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Combined pulsed electric field and high-power ultrasound treatments for microbial inactivation in oil-in-water emulsions

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ABSTRACT

The impact of individual and combined pulsed electric field (PEF) and high power ultrasound (HPU) on the inactivation of different microorganisms in emulsions was investigated. The highest inactivation level using PEF was 2.6, 1.2 and 0.1 log-cycles for *Escherichia coli*, *Aspergillus niger* and *Bacillus pumilus*, respectively, achieved at the highest energy level and temperature (152.3–176.3 kJ/kg and 25 °C). HPU led to the highest reduction (5.4, 4.3 and 0.3 log-cycles for *E. coli*, *A. niger* and *B. pumilus*, respectively) after the longest treatment time studied (3 min). PEF (152.3–176.3 kJ/kg) followed by HPU (3 min) was found to be the most effective sequence, leading to synergistic effects (6.6 and 1.0 log-cycles for *A. niger* and *B. pumilus*, respectively), compared to the individual treatments. PEF-HPU is a promising hurdle technology with which to inactivate vegetative bacteria or fungal spores in emulsions. However, limited inactivation was achieved for bacterial spores.

1. Introduction

Non-thermal technologies for microbial inactivation purposes are considered as an alternative to thermal treatments and have lately been the subject of increased industrial interest. These technologies employ alternative microbial inactivation sources rather than heat, which could reduce the detrimental effects on highly heat-sensitive compounds, and offer higher quality than conventional thermal treatments. Some of these non-thermal technologies are pulsed electric fields (Mosqueda-Melgar, Elez-Martínez, Raybaudi-Massilia, & Martín-Belloso, 2008), high power ultrasound (Piyasena, Mohareb, & McKellar, 2003), high pressure carbon dioxide (Ortuño, Martínez-Pastor, Mulet, & Benedito, 2012) and high hydrostatic processing (Erkmen & Doğan, 2004), among others. Moreover, some of these non-thermal technologies such as pulsed electric fields or high power ultrasound are considered as "green technologies" due to minimal impact exerted on the environment in terms of reduction of water, energy, wastes, etc. (Jambrak, 2018).

The pulsed electric field (PEF) treatment consists of the application of high voltage and short duration electric pulses to a medium placed between two electrodes (Halpin, Cregenzán-Alberti, Whyte, Lyng, & Noci, 2013). Thus, the product is subjected to an electric field whose intensity depends on the voltage across the electrodes and on the geometry of the space between them (Raso et al., 2016). This technology has been shown to be able to inactivate microorganisms when using high electric field strength (>20 kV/cm), while minimally modifying the physicochemical and nutritional properties of the treated products (Barba et al., 2015). The mechanisms for microbial inactivation by PEF are related with an increase in transmembrane potential caused by the external electrical field. When the electrical field strength exceeds the critical threshold value of the transmembrane potential, pores in the cell membrane are formed. This phenomenon is known as electroporation, which can be reversible or irreversible (Spilimbergo, Cappelletti, Tamburini, Ferrentino, & Foladori, 2014). In the case of reversible electroporation, the membrane of the cell temporarily destabilizes and loses its permeability. In addition, the cell can undergo sublethal damage, which is responsible for the subsequent cell death in simultaneous or sequential treatments (Pataro, Ferrentino, Ricciardi, & Ferrari, 2010). In the irreversible electroporation, the cell membrane is irrevocably cracked and the intracellular content is released, leading to microbial inactivation (Palgan et al., 2012). Several authors investigated the use of PEF treatment to inactivate microorganisms in different media, such as water (Pyatkovskyy, Shynkaryk, Mohamed, Yousef, & Sastry, 2018), buffer solutions (Pataro et al., 2010), fruit juices (Hodgins, Mittal, & Griffiths, 2002; Yeom, Streaker, Howard Zhang, & Min, 2000; Yildiz et al., 2019, 2020) or emulsions, such as milk (Michalac, Alvarez, Ji, & Zhang, 2003; Odriozola-Serrano, Bendicho-Porta, & Martín-Belloso, 2006).

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High power ultrasound (HPU) consists of elastic waves of low frequency (20–100 kHz) and high intensity (>1 W/cm^2), which are known to increase heat and mass transfer; therefore, it is used to bring about changes in the products or processes (Contreras, Benedito, Bon, & Garcia-Perez, 2018). One of the significant applications of HPU in food and pharmaceutical applications has been the inactivation of microorganisms (Piyasena et al., 2003). The inactivation mechanisms of HPU are related to cavitation, which consists of the formation, growth and abrupt implosion of bubbles, causing peaks of extremely high temperatures and pressures and mechanical shock that can damage or break the cellular structure of the microorganisms (Cárcel, García-Pérez, Benedito, & Mulet, 2012). Some authors have already studied the inactivation of microorganisms via HPU in different media, such as fruit juices (Evelyn et al., 2016; Evelyn & Silva, 2018), beef slurry, strawberry puree (Evelyn & Silva, 2018), liquid whole egg (Bi et al., 2020) or emulsions, such as milk (Khanal, Anand, & Muthukumarappan, 2014; Scudino et al., 2020).

Both technologies, PEF and HPU, have shown themselves to be of great potential as non-thermal preservation treatments in liquid products (Palgan et al., 2012). However, the individual effects of PEF or HPU treatments on microbial inactivation are usually moderate; therefore, intense conditions or long application times are required to obtain a substantial microbial reduction, which could involve undesirable effects on the quality properties of the treated product, along with some limitations on an industrial scale. In addition, the individual use of PEF and HPU technologies have not been fully successful in inactivating bacterial spores (Fan et al., 2019; Noci, Walkling-Ribeiro, Cronin, Morgan, & Lyng, 2009). The combined use of various non-thermal technologies (hurdle effect) have been proven to enhance the effectiveness as regards microbial inactivation, compared to the individual treatments, leading to additive or synergistic effects. Some authors already studied the combination of PEF and HPU treatments for the purposes of microbial inactivation (Aadil et al., 2018; Halpin et al., 2013; Huang, Mittal, & Griffiths, 2006; Lyu, Huang, Yang, Wang, & Wang, 2016; Palgan et al., 2012; Walkling-Ribeiro, Noci, Cronin, Lyng, & Morgan, 2009; Walkling-Ribeiro, Noci, Riener, et al., 2009). Table 1 shows a list of applications that use PEF, HPU and its combination for the inactivation of different microorganisms in various media. In this regard, Aadil et al. (2018) investigated the effect of a PEF treatment (20 kV/cm and 600 μs) followed by HPU (600 W, 28 kHz and 30 min) on the microorganisms naturally present in grapefruit juice, finding a reduction of 1.9 log-cycles (Total Plate Count) with the combined treatment (1st PEF-2nd HPU) (Table 1), compared to a reduction of 0.5 and 1.5 log-cycles in the individual HPU and PEF treatments, respectively. Noci et al. (2009) studied the reverse combined treatment (1st HPU- 2nd PEF) for the inactivation of *L. innocua* in milk and obtained a reduction of 6.8 log-cycles, compared to 3.3 and 0.6 log-cycles for the individual PEF (40 kV/cm and 50 μs) and HPU (400 W, 80s) treatments, respectively. However, to our knowledge, only a few studies have compared the influence of the order of application of combined PEF and HPU treatments (Huang et al., 2006; Lyu et al., 2016; Palgan et al., 2012), and none of them compared the effectiveness of the combined treatment on microorganisms with different characteristics.

Oil-in-water emulsions are widely used in several industries, including pharmaceuticals, foods, cosmetics and agrochemicals (Muriel Mundo, Zhou, Tan, Liu, & McClements, 2020). Despite that, few studies were found into microbial inactivation in vegetable emulsions using PEF or HPU. Only Dunn (1996), Barsotti, Dumay, Mu, Fernandez Diaz, and Cheftel (2001) and Markus Walkling-Ribeiro, Noci, Cronin, Lyng, and Morgan (2010) stated that emulsions (salad dressings, peanut oil emulsions and coconut milk based smoothies, respectively) could be

Table 1
Applications of pulsed electric field (PEF), high power ultrasound (HPU) and its combination for the inactivation of different microorganisms in various media.

Treatment	Conditions	Microorganism	Medium	Microbial reduction	Reference
PEF	0–30 kV/cm, 0.5–2.5 μs, 50 °C (Exponential waves).	*Escherichia coli*	Nutrient broth	0.2 log-cycles	Yan, Yin, Hao, Liu, and Qiu (2021)
PEF	45 kV/cm, 1 μs, 100 pulses, 30 °C	*Aspergillus niger* spores	Collagen gels	2.0 log-cycles	Griffiths, MacLean, Anderson, MacGregor, and Helen Grant (2012)
PEF	7.5 kV/cm, 5 μS, 1 kHz, 1000 pulses/7.5 kV/cm, 5 μS, 1 kHz, 10,000 pulses.	*Bacillus pumilus* spores	NaCl solution	Negligible/67 ± 8%	Pillet, Formosa-Dague, Baaziz, Dague, and Rols (2016)
PEF	30 kV/cm, 1 μs width, 15 Hz, 150 μs, <56 °C/40 kV/cm, 1 μs width, 15 Hz, 150 μs, <56 °C	*Staphylococcus aureus*	Orange juice	3.0 log-cycles/5.5 log-cycles	Walkling-Ribeiro, Noci, Cronin, Lyng, and Morgan (2009)
PEF	80 mL/min,1 kHz, 20 kV/cm, 600 μs, 40 °C.	Total plate counts (TPC)/ yeasts and molds (Y&M)	Grapefruit juice	1.5 log-cycles (TPC)/ 1.4 log-cycles (Y&M)	Aadil et al. (2018)
HPU	42 kHz, 5–60 min, 20 °C	*Escherichia coli*	Orange juice	~0.4–1.3 log-cycles	Kernou et al. (2021)
HPU	20 kHz, 120 μm, 3 min, 52.5 °C	*Aspergillus flavus* spores	Broth	~0.4 log-cycles	López-Malo, Palou, Jiménez-Fernández, Alzamora, and Guerrero (2005)
HPU	20 kHz, 5 bar, 117 μm, 12 min, 70 °C	*Bacillus subtilis* spores	Distilled water	>99%	Raso, Palop, Pagán, and Condón (1998)
HPU	30 kHz, 5, 10, and 20 min, 55 °C	*Staphylococcus aureus*	Orange juice	0.8, 1.8, and 3.3 Log-cycles	Walkling-Ribeiro, Noci, Cronin, et al. (2009)
HPU	600 W, 28 kHz, 30 min, 20 °C.	Total plate counts (TPC)/ yeasts and molds (Y&M)	Grapefruit juice	0.5 log-cycles (TPC)/0.5 log-cycles (Y&M)	Aadil et al. (2018)
HPU-PEF	HPU: 30 kHz, 10min, 55 °C PEF: 40 kV/cm, 1 μs width, 15 Hz, 150 μs, <56 °C	*Staphylococcus aureus*	Orange juice	6.8 log-cycles	Walkling-Ribeiro, Noci, Cronin, et al. (2009)
HPU-PEF	HPU: 20 kHz, 750 W, 120 min, 35 °C. PEF: 12 kV/cm, 3 μs width, 300 Hz, 120 μs, 35 °C.	*Saccharomyces cerevisiae*	Rice wine	3.7 log-cycles	Lyu et al. (2016)
HPU-PEF	HPU: 100%, 20 kHz, 160 mL/min, 40 W/cm^2, 200 kPa, <52 °C. PEF: 160 mL/min, 34 kV/cm, 32 μs, <35 °C	*Listeria innocua*	Milk-based beverage	5.6 log-cycles	Palgan et al. (2012)
PEF-HPU	PEF: 12 kV/cm, 3 μs width, 200 Hz, 120 μs, 35 °C. HPU: 20 kHz, 750 W, 120 min, 35 °C.	*Saccharomyces cerevisiae*	Rice wine	3.5 log-cycles	Lyu et al. (2016)
PEF-HPU	PEF: 80 mL/min,1 kHz, 20 kV/cm, 600 μs, 40 °C. HPU: 600 W, 28 kHz, 30 min, 20 °C.	Total plate counts (TPC)/ yeasts and molds (Y&M)	Grapefruit juice	1.9 log-cycles (TPC)/ 1.7 log-cycles (Y&M)	Aadil et al. (2018)
PEF-HPU	PEF: 160 mL/min, 34 kV/cm, 32 μs, <35 °C HPU: 100%, 20 kHz, 160 mL/min, 40 W/cm^2,200 kPa, <52 °C	*Listeria innocua*	Milk-based beverage	4.2 log-cycles	Palgan et al. (2012)

pasteurized using PEF with minor physicochemical changes. As regards HPU, only two studies were found into microbial inactivation in vegetable emulsions, specifically peanut milk (Salve, Pegu, & Arya, 2019) and hazelnut milk (Atalar et al., 2019). In addition, although some authors studied the effectiveness of the combined PEF and HPU treatments at inactivating microorganisms in milk-based products (Halpin et al., 2013; Noci et al., 2009; Palgan et al., 2012), no studies have been found for vegetable oil-in-water emulsions. Therefore, the objective of this study was to test the feasibility of individual and combined PEF and HPU treatments in oil-in-water emulsions for the purposes of inactivating different types of microorganisms (vegetative bacteria and fungal and bacterial spores).

2. Materials and methods

2.1. Preparation of emulsions

20% oil-in-water emulsions were prepared to be used as the treatment media. They were elaborated in three steps: mixing, sonication and homogenization. Soybean oil and an emulsifying agent (egg phospholipid) were mixed with a dispersing device (IKA T25 Digital Ultra-Turrax, tool S25N-25G, Germany) at 14,000 rpm for 2 min, 10,200 rpm for 4 min and 10,600 rpm for 4 min. Then the dispersion was slowly poured to the water phase, constituted by deionized water and glycerol, while being mixed using the Ultra-Turrax at 14,000 rpm. Afterwards, the preparation was sonicated for 5 min using the H22 sonotrode and the ultrasound system UP400S (Hielscher, Germany). Finally, the sample was homogenized in two stages (50 bar; 550 bar) with the PANDA Plus 2000 homogenizer (GEA Niro Soavi, Italy).

2.2. Microorganisms

The effectiveness of PEF and HPU treatments has been shown to be dependent on the type of microorganism because of the different composition and structure of their cell walls (Piyasena et al., 2003; Spilimbergo et al., 2014). Therefore, a vegetative bacterium (*Escherichia coli* CECT 101, Spain) and a fungal (*Aspergillus niger* CECT 2807, Spain) and a bacterial (*Bacillus pumilus* CECT 29 T, Spain) spore were used in this study to assess the effectiveness of the inactivation treatments on different types of microorganisms. *E. coli* was selected because it is widely present in nature, including the gastrointestinal tracts of humans. Therefore, its presence in the industry is a good indicator of unfavorable hygienic conditions. *A. niger* was chosen because it is the most abundant filamentous mold found in the environment (Nadumane, Venkatachalam, & Gajaraj, 2016) and, consequently, its presence in contaminated products is not rare. Lastly, *B. pumilus* was selected due to its higher prevalence in contaminated food compared to other *Bacillus* species (From, Hormazabal, & Granum, 2007; Iurlina, Saiz, Fuselli, & Fritz, 2006).

All the microorganisms were prepared to be treated in their most resistant form (growth stage and spore when applicable).

E. coli was inoculated in 50 mL of nutrient broth (Scharlab, Spain) and incubated for 24 h at 37 °C (3,000,957, J.P. Selecta, Spain) at 120 rpm (Rotabit Model 3,000,974, J.P. Selecta, Spain). Then 50 μL of the starter culture were transferred to 50 mL of nutrient both medium and incubated (for 14 h) until reaching the stationary phase; this period of time was established from the growth curves by Gomez-Gomez, Brito-de la Fuente, Gallegos, Garcia-Perez, and Benedito (2020).

A. niger was cultured on Potato Dextrose Agar (Scharlab, Spain) at 25 °C for 7 days. Then the spores were rubbed from the surface of the agar with 10 ml of 0.1% (v/v) Tween 80 and collected. The suspension was kept in a sterile container at 4 °C until use.

B. pumilus cells were sporulated following the methodology of Mafart, Couvert, Gaillard, and Leguerinel (2002), with modifications. A single colony of the bacteria was grown in nutrient broth (Scharlab, Spain) at 30 °C until the stationary phase was reached, according to bibliography (around 24 h) (Han et al., 2017; Liu, Huang, & Feng, 2015). 100 μL of the culture with bacteria were poured on Plate Count Agar (Scharlab, Spain) enriched with $MnSO_4$ (40 mg/L) and $CaCl_2$ (100 mg/L) to enhance the sporulation and incubated at 30 °C for 5–6 days, a period of time in which spores were formed (confirmed with a Thoma counting chamber and an optical microscope). Afterwards, spores were collected by scraping the surface of the agar, suspended in 2 mL of sterile deionized water, and washed three times by centrifugation (8000×*g* for 15 min) (Medifriger BL-S, JP Selecta). The pellet was resuspended in 2 mL of ethanol (50% v/v) and kept at 4 °C for 12 h to eliminate vegetative non-sporulated bacteria. The suspension was once again washed three times by centrifugation. Lastly, the final suspension was distributed into sterile microtubes and kept at 4 °C until use.

2.3. Non-thermal inactivation treatments

Prior to each experiment, the PEF and the HPU systems were sterilized with a disinfectant solution (Diversey Delladet, USA) for 5 min and rinsed with sterile deionized water. 5 mL of the *E. coli* or the *A. niger* spore suspensions were added to 60 mL of the emulsion to reach a cell concentration of 10^7- 10^8 and 10^6–10^7 CFU/mL for *E. coli* and *A. niger* spores, respectively. As for *B. pumilus* spores, the microtubes were heat-shocked at 80 °C for 15 min to eliminate vegetative cells and cooled again to 4 °C before each inactivation treatment. Then, 2 mL of the spore suspension were added to 60 mL of emulsion to reach a concentration of 10^7–10^8 CFU/mL. The resulting treatment media had a conductivity of 1151, 498 and 430 μS/cm for *E. coli*, *A. niger* and *B. pumilus*, respectively.

2.3.1. PEF treatment system

PEF inactivation treatments were performed in a laboratory scale continuous flow unit (Fig. 1). The high voltage pulse generator used was the Epulsus-PM1-10 (Energy pulse systems, Portugal), which produced monopolar square pulses. The emulsion flow was driven by a peristaltic pump (XX8000230, Millipore Corporation, USA) through two parallel plate electrodes in a treatment chamber (groove with a length of 38 mm, height of 3.4 mm and a gap between electrodes of 3.1 mm). Two K-type thermocouples, located at the inlet and outlet of the PEF chamber, were used to measure the initial and the final temperature of the emulsion and a data logger (Fieldlogger, Novus Automation, USA) was used to register the temperature measurements each second.

2.3.2. HPU treatment system

Sonication treatments were performed in a batch system with an ultrasonic processor (UP400St, Hielscher Ultrasonics, Germany) and a Ø14mm sonotrode (s24d14D, Hielscher Ultrasonics, Germany) at 100% of amplitude (160 W measured by the calorimetric method, 24 kHz). The inoculated emulsion (110 mL) was placed in a jacketed beaker, with water circulating at different temperatures: at 40 °C for *E. coli* and *A. niger* to reach a final temperature lower than that for thermal

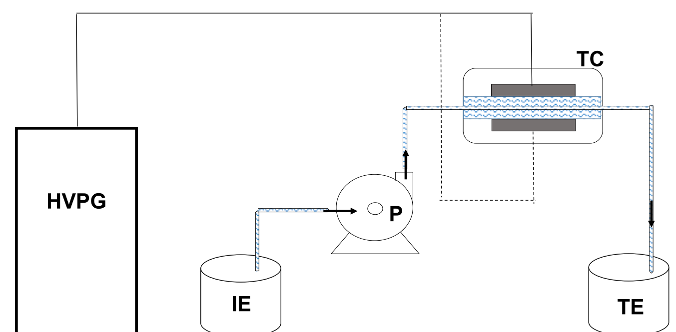

Fig. 1. Schematic diagram of the PEF system. HVPG: high voltage pulse generator. P: pump, TC: treatment chamber, IE: inoculated emulsion, TE: treated emulsion.

inactivation (known for each microorganism though the thermal treatment kinetics); and at 85 °C for *B. pumilus* spores to increase the temperature reached in the HPU treatment, due to the greater resistance of this bacterial spore to the thermal treatments, compared to *E. coli* and *A. niger* spores. A K-type thermocouple was located inside the jacketed beaker to measure the temperature of the emulsion during the treatment (each second), which was recorded with the same data logger used in the PEF treatments.

2.3.3. Treatment conditions

2.3.3.1. E. coli. The effect of different PEF parameters (field strength, treatment time and input temperature of the sample) on inactivation was examined for *E. coli*. For that purpose, the width of the pulse and the pulse repetition frequency were fixed at 5 μs and 50 Hz, respectively. The flow of the pump was set to obtain a PEF treatment time of 90, 130 and 170 μs, (calculated by multiplying the pulse width by the number of pulses received in the treatment chamber), corresponding to 66.6, 46.0 and 35.3 mL/min. As reported by Raso et al. (2016), a treatment field strength of 15–40 kV/cm is required for microbial inactivation. Therefore, in this study, the applied field strength was set at 20, 25 and 30 kV/cm (6200, 7750 and 9300 V, respectively) and the total energy applied on *E. coli* ranged from 41.5 to 176.3 kJ/kg. The experiments were performed at two input temperatures of the emulsion (15 and 25 °C).

HPU treatments were carried out for 2 (HPU2) and 3 min (HPU3). The combination of PEF and HPU technologies were performed in both sequences, PEF-HPU and HPU-PEF. The experiments were done in triplicate.

2.3.3.2. A. niger and B. pumilus spores. The conditions of the most effective PEF and HPU treatments found for *E. coli* were selected to investigate how the individual and the combined treatments affect the inactivation of *A. niger* spores. However, as the total energy applied to the sample by the PEF treatment is related to the conductivity of the treatment sample, a lower energy was applied for the inactivation of *A. niger* spores (76.3 kJ/kg). In order to supply the same total energy as for *E. coli* (176.3 kJ/kg), an additional PEF treatment at 32.3 kV/cm (corresponding to 10,000 V) and 10 μs of pulse width was performed (PEFB) to study the individual and HPU-PEF combined inactivation of *A. niger*. For *B. pumilus* spores only, the PEFB treatment (152.3 kJ/kg) was applied to study both the individual and the combined (PEF-HPU and HPU-PEF) inactivation effectiveness, due to the known greater resistance of bacterial spores to PEF treatments. The experiments were performed in triplicate.

2.4. Thermal treatments

PEF and HPU treatments involve a rise in temperature. In order to separate the temperature effect in the PEF and HPU treatments and to ensure that the inactivation obtained was mostly due to the electroporation mechanisms of PEF and to the cavitation effects of sonication, conventional thermal treatments were conducted at 50 and 60 °C for *E. coli* and *A. niger;* and at 85, 90 °C and 95 °C for *B. pumilus*.

The thermal treatments were performed in a temperature-controlled water bath (1812 Bunsen, Spain). 1.5 mL of inoculated emulsion (the concentration of each microorganism was the same than in the non-thermal treatments) were poured into borosilicate glass tubes of 8 mm in diameter and 70 mm in length (Fiolax, Germany). The tubes were periodically taken from the bath and cooled in ice for immediate analysis. The experiments were carried out in triplicate.

2.5. Microbiological analyses

The cell viability in the emulsions before and after each treatment was determined by the plate count technique. Depending on the expected count, appropriate serial dilutions were prepared with sterile deionized water. 100 μL of the dilution were spread on the surface of PCA (Scharlab, Spain) for *E. coli* and *B. pumilus* and PDA (Scharlab, Spain) for *A. niger* in triplicate and incubated at 37 °C for 24 h, 30 °C for 24 h and 25 °C for 72 h, respectively. The initial microbial load in the sample was also determined following the same procedure. Results were expressed as a logarithm reduction: $\log_{10}(N/N_0)$, where N_0 was the initial population of microorganisms in the untreated emulsion and N the population of microorganisms after the treatment.

2.6. Statistical analysis

Statistical analyses were performed with Statgraphics Centurion XVI (Statpoint Technologies Inc., USA). A multifactorial ANOVA was used to assess the influence of the PEF parameters on the inactivation level of *E. coli*. In addition, a one-way ANOVA was used to determine whether the use of the different non-thermal treatments considered or their combination had a significant effect on the level of inactivation for every microorganism. Fisher's least significant difference (LSD) procedure was used to discriminate among the means ($p < 0.05$).

3. Results and discussion

3.1. E. coli inactivation in emulsion using PEF

Table 2 reports the level of reduction obtained for *E. coli* after the PEF treatment under different conditions of field strength, treatment time and initial temperature of the emulsion. The degree of inactivation was significantly ($p < 0.05$) higher for the greatest electric field intensity (30 kV/cm), the longest treatment times (130 and 170 μs) and the highest input temperature (25 °C). The maximum inactivation level achieved was 2.6 log-cycles for the most intense treatment (30 kV/cm, 170 μs and an input temperature of 25 °C).

The application of an electric field of 30 kV/cm increased the

Table 2
Inactivation of *E. coli* in oil-in-water emulsion after the PEF treatments. Treatment conditions: pulse width of 5 μs, frequency of 50 Hz.

Field strength (kV/cm)	Treatment time (μs)	Number of pulses	Total energy (kJ/kg)	Microbial reduction at 15 °C of inlet temperature (log-cycles)	Microbial reduction at 25 °C of inlet temperature (log-cycles)
30	170	34	176.3	1.3 (0.4)	2.6 (0.4)
25	170	34	122.4	1.3 (0.1)	1.6 (0.4)
20	170	34	78.3	0.9 (0.1)	1.5 (0.4)
30	130	26	135.4	1.1 (0.4)	2.2 (0.3)
25	130	26	94.0	1.0 (0.1)	1.8 (0.2)
20	130	26	60.2	0.8 (0.3)	1.6 (0.3)
30	90	18	93.5	1.0 (0.3)	1.4 (0.6)
25	90	18	64.9	1.2 (0.1)	1.3 (0.3)
20	90	18	41.5	0.6 (0.3)	1.2 (0.5)

All data shown are means of the microbial reduction. Values in brackets are the standard deviations.

inactivation of *E. coli* by 0.2 and 0.5 log-cycles compared to the application of 25 and 20 kV/cm, respectively. These results coincided with those found by other authors. As an example, Spilimbergo, Dehghani, Bertucco, and Foster (2003) also found that the higher the electric field strength (4.5–25 kV/cm), the higher the inactivation levels of *E. coli* in water (0–3.2 log-cycle reduction). Higher inactivation levels (2.25 log-cycles) were obtained by Pataro, De Lisi, Donsì, and Ferrari (2014) when treating *E. coli* in buffer solution at 40 kJ/L and an input temperature of 22 °C, compared to an inactivation of 1.2 log-cycles at 41.5 kJ/kg and 25 °C found in the present study (Table 2). However, these differences could be explained by the different nature of the treatment media, since it is well known that the presence of fat in the media could exert a protective effect on microorganisms against PEF inactivation treatments (Mosqueda-Melgar et al., 2008). Therefore, a more moderate inactivation could be expected in the oil-in-water emulsions than in a simpler medium, such as water or buffer solutions.

As for the input temperature, an average increase in the level of inactivation from 1.0 to 1.7 log-cycles was found for a rise in temperature from 15 to 25 °C. Several authors (Lyu et al., 2016; Raso et al., 2016; Timmermans et al., 2014) have also observed a greater microbial inactivation when the input temperature of the sample increases; in all likelihood, this is due not only to the simple thermal action but also to the fact that the cell membrane becomes more fluid and, therefore, more prone to electroporation. As an example, Lyu et al. (2016) achieved a *S. cerevisiae* inactivation of 2.1 log-cycles when the sample was treated at an initial temperature of 40 °C compared to 1.2 log-cycles at 30 °C. The maximum temperature reached in the present study during the PEF treatment was 50 °C (30 kV/cm, 170 μs and an input temperature of 25 °C), which is a non-lethal temperature for *E. coli* in the emulsion since, as can be observed in Fig. 2A, only 0.4 log-cycles of reduction were achieved in 50 min of thermal treatment. Therefore, although higher temperatures enhanced PEF microbial inactivation, the effect of PEF on the inactivation of *E. coli* was not linked to the temperature rise during the process but to the damage caused by the high voltage electrical pulses. PEF is thought to damage the cell membrane by the induced potential exerted across it. The transmembrane potential inside the membrane cell is 30–70 mV; this increases as an external field is applied until a critical value is reached (70–100 mV), leading to the formation of irreversible pores in the membrane for suitable electric field strength and energy input levels (Pataro et al., 2014; Spilimbergo et al., 2003).

Although no sterile emulsions (2.6 log-cycles out of 8.2 log-cycles for complete inactivation) were obtained under these treatment conditions, higher inactivation levels could be achieved by the application of a combined PEF and HPU treatment (PEF-HPU or HPU-PEF). In addition, the existence of commercial equipment for the continuous treatment of products using PEF or HPU would facilitate the implementation of sequential PEF and HPU treatments in the industry.

3.2. *E. coli* inactivation in emulsions using high power ultrasound

Log-cycle reductions of *E. coli* after different sonication treatment times (2 and 3 min) are shown in Fig. 3. No significant ($p > 0.05$) differences were found between using HPU2 or the most intense PEF treatment (30 kV/cm, 170 μs and 25 °C). However, lengthening the sonication time from 2 to 3 min led to a significant ($p < 0.05$) inactivation boost (from 1.9 to 5.4 log-cycles), with HPU3 becoming the most effective individual treatment, compared to PEF and HPU2. Other authors also found that the longer the sonication time, the greater the *E. coli* inactivation (Ince & Belen, 2001; Piyasena et al., 2003).

HPU2 and HPU3 treatments reached a final temperature of 58.6 and 60 °C, respectively. The level of inactivation achieved by the HPU2 treatment was equal to the level reached in the conventional thermal treatment at 60 °C (1.9 log-cycles in 2 min) (Fig. 2A); therefore, it could be thought that the inactivation obtained by HPU2 could mainly be due to the heating effect. However, when the sonication treatment was

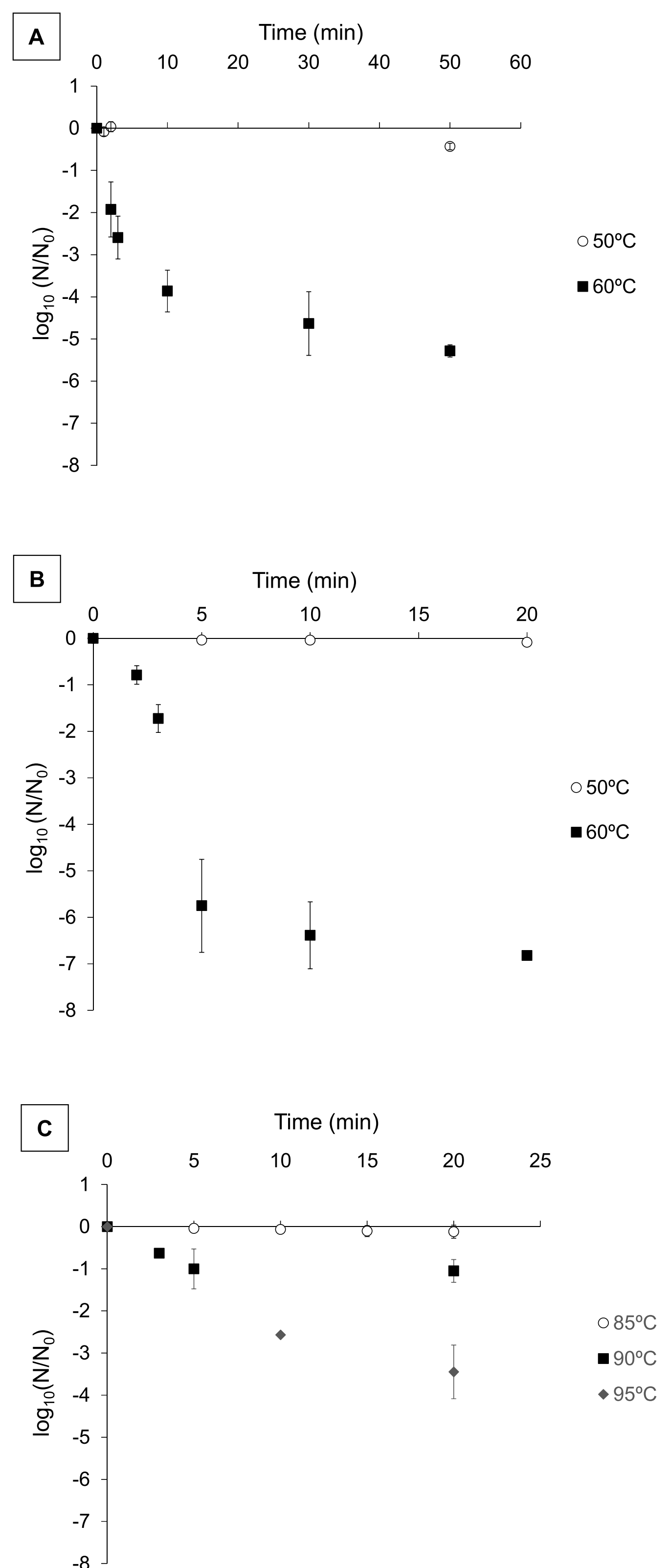

Fig. 2. Inactivation kinetics for the thermal treatment of *E. coli* **(A)**, *A. niger* spores **(B)** and *B. pumilus* spores **(C)** in oil-in-water emulsion.

extended to 3 min, a greater degree of inactivation was reached than in the thermal treatment at 60 °C: 5.4 log-cycles in the HPU3 treatment (Fig. 3) compared to 2.6 log-cycles in the thermal (Fig. 2A). Therefore, it seems that the mechanical cell stress caused by cavitation is dependent on the sonication time and 3 min were required to observe the

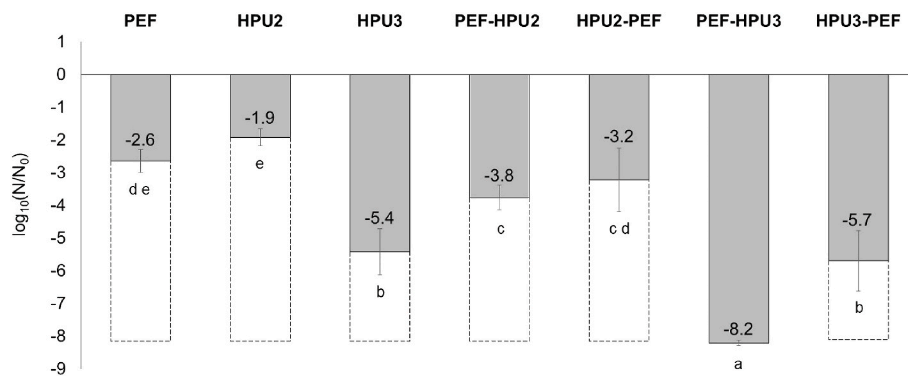

Fig. 3. Inactivation of *E. coli* in oil-in-water emulsion treated by PEF at 30 kV/cm, 170 μs of treatment time, 5 μs of pulse width, 50 Hz and 25 °C of inlet temperature (176.3 kJ/kg), HPU for 2 (HPU2) and 3 min (HPU3) and its combination. Dashed lines indicate complete inactivation. Letters show homogeneous groups established from LSD intervals (95%).

synergistic effect between ultrasound cavitation and heat. Ince and Belen (2001) also observed a moderate inactivation of *E. coli* in buffer solution in the initial 2 min of the HPU treatment (180 W) and, for longer treatments, the inactivation rate increased steeply. Consequently, HPU3 was selected for the inactivation treatments of *A. niger* and *B. pumilus* spores.

3.3. E. coli inactivation in emulsions using a combined PEF and HPU treatment

In Fig. 3, the levels of inactivation of *E. coli* treated with the individual and the sequential PEF and HPU treatments are depicted. The PEF treatment was carried out under the most intense condition (30 kV/cm, 170 μs and 25 °C input temperature), while sonication was performed for 2 and 3 min.

The application of PEF as a pre-treatment (PEF-HPU2 and PEF-HPU3) significantly ($p < 0.05$) increased the inactivation level of *E. coli*, compared to the individual treatments. However, the inactivation level of PEF-HPU2 (3.8 log-cycles) was lower than the addition of the log-reductions of each individual treatment ($2.6 + 1.9 = 4.5$ log-cycles). This could be explained by considering that 3 min of HPU were required to observe any synergistic effect between cavitation and heat, as previously explained. On the contrary, the complete inactivation was reached by the combined PEF-HPU3 treatment (8.2 log-cycles, Fig. 3), which was a slightly higher inactivation level than the theoretical sum of the reductions of each individual treatment ($2.6 + 5.4 = 8.0$ log-cycles).

When HPU was applied first, the combined HPU2-PEF treatment showed non-significant ($p > 0.05$) differences as regards inactivation, compared to the PEF treatment alone. In addition, the level of inactivation achieved in the combined HPU2-PEF treatment (3.2 log-cycles) was lower than the addition of the individual treatments (4.5 log-cycles). This was in accordance with the minimum threshold (>2 min) required to observe inactivation linked to ultrasonic cavitation.

With a longer sonication time in the first stage (HPU3-PEF), the inactivation level significantly ($p < 0.05$) increased from 2.6 to 5.7 log-cycles, compared to the PEF treatment alone, but no differences were found ($p > 0.05$) when employing the individual HPU3. In fact, the level of inactivation of the combined HPU3-PEF treatment was lower than the sum of the two individual treatments ($5.4 + 2.6 = 8.0$ log-cycles), as also observed for the HPU2-PEF treatment. As an example, Walkling-Ribeiro, Noci, Riener, et al. (2009) treated *S. aureus* in orange juice by means of a 10 min HPU treatment followed by a PEF treatment (40 kV/cm for 150 μs), obtaining a slightly smaller reduction than that of the theoretical sum of the two hurdles, as can be observed in Table 1. These authors explained that cavitation may inactivate only the most sensitive cells, leaving the most resistant cells intact for the inactivation brought about by the PEF treatment.

As for the sequence in which the treatments were applied, similar levels of reduction (3.2 and 3.8 log-cycles) were obtained when combining PEF and HPU2, regardless of which of them was applied first, which is consistent with the reduced cavitation effects found in the HPU2 treatment. On the contrary, when combining PEF and HPU3, the sequence of the treatments significantly ($p < 0.05$) affected the inactivation level, being more effective when PEF was carried out as a pre-treatment (from 5.7 log-cycles in the HPU3-PEF treatment to 8.2 in the PEF-HPU3 treatment). Thus, the most intense inactivation was found in the PEF-HPU3 treatment, resulting in the complete inactivation of *E. coli* (8.2 log-cycles). According to literature, one hypothesis could explain the effects of the sequence in the combined PEF and HPU treatments. PEF technology applied as a first hurdle has demonstrated its ability to exert sublethal injuries in the surviving population of microorganisms by damaging the membranes (Mañas & Pagán, 2005), making the microbial cells more sensitive to the subsequent treatment. On the contrary, several authors (Barbosa-Cánovas, Tapia, & Cano, 2005; Mañas & Pagán, 2005; Walkling-Ribeiro, Noci, Riener, et al., 2009) did not detected sublethal injuries in the surviving cells after HPU treatments and described the cavitation effect on inactivation as an "all or nothing" phenomenon, where the most sensitive cells were inactivated, leaving the remaining most resistant cells intact for the subsequent treatment. Thus, lethal synergistic effects should not be expected when HPU is applied as a first hurdle to inactivate vegetative cells.

Only three references were found comparing the influence of the order of application of PEF and HPU treatments on microbial inactivation. On the one hand, Lyu et al. (2016) and Huang et al. (2006) found a similar reduction for both combinations. However, Huang et al. (2006), found no effect on inactivation when the PEF treatment was applied alone; thus, when comparing the sequences of the combined treatments, similar reductions were observed. On the other hand, Palgan et al. (2012) reported that the highest inactivation level was found if the HPU treatment was applied before the PEF (HPU-PEF), conversely to the results of the present study. However, the aforementioned analyses used different media and microorganisms (*S. cerevisiae* in rice wine (Lyu et al., 2016), *S. enteritidis* in liquid whole egg (Huang et al., 2006) and *L. innocua* in a milk-based beverage (Palgan et al., 2012)), indicating that the exact inactivation mechanisms of the combined PEF and HPU treatments is still unclear and the effect of the order of the application of these technologies might depend on the type of microorganism and the food matrix.

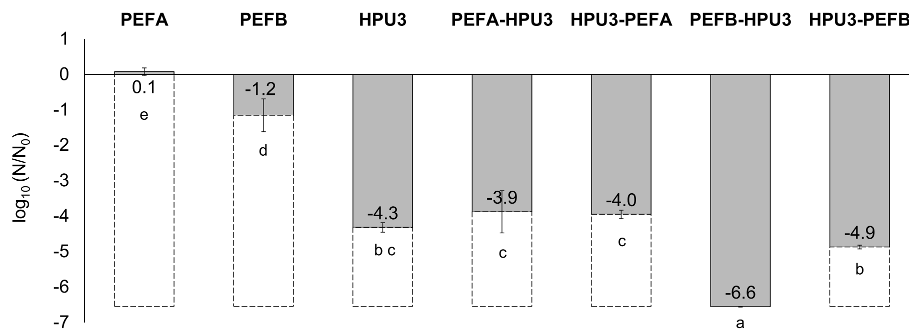

Fig. 4. Inactivation of *A. niger* spores in oil-in-water emulsion treated by PEF at 50 Hz and 25 °C of inlet temperature (PEFA: 30 kV/cm, 170 μs of treatment time, 5 μs of pulse width and 76.3 kJ/kg of total energy and PEFB: 32.3 kV/cm, 340 μs of treatment time, 10 μs of pulse width and 176.3 kJ/kg of total energy), HPU for 3 min (HPU3) and its combination. Dashed lines indicate complete inactivation. Letters show homogeneous groups established from LSD intervals (95%).

3.4. *A. niger* inactivation in emulsions using a combined PEF and HPU treatment

In Fig. 4, the log reductions of *A. niger* spores after the individual and combined PEF and HPU treatments are shown. No inactivation of *A. niger* spores was found after the PEF treatment at 30 kV/cm, a pulse width of 5 μs and an inlet temperature of 25 °C (76.3 kJ/kg of total energy, PEFA). However, increasing the field strength to 32.3 kV/cm and the pulse width to 10 μs (176.3 of total energy, PEFB) significantly ($p < 0.05$) affected the inactivation of *A. niger* spores, since a reduction of 1.2 log-cycles was observed (Fig. 4). *E. coli* treated by PEF at similar total energy levels (78.3 and 176.3 kJ/kg) led to reductions of 1.5 and 2.6 log-cycles, respectively (Table 2), which demonstrates the greater resistance to PEF treatments of *A. niger* spores than *E. coli*. The application of HPU3 led to an inactivation of 4.3 log-cycles (Fig. 4) of the *A. niger* population while only 1.7 log-cycles were reduced in the same length of thermal treatment at 60 °C (Fig. 2B). Therefore, as found for *E. coli*, the inactivation obtained by HPU for 3 min was mainly due to cavitation effects and not to heat. As in the PEF treatments, *A. niger* spores were more resistant to HPU treatment than *E. coli* cells. This greater resistance is linked to the multi-layered cell wall of *Aspergillus* spores, with a different composition and structure to the bacteria cell wall. Specifically, the *A. niger* cell wall is covered by proteins (hydrophobins), which confer a high degree of hydrophobicity to the cell wall protecting the spore (Tischler & Hohl, 2019). Under the protein layer, there is a dense layer composed of melanin, which is known to increase the cell wall rigidity and make the spore remain turgid when exposed to an external stress (Gow, Latge, & Munro, 2017). Therefore, the characteristics of the wall of the *A. niger* spores could be responsible for its greater resistance to electroporation and ultrasonic mechanical stress than the vegetative bacteria.

On the one hand, the application of PEF at 76.3 kJ/kg followed by HPU3 (PEFA-HPU3) did not lead to any beneficial effects on *A. niger* inactivation, compared to the HPU3 treatment alone ($p > 0.05$) (Fig. 4). Non-significant ($p > 0.05$) differences in the level of inactivation were also obtained when the reverse sequence (HPU3-PEFA) was applied. Thereby, not only was the PEF treatment at low energy (PEFA), unable to inactivate *A. niger* spores, but neither did it seem to increase the spores' susceptibility to the subsequent treatment. On the other hand, the combination of high-energy PEF (PEFB 176.3 kJ/kg) followed by HPU3 (PEFB-HPU3) led to the complete inactivation of *A. niger* (6.6 log-cycles), reaching a higher degree of inactivation than the sum of each individual treatment (5.5 log-cycles). Thus, a synergistic effect on inactivation was found, which could be explained by considering that PEF with an energy of 176.3 kJ/kg (PEFB) as a pre-treatment was intense enough to make the cell structure of the fungal spore more sensitive to the subsequent HPU3 treatment. On the contrary, the HPU3-PEFB combination (4.9 log-cycles) did not significantly ($p > 0.05$) increase the inactivation level reached by the individual HPU3 (4.3 log-cycles). This once again illustrates that HPU application as a first hurdle does not lead to sublethal injuries in the microbial cells, as has also been observed for *E. coli* and reported previously.

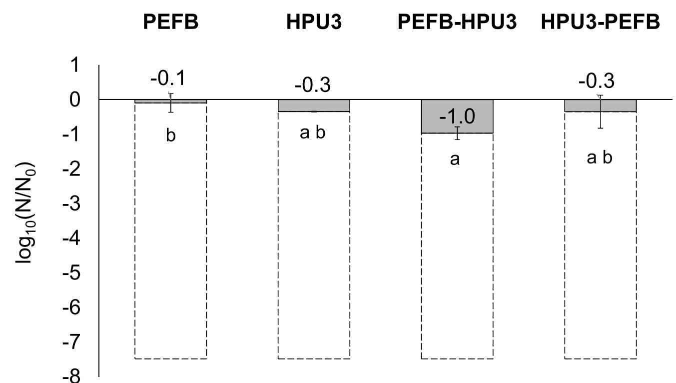

Fig. 5. Inactivation of *B. pumilus* spores in oil-in-water emulsion treated by PEF at 50 Hz and 25 °C of inlet temperature, 32.3 kV/cm, 340 μs of treatment time, 10 μs of pulse width and 152.3 kJ/kg of total energy (PEFB), HPU for 3 min (HPU3) and its combination. Dashed lines indicate complete inactivation. Letters show homogeneous groups established from LSD intervals (95%).

3.5. *B. pumilus* inactivation in emulsions using a combined PEF and HPU treatment

The reduction of *B. pumilus* spores after the individual and combined PEF and HPU treatments is shown in Fig. 5. PEF treatment at 152.3 kJ/kg (PEFB) was not able to inactivate *B. pumilus* spores (0.1 log-cycles reduction). Some authors also studied the PEF inactivation in bacterial spores and either found no effect at all or a very limited one (Devlieghere, Vermeiren, & Debevere, 2004; Heinz, Alvarez, Angersbach, & Knorr, 2001; Noci et al., 2009). As an example, Spilimbergo et al. (2003) found an inactivation of only 0.5 log-cycles when treating *B. cereus* spores in water at room temperature, 25 kV/cm, and 20 pulses applied at 5 s intervals. The application of HPU3, where a peak temperature of 90 °C was reached, led to a reduction of 0.3 log-cycles. However, the 3 min thermal treatment at 90 °C led to a greater reduction (0.6 log-cycles). Therefore, it could be assumed that the inactivation achieved by HPU3 could be mainly associated with the thermal effect. Bacterial spores seem very resistant to cavitation, as reported by previous studies. Fan et al. (2019) required 40 min of HPU at 20 W/mL and 80 °C to achieve a 2.4 log-cycle inactivation of *B. subtilis* spores and Evelyn and Silva (2018) needed 60 min at 0.33 W/g and 75 °C for a reduction of <0.3 and 1.0 log-cycles of *B. cereus* and *C. perfringens* spores, respectively, in beef slurry. The extreme resistance of bacterial spores to PEF and HPU treatments is attributed to the highly resistant mechanical properties of the spore structure (Fan et al., 2019; Reineke &

Mathys, 2020). The spore structure is markedly different from that of the corresponding vegetative cells. Among other things, the main differences are the number of both the layers and constituents of the spore, the dramatic dehydration and the less fluid membrane and cytoplasm, which confer great resistance to different inactivation treatments on the bacterial spore (Black et al., 2007; Feofilova, Ivashechkin, Alekhin, & Sergeeva, 2012; Ishihara, Saito, & Takano, 1999).

As for the vegetative bacteria and the fungal spore, the sequence of the treatments significantly ($p < 0.05$) affected the inactivation level of *B. pumilus* spores, being more effective when PEF was carried out before the HPU treatment (Fig. 5). Non-significant ($p > 0.05$) differences in inactivation were found between the combined HPU3-PEF treatment and the individual ones, and the level of inactivation of the HPU3-PEF treatment was lower (0.3 log-cycles) than the sum of the two individual treatments ($0.3 + 0.1 = 0.4$ log-cycles), as also observed for *E. coli* and *A. niger*. On the contrary, the combined PEFB-HPU3 treatment showed a synergetic effect on the inactivation of *B. pumilus* spores (Fig. 5), since the achieved inactivation (1 log-cycles) was higher than the addition of the individual treatments (0.4 log-cycles). Nevertheless, the level of reduction was low and, therefore, not satisfactory for pasteurization purposes. No studies have been found assessing the effect of the combined PEF and HPU treatments on bacterial spores; therefore, it would be interesting to assess their effect on the inactivation of bacterial spores other than *B. pumilus*. Moreover, as the microbial population in contaminated food and pharma products is usually made up of a 'cocktail' of microorganisms, the resistance of the different microorganisms to the studied inactivation treatments could be affected due to competing aspects between them. Therefore, the results from the present work could not be extrapolated to these 'cocktails' and additional experiments should be performed to evaluate the resistance of the whole mix of microorganisms to the PEF and HPU inactivation treatments. In addition, the combination of PEF and HPU with other emerging non-thermal technologies, such as cold plasma (Liao et al., 2019) or high hydrostatic pressures (Black et al., 2007), could be of great interest.

4. Conclusions

If applied individually for the purposes of inactivating vegetative bacteria and fungal and bacterial spores in oil-in-water emulsions, PEF and HPU treatments only achieved moderate or negligible levels of reduction. The combined HPU-PEF treatment led to lower inactivation levels than that produced by the addition of the individual treatments. On the contrary, the reverse treatment (PEF-HPU) led to there being synergistic effects on the level of inactivation, achieving complete inactivation for *E. coli* and *A. niger* spores. Therefore, the combined PEF-HPU treatment has shown itself to be a promising means of inactivating vegetative bacteria and fungal spores. However, it does not seem feasible for the inactivation of bacterial spores, at least for *B. pumilus*. Further studies should address the impact of the combined treatments on different species of bacterial spores, microorganisms isolated from food and different treatment media must be tested. In addition, future research should analyze the effect of the combined PEF and HPU treatments on the physicochemical properties and stability of the emulsions.

CRediT authorship contribution statement

A. Gomez-Gomez: Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **E. Brito-de la Fuente:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Funding acquisition. **C. Gallegos:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Funding acquisition. **J.V. Garcia-Perez:** Conceptualization, Methodology, Validation, Data curation, Writing – original draft, Writing – review & editing, Supervision. **J. Benedito:** Conceptualization, Methodology, Validation, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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