Microbial inactivation by means of ultrasonic assisted supercritical CO_2. Effect on cell ultrastructure

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Abstract

The effect of ultrasound (HPU) on the supercritical carbon dioxide (SC-CO_2) inactivation of vegetative bacteria (*Escherichia coli, Brevundimonas diminuta*) and a fungal spore (*Aspergillus niger*) at different pressures (100 and 350 bar) and temperatures (35, 50 and 60ºC) was assessed. The effect of SC-CO_2 + HPU on the microbial cell ultrastructure was also evaluated by microscopy techniques (FESEM and TEM). HPU enhanced the SC-CO_2 inactivation treatments, showing an average increase of 4.8, 3.4 and 1.3 log-cycles of reduction for *E. coli*, *B. diminuta* and *A. niger*, respectively. In general, the higher the pressure and temperature, the higher the inactivation. *A. niger* spores were found to be more resistant than vegetative bacteria. Microscopy analysis revealed significant morphological changes, including damaged cell walls, and major alteration and loss of cytoplasmic content. Therefore, the SC-CO_2 + HPU technology appears to be effective for microbial inactivation purposes despite the complexity of the cell wall.

Keywords: supercritical CO_2, high power ultrasound, bacteria, fungal spore, ultrastructure.

1. Introduction

Vegetative bacteria and fungal spores can easily become contaminants of food and pharmaceutical products, leading to product spoilage and causing human disease. In this regard, the assurance of microbiological safety is essential for the industry. Nowadays, thermal treatments are the most common preservation methods in the food and pharmaceutical sectors. In order to prevent heat damage related to thermal treatments and obtain higher quality products, novel non-thermal technologies, applied individually or in combined form, have been investigated and developed during the last few years. Some of these so-called non-thermal technologies are irradiation [1], high power ultrasound [2], pulsed electric fields [3], high hydrostatic pressure [4] and supercritical fluids [5].

The supercritical state of carbon dioxide (SC-CO_2) is reached at moderate pressure and temperature (73.8 bar and 31.1ºC), avoiding the negative thermal effects of traditional preservation methods. In supercritical conditions, CO_2 presents lower viscosity than when in the liquid state and higher density than when in the gaseous state, making SC-CO_2 an excellent solvent that can contribute to the removal of vital components of microbial cells. In this sense, SC-CO_2 has already proven to be an effective method for the inactivation of some microorganisms, minimally affecting the physicochemical properties of the treated products [6–8]. However, SC-CO_2 treatments often require long processing times and/or high temperatures and pressures to provide the necessary microbial reduction that ensures product safety. As an example, more than 75 min were insufficient to achieve the complete inactivation of *E. coli* in apple juice at 32ºC and 100-300 bar [9]. For this reason, it is of great interest to combine the SC-CO_2 treatment with other non-thermal techniques, such as high power ultrasound (HPU), high hydrostatic pressure (HHP) [4], pulsed electric fields (PEF) [10] or the addition of antimicrobial agents, such as hydrogen peroxide [11]. In this regard, the application of HPU to the SC-CO_2 treatments has already been demonstrated to intensify the inactivation of a wide range of vegetative bacteria and yeasts [12,13]. However, the SC-CO_2 + HPU inactivation of filamentous fungal spores has not been explored yet.

The most widely accepted inactivation mechanisms of SC-CO_2 are linked to the diffusion and solubilisation of CO_2 into the external media causing a drop in pH that could damage or alter the microbial cell membrane. Thus, CO_2 penetrates into the cells, reducing the internal pH and extracting intracellular vital components which, eventually, can lead to cell death [14]. When HPU is implemented, the heat and mass transfer processes are enhanced due to cavitation effects, which could increase the CO_2 diffusion rate,

accelerating the SC-CO_2 inactivation mechanisms. In addition, HPU could damage or crack the cell walls of the microorganisms [12,13]. A better understanding of the inactivation mechanisms exerted by the combination of SC-CO_2 and HPU is important in order to find improved strategies with which to guarantee the safety and stability of the treated products, as well as optimize the process conditions or the equipment. For that purpose, the analysis of the treated microbial cells at cellular level using microscopy techniques constitutes a valuable approach. Several authors observed the ultrastructure of microbial cells after SC-CO_2 inactivation treatments and stated that there was a direct relation between the permeabilization of the cell membrane and the inactivation [15,16]. Additionally, Ortuño et al. [17] investigated the effect of the SC-CO_2 + HPU treatment on the intracellular structure of vegetative microorganisms (*E. coli* and *S. cerevisiae*). However, there has been no prior analysis of the cell structural effects linked to the SC-CO_2 + HPU treatment on filamentous fungal spores. Moreover, the analysis of the changes in the external structure of the microbial cells after the SC-CO_2 + HPU treatment is unexplored. Therefore, the objective of this study was to evaluate (i) the intensification of the SC-CO_2 + HPU inactivation of different bacteria (*E. coli* and *B. diminuta*) and a fungal spore (*A. niger*) and (ii) the effect of the inactivation treatment on the external morphology and the intracellular structure of the microbial cells.

2. Materials and methods

2.1. Preparation of the bacterial suspensions: *Escherichia coli* and *Brevundimonas diminuta*

E. coli CECT 101 and *B. diminuta* CECT 313 were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). *E. coli* is a facultative anaerobic gram-negative bacteria with a cell size of around 1 × 3 µm [18], highly common in contaminated food and pharmaceutical products. *B. diminuta* is an aerobic gram-negative bacteria, which is used to test the porosity of pharmaceutical grade filters of 0.2 µm because of its small size [19]: typically of around 0.3 x 1.0 µm [20]. A single colony of each bacterium was inoculated in 50 mL of nutrient broth (Scharlab, Barcelona, Spain) and grown overnight (18-24 h) at 37ºC for *E. coli* and 30ºC for *B. diminuta*, using an incubation chamber (3000957, J.P. Selecta, Spain) and an orbital shaker at 120 rpm (3000974, J.P. Selecta, Spain). 50 µL of the overnight starter culture were transferred to a new growth medium and it was incubated until ensuring the stationary phase was reached, 14 h at 37ºC for *E. coli* and 36 h at 30ºC for *B. diminuta* [21]. After that, 5 mL of the bacterial

suspension in the stationary phase were inoculated in 60 mL of deionized water until a concentration of around 10^8 CFU/mL.

2.2. Preparation of the *Aspergillus niger* spore suspension

A. niger CECT 2807 was also obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). *A. niger* is an aerobic spore-forming filamentous fungi, commonly present in the environment and, thus, usually present in contaminated food and pharmaceutical products [22]. *A. niger* was cultured on Potato Dextrose Agar (Scharlab, Barcelona, Spain) at 25ºC for 7 days. After that, spores were rubbed with 10 ml of 0.1% (v/v) Tween 80, collected and kept at 4ºC until use. Prior to each treatment, 5 mL of the *A. niger* spore suspension were inoculated in 60 mL of deionized water until a concentration of around 10^7 CFU/mL.

2.3. Ultrasonic assisted supercritical fluid inactivation treatments

The inactivation treatments with supercritical carbon dioxide (SC-CO_2) were performed in a supercritical fluids lab-scale equipment and batch mode. A high-power ultrasound (HPU) transducer, embedded in the treatment chamber (600 mL of internal volume) through the cap, was used to perform the combined SC-CO_2 + HPU treatments. The ultrasound system consisted mainly of a high power piezoelectric transducer, a sonotrode and a power generation unit. The power supplied was 50 ± 5 W (I= 250 ± 10 mA; U= 220 ± 5 V) and the frequency was 30 ± 2 kHz. Electrical parameters were measured with a digital power meter (WT210, Yokogawa Electric Corporation, Tokyo, Japan). This system was explained in detail in a previous study [21].

SC-CO_2 and SC-CO_2 + HPU inactivation treatments were carried out on 65 mL of inoculated water at two levels of pressure and temperature for each microorganism. The pressure (pressurization rate of about 70 bar/min) was set at 100 and 350 bar for all the microorganisms, as they are common pressures used in SC-CO_2 microbial inactivation studies [23]. The temperature was set at 35 and 50ºC for *E. coli* and *B. diminuta* in order to select a low temperature (35 ºC), very close to the critical temperature for CO_2 and a mild, but non-lethal, temperature (50ºC) for the vegetative bacteria considered in this study. In this regard, preliminary experiments revealed that no inactivation was found for inoculated *E. coli* and *B. diminuta* in deionized water using a water bath and heating at 50ºC for 50 min. In the case of *A. niger*, treatments were carried out at 50 and 60ºC due

to the known greater resistance of fungal spores to SC-CO_2 compared to vegetative bacteria [23]. Samples of around 2 mL were collected (undergoing a sudden depressurization to atmospheric pressure) during the treatments at different times, depending on the process conditions and type of microorganism. All the experiments were carried out in triplicate.

2.4. Microbiological analyses

The standard plate count was used to measure the number of surviving microorganisms. Serial dilutions of the treated samples were prepared and 100 µL of the appropriate dilutions were spread on PCA for the bacteria and PDA for *A. niger* (Scharlab, Barcelona, Spain) in triplicate. Plates were incubated at 37ºC and 24 h for *E. coli*, 30ºC and 48 h for *B. diminuta* and 25ºC and 72 h for *A. niger*. The initial microbial population in the sample was determined following the same procedure. Results were expressed as $\log_{10}(N/N_0)$, where N_0 represents the number of cells initially inoculated in the deionized water and N the number of cells after treatment.

2.5. Modelling

The non-linear Weibull model following the decimal logarithmic form written by Peleg [24] has been demonstrated to be sufficiently robust for the prediction of microbial inactivation [25], and was used in this study (Eq. (1)).

$$\log_{10}\frac{N}{N_0} = -b\cdot t^{n} \qquad \text{Eq. (1)}$$

where N_0 indicates the initial number of microorganisms in the sample (CFU/mL) , N is the number of microorganisms in the sample after the treatment time t (CFU/mL), n (dimensionless) is the shape factor and b (min^{-n}) is the rate parameter.

The constants of the model (b and n) were computed by minimizing the sum of squared differences between the experimental and predicted levels of inactivation using Solver from Microsoft Excel™. The root mean squared error (RMSE, Eq. 2) and the coefficient of determination (R^2, Eq. 3) were determined to evaluate the goodness of fit.

$$RMSE = \sqrt{\frac{\sum_{k=1}^{z}(y_k - y_k*)^2}{z}} \quad \text{Eq. (2)}$$

$$R^2 = 1 - \frac{S_{yx}{}^2}{S_y{}^2} \quad \text{Eq. (3)}$$

where y and y^* are the experimental and the estimated data, respectively; z is the number of experimental data, and S_{yx} and S_y are the standard deviations of the estimation and the sample deviation, respectively.

The model can be fitted to both downward concave survival curves (n>1) and upward concave curves (n<1); and the log linear curve is a special case where n=1. As described elsewhere [26], the time required to achieve the complete inactivation (t_x) of every microorganism was calculated from Eq. 1 and from the b and n values of the Weibull model obtained for each condition, where x is the average in log-cycles for the complete inactivation of each microorganism (7.9 log-cycles in the case of *E. coli*, 8.1 log-cycles in that of *B. diminuta* and 6.8 log-cycles for *A. niger*).

2.6. Statistical analysis

In order to evaluate the effect of both the treatment conditions (pressure, temperature and use of HPU) and the type of microorganism on the inactivation, a general linear model (GLM) was performed using Statgraphics Centurion XVI (Statpoint Technologies Inc., Warrenton, VA, USA). Fisher's least significant difference (LSD) was used to discriminate among the means ($p<0.05$).

2.7. Electron microscopy observations

The microscopy observations of the microbial cells were performed after and before the SC-CO_2 + HPU treatments. The conditions selected were those that achieved the complete microbial inactivation of each microorganism: 50ºC, 350 bar and 2 min in the case of *E. coli* and *B. diminuta* and 60ºC, 350 bar and 10 min for *A. niger* spores.

In order to observe the external morphology of the microbial cells, a field emission scanning electron microscope (FESEM) was used (ZEISS ULTRA 55, Oxfrod Instruments, Abingdon, UK). To this end, microbial samples were centrifuged at 2600 rpm for 5 min and filtered (0.2 µm of pore diameter). Then, samples were placed in the holder, frozen by immersion in liquid nitrogen and transferred to a cryogenic unit

(PP3010T, Quorum Technologies, East Sussex, UK) to be sublimated and coated with platinum by sputtering at 5 mA for 20 s. Samples were observed at 1 kV at a working distance of between 3-5 mm.

For the observation of the intracellular structure of the microorganisms, transmission electron microscopy (TEM) was used (HT-7800 120 kV, Hitachi High-Technologies, Tokyo, Japan). For this purpose, microbial samples were centrifuged at 2600 rpm for 5 min, fixed with 25 g/L glutaraldehyde solution for 24 h at 4 °C and post-fixed with 20 g/L osmium tetroxide solution for 1.5 h. After that, cells were stabilized with agarose solution (3 g/100mL) at 30ºC and stored at 4ºC for 24 h. The solidified agar with the cells was cut into cubes (3 mm^3), which were fixed with 25 g/L glutaraldehyde solution; post-fixed with 20 g/L osmium tetroxide solution, dehydrated with 300, 500, 700 and 1000 g/kg ethanol, contrasted with 20 g/L uranyl acetate solution and resin-embedded. The blocks obtained were cut into ultrathin sections (0.1 µm) with Reichert-Jung Ultracut ultramicrotome (Leica Microsystems, Wetzlar, Germany), collected in copper grids and stained with 40 g/L lead citrate to be observed at 100 kV.

3. Results and discussion

3.1. Analysis of the inactivation kinetics

3.1.1. SC-CO_2 microbial inactivation

Fig. 1 shows the inactivation kinetics of *E. coli* (A), *B. diminuta* (B) and *A. niger* spores (C) in deionized water for the SC-CO_2 treatments. A high degree of experimental variability was found, as can be observed from the error bars, which could be ascribed to pressure and temperature variations inside the vessel, and to the inherent variability in the microbial growth. Despite the great experimental variability, the Weibull model was satisfactorily fitted to the SC-CO_2 inactivation kinetics, as depicted in Fig. 1. In every case, R^2 was higher than 0.92 and RMSE was lower than 0.64, as shown in Table 1. For some of the inactivation kinetics, the model was not fitted due to the lack of experimental data.

<u>Effect of pressure and temperature</u>

Pressure and temperature had a significant ($p<0.05$) effect on the SC-CO_2 inactivation of *B. diminuta* and *A. niger*. In general terms, the higher the pressure and the temperature, the faster the inactivation. As an example, a reduction of only 4.4 log-cycles of *A. niger* was achieved in 90 min at 100 bar and 50ºC, while the complete inactivation (6.7 log-cycles) was reached in just 15 min at 350 bar and 60ºC (Fig. 1C). However, only

235 the temperature had a significant ($p<0.05$) effect on the inactivation of *E. coli*. For
236 example, complete inactivation (7.9 log-cycles) was reached in 25 min at 35ºC while the
237 same inactivation was achieved in less than 13 min at 50ºC, regardless of the pressure
238 used (Fig. 1A). Moreover, when modelling the inactivation kinetics, less time was
239 generally required to achieve the complete inactivation of the *E. coli, B. diminuta* and
240 *A. niger* population ($t_{7.9}$, $t_{8.1}$ and $t_{6.8}$; respectively), as the pressure and the temperature
241 increased (Table 1). For example, in the case of *A. niger*, 163.7 and 22.1 min were
242 required at 100 bar and 50ºC and at 350 bar and 60ºC, respectively. On the one hand,
243 high temperatures are known to increase CO_2 diffusivity and make cell membranes more
244 fluid, facilitating the penetration of CO_2 [27,28]. On the other hand, high pressures
245 increase the solubility of CO_2 in the media [6]; therefore, there is closer contact between
246 CO_2 and the microbial cell, and the CO_2 penetration into the cells is improved [29,30].

247 Several authors also studied the SC-CO_2 inactivation of *E. coli* and *A. niger* in water
248 solutions [31–35]. However, only one study was found into the inactivation of *B. diminuta*
249 [21]. Noman et al. [31] also found that there was notably greater inactivation of *A. niger*
250 spores at higher pressures and temperatures (e.g. in 60 min and at a temperature of
251 55ºC, microbial reduction increased from 2.8 log-cycles at 300 bar to 4.1 at 350 bar, and
252 at a constant pressure of 350 bar it increased from 2.0 to 4.6 log-cycles at temperatures
253 of 35 and 55ºC, respectively). The inactivation levels achieved by Norman et al. [31] were
254 of a lower magnitude than those in the present study (e.g. 6.8 log-cycles was achieved
255 at 350 bar, 50ºC and 55 min). As for *E. coli*, Dillow et al. [35] reported similar effects of
256 pressure and temperature to those in our study, since the temperature (34 vs 42ºC)
257 affected the inactivation, achieving complete inactivation (around 8.0 log-cycles) in 30
258 min at 34ºC and in 20 min at 42ºC (205 bar), while the influence of pressure was
259 negligible (from 140 to 205 bar at 34ºC). Ortuño et al. [12] also found very similar
260 reductions in the inactivation of *E. coli* in LB Broth, compared to the present results (e.g
261 8.0 log-cycles were achieved at 36ºC and 350 bar in 22 min, while in the present study
262 the same inactivation was found in 24 min at 35ºC and 350 bar). However, these authors
263 found that both temperature (31-41 ºC) and pressure (100-350 bar) influenced microbial
264 inactivation. As regards *B. diminuta*, Gomez-Gomez et al. [21] also found that the higher
265 the pressure and temperature, the higher the inactivation levels in an oil-in-water
266 emulsion. However, the processing time required to achieve the complete inactivation of
267 *B. diminuta* in the emulsions was longer than in the present study under the same
268 conditions (e.g. 40 min were required in the lipid emulsion while only 10 min in water
269 (Fig. 1B) at 350 bar and 50ºC). This was coherent with what can be found in literature,
270 since SC-CO_2 inactivation treatments have proven to be more effective in simple media

than in complex and, in addition, oil is known to protect the microorganisms from different external stresses, including SC-CO_2 [14,21].

Effect of the type of microorganism

As for the resistance of the different microorganisms studied to the SC-CO_2 treatments, significant ($p<0.05$) differences were found, *A. niger* being the most resistant of all three microorganisms. On the contrary, very slight differences were found between *E. coli* and *B. diminuta*. At 50ºC and 350 bar, 55 min were required to achieve the complete inactivation of *A. niger* (6.8 log-cycles), while around 10 min were needed for *E. coli* or *B. diminuta* (around 8 log-cycles). This observation was coherent with the fact that fungal spores are more resistant to SC-CO_2 treatments than vegetative bacteria [23], probably due to their different and more resistant structure. Fungal spores are composed of a multi layered and highly dehydrated structure, which could restrain the CO_2 dissolution and penetration into the spore. Moreover, the structure of *A niger* spores, in particular, include a layer of melanin, which is believed to be related to a higher resistance to environmental stresses [36]. Similarly, Wu et al. [37] achieved a reduction of 4.3 log-cycles for *E. coli* at 78 bar, 35ºC and in 30 min, while only an inactivation of 2.1 log-cycles was obtained for *Absidia coerulea* spores.

3.1.2. SC-CO_2 + HPU microbial inactivation

The combined SC-CO_2 + HPU inactivation kinetics of *E. coli* (A), *B. diminuta* (B) and *A. niger* (C) are shown in Fig. 2. As in the SC-CO_2 kinetics (Fig. 1), the experimental variability was high due to the aforementioned factors and the additional variability related to the behaviour of the HPU transducer under supercritical conditions. The Weibull model fitted the experimental data satisfactorily (Fig. 2), with a R^2 value higher than 0.95 and a RMSE lower than 0.59 (Table 2). In addition, every n value in the treatments with HPU (Table 2) was lower than 1 (from 0.26 to 0.56), which indicated that the shape of every curve was concave upward.

Effect of pressure and temperature

The higher the pressure and temperature, the higher the level of inactivation of the SC-CO_2 + HPU treatments for *B. diminuta* and *A. niger*. However, only the temperature had a significant ($p<0.05$) effect on the inactivation, as also occurred in the SC-CO_2 treatments. With respect to *E. coli* (Fig. 2A), in order to achieve complete inactivation (7.9 log-cycles), raising the temperature from 35 to 50ºC meant that 2.5 min (from 5.5 to 3 min) less were needed, on average; however, only 0.5 min (from 4.5 to 4 min) less were needed when the pressure is raised from 100 to 350 bar. On the other hand, on

305 average, the time required to achieve the complete inactivation of *B. diminuta* (8.1 log-
306 cycles), was shortened from 12 to 6.5 min by raising the temperature from 35 to 50ºC
307 and from 15 to 3.5 min when the pressure was increased from 100 to 350 bar (Fig. 2B).
308 The effect of the temperature was also revealed by the time needed for complete
309 inactivation (t_x) calculated by the Weibull model (Table 2). As an example, for *A. niger*,
310 $t_{6.8}$ was shortened on average from 63.4 min to 23.1 min when the temperature was
311 raised from 50 to 60ºC. Contrary to the results of the present study, some authors found
312 that the increase in the pressure and temperature in the SC-CO_2 + HPU treatments did
313 not affect the microbial inactivation rate, probably because the marked effect of cavitation
314 masked the effects of the other processing conditions [12,17,38]. However, Gomez-
315 Gomez et al. [21] found that the higher the pressure and temperature, the higher the
316 level of SC-CO_2 + HPU inactivation for *E. coli* and *B. diminuta* in oil-in-water emulsions.

317 Effect of HPU and the type of microorganism

318 As in the SC-CO_2 treatments, significant ($p<0.05$) differences were found as regards the
319 resistance of the different microorganisms to the combined SC-CO_2 + HPU treatment.
320 *A. niger* was the most resistant, while *E. coli* and *B. diminuta* showed similar resistance.
321 In fact, the complete inactivation of *E. coli* and *B. diminuta* was achieved in less than 18
322 min, even at low temperatures and pressures (100 bar and 35ºC), whereas for *A. niger*,
323 at least 35 min were required to obtain complete inactivation at the same pressure (100
324 bar) and higher temperatures (50-60ºC), which was considered too long a treatment for
325 industrial applications. The use of a higher pressure (350 bar) and 60ºC was necessary
326 to achieve the complete inactivation of *A. niger* in a shorter time (10 min), which could
327 be considered a reasonable industrial processing time.

328 HPU significantly ($p<0.05$) intensified the SC-CO_2 treatments for all the microorganisms
329 considered in the present study, the effect being milder in the case of *A. niger* spores
330 than for *E. coli* and *B. diminuta* (on average, the increase in the inactivation when HPU
331 was used was 1.3, 4.8 and 3.4 log-cycles, respectively). For instance, as observed in
332 Figs. 1 and 2, the time needed for the complete inactivation of *E. coli* and *B. diminuta* at
333 50ºC and 350 bar was shortened by 19 min when HPU was applied; in the case of
334 *A. niger,* on the other hand, the process was shortened by only 5 min. Thus, the
335 application of HPU reduced the calculated t_x, on average; from 59.1 to 24.8 min (Tables
336 1 and 2). HPU is known to increase the mass and heat transfer under SC-CO_2 conditions
337 [39,40] and, consequently, to enhance both the CO_2 solubilisation in the media and
338 penetration inside the microbial cells [41]. Moreover, HPU could cause cracked or
339 damaged cell walls due to the effects of cavitation [38].

Several authors [12,38,42] also studied the application of HPU during the SC-CO_2 treatments of vegetative cells in liquid media and found that HPU drastically increased the inactivation level.

The inactivation of different microorganisms depended not only on the effect of external stresses, but also on the cell size and morphology [43,44]. Ortuño et al. [17] related the degree of cavitation to the size of the vegetative cells since they observed a stronger effect of cavitation on *S. cerevisiae* (8–10 μm) than on *E. coli* and assumed that there was a greater likelihood of the cavitation bubbles affecting the cell structure when the cells are larger. However, in the present study, there was probably not a big enough difference between the sizes of the *E. coli* and *B. diminuta* cells (a difference of less than 2 μm) to appreciate significant differences in the SC-CO_2 + HPU inactivation between these microorganisms, as also reported by Gomez-Gomez et al. [21]. In the case of *A. niger* spores, the cell size (around 4 μm [45]) is larger than in *E. coli* and *B. diminuta*. However, it is known that molds are generally more resistant to cavitation than vegetative cells because of the differences between the cell wall structure of species [46]. In particular, *A. niger* spores might be more resistant to cavitation due to the greater rigidity of its cell wall due to the presence of melanin [36].

3.2. Analysis of the ultrastructure of microbial cells inactivated by SC-CO_2 + HPU

CryoFESEM and TEM images of the microbial cells after and before the SC-CO_2 + HPU treatment are presented in Figs. 3 and 4, respectively.

The untreated *E. coli* cells showed an intact, well-defined and characteristic rod-shaped structure with a smooth surface (Fig. 3A). The cell wall and the cell membrane presented defined boundaries and were intact, enclosing the cytoplasmic content. In addition, the cell wall can be seen to be attached to the cell membrane (Fig. 4A). As regards the intracellular space, it is observed to be completely and uniformly filled with the cytoplasm (Fig. 4A), with no signs of cytoplasm observed outside the cells. On the contrary, the *E.coli* cells treated with SC-CO_2 + HPU lost their shape (Figs. 3B and 4B), appeared agglomerated and some of them merged, losing their individuality (Fig.3B). The cell walls and membranes were seen to be blurred (Fig. 4B), which indicated that they were partly disintegrated. Inside the cell, empty regions were observed (Fig. 4B), which could be due to a large amount of cytoplasmic content released by the effect of the treatment. This is indicated by the stains being found in the extracellular region (Fig. 4B). Ortuño et al. [17] observed important morphological changes in *E. coli* cells treated with SC-CO_2 + HPU at 350 bar, 36ºC for 5 min. The cytoplasmic space presented big empty regions with an

375 aggregated and unevenly distributed cytoplasm. In addition, the cell wall and membrane
376 appeared disintegrated. On the contrary, after a SC-CO_2 treatment under the same
377 conditions, *E. coli* cells only presented slight changes.
378 *B. diminuta* cells also presented a clear rod-shaped structure with a smooth surface
379 when untreated (Fig. 3C). The cell walls and membranes were intact and well-defined,
380 (Figs. 3C and 4C) enclosing the cytoplasm, which was uniformly distributed in the whole
381 intracellular space (Fig. 4C). Thus, the SC-CO_2 + HPU treatment caused different
382 alterations in the bacteria morphology. The surface appeared irregular with roughness
383 and wrinkles and some bacteria were found merged together (Fig. 3D). The cell walls
384 and membranes were undefined (Fig. 4D), indicating that they were damaged. In
385 addition, empty regions in the intracellular space were observed (Fig. 4D), indicating that
386 a great amount of cytoplasmic content was lost with the treatment. No studies were found
387 analyzing the ultrastructure of *B. diminuta* cells treated with either SC-CO_2 or SC-CO_2 +
388 HPU.
389 *B. diminuta* and *E. coli* cells were both similarly affected by the SC-CO_2 + HPU treatment.
390 As several authors [15,17,47] observed in the case of SC-CO_2 treatments, the
391 morphological structure of the microbial cells, including the cell wall, generally remained
392 almost intact or with only minor alterations, while changes in the intracellular structure,
393 such as an uneven distribution of the cytoplasm, were found. Consequently, it could be
394 stated that the inactivation effect was probably due to an increase in the permeability of
395 the cells exerted by SC-CO_2, instead of to the rupture of the cell wall and membrane. By
396 contrast, the application of HPU could crack or damage cell walls, severely affecting their
397 morphological integrity [17], as observed in the images of the present study (Figs.3 and
398 4).
399 In the case of *A. niger* spores, untreated cells presented a globular shape and a spiny,
400 wrinkled surface (Fig. 3E). The cell wall and membrane showed defined boundaries and
401 uniform thickness (Fig.4E). In addition, in the intracellular region, the organelles were
402 clearly visible and well distributed in a dense cytoplasm (Fig.4E). In contrast, *A. niger*
403 spores treated with SC-CO_2 + HPU demonstrated significant alterations. The shape of
404 the cell changed, since it appeared shrunken and squashed and most of the cells
405 presented a clear cleft in the center (Figs. 3F and 4F). After the treatment, the cell wall
406 appeared much thinner than that of the untreated spores, showing an uneven width with
407 some dissolved areas (Fig. 4F). Moreover, the cell membrane was also thinner and
408 presented an uneven thickness (Fig. 4F). As to the inner region of the spore, it was
409 almost empty due to leakages of cytoplasmic content (Fig. 4F) and the organelles were
410 not visible, indicating that they were completely destroyed after the treatment. Only a
411 small, darker region was found in the cytoplasm, which could be due to the precipitation

412 or aggregation of internal cell components (Fig. 4F). Although no studies were found
413 assessing the ultrastructural changes in *A. niger* spores after SC-CO_2 + HPU treatments,
414 Noman et al. [31] and Efaq et al. [48] observed the morphology of *A. niger* spores treated
415 with SC-CO_2 at 300 bar, 75 °C for 90 min. These authors found completely damaged
416 and deformed spores, with disintegrated cell wall and membrane. Nevertheless, an
417 extremely long process time was used in those studies (90 min) and a higher
418 temperature than in the present study (75ºC vs 60ºC).
419 In general terms, an ultrastructural analysis revealed that for the three microorganisms
420 involved in the present study, the combined SC-CO_2 + HPU treatment damaged the cell
421 walls and affected the permeability of cell membranes, which led to changes in the cell
422 morphology and the release of cytoplasmic content and, consequently, cell death.
423

424 ## 4. Conclusions

425 Ultrasonic application has proven to be an effective way of shortening the inactivation
426 time in SC-CO_2 treatments of the microorganisms studied (two vegetative bacteria and
427 one fungal spore). The higher the pressure and the temperature, the greater the
428 inactivation of *B. diminuta* and *A. niger*. However, it was only the temperature that had a
429 significant effect on *E.coli*. The results obtained confirmed the more marked effect that
430 cavitation has on the SC-CO_2 inactivation of vegetative bacteria, compared to fungal
431 spores.
432 The ultrastructural analysis illustrated the external morphology and intracellular structure
433 of bacteria (*E. coli* and *B. diminuta*) and fungal spores (*A*. *niger*), which definitively
434 contributed to a better understanding of the effects of the SC-CO_2 + HPU treatments.
435 SC-CO_2 + HPU-treated cells presented a deformed shape, partly disintegrated walls and
436 membranes and a leakage of cytoplasmic content, which explains the effectiveness of
437 the SC-CO_2 + HPU treatments for microbial inactivation purposes. Thus, regardless of
438 the type of microorganism (vegetative bacteria or fungal spore) and its different cell wall
439 structure and composition, the SC-CO_2 + HPU treatment caused structural damage
440 leading to cell death. Further research into the ultrastructure of other fungal or bacterial
441 spores in different complex media is required to elucidate the exact inactivation
442 mechanisms of the SC-CO_2 + HPU technology.
443

444 **Acknowledgement**

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446

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621

- HPU increased the SC-CO_2 inactivation of *E. coli*, *B. diminuta* and *A. niger*.
- The higher the pressure and temperature, the greater the SC-CO_2+HPU inactivation.
- *A. niger* spores were found to be more resistant than vegetative bacteria.
- Microscopy analysis revealed significant morphological changes to the microbial cells.
- SC-CO_2 + HPU inactivation was effective for all the microorganisms despite their cell structure.

Fig. 1. Inactivation kinetics of *E. coli* (A), *B.diminuta* (B) and *A. niger* spores (C) in water treated with SC-CO_2. Experimental data (discrete points) and Weibull model (continuous and dashed lines). Error bars show the experimental variability.

Fig. 2. Inactivation kinetics of *E. coli* (A), *B.diminuta* (B) and *A. niger* spores (C) in water treated with SC-CO_2 + HPU. Experimental data (discrete points) and Weibull model (continuous and dashed lines). Error bars show the experimental variability.

Fig. 3. CryoFESEM photographs of *E. coli* (A and B), *B. diminuta* (C and D) and *A. niger* (E and F) untreated and treated by SC-CO_2 + HPU.

Fig. 4. TEM photographs of *E. coli* (A and B), *B. diminuta* (C and D) and *A. niger* (E and F) untreated and treated by SC-CO_2 + HPU.

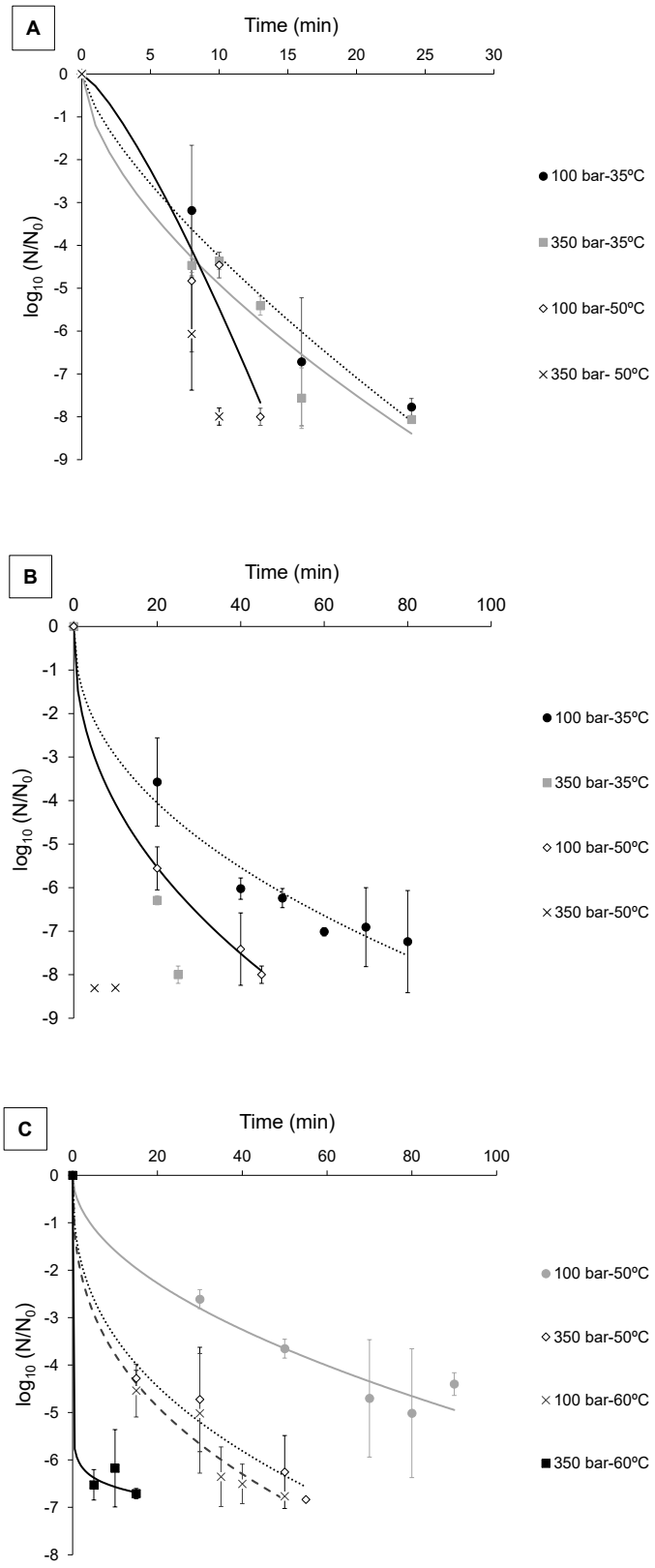
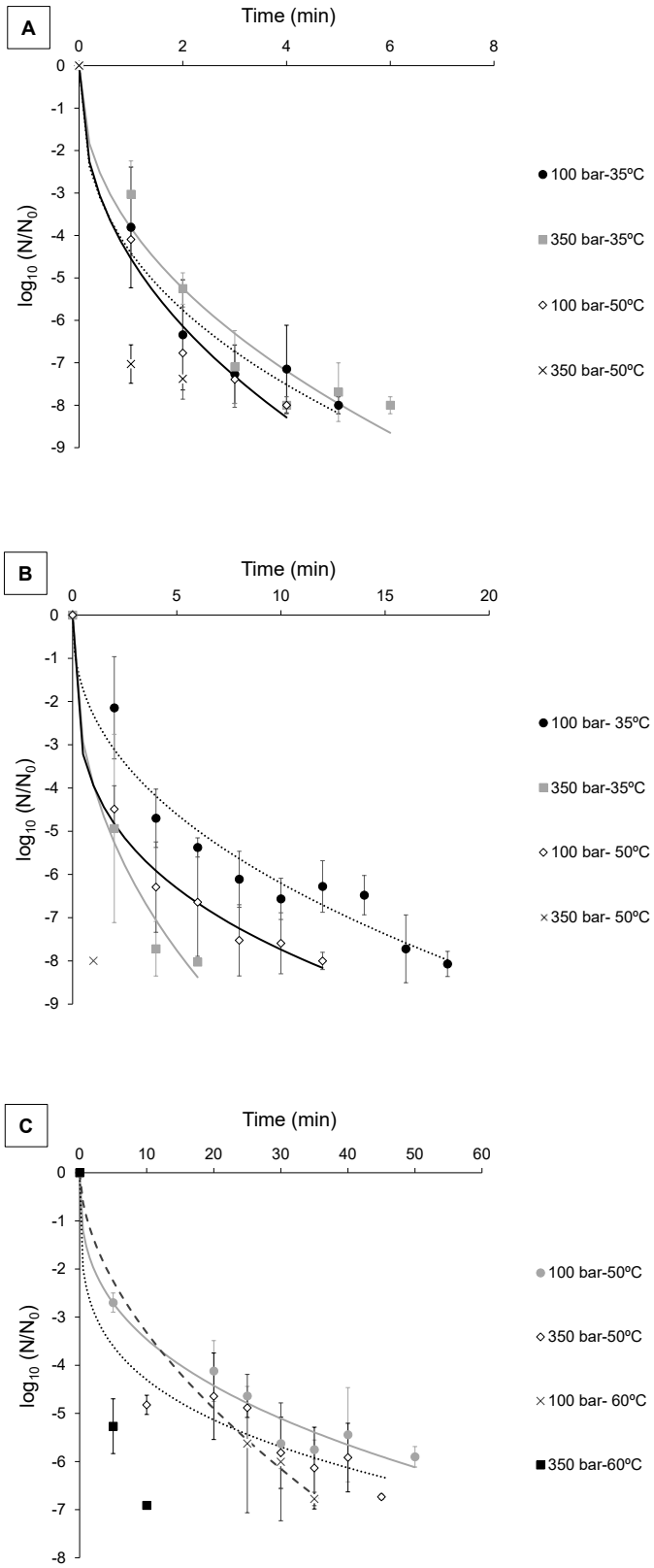

A
Time (min)
0
5
10
15
20
25
30
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
log10 (N/N0)
100 bar-35ºC
350 bar-35ºC
100 bar-50ºC
350 bar- 50ºC
B
Time (min)
0
20
40
60
80
100
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
log10 (N/N0)
100 bar-35ºC
350 bar-35ºC
100 bar-50ºC
350 bar-50ºC
C
Time (min)
0
20
40
60
80
100
0
-1
-2
-3
-4
-5
-6
-7
-8
log10 (N/N0)
100 bar-50ºC
350 bar-50ºC
100 bar-60ºC
350 bar-60ºC

Fig. 1.


A
Time (min)
0
2
4
6
8
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
log10 (N/N0)
100 bar-35ºC
350 bar-35ºC
100 bar-50ºC
350 bar-50ºC
B
Time (min)
0
5
10
15
20
log10 (N/N0)
100 bar- 35ºC
350 bar-35ºC
100 bar- 50ºC
350 bar- 50ºC
C
Time (min)
0
10
20
30
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log10 (N/N0)
100 bar-50ºC
350 bar-50ºC
100 bar- 60ºC
350 bar-60ºC

Fig. 2.

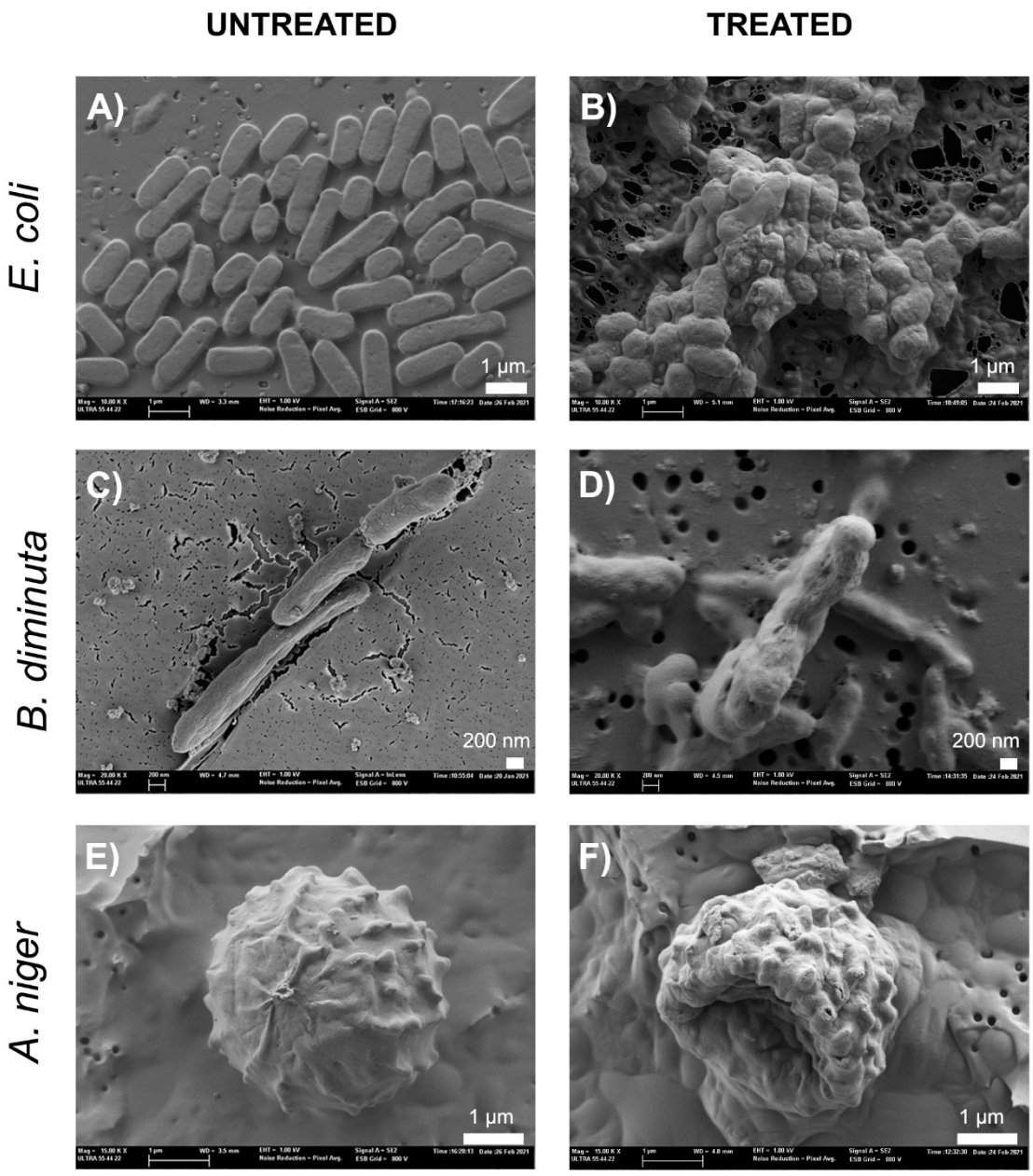
UNTREATED
TREATED
E. coli
B. diminuta
A. niger
A)
B)
C)
D)
E)
F)
1 µm
1 µm
200 nm
200 nm
1 µm
1 µm

Fig. 3.

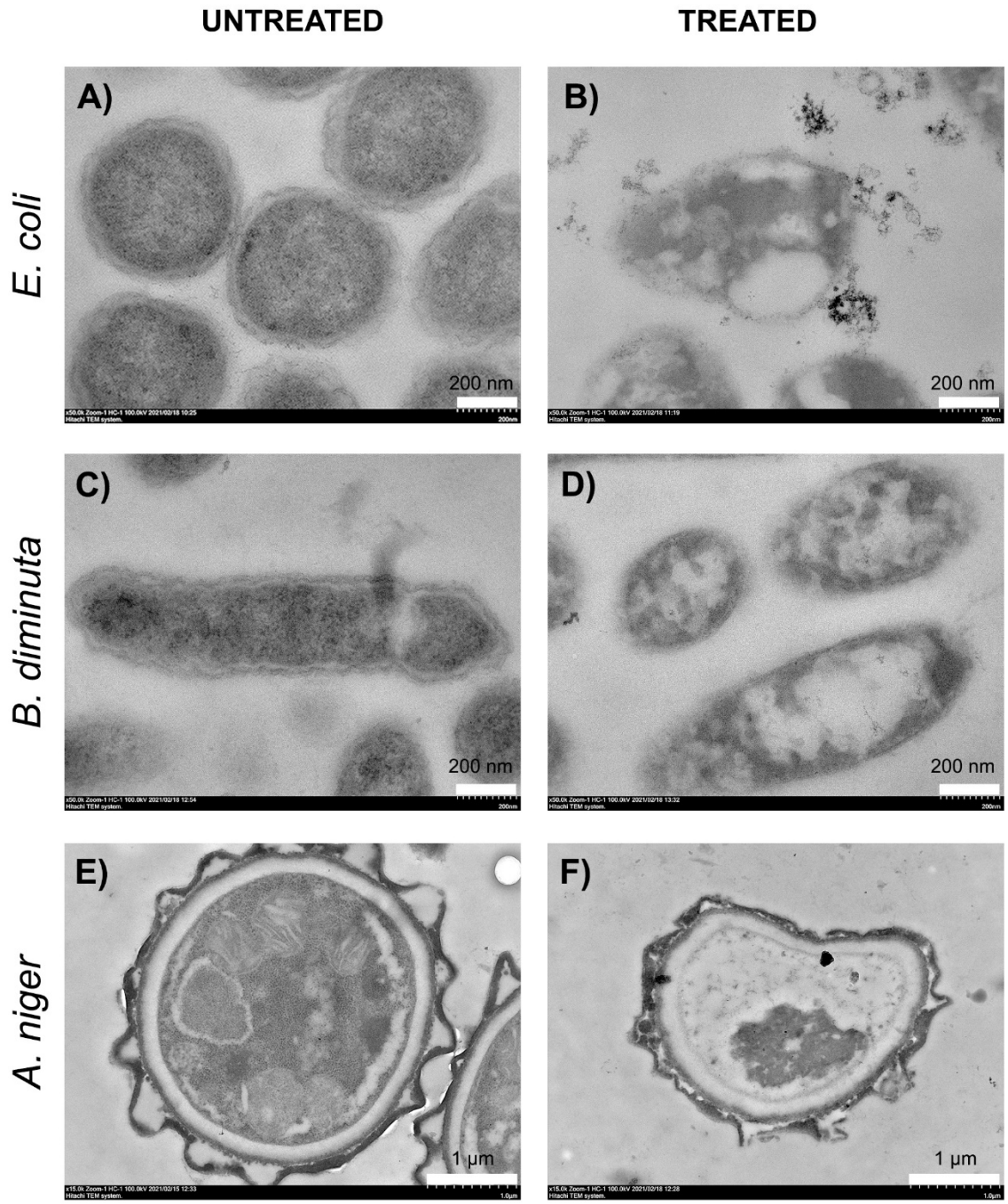
UNTREATED
TREATED
E. coli
B. diminuta
A. niger
A)
B)
C)
D)
E)
F)
200 nm
200 nm
200 nm
200 nm
1 µm
1 µm

Fig. 4.

Table 1. Fitting of Weibull model to SC-CO_2 inactivation kinetics in distilled water. Parameters (b and n), total time for complete inactivation of *E. coli* ($t_{7.9}$; 7.9 log-cycle reduction) , *B. diminuta* ($t_{8.1}$; 8.1 log-cycle reduction) and *A. niger* ($t_{6.8}$; 6.8 log-cycle reduction) and statistical parameters (R^2 and RMSE). Average values and standard errors (in brackets).

Microorganism	Pressure (bar)	Temperature (ºC)	b (min^{-n})	n	$t_{7.9/\ 8.1/\ 6.8}$ (min)	R^2	RMSE
E. coli	100	35	0.84 (0.38)	0.72 (0.16)	22.5	0.97	0.46
E. coli	350	35	1.20 (0.40)	0.61 (0.12)	22.0	0.95	0.52
E. coli	100	50	0.28 (0.33)	1.29 (0.48)	13.3	0.92	0.64
E. coli	350	50	*	*	*	*	*
B. diminuta	100	35	1.06 (0.30)	0.45 (0.07)	91.8	0.98	0.33
B. diminuta	350	35	*	*	*	*	*
B. diminuta	100	50	1.48 (0.13)	0.44 (0.03)	47.6	0.99	0.07
B. diminuta	350	50	*	*	*	*	*
A. niger	100	50	0.48 (0.26)	0.52 (0.13)	163.7	0.96	0.31
A. niger	350	50	1.40 (1.50)	0.38 (0.27)	64.0	0.98	0.28
A. niger	100	60	1.62 (0.48)	0.37 (0.08)	48.3	0.98	0.32
A. niger	350	60	5.95 (0.40)	0.04 (0.02)	22.1	0.99	0.12

* Insufficient experimental data for model fitting

Table 2. Fitting of Weibull model to SC-CO_2 + HPU inactivation kinetics in distilled water. Parameters (b and n), total time for complete inactivation of *E. coli* ($t_{7.9}$; 7.9 log-cycle reduction) , *B. diminuta* ($t_{8.1}$; 8.1 log-cycle reduction) and *A. niger* ($t_{6.8}$; 6.8 log-cycle reduction) and statistical parameters (R^2 and RMSE). Average values and standard errors (in brackets).

Microorganism	Pressure (bar)	Temperature (ºC)	b (min $^{-n}$)	n	$t_{7.9/\ 8.1/\ 6.8}$ (min)	R^2	RMSE
E. coli	100	35	4.42 (0.43)	0.38 (0.08)	4.6	0.97	0.44
E. coli	350	35	3.82 (0.49)	0.46 (0.09)	4.9	0.95	0.59
E. coli	100	50	4.55 (0.40)	0.43 (0.08)	3.6	0.98	0.37
E. coli	350	50	*	*	*	*	*
B. diminuta	100	35	2.31 (0.34)	0.43 (0.06)	18.5	0.95	0.49
B. diminuta	350	35	3.93 (0.69)	0.42 (0.12)	8.0	0.98	0.41
B. diminuta	100	50	3.94 (0.26)	0.29 (0.03)	12.0	0.99	0.23
B. diminuta	350	50	*	*	*	*	*
A. niger	100	50	1.53 (0.27)	0.35 (0.05)	70.9	0.98	0.28
A. niger	350	50	2.39 (0.56)	0.26 (0.07)	55.8	0.96	0.37
A. niger	100	60	0.91 (0.50)	0.56 (0.16)	36.3	0.99	0.09
A. niger	350	60	*	*	*	*	*

* Insufficient experimental data for model fitting