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Power ultrasound decontamination of wastewater from fresh-cut lettuce washing for potential water recycling

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2	recycling
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15 Abstract

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17 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 18 19 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 20 provided under uncontrolled temperature regime. Continuous ultrasound supplied without 21 22 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 23 Salmonella enterica were attained within 5 min of ultrasonication. The heat generated during 24 continuous ultrasound accounted for approximately 58% of the total decontamination effect against 25 L. monocytogenes, while it contributed for 100% to E. coli and S. enterica inactivation. 26

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28 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.

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

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37 **1. Introduction**

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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).

44 The fresh-cut vegetables market has grown considerably in the last few decades in response to an increased 45 demand for fresh-like, healthy and convenient foods. Fresh-cut vegetables production requires intensive use 46 of water to both wash and move vegetables along the production line. In order to secure water supply and 47 protect the environment from the adverse effects of the wastewater discharges (EEC 1991), water recycling in 48 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 49 accepted requirement for safe water disinfection. Wastewater decontamination may be accomplished by means 50 51 of chemical and physical interventions (Casani, Rouhany, & Knøchel, 2005; Olmez & Kretzschmar, 2009). 52 Among these, sodium hypochlorite is the most used due to its low cost and easy use (Olmez & Kretzschmar, 53 2009; Gil, Selma, López-Gálvez, & Allende, 2009). However, not only wastewater containing chlorine has a 54 great environmental impact, but also chlorination disinfection by-products are known to represent a potential 55 risk for human health (Itoh, Gordon, Callan, & Bartram, 2011). Consequently, there is great effort to find 56 suitable technologies to allow wastewater recycling (Casani et al., 2005; Olmez & Kretzschmar, 2009; Artés, 57 Gómez, Aguayo, Escalona, Artés-Hernández, 2009). Power ultrasound has been suggested as a technology 58 alternative to chlorination for wastewater decontamination (Neis & Blume, 2002; Piyasena, Mohareb, & 59 McKellar, 2003). Ultrasound frequencies higher than 20 kHz are actually considered safe, non-toxic and 60 environmentally friendly (Kentish & Ashokkumar, 2011). During ultrasound treatment, cavitation phenomena 61 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 62 well as free radicals and hydrogen peroxide (Leighton, 1994; Mason, Joyce, Phull, & Lorimer, 2003). 63 Ultrasound effectiveness in wastewater decontamination was found to increase with the power input and 64

65 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; 66 67 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 68 chlorination (Drakopoulou, Terzakis, Fountoulakis, Mantzavinos, & Manios, 2009; Ayyildiz, Sanik, & Ileri, 69 70 2011), organic acids (Gómez-López, Gil, Allende, Vanhee, & Selma, 2015), and ultraviolet irradiation (Blume 71 & Neis, 2004; Mason et al., 2003; Naddeo, Land, Belgiorno, & Napoli, 2009; Gómez-López et al. 2015). An 72 increase of microbial sensitivity to ultrasound in combination with temperature increase, experienced with 73 ultrasonic treatment, for wastewater disinfection has been also reported (Madge & Jensen, 2002; Salleh-Mack 74 & Roberts, 2007; Gómez-López, Gil, Allende, Blancke, Schouteten, & Selma, 2014). It has been estimated 75 that the heat generated during ultrasound processing accounted for approximately 52% of the resulting 76 disinfection (Madge & Jensen, 2002).

77 In contrast with the huge number of studies in the literature dealing with ultrasound decontamination of 78 municipal wastewater and effluents as well as model fluids, very few studies investigated ultrasound 79 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 80 effective in inactivating pathogenic bacteria inoculated in fresh-cut lettuce wash water (Elizaquível et al., 81 82 2012), especially in the presence of the residual peroxyacetic acid concentration that can be found in the wash 83 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 84 85 et al. (2014) showed that ultrasound disinfection against *Escherichia coli* O157:H7 inoculated in fresh-cut 86 lettuce wash water can be increased by combination with heating. Reductions of 6 Log of this microorganism 87 were actually achieved after 60 and 20 min of ultrasonication with and without temperature control, 88 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.

92 In this study the efficacy of power ultrasound in decontaminating wastewater deriving from fresh-cut vegetable 93 washing was investigated. To this aim, wastewater obtained by washing fresh-cut lamb's lettuce was subjected 94 to power ultrasound, provided in pulsed or continuous modality, with or without temperature control. The 95 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 96 97 microorganisms were chosen due to their natural occurrence in a water environment and because they are 98 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 99 100 implement strategies for efficient management of water resource in the fresh-cut industry. To this regard, the 101 decontamination efficacy was related to the ultrasound cavitation and heat contributions.

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103 **2.** Materials and methods

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105 2.1. Preparation of fresh-cut vegetable wash water

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107 Lamb's lettuce (*Valerianella locusta* Laterr.) was purchased from a local market. Lettuce leaves were placed 108 into a beaker containing tap water at 18 °C \pm 2 °C (the vegetable-water ratio was 1:30 w/v). After 1 min of 109 washing, water was separated from the leaves by using a domestic salad spinner.

- 110
- 111 2.2. Bacterial strains and inoculum preparation
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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 10⁶ CFU/mL was obtained for each bacteria suspension.
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122 2.3. Power ultrasound treatment

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An ultrasonic processor (Hieschler Ultrasonics GmbH, mod. UP400S, Teltow, Germany) with a titanium horn 124 125 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, 126 E. coli and S. enterica were introduced into 250 mL capacity (110 mm height, 60 mm internal diameter) glass 127 vessels. The tip of the sonicator horn was placed in the centre of the solution, with an immersion depth in the 128 fluid of 10 mm. The ultrasound treatments were performed for increasing lengths of time up to 20 min. During 129 the ultrasonication experiment, the temperature was either controlled using an ice bath, to dissipate the heat 130 131 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 132 133 0.5 s was followed by a pulse interval period of 0.5 s, during which the sonochemical reactor was switched 134 off. Before and after each experiment, the ultrasound probe was disinfected by washing with ethanol followed by through rinsing with sterile water. 135

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137 2.4. Thermal treatment

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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.

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146 2.5. Microbiological analysis

Both naturally present and inoculated microorganisms were quantified at different time intervals during the ultrasound and heat treatments. The wastewater samples were diluted 10 fold with MRD (Oxoid, UK). Total viable count of non inoculated water was enumerated by spreading onto plates with Plate Count Agar (PCA, Oxoid, UK) and incubating at 30 °C for 48 h. *L. monocytogenes* and *S. enterica* concentrations were determined by plating on Palcam Agar (PA, Oxoid, UK) and Xylose Lysine Desoxycholate agar (XLD, Oxoid, UK), respectively, at 37 °C for 48 h, while the Coli ID medium (BioMerieux, Mercy L'Etoile, France) was used for *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. monocytogenes presence and enumerate E. coli. For Salmonella spp., 25 mL of wastewater was diluted with 155 225 mL of Buffered Peptone Water (BPW, Oxoid, UK), homogenised in a Stomacher Lab-Blender 400 (VWR 156 International PBI srl, Milano, Italy) for 2 min and incubated at 37 °C for 24 h. Aliquots of 0.1 mL of BPW 157 were added with 9.9 mL Rappaport Vassiliadis (RV, Oxoid, UK) and incubated at 42-43 °C for 18-24 h. 158 159 Presence/absence of Salmonella spp. was checked by spreading onto XLD agar plates and incubating at 37 °C for 24 h. For L. monocytogenes, 25 mL of wastewater were diluted with 225 mL of Fraser Broth (FB, Oxoid, 160 161 UK), homogenised in a Stomacher for 2 min and incubated at 30 °C for 36-48 h. 1 mL of FB was added with 9 mL of FB and incubated at 37 °C for 24-48 h. Presence/absence of L. monocytogenes was checked by 162 spreading onto PA plates and incubating at 37 °C for 24-48 h. To evaluate the presence of E.coli the Coli ID 163 164 medium at 37 °C for 24 h was used.

In order to investigate whether treatments were responsible for bacteria sub-lethal injury, resuscitation trials were carried out. For each inoculated strain, 10 mL of wastewater was transferred into 10 mL of BHI broth and then incubated at 30 °C for 2h. Afterwards, presence/absence of *L. monocytogenes*, *E. coli* and *S. enterica* was checked by spreading onto PA, Coli ID and XLD agar media, respectively.

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170 *2.6. Temperature measurement*

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The temperature was recorded as a function of time using a copper-constantan thermocouple probe (Ellab,
Denmark), connected to a data-Logger (CHY 502A1, Tersid, Milano, Italy).

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

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$$181 \qquad P = dc_p \left(\frac{\partial T}{\partial t} \right) \tag{1}$$

182

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) was calculated by multiplying the power density value by the duration of the treatment (Hulsmans et al., 2010).

- 186 2.8. Statistical analysis
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Results are averages of two measurements carried out on two replicated samples and are reported as means \pm SD. Analysis of variance (ANOVA) was performed with significance level set to p<0.05 (Statistica for Windows, ver. 5.1, Statsoft Inc. Tulsa, USA, 1997). The Tukey procedure was used to test for differences between means. Linear regression analysis was performed by using Microsoft Excel 2007. The goodness of fitting was evaluated based on visual inspection of residual plots and by the calculation of R² and p.

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- 194 **3. Results and discussion**
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- 196 3.1. Decontamination efficiency of continuous power ultrasound provided under controlled temperature197 regime

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

washed in analogous conditions as those performed in the present study, the microbial count was mainly
 represented by *Pseudomonas* spp, Enterobacteriaceae and total coliforms. No presence of *L. monocytogenes*,
 E. coli and *S. enterica* cells was detected in wastewater.

Wastewater obtained by washing fresh-cut lettuce was subjected to ultrasound treatment for up to 20 min in continuous mode and controlled temperature regime. To avoid temperature increase, the vessel containing the sample was placed into an ice bath to remove the heat generated during the ultrasound process into the fluid. The controlled temperature regime allowed values never exceeding 35 °C to be obtained. The power density transferred from the ultrasound probe into the fluid, quantified calorimetrically using eq. 1, was equal to 270 W/L. Accordingly, the specific acoustic energy values ranged between 15 kJ/L and 314 kJ/L, depending on treatment time.

Fig. 1 shows the decontamination efficiency of continuous power ultrasound provided under controlled 213 temperature regime against the total microbial count as well as L. monocytogenes, E. coli and S. enterica 214 215 inoculated in the wastewater obtained by fresh-cut lettuce washing. Following the ultrasound treatments, Log reductions of the total microbial count as well as L. monocytogenes, E. coli and S. enterica of the wash water 216 217 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 microbial counts as a function of ultrasonication time were 0.127, 218 219 0.09, 0.195 and 0.226 min⁻¹ (0.783<R²<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) reported that reductions of 0.9 and 2.9 Log units of fecal streptococci and E. coli, respectively, were achieved 227 following 60 min at 400 W/L. Similar Log reductions of total coliforms and fecal streptococci in municipal 228 wastewater subjected to 1500 W/L power density were reported by Drakopoulou et al. (2009). Ayyildiz et al. 229 (2011) found that E. coli Log reductions ranged from approximately 0.5 and 1.1 for municipal wastewater 230

processed at 75 to 300 W/L for 10 min. Elizaquivel et al. (2011) reported 2.4 Log reductions of *E. coli* O157:H7
inoculated in fresh-cut vegetable wastewater following 30 min ultrasonication at 280 W/L, while 60 min were
required to achieve complete inactivation (5 Log reductions). Similarly, Gómez-López et al. (2015) reported
that 30 min ultrasound treatment at 280 W/L of wastewaster obtained by lettuce washing allowed 2 Log
reductions for *E. coli* and *S. enterica*, and 1 Log reduction for *L. monocytogenes* to be achieved.

To actually quantify the effect of power ultrasound, the decimal reduction time D_{US} for the inoculated 236 237 pathogenic bacteria was calculated using procedures analogous to those employed in thermal death time studies. In particular, D_{US} was defined as the ultrasonication time needed to reduce the number of 238 239 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.

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3.2. Decontamination efficiency of continuous and pulsed power ultrasound provided under uncontrolled
temperature regime

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In order to study the decontamination potential of combined ultrasound processing with *in situ* generated heat, wastewater obtained by washing fresh-cut lamb's lettuce was subjected to ultrasound treatments under uncontrolled temperature regime. To this purpose, sample temperature was left to rise during the ultrasound process due to heat dissipation. Trials without temperature control were performed in pulsed mode or continuous mode. In the former case, samples were subjected to pulsing at 0.5/0.5 seconds on/off. This modality has been already used to allow to contain the temperature rise during ultrasound process (Madge & Jensen, 2002; Bermúdez-Aguirre & Barbosa-Cánovas, 2012). Fig. 2 shows the time-temperature profiles of 259 wash water during continuous or pulsed ultrasound without temperature control. As expected, temperature 260 increased during treatments, reaching approximately 90 °C after 15 min of continuous ultrasound, whereas 261 temperature values not exceeding 65 °C were recorded for the pulsed modality. In fact, pulsed ultrasound 262 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 263 during the pulsed and continuous power ultrasound processes were of 205 and 572 W/L, respectively. 264 265 Accordingly, the specific acoustic energy values ranged between 60 and 244 kJ/L, and 32 and 687 kJ/L for the 266 pulsed and continuous ultrasound modalities, respectively.

267 Fig. 3 shows the effect of pulsed and continuous power ultrasound provided under uncontrolled temperature 268 regime on the total microbial count of the wastewater obtained by fresh-cut lettuce washing. The effect of heat 269 alone, i.e. generated by providing the water sample the same time-temperature combinations received during 270 the continuous ultrasound without temperature control, on the native microflora is also shown. The Log 271 reductions of the total microbial count of wastewater increased linearly with exposure time (p < 0.05). In 272 particular, the rate constants computed from the slopes of the linear regression of the logarithm of total 273 microbial count vs exposure time were 0.109, 0.147 and 0.142 min⁻¹ (0.711<R²<0.874) for the pulsed 274 ultrasound, continuous ultrasound and heating, respectively. It can be observed that the rate constants of the 275 pulsed and continuous ultrasound increased with increasing levels of power density (205 and 572 W/L, 276 respectively), in agreement with previous findings (Patil, Bourke, Kelly, Frias, & Cullen, 2009; Gao et al., 277 2014). Thus, the lowest Log reductions were attained during pulsed ultrasound. In fact, 20 min of this treatment 278 resulted in 2.4 Log reductions of the total bacterial count. According to the classification suggested by Madge 279 and Jensen (2002), this value accounts for a good disinfection efficiency of the pulsed ultrasound. It is 280 noteworthy that the same Log reduction was achieved by applying continuous power ultrasound with 281 temperature control (Fig. 1). It could be argued that the additional thermal effect produced during the pulsed 282 treatment is likely to compensate the lower cavitation effect generated during the continuous ultrasound 283 process at controlled temperature regime. Microorganisms responded similarly to the continuous ultrasound 284 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, 285 286 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 H₂O₂) mechanisms, temperature rise, occurring during ultrasound, plays an
important role towards microbial inactivation.

291 Fig. 4 shows the Log reductions of the total microbial count in the wastewater derived from washing fresh-cut 292 lettuce as a function of the specific energy generated upon the pulsed and continuous power ultrasound 293 processes without temperature control as well as heating alone. As the specific energy brings together 294 transferred power, time of exposure and treated volume (Hulsmans et al., 2010), it was used as a reference 295 parameter to make possible the comparison. It can be observed that the plots describing the effect of pulsed 296 and continuous power ultrasound on the total bacterial count were almost overlapping, indicating that 297 ultrasound modality (and thus power transferred into the fluid) had barely an effect on the microbial 298 decontamination level, provided that the same energy (and temperature) was achieved. These two plots were 299 in turn nearly on top of that describing the effect of the heating alone on the naturally present microflora. Our 300 results are partially in disagreement with those reported by Madge and Jensen (2002) for fecal coliforms in 301 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 302 303 treatment at increasing doses. The results of the present study clearly show that the specific energy transferred 304 to the system during power ultrasound without temperature control affected the microbial reduction, regardless 305 the ultrasonication modality (pulsed or continuous), and confirmed that the *in situ* generated heat contributed 306 to decontamination.

307 Fig. 5 shows the decontamination efficiency of continuous power ultrasound under uncontrolled temperature 308 regime on wastewater inoculated with L. monocytogenes, E. coli and S. enterica suspensions having initial 309 concentration of approximately 10⁶ CFU/mL. Reductions of 1.0, 1.2 and 5 Log units of L. monocytogenes, E. 310 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 311 with E. coli and S. enterica to heating alone, by providing the same time-temperature combinations received 312 during the continuous ultrasound, 5 Log reductions were also achieved within 5 min and 3 min, respectively. 313 314 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 conditions, temperature never exceeded 50 °C within 3 min of ultrasonication. At this sub-lethal temperature, 316 317 L. monocytogenes cells were subjected to the ultrasound effect alone. On the contrary, as at 5 min of treatment the temperature rose to 65 °C, a contribution to L. monocytogenes reduction of the heat generated during the 318 ultrasound process above this exposure time can be inferred, in agreement with previous studies (Pagan, 319 Manas, Alvarez, & Condon, 1999; Bauman, Martin, & Feng, 2005; Salleh-Mack & Roberts, 2007; Gómez-320 321 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 heating alone. 324

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 regrow, indicating their ability to repair the cellular damage. However, no resuscitation was observed for *L. monocytogenes* cells subjected to longer treatments.

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343

357 4. Conclusions

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The results acquired in this study highlighted the effectiveness of pulsed and continuous power ultrasound in 359 360 decontaminating wastewater derived from fresh-cut production. When ultrasound was provided with 361 temperature control, different capabilities were found among the microorganisms considered (i.e. native 362 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. 363 When ultrasound was applied without temperature control, a 5 Log reduction of the pathogenic bacteria was 364 365 achieved within 5 min. Such a rapid decontamination was attributed to the contribution of *in situ* generated 366 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 367 of this, instead of increasing ultrasound power input and dissipate the heat produced during the treatment, it 368 seems more feasible to apply lower acoustic power densities and exploit the *in situ* generated thermal effect to 369 370 decontaminate wastewater obtained by fresh-cut vegetable washing from heat resistant microorganisms. In the

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

469

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

473

474 Fig. 2. Time-temperature profiles of wastewater from fresh-cut lamb's lettuce washing during pulsed or475 continuous power ultrasound provided under uncontrolled temperature regime.

476

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.

480

481 Fig. 4. Log reductions of total microbial count in wastewater from fresh-cut lamb's lettuce washing as a 482 function of the specific energy generated upon pulsed and continuous power ultrasound without temperature 483 control as well as upon heating provided according to the same time-temperature combinations received during 484 the continuous ultrasound.

485

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.

491

Table 1

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

R ²	control k (L/kJ)	R ²	k (L/kJ)	R ²
R ²	k (L/kJ)	R ²	k (L/kJ)	\mathbb{R}^2
0.830	0.0262	0.070		
0.050	0.0203	0.858	0.0152	0.967
0.979	0.0278	0.892	0.0298	0.843
0.889	0.0449	0.965	0.0477	0.963
	0.889	0.889 0.0449	0.889 0.0449 0.965	0.889 0.0449 0.965 0.0477











