can be observed that the plots describing the effect of pulsed and continuous power ultrasound on the total bacterial count were almost overlapping, indicating that ultrasound modality (and thus power transferred into the fluid) had barely an effect on the microbial decontamination level, provided that the same energy (and temperature) was achieved. These two plots were in turn nearly on top of that describing the effect of the heating alone on the naturally present microflora. Our results are partially in disagreement with those reported by Madge and Jensen (2002) for fecal coliforms in domestic wastewater. In fact, according to these authors, the disinfection efficiency of pulsed and continuous ultrasound was similar up to 60 kJ/L, while the pulsed ultrasound resulted less effective than the continuous treatment at increasing doses. The results of the present study clearly show that the specific energy transferred to the system during power ultrasound without temperature control affected the microbial reduction, regardless the ultrasonication modality (pulsed or continuous), and confirmed that the *in situ* generated heat contributed to decontamination.

Fig. 5 shows the decontamination efficiency of continuous power ultrasound under uncontrolled temperature regime on wastewater inoculated with *L. monocytogenes, E. coli* and *S. enterica* suspensions having initial concentration of approximately 10^6 CFU/mL. Reductions of 1.0, 1.2 and 5 Log units of *L. monocytogenes, E. coli* and *S. enterica* were attained after 3 min of continuous ultrasound, respectively. Complete inactivation of *L. monocytogenes, E. coli* was achieved at 5 min of ultrasound exposure. By subjecting wastewater inoculated with *E. coli* and *S. enterica* to heating alone, by providing the same time-temperature combinations received during the continuous ultrasound, 5 Log reductions were also achieved within 5 min and 3 min, respectively. On the contrary, only 1.7 Log reductions *L. monocytogenes* were attained after 5 min heating, while complete

315 inactivation was achieved following 10 min treatment (Fig. 5). It must be pointed out that in our experimental
316 conditions, temperature never exceeded 50 °C within 3 min of ultrasonication. At this sub-lethal temperature,
317 *L. monocytogenes* cells were subjected to the ultrasound effect alone. On the contrary, as at 5 min of treatment
318 the temperature rose to 65 °C, a contribution to *L. monocytogenes* reduction of the heat generated during the
319 ultrasound process above this exposure time can be inferred, in agreement with previous studies (Pagan,
320 Manas, Alvarez, & Condon, 1999; Bauman, Martin, & Feng, 2005; Salleh-Mack & Roberts, 2007; Gómez-
321 López et al., 2014). Results indicate that the same decontamination efficiency against *E. coli* and *S. enterica*
322 was achieved by providing either ultrasound or heating processes. Only in the case of *L. monocytogenes*
323 different contributions to microbial reduction were found for ultrasound without temperature control and
324 heating alone.
325 To actually differentiate cavitation and heat contributions to bacteria inactivation, *L. monocytogenes*, *E. coli*
326 and *S. enterica* logarithmic cell numbers in wastewater samples were compared in terms of specific energy
327 provided during either the continuous ultrasound treatments with or without temperature control or heating.
328 Table 1 shows the rate constants computed from the slopes of the linear regression ($p<0.005$) of the logarithm
329 of bacterial count *vs* energy values (kJ/L), and the correspondent determination coefficients. The estimated
330 inactivation rate constant for *L. monocytogenes* in wastewater subjected to ultrasound without temperature
331 control was greater than the inactivation rate constants obtained by either heating only or ultrasound under
332 controlled temperature regime. According to Madge and Jensen (2002), these rate constants were used to
333 determine the acoustic and thermal contributions to disinfection. In particular, the former was calculated as the
334 percentage ratio of the rate constants of ultrasonication with and without temperature control; the thermal
335 contribution was computed as the percentage ratio of the rate constants of thermal treatment and ultrasound
336 process without temperature control. The acoustic and thermal contributions to *L. monocytogenes* inactivation
337 were estimated to account for about 22 and 58%, respectively. The remaining 20% of unaccounted contribution
338 can be attributed to synergistic effects. These results are in agreement with data reported by Madge and Jensen
339 (2002) for fecal coliform bacteria in domestic wastewater subjected to ultrasound treatment at 700 W/L with
340 or without temperature control and heating alone. Data of Table 1 also show that the estimated values of
341 inactivation rate constants for *E. coli* and *S. enterica* subjected to continuous ultrasound without temperature
342 control were almost the same of those accounting for the heat treatment alone. In other words, a small

temperature rise (i.e. from 30 °C to 50 °C for *S. enterica*; from 30°C to 63°C for *E. coli*) allowed the disinfection efficiency to be greatly increased. Therefore, in our experimental conditions, the effectiveness of continuous ultrasound carried out without temperature control compared with that provided under controlled temperature regime against *E. coli* and *S. enterica* was mainly due to the thermal contribution, while the acoustic mechanism was negligible. Differences in acoustic and heat contributions observed among *L. monocytogenes, E. coli* and *S. enterica* can be brought back to their different sensitivity to heat and ultrasounds, *L. monocytogenes* being the most resistant (Pagan, Manas, Raso, & Condon, 1999).

To find whether these treatments had reversible or irreversible effects, resuscitation trials were carried out on *L. monocytogenes, E. coli* and *S. enterica* inoculated wastewater already subjected to continuous ultrasound without temperature control or heat treatment. Results showed that *E. coli* and *S. enterica* were irreversibly inactivated by 5 min of both treatments, whereas *L. monocytogenes* cells, although stressed, were able to re-grow, indicating their ability to repair the cellular damage. However, no resuscitation was observed for *L. monocytogenes* cells subjected to longer treatments.

4. Conclusions

The results acquired in this study highlighted the effectiveness of pulsed and continuous power ultrasound in decontaminating wastewater derived from fresh-cut production. When ultrasound was provided with temperature control, different capabilities were found among the microorganisms considered (i.e. native microflora as well as inoculated *L. monocytogenes, E. coli* and *S. enterica)* to withstand physical and chemical effects of cavitation, *L. monocytogenes* and *S. enterica* being the most and the least resistant, respectively. When ultrasound was applied without temperature control, a 5 Log reduction of the pathogenic bacteria was achieved within 5 min. Such a rapid decontamination was attributed to the contribution of *in situ* generated heat during ultrasound treatment. The thermal contribution accounted for 58% for *L. monocytogenes*, while it represented the prevalent mechanism for *E. coli* and *S. enterica*, that are more heat sensitive bacteria. In light of this, instead of increasing ultrasound power input and dissipate the heat produced during the treatment, it seems more feasible to apply lower acoustic power densities and exploit the *in situ* generated thermal effect to decontaminate wastewater obtained by fresh-cut vegetable washing from heat resistant microorganisms. In the

attempt to optimize the wastewater management in the fresh-cut sector, application of power ultrasound in combination with *in situ* generated heat to wastewater decontamination could represent a promising tool for water recycling inside a fresh-cut production. Moreover, besides safety requirements, this technology would also meet cost-effectiveness criteria and existing standards.

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Figure captions

Fig. 1. Log reductions of total microbial count, *L. monocytogenes, E. coli* and *S. enterica* in wastewater obtained by fresh-cut lamb's lettuce washing, subjected to continuous power ultrasound under controlled temperature regime.

Fig. 2. Time-temperature profiles of wastewater from fresh-cut lamb's lettuce washing during pulsed or continuous power ultrasound provided under uncontrolled temperature regime.

Fig. 3. Log reductions of total microbial count in wastewater fresh-cut lamb's lettuce washing subjected to pulsed or continuous power ultrasound under uncontrolled temperature regime, or heating. The latter provided the water sample the same time-temperature combinations received during the continuous ultrasound.

Fig. 4. Log reductions of total microbial count in wastewater from fresh-cut lamb's lettuce washing as a function of the specific energy generated upon pulsed and continuous power ultrasound without temperature control as well as upon heating provided according to the same time-temperature combinations received during the continuous ultrasound.

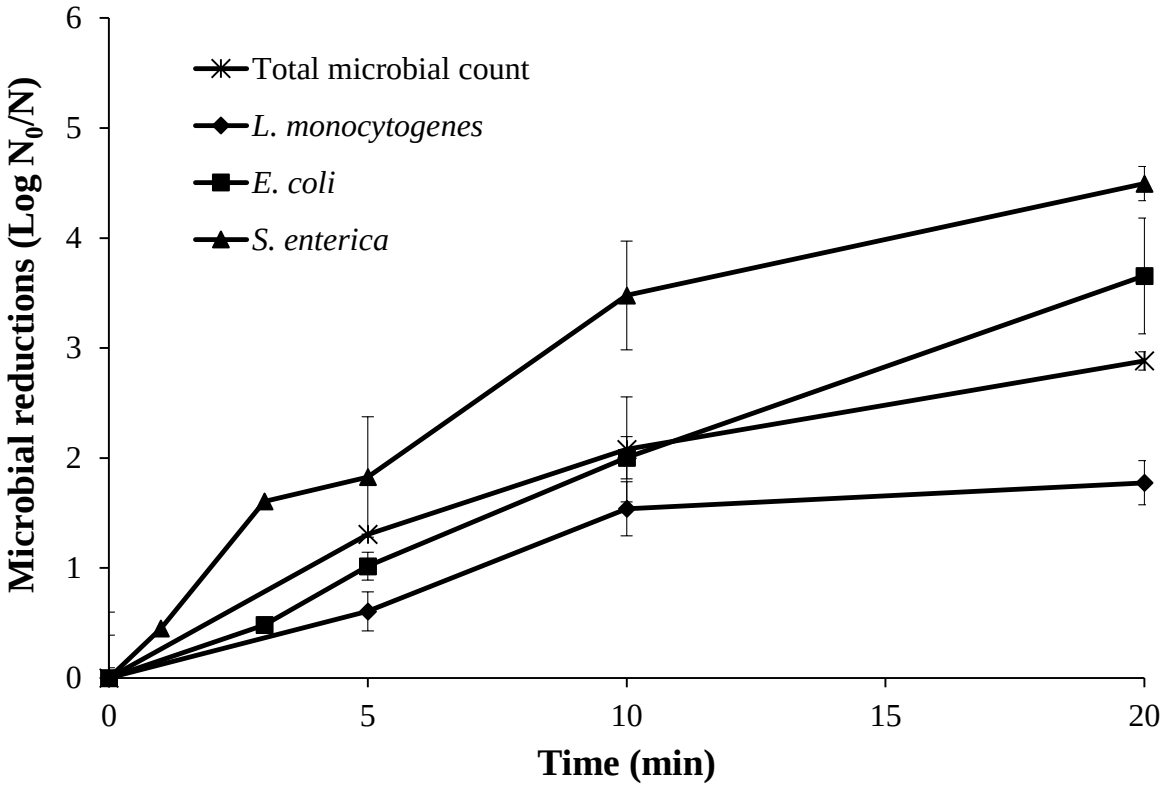
Fig. 5. Log reductions of *L. monocytogenes, E. coli* and *S. enterica* inoculated in wastewater from fresh-cut lamb's lettuce washing as a function of time for continuous power ultrasound under uncontrolled temperature regime. Dashed lines: microbial reduction obtained by subjecting wash water to the sole heat generated by providing the water sample the same time-temperature combinations received during the continuous ultrasound. Asterisk: counts below the detection limit of 1 Log CFU/mL.

Table 1

Rate constants computed from the slopes of the linear regression of the logarithmic cell number of *L. monocytogenes*, *E. coli* and *S. enterica* in wastewater from fresh-cut lamb's lettuce washing subjected to continuous ultrasound processing (US) with or without temperature control or heating *vs* energy values (kJ/L), and correspondent determination coefficients.

	US with temperature control		US without temperature control		Heat only	
	k (L/kJ)	R^2	k (L/kJ)	R^2	k (L/kJ)	R^2
L. monocytogenes	0.0057	0.830	0.0263	0.858	0.0152	0.967
E. coli	0.0125	0.979	0.0278	0.892	0.0298	0.843
S. enterica	0.0144	0.889	0.0449	0.965	0.0477	0.963

$p<0.005$



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507 **Fig. 1**

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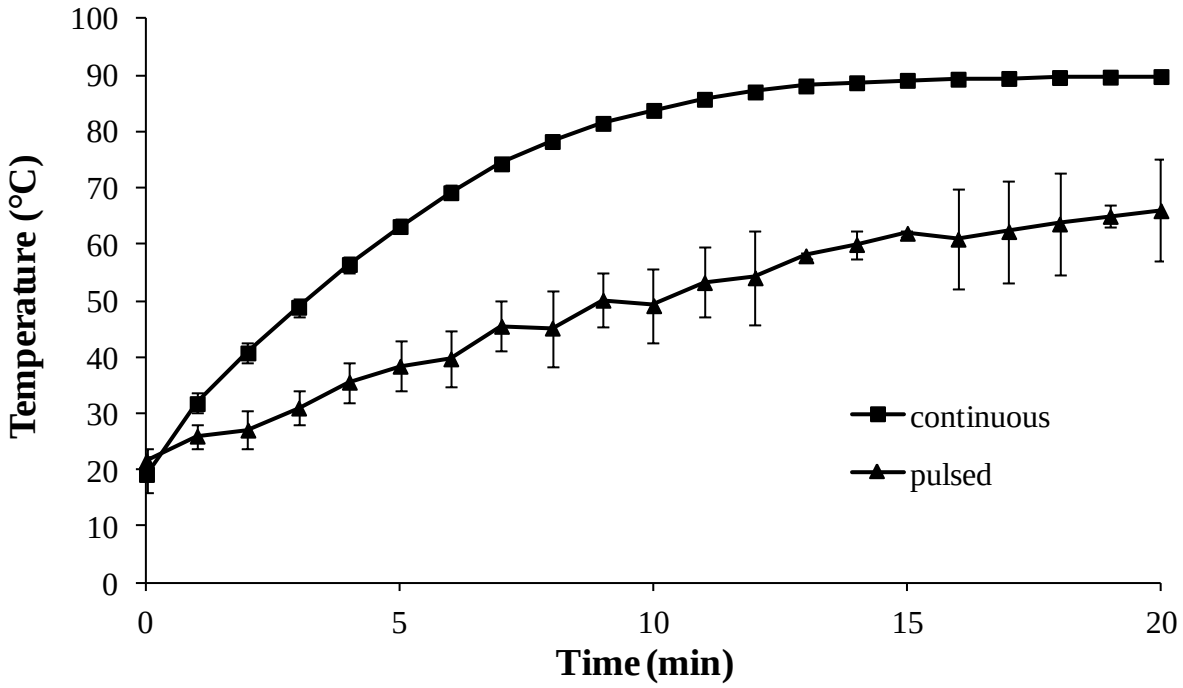

Temperature (°C)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
Time (min)
continuous
pulsed

Fig. 2

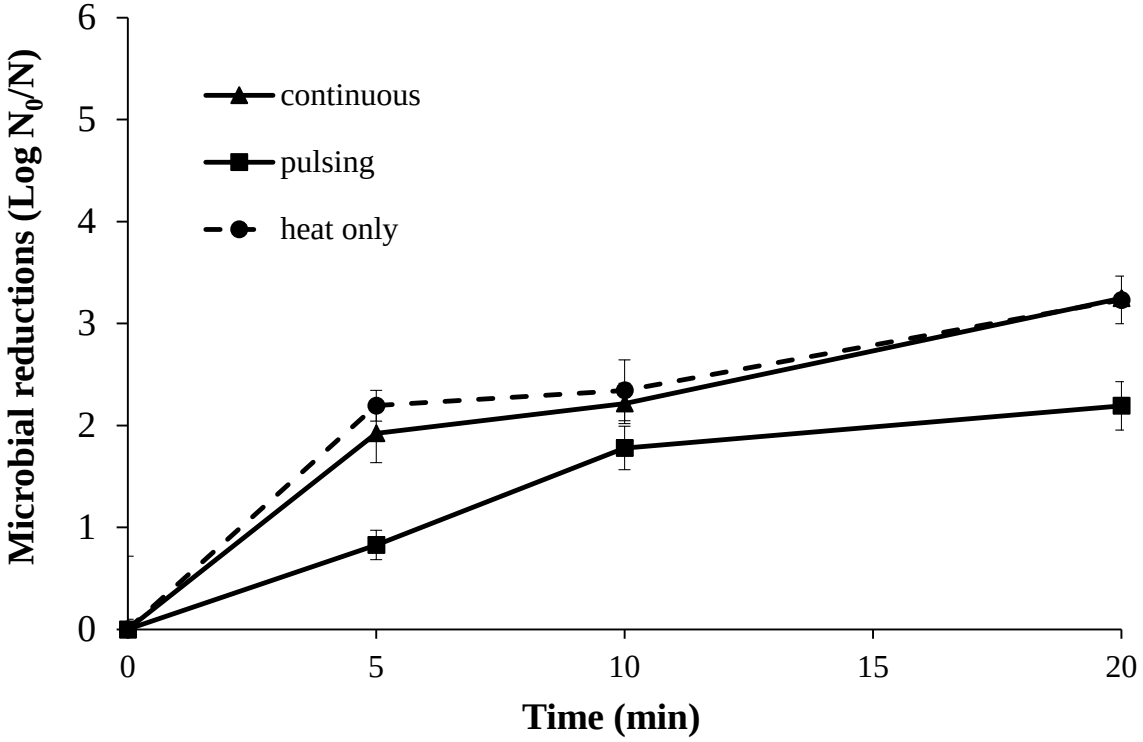
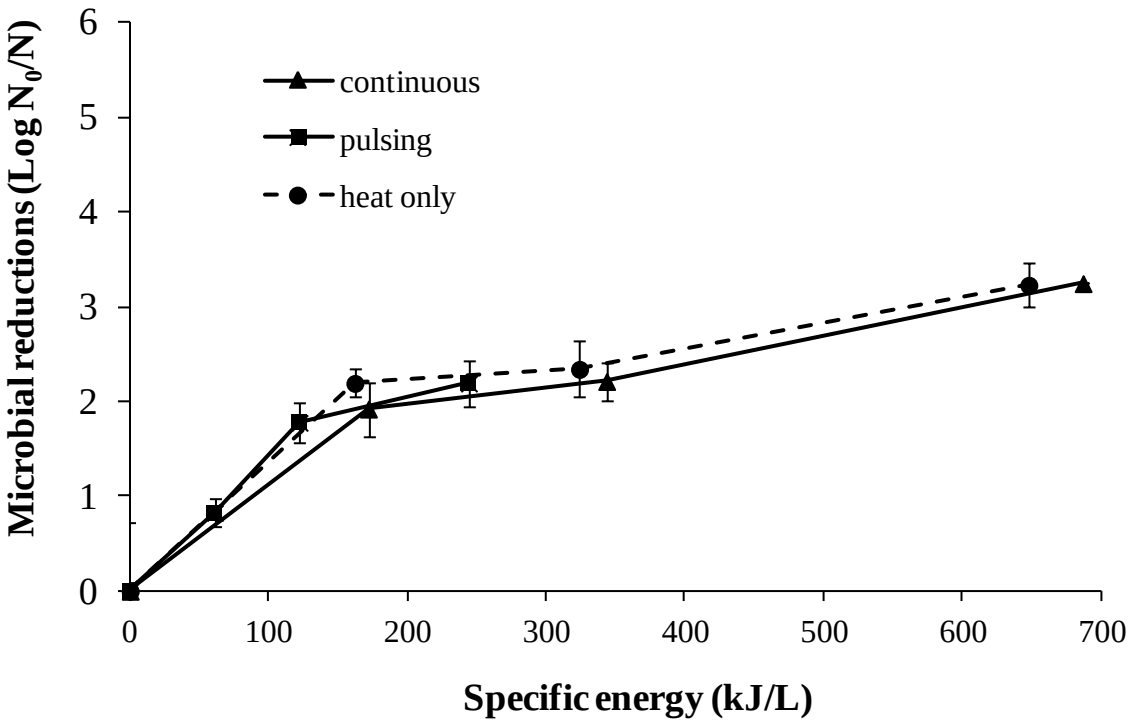

Microbial reductions (Log N_0/N)
6
5
4
3
2
1
0
continuous
pulsing
heat only
0
5
10
15
20
Time (min)

Fig. 3



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540 **Fig. 4**

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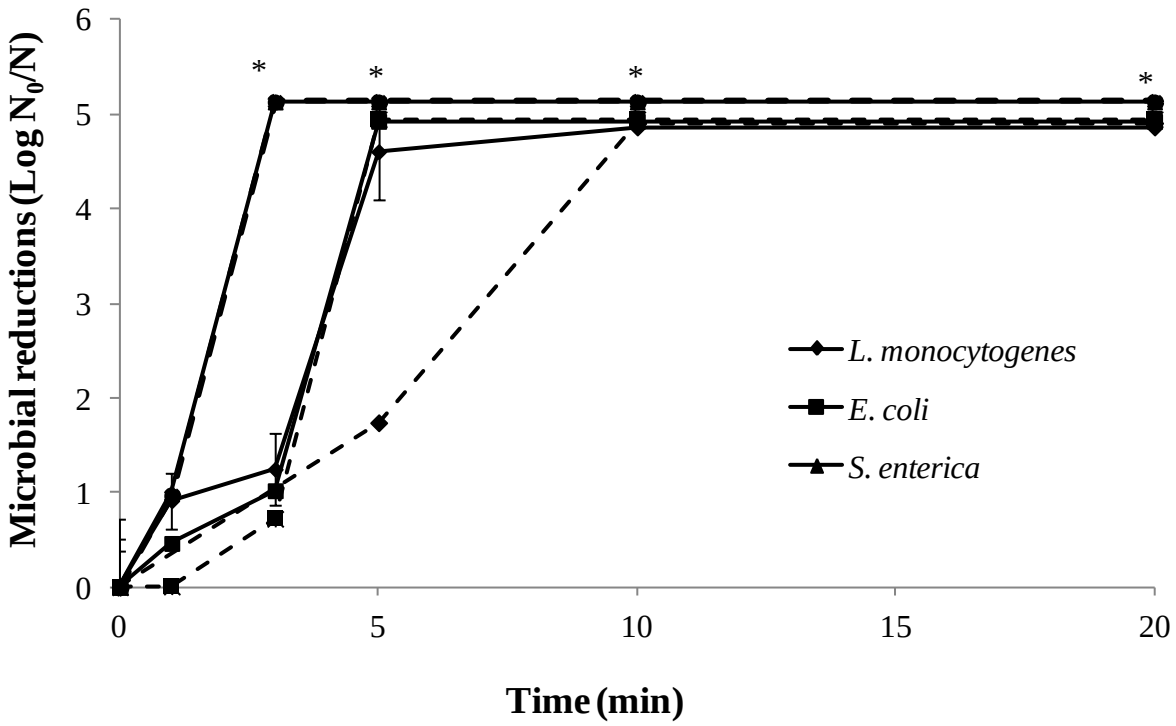
Microbial reductions (Log N_0/N)
Time (min)
L. monocytogenes
E. coli
S. enterica
*
*
*
*
0
1
2
3
4
5
6
0
5
10
15
20

Fig. 5