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Non-thermal pasteurization of lipid emulsions by combined supercritical carbon dioxide and high-power ultrasound treatment

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ABSTRACT

Supercritical carbon dioxide (SC-CO_2) is a novel method for food pasteurization, but there is still room for improvement in terms of the process shortening and its use in products with high oil content. This study addressed the effect of high power ultrasound (HPU) on the intensification of the SC-CO_2 inactivation of *E. coli* and *B. diminuta* in soybean oil-in-water emulsions. Inactivation kinetics were obtained at different pressures (100 and 350 bar), temperatures (35 and 50 °C) and oil contents (0, 10, 20 and 30%) and were satisfactorily described using the Weibull model. The experimental results showed that for SC-CO_2 treatments, the higher the pressure or the temperature, the higher the level of inactivation. Ultrasound greatly intensified the inactivation capacity of SC-CO_2, shortening the process time by approximately 1 order of magnitude (from 50 to 90 min to 5–10 min depending on the microorganism and process conditions). Pressure and temperature also had a significant ($p < 0.05$) effect on SC-CO_2 + HPU inactivation for both bacteria, although the effect was less intense than in the SC-CO_2 treatments. *E. coli* was found to be more resistant than *B. diminuta* in SC-CO_2 treatments, while no differences were found when HPU was applied. HPU decreased the protective effect of oil in the inactivation and similar microbial reductions were obtained regardless of the oil content in the emulsion. Therefore, HPU intensification of SC-CO_2 treatments is a promising alternative to the thermal pasteurization of lipid emulsions with heat sensitive compounds.

1. Introduction

In the food and pharmaceutical industry, microbial inactivation is essential for the purposes of obtaining safe and stable products; to this end, thermal treatment has traditionally been the most widespread technique. However, the use of heat involves some undesirable effects, such as changes in the physico-chemical and organoleptic properties or in the nutrient content. Some of the most common changes brought about by high temperatures are related to colour, taste, flavour, texture, the loss of vitamins or the denaturation of proteins. For that reason, alternative non-thermal methods, in which mild temperatures can be applied, have been studied in recent years [1]. Of these technologies, the use of high-voltage pulsed electric fields [2,3], high hydrostatic pressure [4] or supercritical fluids [5,6] could be cited.

A supercritical fluid is a substance which is above its critical temperature and pressure, shows a high density (similar to that of liquids), has low viscosity (like a gas) and a zero surface tension [7]. Different compounds can be used in their supercritical state, although carbon dioxide is frequently chosen in the food and pharmaceutical microbial inactivation applications. Supercritical carbon dioxide (SC-CO_2) diffuses easily through the microbial cells, causing a pH decrease that modifies their membrane. This fact leads to the extraction of intracellular components that are vital for the cell and eventually promotes the death of the microorganism [8]. Carbon dioxide has the advantage over other compounds of being non-toxic and inexpensive; moreover, its critical temperature (31 °C) and pressure (72.8 bar) are easy to reach. The application of SC-CO_2 has proven to be a satisfactory non-thermal pasteurization technique, which contributes to better preserving the nutrients and organoleptic properties [9]. Ferrentino et al. [10] treated apple pieces in syrup with SC-CO_2 and studied the inactivation of the microorganisms naturally present in the product (mesophilic microorganisms, total coliforms, yeasts and moulds) and polyphenol oxidase enzyme. These authors showed that pH, total acidity, and ascorbic acid content were not affected by the treatment and remained stable for 60 days at 25 °C. Additionally, no noticeable differences in the colour were observed for the treated apples compared

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to the untreated ones.

However, in some cases, long processing times or a high pressure or temperature are needed to guarantee both the product's safety and stability [5]. In order to obtain the required lethality with shorter or milder processes, previous studies assessed the benefits of combining SC-CO_2 with other non-thermal techniques, such as pulsed electric fields (PEF) or high power ultrasound (HPU). Spilimbergo et al. [11] found a synergistic effect when pre-treating *E. coli, S. aureus* and *B. cereus* with PEF before the SC-CO_2 inactivation. *E. coli* and *S. aureus* treated at 25 kV/cm and 10 pulses and subsequently processed by SC-CO_2 at 200 bar and 34 °C for 10 min, were completely inactivated. *B. cereus* spores, although shown to be more resistant, were reduced in 3 log-cycles by sequential PEF (25 kV/cm, 20 pulses) and SC-CO_2 treatment (200 bar, 40 °C for 24 h).

The effect of HPU on microbial inactivation is due to the compression and decompression cycles which generate different phenomena. Thus, mechanical stress caused by ultrasound may induce the violent collapse of air bubbles, a phenomenon known as cavitation. This causes locally intense high temperatures and pressures, with significant shearing and turbulence effects [12], which can affect microbial integrity. However, very high intensities are required for pasteurization when using only ultrasound and a combination with temperature (thermosonication) is often needed. There has been a proven synergistic effect on the inactivation of different microorganisms when simultaneously combining SC-CO_2 and HPU [13,14]. In particular, this combined technology has been used for the inactivation of inoculated *Salmonella enterica* and microbiota in coconut water [15] or *Saccharomyces cerevisiae* in apple juice [16], among others. The application of HPU enhances the contact between SC-CO_2 and the surface of the cells and accelerates the solubilisation rate of CO_2 in the liquid [15]. Due to the vigorous stirring of the medium caused by HPU, the mass transfer between the inner cells and the surrounding SC-CO_2 is also enhanced. Additionally, the cavitation created by HPU causes cell wall damage, which facilitates SC-CO_2 penetration into the cell, causing a drastic drop in the intracellular pH and the extraction of internal components [6]. Furthermore, as the inactivation time is shortened by HPU application, it leads to more cost-effective and environmentally-friendly industrial operations.

Pressure, temperature and treatment time are keyfactors for the microorganism survival rate. In addition, microbial inactivation is also greatly affected by the nature of the suspending media [17]. Whilst several authors observed marked protective effects against external stress on microbial cells in complex physicochemical systems, no protective effect was found in simple solutions [18]. Factors, such as fat, sugar, salt and water contents, or the pH of the suspending medium, may modulate the microbial sensitivity to SC-CO_2 inactivation [17]. Although significant progress has been made in the non-thermal pasteurization of liquid products, the combination of SC-CO_2 and HPU has mostly been tested in fat-free media: products such as juices, with sugars as the main dissolved solutes. In this context, the inactivation of the microbiota in red grapefruit juice [19] and the inactivation of *S. cerevisiae* in YPD Broth, apple and orange juice [20] have been reported. The pasteurization of lipid emulsions has gained interest due to its multiple application in the food, pharmaceutical (e.g. for parenteral nutrition) and cosmetic industries [21,22]. This study, therefore, addressed the feasibility of the pasteurization of soybean oil emulsions using a combination of SC-CO_2 and HPU. The effect of the combined treatment on *Escherichia coli* and *Brevundimonas diminuta* inactivation was assessed and compared to both the SC-CO_2 treatment alone and to a conventional thermal treatment. Moreover, the effect of the fat content in the medium on the microbial resistance to SC-CO_2 and SC-CO_2 + HPU treatments was also evaluated.

2. Materials and methods

2.1. Microorganisms

The lyophilized strains of *Eschericha coli* CECT 101 and *Brevundimonas diminuta* CECT 313 used in this study were obtained from the Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, Spain. *E. coli* is a facultative anaerobic gram-negative bacteria with a size of ~1 × 3 μm [23], very common in contaminated food and pharmaceutical products. *B. diminuta* is an aerobic gram-negative bacteria. It is an opportunistic bacteria, considered of minor clinical importance, used to test the porosity of pharmaceutical grade filters of 0.2 μm because of its small size [24]: typically of ~0.3 × 0.6 μm [25]. The inactivation kinetics of different microorganisms depend not only on the effect of external stresses, such as heat, but also on the membrane structure and the cell size and morphology [26]. Although *B. diminuta* is not considered a significant pathogen and, in general, its virulence is low [27]; it was chosen in this study to compare the resistance to the inactivation treatments considered on microorganisms of differing cell sizes.

2.2. Preparation of the starter culture

Prior to each inactivation treatment, the cultures were refreshed from stock to agar plates and incubated at the suitable temperature and time (37 °C and 24 h for *E. coli* and 30 °C and 48 h for *B. diminuta*). A single fresh colony of each microorganism was inoculated in 50 mL of nutrient broth (Nutrient Broth, Scharlab, Spain) and grown overnight (18–24 h) at 37 °C for *E. coli* and 30 °C for *B. diminuta*, using an incubation chamber (J.P. Selecta, Model 3000957, Barcelona, Spain) and an orbital shaker at 120 rpm (J.P. Selecta, Rotabit Model 3000957, Barcelona, Spain).

2.3. Preparation of bacterial suspensions in the stationary phase

In order to establish the time at which *E. coli* and *B. diminuta* reached the stationary phase, the growth curves were determined (Fig. 1). For that purpose, 50 μL of the overnight starter culture were transferred to a new growth medium and it was incubated at the right temperature for every microorganism. During the growth, two processes were performed: plating on Plate Count Agar (data not shown) and the measurement of optical density at 600 nm (OD_{600}), using a UV–visible spectrophotometer (Thermo Electron Corporation, Helios Gamma Model, Unicam, England). All the measurements were taken in triplicate. Thereby, bacterial suspensions used to inoculate the lipid emulsions were grown 14 h for *E. coli* (37 °C) and 36 h (30 °C) for *B. diminuta* (Fig. 1), to assure that microorganisms reached the stationary phase.

2.4. Preparation of lipid emulsions

The treated samples were emulsions with different oil contents (10, 20 and 30%). Distilled water was used as the control treatment medium (0% soybean oil). Prior to each treatment, the bacterial suspension in the stationary phase was added (5 mL) to the autoclaved emulsion (60 mL) to reach a cell concentration of 10^7–10^8 CFU/mL.

The oil-in-water emulsions were prepared in three stages: mixing with an Ultra-Turrax, sonication and homogenization. Firstly, the lipid phase, formed by soybean oil and egg phospholipid, as the emulsifying agent, was mixed using an Ultra-Turrax (IKA T25 Digital; tool S25N - 25G, Staufen, Germany) at 14000 rpm for 2 min, 10200 rpm for 4 min and 10600 rpm for 4 min. Subsequently, the lipid phase was slowly added to the water phase (deionized water), while being mixed using the Ultra-Turrax at 14000 rpm. Afterwards, samples were sonicated for 5 min with an ultrasound system UP400S (Hielscher, Teltow, Germany), using the H22 sonotrode. Finally, the product was

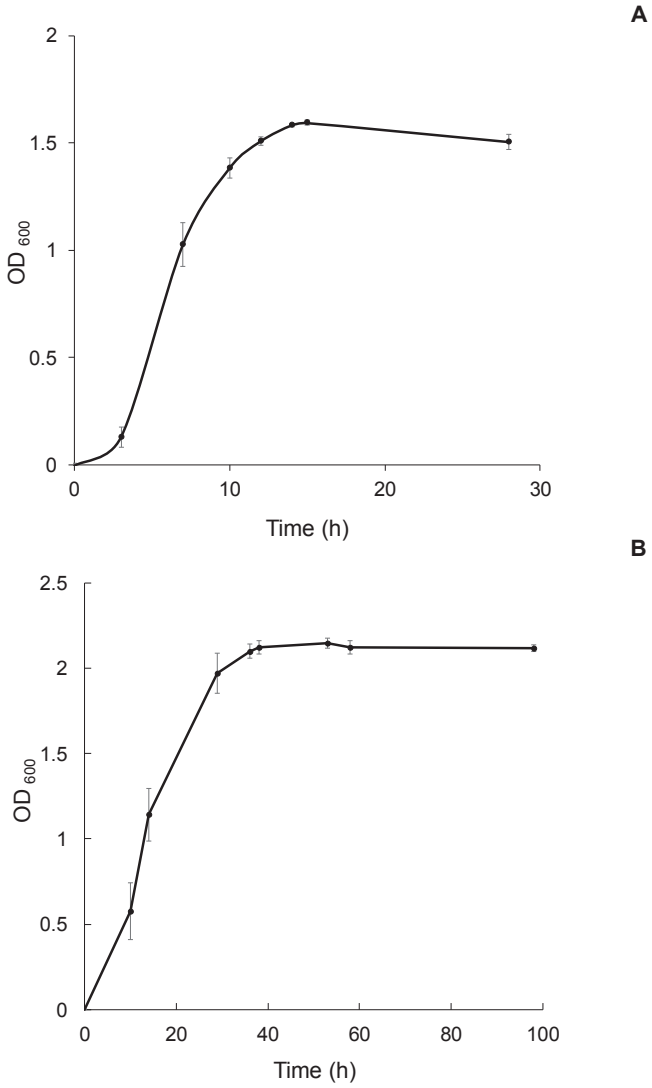

Fig. 1. Growth kinetics of *Escherichia coli* (A) and *Brevundimonas diminuta* (B) monitoring optical density measurements at 600 nm (OD).

homogenized in two stages (50 bar; 550 bar) with the GEA Niro Soavi homogenizer (PANDA Plus 2000, Parma, Italy).

2.5. Thermal treatment

The thermal treatments were performed at 50 ˚C in a temperature controlled water bath (1812, Bunsen, Madrid, Spain). 1.5 mL of sample (20% soybean emulsion at a concentration of 10^7–10^8 CFU/mL of *E. coli* or *B. diminuta*) were poured into borosilicate glass tubes of 8 mm in diameter and 70 mm in length (Fiolax, Germany). The tubes were taken from the bath after 50 min for *E. coli* and after 50 and 70 min for *B. diminuta* and cooled in ice for immediate analysis. The experiments were carried out in triplicate.

2.6. Supercritical fluids and high power ultrasound treatments

The inactivation treatments were performed in custom supercritical fluid lab-scale equipment designed and built by the research team for batch mode operation, which has already been described by Ortuño et al. [20]. The system (Fig. 2) consisted of an inactivation vessel made of stainless steel (5, Fig. 2) with a pressure gauge and a temperature probe, a CO_2 tank stored at room temperature (1, Fig. 2), a chiller reservoir kept at −18 ˚C (2, Fig. 2); a diaphragm metering pump (LDB, LEWA, Japan) to reach the desired pressure in the inactivation vessel (3, Fig. 2) and a thermostatic water bath (4, Fig. 2) to maintain the temperature of the process. The pressure of the vessel was measured with a pressure gauge, the temperature of the vessel (temperature of the treatment) was measured with a temperature probe (K type termopar), both installed in the inactivation vessel. The temperature of the water bath was measured with a pt100 sensor submerged in the bath. All pressure and temperature sensors were connected to digital controllers (E5CK, Omron, Hoofddorp, Netherlands). The controllers of the pressure and the temperature of the treatments were connected to the pump and the thermostatic water bath, respectively. Carbon dioxide was driven from the tank to the chiller reservoir. The liquid CO_2 was fed from the bottom of the reservoir into the vessel (600 mL internal volume) by the pump. Additionally, an ultrasound transducer was attached to the lid of the supercritical fluid vessel. The ultrasound system consisted of a high power ($> 1 W/cm^2$) piezoelectric transducer (6,Fig. 2) made up of two commercial ceramics (8, Fig. 2; 35 mm external diameter; 12.5 mm internal diameter; 5 mm thickness; resonance frequency of 30 kHz; ATU, Spain) and a sonotrode; an insulation system (polypropylene covered with Teflon; 7, Fig. 2) and a power generation unit (10, Fig. 2). The power was 50 W ± 5 W (I = 250 ± 10 mA; U = 220 ± 5 V), measured with a Digital Power Meter, Model WT210 (Yogogawa, Japan) and the frequency was 30 ± 2 kHz.

Five steps were required for each inactivation treatment: plant preparation (disinfection and heating), sample preparation, pressurisation, HPU connection (when needed) and sample extraction. Before every experimental run, the plant was disinfected (Disersey Detalled, Barcelona, Spain) for 5 min, afterwards, the inactivation vessel was rinsed twice with distilled water and once with autoclaved water. The sterile vessel was loaded with the inoculated soybean emulsion (65 mL) and immediately sealed and pressurized. The pressure set-point was reached in less than 5 min. For the combined SC-CO_2 + HPU, the ultrasound system was turned on when the required pressure in the vessel was reached. Throughout the process, temperature and pressure were maintained constant via the thermostatic bath and the pump, respectively. Samples of 2 mL were extracted during each treatment at different times (depending on the conditions of the process, at intervals of 1–10 min) using the sampling tube placed at the bottom of the inactivation vessel. The treated samples were cooled in ice to be immediately analysed.

Inactivation treatments of *E. coli* and *B. diminuta* in 20% oil emulsion were carried out at 100 and 350 bar, and 35 and 50 ˚C. The lowest pressure (100 bar) was chosen because it is close to the critical pressure (73.8 bar) and the highest (350 bar) for being a common pressure used in the SC-CO_2 inactivation studies. On the other hand, 35 ˚C was considered for being close to the critical temperature (31.2 ˚C) and 50 ˚C was selected as a higher temperature that has little thermal effect on the inactivation of the studied microorganisms. In order to study the effect of the oil content in the emulsions on microbial inactivation, emulsions of 10, 20 and 30% of oil and distilled water (0%) were used as the treatment media and conditions of 350 bar and 35 ˚C were selected. All of the treatments were performed with SC-CO_2 and with SC-CO_2 + HPU.

2.7. Microbiological analyses

The treated samples were collected in sterile tubes and the plate count technique was carried out to determine the viability of *E. coli* and *B. diminuta*. Depending on the expected count, appropriate serial dilutions were prepared with sterile distilled water. 100 µL of the dilution were spread on the surface of Plate Count Agar (Scharlab, Barcelona, Spain) in triplicate and incubated at the optimum growth temperature and time for the bacteria (24 h at 37 ˚C for *E. coli* and 48 h at 30 ˚C for *B. diminuta*). The initial microbial load in the sample was also determined following the same procedure.

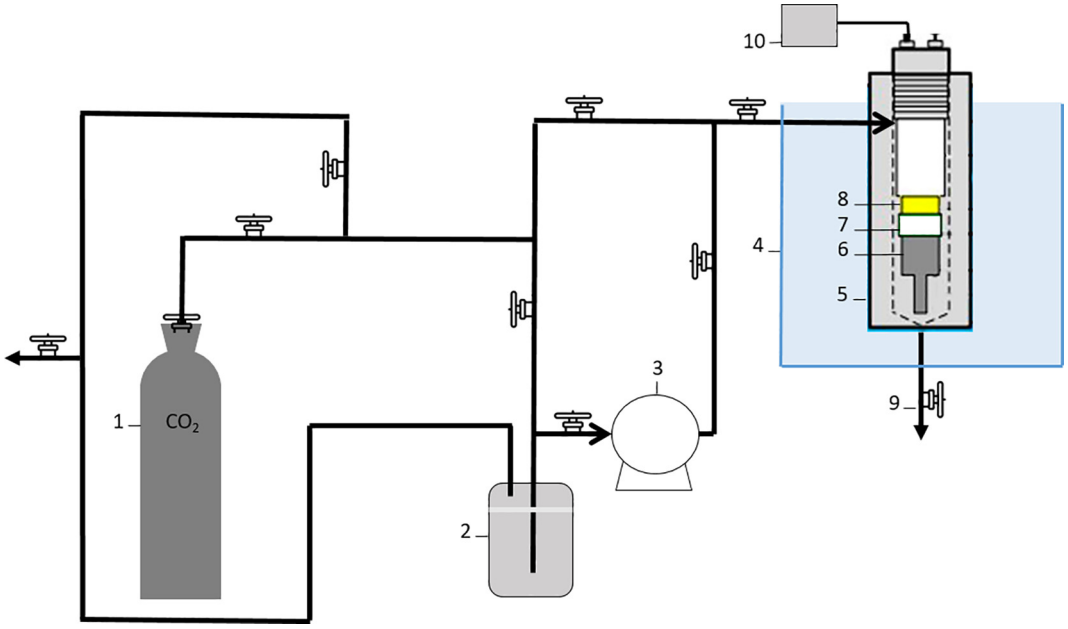

Fig. 2. Supercritical CO_2 treatment system. (1-CO_2 tank, 2-Reservoir, 3-Pump, 4-Bath, 5-Treatment vessel, 6-Transducer, 7-Insulation joint, 8-Ceramics, 9-Sample extraction, 10-Power Generation Unit.

2.8. Modelling

The inactivation kinetics of thermal and non-thermal treatments were described by several authors as first-order kinetics, assuming that microbial populations are homogeneous as regards treatment resistance [28,29]. Nevertheless, some microorganisms show more complex inactivation kinetics, presenting a downward concavity (shoulder) or an upward concavity (tail). Several non-linear models were proposed in order to describe this behaviour, the Weibull model being a simple and sufficiently robust one. Therefore, Weibull distribution was used in this study to describe the microbial inactivation kinetics of *E. coli* and *B. diminuta* (Peleg, 2006) (Eq. (1)).

$$\log_{10}\frac{N}{N_0} = -b\bullet t^n \quad (1)$$

where N_0 is the initial number of colonies of the sample, N the number of colonies in the treated sample at time t. The kinetic constants (b and n) of the model were calculated by minimizing the sum of squared differences between experimental and model predicted data using Solver Microsoft Excel™ tool. Parameter b is a rate parameter which indicates the speed of the microorganism inactivation and n is a fitting parameter that determines the shape of the kinetic curves and their deviation from linearity. When the value of n is higher than 1, the shape of the inactivation curve is concave-downward (shoulder). However, an n value lower than 1 corresponds to concave-upward curves (tailing). When n is equal to 1, the Weibull model conforms a first-order kinetics [30]. 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 of the model and the estimation accuracy.

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

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

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

2.9. Statistical analysis

The statistical package, Statgraphics Centurion XVI, was used to perform a general linear model (GLM) in order to evaluate the effect of both the treatment conditions (pressure and temperature) and the treatment media on the inactivation. Fisher's least significant difference (LSD) procedure was used to discriminate among the means with a 95.0% of confidence ($p < 0.05$). A multifactorial ANOVA was also used to analyse the parameters of the Weibull model.

3. Results and discussion

3.1. Effect of pressure, temperature and high-power ultrasound on microbial inactivation

3.1.1. Effect of pressure and temperature on the SC-CO_2 inactivation of *E. coli* and *B. diminuta*

Fig. 3. shows the inactivation of *E. coli* (A) and *B. diminuta* (B) in a 20% oil-in-water emulsion in SC-CO_2 at different pressures (100 and 350 bar) and temperatures (35 and 50 °C), compared to a conventional thermal treatment at 50 °C. A wide experimental variability was found in the inactivation treatments, which may be ascribed to variations in the microbial growth behaviour and pressure and temperature fluctuations. In general terms, the Weibull model satisfactorily described the SC-CO_2 inactivation kinetics at different pressures and temperatures, as shown in Fig. 3. The R^2 values were higher than 0.91 and the RMSE values were lower than 0.77, except for the *B. diminuta* kinetics at 350 bar and 35 °C ($R^2 = 0.86$ and RMSE = 0.83, Table 1). The thermal inactivation of *E. coli* at 50 min and 50 °C barely reached a reduction of 0.4 log-cycles. *B. diminuta* proved to be slightly more sensitive to heat than *E. coli since*, as shown in Fig. 3B, the thermal treatment led to a 0.9 log-cycle reduction.

Pressure had a significant ($p < 0.05$) effect on the inactivation of both *E. coli* and *B. diminuta*. Treatments at 350 bar were significantly ($p < 0.05$) more effective than at 100 bar for both 35 and 50 °C (Fig. 3). As an example, for *E. coli* at 50 min and 35 °C (Fig. 3A), the inactivation at 350 bar was 2.2 log-cycles higher than at 100 bar. The effect of the pressure was slightly more remarkable at 35 °C than at 50 °C (1.4 log-cycles of difference between 100 and 350 bar at 50 °C). High pressure is known to increase the solubility of CO_2 in the medium.

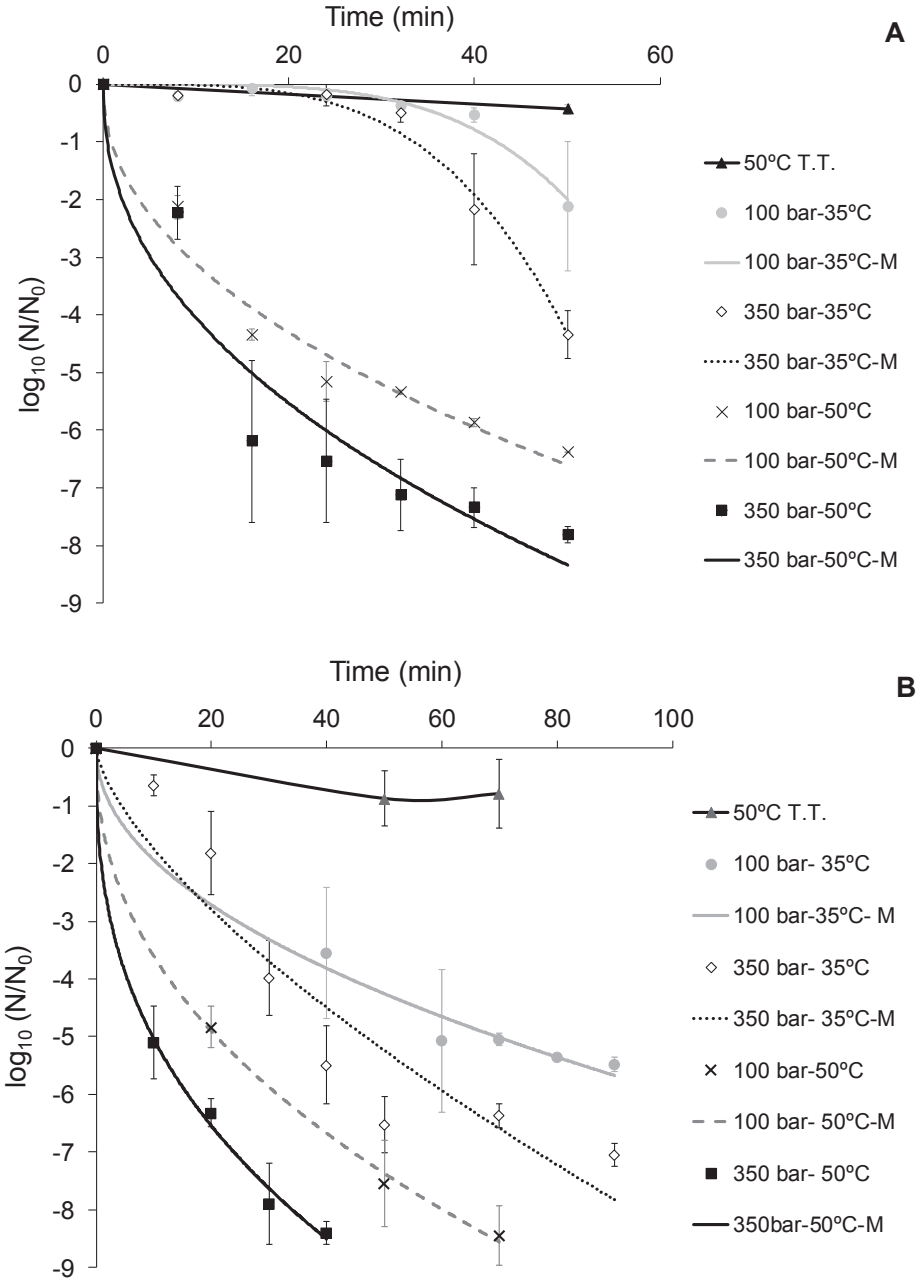

Fig. 3. Inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in 20% oil-in-water emulsion at different pressures (100 and 350 bar) and temperatures (35 and 50 ˚C) using SC-CO_2, compared to conventional thermal treatment (T.T.) at 50 ˚C. Experimental data (discrete points) and Weibull model (continuous and dashed lines).

Table 1
Parameters (b and n) and goodness of fit by using Weibull model in the *E. coli* and *B. diminuta* SC-CO_2 inactivation kinetics for the 20% emulsion at different pressure and temperature conditions. Values in brackets indicate standard errors.

Microorganism	Treatment	Pressure (bar)	Temperature (˚C)	b (min^{-n})		n		R^2	RMSE
E. coli	SC-CO_2	100	35	1.60E−07	(5.81E−07)	4.18	(0.67)	0.95	0.14
E. coli	SC-CO_2	350	35	2.73E−06	(3.21E−06)	3.65	(0.35)	0.98	0.19
E. coli	SC-CO_2	100	50	1.06	(0.27)	0.47	(0.07)	0.96	0.37
E. coli	SC-CO_2	350	50	1.45	(0.60)	0.45	(0.12)	0.91	0.77
B. diminuta	SC-CO_2	100	35	0.63	(0.26)	0.49	(0.10)	0.98	0.22
B. diminuta	SC-CO_2	350	35	0.36	(0.22)	0.68	(0.15)	0.86	0.83
B. diminuta	SC-CO_2	100	50	1.3	(0.15)	0.44	(0.03)	0.99	0.11
B. diminuta	SC-CO_2	350	50	2.11	(0.24)	0.38	(0.03)	0.99	0.17

Therefore, as pressure increases, the contact between CO_2 and the bacteria in the medium is enhanced, allowing a faster microbial inactivation [31]. Ortuño et al. [14] studied the inactivation of *E. coli* in LB medium at 36 °C at different pressures. Thus, at 350 bar, 25 min were needed to achieve 5.0–6.0 log-cycles of reduction; while 50 min were required at 100 bar to achieve the same inactivation level. Hong et al. [32] also reported the relevant effect of the pressure on the inactivation, since 50–55 min were required to inactivate 5.0 log-cycles of *Lactobacillus plantarum* (in MRS broth and phosphate buffer) at 70 bar; while it took only 15–20 min when the pressure was doubled. The effect of pressure on the inactivation kinetics is computed in the b kinetic parameter of the Weibull model since, in general terms, the higher the pressure, the higher the b parameter. In the case of the shape parameter (n), the values found at 100 and 350 °C were similar (Table 1).

The temperature in SC-CO_2 treatments also had a significant ($p < 0.05$) effect on the inactivation of both microorganisms. On average, the temperature rise from 35 to 50 °C at 50 min leads to an increase of 3.0–4.0 log-cycles in the inactivation level, regardless of the microorganism and the pressure. For example, in the inactivation of *B. diminuta* with SC-CO_2 (Fig. 3B), the total inactivation (8.4–8.5 log-cycles) was achieved in less than 70 min at 50 °C, while more than 90 min were required at 35 °C to completely inactivate *B. diminuta*. For both microorganisms, the b parameter of the Weibull model increased on average from 0.25 to 1.48 min^{-n} when the temperature rose from 35 to 50 °C. The more intense inactivation at high temperatures could be explained by the fact that an increase in temperature leads to a lower CO_2 viscosity and higher diffusion rates. In addition, heat increases the membrane permeability and makes cells more sensitive to inactivation [33,34]. Therefore, SC-CO_2 is able to penetrate into the cell membranes faster and to a greater extent at high temperatures, which accelerates the inactivation mechanisms [35].

In Fig. 3A, an initial lag-phase was found in the inactivation kinetics of *E. coli* at 35 °C, during which the inactivation was negligible. This phase is linked to the time required for the CO_2 to dissolve in the liquid medium and to penetrate into the microbial cells and, consequently, to begin the inactivation mechanisms [36]. Once the lag-phase finalized, after approximately 24 min at 35 °C, a faster decrease in the *E. coli* population was observed for both pressures tested (Fig. 3A). On the contrary, when using 50 °C, the lag-phase was not observed (Fig. 3A). The lag-phase is well computed by the n parameter of the Weibull model, whose values are higher than 1 (Table 1); in the case of the *E. coli* treatments at 35 °C, values of 4.18 at 100 bar and 3.65 at 350 bar were found, which illustrates concave downward curves. In contrast, the values of n were lower than 1 in the 50 °C *E. coli* treatments at both pressures, indicating concave upward-shaped curves [37]. Liao et al. [38] reported that the higher the inactivation temperature, the shorter the lag-phase for *E. coli*. Unlike *E. coli* kinetics (Fig. 3A), the inactivation of the *B. diminuta* population did not show an initial lag-phase, regardless of the pressure and temperature applied (Fig. 3B), which might indicate either that CO_2 is able to penetrate into the cells faster than in the case of *E. coli* or a different sensitiveness to CO_2.

B. diminuta was found to be significantly ($p < 0.05$) more sensitive to the SC-CO_2 treatment, compared to *E. coli*. Thereby, the average b parameter of the Weibull model was higher for *B. diminuta* (1.10 min^{-n}) than for *E. coli* (0.63 min^{-n}).

3.1.2. Combined SC-CO_2 + HPU inactivation of E. coli and B. diminuta. Effect of pressure, temperature and high-power ultrasound

Fig. 4 shows the inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in a 20% oil-in-water emulsion for the combined SC-CO_2 + HPU treatment. The experimental variability in the inactivation kinetics with HPU (Fig. 4) was, in general, greater than in the SC-CO_2 kinetics (Fig. 3), due to the additional variability linked to the behaviour of the HPU transducer under supercritical conditions. The use of HPU did not affect the fitting ability of the Weibull model, which also satisfactorily described the inactivation kinetics for both microorganisms at different pressures and temperatures, as illustrated in Fig. 4. Thereby, R^2 values were higher than 0.97 and RMSE values were lower than 0.45 (Table 2).

HPU greatly improved the *E. coli* and *B. diminuta* inactivation by increasing the inactivation rate and the level of microbial reduction (Fig. 4A and B). Thus, while only 10 min were needed to achieve a substantial inactivation in *E. coli* (7.0–8.0 log-cycles) with SC-CO_2 + HPU for every condition of pressure and temperature (Fig. 4A), more than 50 min were required if HPU was not applied (Fig. 3A). In the case of *B. diminuta*, the combined treatment shortened the total inactivation time at 350 bar and 50 °C by 32 min, a time reduction which reached 58 min at 100 bar- 50 °C. The marked effect of HPU on the inactivation rate was well manifested in the Weibull b parameter, since b values in the SC-CO_2 + HPU treatments were significantly ($p < 0.05$) higher (on average, a difference of 2.15 min^{-n}) compared to the treatment under the same conditions without HPU, representing a higher inactivation rate for both bacteria. In the case of the shape parameter of the Weibull model, n values were under 1 for every tested condition (Table 1), since no lag-phases were found.

It is known that HPU generates agitation and cavitation in the medium where it is applied [20]. The strong agitation cause a reduction in the resistance to mass transfer, therefore the contact between the cells and the media is increased. Cavitation refers to the formation, growth and implosion of gas bubbles [12], which has been proven to cause damaged or cracked cell walls, increasing the cell membrane permeation [15]. Thus, when HPU is implemented to SC-CO_2 treatments, the contact between the SC-CO_2 and the bacteria with disrupted cell walls is enhanced, making CO_2 penetration in the cells easier and causing the extraction of vital intracellular components. In addition, the solubilisation of SC-CO_2 is enhanced by the effective agitation of HPU causing a faster drop of the intracellular pH, which accelerates the inactivation mechanisms, causing eventually the cellular death [14,39].

Ortuño et al. [8] contrasted the morphology of *E. coli* cells treated with SC-CO_2 and SC-CO_2 + HPU and reported a disordered distribution of cytoplasm with empty areas in the cells treated with SC-CO_2 + HPU, which indicates changes in the cells, such as damage in the walls and membranes or the loss of cytoplasmic content, due to the cavitation phenomenon of HPU. Contrary to SC-CO_2 treatments, both bacteria exhibited very similar resistance to the treatment, except at 35 °C and 100 bar in which *B. diminuta* was much more resistant than *E. coli*. Thus, no bacteria effect ($p > 0.05$) was found in the b parameter of Weibull.

Ortuño et al. [8] observed a more intense inactivation in *S. cerevisiae* (8–10 μm) than in *E. coli* (1.2–2 μm) when treated with SC-CO_2 + HPU. These authors suggested that the probability of cavitation bubbles affecting the cell structure is higher for *S. cerevisiae* than for *E. coli* due to its larger size (difference of 6.8–8 μm). However, in the present study, the difference in size between *E. coli* and *B. diminuta* is much smaller (a difference of around 2.4 μm), which can partially explain the similar sensitivity of both bacteria to the SCO_2 + HPU treatment.

Visual observation of the emulsions did not show any alteration of the SC-CO_2 + HPU treated samples. However, further studies should be done to analyse the effect of the treatment on the physico-chemical properties (particle size distribution, zeta potential,…) and the stability of the treated emulsions.

Pressure and temperature had a significant ($p < 0.05$) effect on the inactivation of *E. coli* and *B. diminuta* cells treated with combined SC-CO_2 + HPU. In general terms, the higher the pressure and temperature, the faster the inactivation. However, in the case of *E. coli*, the inactivation kinetics at 100 bar were very similar at both temperatures studied (35 and 50 °C) (Fig. 4A), which illustrates a milder temperature effect than in SC-CO_2 treatments. In addition, the inactivation kinetics of *E. coli* at 100 bar and 35 °C were very close to those obtained using 350 bar and 35 °C after 7 min of treatment, which also points to a milder effect of the pressure. Similarly, Ortuño et al. [8] treated *E. coli* in apple juice with SC-CO_2 + HPU, and no significant (p greater than 0.05) differences were found between the conditions applied (100,

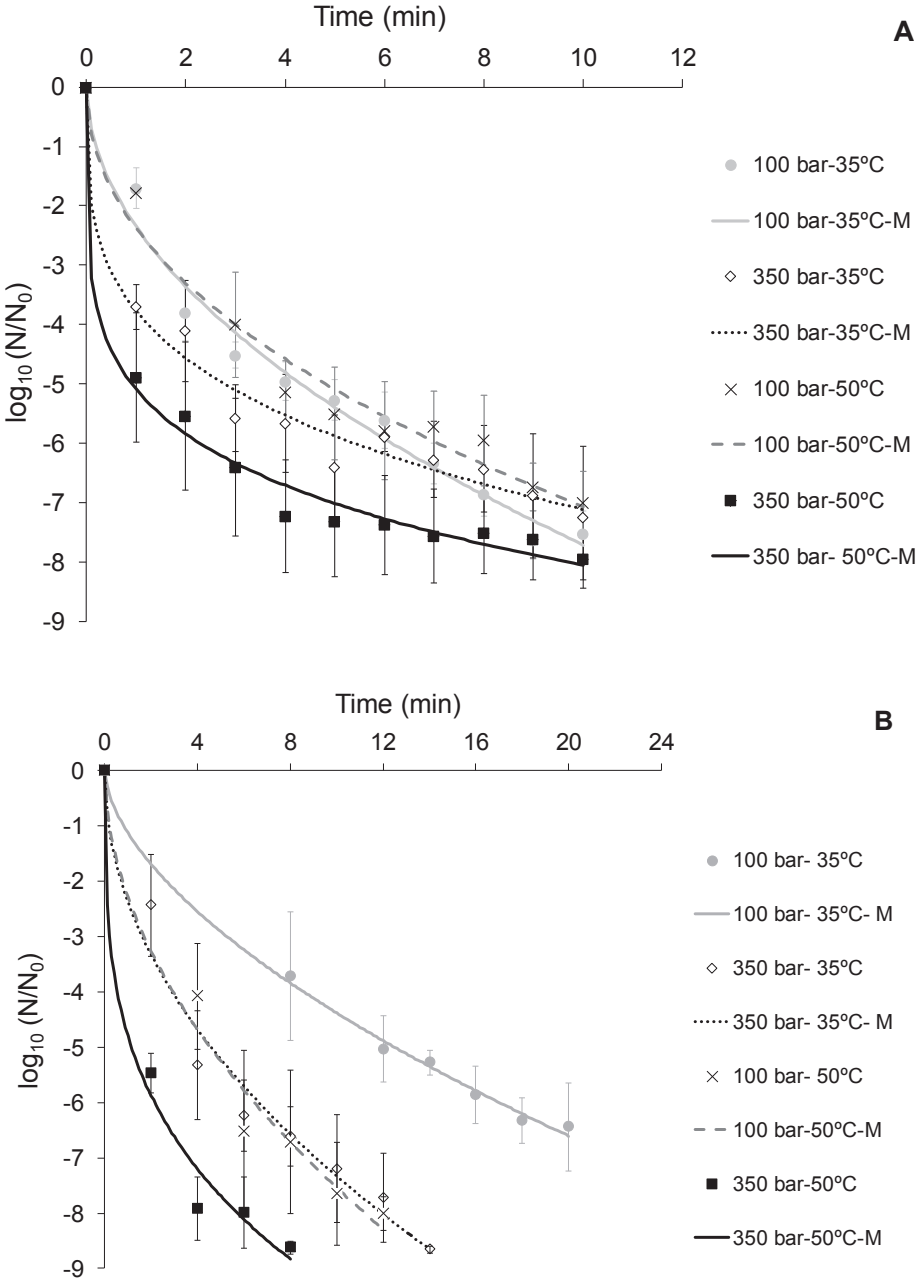

Fig. 4. Inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in 20% oil-in-water emulsion at different pressures (100 and 350 bar) and temperatures (35 and 50 °C) using SC-CO_2 + HPU. Experimental data (discrete points) and Weibull model (continuous and dashed lines).

Table 2
Parameters (b and n) and goodness of fit by using Weibull model in the *E. coli* and *B. diminuta* SC-CO_2 + HPU inactivation kinetics for the 20% emulsion at different pressure and temperature conditions. Values in brackets indicate standard errors.

Microorganism	Treatment	Pressure (bar)	Temperature (°C)	b (min^{-n})		n		R^2	RMSE
E. coli	SC-CO_2 + HPU	100	35	2.35	(0.18)	0.52	(0.04)	0.98	0.31
E. coli	SC-CO_2 + HPU	350	35	3.77	(0.21)	0.28	(0.03)	0.98	0.29
E. coli	SC-CO_2 + HPU	100	50	2.38	(0.24)	0.47	(0.05)	0.97	0.34
E. coli	SC-CO_2 + HPU	350	50	5.09	(0.18)	0.20	(0.02)	0.99	0.24
B. diminuta	SC-CO_2 + HPU	100	35	1.12	(0.12)	0.59	(0.04)	0.99	0.11
B. diminuta	SC-CO_2 + HPU	350	35	2.37	(0.34)	0.49	(0.06)	0.99	0.45
B. diminuta	SC-CO_2 + HPU	100	50	2.28	(0.48)	0.52	(0.10)	0.97	0.41
B. diminuta	SC-CO_2 + HPU	350	50	4.79	(0.56)	0.29	(0.07)	0.98	0.38

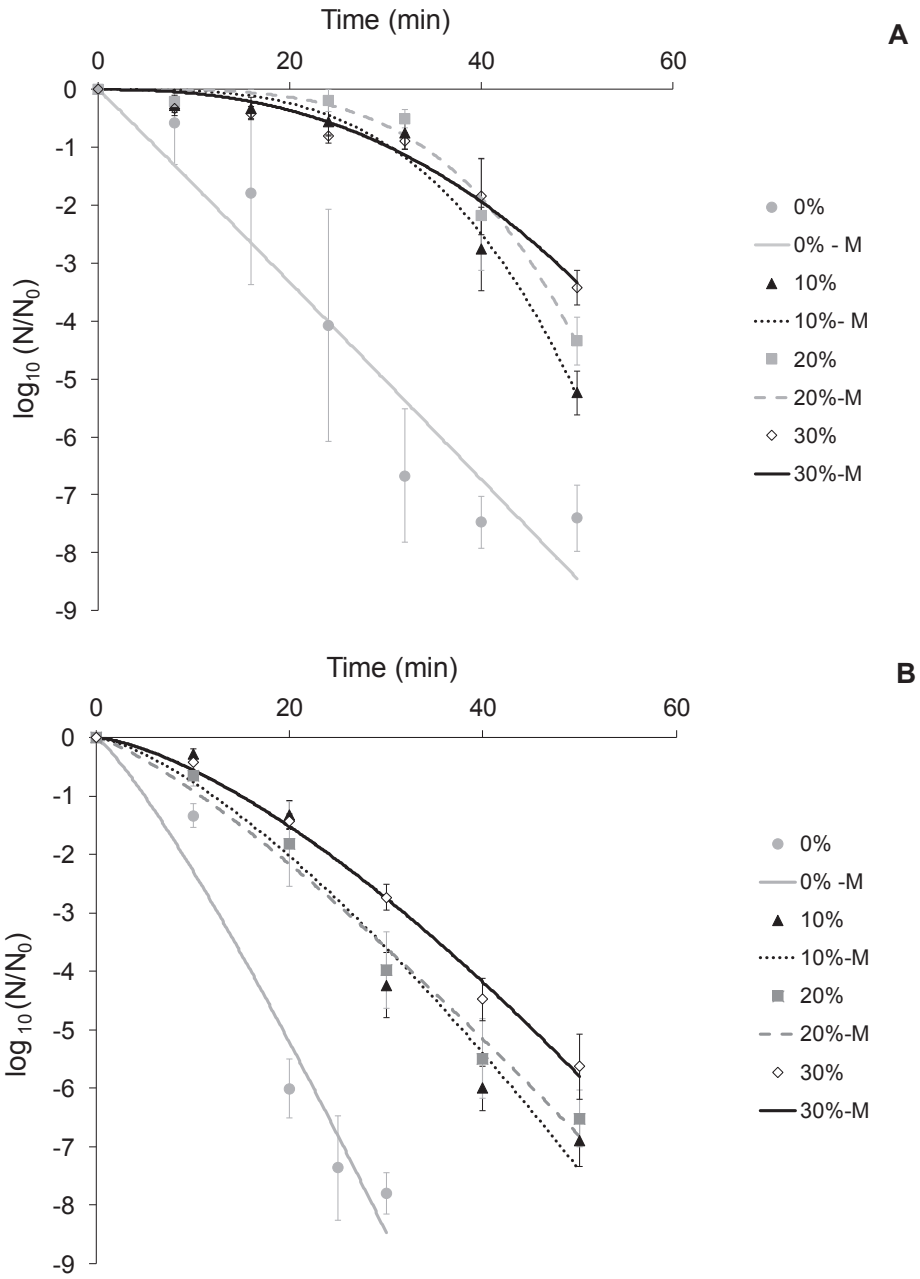

Fig. 5. Inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in oil-in-water emulsions with different oil contents (0, 10, 20 and 30%), using SC-CO_2 at 350 bar and 35 °C. Experimental data (discrete points) and Weibull model (continuous and dashed lines).

225 and 350 bar at 36 °C and 31, 36 and 41 °C at 225 bar). However, in *B. diminuta*, both pressure and temperature effects were more noticeable. The Weibull b parameter highlighted that the effect of the pressure was slightly greater than that of the temperature for both bacteria: as an example for *E. coli*, the average difference between the b values at 100 and 350 bar was 2.1 min^{-n}, while this difference was only 0.7 when the temperature rose from 35 to 50 °C.

3.2. Effect of the medium composition on microbial inactivation

3.2.1. Effect of oil content on the SC-CO_2 inactivation treatments of E. coli and B. diminuta

Numerous analyses have already illustrated that the inactivation rate of microorganisms treated with SC-CO_2 is medium dependent [17]. Several studies reported a strong protective effect on the inactivation of microbial cells in complex food systems, as compared to simple media [17]. Ortuño et al. [14] showed that the total inactivation of *E. coli* in LB broth was achieved in 22 min at 350 bar and 36 °C, while when fruit juices were treated instead of LB broth under the same conditions, the microbial population was only reduced by 0.5–1.0 log-cycles in 25 min. In the same way that the acids and sugars present in fruit juices were found to have a protective effect on the inactivation, the oil content present in the emulsions could have a protective effect on the inactivation of *E. coli* and *B. diminuta.* Fig. 5 shows the inactivation kinetics of *E. coli* and *B. diminuta,* in emulsions with different oil contents (10, 20 and 30%) treated with SC-CO_2 at 350 bar and 35 °C. The lipid emulsion with 0% oil content refers to the water. As in previous cases, the fitting of the inactivation kinetics with the Weibull model was adequate, providing R^2 of over 0.91 and RMSE of under 0.54, except for treatments in water (0%), in which RMSE were slightly higher (Table 3).

The inactivation of both *E. coli* and *B. diminuta* in water (0% oil

Table 3
Parameters (b and n) and goodness of fit by using Weibull model in the *E. coli* and *B. diminuta* inactivation kinetics with SC-CO_2 at 350 bar and 35 °C in the different lipid emulsions (0, 10, 20 and 30%). Values in brackets indicate standard errors.

Microorganism	Treatment	Oil content (%)	b (min^{-n})		n		R^2	RMSE
E. coli	SC-CO_2	0	0.16	(0.13)	1.02	(0.22)	0.91	0.81
E. coli	SC-CO_2	10	9.68E−06	(1.27E−05)	3.38	(0.32)	0.98	0.23
E. coli	SC-CO_2	20	1.29E−06	(2.75E−06)	3.85	(0.36)	0.98	0.18
E. coli	SC-CO_2	30	2.49E−04	(2.88E−04)	2.43	(0.29)	0.96	0.19
B. diminuta	SC-CO_2	0	0.15	(0.14)	1.20	(0.30)	0.94	0.67
B. diminuta	SC-CO_2	10	0.03	(0.03)	1.41	(0.26)	0.96	0.54
B. diminuta	SC-CO_2	20	0.05	(0.03)	1.25	(0.14)	0.99	0.30
B. diminuta	SC-CO_2	30	0.02	(0.01)	1.46	(0.10)	0.99	0.15

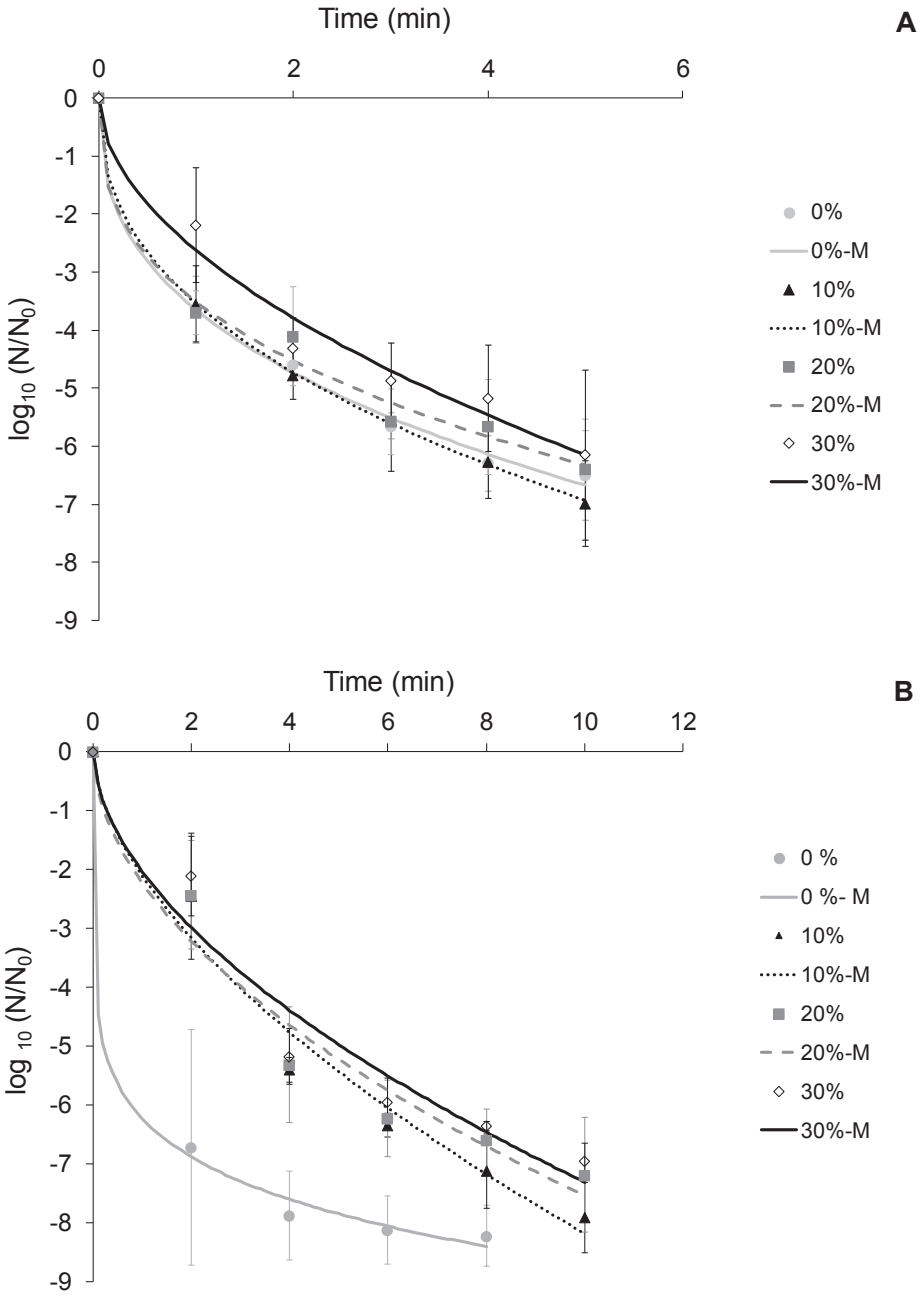

Fig. 6. Inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in the oil-in-water emulsion at different oil contents (0, 10, 20 and 30%) and distilled water using SC-CO_2 + HPU at 350 bar and 35 °C. Experimental data (discrete points) and Weibull model (continuous and dashed lines).

content) was significantly ($p < 0.05$) faster than in the lipid emulsions. Lin et al. [36] suggested that bacterial cells in water are swollen and more accessible to the penetration of CO_2. In addition, the high water content facilitates CO_2 dissolution and acid formation which improves cell permeability and the transport of CO_2 into the cells. In water (0% oil), 50 min (Fig. 5A) and 30 min (Fig. 5B) were enough to achieve an inactivation of 7.4 and 7.8 log-cycles in *E. coli* and *B. diminuta*, respectively. However, when lipid emulsions were treated, an *E. coli* inactivation of between 3.4 and 5.2 log-cycles was achieved in 50 min (Fig. 5A), and of between 2.7 and 4.2 log-cycles for *B. diminuta* in 30 min (Fig. 5B). Equivalent conclusions were drawn from the kinetic Weibull parameter since the b values were much higher in water treatments than in the lipid emulsions. Thus, the b values were of 0.16 min^{-n} for *E. coli* and 0.15 for *B. diminuta* in water, while in the lipid emulsions, an average b value of 8.67E−05 min^{-n} for *E. coli* and 0.03 min^{-n} for *B. diminuta* was identified for the different oil contents. Kobayashi et al. [40] reported that the inactivation of *E. coli* suspended in milk, with pressurized CO_2 at 35, 40, 45 or 50 °C and 40 bar, was less intense than in a physiological saline solution. These authors considered that the contact between CO_2 and the bacteria could be hindered by protein and milkfat, and the inactivation efficiency of CO_2 could decrease due to the buffering capacity of the different components in the solutions. Kim et al. [41] observed a considerably milder inactivation of *L. monocytogenes* in a physiological saline solution, treated at 35 °C, 100 bar and 15 min, due to the addition of oleic acid at different concentrations. Two explanations were proposed by these authors for the purposes of understanding the effect of oil on inactivation. One is that SC-CO_2 is not only solubilized in the lipid bilayer of the membrane but also in the other lipids, which greatly reduces the inactivation rate. The other is that lipid substances also act as a barrier protecting the lipid bilayer of the membranes and hindering the SC-CO_2 penetration and solubilisation. Several authors suggested that bacterial cells grown or suspended in a medium with fat could be biologically or physically affected, with changes either in the structure of cell walls and membranes or in their porosity. Lin et al. [36] found that growing the bacteria in milk increased the resistance of *L. monocytogenes* to further pressurized CO_2 treatments and, the higher the fat content in the milk, the more resistant to CO_2 treatments was the bacteria. Additionally, when CO_2 is injected into the vessel, it is partly dissolved in the water-phase and partly in the oil-phase of the medium [42]. Consequently, less CO_2 will be available in the water phase, which is responsible for the pH decrease and the increase in membrane permeability, which leads to microbial inactivation. Therefore, it can be concluded that the protective effect of the oil observed in the present study was coincided with that found in previous studies into other solutes. In general terms, the higher the oil content, the slower the inactivation. In fact, the percentage of oil promoted significant ($p < 0.05$) differences in the final inactivation levels for both bacteria. These results agree with previous ones reported in Garcia-Gonzalez et al. [43], where there was a reduction in the inactivation degree of *P. fluorescens* treated at 105 bar, 35 °C and 20 min when sunflower oil was added to the control sample (BHI broth supplemented with K_2HPO_4). Whereas a reduction of 6.0 log-cycles was achieved in the control sample, in the samples with 10 and 30% of sunflower oil, decreases of only 3.9 log-cycles and 3.0 log-cycles, respectively, were obtained.

In the inactivation kinetics of *E. coli* (Fig. 5A), a remarkable lag-phase was found for lipid emulsions at different oil contents. However, for *B. diminuta,* the lag-phase was almost negligible (Fig. 5B). This fact was evidenced in the n parameter of Weibull, which ranged from 2.43 to 3.85 for *E. coli*, while it was close to one for for *B. diminuta*. Once again, these results highlight *B. diminuta* exhibits a lower degree of resistance to the SC-CO_2 inactivation treatment than *E. coli.*

3.2.2. Combined SC-CO_2 + HPU inactivation of E. coli and B. diminuta. Effect of oil content

Fig. 6 shows the inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) at 350 bar and 35 °C using the combined SC-CO_2 + HPU treatment with different oil contents. As in SC-CO_2 treatments using the lipid emulsions, the performance of the Weibull model when fitting the inactivation kinetics was noticeable, since R^2 was higher than 0.94 and RMSE was lower than 0.53 (Table 4) for every condition tested.

The application of HPU led to a noticeable increase in the inactivation rate in the SC-CO_2 medium of the lipid emulsions, as observed when Figs. 5 and 6 are compared. When using HPU, only 5 min were needed to achieve 6.2–7.0 log-cycle reductions of *E. coli*, regardless of the oil content in the emulsion (Fig. 6A), while more than 50 min were required with the SC-CO_2 treatment (Fig. 5A). As for *B. diminuta*, a similar effect was found since similar log-cycle reductions were achieved in less than 8 min with HPU application (Fig. 6B), compared to more than 50 min in SC-CO_2 treatments (Fig. 5B). Therefore, the vigorous cavitation and stirring linked to the HPU application has been shown to accelerate the inactivation of the microorganisms. The Weibull b values ranged from 2.63 to 3.65 min^{-n} for *E. coli* and from 2.04 to 6.24 min^{-n} for *B. diminuta*, while for treatments without HPU, the b values were, on average, 0.04 min^{-n} for *E. coli* and 0.06 min^{-n} for *B. diminuta*, Therefore, the rate of inactivation was clearly larger when ultrasound was applied to the SC-CO_2 treatment. In addition, the initial lag phase observed in the *E. coli* kinetics for the lipid emulsions (Fig. 5A), disappeared in the combined SC-CO_2 + HPU treatment (Fig. 6A), which was computed by the Weibull model, providing n values of under 1 in every case. Ortuño et al. [14] also observed that the initial lag phase shown in the inactivation kinetics of *E. coli* with SC-CO_2 disappeared when HPU was applied (225 bar, 31, 36 and 41 °C). Experimental results point to the fact that HPU application perturbs the protective capacity of the oil in the inactivation of both bacteria (Fig. 6) Thus, for *E. coli*, non-significant ($p > 0.05$) differences between any of the treatment media (0, 10, 20 and 30% oil content) were found. While in the case of *B. diminuta* (Fig. 6B), only the inactivation in water was significantly ($p < 0.05$) different from the emulsions. Therefore, the oil content did not significantly affect ($p > 0.05$) the b Weibull parameter. Although no references were found to the effect of the oil content in the combined SC-CO_2 + HPU inactivation, the effect of other solute concentrations has been analysed. Ortuño et al. [14] reported that the marked turbulence caused by ultrasound could mask any effect the nature of the medium may have, since ultrasound application would lead to a faster cellular penetration of SC-CO_2 and an enhanced extraction of vital compounds from cells, regardless of the nature of the media. In conclusion, the HPU sharply intensifies the effectiveness of the process, both facilitating the mass transfer processes and also affecting the cell wall integrity, regardless of the nature of the medium being treated.

4. Conclusions

This study illustrates the fact that the non-thermal pasteurization of *E. coli* and *B. diminuta* in lipid emulsions using SC-CO_2 could be considered as a highly time-consuming process, which could seriously restrict its industrial application. The use of HPU during SCO_2 inactivation led to several benefits. Firstly, HPU greatly accelerated the treatment, shortening the time required for the inactivation by approximately 1 order of magnitude under every condition tested for both bacteria. Secondly, as the effect of pressure and temperature was milder when HPU was applied, less intense process conditions would be necessary. This accounts for a reduction in the cost of the process and could also improve the product quality. Finally, the protective effect of the oil in the inactivation of the lipid emulsions was masked when HPU was applied.

Future studies should address the effect of a combined SC-CO_2 + HPU treatment on the physicochemical properties and stability of the lipid emulsions, as well as assessing the inactivation of more resistant microorganisms, such as gram-positive bacteria, sporulated bacteria or moulds.

Table 4
Parameters (b and n) and goodness of fit by using Weibull model in the *E. coli* and *B. diminuta* inactivation kinetics with SC-CO_2 + HPU at 350 bar and 35 °C in the different lipid emulsions (0, 10, 20 and 30%). Values in brackets indicate standard errors.

Microorganism	Treatment	Oil content (%)	b (min^{-n})		n		R^2	RMSE
E. coli	SC-CO_2 + HPU	0	3.65	(0.12)	0.37	(0.03)	0.99	0.12
E. coli	SC-CO_2 + HPU	10	3.55	(0.04)	0.42	(0.01)	0.99	0.04
E. coli	SC-CO_2 + HPU	20	3.51	(0.24)	0.37	(0.05)	0.98	0.24
E. coli	SC-CO_2 + HPU	30	2.63	(0.28)	0.53	(0.08)	0.97	0.34
B. diminuta	SC-CO_2 + HPU	0	6.24	(0.28)	0.14	(0.03)	0.99	0.17
B. diminuta	SC-CO_2 + HPU	10	2.11	(0.37)	0.59	(0.09)	0.97	0.42
B. diminuta	SC-CO_2 + HPU	20	2.24	(0.47)	0.53	(0.11)	0.96	0.49
B. diminuta	SC-CO_2 + HPU	30	2.04	(0.50)	0.55	(0.12)	0.94	0.53

CRediT authorship contribution statement

Angela Gomez-Gomez: Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing. **Edmundo Brito-de la Fuente:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Funding acquisition. **Críspulo Gallegos:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Funding acquisition. **Jose Vicente Garcia-Perez:** Conceptualization, Methodology, Validation, Data curation, Writing - original draft, Writing - review & editing, Supervision. **Jose Benedito:** Conceptualization, Methodology, Validation, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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