Departamento de Tecnología de Alimentos

UNIVERSITAT
POLITÈCNICA
DE VALÈNCIA

PASTEURIZACIÓN DE EMULSIONES LIPÍDICAS CON CO_2 SUPERCRÍTICO Y ULTRASONIDOS DE POTENCIA

PhD THESIS

Submitted by:

Ángela Gomez Gomez

PhD Supervisors:

Dr. Jose Javier Benedito Fort

Dr. Jose Vicente García Pérez

Valencia, 2021

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTAMENTO DE TECNOLOGÍA DE ALIMENTOS

D. JOSE JAVIER BENEDITO FORT Y D. JOSE VICENTE GARCÍA PÉREZ, AMBOS CATEDRÁTICOS DE UNIVERSIDAD DEL DEPARTAMENTO DE TECNOLOGÍA DE ALIMENTOS DE LA UNIVERSITAT POLITÈCNICA DE VALÈNCIA

CERTIFICAN:

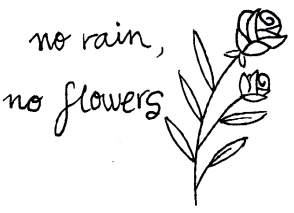
Que la memoria titulada “PASTEURIZACIÓN DE EMULSIONES LIPÍDICAS CON CO_2 SUPERCRÍTICO Y ULTRASONIDOS DE POTENCIA”, presentada por Dña. Ángela Gómez Gómez para aspirar al grado de Doctora en Ciencia, Tecnología y Gestión Alimentaria y realizada bajo nuestra dirección en el Departamento de Tecnología de Alimentos de la Universitat Politècnica de València, cumple las condiciones adecuadas para su aceptación como Tesis Doctoral, por lo que

AUTORIZAN:

A la interesada a su presentación en el Departamento de Tecnología de Alimentos de la Universitat Politècnica de València.

Y para que conste a los efectos oportunos, presentamos la referida memoria firmando el presente certificado en Valencia, 2020.

Fdo. Dr. D. Jose Javier Benedito Fort Fdo. Dr. D. Jose Vicente García Pérez


no rain,
no flowers

Agradecimientos

Es hora de escribir lo más importante, con diferencia significativa ($p<0.05$), de esta Tesis Doctoral: los agradecimientos.

En primer lugar, quiero dar las gracias a mis directores. A José Vicente, por haberme ofrecido la oportunidad de trabajar en el laboratorio ASPA y apoyarme y a José Benedito, por estar siempre ahí, dispuesto a ayudar y por hacer que sea posible el desarrollo de esta tesis.

Gracias a Críspulo y Edmundo, de Fresenius, por vuestra colaboración, apoyo y cercanía.

Al Food Team: Marina, Lola, Virginia, Blanca y Beatriu, porque hemos tenido el "problema" de llevarnos demasiado bien y puede que algunos días hayamos cotorreado más que redactado. Sin vosotras no me lo imagino. Por esos "Tú lo que necesitas es que bajemos a por una flauta de chocolate para que se te pase el disgusto" que hacen que te cambie el día. ¡Me encantáis! Y quiero destacar a Marina, porque desde que nos conocimos no nos hemos separado. Gracias por escuchar siempre tan atenta mis preocupaciones y dar buenos consejos, aunque sea después de esperar un buen rato a que pares de reírte. Tu serenidad, llámala "pachorra en cantidades industriales", yo la necesito. Gracias por compartirla.

A los profesores del grupo ASPA y a todos las personas que han formado parte de él estos años (Ingrid, Nelson, Malike, Paolo, Rubén, Vivian, Paola, Mainé, Alejandro, Nieves…). Por esas salidas a almorzar, dirigidas por Juan, que alegran la mañana. Gracias a Ramón, por arreglar los equipos en tiempo récord. Siento que hayas tenido que tocar de vez en cuando algún que otro bichito.

A Amparo Quiles, que me lleva acompañando desde que empecé la carrera. Gracias por tu apoyo. Haces que trabajar sea tan agradable como tomarse un café con una amiga. A Ana Fuentes y Edgar, porque siempre habéis estado dispuestos a ayudarme.

A mis padres y a las tatitas, por darme consejos: "tú con calma, como ya dijo Daddy Yankee" y proponer soluciones a los problemas, sin duda, muy efectivas: "¿…y has probado a matar a las bacterias con lejía?" Gracias por vuestro apoyo incondicional. ¡Os quiero!

A Edu, porque siempre haces que lo difícil sea más fácil. Gracias por creer más en mí que yo misma.

-Ángela-

ABSTRACT / RESUMEN / RESUM

PASTEURIZATION OF LIPID EMULSIONS WITH SUPERCRITICAL CO_2 AND HIGH POWER ULTRASOUND

Abstract

Oil-in-water emulsions are widely used in the food, pharmaceutical and cosmetics industries. Specifically, lipid emulsions are produced for intravenous nutrition and, consequently, its microbial and physicochemical stability plays a key role. Thermal treatments, usually performed in steam autoclaves at 121ºC and 15-20 min, are generally used for the sterilization in lipid emulsions. However, heating has demonstrated its ability to induce the hydrolysis of lipids and lecithin, which lowers the pH and increases the droplet size. In this sense, non-thermal technologies are emerging in the industry with the aim of achieving microbial stability while avoiding the loss of quality related to heat in thermally treated products. Supercritical carbon dioxide (SC-CO_2) and pulsed electric fields (PEF) are promising non-thermal processing technologies for microbial inactivation. However, these techniques have demonstrated to require high treatment intensities and/or long treatment times to guarantee the product's safety, especially for the inactivation of highly resistant forms of microorganisms (such as spores), or for microorganisms located in complex protecting media (such as oil-based products), which could lead to changes in the physicochemical properties of the processed products, higher costs and greater environmental impact. Therefore, there is still room for the improvement in the use of these novel technologies, in terms of process intensification. Literature has illustrated the capacity of high power ultrasound (HPU) for the intensification of mass and/or heat transfer phenomena. Therefore, its application to non-thermal technologies could be an interesting approach to enhance the microbial inactivation effectiveness.

In this context, the main objective of this PhD Thesis was to evaluate the effect of SC-CO_2, PEF and HPU treatments, applied in individual and combined form, on the inactivation of different microorganisms in oil-in-water emulsions. In order to achieve this goal, on the one hand, the influence of the implementation of HPU to the SC-CO_2 treatments was studied on different types of microorganisms and on media with

different oil content. On the other hand, the effect of the individual and combined PEF and HPU treatments was assessed on different microorganisms.

For that purpose, firstly, the effect of HPU application on SC-CO_2 was studied for the inactivation of two vegetative bacteria (*Escherichia coli* and *Brevundimonas diminuta)* and one fungal spore (*Aspergillus niger*) in a simple medium (water). The SC-CO_2 + HPU treatments were performed at different pressures (100 and 350 bar) and temperatures (35, 50 and 60ºC). Moreover, the ultrastructure of the SC-CO_2 + HPU-inactivated cells was evaluated by microscopy techniques. Secondly, the SC-CO_2 and SC-CO_2 + HPU inactivation was also investigated in oil-in-water emulsions for different types of microorganisms, including vegetative bacteria (*E. coli* and *B. diminuta*), fungal spores (*A. niger*) and bacterial spores (*Clostridium butyricum*, *Bacillus subtilis, Bacillus pumilus* and *Geobacillus stearothermophilus*). The inactivation kinetics were obtained at different pressures (100, 350 and 550 bar) and temperatures (35, 50, 60, 70, 80, 85 and 95ºC). In addition, the effect of the oil content (0, 10, 20 and 30%) in the treating media was investigated for the SC-CO_2 and SC-CO_2 + HPU inactivation of the vegetative bacteria (*E. coli* and *B. diminuta*). Finally, the effect of the SC-CO_2 + HPU treatment conditions (temperature, pressure and time) on the physicochemical properties of the emulsions (appearance, pH, density, droplet size and ζ-potential) was explored using response surface methodology. Thereby, the process conditions that led to a satisfactory microbial inactivation while minimizing the physicochemical changes in the emulsions were sought.

As for PEF and HPU treatments in the 20% oil-in-water emulsions, firstly, experiments for *E.coli* inactivation were performed. PEF treatments were carried out at different energies (from 41.5 to 176.3 kJ/kg) and input temperatures (15 and 25ºC). Moreover, HPU treatments were carried out at two processing times (2 and 3 min). The most effective conditions were selected to assess the effect of the individual and the combined treatments on the inactivation of *E. coli, A. niger* and *B. pumilus* spores. The effect of the sequence of application on the combined treatments (PEF-HPU or HPU-PEF) was studied for the inactivation of the three types of microorganisms.

Experimental results showed that the application of HPU greatly intensified the inactivation capacity of SC-CO_2, shortening the process time for almost all microorganisms and media. For example, for the vegetative bacteria in the 10, 20 and 30% oil content emulsions, the time required for the complete inactivation was shortened by approximately 1 order of magnitude when HPU was applied. HPU probably enhanced the solubilization of CO_2 into the medium and provoked damages in the cell wall, facilitating the penetration of SC-CO_2 into the cells. In this regard, the microscopy analysis of the inactivated vegetative bacteria and fungal spores revealed important morphological changes in the cells, including damaged cell walls, and an important alteration and loss of the cytoplasmic content. Therefore, the combined SC-CO_2 + HPU treatment demonstrated to be effective for microbial inactivation, despite the complexity of the cell wall. Nevertheless, HPU did not improved the SC-CO_2 inactivation of *A. niger* spores in emulsion. For example, at 350 bar and 60ºC, the complete inactivation of the *A.niger* spores was achieved in 10 min, regardless of the use of HPU.

The increase of the pressure in both, the SC-CO_2 and SC-CO_2 + HPU treatments, led to higher inactivation levels, except for *E. coli* in water, where no effect of pressure was found. However, pressures above 350 bar did not seem to exert any additional inactivation, probably because an increase in pressure barely improved the solubility of SC-CO_2.The increase of the temperature had a significant ($p<0.05$) effect for all treatments (thermal, SC-CO_2 and SC-CO_2 + HPU) and microorganisms. For example, in 20 min of thermal, SC-CO_2 and SC-CO_2 + HPU treatments, the inactivation of *B. subtilis* spores increased from 0.1 to 2.7 log-cycles, from 3.1 to 6.2 log-cycles and from 5.4 to 6.7 log-cycles, respectively, from using 85 to 95ºC.

The resistance of the microorganisms to the SC-CO_2 and SC-CO_2 + HPU inactivation treatments was, in order from the less to the most resistant: vegetative bacteria (*E. coli* and *B. diminuta*), fungal spores (*A. niger*), *Clostridium* spores (*C. butyricum)*, *Bacillus* spores (*B. subtilis* and *B. pumilus*) and lastly, *Geobacillus* spores (*G. stearothermophilus*), being the inactivation of the latter unfeasible using the technologies proposed in this work.

Regarding the effect of the medium on microbial inactivation, the presence of oil is known to protect microorganisms and usually more intense treatments are needed, as was observed in the SC-CO_2 inactivation of *E. coli* and *B. diminuta* in water and in emulsions with different oil content (10, 20 and 30 %). However, the application of HPU masked the protective effect exerted by the oil in the emulsions and minimized the differences in microbial inactivation. For example, in 50 min of a SC-CO_2 treatment at 350 bar and 35ºC, an inactivation of 7.4 log-cycles of *E. coli* was achieved in water (0% oil content), while inactivations of 3.4, 4.3 and 5.2 log-cycles were obtained in the 30, 20 and 10% oil content emulsions, respectively. However, in 5 min of SC-CO_2 + HPU treatment at 350 bar and 35ºC, an inactivation of 6.2-7.0 log-cycles of *E. coli* was obtained, regardless of the oil content in the media. On the contrary, for *A. niger* spores no effect of the media (water or the 20% emulsion) on the effectiveness of both, the SC-CO_2 and the SC-CO_2 + HPU inactivation treatments was found and, on average, an inactivation of 4.4 and 4.3 log-cycles were obtained in water or in the emulsion, respectively.

In relation to the effect of the SC-CO_2 + HPU treatments on the quality of the treated emulsions, in general terms, only a mild effect of the process conditions (temperature, pressure and time) was found on the physicochemical properties. Treated emulsions presented, in general, good appearance, minimal changes in density, a good electrostatic stability, a lower pH (from 8.4 to 5.1-5.6) and a larger average droplet size (D[4,3] from 0.365 to 0.338-7.996 µm; D[3,2]: from 0.343 to 0.320-1.505 µm). Hence, by the selection of suitable SC-CO_2 + HPU conditions (e.g. 95ºC, 600 bar and 12.5 min), minimal changes on the physicochemical properties of the emulsions and a satisfactory inactivation for all the studied microorganisms, except for *G. stearothermophilus* spores, can be obtained.

Therefore, SC-CO_2 + HPU technology could be a promising alternative to thermal pasteurization of emulsions in order to better preserve heat sensitive compounds since lower temperatures can be used. However, the process variables of the treatment should be evaluated and selected in order to maintain the quality of the emulsions while inactivating target microorganisms.

Regarding microbial inactivation of emulsions with PEF and HPU treatments, results revealed that the maximum inactivation achieved by the individual PEF treatment, at the highest energy level and input temperature (152.3-176.3 kJ/kg and 25ºC) was 2.6, 1.2 and 0.1 log-cycles for *E.coli, A. niger* and *B. pumilus*, respectively. In addition, the largest inactivation levels achieved by HPU for the longest treatment studied (3 min), were 5.4, 4.3 and 0.3 log-cycles for *E.coli, A. niger* and *B. pumilus*, respectively. Therefore, the complete microbial inactivation in the emulsions was not achieved with the individual treatments. However, when the PEF treatment (152.3-176.3 kJ/kg) was followed by the HPU treatment (3 min), inactivation levels of 8.2, 6.6 and 1.0 log-cycles were obtained for *E. coli*, *A. niger* and *B. pumilus*, respectively, corresponding to the complete *E. coli* and *A. niger* inactivation. Moreover, the combined PEF-HPU treatment presented a synergistic effect, since the inactivation reached was higher than the sum of the individual treatments for all microorganisms. Despite this fact, the inactivation achieved for the bacterial spore (*B. pumilus*) was very limited. On the contrary, the inactivation levels achieved by the reverse treatment (HPU followed by PEF) were lower than the sum of the individual treatments. Thus, the most effective sequence for the combined treatment was the one in which PEF was followed by HPU. In this regard, PEF treatment probably exerted sublethal effects on microorganisms, which made microbial cells more sensitive to the subsequent HPU treatment. Therefore, the combined PEF-HPU treatment demonstrated to be a promising hurdle technology to inactivate vegetative bacteria or fungal spores in emulsions. However, its use was not feasible for bacterial spores in the conditions tested in the present work.

The resistance of the microorganisms to the PEF and HPU treatments, applied individually or in combination, followed the same order found for the SC-CO_2 treatments, being the vegetative bacteria the most sensitive, followed by the fungal spores and lastly, the bacterial spores.

Finally, it can be concluded that, the combination of HPU with SC-CO_2 or PEF generally improved the microbial inactivation. Moreover, in some cases, it was demonstrated that the combination of the treatments achieved synergistic effects. Consequently, reasonable industrial processing times and mild process conditions

could be selected by the use of the studied combined techniques, which could result into a reduction in the processing cost and a lower impact on the quality of the oil-in-water emulsions. However, additional work should be addressed in order to better understanding the inactivation mechanisms exerted by combined SC-CO_2 and HPU treatments and the combined PEF and HPU treatments, especially on fungal and bacterial spores. Moreover, additional research is recommended on the effect of these non-thermal combined technologies on the physicochemical properties of the treated products, in particular for the combined PEF and HPU treatments, which was not covered in the present study.

PASTEURIZACIÓN DE EMULSIONES LIPÍDICAS CON CO_2 SUPERCRÍTICO Y ULTRASONIDOS DE POTENCIA

Resumen

Las emulsiones aceite-en-agua son ampliamente utilizadas en la industria alimentaria, farmacéutica y cosmética. En particular, las emulsiones lipídicas se elaboran para nutrición intravenosa y, en consecuencia, su estabilidad microbiana y fisicoquímica desempeña un papel muy importante. En general, para la esterilización de emulsiones lipídicas se usan tratamientos térmicos, normalmente llevados a cabo en autoclaves de vapor a 121ºC y 15-20 min. Sin embargo, el calentamiento ha demostrado ser capaz de provocar la hidrólisis de lípidos y lecitina, dando lugar a una bajada del pH y a un aumento del tamaño de gota. En este sentido, están surgiendo tecnologías no térmicas en la industria, con el objetivo de llegar a la estabilidad microbiana evitando la pérdida de calidad relacionada con el calor de los productos tratados térmicamente. El dióxido de carbono supercrítico (SC-CO_2) y los campos eléctricos pulsados (PEF) son tecnologías de procesado prometedoras para la inactivación microbiana. Sin embargo, se ha demostrado que estas técnicas requieren altas intensidades y/o largos tiempos de tratamiento para garantizar la seguridad del producto, especialmente para la inactivación de formas altamente resistentes de los microorganismos (como son las esporas), o para microorganismos presentes en medios complejos protectores (como los productos con aceite), lo que podría provocar cambios en las propiedades fisicoquímicas de los productos procesados, mayores costes y un mayor impacto medioambiental. Por lo tanto, todavía existe margen de mejora con respecto al uso de estas nuevas tecnologías en términos de intensificación del proceso. La bibliografía ha descrito la capacidad de los ultrasonidos de potencia (HPU) para intensificar los fenómenos de transferencia de masa y/o calor. Por lo tanto, su aplicación a tecnologías no térmicas podría ser un enfoque interesante para mejorar la eficacia de la inactivación microbiana.

En este contexto, el objetivo principal de esta Tesis Doctoral fue evaluar el efecto de los tratamientos de SC-CO_2, PEF y HPU, aplicados de forma individual y

combinada, sobre la inactivación de diferentes microorganismos en emulsiones aceite-en-agua. Para lograr este objetivo, por una parte, se estudió la influencia de la implementación de HPU en los tratamientos de SC-CO_2 sobre diferentes tipos de microorganismos y sobre medios con diferente contenido en aceite. Por otra parte, se evaluó el efecto de los tratamientos PEF y HPU individuales y combinados sobre diferentes microorganismos.

Para ello, en primer lugar, se estudió el efecto de la aplicación de HPU sobre la inactivación con SC-CO_2 de dos bacterias vegetativas (*Escherichia coli* y *Brevundimonas diminuta*) y una espora fúngica (*Aspergillus niger*), en un medio simple (agua). Los tratamientos con SC-CO_2 + HPU se realizaron a diferentes presiones (100 y 350 bar) y temperaturas (35, 50 y 60ºC). Además, se evaluó la ultraestructura de las células inactivadas mediante SC-CO_2 + HPU con técnicas de microscopía. En segundo lugar, también se investigó la inactivación con SC-CO_2 y SC-CO_2 + HPU en emulsiones aceite-en-agua para diferentes tipos de microorganismos, incluyendo bacterias vegetativas (*E. coli* y *B. diminuta*), esporas fúngicas (*A. niger*) y esporas bacterianas (*Clostridium butyricum, Bacillus subtilis, Bacillus pumilus* y *Geobacillus stearothermophilus*). Las cinéticas de inactivación se obtuvieron a diferentes presiones (100, 350 y 550 bar) y temperaturas (35, 50, 60, 70, 80, 85 y 95ºC). Además, se investigó el efecto del contenido en aceite (0, 10, 20 y 30%) en los medios de tratamiento sobre la inactivación con SC-CO_2 y SC-CO_2 + HPU de bacterias vegetativas (*E. coli* y *B. diminuta*). Finalmente, se exploró el efecto de las condiciones del tratamiento SC-CO_2 + HPU (temperatura, presión y tiempo) sobre las propiedades fisicoquímicas de las emulsiones (apariencia, pH, densidad, tamaño de gota y potencial-ζ) utilizando la metodología de superficie de respuesta. De este modo, se buscaron las condiciones del proceso que condujeran a una inactivación microbiana satisfactoria y, al mismo tiempo, a los mínimos cambios fisicoquímicos en las emulsiones.

En cuanto a los tratamientos de PEF y HPU en las emulsiones de aceite-en-agua al 20%, en primer lugar, se realizaron ensayos para la inactivación de *E. coli*. Los tratamientos con PEF se llevaron a cabo a diferentes niveles de energía (de 41.5 a 176.3 kJ/kg) y temperatura de entrada (15 y 25ºC). Además, los tratamientos con HPU

se realizaron a dos tiempos de procesado (2 y 3 min). Se seleccionaron las condiciones más efectivas para evaluar el efecto de los tratamientos individuales y combinados sobre la inactivación de *E. coli* y de esporas de *A. niger* y *B. pumilus*. También se estudió el efecto de la secuencia de aplicación en los tratamientos combinados (PEF-HPU o HPU-PEF) sobre la inactivación de los tres tipos de microorganismos.

Los resultados experimentales mostraron que la aplicación de HPU intensificó en gran medida la capacidad de inactivación del SC-CO_2, acortando el tiempo del proceso para casi todos los microorganismos y medios. Por ejemplo, para las bacterias vegetativas, en las emulsiones con un contenido en aceite del 10, 20 y 30%, se acortó el tiempo requerido para la inactivación completa en aproximadamente 1 orden de magnitud al aplicar HPU. Probablemente, los HPU aumentaron la solubilización del CO_2 en el medio y provocaron daños en la pared celular, facilitando la penetración del SC-CO_2 en las células. En este sentido, el análisis de la microscopía de las bacterias vegetativas y las esporas fúngicas inactivadas reveló importantes cambios morfológicos en las células, incluyendo paredes celulares dañadas, y una importante alteración y pérdida del contenido citoplasmático. Por lo tanto, el tratamiento combinado SC-CO_2 + HPU demostró ser eficaz para la inactivación microbiana, a pesar de la complejidad de la pared celular. Sin embargo, los HPU no mejoraron la inactivación con SC-CO_2 de las esporas de *A. niger* en la emulsión. Por ejemplo, a 350 bar y 60ºC, la inactivación completa de las esporas de *A. niger* se alcanzó en 10 min, independientemente del uso de HPU.

El aumento de la presión, tanto en los tratamientos con SC-CO_2 como con SC-CO_2 + HPU, dio lugar a mayores niveles de inactivación, a excepción de *E. coli* en agua, donde no se encontró efecto de la presión. Sin embargo, las presiones por encima de 350 bar no parecieron conllevar a una inactivación adicional, probablemente porque un aumento en la presión apenas mejoró la solubilidad del SC-CO_2. El aumento de la temperatura tuvo un efecto significativo ($p<0.05$) para todos los tratamientos (térmicos, SC-CO_2 y SC-CO_2 + HPU) y microorganismos. Por ejemplo, en 20 min de tratamientos térmicos, SC-CO_2 y SC-CO_2 + HPU, la inactivación de las esporas de *B. subtilis* aumentó de 0.1 a 2.7 ciclos-log, de 3.1 a 6.2 ciclos-log y de 5.4 a 6.7 ciclos-log, respectivamente, de usar 85 a 95ºC.

La resistencia de los microorganismos a los tratamientos de inactivación con SC-CO_2 y SC-CO_2 + HPU fue, en orden de menos a más resistente: bacterias vegetativas (*E. coli y B. diminuta*), esporas fúngicas (*A. niger*), esporas de *Clostridium* (*C. butyricum*), esporas de *Bacillus* (*B. subtilis* y *B. pumilus*) y por último, esporas de *Geobacillus* (*G. stearothermophilus*), siendo la inactivación de estas últimas inviable utilizando las tecnologías propuestas en el presente trabajo.

En cuanto al efecto del medio sobre la inactivación microbiana, se sabe que la presencia de aceite protege a los microorganismos y, generalmente, se necesitan tratamientos más intensos, como se observó en la inactivación con SC-CO_2 de *E. coli* y *B. diminuta* en agua y en las emulsiones con diferente contenido en aceite (10, 20 y 30 %). Sin embargo, la aplicación de HPU enmascaró el efecto protector ejercido por el aceite de las emulsiones y minimizó las diferencias en la inactivación microbiana. Por ejemplo, en 50 min del tratamiento con SC-CO_2 a 350 bar y 35ºC, se inactivaron 7.4 ciclos-log de *E. coli* en agua (0% de contenido en aceite), mientras que se inactivaron 3.4, 4.3 y 5.2 ciclos-log en las emulsiones con un 30, 20 y 10% de contenido en aceite, respectivamente. Sin embargo, en 5 min de tratamiento con SC-CO_2 + HPU a 350 bar y 35ºC, se obtuvo una inactivación de 6.2-7.0 ciclos-log para *E. coli*, independientemente del contenido en aceite en el medio. Por el contrario, para las esporas de *A. niger* no se encontró efecto del medio (agua o emulsión al 20%) sobre la efectividad de ambos tratamientos de inactivación, SC-CO_2 y SC-CO_2 + HPU, y, en promedio, se obtuvo una inactivación de 4.4 y 4.3 ciclos-log en agua o en la emulsión, respectivamente.

En relación al efecto de los tratamientos con SC-CO_2 + HPU sobre la calidad de las emulsiones tratadas, en términos generales, solo se encontró un efecto leve de las condiciones del proceso (temperatura, presión y tiempo) sobre las propiedades fisicoquímicas. Las emulsiones tratadas presentaron, en general, buen aspecto, cambios mínimos en la densidad, buena estabilidad electrostática, un pH más bajo (de 8.4 a 5.1-5.6) y un mayor tamaño medio de gota (D[4,3] de 0.365 a 0.338-7.996 µm; D[3.2]: de 0.343 a 0.320-1.505 µm). Por lo tanto, mediante la selección de condiciones de SC-CO_2 + HPU adecuadas (por ejemplo, 95ºC, 600 bar y 12.5 min), se pueden obtener cambios mínimos en las propiedades fisicoquímicas de las emulsiones y una

inactivación satisfactoria para todos los microorganismos estudiados, excepto para las esporas de *G. stearothermophilus*.

Por lo tanto, la tecnología SC-CO_2 + HPU podría ser una alternativa prometedora a la pasteurización térmica de emulsiones que permitiría preservar mejor los compuestos sensibles al calor, ya que se pueden utilizar temperaturas más bajas. Sin embargo, las variables de proceso del tratamiento deben evaluarse y seleccionarse para mantener la calidad de las emulsiones al tiempo que se inactivan los microorganismos diana.

En cuanto a la inactivación microbiana de emulsiones con tratamientos de PEF y HPU, los resultados revelaron que la inactivación máxima alcanzada por el tratamiento individual de PEF, al mayor nivel de energía y temperatura de entrada (152.3-176.3 kJ/kg y 25ºC) fue de 2.6, 1.2 y 0.1 ciclos-log para *E. coli, A. niger y B. pumilus,* respectivamente. Además, los mayores niveles de inactivación alcanzados por HPU para el tratamiento más largo estudiado (3 min), fueron 5.4, 4.3 y 0.3 ciclos-log para *E. coli, A. niger* y *B. pumilus*, respectivamente. Por lo tanto, la inactivación microbiana completa en las emulsiones no se logró con los tratamientos individuales. Sin embargo, cuando el tratamiento de PEF (152.3-176.3 kJ/kg) fue seguido por el tratamiento de HPU (3 min), los niveles de inactivación obtenidos fueron de 8.2, 6.6 y 1.0 ciclos-log para *E. coli, A. niger* y *B. pumilus*, respectivamente, correspondiendo a la inactivación completa de *E. coli* y *A. niger*. Además, el tratamiento combinado PEF-HPU presentó un efecto sinérgico, ya que la inactivación alcanzada fue mayor que la suma de los tratamientos individuales para todos los microorganismos. A pesar de este hecho, la inactivación lograda para la espora bacteriana (*B. pumilus*) fue muy limitada. Por el contrario, los niveles de inactivación alcanzados por el tratamiento inverso (HPU seguido de PEF) fueron inferiores a la suma de los tratamientos individuales. Por lo tanto, la secuencia más eficaz para el tratamiento combinado fue aquella en la cual los PEF fueron seguidos de los HPU. En este sentido, el tratamiento PEF probablemente ejerció efectos subletales sobre los microorganismos, haciendo que las células microbianas estuvieran más sensibles al tratamiento posterior de HPU. Por lo tanto, el tratamiento combinado PEF-HPU demostró ser una tecnología prometedora para inactivar bacterias vegetativas o esporas fúngicas en emulsiones. No obstante, su uso

no fue efectivo para las esporas bacterianas en las condiciones probadas en el presente trabajo.

La resistencia de los microorganismos a los tratamientos de PEF y HPU, aplicados individualmente o en combinación, siguió el mismo orden que el encontrado para los tratamientos de SC-CO_2, siendo las bacterias vegetativas las más sensibles, seguidas de las esporas fúngicas y por último, de las esporas bacterianas.

Finalmente, se puede concluir que, la combinación de HPU con SC-CO_2 o PEF generalmente mejoró la inactivación microbiana. Además, en algunos casos, se demostró que la combinación de los tratamientos logró efectos sinérgicos. En consecuencia, mediante el uso de las técnicas combinadas estudiadas, se podrían seleccionar tiempos de procesamiento industriales razonables y condiciones de proceso suaves, que podría resultar en una reducción en el coste de procesamiento y un menor impacto en la calidad de las emulsiones aceite-en-agua. Sin embargo, para comprender mejor los mecanismos de inactivación ejercidos por los tratamientos combinados de SC-CO_2 y HPU y los tratamientos combinados de PEF y HPU, es necesario realizar un trabajo en mayor profundidad, especialmente en el caso de esporas fúngicas y bacterianas. Además, se recomienda investigar más acerca del efecto de estas tecnologías no térmicas combinadas sobre las propiedades fisicoquímicas de los productos tratados, en particular para los tratamientos combinados de PEF y HPU, aspecto que no ha sido abordado en el presente estudio.

PASTEURITZACIÓ D'EMULSIONS LIPÍDIQUES AMB CO_2 SUPERCRÍTIC I ULTRASONS DE POTÈNCIA

Resum

Les emulsions oli-en-aigua són àmpliament utilitzades en la indústria alimentària, farmacèutica i cosmètica. Concretament, les emulsions lipídiques es produeixen per a nutrició intravenosa i, en conseqüència, la seua estabilitat microbiana i fisicoquímica exerceix un paper important. En general, per a l'esterilització d'emulsions lipídiques s'usen tractaments tèrmics, normalment duts a terme en autoclaus de vapor a 121°C i 15-20 min. No obstant això, el calfament ha demostrat ser capaç de provocar la hidròlisi de lípids i lecitina, donant lloc a una baixada del pH i a un augment de la grandària de gota. En aquest sentit, estan sorgint tecnologies no tèrmiques en la indústria, amb l'objectiu d'arribar a l'estabilitat microbiana mentre s'evita la pèrdua de qualitat dels productes tractats tèrmicament relacionada amb la calor. El diòxid de carboni supercrític (SC-CO_2) i els camps elèctrics polsats (PEF) són tecnologies de processament prometedores per a la inactivació microbiana. No obstant això, s'ha demostrat que aquestes tècniques requereixen altes intensitats i/o llargs temps de tractament per a garantir la seguretat del producte, especialment per a la inactivació de formes altament resistents de microorganismes (com són les espores), o per a microorganismes presents en medis complexos protectors (com els productes amb oli), que podria provocar canvis en les propietats fisicoquímiques dels productes processats, majors costos i un major impacte mediambiental. Per tant, encara hi ha marge de millora respecte a l'ús d'aquestes noves tecnologies en termes d'intensificació del procés. La bibliografia ha il·lustrat la capacitat dels ultrasons de potència (HPU) per a intensificar els fenòmens de transferència de massa i/o calor. Per tant, la seua aplicació a tecnologies no tèrmiques podria ser un enfocament interessant per a millorar l'eficàcia de la inactivació microbiana.

En aquest context, l'objectiu principal d'aquesta Tesi Doctoral va ser avaluar l'efecte dels tractaments de SC-CO_2, PEF i HPU, aplicats de manera individual i combinada, sobre la inactivació de diferents microorganismes en emulsions oli-en-

aigua. Per a aconseguir aquest objectiu, d'una banda, es va estudiar la influència de la implementació d'HPU en els tractaments de SC-CO_2 sobre diferents tipus de microorganismes i sobre medis amb diferent contingut en oli. D'altra banda, es va avaluar l'efecte dels tractaments PEF i HPU individuals i combinats sobre diferents microorganismes.

Per a això, en primer lloc, es va estudiar l'efecte de l'aplicació d'HPU sobre la inactivació amb SC-CO_2 de dos bacteris vegetatius (*Escherichia coli* i *Brevundimonas diminuta*) i una espora fúngica (*Aspergillus niger*) en un medi simple (aigua). Els tractaments amb SC-CO_2 + HPU es van realitzar a diferents pressions (100 i 350 bar) i temperatures (35, 50 i 60°C). A més, la ultraestructura de les cèl·lules inactivades mitjançant SC-CO_2 + HPU va ser avaluada amb tècniques de microscòpia. En segon lloc, la inactivació amb SC-CO_2 i SC-CO_2 + HPU també es va investigar en emulsions oli-en-aigua per a diferents tipus de microorganismes, incloent bacteris vegetatius (*E. coli* i *B. diminuta*), espores fúngiques (*A. niger*) i espores bacterianes (*Clostridium butyricum, Bacillus subtilis, Bacillus pumilus* i *Geobacillus stearothermophilus*). Les cinètiques d'inactivació es van obtindre a diferents pressions (100, 350 i 550 bar) i temperatures (35, 50, 60, 70, 80, 85 i 95°C). A més, es va investigar l'efecte del contingut en oli (0, 10, 20 i 30%) en els medis de tractament sobre la inactivació amb SC-CO_2 i SC-CO_2 + HPU de bacteris vegetatius (*E. coli* i *B. diminuta*). Finalment, es va explorar l'efecte de les condicions del tractament SC-CO_2 + HPU (temperatura, pressió i temps) sobre les propietats fisicoquímiques de les emulsions (aparença, pH, densitat, grandària de gota i potencial-ζ) utilitzant la metodologia de superfície de resposta. D'aquesta manera, es van buscar les condicions del procés que conduïren a una inactivació microbiana satisfactòria i, al mateix temps, a mínims canvis fisicoquímics en les emulsions.

Quant als tractaments de PEF i HPU en les emulsions d'oli-en-aigua al 20%, en primer lloc, es van realitzar experiments per a la inactivació d'*E. coli*. Els tractaments amb PEF es van realitzar a diferents energies (de 41.5 a 176.3 kJ/kg) i temperatures d'entrada (15 i 25 °C). A més, els tractaments amb HPU es van realitzar a dos temps de processament (2 i 3 min). Les condicions més efectives es van seleccionar per a avaluar l'efecte dels tractaments individuals i combinats sobre la inactivació d'*E. coli* i

d'espores de *A. niger* i *B. pumilus*. L'efecte de la seqüència d'aplicació en els tractaments combinats (PEF-HPU o HPU-PEF) es va estudiar per a la inactivació dels tres tipus de microorganismes.

Els resultats experimentals van mostrar que l'aplicació d'HPU va intensificar en gran manera la capacitat d'inactivació del SC-CO_2, acurtant el temps del procés per a quasi tots els microorganismes i medis. Per exemple, per als bacteris vegetatius en les emulsions amb un contingut en oli del 10, 20 i 30% es va acurtar el temps requerit per a la inactivació completa en aproximadament 1 ordre de magnitud en aplicar HPU. Probablement, els HPU van augmentar la solubilització del CO_2 en el medi i van provocar danys en la paret cel·lular, facilitant la penetració del SC-CO_2 en les cèl·lules. En aquest sentit, l'anàlisi de la microscòpia dels bacteris vegetatius i les espores fúngiques inactivades va revelar importants canvis morfològics en les cèl·lules, incloent parets cel·lulars danyades, i una important alteració i pèrdua del contingut citoplasmàtic. Per tant, el tractament combinat SC-CO_2 + HPU va demostrar ser eficaç per a la inactivació microbiana, malgrat la complexitat de la paret cel·lular. No obstant això, els HPU no van millorar la inactivació amb SC-CO_2 de les espores de *A. niger* en una emulsió. Per exemple, a 350 bar i 60 °C, la inactivació completa de les espores de *A. niger* es va aconseguir en 10 min, independentment de l'ús d'HPU.

L'augment de la pressió, tant en els tractaments amb SC-CO_2 com amb SC-CO_2 + HPU, va donar lloc a majors nivells d'inactivació, a excepció d'*E. coli* en aigua, on no es va trobar efecte de la pressió. No obstant això, les pressions per damunt de 350 bar no van semblar comportar a una inactivació addicional, probablement perquè un augment en la pressió a penes va millorar la solubilitat del SC-CO_2. L'augment de la temperatura va tindre un efecte significatiu ($p<0.05$) per a tots els tractaments (tèrmics, SC-CO_2 i SC-CO_2 + HPU) i microorganismes. Per exemple, en 20 min de tractaments tèrmics, SC-CO_2 i SC-CO_2 + HPU, la inactivació de les espores de *B. subtilis* va augmentar de 0.1 a 2.7 cicles-log, de 3.1 a 6.2 cicles-log i de 5.4 a 6.7 cicles-log, respectivament, d'usar 85 a 95 °C.

La resistència dels microorganismes als tractaments d'inactivació amb SC-CO_2 i SC-CO_2 + HPU va ser, en ordre de menys a més resistent: bacteris vegetatius

(*E. coli* i *B. diminuta*), espores fúngiques (*A. niger*), espores de *Clostridium* (*C. butyricum*), espores de *Bacillus* (*B. subtilis* i *B. pumilus*) i finalment, espores de *Geobacillus* (*G. stearothermophilus*), sent la inactivació d'aquestes últimes inviable utilitzant les tecnologies proposades en aquest treball.

Quant a l'efecte del medi sobre la inactivació microbiana, se sap que la presència d'oli protegeix els microorganismes i, generalment, es necessiten tractaments més intensos, com es va observar en la inactivació amb SC-CO_2 d'*E. coli* i *B. diminuta* en aigua i en les emulsions amb diferent contingut en oli (10, 20 i 30%). No obstant això, l'aplicació d'HPU va emmascarar l'efecte protector exercit per l'oli de les emulsions i va minimitzar les diferències en la inactivació microbiana. Per exemple, en 50 min del tractament amb SC-CO_2 a 350 bar i 35°C, es van inactivar 7.4 cicles-log d'*E. coli* en aigua (0% de contingut en oli), mentre que es van inactivar 3.4, 4.3 i 5.2 cicles-log en les emulsions amb un 30, 20 i 10% de contingut en oli, respectivament. No obstant això, en 5 min de tractament amb SC-CO_2 + HPU a 350 bar i 35 °C, es va obtindre una inactivació de 6.2-7.0 cicles-log per a *E. coli*, independentment del contingut en oli en el medi. Per contra, per a les espores de *A. niger* no es va trobar efecte del medi (aigua o emulsió al 20%) sobre l'efectivitat de tots dos tractaments d'inactivació, SC-CO_2 i SC-CO_2 + HPU, i, en mitjana, es va obtindre una inactivació de 4.4 i 4.3 cicles-log en aigua o en l'emulsió, respectivament.

En relació a aquest efecte dels tractaments amb SC-CO_2 + HPU sobre la qualitat de les emulsions tractades, en termes generals, només es va trobar un efecte lleu de les condicions del procés (temperatura, pressió i temps) sobre les propietats fisicoquímiques. Les emulsions tractades van presentar, en general, bon aspecte, canvis mínims en la densitat, bona estabilitat electroestàtica, un pH més baix (de 8.4 a 5.1-5.6) i una major grandària mitjana de gota (D[4,3] de 0.365 a 0.338-7.996 μm; D[3.2]: de 0.343 a 0.320-1.505 μm). Per tant, mitjançant la selecció de condicions de SC-CO_2 + HPU adequades (per exemple, 95 °C, 600 bar i 12.5 min), es poden obtindre canvis mínims en les propietats fisicoquímiques de les emulsions i una inactivació satisfactòria per a tots els microorganismes estudiats, excepte per a les espores de *G. stearothermophilus*.

Per tant, la tecnologia SC-CO_2 + HPU podria ser una alternativa prometedora a la pasteurització tèrmica d'emulsions per a preservar millor els compostos sensibles a la calor, ja que es poden utilitzar temperatures més baixes. No obstant això, les variables de procés del tractament han d'avaluar-se i seleccionar-se per a mantindre la qualitat de les emulsions mentre s'inactiven els microorganismes diana.

Quant a la inactivació microbiana d'emulsions amb tractaments de PEF i HPU, els resultats van revelar que la inactivació màxima aconseguida pel tractament individual de PEF, al major nivell d'energia i temperatura d'entrada (152.3-176.3 kJ/kg i 25°C) va ser de 2.6, 1.2 i 0.1 cicles-log per a *E. coli*, *A. niger* i *B. pumilus*, respectivament. A més, els majors nivells d'inactivació aconseguits per HPU per al tractament més llarg estudiat (3 min), van ser de 5.4, 4.3 i 0.3 cicles-log per a *E. coli, A. niger* i *B. pumilus*, respectivament. Per tant, la inactivació microbiana completa en les emulsions no es va aconseguir amb els tractaments individuals. No obstant això, quan el tractament de PEF (152.3-176.3 kJ/kg) va ser seguit pel tractament d'HPU (3 min), els nivells d'inactivació obtinguts van ser de 8.2, 6.6 i 1.0 cicles-log, per a *E. coli, A. niger* i *B. pumilus*, respectivament, corresponent a la inactivació completa d'*E. coli* i *A. niger*. A més, el tractament combinat PEF-HPU va presentar un efecte sinèrgic, ja que la inactivació aconseguida va ser major que la suma dels tractaments individuals per a tots els microorganismes. Malgrat aquest fet, la inactivació assolida per a l'espora bacteriana (*B. pumilus*) va ser molt limitada. Per contra, els nivells d'inactivació aconseguits pel tractament invers (HPU seguit de PEF) van ser inferiors a la suma dels tractaments individuals. Per tant, la seqüència més eficaç per al tractament combinat va ser aquella en la qual els PEF van ser seguits dels HPU. En aquest sentit, el tractament PEF probablement va exercir efectes subletales sobre els microorganismes, fent que les cèl·lules microbianes estigueren més sensibles al tractament posterior d'HPU. Per tant, el tractament combinat PEF-HPU va demostrar ser una tecnologia prometedora per a inactivar bacteris vegetatius o espores fúngiques en emulsions. No obstant això, el seu ús no va ser factible per a les espores bacterianes en les condicions provades en el present treball.

La resistència dels microorganismes als tractaments de PEF i HPU, aplicats individualment o en combinació, va seguir el mateix ordre que el trobat per als

tractaments de SC-CO_2, sent els bacteris vegetatius els més sensibles, seguides de les espores fúngiques i finalment, de les espores bacterianes.

Finalment, es pot concloure que, la combinació d'HPU amb SC-CO_2 o PEF generalment va millorar la inactivació microbiana. A més, en alguns casos, es va demostrar que la combinació dels tractaments va aconseguir efectes sinèrgics. En conseqüència, mitjançant l'ús de les tècniques combinades estudiades es podrien seleccionar temps de processament industrials raonables i condicions de procés suaus, que podrien resultar en una reducció en el cost de processament i un menor impacte en la qualitat de les emulsions oli-en-aigua. No obstant això, per a comprendre millor els mecanismes d'inactivació exercits pels tractaments combinats de SC-CO_2 i HPU i els tractaments combinats de PEF i HPU, és necessari fer un treball en major profunditat, especialment en espores fúngiques i bacterianes. A més, es recomana investigar més sobre l'efecte d'aquestes tecnologies no tèrmiques combinades sobre les propietats fisicoquímiques dels productes tractats, en particular per als tractaments combinats de PEF i HPU, aspecte que no es van tractar en el present estudi

1. INTRODUCTION

1.1. Microbial inactivation in the food and pharmaceutical industry

The preservation processes in the food and pharmaceutical industry are essential since microbial growth is common and has an effect not only on the product quality but also on the consumer's health. The most widespread preservation method is the thermal treatment. However, the so-called non-thermal technologies, in which its primary effect on microbial inactivation is not caused by the heat, are emerging.

1.1.1. Thermal treatments

Nowadays, thermal sterilization and pasteurization are the most common methods to inactivate microorganisms and enzymes in food and pharmaceutical products. The methods to achieve the complete inactivation of microorganisms, including spores require temperatures higher than 100ºC. Therefore, these treatments have some disadvantages related with the heat damage, such as nutritional loss (e.g. degradation of vitamins), undesirable sensorial changes (e.g. degradation of volatile aroma compounds) and formation of unwanted compounds (e.g. acrolein) (van Boekel et al., 2010).

1.1.2. Non-thermal preservation technologies

In addition to microbial stability, consumers demand high-quality fresh products free of preservatives, with an extended shelf life and at a reasonable cost. Therefore, alternative processing technologies to assure microbiologically stable products, preserving the sensory and nutritional quality, are growing interest. Non-thermal preservation technologies are defined as processes where the application of heat is not the main factor of microbial inactivation. The advantage of these technologies, compared to thermal ones, is potentially a lower impact caused on the physicochemical and nutritional properties of the treated product (Van Impe et al., 2018).

Some of the emerging non-thermal technologies are high pressure processing (HPP), irradiation, pulsed electric fields (PEF), pulsed light, ozonization, high power ultrasound (HPU) and supercritical carbon dioxide (SC-CO_2), among others.

1.1.2.1. High pressure processing

General aspects

High pressure processing (HPP) consists on the application of very high pressure (100 to 600 MPa) on a liquid medium; normally water, which transmits the pressure homogeneously to the treated product. Thus, HPP effectiveness is not affected by the size or shape of the product (Huang et al., 2014). The product is normally contained in a flexible package, which avoids further recontamination.

Inactivation mechanisms

HPP affects noncovalent bonds, producing changes in large molecules such as proteins, polysaccharides, lipids and nucleic acids. Therefore, most of the constituents of the cell are affected, including the cell membrane components, and the metabolic reactions, which are essential for the cell maintenance, are inhibited.

Influence on the product quality

HPP has demonstrated to minimally impact on the physicochemical and sensory properties of the treated products (Li & Farid, 2016). As an example, HPP treatment at ambient temperature had only an slight effect on the anthocyanins content of various fruits and vegetables (Tiwari et al., 2009). However, HPP treatment affects the structure of macromolecules, such as proteins and polysaccharides, which could modify the textural properties of the products or produce loss in nutritional and functional food constituents (Huang et al., 2014). For instance, Monfort et al. (2012) found protein denaturation and coagulation in HPP-treated egg products and Oliveira et al. (2017) found loss of color, increase in hardness and lipid and protein oxidation in fish meat. Nevertheless, the changes in the protein structure could be also beneficial for some foods, such as novel meat products since the gelation properties are enhanced and better texture of the final product is obtained (Rahman et al., 2018).

Limitations

- The structure of proteins and polysaccharides can be altered affecting the quality of the product.
- Lipid oxidation could be accelerated.
- The inactivation of bacterial spores by pressure alone is limited.
- Vegetative bacteria can create resistance to treatments due to long exposition to pressure.
- Since very high pressures are involved, a high investment cost is required.

Applications

HPP technology is widely used in the industry for two main purposes: to provide safe products with an extended shelf life and/or to enhance the physical properties of the treated products. Microbial inactivation has been accomplished in different products, such as meat, dairy products, seafood, fruits, vegetables, and beverages, such as beer or wine (Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Buzrul, 2012; Rahman et al., 2018). HPP has also been used to enhance the texture, color and water holding capacity of meat (Rahman et al., 2018), as a fast deboning process in bivalves and crustaceans (Oliveira et al., 2017) as a cold gelatinizing technology resulting in new product properties (Balakrishna et al., 2020; Larrea-Wachtendorff et al., 2020) and to enhance the coagulation of milk for the preparation of dairy products, such as cheese, yogurt and milky sauces with better technological properties (e.g. lower degree of syneresis, lower titrable acidity and higher firmness) (Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Trujillo, 2002).

1.1.2.2. Irradiation

General aspects

Irradiation is based on the exposure of products to a certain amount of ionizing radiation, which is able to trigger an electron release, producing an electron-deficient

particle (Brewer, 2009). In order to generate irradiation, the most frequent methods are electron beam (e-beam) and X-ray, which are generated by linear accelerators, and gamma-ray, whose source is radionuclides (Co60 or Cs137). Irradiation allows the treatment of packaged products avoiding further recontamination. According to the World Health Organization (WHO), International Atomic Energy Agency (IAEA) and Food and Agriculture Organization (FAO), doses lower than 10 kGy are used to obtain safe irradiated food (World Health Organization, 1994). However, high-dose treatments (10-60 kGy) are used for special purposes, such as the irradiation of food for patients with a low level or lack of immunity and astronauts (Lung et al., 2015).

Inactivation mechanisms

Irradiation action is linked to direct and indirect effects. Direct irradiation causes the microbial DNA, RNA, enzymes and membrane proteins damage and the cell division inhibition. The indirect effects are related to the formation of free radicals generated during the interaction of irradiation with water molecules. The free radicals will combine with each other or with oxygen producing oxidizing agents (Tahergorabi et al., 2012).

Influence on the product quality

Overall, irradiation induces minimal changes on the physicochemical and organoleptic properties and nutrient content compared to conventional thermal processing (Tiwari et al., 2009). However, some reactions between components could trigger lipid oxidation, breakdown of proteins, reduction in vitamin content or changes in color, odor, and flavor in the product (Brewer, 2009; Lewis et al., 2002; Tiwari et al., 2009). As an example, Mendes et al. (2020) irradiated tomatoes with gamma rays (1 kGy) and no changes in attributes, such as pH and peel color were found. However, irradiated tomatoes were softer than the fresh ones. Moreover, Elias et al. (2020) irradiated raspberries (E-beam at 3 kGy) and, although the content of vitamin C was reduced, no changes in the phenolic content and antioxidant activity were found.

Limitations

- Consumers reject irradiation since, misguidedly, irradiated products are considered as non-safe.
- The doses of irradiation able to inactivate spores are not permitted in the food industry (Li & Farid, 2016).
- Irradiation technology can be only used in authorized facilities and in some products.
- The use of radionuclides requires protection facilities and the treatment of radioactive waste, which increase the cost of the technology. In addition, there is a potential health issue for the individuals exposed to the irradiation source (Li & Farid, 2016).
- E-beam has a relatively low penetration capacity (<10 cm), compared to X-ray (20 cm) and gamma-ray (40 cm) (Li & Farid, 2016; Tahergorabi et al., 2012).

Applications

Irradiation has been used for the microbial and enzyme inactivation of food products such as spices, meat, cereals, fruits, and vegetables (Brewer, 2009; Lung et al., 2015; Pan et al., 2020), air and surfaces (Li & Farid, 2016) and medicinal devices (European Medicines Agency, 2016). In addition, low doses of irradiation have been used to inhibit the germination of potato, onion and garlic, delay the ripening rate of fruits and vegetables or for insect disinfestation in products such as spices or cereals (Lung et al., 2015).

1.1.2.3. Supercritical fluids

General aspects

A fluid is under supercritical conditions when it is at a pressure and temperature above its critical point. In supercritical conditions, there is no distinction between the liquid and the gas phase (Fig.1). Supercritical fluids (SCF) have a lower viscosity and

a higher diffusion capacity than a liquid fluid, which leads to higher mass transfer (Sihvonen et al., 1999). In addition, the dissolving power depends on the density which, unlike in the liquid phase, can be easily adjusted by changing the temperature or pressure. Those properties make SCF a favorable medium for extraction and separation techniques, which has been the main application of this technology. In addition, SCF could be more effective than in the gaseous or liquid state for penetrating inside the microbial cells, extracting vital components and provoking their inactivation (Garcia-Gonzalez et al., 2007). The most widely used fluid in SCF is CO_2 due to its advantageous characteristics: non-toxic, non-flammable, non-corrosive, colorless, inexpensive and leaves no residues. In addition, the critical values of pressure and temperature are relatively low (72.8 bar and 31.1ºC, respectively) and therefore, industrially easy to reach.

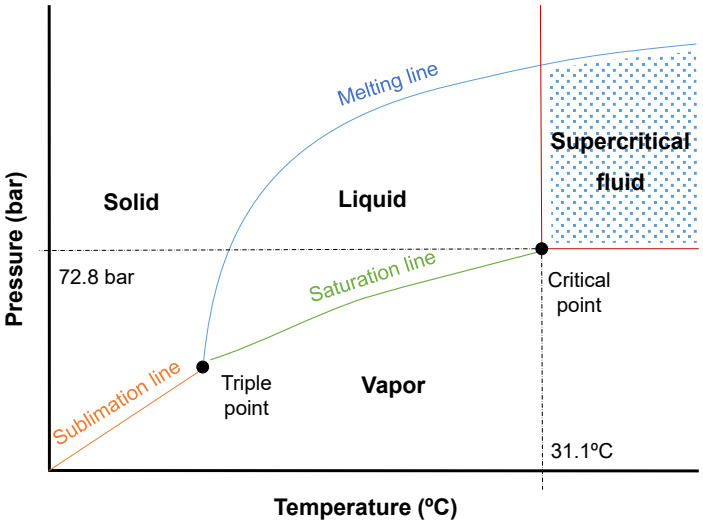


Fig. 1. Temperature-pressure diagram of carbon dioxide.

Inactivation mechanisms

Inactivation mechanisms for vegetative cells

CO_2 dissolves in the aqueous medium and transforms into carbonic acid (H_2CO_3), which dissociates into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and hydrogen (H^+) ions. The release of hydrogen ions lowers the pH of the medium, which may provoke the inhibition of the microbial growth and a high energy consumption of the microbial cells to maintain the pH homeostasis, which decreases its resistance to inactivation. In addition, the decrease of the extracellular pH could damage the structure of the cells membrane increasing its permeability, which facilitates the penetration of CO_2 into the microbial cells. In turn, CO_2, due to its hydrophobic and fat-soluble nature, can diffuse, accumulate and extract lipids from the cellular membrane increasing its fluidity, which also enhances the permeability of the cells membrane (Ribeiro et al., 2020). Once inside the microbial cells, CO_2 accumulates in the cytoplasm, lowering the internal pH and causing a collapse in the buffering system of the microbial cells, and the denaturation of enzymes, which are vital for metabolic and regulatory routes of the microbial cells. The accumulation of CO_2 and HCO_3^- inside the cells could directly affect the cells metabolism by affecting the anion-sensitive sites of key enzymes, displacing equilibriums and inhibiting the carboxylation and decarboxylation metabolic reactions, which are essential for the glucogenesis and the synthesis of amino acids and nucleic acids. Moreover, CO_3^{2-} inside the cell could precipitate together with inorganic electrolytes (such as Ca_2^+ or Mg_2^+), which are essential for the regulation of cellular activities such as the maintenance of the osmotic balance. The increase of the membrane fluidity along with the high solvating power of SC-CO_2 allows the extraction of vital cellular constituents. Finally, microbial death can also occur by the burst of the cells due to the abrupt depressurization of the supercritical CO_2 system (Ribeiro et al., 2020). It must be mentioned that the described inactivation mechanisms may be interrelated and not occur consecutively.

Inactivation mechanisms for fungal and bacterial spores

The complex, highly dehydrated and robust structure of spores provide them a strong resistance to SC-CO_2 treatments since the penetration and dissolution of CO_2 into the cell could be restrained. There is not a clear explanation for the inactivation of spores by SC-CO_2. However, there are two main hypotheses, one being related to the germination of spores, which makes cells more sensitive (Spilimbergo et al., 2003) and the other, related with the direct damage on the spore structure initially exerted by the high temperature usually applied (normally, higher than 60ºC), which makes the spore more permeable to SC-CO_2 (Rao et al., 2015; Soares et al., 2019).

Influence on the product quality

In general terms, it could be considered that SC-CO_2 treated liquid products retain the sensory, nutritional, and physical properties from the fresh product (Damar & Balaban, 2006). For fruit juices, one of the most studied product, negligible changes on the physicochemical properties were found after the SC-CO_2 treatment (Ferrentino et al., 2009). In addition, sensory evaluation showed that juices treated with SC-CO_2 were preferred than pasteurized ones and were indistinguishable from untreated juices (Gasperi et al., 2009). In some studies, treated juices were even improved in terms of colour, formation and stability of cloud and retention of antioxidants (Arreola et al., 1991; Kincal et al., 2006). On the contrary, physicochemical changes were found in more complex medium, such as milk (Watanabe et al., 2003). As for solid products, longer processing times are usually required to obtain an acceptable microbial reduction due to the more difficult diffusion of CO_2 though the product. Therefore, solid products can be more adversely affected by SC-CO_2 treatments compared to liquid ones. In this regard, the treatment of whole fruits and vegetables to inhibit mould growth caused severe tissue damage even at low pressures (Haas et al., 1989).

Limitations

- Sometimes, long processing times or high temperatures and pressures are required to achieve a satisfactory level of microbial inactivation, which leads to an increase in process cost and a decrease in product quality (Ortuño et al., 2012).
- SC-CO_2 under mild temperatures and pressures does not inactivate bacterial spores (Ribeiro et al., 2020).
- In solid products, the treatment only can be performed in a batch system. In addition, CO_2 diffusivity through the product is complicated.
- High cost of the equipment and requirement of security measures are demanded for operating at high pressures.

Applications

The most studied application of SC-CO_2 has been the extraction and fractionation. Thus, high added value components, such as essential oils, can be extracted; or unwanted compounds, such as caffeine of coffee and tea, flavor, cholesterol or alcohol, can be removed (Sihvonen et al., 1999). Regarding the inactivation of microorganisms and enzymes, SC-CO_2 has been used in different types of food media such as juices, fruits, vegetables and rice (Ferrentino & Spilimbergo, 2017; Gasperi et al., 2009; Ribeiro et al., 2020). Moreover, it has also been used for the sterilization of biomedical materials, such as implantable devices and allograft tissues (Ribeiro et al., 2020). Other applications that should be mentioned include the microencapsulation of molecules for the development of pharmaceutical formulations (Sahena et al., 2009), the supercritical fluids chromatography for the quantification of heat-labile non-volatile components (Sihvonen et al., 1999) or the destruction of insect eggs, larva or beetles (Perrut, 2012).

1.1.2.4. High power ultrasound

General aspects

Ultrasound is mechanical waves with frequencies that exceed the limit of human hearing (>20 kHz) which require an elastic medium to propagate through it. An ultrasound system is composed of a power generator, which produce the electrical signal that is converted into vibration by a transducer. In high intensity (>1 W/cm^2) or low frequency (20- 100 kHz) ultrasound, also called high power ultrasound (HPU), the vibration is transmitted to the medium exerting an effect on it. HPU is used for the improvement of heat and/or mass transfer processes and can be used for microbial inactivation purposes.

Inactivation mechanisms

HPU waves produce alternating compression and decompression of the media. When ultrasonic power reaches a threshold in the decompression cycles, air bubbles may cavitate. During cavitation, these bubbles could maintain a stable increasing and decreasing size generating microagitation of the medium (stable cavitation, Fig. 2) or could grow and collapse generating very high local temperatures (5000 K) and pressures (up to 100 MPa), high energy shear waves and turbulence (unstable cavitation, Fig. 2). Consequently, HPU has demonstrated its capacity to intensify mass and/or heat transfer phenomena. In addition, the implosion of the bubbles creates microjets towards the solid surfaces. All this phenomena could cause damage and/ or cracks on the microbial cell membranes (Cárcel et al., 2012).

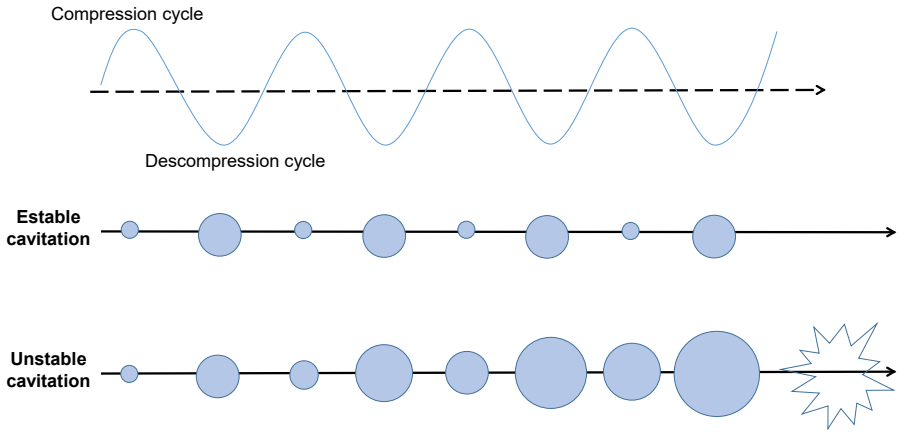


Fig. 2. Stable and unstable cavitation.

Influence on the product quality

Overall, the organoleptic and nutritional properties of products treated with HPU are not different to the ones thermally treated (Sulaiman et al., 2017). In addition, HPU may induce beneficial effects, such as degassing the medium, which could have some advantages related with the presence of oxygen and the degradation of antioxidants. Thus, Sulaiman et al. (2017) and Knorr et al. (2004) found higher retention of the antioxidant activity in apple and orange juice, respectively. Other beneficial effects in fruit juices are the improved cloud stability due to a reduced activity of enzymes or preferred taste (Knorr et al., 2004; Paniwnyk, 2017; Walkling-Ribeiro, Noci, Cronin, et al., 2009). However, some authors affirmed that free radicals produced during HPU processing could react with other constituents of the product and lead to the degradation of components, such as carotenoids and phenolic and antioxidant compounds in juices (Awad et al., 2012; Paniwnyk, 2017) and to the formation of off-flavors (Sulaiman et al., 2017).

Limitations

- Usually, HPU treatment alone requires very high intensities to achieve acceptable levels of microbial inactivation, leading to overheating of the product and, consequently, to the degradation of heat-sensitive components.
- The effect of HPU alone has demonstrated to be insufficient to inactivate bacterial spores (Fan et al., 2019). For this reason, HPU can be assisted with pressure (manosonication), temperature (thermosonication) or both (mano-thermosonication) (Awad et al., 2012).
- Free radicals can be formed during HPU processing, which could led to oxidation reactions (Yildiz et al., 2020). However, these reactions can be prevented by the reduction of the time and temperature of the HPU treatment (Paniwnyk, 2017).

Applications

The main applications of HPU in industrial processes are linked to the enhancement of heat or mass transfer operations. HPU, usually in combination with heat and/or pressure, has been used for microbial and enzyme inactivation in different products including fruit and vegetable juices, milk, liquid whole egg, etc. (Paniwnyk, 2017; Piyasena et al., 2003). In addition, other applications are cleaning of equipment, homogenization and emulsification (Villamiel & de Jong, 2000), enhancement of dehydration or extraction processes (Santacatalina et al., 2016), enhancement of heat transfer in heat exchangers (Gondrexon et al., 2015), degassing of liquids, cutting or induction of oxidation/reduction reactions (Knorr et al., 2004).

1.1.2.5. Pulsed electric fields

General aspects

A pulsed electric field (PEF) system consists of a generator and a treatment chamber with electrodes. PEF treatment involves the application of a sequence of high voltage and short duration (in the order of μs) electric pulses to a product that is placed between or passes through two electrodes. Consequently, the material is exposed to

an electric field whose intensity depends on the voltage across the electrodes and the geometry of the chamber and the electrodes. The electric field strength and the total specific energy are the main process parameters used to define and compare PEF treatments (Raso et al., 2016). The electric field strength is the field strength (kV/cm) locally existent in the treatment chamber during the sample processing. In parallel plate electrodes, the electric field between the electrodes is considered homogeneous and therefore, it is estimated by dividing the voltage between the electrodes by the electrode distance. The total specific energy can be calculated by multiplying the electrical energy supplied in the chamber by each pulse, by the number of pulses applied.

Inactivation mechanisms

The application of an external electric field to biological cells induces alterations of their transmembrane voltage which leads to electrocompressive forces that are balanced by elastic deformations of the cell membrane (Pataro et al., 2010). Whether the external electric field exceeds critical levels (>1 V), the breakdown of the membrane occurs, which cause the formation of reversible or irreversible pores, mechanism called electroporation (Palgan et al., 2012; Spilimbergo et al., 2014). In the reversible electroporation (Fig. 3), the membrane of the cell temporally destabilizes and increase its permeability. In addition, the cell can undergo sublethal damages (Pataro et al., 2010). In the irreversible electroporation (Fig. 3), the cell membrane is irrevocably cracked and the intracellular content is released, leading to microbial inactivation (Palgan et al., 2012).

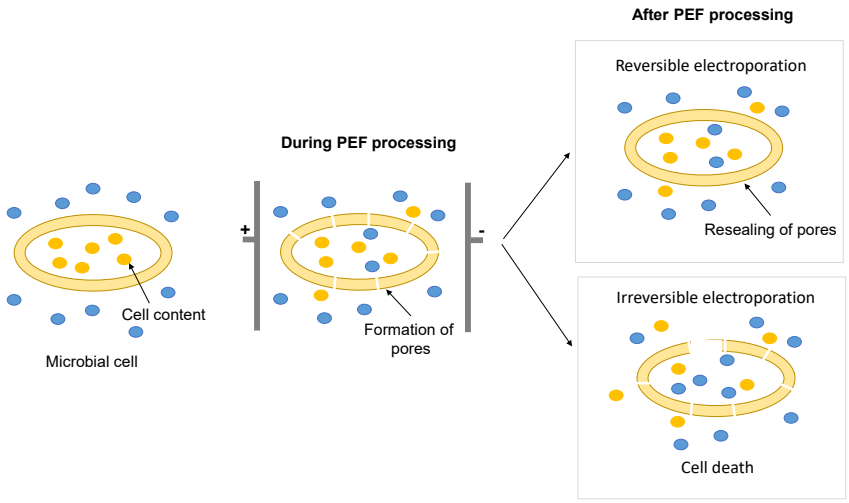


Fig. 3. Schematic representation of the electroporation of a cell membrane.

Influence on the product quality

Overall, PEF treatments for microbial inactivation purposes do not cause significant changes in the physicochemical and sensory properties of the treated products. Several authors (Dunn, 1996; Hodgins et al., 2002; Yeom et al., 2000) found no losses in flavor, color or nutrients and similar °Brix and pH values in PEF-treated fruit juices, compared to the heat-treated ones. In addition, the amount of ascorbic acid was minimally reduced after the PEF treatment or even increased in some cases. Regarding sensory properties, Dunn (1996) found the taste of orange juice treated with PEF similar to the untreated one. However, in some studies migration of electrode materials to the processed products were found and metallic flavor was detected (Yang et al., 2016). As an example, Evrendilek et al. (2004) found a significant increase in the concentration of metal ions (Fe, Cr, Zn, and Mn) in beer, which led to a noticeable different flavor.

Limitations

- Metal ions can be released from the electrode to the treated product (Yang et al., 2016).
- PEF treatment is ineffective for the inactivation of bacterial spores (Heinz et al., 2001; Noci et al., 2009).
- PEF treatment can be applied to a limited number of products (only to fluids). The treatment of liquids with particles or gas bubbles leads to dielectric breakdown due to the differences in the dielectric constants between liquid, gas and solid particles (Jeyamkondan et al., 1999). In addition, only products with conductivities and viscosities comprised in a suitable range can be treated. The treatment of high conductivity products leads to a high increase in temperature, which could provoke short-circuit and the implementation of a cooling system is difficult. The treatment of high viscosity products results in a decrease of the PEF effect, being sometimes inexistent (Heinz et al., 2001; Huang et al., 2006; Paniagua-Martínez et al., 2018). Moreover, sometimes, products such as water-in-oil emulsions cannot be treated since the continuous oil phase prevents the induction of an electric field in the dispersed water phase. However, the use of high frequencies could solve this problem (Mastwijk & Bartels, 2007).
- There are few companies in the market that manufacture equipment for processing product streams at an industrial level (Raso et al., 2016).

Applications

PEF treatment has been used for microbial and enzymes inactivation purposes in several matrices such as water, alcoholic beverages, fruit and vegetable juices and milk (Heinz et al., 2001; Li et al., 2008; Xiang et al., 2011). Moreover, PEF can be used as a pretreatment to enhance the efficiency of processes related with mass and heat transfer, since it has demonstrated to reduce the temperature and time or the amount of solvents required in the subsequent processes. Therefore, heat-sensitive compounds are better retained and higher quality products can be obtained (Llavata et

al., 2020). Some examples of those processes are the extraction of water, juices, solutes or high-added value compounds from biological matrices; the drying of fruits and vegetables or freezing and thawing processes (Barba et al., 2015). In addition, reversible electroporation is used in molecular biology and clinical biotechnological applications in order to introduce DNA vectors, oligonucleotides, antibodies or drugs into the cytoplasm of the cell and after that, to have the cell membrane resealed (Raso et al., 2016).

1.1.2.6. Combined treatments

A considerable number of the limitations described for the aforementioned non-thermal technologies can be solved by their combination. Often, each individual technology applied in a combined treatment leads to an additional stress on the microorganisms, resulting in a higher effect on inactivation compared to the individual treatments (hurdle approach). Thus, additive or synergistic effects can be obtained. This allows to reduce the intensities of the individual treatments, which, in many cases, involves less consumed energy and higher quality of the treated products (Martín-Belloso & Sobrino-López, 2011). In addition, bacterial spores are more prone to be inactivated by combined treatments (Fan et al., 2019).

Among the combinations of non-thermal treatments for microbial inactivation, some authors investigated the sequential or simultaneous processing with pressurized **CO_2 and HPP**. As an example, Park et al. (2002) obtained the complete inactivation of aerobic microorganisms (8 log-cycles of reduction) in carrot juice treated with CO_2 at 50 bar, 5ºC and 5 min followed by HPP at 3000 bar, 25ºC and 5 min; while only 4 and 3 log-cycles were reduced applying the individual CO_2 or HPP treatments for 10 min, respectively. The effect of HPP is known to be increased at high temperatures and low pH. Therefore, probably, pressurized CO_2 increased the effect of the subsequent treatment by lowering the pH. Ortuño et al. (2013) found that the simultaneous application of HPP and SC-CO_2 also synergistically enhanced the inactivation of enzymes in feijoa puree.

To our knowledge, there are no references in the literature studying the sequential combination of **SC-CO_2 and HPU** treatments for microbial inactivation. Moreover, only two research groups (Universitat Politècnica de València and University of Padova) are currently studding the simultaneous combination of SC-CO_2 and HPU treatments for microbial inactivation, probably due to the difficulty of implementing an ultrasound system with the electrical connections in contact with a highly conductor media, which can provoke electrical short-circuits (Ortuño et al., 2012). The application of HPU intensified the inactivation effectiveness of SC-CO_2, shortening the process time to inactivate microbial cells in different media such as *E. coli* in fresh cut carrot (Ferrentino & Spilimbergo, 2016), microorganisms naturally present in coconut water (Cappelletti et al., 2014) or *S. cerevisiae* in YPD broth, apple juice and orange juice (Ortuño, Martínez-Pastor, et al., 2013). HPU cause vigorous agitation of the medium, which enhances mass transfer and facilitates the dissolution of CO_2 in the liquid phase. In addition, the cavitation produced by HPU could damage or crack the cell walls, enhancing the penetration of SC-CO_2 into the cells and facilitating the extraction of vital components.

The combination of **SC-CO_2 and PEF** has also been investigated for microbial inactivation purposes. Pataro et al. (2010, 2014) studied the effect of its sequential combination, PEF being applied as a pretreatment stage, followed by the SC-CO_2 treatment, in a batch (Pataro et al., 2010) and in a continuous flow (Pataro et al., 2014) system to inactivate *S. cerevisiae* and *E. coli*, respectively. In both cases, a synergistic effect was observed, probably due to the fact that electroporation increases the permeability of the cell membrane, enhancing the subsequent penetration of CO_2 into the cell and accelerating the collapse of the cells.

Some studies were also found investigating combined **PEF and HPP** treatments. Rzoska et al. (2015) showed the enhancement of the inactivation of *S. cerevisiae* in a water-agar suspension by the simultaneous application of HPP and PEF (200 MPa and 10 kV/cm), compared to the single treatments, allowing the use of lower pressures and lower electric field than for the individual treatments. The sequential combination of PEF followed by HHP treatments to inactivate *B. subtilis* spores in buffer

solution also led to higher inactivation levels compared to each treatment applied independently (Sasagawa et al., 2006).

Promising inactivation results were also found for the sequential **PEF and HPU** treatments. Both alternatives in terms of sequence, PEF-HPU (Aadil et al., 2018; Palgan et al., 2012) and HPU-PEF treatments (Noci et al., 2009; Walkling-Ribeiro, Noci, Riener, et al., 2009) were found in the literature. As an example, Lyu et al. (2016) inactivated 3.7 (with HPU followed by PEF) and 3.5 log-cycles (with PEF followed by HPU) of *S. cerevisiae* with the combined treatments, while only 0.8 and 2.9 log-cycles were achieved with the individual HPU and PEF treatments, respectively. However, to our knowledge, only three studies (Huang et al., 2006; Lyu et al., 2016; Palgan et al., 2012) compared the influence of the sequence of the application in PEF and HPU combined treatments.

Nevertheless, scarce studies comparing the effectiveness of the combined non-thermal treatments on different microorganisms are found in the literature. For example, no references were found exploring the effect of SC-CO_2 and HPU or PEF and HPU treatments on filamentous fungal or bacterial spores inoculated in a liquid media. Therefore, more research is needed for seeking the most efficient and cost-effective combination for each application, while maintaining the quality of the final treated product

1.2. Emulsions

1.2.1. General aspects

An emulsion consists of two immiscible liquid phases, one being dispersed as small droplets in the other acting as a continuous phase. In the food, pharmaceutical and cosmetic industry, emulsions are widely distributed and are usually composed by an oil and a water phase, termed as oil-in-water (O/W) emulsion when the oil phase droplets are dispersed in the water phase and water-in-oil (W/O) emulsion when the water phase droplets are dispersed in the oil phase (Dong et al., 2016). Another emulsions, formed by aqueous droplets trapped inside oil phase droplets, which are

dispersed in an aqueous solution (W/O/W) and vice versa (O/W/O), can also be formed. As the contact between the two immiscible phases is unfavorable and the density is different, emulsions are thermodynamically unstable systems. Stabilizers such as emulsifiers or texture modifiers can be used to enhance the kinetic stability of emulsions (McClements, 2015). Emulsifiers (commonly phospholipids, small surfactants molecules, proteins and polysaccharides) can be adsorbed in the oil-water interface of the droplets providing protective coating (by electrostatic double-layer repulsive force or by steric barrier between the dispersed droplets) preventing the droplets from aggregation. In addition, emulsifiers reduce the interfacial tension, facilitating the formation of smaller droplets during homogenization. Moreover, texture modifiers (such as starch, pectin, alginate, carrageenan or gelatin) are substances used to slow down the droplets movement by increasing the viscosity or by the formation of a gel network within the continuous phase (McClements, 2007).

1.2.2. Use in the industry

In the food industry, many lipid-rich products are in the form of emulsions, being the most common the O/W systems. Some examples are milk, cream, mayonnaise, soft drinks, nutritional beverages, dressings, soups and sauces. However, there are also some food products which are W/O emulsions, such as butter or margarine. In addition, emulsions can be implemented in the food industry to elaborate products with a higher satiating power while maintaining or enhancing the organoleptic properties (Lett et al., 2015). As an example, W/O/W emulsions have been elaborated to create fat-reduced emulsions, by replacing part of the oil phase with water droplets (Liu et al., 2020). Some of the fats/oils commonly used for the elaboration of food emulsions are milk cream and vegetable oils, such as corn, soybean, sunflower or olive oil, among others.

In the pharmaceutical and cosmetic industry, emulsions are also very common. Some examples are emulsions for topical or parenteral administration. In topical pharmaceutical and cosmetics emulsions, such as lotions, sun care products, moistening, and anti-ageing creams, small size of droplets has demonstrated to

enhance skin protection, minimize the loss of trans epidermal water and enhance the penetration of active ingredients (Azmi et al., 2019). Regarding emulsions for intravenous nutrition (normally O/W), which are mainly used as a source of essential fatty acids and calories, the microbial sterility, the emulsion stability and the droplet size are of great importance. Droplet sizes of 0.25-0.50 μm are required to simulate natural chylomicrons, which are lipoproteins that carry dietary lipids from the intestine to other parts of the body. Moreover, higher than 5 μm droplets involve a risk of obstruction of the pulmonary arterioles (Driscoll, 2006; Pertkiewicz et al., 2009). Therefore, only a percentage of 0.05% w/v of droplets exceeding 5 μm is allowed in intravenously administered emulsions (Gallegos et al., 2012). Some of the oils commonly used for the elaboration of intravenous emulsions are soybean oil, olive oil and fish oil.

Emulsions are also used in other industries. For instance, emulsions can be implemented in paints to decrease the viscosity and enhance the spreading and drying processes or in chemicals to control reactions by reducing the contact between reagents (Mikkonen, 2020). Emulsions have also been used to encapsulate bioactive substances, including probiotics, vitamins, minerals, fatty acids and antioxidants. Encapsulation aims to control the release of target compounds or to encapsulate undesirable substances and to mask unpleasant flavors. This application constitutes a cross-sectional aspect of interest in food, pharmaceutical, cosmetic or agrochemical industries (Liu et al., 2020b; Muriel Mundo et al., 2020).

1.2.3. Physicochemical properties and stability

“Stability” refers to the ability of an emulsion to maintain unaltered its physicochemical properties throughout a defined period of time. Emulsions can be unstable due to different physicochemical mechanisms, all of them being often interrelated (Fig.4). Some of them are:

- creaming and sedimentation, where droplets move upward or downward, because its density is lower or higher than that of the surrounding liquid.

- flocculation, where two or more droplets aggregate together retaining its individual integrity.
- coalescence, where two or more droplets merge together and form one larger droplet.
- Ostwald ripening, where larger droplets get larger at the expense of smaller droplets.
- phase inversion, where an O/W emulsion become a W/O emulsion or vice versa.

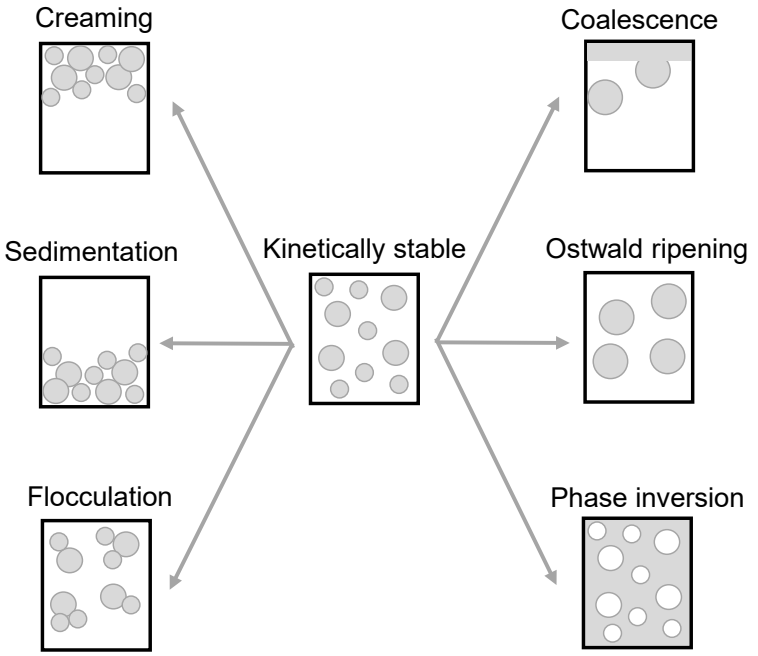


Fig. 4. Most common instability mechanisms in emulsions

Physical stability of oil-in-water emulsions is highly influenced by the droplets concentration, size, charge and interactions between them. Small size droplets and narrow size distributions should contribute positively to the emulsion stability (Desrumaux & Marcand, 2002). In fact, emulsions with a droplet size lower than 0.5

μm, are known to own a great stability, since flocculation is prevented (Anton et al., 2008). On the contrary, emulsions with large droplet size usually have a strong tendency to gravitacional separation, such as creaming (Azmi et al., 2019). The droplet charge is usually determined in terms of zeta potential, which is the difference in the electrical potential between the static coat of the dispersion medium attached to the dispersed droplets and the mobile dispersion medium. A high zeta potential, negative or positive, means that the emulsion is electrostatically stabilized while a low zeta potential leads to instability (Lu & Gao, 2010). The surrounding pH also affects the stability of emulsions since may reduce the repulsive forces between the particles resulting in flocculation, which may lead to coalescence and larger droplet formation (Pertkiewicz et al., 2009). Hence, the stability of emulsions may be measured by methods determining visible signs of instability (creaming), particle size and size distribution, droplet charge and pH.

1.2.4. Non-thermal inactivation

Due to the multiple applications of the emulsions, its pasteurization has gained interest in the industry (food, pharmaceutical, cosmetics, etc.). Moreover, in some pharmaceutical preparations, such as for parenteral and ophthalmic products, the assurance of sterility is of vital importance (Ribeiro et al., 2020). Thermal treatments (for pasteurization or sterilization) have been traditionally used to ensure safety and to extend the shelf life of food, pharmaceutical and cosmetic emulsions. However, high temperatures could adversely affect the stability of emulsions since many of the ingredients used in the emulsions are heat-sensitive; proteins, surfactants and polysaccharides being some examples. In addition, changes in the physicochemical properties such as pH, density or droplet size, in the organoleptic properties such as color, texture, and flavor and a decrease in the nutritional composition, such as loss of vitamins may occur, resulting in a decrease in the quality of the emulsions. Consequently, the microbial inactivation in emulsions by using non-thermal treatments becomes of great interest.

1.2.4.1. High pressure processing

Several authors investigated the effects of HPP on oil-in-water emulsions and found generally, satisfactory levels of microbial inactivation (Anton et al., 2001). In addition, some of them studied the possibility of using the high-pressure homogenization step, usually employed for the preparation of emulsions, to decrease the initial microbial content (Diels & Michiels, 2006). Thus, the homogenization step could avoid or reduce the intensity of the subsequent process for pasteurization/sterilization, obtaining better quality products and reducing the cost of the process (Diels & Michiels, 2006; Dong et al., 2016). However, diverse results about the HPP effect on the properties of emulsions (such as stability and rheology) were reported. Liu et al. (2020) achieved the same level of reduction (2.6 log-cycles) for the mesophilic bacteria in milk for both HPP (600 MPa for 5 min) and thermal treatment (72 °C for 15 s) and similar aroma profiles and in-vitro protein digestion were found. However, a higher denaturalization of the protein β-lactoglobulin was found in the HPP-treated milk. Anton et al. (2001) achieved the complete inactivation of the bacteria, molds and yeasts naturally present in sunflower oil emulsions (pH of 3 and 7) with an HPP treatment at 500 MPa, 10ºC and 10 min and no physicochemical changes were found, except for an increase in viscosity in the emulsions at pH 7. However, some studies demonstrated that the application of high pressures (>650 MPa) made emulsions more prone to lipid oxidation and physical instability (Gharibzahedi et al., 2019).

1.2.4.2. Irradiation

As for the use of irradiation for the microbial inactivation in emulsions, few studies were found. Oil is very susceptible to the oxidation by the free radicals formed in the irradiation process. Therefore, the generation of undesirable organoleptic properties is likely to occur in emulsions (Grandison, 2012). Jo and Ahn (2000) investigated the effect of irradiation (doses from 2.5 to 10 kGy) in various soybean oil emulsions and found an accelerated lipid oxidation along with off-flavors. Low doses of irradiation (2-3 kGy of gamma irradiation) achieved between 2 and 3 log-cycles of

reduction of mesophilic bacteria in milk and dairy products, such as ice creams, although noticeable rancid off-flavors were also reported (de Oliveira Silva et al., 2015; Kamat et al., 2000). Moreover, to inactivate some enzymes present in milk, such as phosphatase, about 5-10 times the dose required for the inactivation of microorganisms were needed, which makes the technology unsuitable for this purpose (Ahmad et al., 2019).

1.2.4.3. Supercritical fluids

Regarding SC-CO_2 microbial inactivation in emulsions, only studies in milk were found. Werner & Hotchkiss (2006) achieved reductions of 5.4 and 5.0 log-cycles for native microorganisms and inoculated *P. fluorescens* in milk treated at 207 bar, 35°C and 10 min, while no effect was found in spores. Watanabe et al. (2003) reported milk coagulation after the treatment (300 bar, 95ºC and 120 min). Some authors explained that the SC-CO_2 could destabilize casein micelles since the carbonic acid forms binds with calcium ions (Amaral et al., 2017; Bonnaillie & Tomasula, 2015). In addition, acidification by the dissolution of CO_2 in the milk could affect the proteins since the ionic and electrostatic interactions within the casein micelles and the whey protein could change (Bonnaillie & Tomasula, 2015).

1.2.4.4. High power ultrasound

HPU technology, usually applied along with heat (thermosonication), has proven to be effective for the inactivation of microorganisms in milk and vegetable emulsions. Moreover, in some studies, HPU applied to inactivate microorganisms, simultaneously reduced the size of large oil droplets (Bermúdez-Aguirre et al., 2009; Cameron et al., 2009; Salve et al., 2019). Cameron et al. (2009) successfully inactivated *E. coli*, *P. fluorescens* and *L. monocytogenes* in milk with HPU for 6-10 min and obtained a decreased fat globule size while no important changes on pH, lactic acid and protein and lactose content were found. Salve et al. (2019) achieved an inactivation of 0.5-1.1 log-cycles of mesophilic microorganisms present in peanut milk after 3 min of HPU treatment while found some improved attributes, which could

prevent phase separation, namely smaller particle and oil droplet size, higher content of hydrolyzed protein and better sedimentation index. Atalar et al. (2019) obtained the complete inactivation of the microorganisms naturally present in hazelnut milk (75ºC, 15-25 min, 60-80% amplitude), finding an improved quality in terms of appearance, syneresis, sedimentation, viscosity and consistency. However, lipid oxidation related with cavitation has also been reported in emulsions. For example, unpleasant off-flavors and metallic and rancid smell have been reported in sunflower oil, sunflower oil emulsions (Chemat et al., 2004) and in dairy products (Awad et al., 2012).

1.2.4.5. Pulsed electric fields

Several authors investigated the PEF treatment for the microbial inactivation in milk. Dunn (1996), Michalac et al. (2003) and Odriozola-Serrano et al. (2006) inactivated between 0.3 and 6 log-cycles of microorganism naturally present in milk and inoculated *L. innocua*, *P. fluorescens*, *L. lactis* and *B. cereus*, reporting no changes in the physicochemical properties, namely pH, density, color, fat integrity, calcium distribution, free fatty acid content, proteins structure, moisture, droplet size and electrical conductivity. Moreover, less flavor degradation, compared to the milk treated with heat was reported by Dunn (1996). However, Xiang et al. (2011) found changes in the rheological and protein structure in skimmed milk. Apart from milk, PEF technology was also studied for microbial inactivation in some vegetable emulsions. Dunn (1996) affirmed that salad dressings can be PEF-pasteurized without affecting the emulsion integrity. Walkling-Ribeiro et al. (2010) reduced 3.4 log-cycles of bacteria and 4 log-cycles of yeasts and molds naturally present in coconut milk based smoothies, obtaining similar physicochemical and organoleptic properties than in the thermally pasteurized products. Moreover, Barsotti et al. (2001) treated different types of oil-in-water emulsions (milk, 35% fat dairy cream and 30% peanut oil emulsion) with PEF and the size distribution of the oil droplets was not markedly changed.

1.3. Justification of the research

A great number of products in the food, pharmaceutical and cosmetics industry are based on emulsions. Among the emulsions used in the pharmaceutical industry, oil-in-water parenteral formulations are routinely used for the intravenous nutrition of patients with gastrointestinal disorders (also named lipid emulsions) (Amran et al., 2019). Microbial contamination or changes in the physicochemical properties, could cause very serious complications in parenteral nutrition. For example, an emulsion droplet size larger than the capillaries diameter could led to vascular occlusion. Therefore, the assurance of safe parenteral emulsions, in terms of sterility and physicochemical stability, is considered of vital importance (Montejo et al., 2000; Takagi et al., 1989). In this sense, the United States Pharmacopeia (USP) sets pharmaceutical requirements on pH, free fatty acids and droplet size (in terms of mean droplet size and population of droplets larger than 0.5 μm), among others (Driscoll, 2006; Gallegos et al., 2012; Wanten, 2015). As for sterility, parental emulsions are usually heat sterilized in steam autoclaves at 121ºC (Montejo et al., 2000; Riera et al., 2018). However, high temperatures are known to negatively affect heat-sensitive compounds and physicochemical properties of the emulsions (Chansiri et al., 1999). Specifically in parenteral emulsions, heat sterilization causes the hydrolysis of lipids and lecithin, which leads to negative effects, such as the liberation of free fatty acids, which lowers the pH, and an increase in the mean droplet size (Chansiri et al., 1999; Chaturvedi et al., 1992; Floyd, 1999; Hippalgaonkar et al., 2010). One alternative to eliminate or minimize the heat input is the one based on filtration techniques using sterilize grade filters and further aseptic filling. However, this method is less effective eliminating microorganisms than thermal sterilization and its use is limited to emulsions with low viscosity and droplet size below 0.2 μm. Moreover, filters could potentially retain some interesting solutes, provoke contaminations and affect the physicochemical stability of the emulsions (Floyd, 1999; Hippalgaonkar et al., 2010; Lidgate et al., 1992). In addition to filtration, no literature has been found studying other non-thermal alternatives for microbial inactivation in parenteral emulsions.

Emerging technologies, such as SC-CO_2, HPU or PEF, have already demonstrated success on microbial inactivation in a wide range of products, while partially avoiding or minimizing the detrimental effects linked to high temperatures. Nevertheless, for some resistant microorganism (mainly spores) or some complex media, such as fat-rich products (e.g. lipid emulsions), the individual non-thermal treatments could be ineffective or require extremely long process times or intensities to achieve a satisfactory level of inactivation. These extreme treatments could lead to changes in the physicochemical properties of the product and high process cost. For this reason, the combination of different non-thermal technologies could be promising for the microbial inactivation in parenteral emulsions. Despite its importance, scarce literature was found about the effectiveness of combined non-thermal technologies on the microbial inactivation in emulsions. Specifically, no studies of combined SC-CO_2, PEF or HPU treatments were found on vegetable oil-in-water emulsions, including parenteral emulsions, and the effect of the oil content on the effectiveness of these combined treatments has not been addressed elsewhere. In addition, no studies assessed the combined SC-CO_2, PEF or HPU inactivation of fungal and bacterial spores inoculated in liquid emulsions, or compared the resistance of different types of microorganisms (such as vegetative bacteria and fungal and bacterial spores). As for the physicochemical properties of the treated product, the effect of the SC-CO_2 + HPU treatment on the quality of emulsions has not been previously analyzed. Therefore, the assessment of non-thermal inactivation technologies to guarantee safe parenteral emulsions in terms of both, microbial and physicochemical stability, is of great interest for the pharmaceutical industry and its exploration is fully justified. In this context, this Doctoral Thesis has been carried out in the frame of the project ‘Assessment of the non-thermal pasteurization of lipid emulsions in supercritical CO2 intensified by power ultrasound’ funded by the pharmaceutical company Fresenius Kabi Deutschland GmbH.

2. OBJECTIVES

The main objective of this PhD Thesis was to evaluate the feasibility of different emerging non-thermal technologies, applied in individual and combined form, to inactivate different microorganisms in oil-in-water emulsions.

In order to achieve this goal, the following particular objectives were established:

1. To determine the effect of the application of high power ultrasound (HPU) on supercritical carbon dioxide (SC-CO_2) treatments on the inactivation of vegetative bacteria (*E. coli* and *B. diminuta*) and a fungal spore (*A. niger*) in a simple medium (water).
2. To analyze the microstructural changes on the microbial cells (*E. coli*, *B. diminuta* and *A. niger*) after the SC-CO_2 + HPU treatments in water.
3. To address the application of SC-CO_2 + HPU treatments for the inactivation of vegetative bacteria (*E. coli* and *B. diminuta*) in a complex lipid-rich medium (20% oil-in-water emulsions).
4. To study the effect of the oil content in the media (water and oil-in-water emulsions with a 10, 20 and 30% of soybean oil) on the SC-CO_2 and SC-CO_2 + HPU inactivation treatments of vegetative bacteria (*E. coli* and *B. diminuta*).
5. To explore the differences on the resistance to SC-CO_2 and SC-CO_2 + HPU treatments between a fungal (*A. niger*) and an anaerobic bacterial spore (*C. butyricum*) in 20% oil-in-water emulsions.
6. To determine the effect of SC-CO_2 + HPU treatments on the inactivation of aerobic bacterial spores (*B. subtilis, B. pumilus* and *G. stearothermophilus*) in 20% oil-in-water emulsions.
7. To evaluate the effect of SC-CO_2 + HPU treatments on the physicochemical properties (appearance, pH, density, droplet size and droplet charge) of 20% oil-in-water emulsions.

8. To evaluate the effect of individual and sequentially combined pulsed electric field (PEF) and HPU treatments on the inactivation of a vegetative bacteria (*E. coli*) a fungal spore (*A. niger*) and a bacterial (*B. pumilus*) spore in 20% oil-in-water emulsion.

3. METHODOLOGY

3.1. Working plan

The working plan of the present PhD Thesis (Fig. 1) was designed based on the objectives stablished. Thus, the experimental plan was divided into five parts giving rise to the two chapters in which the Results and Discussion section has been structured. These chapters correspond to the two different combinations of non-thermal technologies for microbial inactivation used, supercritical carbon dioxide and high power ultrasound (**Chapter 1**) and pulsed electric field and high power ultrasound (**Chapter 2**).

The first chapter is composed by four parts. Firstly, the feasibility of SC-CO_2 treatments (with and without the application of high power ultrasound) to inactivate three different microorganisms; two vegetative bacteria (*E. coli* and *B. diminuta*) and a fungal spore (*A. niger*) was assessed in simple media (water). In addition, the microstructural damage on the microorganisms' cells after the SC-CO_2 + HPU treatments was observed by using cryoFESEM and TEM (Part 1.1, Chapter 1). Secondly, the same treatments were performed to inactivate *E. coli* and *B. diminuta* in a 20% oil-in-water emulsion. The treating medium is known to affect the effectiveness of inactivation treatment. Therefore, the effect of different oil content in emulsions on the inactivation kinetics was analyzed. For this purpose, 10, 20 and 30% oil content emulsions were prepared (Part 1.2, Chapter 1). Thirdly, SC-CO_2 treatments (with and without the application of high power ultrasound) were performed on spores of *A. niger* and *C. butyricum* in a 20% oil-in-water emulsions with the aim of assessing the effect of the inactivation treatments on fungal and bacterial spores (Part 1.3, Chapter 1). Finally, SC-CO_2 + HPU treatments were performed on different *Bacillus* and *Geobacillus* spores (*B. subtilis, B. pumilus* and *G. stearothermophilus*) and the effect of the treatment conditions (pressure, temperature and time) on the physicochemical properties of the emulsions (pH, density, size of droplets and ζ-potential) was analyzed (Part 1.4, Chapter 1). The inactivation kinetics from Chapter 1 were described by the Weibull model. Moreover, the effect of the treatment conditions (pressure, temperature, time and use of ultrasound), the treating medium and the type of microorganism on microbial inactivation was analyzed by a General Linear Model.

In the fifth part of the experimental plan (Part 2.1, Chapter 2) the effectiveness of the individual and the combined PEF and HPU treatments (in both sequences, PEF + HPU and HPU + PEF) on the inactivation of a vegetative bacteria (*E. coli*), a fungal spore (*A. niger*) and a bacterial spore (*B. pumilus*), in 20% oil-in-water emulsions, was assessed. The effect of the treatment conditions (for the PEF treatments, field strength, treatment time and input temperature of the sample and for the HPU treatments, the treatment time), the sequence (PEF + HPU or HPU + PEF) and the type of microorganism on microbial inactivation was analyzed by ANOVA.

In the Results and Discussion section of this PhD Thesis, the specific methodology used in each results' subsection is described in detail. Consequently, in this Methodology section, the material and methods will be only outlined.

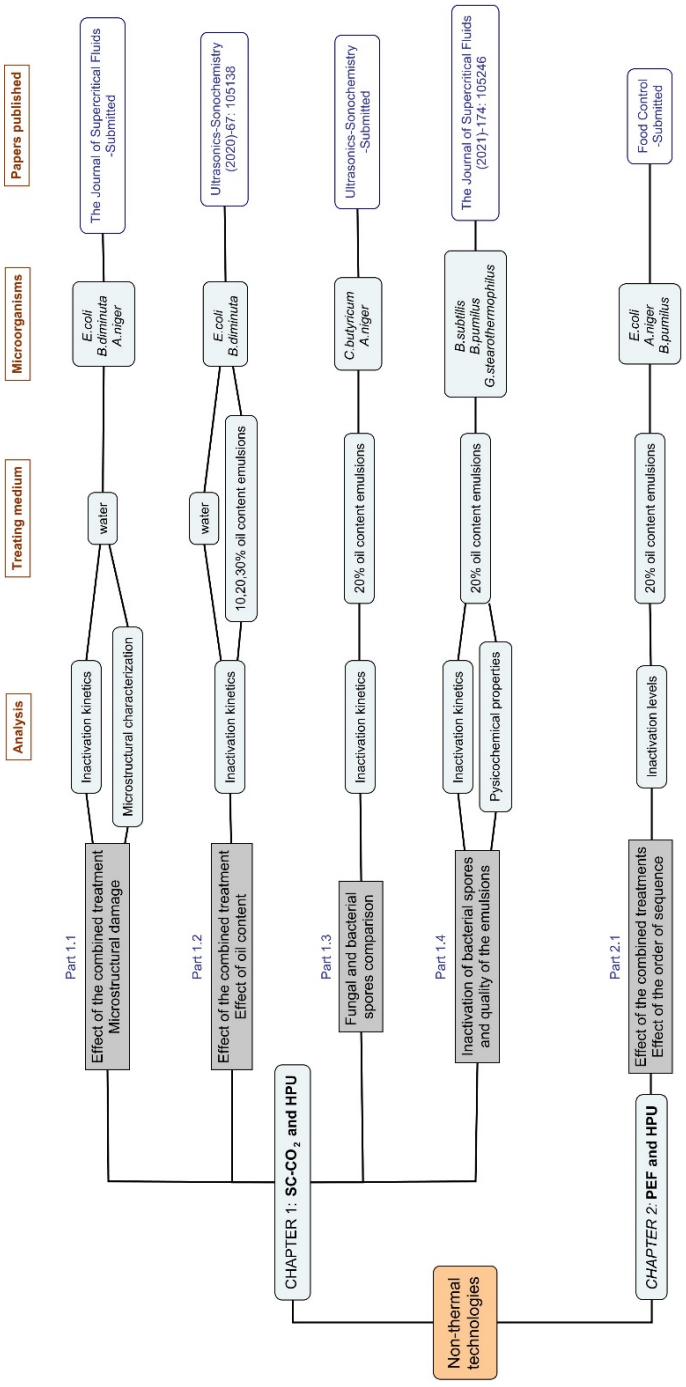


Fig. 1. Working plan

3.2. Preparation of microorganisms

All the strains of the microorganisms used in this PhD Thesis were obtained lyophilized from the Spanish Type Culture Collection (CECT, Valencia, Spain). The bacteria were: *Eschericha coli* CECT 101, *Brevundimonas diminuta* CECT 313, *Clostridium butyricum* CECT 361T, *Bacillus subtilis* CECT 356*, Bacillus pumilus* CECT 29T and *Geobacillus stearothermophilus* CECT 43 T. The filamentous fungi was *Aspergillus niger* CECT 2807.

E. coli and *B. diminuta* were selected as gram-negative bacteria (vegetative bacteria). *E. coli* because it is commonly present in contaminated products and *B. diminuta* because of its especially small cell size. *C. butyricum*, *B. subtilis*, *B. pumilus* and *G. stearothermophilus* were selected as gram-positive bacteria, able to form spores. *C. butyricum* was used to study a spore with anaerobic growth and *B. subtilis*, *B. pumilus* and *G. stearothermophilus*, to study spores with aerobic growth, already used as biological indicators for other inactivation treatments. Lastly, *A. niger* was selected as a spore-forming filamentous fungi, very common in contaminated products.

-Vegetative bacteria:

A single colony of *E. coli* or *B. diminuta* was inoculated in 50 mL of Nutrient Broth (Scharlab, Barcelona, Spain) and grown overnight (18-24 h) at 37 and 30ºC, respectively, while shaking using an incubation chamber and an orbital shaker (J.P. Selecta, Barcelona, Spain). It is known that the culture growth stage affects the resistance to treatments of vegetative microorganisms, being more resistant in the stationary phase compared to the initial growth phases (Ortuño et al., 2012). Therefore, 50 µL of the overnight culture were transferred to a new 50 mL of Nutrient Broth to be grown until the stationary phase was reached. The time at which *E. coli* and *B. diminuta* reached the stationary phase was stablished by plating on Plate Count Agar (data not shown) and measuring the optical density at 600 nm (Fig. 2), using a UV-visible spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). All the measurements were taken in triplicate.

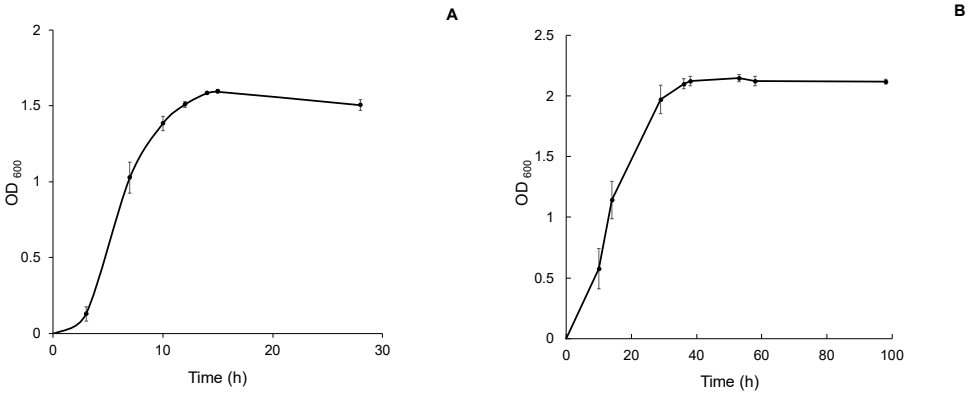


Fig. 2. Growth kinetics of *E. coli* (A) and *B. diminuta* (B) monitoring by optical density measurements at 600 nm (OD_{600}).

Thereby, in order to assure that *E. coli* and *B. diminuta* reached the stationary phase, the bacterial suspensions used to inoculate the media (water or emulsions) were cultivated for 14 h at 37ºC and for 36 h at 30ºC, respectively.

-Fungal spore:

A. niger was first cultured on Potato Dextrose Agar (Scharlab, Barcelona, Spain) at 25ºC for 7 days, time at which a high fungal spore concentration is formed (Reverter-Carrión et al., 2018). After that, the spores were rubbed and collected from the agar with 10 mL of 0.1% (v/v) Tween 80. The suspension was kept in a sterile container at 4ºC until use.

-Bacterial spores:

Bacillus, Geobacillus and *Clostridium spp.* were sporulated following the methodology of Mafart et al. (2002) with modifications. A single colony of *C. butyricum, B. subtilis, B. pumilus* or *G. stearothermophilus* was cultivated in its growing medium at optimum temperature until reaching the stationary phase according to previous literature (Table 1). After that, 100 µL of the bacterial suspension were incubated for 5-6 days on agar (Table 1) enriched with $MnSO_4$ (40 mg/L) and $CaCl_2$ (100 mg/L) to

enhance the sporulation. Spores were then collected by scraping the surface of the agar, suspended in 2 mL of sterile deionized water, and washed three times by centrifugation at 8000 x g for 15 min (Medifriger BL-S, JP Selecta, Barcelona, Spain). The pellet was resuspended in 2 mL of ethanol (50% v/v) and kept at 4°C for 12 h. Then, the suspension was washed again three times, distributed into sterile microtubes and kept at 4°C until use. Before being treated, the microtubes were heat-shocked at 80°C for 15 min and cooled again (Ávila et al., 2014; Spilimbergo et al., 2003). *B. subtilis, B. pumilus* and *G. stearothermophilus* were aerobically sporulated, while *C. butyricum* was grown and sporulated in anaerobic conditions, with airtight incubation containers and CO_2 generating systems (Oxoid, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Table 1. Medium, temperature and time conditions for bacterial growth and sporulation.

Bacteria	Growing broth	Agar medium	Temperature	Time for stationary phase
C. butyricum	RCM	RCA*	37ºC	36 h (Kong et al., 2006; *NCBI*, 2021)
B. subtilis	NB	PCA*	30ºC	24 h (Lee et al., 2011; Mondal et al., 2015)
B. pumilus	NB	PCA*	30ºC	24 h (Han et al., 2017; Liu et al., 2015).
G. stearothermophilus	TSM	TSA*	50ºC	24 h (Hetzer et al., 2006)

*$MnSO_4$ (40 mg/L) and $CaCl_2$ (100 mg/L) was added to the agar to enhance the sporulation. (RCM: Reinforced Clostridial Medium; RCA: Reinforced Clostridial Agar; NB: Nutrient Broth; PCA: Plate Count Agar; TSM: Tryptic Soy Medium; TSA: Tryptic Soy Agar).

3.3. Preparation of oil-in-water emulsions

Oil-in-water emulsions were prepared with different soybean oil contents (10, 20 and 30%). Deionized water was used as the control treatment medium (0% oil content). The emulsions were prepared in three stages: mixing, sonication and homogenization. The lipid phase was formed by soybean oil and egg phospholipid and

the water phase was constituted by deionized water and glycerol. The lipid phase was prepared using a disperser device (IKA T25 Digital, Ultra-Turrax, tool S25N -25G, Staufen, Germany) at 14000 rpm for 2 min, 10200 rpm for 4 min and 10600 rpm for 4 min and after that, it was slowly added to the water phase, while being mixed at 14000 rpm. The mix was sonicated for 5 min with the UP400S ultrasound system and the H22 sonotrode (Hielscher, Teltow, Germany). Finally, the product was homogenized in two stages (50 bar; 550 bar) with the PANDA Plus 2000 homogenizer (GEA Niro Soavi, Parma, Italy).

3.4. Inoculation

Prior to each treatment, the sample was prepared by inoculating the vegetative cell, fungal spore or bacterial spore suspension in sterile deionized water or oil-in-water emulsion, to reach a cell concentration of 10^7-10^8 CFU/mL for *E. coli* and *B. diminuta*, 10^6-10^7 CFU/mL for *A. niger* spores, 10^4-10^5 CFU/mL for *C. butyricum* spores, 10^6-10^8 CFU/mL for *B. subtilis spores*, 10^7-10^8 CFU/mL for *B. pumilus* spores and 10^5-10^6 CFU/mL for *G. stearothermophilus* spores.

3.5. Thermal treatments

1.5 mL of inoculated medium were poured into sterile glass tubes (Fiolax, 8 mm in diameter and 70 mm in length, Schott, Mainz, Germany) and placed in a temperature-controlled water bath (1812, Bunsen, Madrid, Spain). The tubes were taken from the bath after different times, depending on the microorganism and the temperature of the treatment and placed in ice until the microbial analysis was performed. The experiments were carried out in triplicate.

3.6. Non-thermal treatments

In this section, the equipment and conditions will be only outlined since further details can be found in the Materials & Methods subsection of the different parts of Results and discussion section. Before use, any non-thermal processing system used

in this PhD Thesis was sterilized for 5 min with a disinfectant solution (Diversey Delladet, South Carolina, USA).

3.6.1. Supercritical carbon dioxide and high power ultrasound

Supercritical carbon dioxide equipment

A supercritical fluid lab-scale equipment designed and built by the research team for batch mode operation was used. The system (Fig. 3) consisted of an inactivation vessel (5, Fig. 3) submerged in a thermostatic water bath (4, Fig. 3), to maintain the temperature of the process, a CO_2 tank (1, Fig. 3), a chiller reservoir kept at −18°C (2, Fig. 3) and a diaphragm metering pump (LDB, LEWA, Tokyo, Japan) to reach the desired pressure in the inactivation vessel (3, Fig. 3). To measure the temperature and pressure of the sample throughout the process, a pressure gauge and a K-type thermocouple were installed inside the vessel. The vessel was loaded with the inoculated sample (65 or 62 mL, depending on the microorganism) and immediately sealed and pressurized with CO_2. During the treatment, sample was extracted at different times. Both, the treatment conditions (pressure, temperature and time) and the sampling times were chosen according to the microorganism to be treated.

Ultrasonic system

The ultrasound system consisted mainly of a high power (> 1W/cm^2) piezoelectric transducer (6, Fig. 3) made up of two commercial ceramics (8, Fig. 3; resonance frequency of 30 kHz; ATU, Spain), a sonotrode and a power generation unit (10, Fig. 3). The power was 30 ± 5 W or 50 ± 5 W (I: 250 ± 10 mA; U: 220 ± 5 V) and the frequency was 30 ± 2 kHz, measured with a Digital Power Meter (WT210, Yokogawa Electric Corporation, Tokyo, Japan). The transducer was attached to the lid of the treatment vessel to sonicate directly the SC-CO_2. When the required pressure was reached in the treatment vessel, the HPU system was connected.

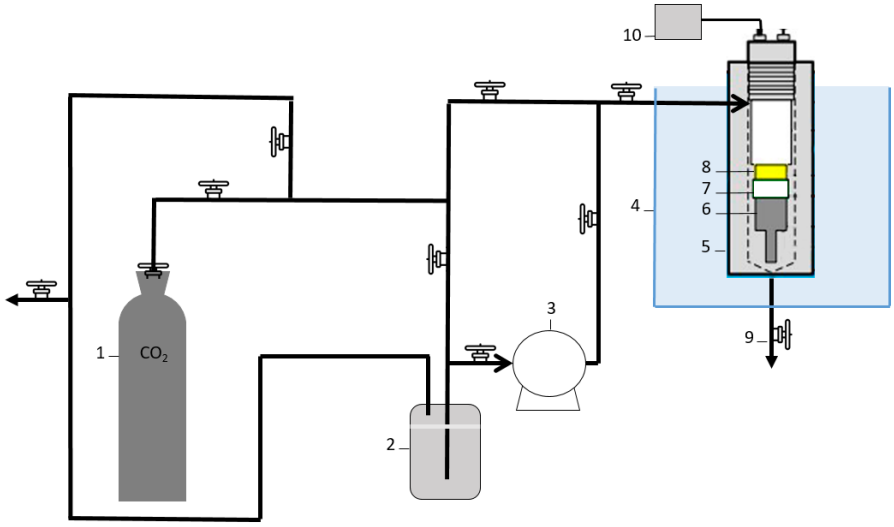


Fig. 3. Supercritical CO_2 treatment system. (1-CO_2 tank, 2-Reservoir, 3-Pump, 4-Bath, 5-Treatment vessel, 6-Transducer, 7-Insulation joint, 8-Ceramics, 9-Vessel output for sample extraction, 10-Power Generation Unit).

3.6.2. Pulsed electric field and high power ultrasound

Four different inactivation treatments were carried out:

- PEF
- HPU
- PEF followed by HPU (PEF-HPU)
- HPU followed by PEF (HPU-PEF)

In the combined treatments (PEF-HPU and HPU-PEF), the sample was processed by the first treatment and immediately collected in a sterile container, cooled to reach the initial temperature and treated again.

PEF treatments

A continuous flow lab-scale system (Fig. 4), which consisted of a high voltage pulse generator (Epulsus-PM1-10, Energy pulse systems, Lisboa, Portugal) and a

treatment chamber with two parallel plate electrodes (electrode gap of 31 mm) was used for the application of monopolar square-shaped pulses. The inoculated emulsion was circulated through the chamber by a peristaltic pump (XX8000230, Millipore Corporation, Massachusetts, USA). Two K-type thermocouples, located at the inlet and outlet of the PEF chamber were used to measure the temperature variation caused by the treatment.

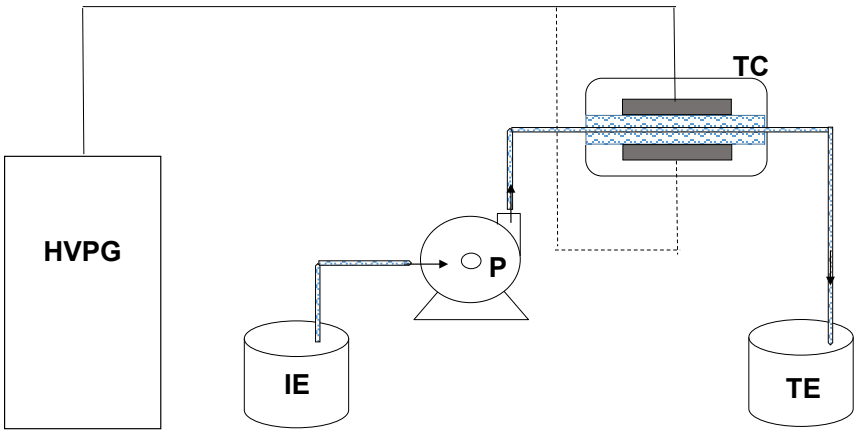


Fig. 4. Schematic diagram of the PEF system. HVPG: high voltage pulse generator. P: pump, TC: treatment chamber, IE: inoculated emulsion, TE: treated emulsion.

HPU treatments

An UP400St ultrasonic processor (100% of amplitude, 400 W, 24 kHz) with the s24d14D sonotrode (Hielscher Ultrasonics, Teltow, Germany) was used for microbial inactivation in batch mode. The inoculated emulsion was placed in a jacketed beaker where water was circulated to control the temperature of the treatment. A K-type thermocouple was located in the beaker to measure the temperature of the emulsion during the treatment.

3.7. Microbiological analyses

The viability of the microorganisms after the inactivation treatments was evaluated by means of standard plate count. Depending on the expected count, appropriate serial dilutions were prepared with sterile deionized water. For the microorganisms able to grow in aerobic conditions (*E. coli, B. diminuta, A. niger, B. subtilis, B. pumilus* and *G. stearothermophilus*), 100 µL of the dilution were spread on the surface of the agar (PDA for *A. niger*, TSA for *G. stearothermophilus* and PCA for *E. coli, B. diminuta, B. subtilis* and *B. pumilus*) in triplicate. For *C. butyricum*, 500 µL of the dilution were poured on to empty plates in triplicate and the melted RCA agar was added to each plate. Plates were incubated (in anaerobic atmosphere for *C. butyricum*) at the optimum growth temperature and time (detailed conditions for each microorganism can be found in each part of the Results and Discussion section). The initial microbial population in the sample was also determined following the same procedures. The results were expressed as $\log_{10}(N/N_0)$, where N_0 is the initial number of cells in the untreated sample and N is the number of cells in the treated sample.

3.8. Modelling of microbial inactivation

Microbial inactivation kinetics were described by the Weibull model (Eq. 1), which is an empirical non-lineal model commonly used for describing inactivation kinetics of thermal and non-thermal inactivation treatments (Chen & Hoover, 2004; Peleg, 2006).

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

where N_0 (CFU/mL) is the initial microbial population in the sample, N the number of survival microorganism in the treated sample at time t (CFU/mL), b (min^{-n}) is a rate parameter and n is the shape parameter of the kinetic curve.

The kinetic constants (b and n) of the model were calculated by minimizing the sum of squared differences between experimental and the predicted data using Solver Microsoft Excel™ tool. The goodness of fit of the model was assessed by computing the root mean squared error (RMSE, Eq. 2) and the coefficient of determination (R^2, Eq. 3).

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

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

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

3.9. Ultrastructural analyses

The microscopy analyses of *E. coli*, *B. diminuta* and *A. niger* cells were performed by electron microscopy, after and before the SC-CO_2 + HPU treatments (Chapter 1, Part 1.1). The conditions selected were those that achieved the complete inactivation for each microorganism.

In order to observe the morphology and surface of the microbial cell walls, a field emission scanning electron microscope (FESEM) was used (ZEISS ULTRA 55, Oxfrod Instruments, Abingdon, UK). To this end, microbial samples were centrifuged, filtered and placed in the holder, frozen by immersion in liquid nitrogen and transferred to a cryogenic unit (PP3010T, Quorum Technologies, East Sussex, UK) to be sublimated and coated with platinum. Samples were observed at 1 kV at a working distance of between 3-5 mm. For the observation of the intracellular structure of the microorganisms, a transmission electron microscopy (TEM) was used (HT-7800 120

kV, Hitachi High-Technologies, Tokyo, Japan). For this purpose, microbial samples were centrifuged and fixed with glutaraldehyde solution for 24 h at 4 °C and osmium tetroxide solution for 1.5 h. After that, cells were stabilized with agarose solution at 30ºC and stored at 4ºC for 24 h. The solidified agar with the cells was cut into cubes (3 mm^3), which were fixed with glutaraldehyde solution and osmium tetroxide solution, dehydrated, contrasted with uranyl acetate solution and resin-embedded. The blocks obtained were cut in ultrathin sections (0.1 μm) with Reichert-Jung Ultracut ultramicrotome (Leica Microsystems, Wetzlar, Germany), collected in copper grids and stained with lead citrate to be observed at 100 kV.

More details on the procedure of the ultrastructural analyses can be found in the Materials & Methods subsection of part 1.1 (Chapter 1).

3.10. Physicochemical properties of the emulsions treated with SC-CO_2 + HPU

The physicochemical properties (appearance, pH, density, droplet size and zeta potential) were measured in triplicate before and after the SC-CO_2 + HPU treatments at the different conditions considered in a Box-Behnken experimental design. Three process variables were considered: pressure (from 100 to 600 bar), temperature (from 55 to 95ºC) and time (from 5 to 20 min). The design involved three levels for each factor and three replications at the center point (15 experimental runs).

3.10.1. Appearance, pH and density

The appearance of the samples was visually evaluated with the HLWC 111 fluorescent lamp (Waldmann, Germany) and signs of instability were searched. The pH was measured with a digital pH-meter (pHenomenal 1000, VWR, USA) and the density was measured using a densitometer (densito 30PX Mettler Toledo, Switzerland).

3.10.2. Droplet size and ζ -potential

Analyses of droplet size (D[4,3] and D[3,2]) were carried out using a laser diffractometer (Mastersizer 2000, Malvern Instruments Ltd, Worcestershire,UK). The Mie theory was applied considering a refractive index of 1.456 and absorption of 0.01. The emulsions were diluted in deionized water until an obscuration rate of 5-12% was obtained. ζ-potential was obtained by using a Zetasizer (Nano ZS, Malvern Instruments Ltd, Worcestershire, UK). Emulsions were diluted with deionized water at a concentration of 0.001% v/v. The Smoluchowsky model was used to transform the electrophoretic mobility of the droplets into ζ-potential values.

3.11. Statistical analyses

Statistical analyses were performed with Statgraphics Centurion XVI (Statpoint Technologies Inc., Warrenton, VA, USA). The inactivation kinetics obtained in the SC-CO_2 and SC-CO_2 + HPU treatments (Results subsections of **Chapter 1)** were analysed with a general linear model (GLM) in order to evaluate the effect of the treatment conditions (temperature, pressure, time and use of HPU) and the type of microorganism on the inactivation. Fisher's least significant difference (LSD) procedure was used to discriminate among the means ($p<0.05$). In order to evaluate the effect of the SC-CO_2 + HPU treatments on the physicochemical properties of emulsions (Part 1.4, Chapter 1), a Box-Behnken experimental design for response surface methodology (second-order polynomial model) was employed.

In the Results subsection of **Chapter 2**, a multifactorial ANOVA was used to evaluate if the operating conditions in the PEF treatments had a significant ($p<0.05$) influence on the inactivation of *E. coli* and the least significant difference (LSD) intervals were estimated to discriminate among the means ($p<0.05$). In addition, a one-way ANOVA was used to determine if the use of the different treatments (PEF and HPU) or its combination had a significant effect on the level of inactivation for every microorganism (*E. coli, A. niger* and *B. pumilus*). Fisher's least significant difference (LSD) procedure was used to discriminate among the means ($p<0.05$)

4. RESULTS AND DISCUSSION

CHAPTER 1

SC-CO_2 + HPU microbial inactivation

Journal of Supercritical Fluids (2021)-Submitted

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

Angela Gomez-Gomez[1], Edmundo Brito-de la Fuente[2], Críspulo Gallegos[2], Jose Vicente Garcia-Perez[1], Amparo Quiles[1] and Jose Benedito[1]

[1]Grupo ASPA, Departamento de Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, València E46022, Spain

[2]Fresenius-Kabi Deutschland GmbH, Product and Process Engineering Center, Pharmaceuticals & Device Division, Bad Homburg, Germany

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

Abstract

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

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

1. Introduction

Vegetative bacteria and fungal spores can easily become contaminants of food and pharmaceutical products, leading to product spoilage and causing human disease. In this regard, the assurance of microbiological safety is essential for the industry. Nowadays, thermal treatments are the most common preservation methods in the food and pharmaceutical sectors. In order to prevent heat damage related to thermal treatments and obtain higher quality products, novel non-thermal technologies, applied individually or in combined form, have been investigated and developed during the last few years. Some of these so-called non-thermal technologies are irradiation (Lung et al., 2015), high power ultrasound (Knorr et al., 2004), pulsed electric fields (Spilimbergo et al., 2003), high hydrostatic pressure (Ortuño, Duong, et al., 2013) and supercritical fluids (Ortuño et al., 2012a).

The supercritical state of carbon dioxide (SC-CO_2) is reached at moderate pressure and temperature (72.8 bar and 31.1ºC), avoiding the negative thermal effects of traditional preservation methods. In supercritical conditions, CO_2 presents lower viscosity than when in the liquid state and higher density than when in the gaseous state, making SC-CO_2 an excellent solvent that can contribute to the removal of vital components of microbial cells. In this sense, SC-CO_2 has already proven to be an effective method for the inactivation of some microorganisms, minimally affecting the physicochemical properties of the treated products (Damar & Balaban, 2006; Ferrentino et al., 2009; Gasperi et al., 2009). However, SC-CO_2 treatments often require long processing times and/or high temperatures and pressures to provide the necessary microbial reduction that ensures product safety. As an example, more than 75 min were insufficient to achieve the complete inactivation of *E. coli* in apple juice at 32ºC and 100-300 bar (Liao et al., 2008). For this reason, it is of great interest to combine the SC-CO_2 treatment with other non-thermal techniques, such as high power ultrasound (HPU), high hydrostatic pressure (HHP) (Ortuño, Duong, et al., 2013), pulsed electric fields (PEF) (Pataro et al., 2010) or the addition of antimicrobial agents, such as hydrogen peroxide (Shieh et al., 2009). In this regard, the application of HPU to the SC-CO_2 treatments has already been demonstrated to intensify the inactivation

of a wide range of vegetative bacteria and yeasts (Ferrentino & Spilimbergo, 2016; Ortuño et al., 2012b). However, the SC-CO_2 + HPU inactivation of filamentous fungal spores has not been explored yet.

The most widely accepted inactivation mechanisms of SC-CO_2 are linked to the diffusion and solubilisation of CO_2 into the external media causing a drop in pH that could damage or alter the microbial cell membrane. Thus, CO_2 penetrates into the cells, reducing the internal pH and extracting intracellular vital components which, eventually, can lead to cell death (Garcia-Gonzalez et al., 2009). When HPU is implemented, the heat and mass transfer processes are enhanced due to cavitation effects, which could increase the CO_2 diffusion rate, accelerating the SC-CO_2 inactivation mechanisms. In addition, HPU could damage or crack the cell walls of the microorganisms (Ferrentino & Spilimbergo, 2016; Ortuño et al., 2012b). A better understanding of the inactivation mechanisms exerted by the combination of SC-CO_2 and HPU is important in order to find improved strategies with which to guarantee the safety and stability of the treated products, as well as optimize the process conditions or the equipment. For that purpose, the analysis of the treated microbial cells at cellular level using microscopy techniques constitutes a valuable approach. Several authors observed the ultrastructure of microbial cells after SC-CO_2 inactivation treatments and stated that there was a direct relation between the permeabilization of the cell membrane and the inactivation (Garcia-Gonzalez et al., 2010; Liao et al., 2010). Additionally, Ortuño et al. (2014) investigated the effect of the SC-CO_2 + HPU treatment on the intracellular structure of vegetative microorganisms (*E. coli* and *S. cerevisiae*). However, there has been no prior analysis of the cell structural effects linked to the SC-CO_2 + HPU treatment on filamentous fungal spores. Moreover, the analysis of the changes in the external structure of the microbial cells after the SC-CO_2 + HPU treatment is unexplored. Therefore, the objective of this study was to evaluate (i) the intensification of the SC-CO_2 + HPU inactivation of different bacteria (*E. coli* and *B. diminuta*) and a fungal spore (*A. niger*) and (ii) the effect of the inactivation treatment on the external morphology and the intracellular structure of the microbial cells.

2. Materials and methods

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

E. coli CECT 101 and *B. diminuta* CECT 313 were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). *E. coli* is a facultative anaerobic gram-negative bacteria with a cell size of around 1 × 3 µm (Reshes et al., 2008), highly common in contaminated food and pharmaceutical products. *B. diminuta* is an aerobic gram-negative bacteria, which is used to test the porosity of pharmaceutical grade filters of 0.2 µm because of its small size (Sundaram et al., 2001): typically of around 0.3 x 1.0 µm (Madaeni, 1999). A single colony of each bacterium was inoculated in 50 mL of nutrient broth (Scharlab, Barcelona, Spain) and grown overnight (18-24 h) at 37ºC for *E. coli* and 30ºC for *B. diminuta*, using an incubation chamber (3000957, J.P. Selecta, Spain) and an orbital shaker at 120 rpm (3000974, J.P. Selecta, Spain). 50 µL of the overnight starter culture were transferred to a new growth medium and it was incubated until ensuring the stationary phase was reached, 14 h at 37ºC for *E. coli* and 36 h at 30ºC for *B. diminuta* (Gomez-Gomez et al., 2020). After that, 5 mL of the bacterial suspension in the stationary phase were inoculated in 60 mL of deionized water until a concentration of around 10^8 CFU/mL.

2.2. Preparation of the *Aspergillus niger* spore suspension

A. niger CECT 2807 was also obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). *A. niger* is an aerobic spore-forming filamentous fungi, commonly present in the environment and, thus, usually present in contaminated food and pharmaceutical products (Shimoda et al., 2002). *A. niger* was cultured on Potato Dextrose Agar (Scharlab, Barcelona, Spain) at 25ºC for 7 days. After that, spores were rubbed with 10 ml of 0.1% (v/v) Tween 80, collected and kept at 4ºC until use. Prior to each treatment, 5 mL of the *A. niger* spore suspension were inoculated in 60 mL of deionized water until a concentration of around 10^7 CFU/mL.

2.3. Ultrasonic assisted supercritical fluid inactivation treatments

The inactivation treatments with supercritical carbon dioxide (SC-CO_2) were performed in a supercritical fluids lab-scale equipment and batch mode. A high-power ultrasound (HPU) transducer, embedded in the treatment chamber through the cap, was used to perform the combined SC-CO_2 + HPU treatments. The ultrasound system consisted mainly of a high power piezoelectric transducer, a sonotrode and a power generation unit. The power supplied was 50 ± 5 W (I= 250 ± 10 mA; U= 220 ± 5 V) and the frequency was 30 ± 2 kHz. Electrical parameters were measured with a digital power meter (WT210, Yokogawa Electric Corporation, Tokyo, Japan). This system was explained in detail in a previous study (Gomez-Gomez et al., 2020).

SC-CO_2 and SC-CO_2 + HPU inactivation treatments were carried out at two levels of pressure and temperature for each microorganism. The pressure was set at 100 and 350 bar for all the microorganisms, as they are common pressures used in SC-CO_2 microbial inactivation studies (Soares et al., 2019). The temperature was set at 35 and 50ºC for *E. coli* and *B. diminuta* in order to select a low temperature (35 ºC), very close to the critical temperature for CO_2 and a mild, but non-lethal, temperature (50ºC) for the vegetative bacteria considered in this study. In this regard, preliminary experiments revealed that no inactivation was found for inoculated *E. coli* and *B. diminuta* in deionized water using a water bath and heating at 50ºC for 50 min. In the case of *A. niger*, treatments were carried out at 50 and 60ºC due to the known greater resistance of fungal spores to SC-CO_2 compared to vegetative bacteria (Soares et al., 2019). Samples of around 2 mL were collected during the treatments at different times, depending on the process conditions and type of microorganism. All the experiments were carried out in triplicate.

2.4. Microbiological analyses

The standard plate count was used to measure the number of surviving microorganisms. Serial dilutions of the treated samples were prepared and 100 µL of the appropriate dilutions were spread on PCA for the bacteria and PDA for *A. niger* (Scharlab, Barcelona, Spain) in triplicate. Plates were incubated at 37ºC and 24 h for

E. coli, 30ºC and 48 h for *B. diminuta* and 25ºC and 72 h for *A. niger*. The initial microbial population in the sample was determined following the same procedure. Results were expressed as $\log_{10}(N/N_0)$, where N_0 represents the number of cells initially inoculated in the deionized water and N the number of cells after treatment.

2.5. Modelling

The non-linear Weibull model following the decimal logarithmic form written by Peleg (1999) has been demonstrated to be sufficiently robust for the prediction of microbial inactivation (Peleg, 2006), and was used in this study (Eq. (1)).

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

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

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

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

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

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

The model can be fitted to both downward concave survival curves (n>1) and upward concave curves (n<1); and the log linear curve is a special case where n=1. As described elsewhere (Gomez-Gomez et al., 2021), the time required to achieve the complete inactivation (t_x) of every microorganism was calculated from Eq. 1 and from the b and n values of the Weibull model obtained for each condition, where x is the average in log-cycles for the complete inactivation of each microorganism (7.9 log-cycles in the case of *E. coli*, 8.1 log-cycles in that of *B. diminuta* and 6.8 log-cycles for *A. niger*).

2.6. Statistical analysis

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

2.7. Electron microscopy analyses

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

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

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

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

3. Results and discussion

3.1. Analysis of the inactivation kinetics

3.1.1. SC-CO_2 microbial inactivation

Fig. 1 shows the inactivation kinetics of *E. coli* (A), *B. diminuta* (B) and *A. niger* spores (C) in deionized water for the SC-CO_2 treatments. A high degree of experimental variability was found, as can be observed from the error bars, which could be ascribed to pressure and temperature variations inside the vessel, and to the inherent variability in the microbial growth. Despite the great experimental variability, the Weibull model was satisfactorily fitted to the SC-CO_2 inactivation kinetics, as depicted in Fig. 1. In every case, R^2 was higher than 0.92 and RMSE was lower than 0.64, as shown in Table

1. For some of the inactivation kinetics, the model was not fitted due to the lack of experimental data.

Effect of pressure and temperature

Pressure and temperature had a significant ($p<0.05$) effect on the SC-CO_2 inactivation of *B. diminuta* and *A. niger*. In general terms, the higher the pressure and the temperature, the faster the inactivation. As an example, a reduction of only 4.4 log-cycles of *A. niger* was achieved in 90 min at 100 bar and 50ºC, while the complete inactivation (6.7 log-cycles) was reached in just 15 min at 350 bar and 60ºC (Fig. 1C). However, only the temperature had a significant ($p<0.05$) effect on the inactivation of *E. coli*. For example, complete inactivation (7.9 log-cycles) was reached in 25 min at 35ºC while the same inactivation was achieved in less than 13 min at 50ºC, regardless of the pressure used (Fig. 1A). Moreover, when modelling the inactivation kinetics, less time was generally required to achieve the complete inactivation of the *E. coli, B. diminuta* and *A. niger* population ($t_{7.9}$, $t_{8.1}$ and $t_{6.8}$; respectively), as the pressure and the temperature increased (Table 1). For example, in the case of *A. niger*, 163.7 and 22.1 min were required at 100 bar and 50ºC and at 350 bar and 60ºC, respectively. On the one hand, high temperatures are known to increase CO_2 diffusivity and make cell membranes more fluid, facilitating the penetration of CO_2 (Ferrentino & Spilimbergo, 2015; Hong et al., 1999). On the other hand, high pressures increase the solubility of CO_2 in the media; therefore, there is closer contact between CO_2 and the microbial cell, and the CO_2 penetration into the cells is improved (Hong et al., 1997; Liao et al., 2007).

Several authors also studied the SC-CO_2 inactivation of *E. coli* and *A. niger* in water solutions (Ballestra & Cuq, 1998; Dillow et al., 1999; Kobayashi et al., 2007; Noman et al., 2018; Yuk et al., 2009). However, only one study was found into the inactivation of *B. diminuta* (Gomez-Gomez et al., 2020). Noman et al. (2018) also found that there was notably greater inactivation of *A. niger* spores at higher pressures and temperatures (e.g. in 60 min and at a temperature of 55ºC, microbial reduction increased from 2.8 log-cycles at 300 bar to 4.1 at 350 bar, and at a constant pressure

of 350 bar it increased from 2.0 to 4.6 log-cycles at temperatures of 35 and 55ºC, respectively). The inactivation levels achieved by Noman et al. (2018) were of a lower magnitude than those in the present study (e.g. 6.8 log-cycles was achieved at 350 bar, 50ºC and 55 min). As for *E. coli*, Dillow et al. (1999) reported similar effects of pressure and temperature to those in our study, since the temperature (34 vs 42ºC) affected the inactivation, achieving complete inactivation (around 8.0 log-cycles) in 30 min at 34ºC and in 20 min at 42ºC (205 bar), while the influence of pressure was negligible (from 140 to 205 bar at 34ºC). Ortuño et al. (2012b) also found very similar reductions in the inactivation of *E. coli* in LB Broth, compared to the present results (e.g 8.0 log-cycles were achieved at 36ºC and 350 bar in 22 min, while in the present study the same inactivation was found in 24 min at 35ºC and 350 bar). However, these authors found that both temperature (31-41 ºC) and pressure (100-350 bar) influenced microbial inactivation. As regards *B. diminuta*, Gomez-Gomez et al. (2020) also found that the higher the pressure and temperature, the higher the inactivation levels in an oil-in-water emulsion. However, the processing time required to achieve the complete inactivation of *B. diminuta* in the emulsions was longer than in the present study under the same conditions (e.g. 40 min were required in the lipid emulsion while only 10 min in water (Fig. 1B) at 350 bar and 50ºC). This was coherent with what can be found in literature, since SC-CO_2 inactivation treatments have proven to be more effective in simple media than in complex and, in addition, oil is known to protect the microorganisms from different external stresses, including SC-CO_2 (Garcia-Gonzalez et al., 2009; Gomez-Gomez et al., 2020).

Effect of the type of microorganism

As for the resistance of the different microorganisms studied to the SC-CO_2 treatments, significant ($p<0.05$) differences were found, *A. niger* being the most resistant of all three microorganisms. On the contrary, very slight differences were found between *E. coli* and *B. diminuta*. At 50ºC and 350 bar, 55 min were required to achieve the complete inactivation of *A. niger* (6.8 log-cycles), while around 10 min were needed for *E. coli* or *B. diminuta* (around 8 log-cycles). This observation was coherent

with the fact that fungal spores are more resistant to SC-CO_2 treatments than vegetative bacteria (Soares et al., 2019), probably due to their different and more resistant structure. Fungal spores are composed of a multi layered and highly dehydrated structure, which could restrain the CO_2 dissolution and penetration into the spore. Moreover, the structure of *A niger* spores, in particular, include a layer of melanin, which is believed to be related to a higher resistance to environmental stresses (Tischler & Hohl, 2019). Similarly, Wu et al. (2007) achieved a reduction of 4.3 log-cycles for *E. coli* at 78 bar, 35ºC and in 30 min, while only an inactivation of 2.1 log-cycles was obtained for *Absidia coerulea* spores.

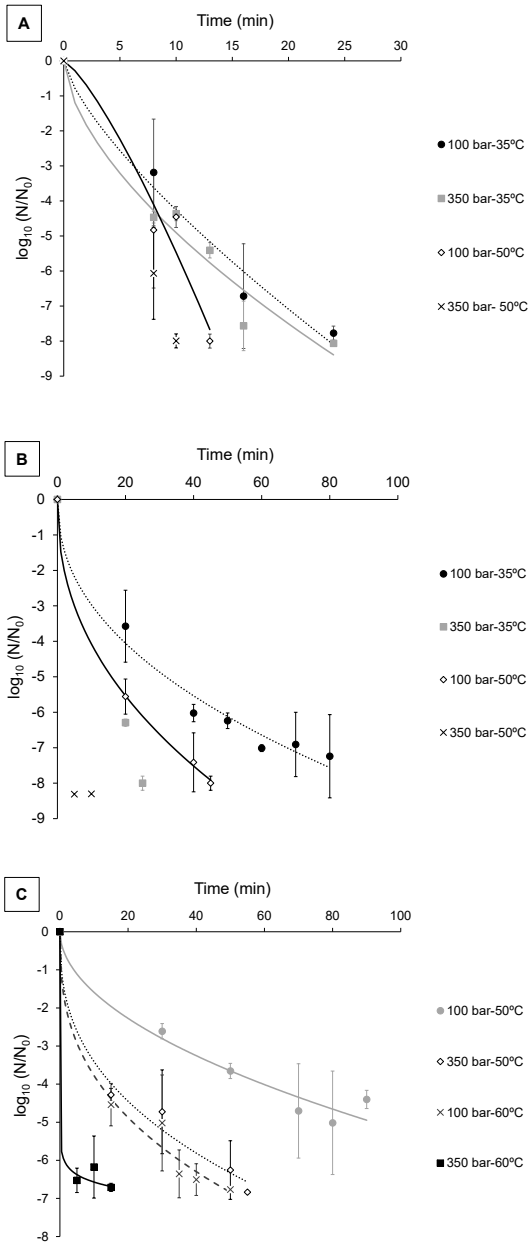


Fig. 1. Inactivation kinetics of *E. coli* (A), *B.diminuta* (B) and *A. niger* spores (C) in water treated with SC-CO_2. Experimental data (discrete points) and Weibull model (continuous and dashed lines). Error bars show the experimental variability.

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

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

* Insufficient experimental data for model fitting

3.1.2. SC-CO_2 + HPU microbial inactivation

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

Effect of pressure and temperature

The higher the pressure and temperature, the higher the level of inactivation of the SC-CO_2 + HPU treatments for *B. diminuta* and *A. niger*. However, only the temperature had a significant ($p<0.05$) effect on the inactivation, as also occurred in the SC-CO_2 treatments. With respect to *E. coli* (Fig. 2A), in order to achieve complete inactivation (7.9 log-cycles), raising the temperature from 35 to 50ºC meant that 2.5 min (from 5.5 to 3 min) less were needed, on average; however, only 0.5 min (from 4.5 to 4 min) less were needed when the pressure is raised from 100 to 350 bar. On the other hand, on average, the time required to achieve the complete inactivation of *B. diminuta* (8.1 log-cycles), was shortened from 12 to 6.5 min by raising the temperature from 35 to 50ºC and from 15 to 3.5 min when the pressure was increased from 100 to 350 bar (Fig. 2B). The effect of the temperature was also revealed by the time needed for complete inactivation (t_x) calculated by the Weibull model (Table 2). As an example, for *A. niger*, $t_{6.8}$ was shortened on average from 63.4 min to 23.1 min when the temperature was raised from 50 to 60ºC. Contrary to the results of the present study, some authors found that the increase in the pressure and temperature in the SC-CO_2 + HPU treatments did not affect the microbial inactivation rate, probably because the marked effect of cavitation masked the effects of the other processing conditions (Ortuño et al., 2012b, 2014; Ortuño, Martínez-Pastor, et al., 2013). However, Gomez-Gomez et al. (2020) found that the higher the pressure and temperature, the higher the level of SC-CO_2 + HPU inactivation for *E. coli* and *B. diminuta* in oil-in-water emulsions.

Effect of HPU and the type of microorganism

As in the SC-CO_2 treatments, significant ($p<0.05$) differences were found as regards the resistance of the different microorganisms to the combined SC-CO_2 + HPU treatment. *A. niger* was the most resistant, while *E. coli* and *B. diminuta* showed similar resistance. In fact, the complete inactivation of *E. coli* and *B. diminuta* was achieved in less than 18 min, even at low temperatures and pressures (100 bar and 35ºC), whereas for *A. niger*, at least 35 min were required to obtain complete inactivation at the same

pressure (100 bar) and higher temperatures (50-60ºC), which was considered too long a treatment for industrial applications. The use of a higher pressure (350 bar) and 60ºC was necessary to achieve the complete inactivation of *A. niger* in a shorter time (10 min), which could be considered a reasonable industrial processing time.

HPU significantly ($p<0.05$) intensified the SC-CO_2 treatments for all the microorganisms considered in the present study, the effect being milder in the case of *A. niger* spores than for *E. coli* and *B. diminuta* (on average, the increase in the inactivation when HPU was used was 1.3, 4.8 and 3.4 log-cycles, respectively). For instance, as observed in Figs. 1 and 2, the time needed for the complete inactivation of *E. coli* and *B. diminuta* at 50ºC and 350 bar was shortened by 19 min when HPU was applied; in the case of *A. niger,* on the other hand*,* the process was shortened by only 5 min. Thus, the application of HPU reduced the calculated t_x, on average; from 59.1 to 24.8 min (Tables 1 and 2). HPU is known to increase the mass and heat transfer under SC-CO_2 conditions (Cárcel et al., 2012; Gao et al., 2009) and, consequently, to enhance both the CO_2 solubilisation in the media and penetration inside the microbial cells (Garcia-Gonzalez et al., 2007). Moreover, HPU could cause cracked or damaged cell walls due to the effects of cavitation (Ortuño, Martínez-Pastor, et al., 2013).

Several authors (Ortuño et al., 2012b; Ortuño, Martínez-Pastor, et al., 2013; Paniagua-Martínez et al., 2016) also studied the application of HPU during the SC-CO_2 treatments of vegetative cells in liquid media and found that HPU drastically increased the inactivation level.

The inactivation of different microorganisms depended not only on the effect of external stresses, but also on the cell size and morphology (Chemat et al., 2011; Tonyali et al., 2019). Ortuño et al. (2014) related the degree of cavitation to the size of the vegetative cells since they observed a stronger effect of cavitation on *S. cerevisiae* (8–10 μm) than on *E. coli* and assumed that there was a greater likelihood of the cavitation bubbles affecting the cell structure when the cells are larger. However, in the present study, there was probably not a big enough difference between the sizes of the *E. coli* and *B. diminuta* cells (a difference of less than 2 μm) to appreciate significant differences in the SC-CO_2 + HPU inactivation between these microorganisms, as also

reported by Gomez-Gomez et al. (2020). In the case of *A. niger* spores, the cell size (around 4 μm (Teertstra et al., 2017)) is larger than in *E. coli* and *B. diminuta*. However, it is known that molds are generally more resistant to cavitation than vegetative cells because of the differences between the cell wall structure of species (López-Malo et al., 2005). In particular, *A. niger* spores might be more resistant to cavitation due to the greater rigidity of its cell wall due to the presence of melanin (Tischler & Hohl, 2019).

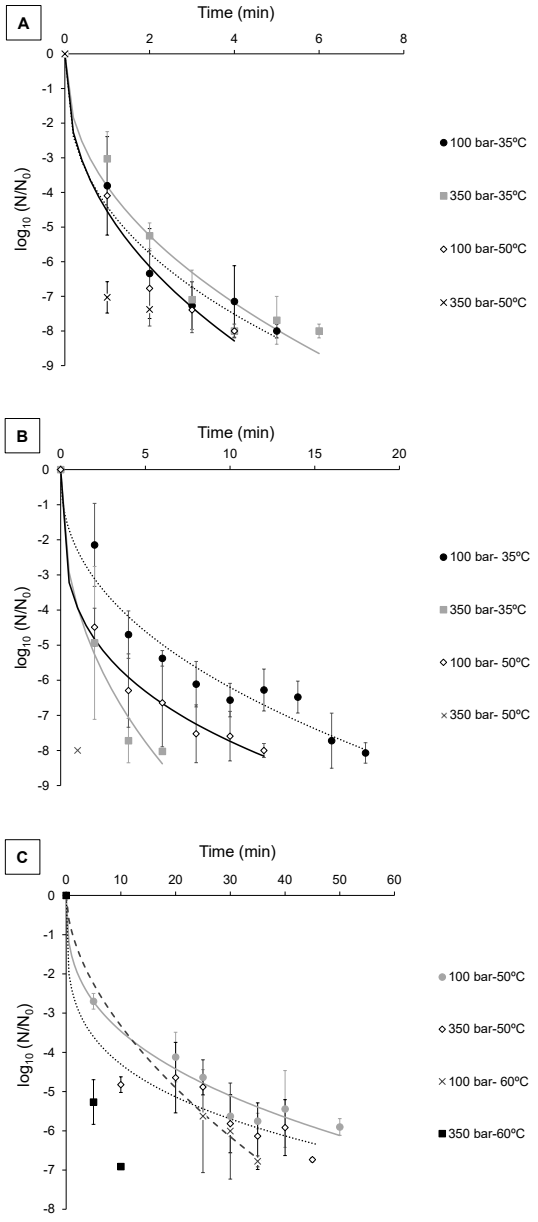


Fig. 2. Inactivation kinetics of *E. coli* (A), *B.diminuta* (B) and *A. niger* spores (C) in water treated with SC-CO_2 + HPU. Experimental data (discrete points) and Weibull model (continuous and dashed lines). Error bars show the experimental variability.

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

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

* Insufficient experimental data for model fitting

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

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

The untreated *E. coli* cells showed an intact, well-defined and characteristic rod-shaped structure with a smooth surface (Fig. 3A). The cell wall and the cell membrane presented defined boundaries and were intact, enclosing the cytoplasmic content. In addition, the cell wall can be seen to be attached to the cell membrane (Fig. 4A). As regards the intracellular space, it is observed to be completely and uniformly filled with the cytoplasm (Fig. 4A), with no signs of cytoplasm observed outside the

cells. On the contrary, the *E.coli* cells treated with SC-CO_2 + HPU lost their shape (Figs. 3B and 4B), appeared agglomerated and some of them merged, losing their individuality (Fig.3B). The cell walls and membranes were seen to be blurred (Fig. 4B), which indicated that they were partly disintegrated. Inside the cell, empty regions were observed (Fig. 4B), which could be due to a large amount of cytoplasmic content released by the effect of the treatment. This is indicated by the stains being found in the extracellular region (Fig. 4B). Ortuño et al. (2014) observed important morphological changes in *E. coli* cells treated with SC-CO_2 + HPU at 350 bar, 36ºC for 5 min. The cytoplasmic space presented big empty regions with an aggregated and unevenly distributed cytoplasm. In addition, the cell wall and membrane appeared disintegrated. On the contrary, after a SC-CO_2 treatment under the same conditions, *E. coli* cells only presented slight changes.

B. diminuta cells also presented a clear rod-shaped structure with a smooth surface when untreated (Fig. 3C). The cell walls and membranes were intact and well-defined, (Figs. 3C and 4C) enclosing the cytoplasm, which was uniformly distributed in the whole intracellular space (Fig. 4C). Thus, the SC-CO_2 + HPU treatment caused different alterations in the bacteria morphology. The surface appeared irregular with roughness and wrinkles and some bacteria were found merged together (Fig. 3D). The cell walls and membranes were undefined (Fig. 4D), indicating that they were damaged. In addition, empty regions in the intracellular space were observed (Fig. 4D), indicating that a great amount of cytoplasmic content was lost with the treatment. No studies were found analyzing the ultrastructure of *B. diminuta* cells treated with either SC-CO_2 or SC-CO_2 + HPU.

B. diminuta and *E. coli* cells were both similarly affected by the SC-CO_2 + HPU treatment. As several authors (Garcia-Gonzalez et al., 2010; Ortuño et al., 2014; Spilimbergo et al., 2007) observed in the case of SC-CO_2 treatments, the morphological structure of the microbial cells, including the cell wall, generally remained almost intact or with only minor alterations, while changes in the intracellular structure, such as an uneven distribution of the cytoplasm, were found. Consequently, it could be stated that the inactivation effect was probably due to an increase in the permeability of the cells exerted by SC-CO_2, instead of to the rupture of the cell wall and membrane. By

contrast, the application of HPU could crack or damage cell walls, severely affecting their morphological integrity (Ortuño et al., 2014), as observed in the images of the present study (Figs.3 and 4).

In the case of *A. niger* spores, untreated cells presented a globular shape and a spiny, wrinkled surface (Fig. 3E). The cell wall and membrane showed defined boundaries and uniform thickness (Fig.4E). In addition, in the intracellular region, the organelles were clearly visible and well distributed in a dense cytoplasm (Fig.4E). In contrast, *A. niger* spores treated with SC-CO_2 + HPU demonstrated significant alterations. The shape of the cell changed, since it appeared shrunken and squashed and most of the cells presented a clear cleft in the center (Figs. 3F and 4F). After the treatment, the cell wall appeared much thinner than that of the untreated spores, showing an uneven width with some dissolved areas (Fig. 4F). Moreover, the cell membrane was also thinner and presented an uneven thickness (Fig. 4F). As to the inner region of the spore, it was almost empty due to leakages of cytoplasmic content (Fig. 4F) and the organelles were not visible, indicating that they were completely destroyed after the treatment. Only a small, darker region was found in the cytoplasm, which could be due to the precipitation or aggregation of internal cell components (Fig. 4F). Although no studies were found assessing the ultrastructural changes in *A. niger* spores after SC-CO_2 + HPU treatments, Noman et al. (2018) and Efaq et al. (2017) observed the morphology of *A. niger* spores treated with SC-CO_2 at 300 bar, 75 °C for 90 min. These authors found completely damaged and deformed spores, with disintegrated cell wall and membrane. Nevertheless, an extremely long process time was used in those studies (90 min) and a higher temperature than in the present study (75ºC vs 60ºC).

In general terms, an ultrastructural analysis revealed that for the three microorganisms involved in the present study, the combined SC-CO_2 + HPU treatment damaged the cell walls and affected the permeability of cell membranes, which led to changes in the cell morphology and the release of cytoplasmic content and, consequently, cell death.

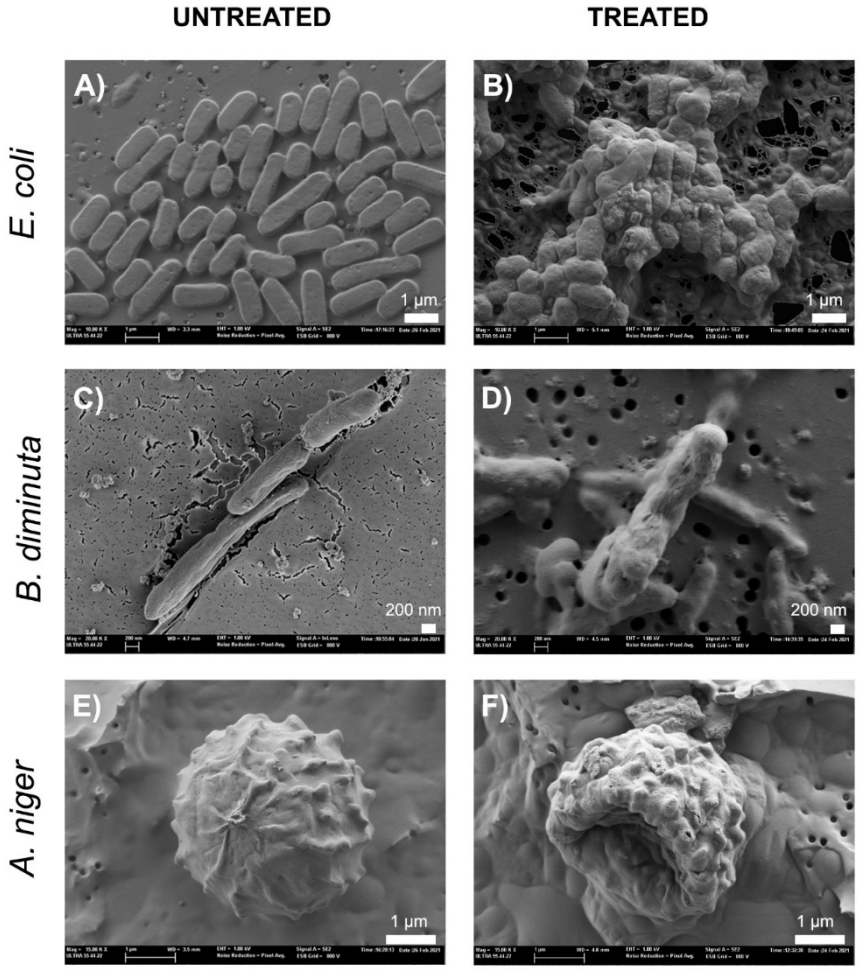


Fig. 3. CryoFESEM photographs of *E. coli* (A and B), *B. diminuta* (C and D) and *A. niger (E and F)* untreated and treated by SC-CO_2 + HPU.

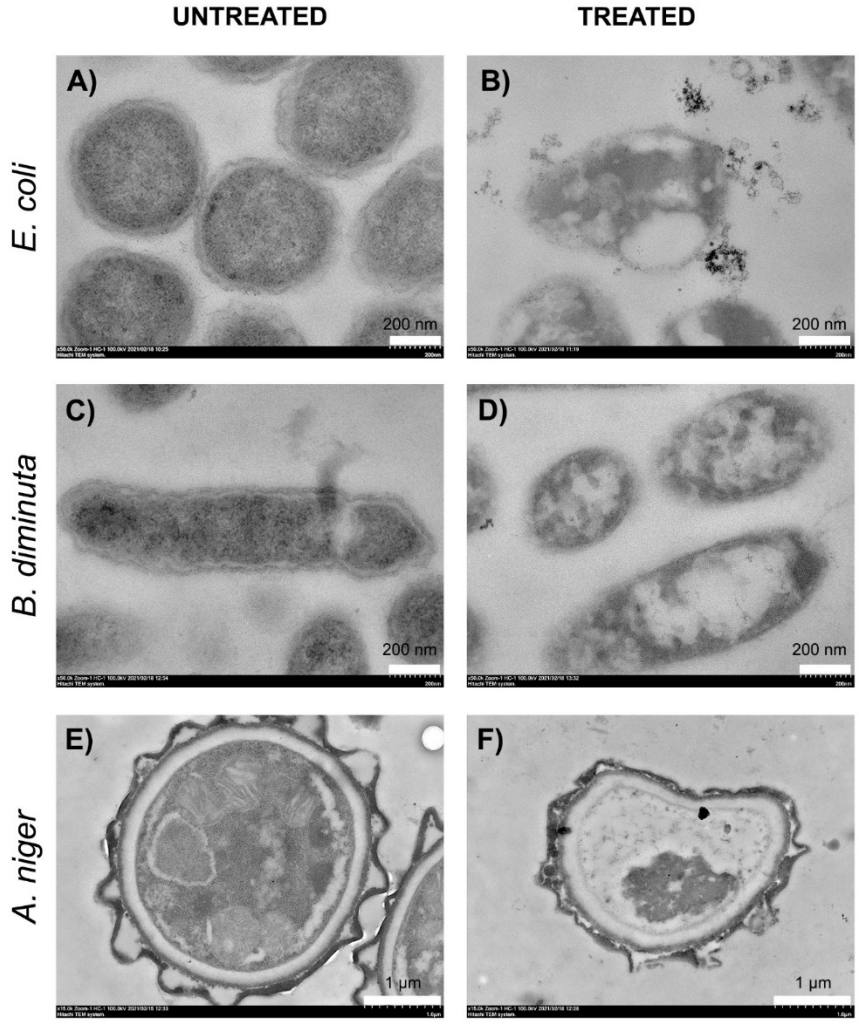


Fig. 4. TEM photographs of *E. coli* (A and B)*, B. diminuta* (C and D) and *A. niger (E and F) untreated and* treated by SC-CO_2 + HPU.

4. Conclusions

Ultrasonic application has proven to be an effective way of shortening the inactivation time in SC-CO_2 treatments of the microorganisms studied (two vegetative bacteria and one fungal spore). The higher the pressure and the temperature, the

greater the inactivation of *B. diminuta* and *A. niger*. However, it was only the temperature that had a significant effect on *E.coli*. The results obtained confirmed the more marked effect that cavitation has on the SC-CO_2 inactivation of vegetative bacteria, compared to fungal spores.

The ultrastructural analysis illustrated the external morphology and intracellular structure of bacteria (*E. coli* and *B. diminuta*) and fungal spores (*A. niger*), which definitively contributed to a better understanding of the effects of the SC-CO_2 + HPU treatments. SC-CO_2 + HPU-treated cells presented a deformed shape, partly disintegrated walls and membranes and a leakage of cytoplasmic content, which explains the effectiveness of the SC-CO_2 + HPU treatments for microbial inactivation purposes. Thus, regardless of the type of microorganism (vegetative bacteria or fungal spore) and its different cell wall structure and composition, the SC-CO_2 + HPU treatment caused structural damage leading to cell death. Further research into the ultrastructure of other fungal or bacterial spores in different complex media is required to elucidate the exact inactivation mechanisms of the SC-CO_2 + HPU technology.

Acknowledgement

The authors acknowledge the financial support from Fresenius Kabi.

References

Ballestra, P., & Cuq, J. L. (1998). Influence of pressurized carbon dioxide on the thermal inactivation of bacterial and fungal spores. *LWT - Food Science and Technology*, *31*(1), 84–88. https://doi.org/10.1006/fstl.1997.0299

Cárcel, J. A., García-Pérez, J. V., Benedito, J., & Mulet, A. (2012). Food process innovation through new technologies: Use of ultrasound. *Journal of Food Engineering*, *110*(2), 200–207. https://doi.org/10.1016/J.JFOODENG.2011.05.038

Chemat, F., Zill-E-Huma, & Khan, M. K. (2011). Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry*, *18*(4), 813–835. https://doi.org/10.1016/j.ultsonch.2010.11.023

Damar, S., & Balaban, M. O. (2006). Review of dense phase CO2 technology: microbial and enzyme inactivation, and effects on food quality. *Journal of Food Science*, *71*(1), 1–11.

Dillow, A. K., Dehghani, F., Hrkach, J. S., Foster, N. R., & Langer, R. (1999). Bacterial inactivation by using near- and supercritical carbon dioxide. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(18), 10344–10348. https://doi.org/10.1073/pnas.96.18.10344

Efaq, A. N., Nik Norulaini, N. N. N., Nagao, H., Al-Gheethi, A. A., & Ab. Kadir, M. O. (2017). Inactivation of Aspergillus spores in clinical wastes by supercritical carbon dioxide. *Arabian Journal for Science and Engineering*, *42*(1), 39–51. https://doi.org/10.1007/s13369-016-2087-5

Ferrentino, G., Plaza, M. L., Ramirez-Rodrigues, M., Ferrari, G., & Balaban, M. O. (2009). Effects of dense phase carbon dioxide pasteurization on the physical and quality attributes of a red grapefruit juice. *Journal of Food Science*, *74*(6). https://doi.org/10.1111/j.1750-3841.2009.01250.x

Ferrentino, G., & Spilimbergo, S. (2015). High pressure carbon dioxide combined with high power ultrasound pasteurization of fresh cut carrot. *The Journal of Supercritical Fluids*, *105*, 170–178.

https://doi.org/10.1016/J.SUPFLU.2014.12.014

Ferrentino, G., & Spilimbergo, S. (2016). A combined high pressure carbon dioxide and high power ultrasound treatment for the microbial stabilization of cooked ham. *Journal of Food Engineering*, *174*, 47–55. https://doi.org/10.1016/J.JFOODENG.2015.11.013

Gao, Y., Nagy, B., Liu, X., Simándi, B., & Wang, Q. (2009). Supercritical CO2 extraction of lutein esters from marigold (Tagetes erecta L.) enhanced by ultrasound. *The Journal of Supercritical Fluids*, *49*(3), 345–350. https://doi.org/10.1016/J.SUPFLU.2009.02.006

Garcia-Gonzalez, L., Geeraerd, A. H., Elst, K., Van Ginneken, L., Van Impe, J. F., & Devlieghere, F. (2009). Influence of type of microorganism, food ingredients and food properties on high-pressure carbon dioxide inactivation of microorganisms. *International Journal of Food Microbiology*, *129*(3), 253–263. https://doi.org/10.1016/j.ijfoodmicro.2008.12.005

Garcia-Gonzalez, L., Geeraerd, A. H., Mast, J., Briers, Y., Elst, K., Van Ginneken, L., Van Impe, J. F., & Devlieghere, F. (2010). Membrane permeabilization and cellular death of Escherichia coli, Listeria monocytogenes and Saccharomyces cerevisiae as induced by high pressure carbon dioxide treatment. *Food Microbiology*, *27*(4), 541–549. https://doi.org/10.1016/j.fm.2009.12.004

Garcia-Gonzalez, L., Geeraerd, A. H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Van Impe, J. F., & Devlieghere, F. (2007). High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. In *International Journal of Food Microbiology* (Vol. 117, Issue 1, pp. 1–28). https://doi.org/10.1016/j.ijfoodmicro.2007.02.018

Gasperi, F., Aprea, E., Biasioli, F., Carlin, S., Endrizzi, I., Pirretti, G., & Spilimbergo, S. (2009). Effects of supercritical CO2 and N2O pasteurisation on the quality of fresh apple juice. *Food Chemistry*, *115*(1), 129–136. https://doi.org/10.1016/j.foodchem.2008.11.078

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., &

Benedito, J. (2020). Non-thermal pasteurization of lipid emulsions by combined supercritical carbon dioxide and high-power ultrasound treatment. *Ultrasonics Sonochemistry*, *67*, 105–138. https://doi.org/10.1016/J.ULTSONCH.2020.105138

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2021). Ultrasonic-assisted supercritical CO2 inactivation of bacterial spores and effect on the physicochemical properties of oil-in-water emulsions. *The Journal of Supercritical Fluids*, *174*(March), 105246. https://doi.org/10.1016/j.supflu.2021.105246

Hong, S. I., Park, W. S., & Pyun, Y. R. (1997). Inactivation of Lactobacillus sp. from Kimchi by high pressure carbon dioxide. *LWT - Food Science and Technology*, *30*(7), 681–685. https://doi.org/10.1006/FSTL.1997.0250

Hong, S. I., Park, W. S., & Pyun, Y. R. (1999). Non-thermal inactivation of Lactobacillus plantarum as influenced by pressure and temperature of pressurized carbon dioxide. *International Journal of Food Science and Technology*, *34*(2), 125–130. https://doi.org/10.1046/j.1365-2621.1999.00241.x

Knorr, D., Zenker, M., Heinz, V., & Lee, D. U. (2004). Applications and potential of ultrasonics in food processing. *Trends in Food Science and Technology*, *15*(5), 261–266. https://doi.org/10.1016/j.tifs.2003.12.001

Kobayashi, F., Hayata, Y., Kohara, K., Muto, N., & Osajima, Y. (2007). Application of supercritical CO2 bubbling to inactivate E. coli and coliform bacteria in drinking water. *Food Science and Technology Research*, *13*(1), 20–22. https://doi.org/10.3136/fstr.13.20

Liao, H., Hu, X., Liao, X., Chen, F., & Wu, J. (2007). Inactivation of Escherichia coli inoculated into cloudy apple juice exposed to dense phase carbon dioxide. *International Journal of Food Microbiology*, *118*(2), 126–131. https://doi.org/10.1016/J.IJFOODMICRO.2007.06.018

Liao, H., Zhang, F., Liao, X., Hu, X., Chen, Y., & Deng, L. (2010). Analysis of Escherichia coli cell damage induced by HPCD using microscopies and

fluorescent staining. *International Journal of Food Microbiology*, *144*(1), 169–176. https://doi.org/10.1016/j.ijfoodmicro.2010.09.017

Liao, H., Zhang, Y., Hu, X., Liao, X., & Wu, J. (2008). Behavior of inactivation kinetics of Escherichia coli by dense phase carbon dioxide. *International Journal of Food Microbiology*, *126*(1–2), 93–97. https://doi.org/10.1016/J.IJFOODMICRO.2008.05.008

López-Malo, A., Palou, E., Jiménez-Fernández, M., Alzamora, S. M., & Guerrero, S. (2005). Multifactorial fungal inactivation combining thermosonication and antimicrobials. *Journal of Food Engineering*, *67*(1–2), 87–93. https://doi.org/10.1016/j.jfoodeng.2004.05.072

Lung, H. M., Cheng, Y. C., Chang, Y. H., Huang, H. W., Yang, B. B., & Wang, C. Y. (2015). Microbial decontamination of food by electron beam irradiation. *Trends in Food Science and Technology*, *44*(1), 66–78. https://doi.org/10.1016/j.tifs.2015.03.005

Madaeni, S. S. (1999). The application of membrane technology for water disinfection. *Water Research*, *33*(2), 301–308. https://doi.org/10.1016/S0043-1354(98)00212-7

Noman, E., Norulaini Nik Ab Rahman, N., Al-Gheethi, A., Nagao, H., Talip, B. A., & Ab. Kadir, O. (2018). Selection of inactivation medium for fungal spores in clinical wastes by supercritical carbon dioxide. *Environmental Science and Pollution Research*, *25*(22), 21682–21692. https://doi.org/10.1007/s11356-018-2335-1

Ortuño, C., Duong, T., Balaban, M., & Benedito, J. (2013). Combined high hydrostatic pressure and carbon dioxide inactivation of pectin methylesterase, polyphenol oxidase and peroxidase in feijoa puree. *Journal of Supercritical Fluids*, *82*, 56–62. https://doi.org/10.1016/j.supflu.2013.06.005

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012a). Supercritical carbon dioxide inactivation of Escherichia coli and Saccharomyces cerevisiae in different growth stages. *The Journal of Supercritical Fluids*, *63*, 8–15. https://doi.org/10.1016/J.SUPFLU.2011.12.022

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012b). An ultrasound-enhanced system for microbial inactivation using supercritical carbon dioxide. *Innovative Food Science & Emerging Technologies*, *15*, 31–37. https://doi.org/10.1016/J.IFSET.2012.02.006

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2013). Application of high power ultrasound in the supercritical carbon dioxide inactivation of Saccharomyces cerevisiae. *Food Research International*, *51*(2), 474–481. https://doi.org/10.1016/J.FOODRES.2013.01.041

Ortuño, C., Quiles, A., & Benedito, J. (2014). Inactivation kinetics and cell morphology of E. coli and S. cerevisiae treated with ultrasound-assisted supercritical CO2. *Food Research International*, *62*, 955–964. https://doi.org/10.1016/J.FOODRES.2014.05.012

Paniagua-Martínez, I., Mulet, A., García-Alvarado, M. A., & Benedito, J. (2016). Ultrasound-assisted supercritical CO2 treatment in continuous regime: Application in Saccharomyces cerevisiae inactivation. *Journal of Food Engineering*, *181*, 42–49. https://doi.org/10.1016/J.JFOODENG.2016.02.024

Pataro, G., Ferrentino, G., Ricciardi, C., & Ferrari, G. (2010). Pulsed electric fields assisted microbial inactivation of S. cerevisiae cells by high pressure carbon dioxide. *Journal of Supercritical Fluids*, *54*(1), 120–128. https://doi.org/10.1016/j.supflu.2010.04.003

Peleg, M. (1999). On calculating sterility in thermal and non-thermal preservation methods. *Food Research International*, *32*(4), 271–278. https://doi.org/10.1016/S0963-9969(99)00081-2

Peleg, M. (2006). *Models for predicting growth and inactivation. Advanced quantitative microbiology for foods and biosystems*.

Reshes, G., Vanounou, S., Fishov, I., & Feingold, M. (2008). Cell shape dynamics in Escherichia coli. *Biophysical Journal*, *94*(1), 251–264. https://doi.org/10.1529/BIOPHYSJ.107.104398

Shieh, E., Paszczynski, A., Wai, C. M., Lang, Q., & Crawford, R. L. (2009). Sterilization

of Bacillus pumilus spores using supercritical fluid carbon dioxide containing various modifier solutions. *Journal of Microbiological Methods*, *76*(3), 247–252. https://doi.org/10.1016/j.mimet.2008.11.005

Shimoda, M., Kago, H., Kojima, N., Miyake, M., Osajima, & Hayakawa, I. (2002). Accelerated death kinetics of Aspergillus niger spores under high-pressure carbonation. *Applied and Environmental Microbiology*, *68*(8), 4162–4167. https://doi.org/10.1128/AEM.68.8.4162

Soares, G. C., Learmonth, D. A., Vallejo, M. C., Davila, S. P., González, P., Sousa, R. A., & Oliveira, A. L. (2019). Supercritical CO2 technology: The next standard sterilization technique? *Materials Science and Engineering: C*, *99*, 520–540. https://doi.org/10.1016/J.MSEC.2019.01.121

Spilimbergo, S., Dehghani, F., Bertucco, A., & Foster, N. R. (2003). Inactivation of bacteria and spores by pulse electric field and high pressure CO2 at low temperature. *Biotechnology and Bioengineering*, *82*(1), 118–125. https://doi.org/10.1002/bit.10554

Spilimbergo, S., Mantoan, D., & Dalser, A. (2007). Supercritical gases pasteurization of apple juice. *The Journal of Supercritical Fluids*, *40*(3), 485–489. https://doi.org/10.1016/J.SUPFLU.2006.07.013

Sundaram, S., Eisenhuth, J., Howard, G., & Brandwein, H. (2001). Retention of Water-Borne Bacteria by Membrane Filters Part I: Bacterial Challenge Tests on 0.2 and 0.22 Micron Rated Filters. *PDA Journal of Pharmaceutical Science and Technology*, *55*(2), 65–86.

Teertstra, W. R., Tegelaar, M., Dijksterhuis, J., Golovina, E. A., Ohm, R. A., & Wösten, H. A. B. (2017). Maturation of conidia on conidiophores of Aspergillus niger. *Fungal Genetics and Biology*, *98*, 61–70. https://doi.org/10.1016/j.fgb.2016.12.005

Tischler, B. Y., & Hohl, T. M. (2019). Menacing mold: recent advances in Aspergillus pathogenesis and host defense. *Journal of Molecular Biology*, *431*(21), 4229–4246. https://doi.org/10.1016/j.jmb.2019.03.027

Tonyali, B., McDaniel, A., Trinetta, V., & Yucel, U. (2019). Evaluation of heating effects on the morphology and membrane structure of Escherichia coli using electron paramagnetic resonance spectroscopy. *Biophysical Chemistry*, *252*, 106191. https://doi.org/10.1016/J.BPC.2019.106191

Wu, Y., Yao, S. J., & Guan, Y. X. (2007). Inactivation of microorganisms in carbon dioxide at elevated pressure and ambient temperature. *Industrial and Engineering Chemistry Research*, *46*(19), 6345–6352. https://doi.org/10.1021/ie0702330

Yuk, H. G., Geveke, D. J., & Zhang, H. Q. (2009). Non-thermal inactivation of Escherichia coli K12 in buffered peptone water using a pilot-plant scale supercritical carbon dioxide system with a gas-liquid porous metal contactor. *Food Control*, *20*(9), 847–851. https://doi.org/10.1016/j.foodcont.2008.10.004

Ultrasonics - Sonochemistry 67 (2020) 105-138

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

Angela Gomez-Gomez[1], Edmundo Brito-de la Fuente[2], Críspulo Gallegos[2], Jose Vicente Garcia-Perez[1], Jose Benedito[1]

[1]Grupo ASPA, Departamento de Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, València E46022, Spain

[2]Fresenius-Kabi Deutschland GmbH, Product and Process Engineering Center, Pharmaceuticals & Device Division, Bad Homburg, Germany

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

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 50ºC) 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 50-90 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.

Keywords: supercritical carbon dioxide, high power ultrasound, *Escherichia coli*, *Brevundimonas diminuta*, lipid emulsions

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 (Li & Farid, 2016). Of these technologies, the use of high-voltage pulsed electric fields (Heinz et al., 2001; Raso et al., 2016), high hydrostatic pressure (Monfort et al., 2012) or supercritical fluids (Ortuño et al., 2012a; Paniagua-Martínez et al., 2018) 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 (Soares et al., 2019). 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 (Ortuño et al., 2014). Carbon dioxide has the advantage over other compounds of being non-toxic and inexpensive; moreover, its critical temperature (31ºC) 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 (Amaral et al., 2017). Ferrentino & Spilimbergo (2017) 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 25ºC. Additionally, no noticeable differences in the colour were observed for the treated apples compared 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 (Ortuño et al., 2012a). 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. (2003) 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, 40ºC 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 (Cárcel et al., 2012), 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 (Ferrentino & Spilimbergo, 2015; Ortuño et al., 2012b). In particular, this combined technology has been used for the inactivation of inoculated *Salmonella enterica* and microbiota in coconut water (Cappelletti et al., 2014) or *Saccharomyces cerevisiae* in apple juice (Paniagua-Martínez et al., 2016), 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 (Cappelletti et al., 2014). 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 (Paniagua-Martínez et al., 2018). 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 key factors for the microorganism survival rate. In addition, microbial inactivation is also greatly affected by the nature of the suspending media (Garcia-Gonzalez et al., 2007). 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 (Wei et al., 1991). 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 (Garcia-Gonzalez et al., 2007). 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 (Ferrentino et al., 2009) and the inactivation of *S. cerevisiae* in YPD Broth, apple and orange juice (Ortuño et al., 2013) 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 (Anton et al., 2008; Schwarz et al., 1994). 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 x 3 μm (Reshes et al., 2008), 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 (Sundaram et al., 2001): typically of ~0.3 × 0.6 μm (Sandle, 2013). 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 (Tonyali et al., 2019). Although *B. diminuta* is not considered a significant pathogen and, in general, its virulence is low (Ryan & Pembroke, 2018); 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 (37ºC and 24 h for *E. coli* and 30ºC 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 37ºC for *E. coli* and 30ºC 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 3000974, 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* (37ºC) and 36 h (30ºC) for *B. diminuta* (Fig. 1), to assure that microorganisms reached the stationary phase.

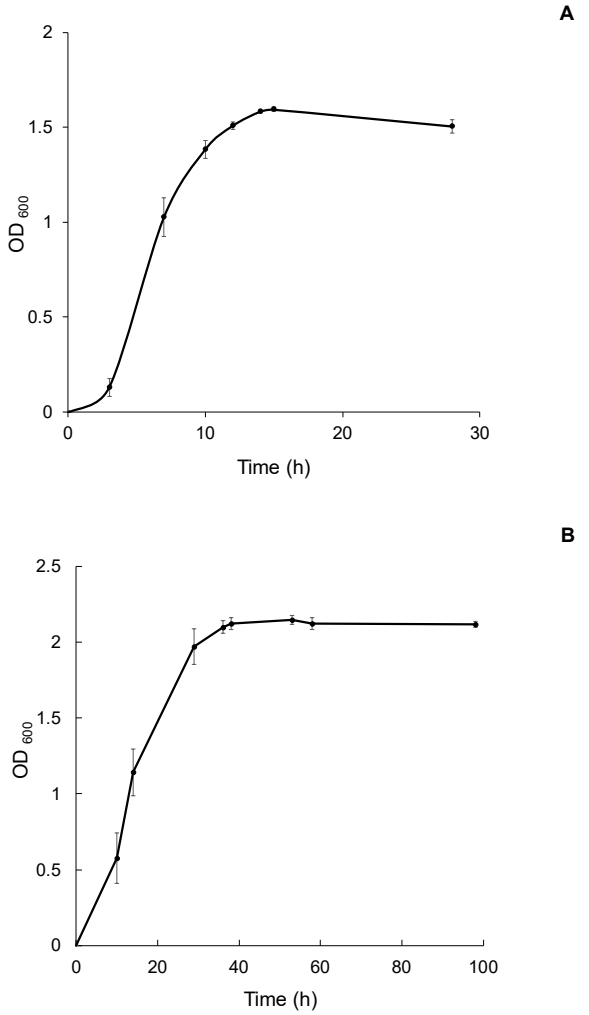


Fig. 1. Growth kinetics of *Escherichia coli* (A) and *Brevundimonas diminuta* (B) monitoring optical density measurements at 600 nm (OD).

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

2.5. Thermal treatment

The thermal treatments were performed at 50ºC 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. (Ortuño et al., 2013). 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 -18ºC (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 (>1W/ 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 ± 5V), 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 50ºC. 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, 35ºC was considered for being close to the critical temperature (31.2ºC) and 50ºC 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 35ºC were selected. All of the treatments were performed with SC-CO_2 and with SC-CO_2 + HPU.

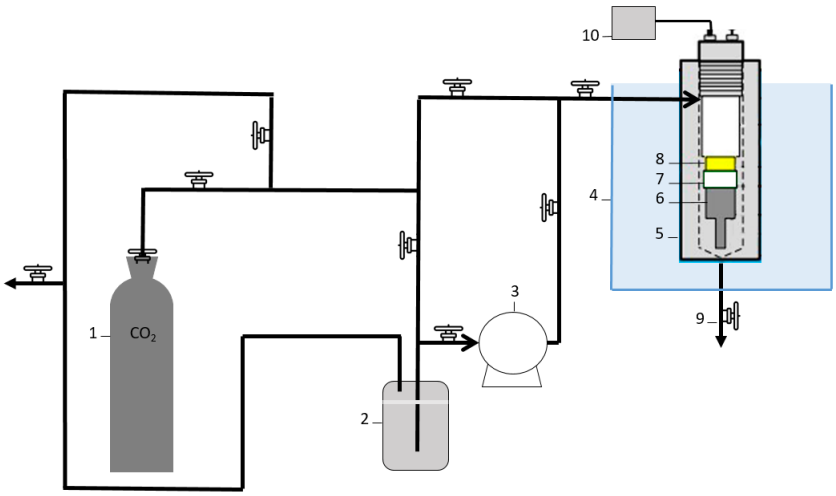


Fig. 2. Supercritical CO_2 treatment system. (1-CO_2 tank, 2-Reservoir, 3-Pump, 4-Bath, 5-Treatment vessel, 6-Transducer, 7-Insulation joint, 8-Ceramics, 9-Sample extraction, 10-Power Generation Unit.

2.7. 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 ºC for *E. coli* and 48 h at 30º C for *B. diminuta*). The initial microbial load in the sample was also determined following the same procedure.

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 (Corradini & Peleg, 2012; Mafart et al., 2002). 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).

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

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 (Jiao et al., 2019). 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.

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

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

where y and y* are the experimental and the estimated data, respectively; z is the number of experimental 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 50ºC), compared to a conventional thermal treatment at 50ºC. 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 35ºC (R^2 =0.86 and RMSE=0.83, Table 1). The thermal inactivation of *E. coli* at 50 min and 50ºC 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 50ºC (Fig. 3). As an example, for *E. coli* at 50 min and 35ºC (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 35ºC than at 50ºC (1.4 log-cycles of difference between 100 and 350 bar at 50ºC). High pressure is known to increase the solubility of CO_2 in the medium. Therefore, as pressure increases, the contact between CO_2 and the bacteria in the medium is enhanced, allowing a faster microbial inactivation (Liao et al., 2007). Ortuño et al. (2012b) studied the inactivation of *E. coli* in LB medium at 36ºC 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. (1999) 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 350ºC 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 50ºC 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 50ºC, while more than 90 min were required at 35ºC 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 50ºC. 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 (Boziaris et al., 1998; Tsuchido et al., 1985). 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 (Spilimbergo & Bertucco, 2003).

In Fig. 3A, an initial lag-phase was found in the inactivation kinetics of *E. coli* at 35ºC, 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 (Lin et al., 1994). Once the lag-phase finalized, after approximately 24 min at 35ºC, a faster decrease in the *E. coli* population was observed for both pressures tested (Fig. 3A). On the contrary, when using 50ºC, 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 35ºC, 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 50ºC *E. coli* treatments at both pressures, indicating concave

upward-shaped curves (Peleg, 2006). Liao et al. (2008) 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}).

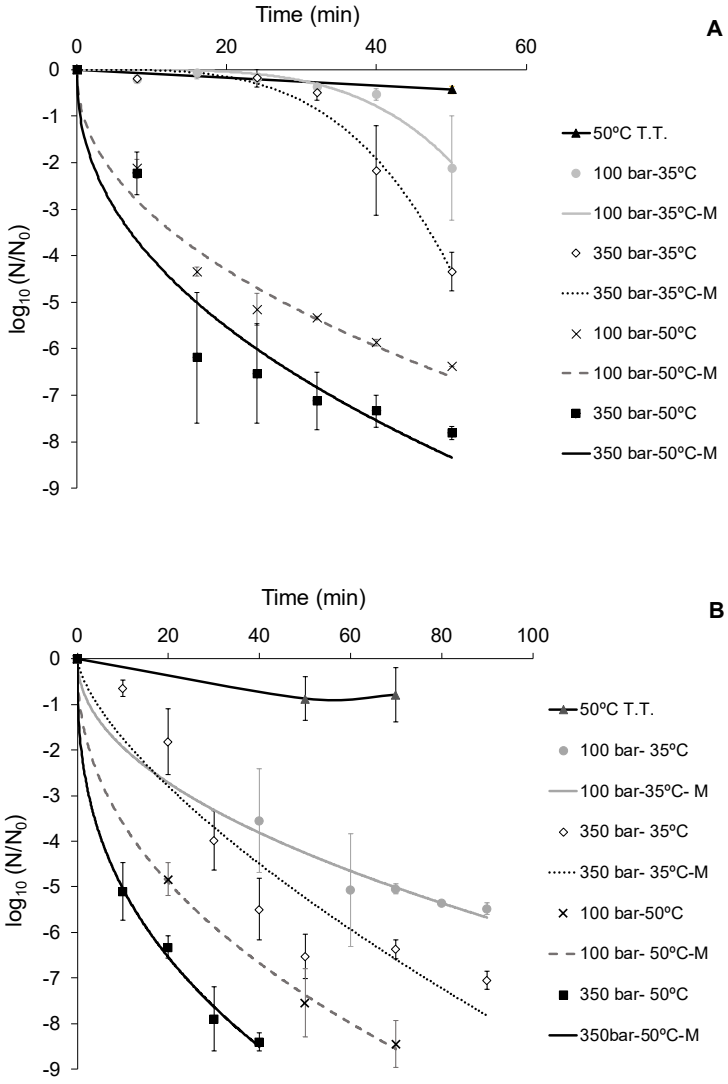


Fig. 3. Inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in 20% oil-water emulsion at different pressure (100 and 350 bar) and temperature (35 and 50ºC) using SC-CO_2, compared to conventional thermal treatment (T.T.) at 50ºC. Experimental data (discrete points) and Weibull model (continuous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

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 (Figs. 4A and 4B). 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 50ºC by 32 min, a time reduction which reached 58 min at 100 bar- 50ºC. 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 (Ortuño et al., 2013). 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 (Cárcel et al., 2012), which has been proven to cause damaged or cracked cell walls, increasing the cell membrane permeation (Cappelletti et al., 2014). 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 (Benedito et al., 2015; Ortuño et al., 2012b).

Ortuño et al. (2014) 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 35ºC 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. (2014) 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 physicochemical 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 50ºC) (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 35ºC were very close to those obtained using 350 bar and 35ºC after 7 min of treatment, which also points to a milder effect of the pressure. Similarly, Ortuño et al. (2014) treated *E. coli* in apple juice with SC-CO_2 + HPU, and no significant ($p>0.05$) differences were found between the conditions applied (100, 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 50ºC.

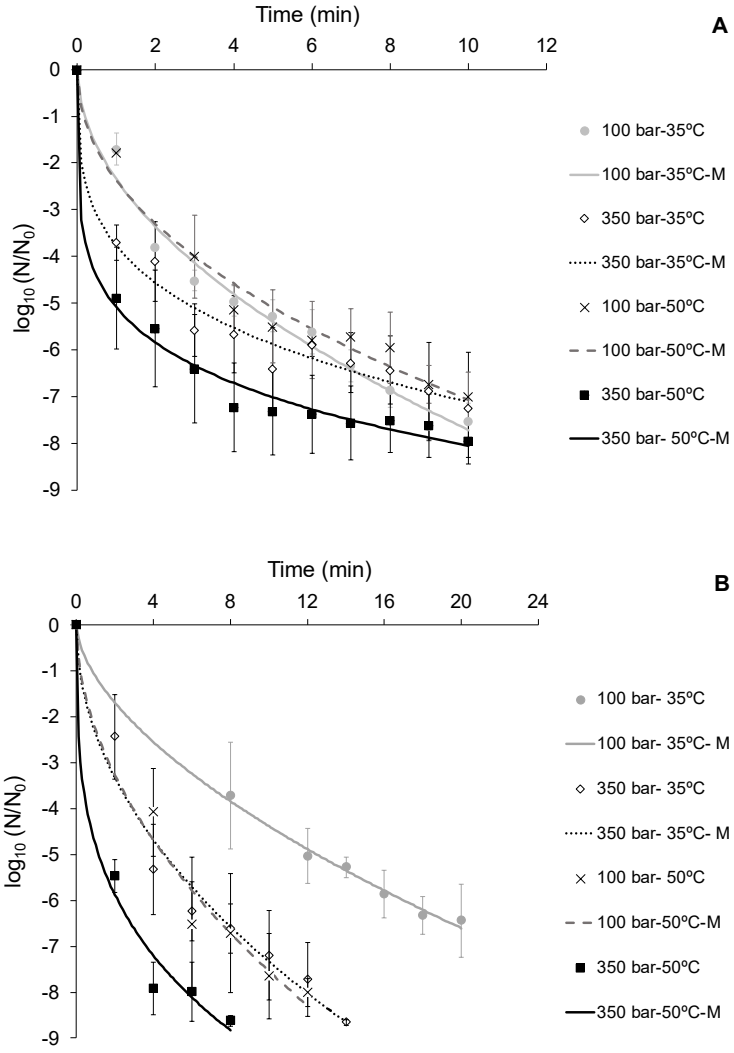


Fig. 4. Inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in 20% oil-water emulsion at different pressure (100 and 350 bar) and temperature (35 and 50ºC) using SC-CO_2 + HPU. Experimental data (discrete points) and Weibull model (continuous and dashed lines).

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 (ºC)	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

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 (Garcia-Gonzalez et al., 2007). Several studies reported a strong protective effect on the inactivation of microbial cells in complex food systems, as compared to simple media (Garcia-Gonzalez et al., 2007). Ortuño et al. (2012b) showed that the total inactivation of *E. coli* in LB broth was achieved in 22 min at 350 bar and 36ºC, 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 35ºC. 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 content) was significantly ($p<0.05$) faster than in the lipid emulsions. Lin et al. (1994) 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 3.4-5.2 log-cycles was achieved in 50 min (Fig. 5A), and of between 2.7-4.2 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. (Kobayashi et al., 2016) reported that the inactivation of *E. coli* suspended in milk, with pressurized CO_2 at 35, 40, 45 or 50ºC 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. (Kim et al., 2008) observed a considerably milder inactivation of *L. monocytogenes* in a physiological saline solution, treated at 35ºC,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. (1994) 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 (Devlieghere et al., 1998). 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. (2009), where there was a reduction in the inactivation degree of *P. fluorescens* treated at 105 bar, 35ºC 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.*

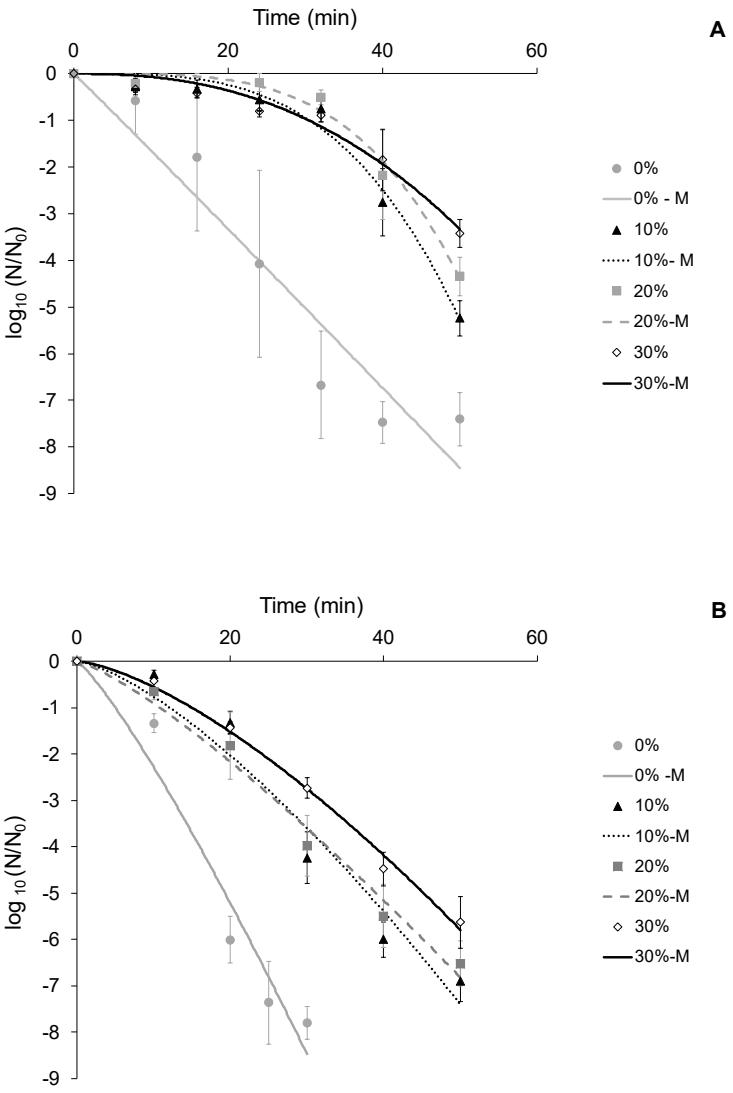


Fig. 5. Inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in oil-water emulsions with different oil contents (0, 10%, 20% and 30%), using SC-CO_2 at 350 bar and 35ºC. Experimental data (discrete points) and Weibull model (continuous and dashed lines).

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 35ºC 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

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 35ºC 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. (2012b) 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 41ºC). 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. (2012b) 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.

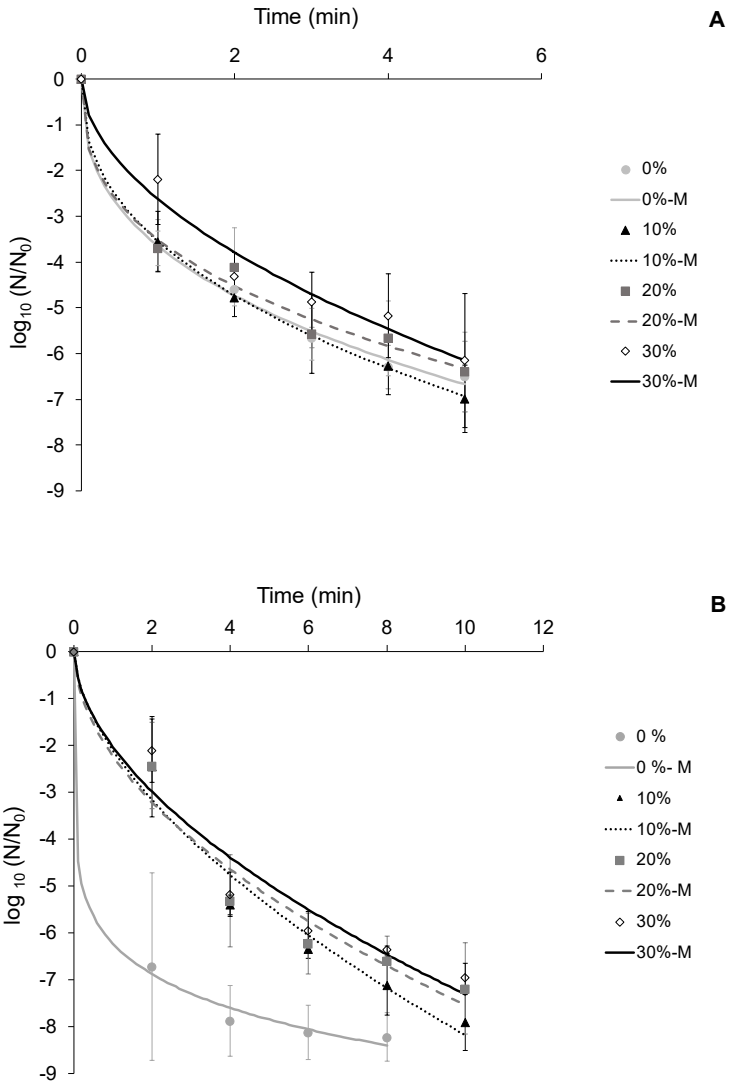


Fig. 6. Inactivation kinetics of *E. coli* (A) and *B. diminuta* (B) in the oil-water emulsion at different oil content (0, 10%, 20% and 30%) and distilled water using SC-CO_2 + HPU at 350 bar and 35ºC. Experimental data (discrete points) and Weibull model (continuous and dashed lines).

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 35ºC 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

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.

Acknowledgements

The authors acknowledge the financial support from Fresenius Kabi.

References

Amaral, G. V., Silva, E. K., Cavalcanti, R. N., Cappato, L. P., Guimaraes, J. T., Alvarenga, V. O., Esmerino, E. A., Portela, J. B., Sant' Ana, A. S., Freitas, M. Q., Silva, M. C., Raices, R. S. L., Meireles, M. A. A., & Cruz, A. G. (2017). Dairy processing using supercritical carbon dioxide technology: Theoretical fundamentals, quality and safety aspects. *Trends in Food Science and Technology*, *64*, 94–101. https://doi.org/10.1016/j.tifs.2017.04.004

Anton, N., Benoit, J.-P., & Saulnier, P. (2008). Design and production of nanoparticles formulated from nano-emulsion templates—A review. *Journal of Controlled Release*, *128*(3), 185–199. https://doi.org/10.1016/J.JCONREL.2008.02.007

Benedito, J., Ortuño, C., Castillo-Zamudio, R. I., & Mulet, A. (2015). Microbial Inactivation by Ultrasound Assisted Supercritical Fluids. *Physics Procedia*, *70*, 824–827. https://doi.org/10.1016/J.PHPRO.2015.08.168

Boziaris, I. S., Humpheson, L., & Adams, M. R. (1998). Effect of nisin on heat injury and inactivation of Salmonella enteritidis PT4. *International Journal of Food Microbiology*, *43*(1–2), 7–13. https://doi.org/10.1016/S0168-1605(98)00083-X

Cappelletti, M., Ferrentino, G., & Spilimbergo, S. (2014). Supercritical carbon dioxide combined with high power ultrasound: An effective method for the pasteurization of coconut water. *The Journal of Supercritical Fluids*, *92*, 257–263. https://doi.org/10.1016/J.SUPFLU.2014.06.010

Cárcel, J. A., García-Pérez, J. V., Benedito, J., & Mulet, A. (2012). Food process innovation through new technologies: Use of ultrasound. *Journal of Food Engineering*, *110*(2), 200–207. https://doi.org/10.1016/J.JFOODENG.2011.05.038

Corradini, M. G., & Peleg, M. (2012). The kinetics of microbial inactivation by carbon dioxide under high pressure. In *Dense Phase Carbon Dioxide: Food and Pharmaceutical Applications* (pp. 135–155).

Devlieghere, F., Debevere, J., & Van Impe, J. (1998). Concentration of carbon dioxide in the water-phase as a parameter to model the effect of a modified atmosphere

on microorganisms. *International Journal of Food Microbiology*, *43*(1–2), 105–113. https://doi.org/10.1016/S0168-1605(98)00101-9

Ferrentino, G., Plaza, M. L., Ramirez-Rodrigues, M., Ferrari, G., & Balaban, M. O. (2009). Effects of dense phase carbon dioxide pasteurization on the physical and quality attributes of a red grapefruit juice. *Journal of Food Science*, *74*(6). https://doi.org/10.1111/j.1750-3841.2009.01250.x

Ferrentino, G., & Spilimbergo, S. (2015). High pressure carbon dioxide combined with high power ultrasound pasteurization of fresh cut carrot. *The Journal of Supercritical Fluids*, *105*, 170–178. https://doi.org/10.1016/J.SUPFLU.2014.12.014

Ferrentino, G., & Spilimbergo, S. (2017). Non-thermal pasteurization of apples in syrup with dense phase carbon dioxide. *Journal of Food Engineering*, *207*, 18–23. https://doi.org/10.1016/j.jfoodeng.2017.03.014

Garcia-Gonzalez, L., Geeraerd, A. H., Elst, K., Van Ginneken, L., Van Impe, J. F., & Devlieghere, F. (2009). Influence of type of microorganism, food ingredients and food properties on high-pressure carbon dioxide inactivation of microorganisms. *International Journal of Food Microbiology*, *129*(3), 253–263. https://doi.org/10.1016/j.ijfoodmicro.2008.12.005

Garcia-Gonzalez, L., Geeraerd, A. H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Van Impe, J. F., & Devlieghere, F. (2007). High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. In *International Journal of Food Microbiology* (Vol. 117, Issue 1, pp. 1–28). https://doi.org/10.1016/j.ijfoodmicro.2007.02.018

Heinz, V., Alvarez, I., Angersbach, A., & Knorr, D. (2001). Preservation of liquid foods by high intensity pulsed electric fields—basic concepts for process design. *Trends in Food Science & Technology*, *12*(3–4), 103–111. https://doi.org/10.1016/S0924-2244(01)00064-4

Hong, S. I., Park, W. S., & Pyun, Y. R. (1999). Non-thermal inactivation of Lactobacillus plantarum as influenced by pressure and temperature of pressurized carbon

dioxide. *International Journal of Food Science and Technology*, *34*(2), 125–130. https://doi.org/10.1046/j.1365-2621.1999.00241.x

Jiao, S., Zhang, H., Hu, S., & Zhao, Y. (2019). Radio frequency inactivation kinetics of Bacillus cereus spores in red pepper powder with different initial water activity. *Food Control*, *105*, 174–179. https://doi.org/10.1016/J.FOODCONT.2019.05.038

Kim, S. R., Park, H. J., Yim, D. S., Kim, H. T., Choi, I.-G., & Kim, K. H. (2008). Analysis of survival rates and cellular fatty acid profiles of Listeria monocytogenes treated with supercritical carbon dioxide under the influence of cosolvents. *Journal of Microbiological Methods*, *75*(1), 47–54. https://doi.org/10.1016/J.MIMET.2008.04.012

Kobayashi, F., Odake, S., Miura, T., & Akuzawa, R. (2016). Pasteurization and changes of casein and free amino acid contents of bovine milk by low-pressure CO2 microbubbles. *LWT - Food Science and Technology*, *71*, 221–226. https://doi.org/10.1016/j.lwt.2016.03.042

Li, X., & Farid, M. (2016). A review on recent development in non-conventional food sterilization technologies. *Journal of Food Engineering*, *182*, 33–45. https://doi.org/10.1016/J.JFOODENG.2016.02.026

Liao, H., Hu, X., Liao, X., Chen, F., & Wu, J. (2007). Inactivation of Escherichia coli inoculated into cloudy apple juice exposed to dense phase carbon dioxide. *International Journal of Food Microbiology*, *118*(2), 126–131. https://doi.org/10.1016/J.IJFOODMICRO.2007.06.018

Liao, H., Zhang, Y., Hu, X., Liao, X., & Wu, J. (2008). Behavior of inactivation kinetics of Escherichia coli by dense phase carbon dioxide. *International Journal of Food Microbiology*, *126*(1–2), 93–97. https://doi.org/10.1016/J.IJFOODMICRO.2008.05.008

Lin, H., Cao, N., & Chen, L. (1994). Antimicrobial effect of pressurized carbon dioxide on Listeria monocytogenes. *Journal of Food Science*, *59*(3), 657–659. https://doi.org/10.1111/j.1365-2621.1994.tb05587.x

Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in

thermal preservation methods: application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, *72*(1–2), 107–113. https://doi.org/10.1016/S0168-1605(01)00624-9

Monfort, S., Ramos, S., Meneses, N., Knorr, D., Raso, J., & Álvarez, I. (2012). Design and evaluation of a high hydrostatic pressure combined process for pasteurization of liquid whole egg. *Innovative Food Science & Emerging Technologies*, *14*, 1–10. https://doi.org/10.1016/J.IFSET.2012.01.004

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012a). Supercritical carbon dioxide inactivation of Escherichia coli and Saccharomyces cerevisiae in different growth stages. *The Journal of Supercritical Fluids*, *63*, 8–15. https://doi.org/10.1016/J.SUPFLU.2011.12.022

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012b). An ultrasound-enhanced system for microbial inactivation using supercritical carbon dioxide. *Innovative Food Science & Emerging Technologies*, *15*, 31–37. https://doi.org/10.1016/J.IFSET.2012.02.006

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2013). Application of high power ultrasound in the supercritical carbon dioxide inactivation of Saccharomyces cerevisiae. *Food Research International*, *51*(2), 474–481. https://doi.org/10.1016/J.FOODRES.2013.01.041

Ortuño, C., Quiles, A., & Benedito, J. (2014). Inactivation kinetics and cell morphology of E. coli and S. cerevisiae treated with ultrasound-assisted supercritical CO2. *Food Research International*, *62*, 955–964. https://doi.org/10.1016/J.FOODRES.2014.05.012

Paniagua-Martínez, I., Mulet, A., García-Alvarado, M. A., & Benedito, J. (2016). Ultrasound-assisted supercritical CO2 treatment in continuous regime: Application in Saccharomyces cerevisiae inactivation. *Journal of Food Engineering*, *181*, 42–49. https://doi.org/10.1016/J.JFOODENG.2016.02.024

Paniagua-Martínez, I., Mulet, A., García-Alvarado, M. A., & Benedito, J. (2018). Orange juice processing using a continuous flow ultrasound-assisted supercritical CO2

system: Microbiota inactivation and product quality. *Innovative Food Science & Emerging Technologies*, *47*, 362–370. https://doi.org/10.1016/J.IFSET.2018.03.024

Peleg, M. (2006). *Models for predicting growth and inactivation. Advanced quantitative microbiology for foods and biosystems*.

Raso, J., Frey, W., Ferrari, G., Pataro, G., Knorr, D., Teissie, J., & Miklavčič, D. (2016). Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes. *Innovative Food Science and Emerging Technologies*. https://doi.org/10.1016/j.ifset.2016.08.003

Reshes, G., Vanounou, S., Fishov, I., & Feingold, M. (2008). Cell shape dynamics in Escherichia coli. *Biophysical Journal*, *94*(1), 251–264. https://doi.org/10.1529/BIOPHYSJ.107.104398

Ryan, M. P., & Pembroke, J. T. (2018). Brevundimonas spp: Emerging global opportunistic pathogens. *Virulence*, *9*(1), 480–493. https://doi.org/10.1080/21505594.2017.1419116

Sandle, T. (2013). Sterilization by filtration. In *Sterility, Sterilisation and Sterility Assurance for Pharmaceuticals*. Woodhead Publishing. https://doi.org/10.1533/9781908818638.143

Schwarz, C., Mehnert, W., Lucks, J. S., & Müller, R. H. (1994). Solid lipid nanoparticles (SLN) for controlled drug delivery. I. Production, characterization and sterilization. *Journal of Controlled Release*, *30*(1), 83–96. https://doi.org/10.1016/0168-3659(94)90047-7

Soares, G. C., Learmonth, D. A., Vallejo, M. C., Davila, S. P., González, P., Sousa, R. A., & Oliveira, A. L. (2019). Supercritical CO2 technology: The next standard sterilization technique? *Materials Science and Engineering: C*, *99*, 520–540. https://doi.org/10.1016/J.MSEC.2019.01.121

Spilimbergo, S., & Bertucco, A. (2003). Non-thermal bacteria inactivation with dense CO2. *Biotechnology and Bioengineering*, *84*(6), 627–638.

https://doi.org/10.1002/bit.10783

Spilimbergo, S, Dehghani, F., Bertucco, A., & Foster, N. R. (2003). Inactivation of bacteria and spores by pulse electric field and high pressure CO2 at low temperature. *Biotechnology and Bioengineering*, *82*(1), 118–125. https://doi.org/10.1002/bit.10554

Sundaram, S., Eisenhuth, J., Howard, G., & Brandwein, H. (2001). Retention of Water-Borne Bacteria by Membrane Filters Part I: Bacterial Challenge Tests on 0.2 and 0.22 Micron Rated Filters. *PDA Journal of Pharmaceutical Science and Technology*, *55*(2), 65–86.

Tonyali, B., McDaniel, A., Trinetta, V., & Yucel, U. (2019). Evaluation of heating effects on the morphology and membrane structure of Escherichia coli using electron paramagnetic resonance spectroscopy. *Biophysical Chemistry*, *252*, 106191. https://doi.org/10.1016/J.BPC.2019.106191

Tsuchido, T., Katsui, N., Takeuchi, A., Takano, M., & Shibasaki, I. (1985). Destruction of the outer membrane permeability barrier of Escherichia coli by heat treatment. *Applied and Environmental Microbiology*, *50*(2), 298–303.

Wei, C. I., Balaban, M. O., Fernando, S. Y., & Peplow, A. J. (1991). Bacterial effect of high pressure CO2 treatment on foods spiked with Listeria or Salmonella. *Journal of Food Protection*, *54*(3), 189–193. https://doi.org/10.4315/0362-028x-54.3.189

Ultrasonics – Sonochemistry 76 (2021) 105-636

Combination of supercritical CO_2 and high-power ultrasound for the inactivation of fungal and bacterial spores in lipid emulsions

Angela Gomez-Gomez[1], Edmundo Brito-de la Fuente[2], Críspulo Gallegos[2], Jose Vicente Garcia-Perez[1], Jose Benedito[1]

[1]Grupo ASPA, Departamento de Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, València E46022, Spain

[2]Fresenius-Kabi Deutschland GmbH, Product and Process Engineering Center, Pharmaceuticals & Device Division, Bad Homburg, German

Combination of supercritical CO_2 and high-power ultrasound for the inactivation of fungal and bacterial spores in lipid emulsions

Abstract

For the first time, this study addresses the intensification of supercritical carbon dioxide (SC-CO_2) treatments using high-power ultrasound (HPU) for the inactivation of fungal (*Aspergillus niger*) and bacterial (*Clostridium butyricum*) spores in oil-in-water emulsions. The inactivation kinetics were analyzed at different pressures (100, 350 and 550 bar) and temperatures (50, 60, 70, 80, 85 °C), depending on the microorganism, and compared to the conventional thermal treatment. The inactivation kinetics were satisfactorily described using the Weibull model.

Experimental results showed that SC-CO_2 enhanced the inactivation level of both spores when compared to thermal treatments. Bacterial spores (*C. butyricum)* were found to be more resistant to SC-CO_2 + HPU, than fungal (*A. niger)* ones, as also observed in the thermal and SC-CO_2 treatments. The application of HPU intensified the SC-CO_2 inactivation of *C. butyricum* spores, e.g. shortening the total inactivation time from 10 to 3 min at 85ºC. However, HPU did not affect the SC-CO_2 inactivation of *A. niger* spores. The study into the effect of a combined SC-CO_2 + HPU treatment has to be necessarily extended to other fungal and bacterial spores, and future studies should elucidate the impact of HPU application on the emulsion's stability.

Keywords: spores, inactivation, supercritical fluids, ultrasound, emulsions

1. Introduction

The inactivation of fungal and bacterial spores plays a relevant role in the food and pharmaceutical industry due to the fact that spores may cause product spoilage and related diseases. Spores are known to be highly resistant to many processing treatments, such as heating, drying, radiation or chemicals, among others.

The most common technology used to inactivate all types of microorganisms, including spores, has been moist heat at high temperatures (≥121ºC). However, many disadvantages are linked to the use of high temperatures, such as changes in the nutritive or organoleptic properties of the treated products.

The use of supercritical CO_2 (SC-CO_2) has been investigated as an alternative technology for the purposes of microbial inactivation (Omar et al., 2018). CO_2 is non-toxic, nonflammable, cheap, and its critical temperature (31ºC) and pressure (73.8 bar) are easy to reach. Moreover, SC-CO_2 has liquid-like density, gas-like diffusivity and viscosity, and zero surface tension, which provides CO_2 with excellent transport properties. SC-CO_2 has been seen to perform well in the inactivation of vegetative cells, such as *E. coli, S. cerevisiae* (Ortuño et al., 2012a) or *B. diminuta,* at mild temperatures (35-50ºC) (Gomez-Gomez et al., 2020). The inactivation mechanisms of SC-CO_2 on vegetative cells have been extensively studied. In short, SC-CO_2 dissolves in the media causing acidification that modifies the membrane of the microbial cells, increasing the permeability; thus, SC-CO_2 easily diffuses into the inner cell. As a result, the vital intracellular components of the cell are extracted, which leads to cell death (Ortuño et al., 2014). However, SC-CO_2 inactivation mechanisms in spores are not yet fully elucidated. Spore structure is different compared to that of vegetative cells, with one of the main differences being the extreme dehydration of the spores (Feofilova et al., 2012; Ishihara et al., 1999).

In order to maintain product quality standards in SC-CO_2 treatments, it is advisable to operate at the lowest possible temperature and pressure for the shortest time, while preserving product safety. However, the use of SC-CO_2 at mild temperatures (<50ºC) is insufficient to inactivate fungal and bacterial spores and high pressures and temperatures and long times are required (Garcia-Gonzalez et al., 2009;

Kamihira et al., 1987; Rao et al., 2015). Consequently, the damage of heat sensitive components in the product and the increase in the process cost hinders the use of SC-CO_2 for spore inactivation.

SC-CO_2 treatment can be intensified by the application of high-power ultrasound (HPU). The effect of HPU on microbial inactivation is mainly linked to the violent collapse of microbubbles, known as cavitation (Bi et al., 2020). Locally intense high temperatures and pressures, with significant shearing and turbulence effects, are caused by cavitation (Cárcel et al., 2012), which can affect microbial integrity. In addition, both the contact between SC-CO_2 and the surface of the cells and the SC-CO_2 penetration into the cell are enhanced (Cappelletti et al., 2014). The coupling of HPU to the SC-CO_2 treatment has been demonstrated to shorten the inactivation time for vegetative cells located in fat-free media (Ferrentino & Spilimbergo, 2016; Ortuño et al., 2012b). However, there are no references to the combined SC-CO_2 + HPU treatment for the inactivation of fungal and bacterial spores in lipid media. Only Michelino et al. (2018) dealt with the SC-CO_2 + HPU inactivation of bacterial spores naturally present in a solid product (coriander) and revealed an enhanced inactivation when HPU was applied. It is well known that oils/fats can hinder SC-CO_2 microbial inactivation; thus, due to the importance of oil-in-water emulsions in the food and pharmaceutical industry, it would be of great interest to find alternative non-thermal treatments able to achieve a noticeable spore reduction in this type of product. Therefore, the aim of this study was to assess the feasibility of intensifying the SC-CO_2 inactivation of fungal (*Aspergillus niger*) and bacterial (*Clostridium butyricum*) spores in oil-in-water emulsions by using high-power ultrasound. *A. niger* is a spore-forming mesophilic and aerobic filamentous fungi, common in contaminated food and pharmaceutical products (Shimoda et al., 2002) and widely distributed in the environment. It is also an opportunistic fungus causing otomycosis and implicated in nosocomial infections (Kar et al., 2019). *C. butyricum* is an anaerobic gram-positive bacterium, which forms spores as a mechanism of resistance to stress factors. It is a spoilage bacterium capable of growing and forming butyric acid in food and pharmaceutical products (Ghoddusi & Sherburn, 2010).

2. Materials and methods

2.1. Preparation of the oil-in-water emulsion

The treated samples were oil-in-water emulsions with 20 % soybean oil content. The emulsions were prepared in three stages: mixing with an Ultra-Turrax, sonication and homogenization. Firstly, the lipid phase, consisting of soybean oil and egg phospholipid, was mixed using a disperser device (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 and glycerol), while being mixed again at 14000 rpm. Afterwards, samples were sonicated for 5 min (UP400S, Hielscher, Teltow, Germany) using the H22-type sonotrode. Finally, the product was homogenized in two stages (50 bar; 550 bar) using a high-pressure homogenizer (PANDA Plus 2000, GEA Niro Soavi, Parma, Italy).

2.2. Preparation of the *Aspergillus niger* and *Clostridium butyricum* spore suspension

The lyophilized strains of *Aspergillus niger* CECT 2807 and *Clostridium butyricum* CECT 361T used in this study were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain).

A. niger was cultured on Potato Dextrose Agar (PDA, Scharlab, Barcelona, Spain) at 25ºC for 7 days. Afterwards, 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 4ºC until use. Finally, prior to each treatment, 5 mL of the *A. niger* spore suspension were inoculated in a 20% soybean emulsion (60 mL) until an *A. niger* spore concentration of 10^6- 10^7 CFU/mL. *C. butyricum* was sporulated following the methodology of Mafart et al. (2002) with modifications. A single colony of *C. butyricum* was anaerobically pre-cultivated in Reinforced Clostridial Medium (RCM, Scharlab, Barcelona, Spain) at 37ºC until the stationary phase was reached (36h). Anaerobic conditions were achieved with incubation containers with a CO_2 gas generating system

(Oxoid, Thermo Fisher Scientific, Waltham, Massachusetts, USA). An anaerobic indicator (Oxoid, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to monitor the anaerobic conditions. 100 µL of the *C. butyricum* culture were poured into Reinforced Clostridial Agar (RCA, Scharlab, Barcelona, Spain) enriched with $MnSO_4$ (40 mg/L) and $CaCl_2$ (100 mg/L) to enhance the sporulation. The plates were anaerobically incubated at 37ºC for 5-6 days, during which time spores were formed (determined 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 distilled water, and washed three times by centrifugation (8000x g for 15 min) (Medifriger BL-S, JP Selecta, Barcelona, Spain). 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. Lastly, the suspension was washed again three times by centrifugation, distributed into sterile Eppendorf microtubes and kept at 4°C until use. Before being treated, the microtubes were heat-shocked at 80°C for 15 min to eliminate vegetative cells and cooled again at 4ºC. Prior to each treatment, the spore suspension was added (2 mL) to the autoclaved emulsion (60 mL) to reach a cell concentration of 10^4-10^5 CFU/mL.

2.3. Thermal treatment

The thermal treatments for *A. niger* inoculated in the emulsion were performed at 50, 60 and 70ºC. The thermal treatments for *C. butyricum* inoculated in the emulsion were performed at 70 and 85ºC in a temperature-controlled water bath (1812, Bunsen, Madrid, Spain). 1.5 mL of sample (emulsion with a concentration of 10^6 -10^7 CFU/mL of *A. niger* or 10^4-10^5 CFU/mL of *C. butyricum*) were poured into borosilicate glass tubes, 8 mm in diameter and 70 mm in length (Fiolax, DWK, Wertheim/Main, Germany). The tubes were taken out of the bath after different times, ranging from 5 s to 30 min, depending on the microorganism and the temperature of the treatment. The samples were placed in ice until analysed. The experiments were carried out in triplicate.

2.4. Ultrasonic-assisted supercritical fluid treatments

The supercritical carbon dioxide (SC-CO_2) treatments for the purposes of inactivating *A. niger* and *C. butyricum* were carried out using batch lab-scale equipment already described by Gomez-Gomez et al. (2020) (Fig. 1). Briefly, the system consisted of a CO_2 tank (1, Fig. 1), a chiller reservoir (2, Fig. 1); a diaphragm metering pump (LDB, LEWA, Tokyo, Japan) (3, Fig. 1), a thermostatic water bath (4, Fig. 1) and an inactivation vessel (5, Fig. 1). Additionally, a high-power ultrasound (HPU) transducer (Benedito et al., 2011) was attached to the vessel lid to perform the combined SC-CO_2 + HPU treatments. The ultrasound system mainly consisted of a high power (>1W/cm^2) piezoelectric transducer (6, Fig. 1), a sonotrode and a power generation unit (10, Fig. 1). The power was 50 ± 5 W (I= 250 ± 10 mA; U= 220 ± 5V), and the frequency was 30 ± 2 kHz (WT210, Yokogawa Electric Corporation, Tokyo, Japan).

Treatments were extended up to 50 min and samples of 2 mL were taken at different time intervals, ranging from 5 s to 20 min. Both treatment and sampling time were chosen depending on the microorganisms and process conditions. The treated samples were immediately cooled in ice before analysis.

For *A. niger*, SC-CO_2 and SC-CO_2 + HPU inactivation treatments were carried out combining two different pressures (100 and 350 bar) and temperatures (50 and 60ºC). The lowest pressure (100 bar) was chosen because it is close to the critical pressure (73.8 bar) and the highest (350 bar) as it is a common pressure used in SC-CO_2 inactivation studies (Soares et al., 2019). 50ºC was selected as a mild temperature that has little thermal effect on the inactivation of the studied microorganism (Ballestra & Cuq, 1998) and 60ºC was selected to study the effect a higher temperature has on the inactivation. Moreover, treatments at 70ºC were tested at different pressure levels (100, 350 and 550 bar) to explore more extreme conditions, which could provide larger inactivation levels. In the case of *C. butyricum,* and due to the greater thermal resistance of bacterial spores than fungal ones (Soares et al., 2019), more extreme conditions of temperature and pressure were tested. Thus, SC-CO_2 and SC-CO_2 + HPU inactivation treatments were performed at 60, 70, 80 and 85ºC and 100, 350 and 550 bar. All the experiments were performed in triplicate.

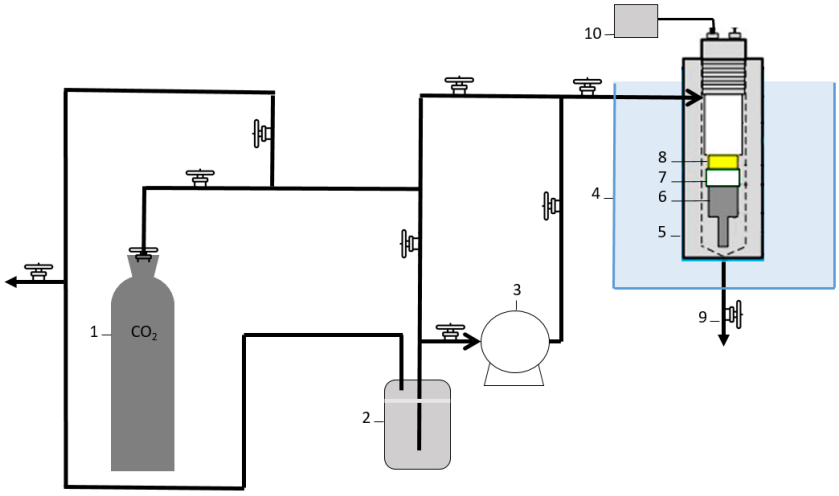


Fig. 1. Supercritical CO_2 treatment system. (1-CO_2 tank, 2-Reservoir, 3-Pump, 4-Bath, 5-Treatment vessel, 6-Transducer, 7-Insulation joint, 8-Ceramics, 9-Sample extraction, 10-Power Generation Unit).

2.5. Microbiological analyses

A. niger and *C. butyricum* spores were quantified by means of standard plate count techniques. Depending on the expected count, appropriate serial dilutions were prepared with sterile distilled water. For *A. niger*, 100 µL of the dilution were spread on the surface of PDA (Scharlab, Barcelona, Spain) in triplicate and incubated at 25ºC for 72 h. The initial *A. niger* population in the sample was also determined following the same procedure. For *C. butyricum*, 500 µL of the dilution were poured on to empty plates in triplicate and melted RCA (Scharlab, Barcelona, Spain) was added to each plate. Plates were anaerobically incubated at 37ºC for 15 h. The initial *C. butyricum* spore population in the sample was also determined following the same procedure.

2.6. Modelling

Microbial inactivation kinetics are usually considered as first-order kinetics (Corradini & Peleg, 2012; Mafart et al., 2002). However, a survival curve is the

cumulative temporal distribution of mortality events and can exhibit a wide variety of shapes. Thus, several models have been proposed to describe this behaviour, the Weibull model being a robust one (Peleg, 2006). Therefore, Weibull distribution was used in this study to describe the microbial inactivation kinetics of *A. niger* and *C. butyricum*, computing the log-cycle reduction in the number of viable cells (N), using Eq. 1.

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

where N_0 (CFU/mL) represents the initial number of spores in the sample, N is the number of spores in the sample (CFU/mL) at treatment time t, n is the shape factor and b is the rate parameter (min^{-n}). The kinetic constants (b and n) of the model were calculated by minimizing the sum of squared differences between experimental and predicted inactivation level using Solver Microsoft Excel™ tool. According to this model, an upward concavity is manifested for n < 1 (tailing) and a downward concavity for n > 1 (shoulder). The traditional 'first-order kinetics' is just a special case of the model, with n = 1 (Jiao et al., 2019).

The root mean squared error (RMSE, Eq. 2) and the coefficient of determination (R^2, Eq. 3) were computed to evaluate the estimation accuracy and the model's goodness of fit.

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

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

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 sample deviation, respectively.

Several authors (Deen & Diez-Gonzalez, 2019; Jiao et al., 2019) have compared the parameters (b and n) of the Weibull model as independent values, but they are mathematically related since the units of b rate parameter are min^{-n}. Thus, when comparing two different inactivation treatments, a higher value of b in one of them does not directly involve a faster inactivation, since a lower n can diminish the microbial inactivation rate in favor of the other treatment. In this regard, some authors have fixed the shape parameter (n) at an average value and estimated only the rate parameter b (Baril et al., 2011; Couvert et al., 2005). However, this estimation is only acceptable when there is no influence of the studied conditions (pressure and temperature of the treatment, type of microorganism, treated media) on the shape of the inactivation kinetics. Therefore, in order to use the model to compare the effect of the different variables (temperature, pressure, use of HPU and microorganism) on microbial inactivation, the time required to achieve complete inactivation (t_x) was calculated from Eq.1 and the b and n values of Weibull model obtained for each condition (Tables 1 and 2), where x is the average number of log-cycles of total inactivation for every microorganism (6.8 log-cycles for *A. niger* and 4.8 log-cycles for *C. butyricum*).

2.7. Statistical analysis

Statgraphics Centurion XVI was used to perform a general linear model (GLM) in order to evaluate the effect of the treatment conditions (pressure, temperature and use of HPU) and the type of microorganism on the inactivation. Fisher's least significant difference (LSD) procedure was used to discriminate among the means with a 95.0 % confidence level ($p<0.05$).

3. Results and discussion

3.1. SC-CO_2 inactivation of *A. niger* spores in oil-in-water emulsions

Fig. 2A shows the inactivation of *A. niger* spores in the 20% oil-in-water emulsion treated with SC-CO_2 at different pressures (100 and 350 bar) and temperatures (50 and 60ºC), compared to the conventional thermal treatments at 50 and 60ºC. The experimental variability found in the inactivation treatments may be ascribed to possible pressure and temperature fluctuations and a variability in microbial growth behaviour. The fitting of the Weibull model to the SC-CO_2 kinetics was satisfactory, providing R^2 of over 0.97 and RMSE of under 0.199 (Table 1). All n Weibull parameter values were lower than 1, which reveals that the shape of the inactivation kinetics was concave-upward, except for the treatment at 100 bar and 50ºC, with an n value of 1.04, which was close to the linear behaviour.

SC-CO_2 vs thermal treatments

The thermal inactivation of *A. niger* after 50 min and at 50ºC was negligible (a reduction of less than 0.2 log-cycles), while at 60ºC, 6.8 log-cycles were achieved after 20 min. In SC-CO_2 treatments at 50ºC, an average of 5.1 log-cycles for 100 and 350 bar were inactivated after 40 min (Fig. 2A). However, in the treatments at 60ºC, only the use of 350 bar allowed for a slightly ($p<0.05$) faster inactivation than the thermal treatment (Fig.2A). Ballestra & Cuq (1998) demonstrated a greater lethal effect in the case of *A. niger* spores in Ringer solution with saccharose treated with CO_2 at 50 bar and 50ºC (D-value of 46 min), compared to the thermal treatment (D-values of more than 300 min). On the contrary, when using 60ºC, these authors found no noticeable differences between the thermal and the pressurized CO_2 treatments, which coincides with the experimental results depicted in Fig. 2A. Ballestra & Cuq (1998) manifested that at high temperatures, the antimicrobial effect of pressurized CO_2 could be masked by the lethal effect of heat.

Effects of pressure and temperature

The temperature had a significant ($p<0.05$) effect on inactivation in both thermal and SC-CO_2 treatments. The time required to reach complete inactivation (on average, 6.8 log-cycles of reduction; $t_{6.8}$) was computed using the Weibull model in order to compare the performances of the different treatments (Table 1). For the thermal treatments, values of 7588 min and 25.4 min at 50 and 60ºC, respectively, were computed. For the SC-CO_2 treatments, the average $t_{6.8}$ values were 58.3 min at 50ºC and 24.6 min at 60ºC. Therefore, *A. niger* inactivation kinetics in oil-in-water emulsions was highly temperature dependent. The increase in temperature is known to decrease the CO_2 viscosity and, therefore, to facilitate its diffusion in the media. The temperature also affects the integrity of the cell wall of the fungal spores, facilitating the penetration of CO_2 and the hydration of the cell structure (Noman et al., 2018).

The pressure also had a significant ($p<0.05$) effect, although milder than the temperature. On average, a rise in pressure from 100 to 350 bar for a treatment of 20 min led to an increase of between 4.2 and 5.1 log-cycles in the inactivation level of *A. niger* (Fig. 2A). The pressure shortened the $t_{6.8}$ computed by the Weibull model (Table 1), lasting on average 56.0 min at 100 bar and 27.9 min at 350 bar. The rise in pressure is known to increase the solubility of CO_2 in the suspension; therefore, both the acidification of the external medium and the contact between CO_2 and the microbial cells are improved, which facilitates the CO_2 penetration into the cells (Ceni et al., 2016; Liao et al., 2007).

Noman et al. (2018) and Shimoda et al. (2002) also found that the inactivation of *A. niger* spores was more noticeable at high temperatures and pressures in distilled water and a saline solution, respectively. However, different results were obtained by Calvo et al. (2007) for *A. niger* inoculated in milled dried cocoa since the increase in the temperature (from 40 to 80ºC) or the pressure (from 130 to 300 bar) did not improve the inactivation. This distinction was probably due to the sizeable differences in the nature of the treated medium, since Calvo et al. (2007) conducted the experiments in a solid medium. The composition and nature of the treated medium may modulate its protective effect on the microorganism. In fact, marked protective effects to inactivation

treatments were observed in complex physicochemical samples, compared to simpler media (Garcia-Gonzalez et al., 2007). As an example, Noman et al. (2018) observed *A. niger* spores to be completely inactivated (6.0 log-cycles of reduction) in distilled water at 75ºC, 300 bar and after 90 min; in contrast, the maximum inactivation level in a seawater medium and normal saline was 5.5 log-cycles. No references have been found to the inactivation of *A. niger* in lipid media. However, several authors found there was a protective effect exerted by the oil content in the treating media against subcritical CO_2 or SC-CO_2 treatments for other microorganisms, such as vegetative bacteria (Garcia-Gonzalez et al., 2009; Gomez-Gomez et al., 2020).

3.2. SC-CO_2 + HPU inactivation of *A. niger* spores in oil-in-water emulsions

Fig. 2B shows the SC-CO_2 + HPU inactivation kinetics of *A. niger* spores in the oil-in-water emulsion at 50 and 60ºC and 100 and 350 bar. The Weibull model satisfactorily described the inactivation kinetics since R^2 was higher than 0.98 and RMSE was lower than 0.301 (Table 1). In the case of the shape parameter (n), all the values were lower than 1 (Table 1), indicating the kinetics were concave-upward shaped. Since the process times required to achieve emulsion sterilization were longer than expected, higher temperatures and pressure levels (70ºC and up to 550 bar) were assessed for the combined SC-CO_2+ HPU treatment (Fig. 3).

Effect of temperature and pressure

As in the SC-CO_2 inactivation treatments (Fig. 2A), both temperature and pressure significantly ($p<0.05$) affected the inactivation level of SC-CO_2 + HPU treatments at 50 and 60ºC (Fig. 2B), the temperature effect being greater than that of the pressure. An increase in temperature from 50 to 60ºC implied the shortening of the time needed for total inactivation from 45 to 17.5 min, whereas an increase in pressure from 100 to 350 bar only led to a reduction from 35 to 27.5 min (Fig. 2B). When ultrasound was applied, the effect of the pressure and temperature on inactivation kinetics was milder compared to that in the SC-CO_2 treatments. Gomez-Gomez et al.

(2020) reported similar results for the inactivation of *E. coli* and *B. diminuta* in oil-in-water emulsions. However, several studies using SC-CO_2 + HPU to inactivate microorganisms found that an increase in both the pressure and temperature of the process did not increase the inactivation rate, probably because the marked influence of HPU masked the effects (Ortuño et al., 2012b, 2013). In this sense, although Ortuño et al. (2014) found that neither pressure (100-350 bar at 36ºC) nor temperature (31-41ºC at 225 bar) had any effect on the inactivation level of *E. coli* in apple juice, the effects were significant ($p<0.05$) for *S. cerevisiae*. Therefore, the effect of pressure and temperature on SC-CO_2 + HPU inactivation depends not only on the treating media but also on the microorganism considered. The effect of the temperature still remained strong at 70ºC, since the total inactivation time was shortened from less than 3 min (Fig. 3) to the 10-25 min required at 60ºC (Fig. 2B). As for the pressure, *A. niger* spores were completely inactivated (6.8 log-cycles) after a longer time (3 min) at 70ºC and 100 bar, compared to higher pressures (350 bar and 550 bar), where similar results were found and the complete inactivation was achieved in only 5 s (Fig. 3). Therefore, 5 s treatments at 350 bar could be implemented in the industry for emulsion pasteurization, which could improve the quality of the treated products, properly preserving heat sensitive compounds.

Effect of HPU

Unlike previous studies into inactivation, where the implementation of HPU in the SC-CO_2 treatment drastically shortened the processing times (Gomez-Gomez et al., 2020; Ortuño et al., 2012b, 2013), in the present study, HPU did not significantly ($p>0.05$) affect the SC-CO_2 inactivation kinetics of *A. niger* (Fig. 2B). For example, at 350 bar, 50ºC and after 45 min, similar reductions were achieved without (Fig. 2A) or with HPU (Fig. 2B) (6.2 and 6.8 log-cycles, respectively). Moreover, no microbial count was detected after 10 min at 350 bar and 60ºC, regardless of the use of HPU (Fig. 2AB). As regards the thermal treatment at 70ºC (Fig. 3), a slower rate of inactivation was shown at the beginning of the treatment (until 30 s of process), compared to the SC-CO_2 + HPU treatments. However, in the following 30 s of the thermal treatment, the

inactivation rate sped up to reach a similar inactivation level as in the SC-CO_2 + HPU treatment at 100 bar and, no plate count was detected after 3 min for either treatment. Therefore, at high temperatures (60 and 70ºC), similar inactivation levels are reached in the thermal treatment and the SC-CO_2 + HPU treatment at 100 bar.

No references were found to the application of HPU to the SC-CO_2 treatment of inoculated filamentous fungi. However, the results obtained would show that the inactivation of the *A. niger* fungal spores was limited by the CO_2 penetration through the *A. niger* spore structure. In addition, the expected effect of the ultrasound cavitation on the spore integrity was not found. In fact, the *A. niger* spore could be more resistant to cavitation than other microorganisms due to the differences in the composition of its multilayer cell wall. *A. niger* conidium cell is composed of a multi layered polysaccharide-rich wall, covered by a proteinaceous, highly hydrophobic layer of rodlets (hydrophobins), which conceals an underlying, dense, pigmented layer, composed of melanin (Tischler & Hohl, 2019). Melanin is related to an adaptation of fungi whereby they are able to resist environmental stress since this pigment is known to increase cell wall rigidity, which could improve its resistance to ultrasonic mechanical stress.

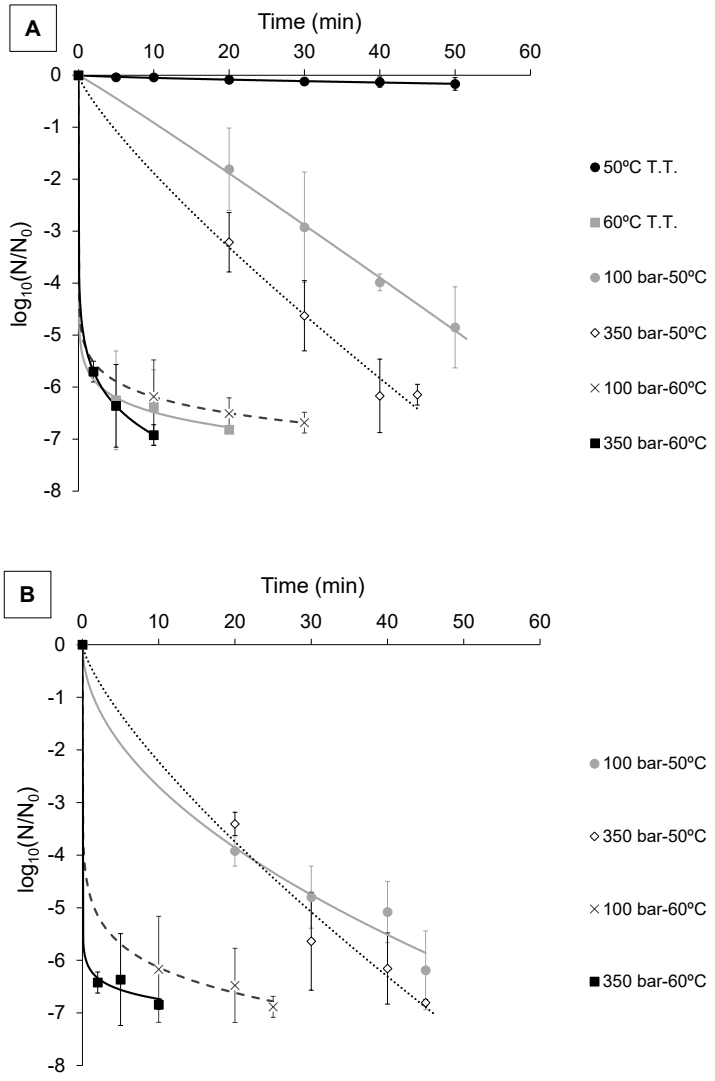


Fig. 2. Inactivation kinetics of *A. niger* in oil-in-water emulsion treated using thermal (T.T.) and SC-CO_2 treatments **(A)** and through SC-CO_2 + HPU treatments **(B)** at different pressures (100 and 350 bar) and temperatures (50 and 60ºC). Experimental data (discrete points) and Weibull model (continuous and dashed lines).

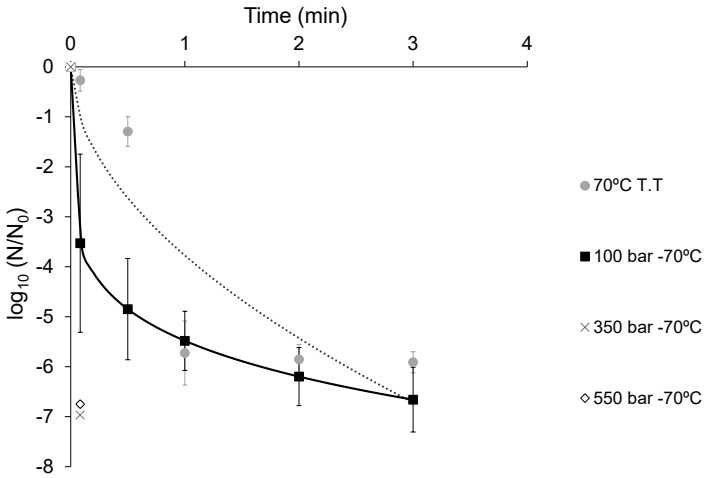


Fig. 3. Inactivation kinetics of *A. niger* in oil-in-water emulsion at 70ºC treated with SC-CO_2 + HPU at different pressures (100, 350 and 550 bar) and by thermal treatment (T.T.). Experimental data (discrete points) and Weibull model (continuous and dashed lines).

Table 1. Parameters (b and n), total time for complete inactivation ($t_{6.8}$; 6.8 log-cycle reduction) and goodness of fit by using the Weibull model for thermal, SC-CO_2 and SC-CO_2 + HPU inactivation kinetics of *A. niger* in oil-in-water emulsions under different pressure and temperature conditions. Values in brackets indicate standard errors.

Treatment	Temperature (ºC)	Pressure (bar)	b (min^{-n})	n	$t_{6.8}$ (min)	R^2	RMSE
Thermal	50	-	0.01 (0.002)	0.73 (0.06)	7588.4	0.99	0.006
Thermal	60	-	5.60 (0.18)	0.06 (0.01)	25.4	0.99	0.059
Thermal	70	-	3.77 (0.88)	0.52 (0.26)	3.1	0.80	1.079
SC-CO_2	50	100	0.08 (0.01	1.04 (0.04)	71.6	0.99	0.064
SC-CO_2	50	350	0.29 (0.10)	0.82 (0.10)	46.9	0.97	0.199
SC-CO_2	60	100	5.25 (0.03)	0.07 (0.002)	40.3	0.99	0.006
SC-CO_2	60	350	5.24 (0.004)	0.12 (0.0004)	8.8	0.99	0.003
SC-CO_2 + HPU	50	100	0.82 (0.33)	0.52 (0.11)	58.4	0.98	0.246
SC-CO_2 + HPU	50	350	0.39 (0.19)	0.75 (0.13)	45.2	0.98	0.301
SC-CO_2 + HPU	60	100	4.77 (0.39)	0.11 (0.33)	25.1	0.99	0.089
SC-CO_2 + HPU	60	350	6.17 (0.23)	0.04 (0.02)	11.4	0.99	0.119
SC-CO_2 + HPU	70	100	5.48 (0.17)	0.18 (0.03)	3.3	0.98	0.313
SC-CO_2 + HPU	70	350	*	*	*	*	*
SC-CO_2 + HPU	70	550	*	*	*	*	*

* Not enough experimental data for model fitting

3.3. SC-CO_2 inactivation of *C. butyricum* spores in oil-in-water emulsions

Fig. 4 shows the inactivation kinetics of *C. butyricum* in a 20 % oil-in-water emulsion for the SC-CO_2 treatment, at 70ºC (A) and 85ºC (B) and 550 bar, compared to the conventional thermal treatment at 70 and 85ºC. The fitting of the Weibull model to the SC-CO_2 inactivation kinetics was highly satisfactory at 70ºC, providing R^2 higher than 0.99 and RMSE lower than 0.097 (Table 2), while not enough experimental data was obtained for the fitting at 85ºC.

The fact that *C. butyricum* spores displayed greater heat resistance than *A. niger* spores was remarkable. In the thermal treatments at 70ºC, no *A. niger* count was detected after 3 min, while no inactivation was found for *C. butyricum* under the same conditions. As for the SC-CO_2 treatments, C*. butyricum* spores were also

significantly ($p<0.05$) more resistant than *A. niger* spores. *A. niger* was completely inactivated (6.8 log-cycles) after 10 min of SC-CO_2 treatment at 60ºC and 350 bar, while only 0.5 log-cycles of *C. butyricum* spores were reduced for the same treatment time at a higher temperature (70ºC) and pressure (550 bar).

SC-CO_2 vs thermal treatments

The SC-CO_2 treatment at 550 bar significantly ($p<0.05$) enhanced the inactivation, compared to the thermal treatment alone (Fig. 4), since no inactivation was obtained in the thermal treatment at 70ºC while 3.2 log-cycles were reduced in the SC-CO_2 treatment at 70ºC after 50 min (Fig. 4A). In addition, only 0.7 log-cycles were achieved after 10 min of the thermal treatment at 85ºC, while no microbial count was detected in the SC-CO_2 one (Fig. 4B). The results obtained clearly indicated that CO_2 has a major role in *C. butyricum* spore inactivation.

Few studies have addressed the SC-CO_2 inactivation of *Bacillus* spores, such as *B. subtilis* (Ballestra & Cuq, 1998; Rao et al., 2015; Spilimbergo et al., 2003) or *B. pumilus* (Zhang et al., 2006). However, for the first time, this study addresses the SC-CO_2 inactivation of *Clostridium* spp. Only Haas et al. (1989) studied the inactivation of *C. sporogenes* spores using pressurized CO_2 at low pressure (55 bar).

Effect of temperature

As for the temperature, the use of 70 or 85ºC in the SC-CO_2 treatments significantly ($p<0.05$) affected to what extent *C. butyricum* was reduced. In Fig. 4, the SC-CO_2 inactivation kinetics showed that after only 10 min, *C. butyricum* spores were completely inactivated at 85ºC (B), while at 70ºC (A), the inactivation achieved after 10 min was only of 0.5 log-cycles. Haas et al. (1989) also found a clear temperature effect when treating *C. sporogenes* spores (7.8 log CFU/mL) suspended in thioglycolate broth with CO_2 at 55 bar, since substantial levels of inactivation were achieved at 70ºC after 120 min (from 0.8 to 7.8 log-cycles, depending on the pH of the media), while no inactivation was found at 60ºC. The effect of high temperatures has been widely

investigated for the SC-CO_2 inactivation of vegetative bacterial cells, where SC-CO_2 is able to penetrate into the cell membranes faster, accelerating the inactivation mechanisms (Spilimbergo & Bertucco, 2003). However, the complex and resistant structure of bacterial spores could not be compatible with those mechanisms (Ballestra & Cuq, 1998; Zhang et al., 2006) because the CO_2 penetration and dissolution into the spore could be restrained, as its structure is dehydrated (Ishihara et al., 1999). There is no clear explanation for the inactivation mechanisms for bacterial spores. One of the hypotheses was that spores firstly have to be activated so as to germinate before being inactivated (Spilimbergo & Bertucco, 2003). As Spilimbergo et al. (2003) explained, the effect of the CO_2 acidification along with a certain spore-dependent temperature could be sufficient to promote the activation, which leads to a destruction of the spore coat and the subsequent hydration of the spore structure, becoming more sensitive to CO_2 treatments (Rao et al., 2015). Another widely accepted mechanism is related with changes in the spore structure (Soares et al., 2019) induced by the effect of the temperature along with SC-CO_2, causing damage to the spore envelope until the inner membrane is modified and its permeability increased. Thus, the core spore could be hydrated and the spores could lose their resistance (Rao et al., 2015).

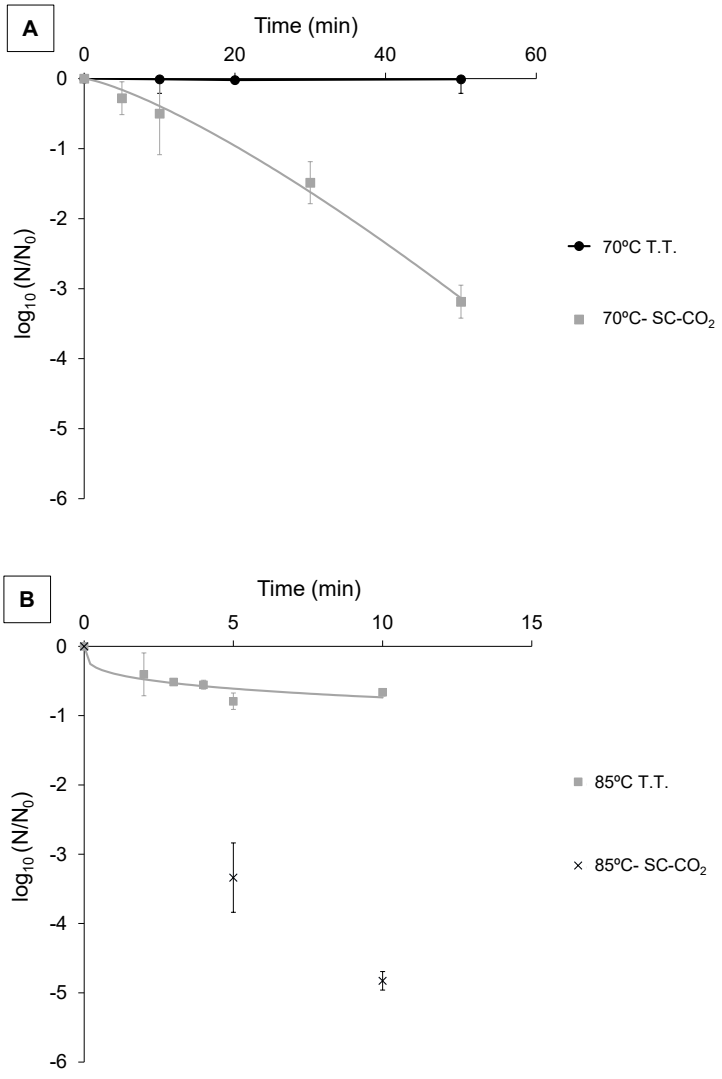


Fig. 4. Inactivation kinetics of *C. butyricum* spores in oil-in-water emulsion treated with SC-CO_2 at 550 bar and 70ºC **(A)** and 85ºC **(B)** and using thermal treatments (T.T.). Experimental data (discrete points) and Weibull model (continuous line).

3.4. SC-CO_2 + HPU inactivation of *C. butyricum* spores in oil-in-water emulsions

Figs. 5 and 6 show the inactivation kinetics of *C. butyricum* in a 20% oil-in-water emulsion for the combined SC-CO_2 + HPU treatment at different temperatures and pressures. The fitting of the kinetics with the Weibull model was appropriate, providing R^2 higher than 0.94 and RMSE lower than 0.385 in every case (Table 2).

Effect of temperature and pressure

As in the SC-CO_2 treatments (Fig. 4), the higher the temperature, the higher the level of SC-CO_2 + HPU inactivation (Fig. 5). As an example, at the lowest studied temperature (60ºC), a reduction of 1.4 log-cycles was achieved after 50 min, which should be considered as a weak inactivation level for a highly time-consuming treatment with HPU. On the contrary, at 85ºC, no microbial count was achieved after a treatment of only 3 min. The temperature effect was also computed by the $t_{4.8}$, which shortened as the temperature rose (Table 2), being 94.8, 52.9 and 15.2 at 60, 70 and 80 ºC, respectively. In the kinetics at 60ºC and 550 bar (Fig. 5), an initial lag-phase (of around 15 min) was found, a phase which was not observed at higher temperatures (70 and 80 ºC). This could mean that at temperatures higher than 60ºC, the heat along with the decrease in the pH of the media exerted by the SC-CO_2 and the effect of HPU are able to damage the cortex of the spore immediately, making it accessible for CO_2. The lag-phase in the SC-CO_2 + HPU treatments at 550 bar (Fig.5) was also well computed by the n parameter of the Weibull model (Table 2) since it was only higher than 1 for the treatment at 60ºC (2.06).

As regards pressure, whether 350 or 550 bar was used was found to have no significant ($p>0.05$) effects on the SC-CO_2 + HPU inactivation at 60ºC (Fig. 6A). In addition to 350 bar and 550 bar, a lower pressure (100 bar) was investigated in the treatments at 85ºC (Fig. 6B). In this case, the pressure had a significant ($p<0.05$) effect on the inactivation. After 2 min, 2.0 log-cycles of reduction were achieved at 100 bar, 3.2 log-cycles at 350 bar and 4.1 log-cycles at 550 bar. However, after 3 min, no microbial count was obtained either at 350 or 550 bar. Therefore, high pressures barely improved CO_2 solubility in oil-in-water emulsions. Ballestra & Cuq (1998) postulated

that if the spore structure is already altered by the action of the SC-CO_2 and temperature, an increase in pressure can lead to an increase in the amount of CO_2 passing through the membrane and to a decrease in the internal pH.

Effect of HPU

The application of HPU had a significant ($p<0.05$) effect on the inactivation at both studied temperatures (70 and 85ºC). In the treatments at 70ºC with HPU (Fig.5), no microbial count was obtained after 50 min, while only 3.2 log-cycles were reduced when HPU was not applied (Fig.4A). In addition, at 70ºC, the kinetics changed from a downward concavity shape ($n>1$) in the treatment without HPU (Fig.4A) to upward concavity behavior ($n<1$) in the treatment with HPU (Fig.5). At 85ºC, the time required for no microbial count was shortened from 10 (Fig. 4B) to only 3 min (Fig. 5) when ultrasound was applied.

Numerous studies have already demonstrated the high effect of HPU on SC-CO_2 treatments in vegetative microorganisms (Michelino et al., 2018; Ortuño et al., 2013; Paniagua-Martínez et al., 2018). As for bacterial spores, only Michelino et al. (2018) studied the effect of the combined SC-CO_2 + HPU (40W) treatment. An inactivation of 1.6 log-cycles of mesophilic bacterial spores from fresh coriander (with an initial load, naturally present in the product, of 3.6 log CFU/g) was achieved at 40 or 50ºC and 100 bar only during the pressurization (20 min) and depressurization (40 min), while no spore inactivation was obtained when HPU was not applied.

As concerns the effect of HPU on the SC-CO_2 treatments, although a remarkable difference was found between the microorganisms analyzed in the present study, it was only remarkable in the case of the inactivation of *C. butyricum* spores while no significant effect of HPU was found for the inactivation of *A. niger* spores. Therefore, it seems that the differences between the cell wall of fungal (*A. niger*) and bacterial spores (*C. butyricum*) contribute not only to the roles of temperature, pressure and time but also to the effect that HPU has on the inactivation process (Noman et al., 2018). Although no previous studies have compared the resistance of bacterial and fungal spores to SC-CO_2 + HPU treatments, the objective of Michelino et al. (2018) was

to compare bacterial spores with the yeast and molds naturally present in coriander; however, yeast and molds were already completely inactivated in the SC-CO_2 treatment without HPU. In addition, other authors compared the resistance of different bacterial spores to thermosonication treatments (70–75 °C, up to 60 min) and found a negligible effect on *A. acidoterrestris* and *C. perfringens* spores, while for *B. cereus* the effect was remarkable in beef slurry (Evelyn & Silva, 2018).

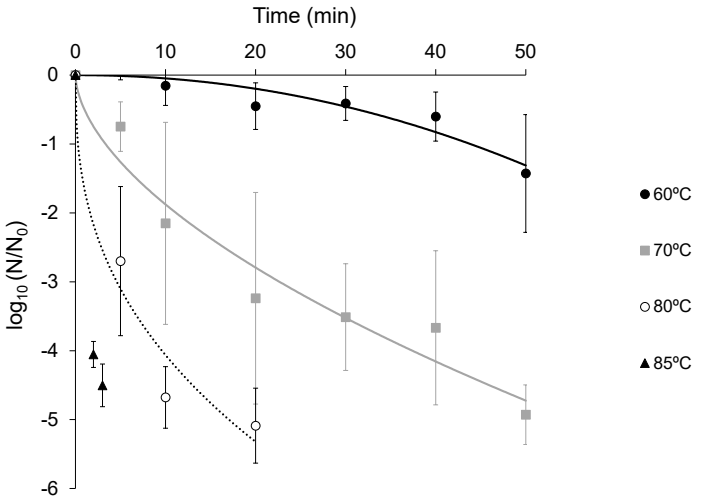


Fig. 5. Inactivation kinetics of *C. butyricum* spores in oil-in-water emulsion treated with SC-CO_2 + HPU at 550 bar and four different temperatures (60, 70, 80 and 85ºC). Experimental data (discrete points) and Weibull model (continuous and dashed lines).

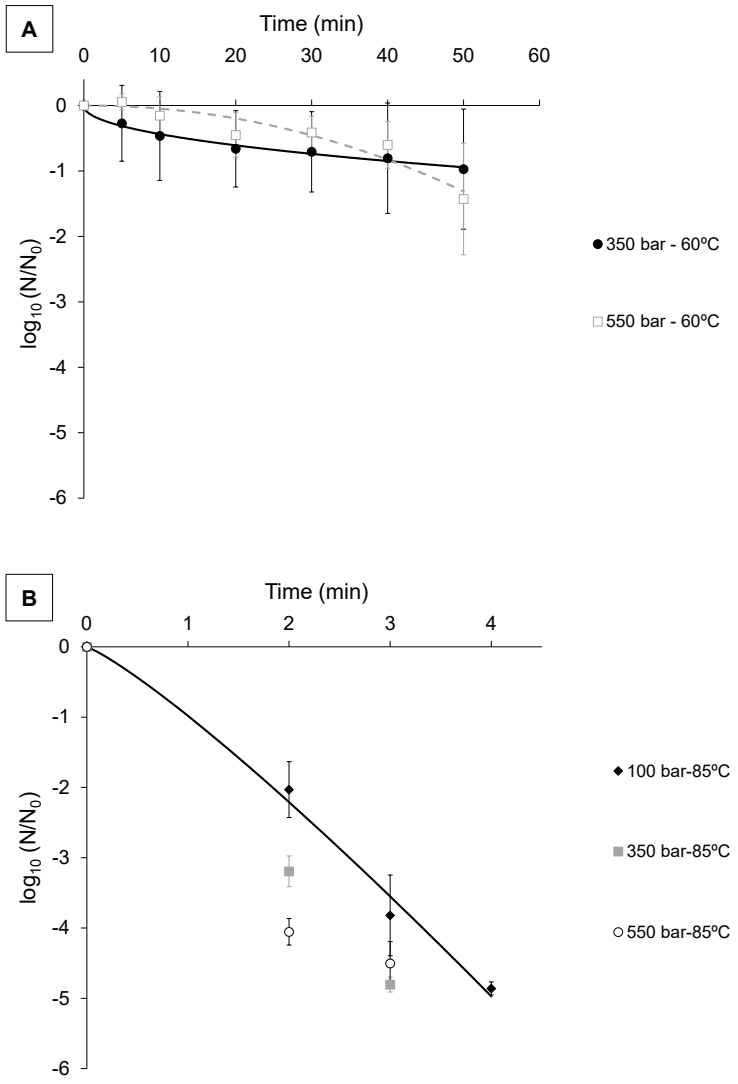


Fig. 6. Inactivation kinetics of *C. butyricum* spores in oil-in-water emulsion treated with SC-CO_2 + HPU at 350 and 550 bar at 60ºC **(A)** and at 100, 350 and 550 bar at 85ºC **(B)**. Experimental data (discrete points) and Weibull model (continuous line).

Table 2. Parameters (b and n), total time for complete inactivation ($t_{4.8}$; 4.8 log-cycles of reduction) and goodness of fit by using Weibull model for thermal, SC-CO_2 and SC-CO_2 + HPU inactivation kinetics of *C. butyricum* spores in oil-in-water emulsions under different pressure and temperature conditions. Values in brackets indicate standard errors.

Treatment	Temperature (ºC)	P (bar)	b (min^{-n})	n	$t_{4.8}$ (min)	R^2	RMSE
Thermal	70	-	1.33E-02	1.00E-02	-	0.50	0.004
Thermal	85	-	0.39 (0.09)	0.27 (0.14)	9751.8	0.85	0.086
SC-CO_2	70	550	0.02 (0.01)	1.29 (0.12)	70.0	0.99	0.097
SC-CO_2	85	550	*	*	*	*	*
SC-CO_2 + HPU	60	550	4.07E-04 (0.001)	2.06 (0.53)	94.8	0.94	0.144
SC-CO_2 + HPU	70	550	0.50 (0.17)	0.57 (0.10)	52.9	0.95	0.342
SC-CO_2 + HPU	80	550	1.66 (0.60)	0.39 (0.14)	15.2	0.95	0.385
SC-CO_2 + HPU	85	550	*	*	*	*	*
SC-CO_2 + HPU	60	350	0.14 (0.02)	0.48 (0.04)	1578.2	0.98	0.036
SC-CO_2 + HPU	85	100	0.98 (0.20)	1.17 (0.16)	3.9	0.99	0.171
SC-CO_2 + HPU	85	350	*	*	*	*	*

* Not enough experimental data for model fitting

4. Conclusions

The present study demonstrated that the industrial application of the low temperature pasteurization (< 60ºC) of fungal and bacterial spores in oil-in-water emulsions using SC-CO_2 was not feasible due to the low inactivation rate. *A. niger* spores were more sensitive to the SC-CO_2 treatments than *C. butyricum* spores and the application of HPU only intensified the inactivation of the *C. butyricum* spores, thus illustrating for the first time the different resistance of bacterial and fungal spores to the combined SC-CO_2 + HPU. The performance of the SC-CO_2 + HPU inactivation treatments on *C. butyricum* spores was affected by the temperature (from 60 to 85ºC), while pressure levels above 350 bar did not improve the inactivation. Additional studies should evaluate the effect of the combined SC-CO_2 + HPU treatment on other spores and address its effect on the quality properties of the oil-in-water emulsions.

Acknowledgements

The authors acknowledge the financial support from Fresenius Kabi.

References

Ballestra, P., & Cuq, J. L. (1998). Influence of pressurized carbon dioxide on the thermal inactivation of bacterial and fungal spores. *LWT - Food Science and Technology*, *31*(1), 84–88. https://doi.org/10.1006/fstl.1997.0299

Baril, E., Coroller, L., Postollec, F., Leguerinel, I., Boulais, C., Carlin, F., & Mafart, P. (2011). The wet-heat resistance of Bacillus weihenstephanensis KBAB4 spores produced in a two-step sporulation process depends on sporulation temperature but not on previous cell history. *International Journal of Food Microbiology*, *146*(1), 57–62. https://doi.org/10.1016/J.IJFOODMICRO.2011.01.042

Benedito, J., Martínez-Pastor, M. T., Mulet, A., Ortuño, C., & Peña, R. (2011). Procedure of inactivation microorganisms by combination of supercritical fluids and ultrasound. *Spain. Patent*, *P201131099*.

Bi, X., Wang, X., Chen, Y., Chen, L., Xing, Y., & Che, Z. (2020). Effects of combination treatments of lysozyme and high power ultrasound on the Salmonella typhimurium inactivation and quality of liquid whole egg. *Ultrasonics Sonochemistry*, *60*, 104763. https://doi.org/10.1016/J.ULTSONCH.2019.104763

Calvo, L., Muguerza, B., & Cienfuegos-Jovellanos, E. (2007). Microbial inactivation and butter extraction in a cocoa derivative using high pressure CO2. *The Journal of Supercritical Fluids*, *42*(1), 80–87. https://doi.org/10.1016/J.SUPFLU.2007.01.009

Cappelletti, M., Ferrentino, G., & Spilimbergo, S. (2014). Supercritical carbon dioxide combined with high power ultrasound: An effective method for the pasteurization of coconut water. *The Journal of Supercritical Fluids*, *92*, 257–263. https://doi.org/10.1016/J.SUPFLU.2014.06.010

Cárcel, J. A., García-Pérez, J. V., Benedito, J., & Mulet, A. (2012). Food process innovation through new technologies: Use of ultrasound. *Journal of Food Engineering*, *110*(2), 200–207. https://doi.org/10.1016/J.JFOODENG.2011.05.038

Ceni, G., Fernandes Silva, M., Valério, C., Cansian, R. L., Oliveira, J. V., Dalla Rosa,

C., & Mazutti, M. A. (2016). Continuous inactivation of alkaline phosphatase and Escherichia coli in milk using compressed carbon dioxide as inactivating agent. *Journal of CO2 Utilization*, *13*, 24–28. https://doi.org/10.1016/j.jcou.2015.11.003

Corradini, M. G., & Peleg, M. (2012). The kinetics of microbial inactivation by carbon dioxide under high pressure. In *Dense Phase Carbon Dioxide: Food and Pharmaceutical Applications* (pp. 135–155).

Couvert, O., Gaillard, S., Savy, N., Mafart, P., & Leguérinel, I. (2005). Survival curves of heated bacterial spores: effect of environmental factors on Weibull parameters. *International Journal of Food Microbiology*, *101*(1), 73–81. https://doi.org/10.1016/J.IJFOODMICRO.2004.10.048

Deen, B., & Diez-Gonzalez, F. (2019). Assessment of Pediococcus acidilactici ATCC 8042 as potential Salmonella surrogate for thermal treatments of toasted oats cereal and peanut butter. *Food Microbiology*, *83*, 187–192. https://doi.org/10.1016/J.FM.2019.05.015

Evelyn, & Silva, F. V. M. (2018). Differences in the resistance of microbial spores to thermosonication, high pressure thermal processing and thermal treatment alone. *Journal of Food Engineering*, *222*, 292–297. https://doi.org/10.1016/J.JFOODENG.2017.11.037

Feofilova, E. P., Ivashechkin, A. A., Alekhin, A. I., & Sergeeva, Y. E. (2012). Fungal spores: Dormancy, germination, chemical composition, and role in biotechnology (review). *Applied Biochemistry and Microbiology*, *48*(1), 1–11. https://doi.org/10.1134/S0003683812010048

Ferrentino, G., & Spilimbergo, S. (2016). A combined high pressure carbon dioxide and high power ultrasound treatment for the microbial stabilization of cooked ham. *Journal of Food Engineering*, *174*, 47–55. https://doi.org/10.1016/J.JFOODENG.2015.11.013

Garcia-Gonzalez, L., Geeraerd, A. H., Elst, K., Van Ginneken, L., Van Impe, J. F., & Devlieghere, F. (2009). Influence of type of microorganism, food ingredients and food properties on high-pressure carbon dioxide inactivation of microorganisms.

International Journal of Food Microbiology, *129*(3), 253–263. https://doi.org/10.1016/j.ijfoodmicro.2008.12.005

Garcia-Gonzalez, L., Geeraerd, A. H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Van Impe, J. F., & Devlieghere, F. (2007). High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. In *International Journal of Food Microbiology* (Vol. 117, Issue 1, pp. 1–28). https://doi.org/10.1016/j.ijfoodmicro.2007.02.018

Ghoddusi, H. B., & Sherburn, R. (2010). Preliminary study on the isolation of Clostridium butyricum strains from natural sources in the UK and screening the isolates for presence of the type E botulinal toxin gene. *International Journal of Food Microbiology*, *142*(1–2), 202–206. https://doi.org/10.1016/j.ijfoodmicro.2010.06.028

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2020). Non-thermal pasteurization of lipid emulsions by combined supercritical carbon dioxide and high-power ultrasound treatment. *Ultrasonics Sonochemistry*, *67*, 105–138. https://doi.org/10.1016/J.ULTSONCH.2020.105138

Haas, G. J., Prescott, H. E., Dudley, E., Dik, R., Hintlian, C., & Keane, L. (1989). Inactivation of Microorganisms By Carbon Dioxide Under Pressure. *Journal of Food Safety*, *9*(4), 253–265. https://doi.org/10.1111/j.1745-4565.1989.tb00525.x

Ishihara, Y., Saito, H., & Takano, J. (1999). Differences in the surface membranes and water content between the vegetative cells and spores of Bacillus subtilis. *Cell Biochemistry and Function*, *17*(1), 9–13. https://doi.org/10.1002/(SICI)1099-0844(199903)17:1<9::AID-CBF803>3.0.CO;2-9

Jiao, S., Zhang, H., Hu, S., & Zhao, Y. (2019). Radio frequency inactivation kinetics of Bacillus cereus spores in red pepper powder with different initial water activity. *Food Control*, *105*, 174–179. https://doi.org/10.1016/J.FOODCONT.2019.05.038

Kamihira, M., Taniguchi, M., & Kobayashi, T. (1987). Sterilization of microorganisms with supercritical carbon dioxide. *Agricultural and Biological Chemistry*, *51*(2),

407–412. https://doi.org/10.1080/00021369.1987.10868053

Kar, S., Mujumdar, A. S., & Sutar, P. P. (2019). Aspergillus niger inactivation in microwave rotary drum drying of whole garlic bulbs and effect on quality of dried garlic powder. *Drying Technology*, *37*(12), 1528–1540. https://doi.org/10.1080/07373937.2018.1517777

Liao, H., Hu, X., Liao, X., Chen, F., & Wu, J. (2007). Inactivation of Escherichia coli inoculated into cloudy apple juice exposed to dense phase carbon dioxide. *International Journal of Food Microbiology*, *118*(2), 126–131. https://doi.org/10.1016/J.IJFOODMICRO.2007.06.018

Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, *72*(1–2), 107–113. https://doi.org/10.1016/S0168-1605(01)00624-9

Michelino, F., Zambon, A., Vizzotto, M. T., Cozzi, S., & Spilimbergo, S. (2018). High power ultrasound combined with supercritical carbon dioxide for the drying and microbial inactivation of coriander. *Journal of CO2 Utilization*, *24*, 516–521. https://doi.org/10.1016/J.JCOU.2018.02.010

Noman, E., Norulaini Nik Ab Rahman, N., Al-Gheethi, A., Nagao, H., Talip, B. A., & Ab. Kadir, O. (2018). Selection of inactivation medium for fungal spores in clinical wastes by supercritical carbon dioxide. *Environmental Science and Pollution Research*, *25*(22), 21682–21692. https://doi.org/10.1007/s11356-018-2335-1

Omar, A. K. M., Tengku Norsalwani, T. L., Asmah, M. S., Badrulhisham, Z. Y., Easa, A. M., Omar, F. M., Hossain, M. S., Zuknik, M. H., & Nik Norulaini, N. A. (2018). Implementation of the supercritical carbon dioxide technology in oil palm fresh fruits bunch sterilization: A review. *Journal of CO2 Utilization*, *25*(March), 205–215. https://doi.org/10.1016/j.jcou.2018.03.021

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012a). Supercritical carbon dioxide inactivation of Escherichia coli and Saccharomyces cerevisiae in different growth stages. *The Journal of Supercritical Fluids*, *63*, 8–15.

https://doi.org/10.1016/J.SUPFLU.2011.12.022

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012b). An ultrasound-enhanced system for microbial inactivation using supercritical carbon dioxide. *Innovative Food Science & Emerging Technologies*, *15*, 31–37. https://doi.org/10.1016/J.IFSET.2012.02.006

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2013). Application of high power ultrasound in the supercritical carbon dioxide inactivation of Saccharomyces cerevisiae. *Food Research International*, *51*(2), 474–481. https://doi.org/10.1016/J.FOODRES.2013.01.041

Ortuño, C., Quiles, A., & Benedito, J. (2014). Inactivation kinetics and cell morphology of E. coli and S. cerevisiae treated with ultrasound-assisted supercritical CO2. *Food Research International*, *62*, 955–964. https://doi.org/10.1016/J.FOODRES.2014.05.012

Paniagua-Martínez, I., Mulet, A., García-Alvarado, M. A., & Benedito, J. (2018). Orange juice processing using a continuous flow ultrasound-assisted supercritical CO2 system: Microbiota inactivation and product quality. *Innovative Food Science & Emerging Technologies*, *47*, 362–370. https://doi.org/10.1016/J.IFSET.2018.03.024

Peleg, M. (2006). *Models for predicting growth and inactivation. Advanced quantitative microbiology for foods and biosystems*.

Rao, L., Xu, Z., Wang, Y., Zhao, F., Hu, X., & Liao, X. (2015). Inactivation of Bacillus subtilis spores by high pressure CO2 with high temperature. *International Journal of Food Microbiology*, *205*, 73–80. https://doi.org/10.1016/J.IJFOODMICRO.2015.04.012

Shimoda, M., Kago, H., Kojima, N., Miyake, M., Osajima, & Hayakawa, I. (2002). Accelerated death kinetics of Aspergillus niger spores under high-pressure carbonation. *Applied and Environmental Microbiology*, *68*(8), 4162–4167. https://doi.org/10.1128/AEM.68.8.4162

Soares, G. C., Learmonth, D. A., Vallejo, M. C., Davila, S. P., González, P., Sousa, R.

A., & Oliveira, A. L. (2019). Supercritical CO2 technology: The next standard sterilization technique? *Materials Science and Engineering: C*, *99*, 520–540. https://doi.org/10.1016/J.MSEC.2019.01.121

Spilimbergo, S., & Bertucco, A. (2003). Non-thermal bacteria inactivation with dense CO2. *Biotechnology and Bioengineering*, *84*(6), 627–638. https://doi.org/10.1002/bit.10783

Spilimbergo, S., Bertucco, A., Lauro, F. M., & Bertoloni, G. (2003). Inactivation of Bacillus subtilis spores by supercritical CO2 treatment. *Innovative Food Science and Emerging Technologies*, *4*(2), 161–165. https://doi.org/10.1016/S1466-8564(02)00089-9

Tischler, B. Y., & Hohl, T. M. (2019). Menacing mold: recent advances in Aspergillus pathogenesis and host defense. *Journal of Molecular Biology*, *431*(21), 4229–4246. https://doi.org/10.1016/j.jmb.2019.03.027

Zhang, J., Burrows, S., Gleason, C., Matthews, M. A., Drews, M. J., LaBerge, M., & An, Y. H. (2006). Sterilizing Bacillus pumilus spores using supercritical carbon dioxide. *Journal of Microbiological Methods*, *66*(3), 479–485. https://doi.org/10.1016/j.mimet.2006.01.012

The Journal of Supercritical Fluids 174 (2021) 105-246

Ultrasonic-assisted supercritical CO_2 inactivation of bacterial spores and effect on the physicochemical properties of oil-in-water emulsions

Angela Gomez-Gomez[1], Edmundo Brito-de la Fuente[2], Críspulo Gallegos[2], Jose Vicente Garcia-Perez[1], Jose Benedito[1]

[1]Grupo ASPA, Departamento de Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, València E46022, Spain

[2]Fresenius-Kabi Deutschland GmbH, Product and Process Engineering Center, Pharmaceuticals & Device Division, Bad Homburg, German

Ultrasonic-assisted supercritical CO_2 inactivation of bacterial spores and effect on the physicochemical properties of oil-in-water emulsions

Abstract

A combined supercritical carbon dioxide and high power ultrasound treatment (SC-CO_2 + HPU) to inactivate spores while minimizing the physicochemical changes in emulsions was investigated. The inactivation kinetics were obtained for *Bacillus subtilis, Bacillus pumilus* and *Geobacillus stearothermophilus* spores and the effect of the treatment conditions on the quality of the emulsions was explored using response surface methodology. The treatment for 20 min at 95ºC and 350 bar was effective at inactivating *B. subtilis* and *B. pumilus.* Treated emulsions showed, in general, minimal changes in density and good stability, although the pH decreased and the droplet size increased. The treatment at 600 bar, 95ºC and 12.5 min permitted satisfactory *B. subtilis* and *B. pumilus* inactivation with minimal physicochemical changes in the emulsions. Therefore, the use of SC-CO_2 + HPU could be a viable alternative for the preservation of emulsions using temperatures lower than those of thermal treatments.

Keywords: supercritical CO_2; high power ultrasound; inactivation; spores; emulsion; quality.

1. Introduction

Microbial safety is essential in the food and pharmaceutical industries. Specifically, spore contamination is a major industrial risk since spores are the most resistant form of bacteria due to the fact that their structure is different to that of vegetative cells (Dong et al., 2016), more complex and reinforced . Some gram-positive bacteria, such as those from the *Bacillus*, *Geobacillus* (aerobic) and *Clostridium* (anaerobic) genus, are able to form spores as a mechanism of resistance to stress factors, being able to survive under extreme chemical and physical environments (Spilimbergo et al., 2003; Watanabe et al., 2003). Therefore, treatments in intense process conditions are required to reach satisfactory levels of bacterial spore inactivation. In this regard, some species of bacterial spores have been used as biological indicators for sterilization procedures: *Bacillus subtilis* for dry heat and ethylene oxide sterilization, *Bacillus pumilus* for irradiation and *Geobacillus stearothermophilus* for steam sterilization and formaldehyde sterilization (Spicher, 1988; Y. Zhang et al., 2018).

In addition to the microbial safety, consumers also demand high-quality processed products. In this sense, non-thermal preservation technologies are emerging, which are defined as processes where the application of heat is not the main cause of microbial inactivation. Therefore, the advantage of these technologies over thermal technologies is the milder impact on the physicochemical and nutritional properties of the treated product. Among others, some examples of non-thermal technologies are high hydrostatic pressure (Bermúdez-Aguirre & Barbosa-Cánovas, 2011), irradiation (Lewis et al., 2002), electric fields (Michalac et al., 2003), high power ultrasound (HPU) (Evelyn & Silva, 2015) and supercritical carbon dioxide (SC-CO_2) (Ribeiro et al., 2020). SC-CO_2 has shown itself to be effective at inactivating vegetative cells (Gomez-Gomez et al., 2020; Ortuño et al., 2012). However, the complex, highly dehydrated and robust structure of spores makes them highly resistant to SC-CO_2 treatments and, usually, the increase of the permeability of the spore coat is required to allow SC-CO_2 to exert its inactivating effect. In this sense, the implementation of high temperatures, normally over 60ºC, has shown to enhance the permeability to SC-CO_2

in bacterial spores (Rao et al., 2015). Nonetheless, long processing times are still necessary. In this regard, 40 min and 30 min were needed to completely inactivate *Alicyclobacillus acidoterrestris* spores at 65ºC and 100 bar and at 70°C and 80 bar, respectively (Bae et al., 2009); 60 min to inactivate less than 1 log-cycle of *Bacillus subtilis* spores at 66-77ºC and 200 bar (Rao et al., 2015) and 60 min for a 6 log-cycle reduction of *Bacillus subtilis* spores at 55°C and 300 bar (Ishikawa et al., 1997). In this regard, the combination of SC-CO_2 and medium-high temperatures with other non-thermal technology could be essential to enhance the SC-CO_2 effectiveness for spore inactivation.

High intensity (> 1 W/cm^2) and low frequency (20-100 kHz) ultrasound, also called high power ultrasound (HPU), is made up of acoustic waves whose vibration is transmitted through the medium exerting an effect on the product or process (Cárcel et al., 2012). HPU has been used for microbial inactivation purposes, although its application alone has been shown to be insufficient for the inactivation of bacterial spores (Fan et al., 2019). It has been demonstrated that the simultaneous application of HPU to the SC-CO_2 treatment enhances mass and heat transfer processes, facilitating the SC-CO_2 inactivation mechanisms. In addition, the cavitation produced by HPU could cause the degradation of the external layers of the spore structure (Mañas & Pagán, 2005), enhancing the penetration of SC-CO_2 into the cells. Only one reference to the combined SC-CO_2 + HPU treatment for the inactivation of naturally present mesophilic bacterial spores during coriander drying was found (Michelino et al., 2018). Moreover, most of the studies into SC-CO_2 + HPU inactivation mainly focus on microbial inactivation, commonly postponing the analysis of the quality of the treated product. A few studies can be found analysing the quality of the SC-CO_2 + HPU treated products. In this sense, Paniagua-Martinez et al. (2018a & 2018b) treated fruit juices and found small differences in the physicochemical properties and even discovered an enhancement in the quality of some attributes, such as in the quantity of phenolic compounds, the antioxidant capacity or the stability of the juices, compared to the thermally treated juices. Working on chicken, Morbiato et al. (2019) were able to simultaneously drying-inactivate (the naturally present and inoculated *S. enterica*) the raw meat, while maintaining its nutritional properties. Lastly, Ferrentino & Spilimbergo

(2015) found no differences in the pH and total acidity of carrot when compared to the untreated and, although changes in texture were found, those were also observed in the thermally treated carrot. Instead, a noticeable off taste was perceived in the SC-CO_2 + HPU treated carrot.

Interest in microbial inactivation in commercial emulsions has increased due to the multiple industrial applications of emulsions (food, pharmaceutical, cosmetic, etc.). An emulsion is composed of two immiscible liquids, one dispersed in the other. Consequently, emulsions, except microemulsions (Lu & Gao, 2010), are thermodynamically unstable systems and can be destabilized by means of a wide variety of mechanisms, such as gravitational separation, droplet aggregation, chemical and microbiological processes or applied mechanical forces (McClements, 2015). Gomez-Gomez et al. (2020) investigated the SC-CO_2 + HPU inactivation of vegetative bacteria in oil-in-water emulsions. Nevertheless, no studies were found into the changes in the physicochemical properties after SC-CO_2 + HPU treatments in emulsions. Therefore, the objective of the present study was to assess the feasibility of using the SC-CO_2 + HPU treatment at high temperatures in the inactivation of different bacterial spores in oil-in-water emulsions and to analyze the effect of the SC-CO_2 + HPU treatment on its physicochemical properties.

2. Materials and methods

2.1. Preparation of the lipid emulsion

The treatment media were oil-in-water emulsions with a soybean oil content of 20 % and egg phospholipid as the emulsifying agent. The emulsions were prepared in three stages: mixture with a disperser device, sonication and homogenization, following the procedure described by Gomez-Gomez et al. (2020)

2.2. Preparation of the bacterial spore suspensions

B. subtilis (CECT 356), *B. pumilus* (CECT 29T) and *G. stearothermophilus* (CECT 43 T) were obtained from the Spanish Type Culture Collection (CECT), Spain.

Bacterial spores were obtained following the methodology of Mafart et al. (2002), with modifications. A single colony of the bacteria was cultivated on Nutrient Broth (Scharlab, Barcelona, Spain) for *B. subtilis* and *B. puminus* at 30ºC and in Tryptic Soy Broth (Scharlab, Barcelona, Spain) for *G. stearothermophilus* at 50ºC until the stationary phase was reached (around 24 h (Han et al., 2017; Hetzer et al., 2006; Lee et al., 2011; Mondal et al., 2015)). 100 µL of the suspension were poured on agar (Plate Count Agar for *B. subtilis* and *B. pumilus* and Tryptic Soy Agar for *G. stearothermophilus*) enriched with $MnSO_4$ (40 mg/L) and $CaCl_2$ (100 mg/L) to enhance the sporulation. The plates were incubated at the required temperature for each bacteria for 5-6 days, a time during which spores were formed (determined with a Thoma counting chamber and an optical microscope). Afterwards, the spores were collected by scraping the surface of the agar, suspended in deionized water, and washed three times by centrifugation (Medifriger BL-S, JP Selecta, Barcelona, Spain) at 8000x g for 15 min. The pellet was suspended in 2 mL ethanol (50% v/v) and kept at 4°C for 12 h to eliminate vegetative non-sporulated bacteria. Afterwards, the suspension was washed again three times by centrifugation. Lastly, the final suspension was distributed in sterile microtubes and kept at 4°C until use. Prior to each inactivation treatment, the microtubes were heat-shocked at 80°C for 15 min to eliminate the remaining vegetative cells (Ávila et al., 2014; Spilimbergo et al., 2003) and cooled at 4ºC. After that, 2 mL of the spore suspension were added to the autoclaved emulsion (60 mL) to reach a cell concentration of 10^6-10^8 CFU /mL for *B. subtilis*, 10^7-10^8 CFU /mL for *B. pumilus* and 10^5-10^6 CFU /mL for *G. stearothermophilus*.

2.3. Thermal treatment

The thermal treatments were performed at 85 and 95ºC for *B. subtilis* and at 95ºC for *B. pumilus* and *G. stearothermophilus* in a temperature-controlled water bath (1812, Bunsen, Madrid, Spain). A 1.5 mL sample (emulsion with bacterial spore suspension) was 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 5, 10, 15 and 20

min for *B. subtilis* and after 10 and 20 min for *B. pumilus* and *G. stearothermophilus.* The samples were cooled in ice for immediate analysis. The experiments were carried out in triplicate.

2.4. Ultrasonic assisted supercritical CO_2 processing

Batch lab-scale equipment, designed and built by the research group to operate with supercritical fluids, was used for the SC-CO_2 and SC-CO_2 + HPU treatments. The main components of the system are a tank of carbon dioxide, a chiller reservoir, a diaphragm metering pump (LDB, LEWA, Japan), an inactivation vessel (600 mL of internal volume) and a thermostatic water bath. For the inactivation treatments, 62 mL of sample (emulsion with bacterial spore suspension) were introduced in the vessel. Additionally, a HPU transducer was attached to the lid of the vessel to carry out the combined SC-CO_2 + HPU treatments (Benedito et al., 2011). The ultrasound system consisted essentially of a high power (>1W/cm^2) piezoelectric transducer, a sonotrode and a power generation unit. The power was 35 W ± 5 W (I= 250 ± 10 mA; U= 220 ± 5V) and the frequency was 30 ± 2 kHz, both measured using a digital power meter (WT210, Yokogawa Electric Corporation, Tokyo, Japan). HPU was connected when the desired pressure was reached in the vessel. The equipment and procedure described in detail can be found in Gomez-Gomez et al. (2020).

For *B. subtilis*, SC-CO_2 and SC-CO_2 + HPU inactivation treatments were carried out at 85 and 95ºC and 350 bar. For *B. pumilus* and *G. stearothermophilus*, SC-CO_2 + HPU inactivation treatments were only performed at the highest temperature (95ºC). 85 and 95ºC were selected because bacterial spores are known to be extremely resistant to heat and unaffected by SC-CO_2 at mild temperatures (T<60ºC) (Rao et al., 2015). In turn, the inactivation of bacterial spores is known to be drastically improved at temperatures above 70ºC (Perrut, 2012). The pressure (350 bar) was chosen for being commonly used in SC-CO_2 inactivation studies (Soares et al., 2019). In addition, to test a condition which could provide a greater level of *G. stearothermophilus* spore reduction, a higher pressure (550 bar) was tested for the SC-CO_2 + HPU treatments. The time of the experiments was limited to 20 min because longer SC-CO_2 + HPU

treatments are not desirable for industrial processes. Samples of around 2 mL were drawn at intervals of 2-5 min and were cooled in ice before analysis. Each treatment condition was applied in triplicate.

2.5. Microbiological analyses

The bacterial spores were quantified by means of the standard plate count technique, before and after the treatments. For that purpose, 100 µL of the sample dilutions were spread on agar (Plate Count Agar for *B. subtilis* and *B. pumilus* and Tryptic Soy Agar for *G. stearothermophilus*) in triplicate and incubated for 24 h at 30ºC, for the *B. subilis* and *B. pumilus* spores, and at 50ºC, for the *G. stearothermophilus* spores. Microbial inactivation was reported as log (N/N_0) versus time, where N (CFU/mL) was the number of spores after different times of the treatment and N_0 the number of spores in the untreated emulsion.

2.6. Modelling

The Weibull model, which is a robust, empirical, non-linear model successfully applied to inactivation kinetics (Peleg, 2006), was used in the decimal logarithmic form to describe the inactivation of the spores (Eq. 1).

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

where N_0 is the number of spores counted in the untreated emulsion (CFU/mL), N is the number of spores in the emulsion (CFU/mL) after the treatment time t, n is the shape factor and b is the rate parameter.

The kinetic constants of the model (b and n) were calculated by minimizing the sum of the squared differences between the experimental and predicted data using the Solver Microsoft Excel™ tool. According to this model, $n < 1$ and $n > 1$ correspond to survival curves with concave-upwards (tailings) and concave-downwards (shoulders),

respectively; while n =1 represents the traditional first-order kinetics (Jiao et al., 2019). 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.

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

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

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

The Weibull parameters (b and n) are simultaneously identified and are both affected by the inactivation velocity. Therefore, for model analysis, they should not be considered as independent values. Thus, when two different inactivation treatments are compared, a higher value of b in one of them does not directly involve a faster inactivation since a lower n value can diminish the microbial inactivation rate in favor of the other treatment. Therefore, in order to use the model to compare the effect of the different variables on microbial inactivation, the time required to achieve complete inactivation (t_x) was calculated from Eq. 1 and the b and n values of the Weibull model were identified for each condition, where x was the average of the total inactivation for every bacterial spore (6.8 log-cycles for *B. subtilis*, 8.1 for *B. pumilus* and 5.3 for *G. stearothermophilus*).

2.7. Statistical analysis of microbial kinetics

The statistical package Statgraphics Centurion XVI (Statpoint Technologies Inc., Warrenton, VA, USA) was used to perform a general linear model (GLM) in order to evaluate the effect of both the treatment conditions (temperature, pressure and use of HPU) and the different bacterial spores on the level of inactivation and the LSD (Least Significant Differences) were identified to discriminate among the means ($p<0.05$).

2.8. Effect of the treatments on the physicochemical properties of the emulsions. Experimental design and response surface analysis

In addition to studying the effectiveness of SC-CO_2 + HPU treatments on the inactivation of the bacterial spores, the effect of these treatments on the physicochemical properties of the emulsions was assessed. For that purpose, a Box-Behnken experimental design for response surface methodology (second-order polynomial model) was employed, using Statgraphics Centurion XVI (Statpoint Technologies Inc., Warrenton, VA, USA). Three process variables were considered: pressure (range from 100 to 600 bar), temperature (range from 55 to 95ºC) and time (range from 5 to 20 min). The design had 15 experimental runs, which involved three levels for each factor and three replications at the center point (Table 2).

2.9. Physicochemical analysis of the oil-in-water emulsions

The quality of emulsions is heavily influenced by the concentration, size, charge and interactions of the droplets. Hence, physical stability was measured by methods determining visible aggregation (creaming), droplet size, ζ-potential and pH (McClements, 2007). The physicochemical properties were measured before and after the SC-CO_2 + HPU treatments under the different conditions considered in the experimental design (Table 2). All of the measurements were taken at 25 °C in triplicate.

2.9.1. Appearance

The appearance of the samples was visually evaluated with the aid of a fluorescent lamp (HLWC 111, Waldmann, Germany), searching for signs of instability, such as visible oil droplets on the surface or creaming.

2.9.2. pH and density

The pH was measured using a digital pH-meter (pHenomenal 1000, VWR, USA) after calibration with commercial buffer solutions at pH 4, 7 and 10. The density was measured with a densitometer (densito 30PX Mettler Toledo, Switzerland), which uses the oscillating body method.

2.9.3. Droplet size

The mean droplet size was expressed as volume–length diameter (D[4,3]) and area–volume mean diameter (D[3,2]). D[4,3] and D[3,2] were measured using a laser diffractometer (Mastersizer 2000, Malvern Instruments, UK). The emulsions were diluted in deionized water until an obscuration rate of 5-12% was obtained and the Mie theory was applied considering a refractive index of 1.456, and absorption of 0.01.

2.9.4. ζ-potential

The surface charge of the droplets was established by ζ-potential using a Zetasizer (Nano ZS, Malvern Instruments, UK), which transforms the electrophoretic mobility of the droplets by applying the Smoluchowski model to ζ-potential values. For this purpose, dilutions of the emulsions were prepared in deionized water at a concentration of 0.001% v/v.

3. Results and discussion

3.1. SC-CO_2 and SC-CO_2 + HPU treatments of *B. subtilis* spores

Fig. 1 shows the inactivation kinetics of *B. subtilis* spores inoculated in an oil-in-water emulsion with SC-CO_2 (350 bar) and SC-CO_2 + HPU (350 bar) treatments at 85 and 95ºC. The Weibull model satisfactorily described the SC-CO_2 and SC-CO_2 + HPU inactivation kinetics, as shown in Fig. 1, with R^2 values of over 0.98 and RMSE values lower than 0.25. On the contrary, for the thermal treatment kinetics, the Weibull model only fitted well in the 95ºC treatment (R^2 0.99 and RMSE 0.07) and almost no inactivation (a reduction of 0.1 log-cycles) was achieved at 85°C in 20 min (Fig. 1A).

A significantly ($p<0.05$) higher level of inactivation was achieved when the *B. subtilis* spores were treated with SC-CO_2 than when the thermal treatment was used. In addition, the temperature had a significant ($p<0.05$) effect on inactivation in both the thermal and the SC-CO_2 treatments. At 95ºC, the reduction in 20 min varied from 2.7 log-cycles in the thermal treatment to 6.2 log-cycles in the SC-CO_2 (Fig.1B). According to the Weibull model, the time needed for the complete inactivation of *B. subtilis* spores ($t_{6.8}$) in the SC-CO_2 treatments was 28.5 min at 85ºC and 22.0 min at 95ºC (Table 1). In addition, the shape of the SC-CO_2 kinetics also varied according to the temperature since, as observed in Fig. 1, at 85ºC (A) the inactivation was negligible in the initial 5 min of treatment (an inactivation of 0.04 log-cycles), while at 95ºC (B) a rapid inactivation was obtained for the same time (a reduction of 3.5 log-cycles). The shape parameter of the Weibull model (n) successfully represented the observed shapes of the kinetics, since n was higher than 1 (2.22) for the SC-CO_2 treatment at 85ºC, linked to a downward concavity and lower than 1 (0.41) for the SC-CO_2 treatment at 95ºC, related to an upward concavity. Therefore, the SC-CO_2 inactivation kinetics of *B. subtilis* in oil-in-water emulsions was highly temperature dependent.

Several authors have reported higher levels of *B. subtilis* spore inactivation using SC-CO_2 than when a conventional thermal treatment was used at the same temperature (Ballestra & Cuq, 1998; Spilimbergo et al., 2003). As an example, Spilimbergo et al. (2003) achieved complete inactivation (7.0 log-cycles) at 75ºC, 70 bar and in 2 h in a saline solution, while no inactivation was obtained in the thermal

treatment with the same time-temperature combination. As regards the temperature effect, Ballestra & Cuq (1998) also observed that a rise in temperature brought about higher inactivation levels for *B. subtilis* spores in a saline solution treated with CO_2 at 50 bar (e.g. in 45 min, less than 2.0 log-cycles were reduced at 80ºC while around 3.0 log-cycles were inactivated at 90ºC). In addition, Rao et al., (2015) completely inactivated (a reduction of 7.0 log-cycles) *B. subtilis* spores in deionized water with SC-CO_2 at 86ºC and at 91ºC (100-150 bar) in 60 min, while less than 1.0 log-cycle was reduced at temperatures of under 77ºC.

High temperatures are known to enhance the diffusivity of CO_2 and the fluidity of the cell wall in vegetative microorganisms; thus, at high temperatures SC-CO_2 is able to penetrate into the cell membranes faster, enhancing the inactivation effect (Spilimbergo & Bertucco, 2003). Nevertheless, the inactivation mechanisms of SC-CO_2 for vegetative cells might not be applicable to bacterial spores (Ballestra & Cuq, 1998; Zhang et al., 2006). Spores have a complex and extremely dehydrated structure (Ishihara et al., 1999), which makes it difficult for CO_2 to penetrate through the spore structure. The inactivation mechanisms for bacterial spores need to be elucidated, but some authors have explained that spores firstly need to be activated and germinated before being inactivated (Spilimbergo & Bertucco, 2003). The activation, which damages the coating and leads to the successive hydration of the spore structure, could be achieved with the CO_2 acidification along with a certain spore-dependent temperature (Spilimbergo et al., 2003). However, the findings of Rao et al. (2016) suggested that the SC-CO_2 inactivation of *B. subtilis* spores was achieved through the direct damage of the spore structure caused by the effect of high temperatures along with SC-CO_2. Thus, the spore envelope is damaged by the process, which leads to the alteration of the inner membrane and the loss in spore resistance to treatments (Rao et al., 2015). Another way of increasing spore coat permeability is the exposure of the spore to chemicals (White et al., 2006), but this option is less attractive for food and pharmaceutical products.

3.1.1. Effect of HPU

The application of HPU had a significant ($p<0.05$) effect on the lethality of the SC-CO_2 treatments, the effect being greater at the lowest temperature (85ºC). In 20 min, the level of inactivation for the *B. subtilis* spores in SC-CO_2 was increased by 2.3 and 0.5 log-cycles by using HPU at 85 and 95ºC, respectively. In addition, the application of HPU in the 85ºC treatment changed the shape parameter of the curves from $n>1$ (concave downward) in the SC-CO_2 treatment to $n<1$ (concave upward) in the SC-CO_2 + HPU treatment (Table 1), as also observed in Fig. 1A. As in the SC-CO_2 treatments, the temperature had a significant ($p<0.05$) effect on the inactivation with SC-CO_2+HPU. For example, in a treatment time of 10 min, 3.7 log-cycles were reduced at 85ºC while at 95ºC there was a reduction of 6.3 log-cycles. The temperature in the SC-CO_2 + HPU inactivation kinetics also affected the $t_{6.8}$ (Table 1), since it was 26.5 min at 85ºC and 18.3 min at 95 ºC.

HPU produces the alternating compression and decompression of the medium along with the formation of cavitation bubbles. These bubbles give rise to a vigorous agitation of the medium and can collapse, causing high local temperatures and pressures, which produce, in turn, high located energy shear waves (Cárcel et al., 2012). The agitation and implosion of the bubbles reduce the resistance to mass and heat transfer processes (Contreras et al., 2018) and, in addition, could damage the structure of the spores, affecting its permeability (Mañas & Pagán, 2005; Palacios et al., 1991). The increase in spore permeability could lead both to a release of calcium dipicolinic acid (DPA) and other substances from the spore core and also to the rehydration of the structure, processes related with the loss of the extreme resistance of *bacterial* spores (Black et al., 2007; Palacios et al., 1991; Setlow, 2006). In fact, several authors observed that a HPU pretreatment enhanced the sensitivity of the spores in the subsequent thermal treatments (Ansari et al., 2017; Burgos et al., 1972; Evelyn & Silva, 2015; Ordoñez & Burgos, 1976; Palacios et al., 1991). Therefore, the application of HPU to the SC-CO_2 treatments was expected to enhance the solubilization and penetration of CO_2 and to accelerate the SC-CO_2 inactivation mechanisms. Numerous studies have already demonstrated the marked effect of HPU on SC-CO_2 treatments for vegetative microorganisms (Michelino et al., 2018; Ortuño et

al., 2013; Paniagua-Martínez et al., 2018). However, as regards bacterial spores treated with SC-CO_2 + HPU, only Michelino et al. (2018) studied the effect of the combined SC-CO_2 + HPU (40W) treatment on the inactivation of mesophilic bacterial spores in fresh coriander at 100 bar and two temperatures (40 and 50ºC). An inactivation of 1.6 log-cycles was obtained during the pressurization (20 min) and depressurization (40 min) stages with HPU, while no spore inactivation was obtained when HPU was not applied. In contrast to our results, no significant ($p>0.05$) effect of the temperature was found by these authors. The different influence of temperature on microbial inactivation between Michelino et al. (2018) and the present work, could be due to the differences in the temperature range, media, equipment and nature of bacterial spores.

As in the present study the *B. subtilis* spores were found to be very resistant to the SC-CO_2 + HPU treatment at high temperatures, and based on preliminary experiments, the remaining bacterial spores were only treated with HPU at 95ºC (the highest temperature the equipment can reach).

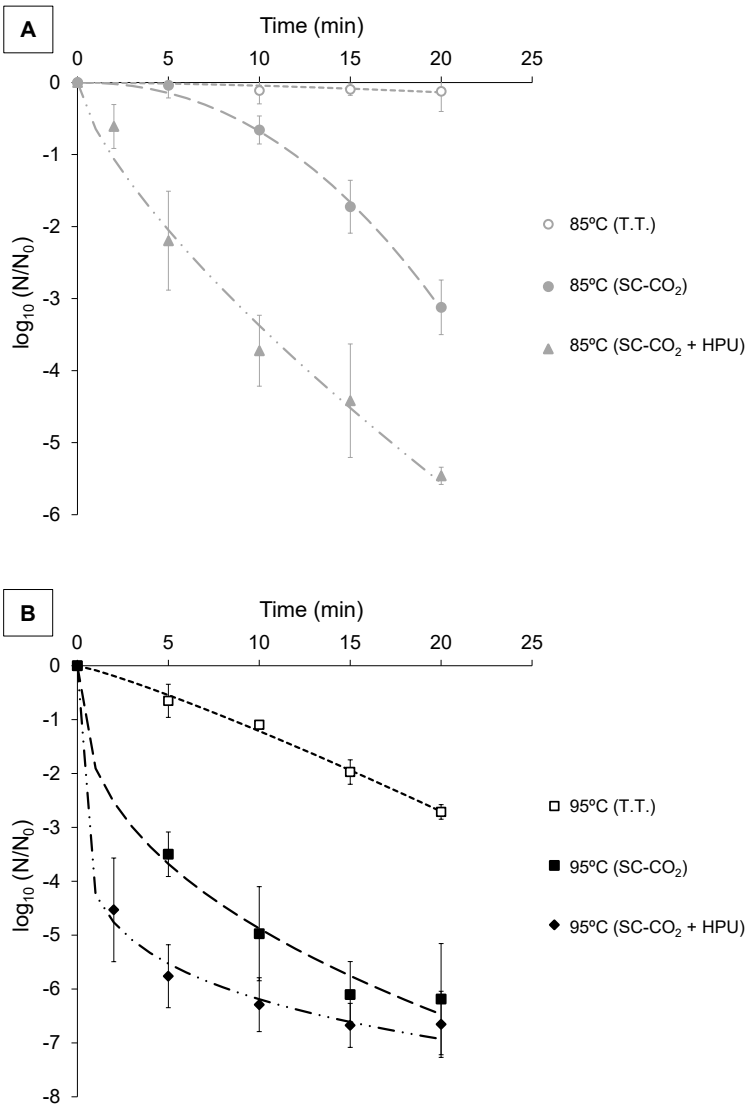


Fig 1. Inactivation kinetics of *B. subtilis* spores in oil-in-water emulsion: thermal (T.T.) at 85 (A) and 95ºC (B) and SC-CO_2 and SC-CO_2 + HPU treatments at 350 bar and 85 (A) and 95ºC (B). Experimental data (discrete points) and the Weibull model (dashed lines).

Table 1. Modelling of inactivation kinetics using the Weibull equation. Fitting parameters (b and n), time for complete inactivation ($t_{6.8}$; a reduction of 6.8 log-cycles for *B. subtilis*; $t_{8.1}$; 8.1 log-cycles for *B. pumilus* and $t_{5.3}$; 5.3 for *G. stearothermophilus*) and goodness of fit. Values in brackets indicate standard errors.

Spore	Treatment	Temperature (ºC)	Pressure (bar)	b (min^{-n})	n	$t_{6.8}$/ $t_{8.1}$/ $t_{5.3}$ (min)	R^2	RMSE
B. subtilis	Thermal	85	-	9.96E-04 (0.01)	1.64 (2.02)	217.4	0.33	0.06
B. subtilis	Thermal	95	-	0.09 (0.02)	1.16 (0.09)	41.5	0.99	0.07
B. subtilis	SC-CO_2	85	350	4.00E-3 (1.40E-3)	2.22 (0.11)	28.5	0.99	6.00E-02
B. subtilis	SC-CO_2	95	350	1.91 (0.30)	0.41 (0.06)	22.0	0.99	0.22
B. subtilis	SC-CO_2 + HPU	85	350	0.64 (0.14)	0.72 (0.08)	26.5	0.98	0.25
B. subtilis	SC-CO_2 + HPU	95	350	4.26 (0.22)	0.16 (0.02)	18.3	0.99	0.18
B. pumilus	Thermal	95	-	*	*	*	*	*
B. pumilus	SC-CO_2 + HPU	95	350	3.70 (0.45)	0.22(0.05)	35.8	0.99	0.22
G. stearothermophilus	Thermal	95	-	*	*	*	*	*
G. stearothermophilus	SC-CO_2 + HPU	95	350	*	*	*	*	*
G. stearothermophilus	SC-CO_2 + HPU	95	550	*	*	*	*	*

* Not enough experimental data for model fitting purposes

3.2. SC-CO_2 + HPU treatment of *B. pumilus* spores

The Weibull model satisfactorily described the SC-CO_2 + HPU inactivation kinetics, as shown in Fig. 2. The R^2 value was 0.99 and the RMSE value 0.22 (Table 1). The inactivation of the *B. pumilus* spores with SC-CO_2 + HPU was significantly ($p<0.05$) more efficient than the thermal treatment at the same temperature, since the inactivation level of the SC-CO_2 + HPU treatment in 20 min was 7.1 log-cycles while only 3.5 log-cycles were achieved in the thermal treatment. As in the SC-CO_2 + HPU treatments for *B. subtilis*, the shape parameter of Weibull (n) was also lower than 1, which is linked to concave upward curves, as observed in Fig. 2.

No previous data was found concerning the effects of SC-CO_2 + HPU on the inactivation of *B. pumilus* spores and scarce data is available on the effects of individual SC-CO_2 treatments. Zhang et al. (2006) studied the inactivation of *B. pumilus* spores in medical devices using SC-CO_2 and found a maximum reduction of around 3 log-cycles at 275 bar and 60-80ºC in 4 h, which was a very poor inactivation over an

extremely long time. These authors added H_2O_2 to intensify the SC-CO_2 treatment and complete inactivation was achieved at 60ºC, 275 bar and 4h.

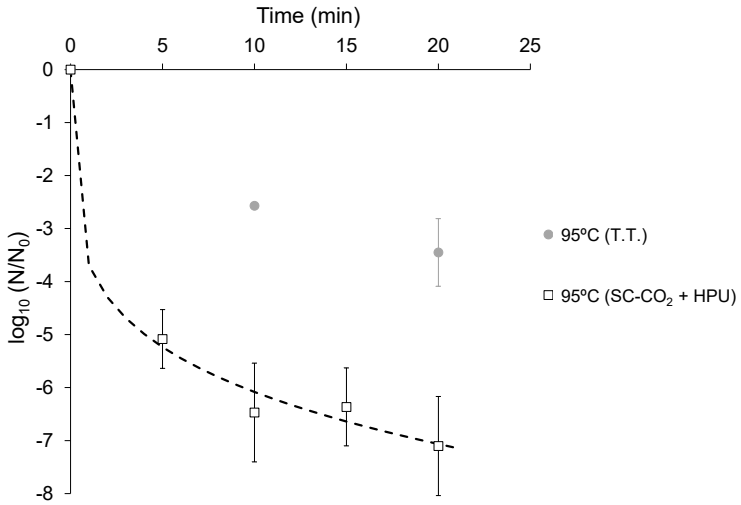


Fig. 2. Inactivation kinetics of *B. pumilus* spores in oil-in-water emulsion: thermal (T.T.) at 95ºC and a SC-CO_2 + HPU treatment at 350 bar and 95ºC. Experimental data (discrete points) and the Weibull model (dashed lines).

3.3. SC-CO_2 + HPU treatment of *G. stearothermophilus* spores

The 20 min thermal treatment at 95ºC did not achieve satisfactory inactivation levels for *G. stearothermophilus* (Fig. 3). In fact, the spore load slightly increased (1 log-cycles), which shows that 95ºC could be an appropriate growth temperature for *G. stearothermophilus* spores. The combined SC-CO_2 + HPU at 95ºC did not significantly ($p>0.05$) affect the *G. stearothermophilus* spore count and negligible reductions were achieved in 20 min (<0.5 log-cycles), regardless of the pressure. No references were found to the inactivation of *G. stearothermophilus* by SC-CO_2 + HPU. However, some authors studied the inactivation of the individual treatments. In this sense, Palacios et al. (1991) treated *G. stearothermophilus* in water with HPU (20 kHz,

120 W) at 12ºC and for 30 min and found no lethal effects; neither did Watanabe et al. (2003), who treated *G. stearothermophilus* spores using a conventional thermal treatment at 95ºC for 120 min and found no inactivation. However, although no inactivation in the SC-CO_2 treatment (300 bar and 120 min) was found at temperatures from 35 to 85ºC, the spores were reduced 5 log-cycles at 95ºC (Watanabe et al., 2003). Therefore, it seems that long processing times (100 min more than in our treatments) and high temperatures are required to obtain a significant effect of the SC-CO_2 treatment on the inactivation of *G. stearothermophilus* spores. However, processing times of the SC-CO_2 + HPU treatments of over 20 min do not seem feasible for industrial purposes. Therefore, from the obtained results, it could be concluded that the SC-CO_2 + HPU treatment was not adequate to inactivate *G. stearothermophilus* spores. For biomedical applications, the combination of SC-CO_2 with chemical additives can be a feasible approach for *G. stearothermophilus* spores inactivation (White et al., 2006).

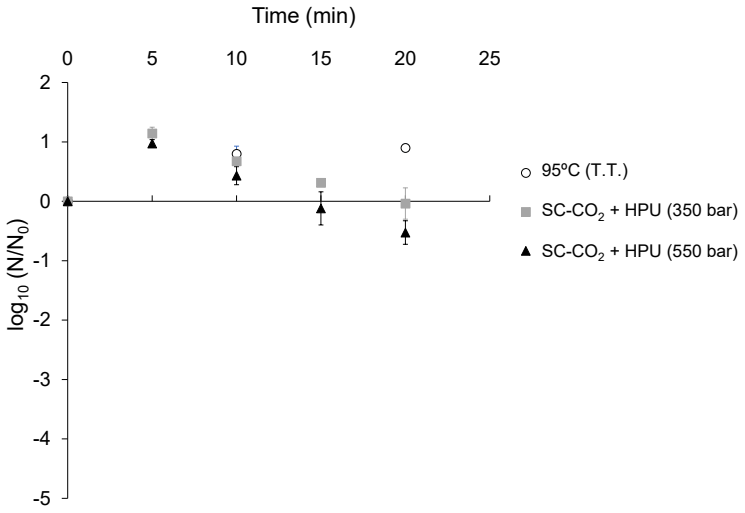


Fig. 3. Inactivation kinetics of *G. stearothermophilus* spores in oil-in-water emulsion: thermal (T.T.) at 95ºC and SC-CO_2 + HPU treatments at 350 and 550 bar and 95ºC. Experimental data (discrete points).

3.4. Effect of the type of spores on the SC-CO_2 + HPU treatment

Significant ($p<0.05$) differences were found as regards the resistance of the different bacterial spores considered in this study to both thermal and SC-CO_2 + HPU treatments at 95ºC. *G. stearothermophilus* spores were found to be the most resistant to inactivation treatments. However, no significant ($p>0.05$) differences were found between the resistance of *B. subtilis* and *B. pumilus* to thermal and SC-CO_2 + HPU treatments. Da Silva et al. (2016) also found that *G. stearothermophilus* spores had a greater resistance than *B. subtilis* treated with SC-CO_2 at 60ºC and 300 bar; this was to be expected since *G. stearothermophilus* is considered one of the most resistant microorganisms and is often used as a biological indicator of thermal processes, such as sterilization by steam under pressure (Periago et al., 1998).

3.5. Effect of the SC-CO_2 + HPU treatments on the physicochemical properties of the emulsions.

3.5.1. Appearance of the emulsions treated with SC-CO_2 + HPU

Most of the volume of the emulsions extracted from the vessel after the SC-CO_2 + HPU treatments presented no apparent signs of destabilization and, therefore, the appearance was similar to the original emulsions. However, when the last aliquot (2 mL) of the treated emulsions was extracted from the vessel, oil separation on the surface of the emulsions was visible under every condition, except for the 12.5 min treatment at 100 bar and 55ºC and the 5 min treatment at 100 bar and 75ºC (Runs 2 and 6, Table 2), where no destabilization signs were identified with the fluorescent lamp (Fig. 4). Thus, for a correct emulsion treatment, this fraction should be removed after the process. It is known that CO_2 is more soluble in lipids than in water or aqueous solutions (Bonnaillie & Tomasula, 2015). Therefore, CO_2 in supercritical conditions could probably be dissolved in the oil droplets of the emulsion during the treatment (Jakobsen et al., 2009). However, when the treated emulsions are extracted from the vessel, a severe depressurization occurs, and the supercritical CO_2 quickly becomes gaseous CO_2, which could break the oil-water interface leading to the coalescence of the droplets and the release of a small part of the oil (Ling et al., 2016). As regards

HPU, it is known that shear forces can reduce the size of the droplet to a critical shear stress when the coalescence of droplets may occur, a mechanism known as over-processing, which is dependent on the ultrasonic power and processing time (Kentish et al., 2008; Mahdi Jafari et al., 2006). In fact, although HPU has been demonstrated to be an efficient technique for emulsification purposes (Chemat et al., 2011), some authors applied HPU to enhance aggregation and to facilitate the separation of the oil and the aqueous phases in emulsions, such as milk (Juliano et al., 2011) or canola oil emulsions (Nii et al., 2009).

No previous literature has addressed the effect of the SC-CO_2 + HPU treatment on the quality of oil-in-water emulsions. However, there are a few studies assessing the effect of the individual treatments on the quality of food emulsions. In this sense, Watanabe et al. (2003) found that the 120 min SC-CO_2 treatment at 95ºC and 300 bar resulted in milk coagulation. In this case, SC-CO_2 could affect milk protein since the carbonic acid forms bonds with calcium ions, which may destabilize the casein micelles (Amaral et al., 2017). Moreover, the lower pH may change the ionic and electrostatic interactions within the casein micelles and the whey proteins (Bonnaillie & Tomasula, 2015). On the contrary, several studies have shown promising results in terms of the quality of vegetable emulsions after the application of HPU for microbial inactivation purposes. For example, rice milk beverages showed an increased cloud index, which indicates the stability of the particles dispersed in the suspension (Amador-Espejo et al., 2020); peanut milk showed an increase in the hydrolyzed protein content, a better sedimentation index and smaller particle and oil droplets, which could prevent phase separation (Salve et al., 2019); and hazelnut milks showed an increase in the content of total phenolic compounds, and improved antioxidant activity, appearance, syneresis, sedimentation, viscosity and consistency (Atalar et al., 2019).

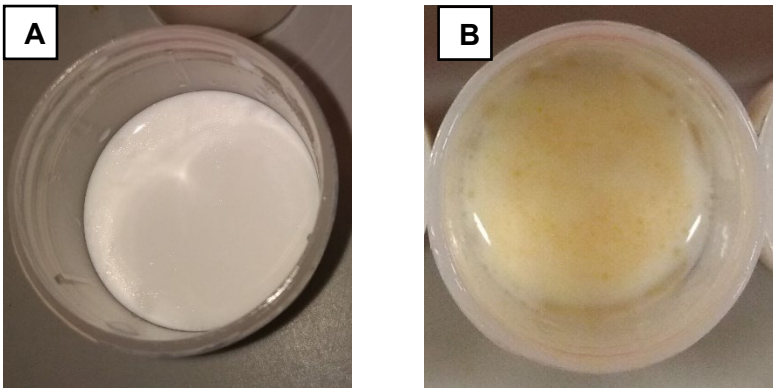


Fig. 4. Appearance of emulsions treated at 100 bar, 55ºC and for 2.5 min; run 2 (A, no destabilization) and at 350 bar, 55ºC and for 20 min; run 3 (B, oil separation).

3.5.2. pH, density, droplet size and ζ-potential of the emulsions treated with SC-CO_2 + HPU

The physicochemical measurements of the original untreated emulsion were: pH: 8.4 ± 0.4, density: 0.987 ± 0.001 g/cm^3, D[4,3]: 0.365 ± 0.021 μm, D[3,2]: 0.343 ± 0.014 μm and ζ-potential: -41.9 ± 4.7 mV. The physicochemical measurements of the treated emulsions are reported in Table 2. Response surface models were fitted to the studied variables (pH, density, D[4,3], D[3,2] and ζ-potential), but were only significant ($p<0.05$) for D[3,2]. The R^2 of the response surface models ranged from 0.71 for pH to 0.90 for D[3,2]. This poor fit shows the highly variable effect of the SC-CO_2 + HPU treatments on the physicochemical properties of the emulsions.

3.5.2.1. pH

As observed in Table 2, the pH was reduced after every SC-CO_2 + HPU treatment, being between 2.8 and 3.3 lower than the untreated emulsion. Moreover, the pH was not restored to its initial value after resting (4 h) for the purposes of degassing after the treatment. A pH decrease in the medium where the droplets are dispersed may reduce the repulsive forces between the droplets leading to aggregation, which could result in a loss of emulsion stability (Pertkiewicz et al., 2009).

However, in the present study, the pH was not significantly ($p>0.05$) related to the droplet size or the ζ-potential of the emulsions.

It is known that most of the CO_2 dissolved in water remains in the solvated form of CO_2 while a small fraction reacts with water to form carbonic acid (H_2CO_3). A portion of H_2CO_3 dissociates to H^+ and HCO_3^-, which can further dissociate to CO_3^{2-} and H^+, leading to a decrease in pH. Moreover, cavitation could enhance the decrease in pH since it is known that agitation increases the rate of CO_2 decomposition into carbonic acid (Bonnaillie & Tomasula, 2015). Martin et al. (2003) also observed a pH decrease (from 6.3 to 4.7-5.0) after injecting CO_2 into raw milk. On the contrary, Paniagua-Martínez et al., (2018) found no changes in the pH of orange juice processed with SC-CO_2 + HPU at 31-41ºC and 100 bar, compared to the untreated juice. These authors suggested it could be due to both the short process time (3.06 min) and the low initial pH of the juice (3.6-3.7), since, at low pH values, the dissociation of the carbonic acid formed by the dissolution of the CO_2 into the juice could be hindered (Zhou et al., 2009). Unlike Paniagua-Martinez et al. (2018), the time of the process in the present study was longer (from 5 to 20 min) and the emulsions presented an initial pH much higher (8.4). Consequently, the dissociation of carbonic acid could be facilitated, lowering the pH of the medium.

3.5.2.2. Density

As shown in Table 2, the changes found between the density of the untreated emulsion (0.987 g/cm^3) and the treated (0.985-1.007 g/cm^3) were minimal. Soybean oil and deionized water have different density values, the oil density being lower than that of the water. Therefore, a high density could be related to the oil separation in the emulsion.

3.5.2.3. Droplet size

D[4,3] increased, on average, from 0.365 (untreated) to 1.713 μm in the treated emulsions, except for the 20 min treatment at 600 bar and 75ºC (run 7) in which a lower

value was obtained (0.338 µm). D[3,2] also increased, on average, from 0.343 to 0.731 µm in the treated emulsions, except for the 20 min treatment at 600 bar and 75ºC (0.320 µm, run 7 Table 2) and the 12.5 min treatment at 600 bar and 95ºC (0.340 µm, run 13 Table 2). An emulsion containing small droplets improves its stability. In fact, emulsions with a droplet size of <0.5 µm are known to be highly stable (Anton et al., 2008). On the contrary, emulsions with large droplets usually have a strong tendency towards coalescence or Ostwald ripening (Azmi et al., 2019).

The only significant ($p<0.05$) model found to explain the effect of the process variables on the droplet size was for D[3,2] (R^2 0.90). In the Pareto Chart (Fig. 5), the decreasing order of importance of the process variables can be found. The pressure and its interaction with temperature were found to be significant ($p<0.05$) factors.

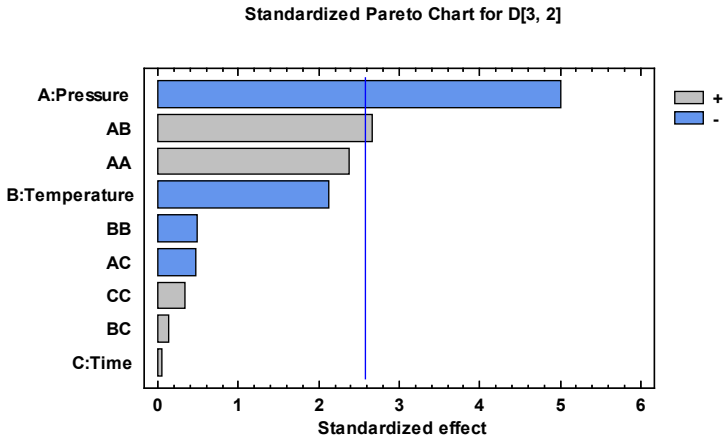


Fig. 5. Pareto chart of the standardized effects (bars) in decreasing order of importance for D[3,2]. Effects crossing the vertical line are statistically significant at the 95.0% confidence level (A: pressure, B: temperature and C: time).

3.5.2.4. ζ-potential

ζ-potential is the difference between the electrical potential of the static coat of the dispersion medium attached to the dispersed droplets and the mobile dispersion medium. Generally, absolute ζ-potential values higher than or equal to 30 mV indicates that the electrostatic repulsion among droplets contributes to preventing their aggregation (Ribes et al., 2017). However, low absolute ζ-potential values indicate that emulsions tend towards destabilization (Lu & Gao, 2010). In the present study, all of the treated emulsions showed stability (≤ -36.90 mV), except the emulsion treated at 100 bar, 95ºC for 12.5 min, in which a ζ-potential of -10.04 mV was obtained (Table 2). All of the ζ-potential values were negative due to the negative charge provided by the layer of phospholipids around the oil droplets (Pertkiewicz et al., 2009).

Table 2. Box-Behnken experimental design and physicochemical properties of the emulsions treated with SC-CO_2 + HPU.

Run	Pressure (bar)	Temperature (ºC)	Time (min)	pH	Density (g/cm^3)	D[4,3] (μm)	D[3,2] (μm)	ζ-potential (mV)
1	350	55	5	5.1	0.998	2.312	0.546	-49.47
2	100	55	12.5	5.3	0.985	3.420	2.018	-43.13
3	350	55	20	5.5	0.995	1.090	0.475	-36.90
4	600	55	12.5	5.2	1.007	0.856	0.426	-39.63
5	350	75	12.5	5.2	0.991	0.957	0.494	-42.47
6	100	75	5	5.2	0.991	2.386	1.397	-38.60
7	600	75	20	5.2	0.998	0.338	0.320	-40.03
8	600	75	5	5.3	0.995	0.645	0.397	-46.83
9	350	75	12.5	5.3	0.990	1.047	0.513	-41.43
10	350	75	12.5	5.2	0.989	1.334	0.586	-54.97
11	100	75	20	5.1	1.001	7.996	1.505	-42.50
12	350	95	20	5.3	0.991	0.998	0.505	-44.70
13	600	95	12.5	5.5	0.997	0.430	0.340	-51.43
14	350	95	5	5.4	0.992	0.949	0.503	-48.90
15	100	95	12.5	5.6	1.004	0.911	0.441	-10.04

4. Conclusions

The inactivation of bacterial spores is highly complex. The higher the temperature of the treatments (thermal, SC-CO_2 and SC-CO_2 + HPU), the greater the inactivation level of *B. subtilis* spores. The SC-CO_2 treatments were more effective at inactivating *B. subtilis* spores than the thermal treatments. Compared to the thermal treatments, the degree of inactivation of the *B. subtilis* and *B. pumilus* spores achieved by the combined SC-CO_2 + HPU treatment was 2.5 times greater and twice as great, respectively. No differences were found between the resistances of *B. subtilis* and *B. pumilus* spores to treatments. On the contrary, the SC-CO_2 + HPU treatment did not significantly affect the inactivation level obtained for *G. stearothermophilus* spores, compared to the thermal treatment at 95ºC and nor did the use of higher pressures increase the effectiveness of the treatment.

A decrease in the pH and an increase in the droplet size of the emulsions was caused by the SC-CO_2 + HPU treatment. Nevertheless, no changes in density or ζ-potential were documented. In general terms, for the SC-CO_2 + HPU inactivation treatment, only a mild effect of the process variables (temperature, pressure and time) was found on quality.

Suitable conditions of pressure, temperature and time permitted the quality and stability of the treated emulsions to be maintained while achieving satisfactory inactivation levels for two of the three bacterial spores studied (*B. subtilis* and *B. pumilus*). In view of a possible industrial application of the SC-CO_2 + HPU technology, suitable process conditions should be sought bearing in mind the target microorganisms and the expected droplet size.

Acknowledgements

The authors acknowledge the financial support from Fresenius Kabi.

References

Amador-Espejo, G. G., Chávez-Ocegueda, J., Cruz-Cansino, N., Suárez-Jacobo, A., Gutiérrez-Martínez, P., Valencia-Flores, D., & Velázquez Estrada, R. (2020). Thermosonication parameter effects on physicochemical changes, microbial and enzymatic inactivation of fruit smoothie. *Journal of Food Science and Technology*, *57*(5), 1680–1688. https://doi.org/10.1007/s13197-019-04201-x

Amaral, G. V., Silva, E. K., Cavalcanti, R. N., Cappato, L. P., Guimaraes, J. T., Alvarenga, V. O., Esmerino, E. A., Portela, J. B., Sant' Ana, A. S., Freitas, M. Q., Silva, M. C., Raices, R. S. L., Meireles, M. A. A., & Cruz, A. G. (2017). Dairy processing using supercritical carbon dioxide technology: Theoretical fundamentals, quality and safety aspects. *Trends in Food Science and Technology*, *64*, 94–101. https://doi.org/10.1016/j.tifs.2017.04.004

Ansari, J. A., Ismail, M., & Farid, M. (2017). Investigation of the use of ultrasonication followed by heat for spore inactivation. *Food and Bioproducts Processing*, *104*, 32–39. https://doi.org/10.1016/j.fbp.2017.04.005

Anton, N., Benoit, J.-P., & Saulnier, P. (2008). Design and production of nanoparticles formulated from nano-emulsion templates—A review. *Journal of Controlled Release*, *128*(3), 185–199. https://doi.org/10.1016/J.JCONREL.2008.02.007

Atalar, I., Gul, O., Saricaoglu, F. T., Besir, A., Gul, L. B., & Yazici, F. (2019). Influence of thermosonication (TS) process on the quality parameters of high pressure homogenized hazelnut milk from hazelnut oil by-products. *Journal of Food Science and Technology*, *56*(3), 1405–1415. https://doi.org/10.1007/s13197-019-03619-7

Ávila, M., Gómez-Torres, N., Hernández, M., & Garde, S. (2014). Inhibitory activity of reuterin, nisin, lysozyme and nitrite against vegetative cells and spores of dairy-related Clostridium species. *International Journal of Food Microbiology*, *172*, 70–75. https://doi.org/10.1016/j.ijfoodmicro.2013.12.002

Azmi, N. A. N., Elgharbawy, A. A. M., Motlagh, S. R., Samsudin, N., & Salleh, H. M. (2019). Nanoemulsions: Factory for food, pharmaceutical and cosmetics.

Processes, *7*(9). https://doi.org/10.3390/pr7090617

Bae, Y. Y., Lee, H. J., Kim, S. A., & Rhee, M. S. (2009). Inactivation of Alicyclobacillus acidoterrestris spores in apple juice by supercritical carbon dioxide. *International Journal of Food Microbiology*, *136*(1), 95–100. https://doi.org/10.1016/j.ijfoodmicro.2009.09.015

Ballestra, P., & Cuq, J. L. (1998). Influence of pressurized carbon dioxide on the thermal inactivation of bacterial and fungal spores. *LWT - Food Science and Technology*, *31*(1), 84–88. https://doi.org/10.1006/fstl.1997.0299

Benedito, J., Martínez-Pastor, M. T., Mulet, A., Ortuño, C., & Peña, R. (2011). Procedure of inactivation microorganisms by combination of supercritical fluids and ultrasound. *Spain. Patent*, *P201131099*.

Bermúdez-Aguirre, D., & Barbosa-Cánovas, G. V. (2011). An update on high hydrostatic pressure, from the laboratory to industrial applications. *Food Engineering Reviews*, *3*(1), 44–61. https://doi.org/10.1007/s12393-010-9030-4

Black, E. P., Setlow, P., Hocking, A. D., Stewart, C. M., Kelly, A. L., & Hoover, D. G. (2007). Response of spores to high-pressure processing. In *Comprehensive reviews in food science and food safety: Vol. 6 (4)* (pp. 103–119).

Bonnaillie, L. M., & Tomasula, P. M. (2015). Carbon dioxide: An alternative processing method for milk. *Emerging Dairy Processing Technologies: Opportunities for the Dairy Industry*, 205–250. https://doi.org/10.1002/9781118560471.ch8

Burgos, J., Ordoñez, J. A., & Sala, F. (1972). Effect of ultrasonic waves on the heart resistance of Bacillus cereus and Bacillus licheniformis spores. *Applied and Environmental Microbiology*, *24*(3), 497–498. https://doi.org/10.1128/aem.32.1.183-184.1976

Cárcel, J. A., García-Pérez, J. V., Benedito, J., & Mulet, A. (2012). Food process innovation through new technologies: Use of ultrasound. *Journal of Food Engineering*, *110*(2), 200–207. https://doi.org/10.1016/J.JFOODENG.2011.05.038

Chemat, F., Zill-E-Huma, & Khan, M. K. (2011). Applications of ultrasound in food

technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry*, *18*(4), 813–835. https://doi.org/10.1016/j.ultsonch.2010.11.023

Contreras, M., Benedito, J., Bon, J., & Garcia-Perez, J. V. (2018). Intensification of heat transfer during mild thermal treatment of dry-cured ham by using airborne ultrasound. *Ultrasonics Sonochemistry*, *41*(June 2017), 206–212. https://doi.org/10.1016/j.ultsonch.2017.09.019

da Silva, M. A., de Araujo, A. P., de Souza Ferreira, J., & Kieckbusch, T. G. (2016). Inactivation of Bacillus subtilis and Geobacillus stearothermophilus inoculated over metal surfaces using supercritical CO2 process and nisin. *The Journal of Supercritical Fluids*, *109*, 87–94. https://doi.org/10.1016/J.SUPFLU.2015.11.013

Dong, P., Georget, E., Aganovic, K., Heinz, V., & Mathys, A. (2016). Inactivation of Bacillus amyloliquefaciens spores by continuous high-pressure-assisted thermal sterilization in an oil-in-water (o/w) emulsion with 10 % soybean oil. *European Food Research and Technology*, *242*(6), 935–942. https://doi.org/10.1007/s00217-015-2600-1

Evelyn, & Silva, F. V. M. (2015). Use of power ultrasound to enhance the thermal inactivation of Clostridium perfringens spores in beef slurry. *International Journal of Food Microbiology*, *206*, 17–23. https://doi.org/10.1016/J.IJFOODMICRO.2015.04.013

Fan, L., Hou, F., Muhammad, A. I., Ruiling, L. V., Watharkar, R. B., Guo, M., Ding, T., & Liu, D. (2019). Synergistic inactivation and mechanism of thermal and ultrasound treatments against Bacillus subtilis spores. *Food Research International*, *116*(September 2018), 1094–1102. https://doi.org/10.1016/j.foodres.2018.09.052

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2020). Non-thermal pasteurization of lipid emulsions by combined supercritical carbon dioxide and high-power ultrasound treatment. *Ultrasonics Sonochemistry*, *67*, 105–138. https://doi.org/10.1016/J.ULTSONCH.2020.105138

Han, L. L., Shao, H. H., Liu, Y. C., Liu, G., Xie, C. Y., Cheng, X. J., Wang, H. Y., Tan, X. M., & Feng, H. (2017). Transcriptome profiling analysis reveals metabolic changes across various growth phases in Bacillus pumilus BA06. *BMC Microbiology*, *17*(1), 1–13. https://doi.org/10.1186/s12866-017-1066-7

Hetzer, A., Daughney, C. J., & Morgan, H. W. (2006). Cadmium ion biosorption by the thermophilic bacteria Geobacillus stearothermophilus and G. thermocatenulatus. *Applied and Environmental Microbiology*, *72*(6), 4020–4027. https://doi.org/10.1128/AEM.00295-06

Ishihara, Y., Saito, H., & Takano, J. (1999). Differences in the surface membranes and water content between the vegetative cells and spores of Bacillus subtilis. *Cell Biochemistry and Function*, *17*(1), 9–13. https://doi.org/10.1002/(SICI)1099-0844(199903)17:1<9::AID-CBF803>3.0.CO;2-9

Ishikawa, H., Shimoda, M., Tamaya, K., Yonekura, A., Kawano, T., & Osajima, Y. (1997). Inactivation of Bacillus spores by the supercritical carbon dioxide micro-bubble method. *Bioscience, Biotechnology and Biochemistry*, *61*(6), 1022–1023. https://doi.org/10.1271/bbb.61.1022

Jakobsen, M., Jensen, P. N., & Risbo, J. (2009). Assessment of carbon dioxide solubility coefficients for semihard cheeses: The effect of temperature and fat content. *European Food Research and Technology*, *229*(2), 287–294. https://doi.org/10.1007/s00217-009-1059-3

Jiao, S., Zhang, H., Hu, S., & Zhao, Y. (2019). Radio frequency inactivation kinetics of Bacillus cereus spores in red pepper powder with different initial water activity. *Food Control*, *105*, 174–179. https://doi.org/10.1016/J.FOODCONT.2019.05.038

Juliano, P., Kutter, A., Cheng, L. J., Swiergon, P., Mawson, R., & Augustin, M. A. (2011). Enhanced creaming of milk fat globules in milk emulsions by the application of ultrasound and detection by means of optical methods. *Ultrasonics Sonochemistry*, *18*(5), 963–973. https://doi.org/10.1016/j.ultsonch.2011.03.003

Kentish, S., Wooster, T. J., Ashokkumar, M., Balachandran, S., Mawson, R., & Simons, L. (2008). The use of ultrasonics for nanoemulsion preparation. *Innovative Food*

Science and Emerging Technologies, *9*(2), 170–175. https://doi.org/10.1016/j.ifset.2007.07.005

Lee, A. J., Byun, B. Y., Kang, D. H., Tang, J., Kim, Y. W., Hwang, H. J., & Mah, J. H. (2011). The ability of zinc to inhibit the sporulation and viability of Clostridium sporogenes and growth of other bacteria. *International Journal of Food Science and Technology*, *46*(7), 1494–1501. https://doi.org/10.1111/j.1365-2621.2011.02644.x

Lewis, S. J., Velasquez, A., Cuppett, S. L., & McKee. (2002). Effect of electron beam irradiation on poultry meat safety and quality. *Poultry Science*, *81*(6), 896–903. https://doi.org/https://doi.org/10.1093/ps/81.6.896

Ling, N. N. A., Haber, A., Hughes, T. J., Graham, B. F., May, E. F., Fridjonsson, E. O., & Johns, M. L. (2016). NMR studies of the effect of CO2 on oilfield emulsion stability. *Energy and Fuels*, *30*(7), 5555–5562. https://doi.org/10.1021/acs.energyfuels.6b00782

Lu, G. W., & Gao, P. (2010). Emulsions and microemulsions for topical and transdermal drug delivery. In *Handbook of Non-Invasive Drug Delivery Systems* (pp. 59–94). https://doi.org/10.1016/b978-0-8155-2025-2.10003-4

Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, *72*(1–2), 107–113. https://doi.org/10.1016/S0168-1605(01)00624-9

Mahdi Jafari, S., He, Y., & Bhandari, B. (2006). Nano-emulsion production by sonication and microfluidization - A comparison. *International Journal of Food Properties*, *9*(3), 475–485. https://doi.org/10.1080/10942910600596464

Mañas, P., & Pagán, R. (2005). Microbial inactivation by new technologies of food preservation. *Journal of Applied Microbiology*, *98*(6), 1387–1399. https://doi.org/10.1111/j.1365-2672.2005.02561.x

Martin, J. D., Werner, B. G., & Hotchkiss, J. H. (2003). Effects of carbon dioxide on bacterial growth parameters in milk as measured by conductivity. *Journal of Dairy*

Science, *86*(6), 1932–1940. https://doi.org/10.3168/jds.S0022-0302(03)73780-1

McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition*, *47*(7), 611–649. https://doi.org/10.1080/10408390701289292

McClements, D. J. (2015). *Food emulsions. Principles, practices and techniques*. CRC press.

Michalac, S., Alvarez, V., Ji, T., & Zhang, Q. H. (2003). Inactivation of selected microorganisms and properties of pulsed electric field processed milk. *Journal of Food Processing and Preservation*, *27*(2), 137–151. https://doi.org/10.1111/j.1745-4549.2003.tb00507.x

Michelino, F., Zambon, A., Vizzotto, M. T., Cozzi, S., & Spilimbergo, S. (2018). High power ultrasound combined with supercritical carbon dioxide for the drying and microbial inactivation of coriander. *Journal of CO2 Utilization*, *24*, 516–521. https://doi.org/10.1016/J.JCOU.2018.02.010

Mondal, D., Bhowmick, B., Maity, D., Mollick, M. M. R., Rana, D., Rangarajan, V., Sen, R., & Chattopadhyay, D. (2015). Investigation on sodium benzoate release from poly(butylene adipate-co-terephthalate)/organoclay/sodium benzoate based nanocomposite film and their antimicrobial activity. *Journal of Food Science*, *80*(3), E602–E609. https://doi.org/10.1111/1750-3841.12745

Nii, S., Kikumoto, S., & Tokuyama, H. (2009). Quantitative approach to ultrasonic emulsion separation. *Ultrasonics Sonochemistry*, *16*(1), 145–149. https://doi.org/10.1016/j.ultsonch.2008.07.005

Ordoñez, J. A., & Burgos, J. (1976). Effect of ultrasonic waves on the heat resistance of bacillus spores. *Journal of the American Oil Chemists Society*, *32*(1), 183–184. https://doi.org/10.1007/BF02643337

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012). Supercritical carbon dioxide inactivation of Escherichia coli and Saccharomyces cerevisiae in different growth stages. *The Journal of Supercritical Fluids*, *63*, 8–15. https://doi.org/10.1016/J.SUPFLU.2011.12.022

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2013). Application of high power ultrasound in the supercritical carbon dioxide inactivation of Saccharomyces cerevisiae. *Food Research International*, *51*(2), 474–481. https://doi.org/10.1016/J.FOODRES.2013.01.041

Palacios, P., Burgos, J., Hoz, L., Sanz, B., & Ordóñez, J. A. (1991). Study of substances released by ultrasonic treatment from Bacillus stearothermophilus spores. *Journal of Applied Bacteriology*, *71*(5), 445–451. https://doi.org/10.1111/j.1365-2672.1991.tb03815.x

Paniagua-Martínez, I., Mulet, A., García-Alvarado, M. A., & Benedito, J. (2018). Orange juice processing using a continuous flow ultrasound-assisted supercritical CO2 system: Microbiota inactivation and product quality. *Innovative Food Science & Emerging Technologies*, *47*, 362–370. https://doi.org/10.1016/J.IFSET.2018.03.024

Peleg, M. (2006). *Models for predicting growth and inactivation. Advanced quantitative microbiology for foods and biosystems*.

Periago, P. M., Fernández, P. S., Ocio, M. J., & Martinez, A. (1998). Apparent thermal resistance of Bacillus stearothermophilus spores recovered under anaerobic conditions. *European Food Research and Technology*, *206*(1), 63–67. https://doi.org/10.1007/s002170050215

Perrut, M. (2012). Sterilization and virus inactivation by supercritical fluids (a review). *The Journal of Supercritical Fluids*, *66*, 359–371. https://doi.org/10.1016/J.SUPFLU.2011.07.007

Pertkiewicz, M., Cosslett, A., Mühlebach, S., & Dudrick, S. J. (2009). Basics in clinical nutrition: Stability of parenteral nutrition admixtures. *E-SPEN*, *4*(3), 117–119. https://doi.org/10.1016/j.eclnm.2009.01.010

Rao, L., Xu, Z., Wang, Y., Zhao, F., Hu, X., & Liao, X. (2015). Inactivation of Bacillus subtilis spores by high pressure CO2 with high temperature. *International Journal of Food Microbiology*, *205*, 73–80. https://doi.org/10.1016/J.IJFOODMICRO.2015.04.012

Rao, L., Zhao, F., Wang, Y., Chen, F., Hu, X., & Liao, X. (2016). Investigating the inactivation mechanism of Bacillus subtilis spores by high pressure CO2. *Frontiers in Microbiology*, *7*(SEP), 1–12. https://doi.org/10.3389/fmicb.2016.01411

Ribeiro, N., Soares, G. C., Santos-Rosales, V., Concheiro, A., Alvarez-Lorenzo, C., García-González, C. A., & Oliveira, A. L. (2020). A new era for sterilization based on supercritical CO2 technology. *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, *108*(2), 399–428. https://doi.org/10.1002/jbm.b.34398

Ribes, S., Fuentes, A., Talens, P., Barat, J. M., Ferrari, G., & Donsì, F. (2017). Influence of emulsifier type on the antifungal activity of cinnamon leaf, lemon and bergamot oil nanoemulsions against Aspergillus niger. *Food Control*, *73*, 784–795. https://doi.org/10.1016/j.foodcont.2016.09.044

Salve, A. R., Pegu, K., & Arya, S. S. (2019). Comparative assessment of high-intensity ultrasound and hydrodynamic cavitation processing on physico-chemical properties and microbial inactivation of peanut milk. *Ultrasonics Sonochemistry*, *59*(August), 104728. https://doi.org/10.1016/j.ultsonch.2019.104728

Setlow, P. (2006). Spores of Bacillus subtilis: Their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, *101*(3), 514–525. https://doi.org/10.1111/j.1365-2672.2005.02736.x

Soares, G. C., Learmonth, D. A., Vallejo, M. C., Davila, S. P., González, P., Sousa, R. A., & Oliveira, A. L. (2019). Supercritical CO2 technology: The next standard sterilization technique? *Materials Science and Engineering: C*, *99*, 520–540. https://doi.org/10.1016/J.MSEC.2019.01.121

Spicher, G. (1988). Biological indicators and monitoring systems for validation and cycle control of sterilization processes. *Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene - Abt. 1 Orig. A*, *267*(4), 463–484. https://doi.org/10.1016/S0176-6724(88)80029-4

Spilimbergo, S., & Bertucco, A. (2003). Non-thermal bacteria inactivation with dense CO2. *Biotechnology and Bioengineering*, *84*(6), 627–638.

https://doi.org/10.1002/bit.10783

Spilimbergo, S., Bertucco, A., Lauro, F. M., & Bertoloni, G. (2003). Inactivation of Bacillus subtilis spores by supercritical CO2 treatment. *Innovative Food Science and Emerging Technologies*, *4*(2), 161–165. https://doi.org/10.1016/S1466-8564(02)00089-9

Watanabe, T., Furukawa, S., Hirata, J., Koyama, T., Orihara, H., & Yamasaki, M. (2003). Inactivation of Geobacillus stearothermophilus spores by high-pressure carbon dioxide treatment. *Cambridge University Press*, *69*(12), 7124–7129. https://doi.org/10.1128/AEM.69.12.7124

White, A., Burns, D., & Christensen, T. W. (2006). Effective terminal sterilization using supercritical carbon dioxide. *Journal of Biotechnology*, *123*(4), 504–515. https://doi.org/10.1016/j.jbiotec.2005.12.033

Zhang, J., Burrows, S., Gleason, C., Matthews, M. A., Drews, M. J., LaBerge, M., & An, Y. H. (2006). Sterilizing Bacillus pumilus spores using supercritical carbon dioxide. *Journal of Microbiological Methods*, *66*(3), 479–485. https://doi.org/10.1016/j.mimet.2006.01.012

Zhang, Y., Moeller, R., Tran, S., Dubovcova, B., Akepsimaidis, G., Meneses, N., Drissner, D., & Mathys, A. (2018). Geobacillus and Bacillus spore inactivation by low energy electron beam technology: Resistance and influencing factors. *Frontiers in Microbiology*, *9*(2720). https://doi.org/10.3389/fmicb.2018.02720

Zhou, L., Wang, Y., Hu, X., Wu, J., & Liao, X. (2009). Effect of high pressure carbon dioxide on the quality of carrot juice. *Innovative Food Science and Emerging Technologies*, *10*(3), 321–327. https://doi.org/10.1016/j.ifset.2009.01.002

CHAPTER 2

PEF and HPU microbial inactivation

Food Control 130 (2021) 108-348

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

Angela Gomez-Gomez[1], Edmundo Brito-de la Fuente[2], Críspulo Gallegos[2], Jose Vicente Garcia-Perez[1], Jose Benedito[1]

[1]Grupo ASPA, Departamento de Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, València E46022, Spain

[2]Fresenius-Kabi Deutschland GmbH, Product and Process Engineering Center, Pharmaceuticals & Device Division, Bad Homburg, German

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

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 25ºC). 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.

Keywords: fungal spores, bacterial spores, pulsed electric fields, high-power ultrasound, emulsions.

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 et al., 2008), high power ultrasound (Piyasena et al., 2003), high pressure carbon dioxide (Ortuño et al., 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 et al., 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 et al., 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 et al., 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 et al., 2018), buffer solutions (Pataro et al., 2010), fruit juices (Hodgins et al., 2002; Yeom et al., 2000; Yildiz et al., 2019, 2020) or emulsions, such as milk (Michalac et al., 2003; Odriozola-Serrano et al., 2006).

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 et al., 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 et al., 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 et al., 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 et al., 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 et

al., 2006; Lyu et al., 2016; Palgan et al., 2012; Walkling-Ribeiro, Noci, Cronin, et al., 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 50μs) and HPU (400W, 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 et al., 2020). Despite that, few studies were found into microbial inactivation in vegetable emulsions using PEF or HPU. Only Dunn (1996), Barsotti et al. (2001) and Markus Walkling-Ribeiro et al. (2010) stated that emulsions (salad dressings, peanut oil emulsions and coconut milk based smoothies, respectively) could be 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 et al., 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).

Table 1. Pulsed electric field (PEF) and high power ultrasound (HPU) inactivation treatments and its combination on different microorganisms and media.

Treatment	Conditions	Microorganism	Medium	Microbial reduction	Reference
PEF	0-30 kV/cm, 0.5-2.5 μs, 50ºC (Exponential waves).	*Escherichia coli*	Nutrient broth	0.2 log-cycles	(Yan et al., 2021)
PEF	45 kV/cm, 1 μs, 100 pulses, 30ºC	*Aspergillus niger* spores	Collagen gels	2.0 log-cycles	(Griffiths et al., 2012)
PEF	7.5 kV/cm, 5 μS, 1kHz, 1000 pulses / 7.5 kV/cm, 5 μS, 1kHz, 10000 pulses.	*Bacillus pumilus* spores	NaCl solution	Negligible / 67± 8%	(Pillet et al., 2016)
PEF	30 kV/cm, 1 μs width, 15 Hz, 150 μs, <56ºC / 40 kV/cm, 1 μs width, 15 Hz, 150 μs, <56ºC	*Staphylococcus aureus*	Orange juice	3.0 log-cycles / 5.5 log-cycles	(Walkling-Ribeiro et al., 2009)
PEF	80 mL/min,1 kHz, 20 kV/ cm, 600 μs, 40ºC.	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, 20ºC	*Escherichia coli*	Orange juice	~0.4- 1.3 log-cycles	(Kernou et al., 2021)
HPU	20 kHz, 120 μm, 3 min, 52.5ºC	*Aspergillus flavus* spores	Broth	~0.4 log-cycles	(López-Malo et al., 2005)
HPU	20 kHz, 5 bar,117 μm, 12 min, 70°C	*Bacillus subtilis* spores	Distilled water	>99%	(Raso et al., 1998)
HPU	30 kHz, 5, 10, and 20 min, 55ºC	*Staphylococcus aureus*	Orange juice	0.8, 1.8, and 3.3 log-cycles	(Walkling-Ribeiro et al., 2009)
HPU	600 W, 28 kHz, 30 min, 20ºC.	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, 55ºC PEF: 40 kV/cm, 1 μs width, 15 Hz, 150 μs, <56ºC	*Staphylococcus aureus*	Orange juice	6.8 log-cycles	(Walkling-Ribeiro 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, <52ºC. PEF: 160 mL/min, 34 kV/cm, 32 μs, <35ºC	*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, 40ºC. HPU: 600 W, 28 kHz, 30 min, 20ºC.	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, <35ºC HPU: 100%, 20 kHz, 160 mL/min, 40 W/cm^2 ,200 kPa, <52ºC	*Listeria innocua*	Milk-based beverage	4.2 log-cycles	(Palgan et al., 2012)

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 14000 rpm for 2 min, 10200 rpm for 4 min and 10600 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 14000 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 29T, 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 et al., 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 et al., 2007; Iurlina et al., 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 37ºC (3000957, J.P. Selecta, Spain) at 120 rpm (Rotabit Model 3000974, J.P. Selecta, Spain). Then 50 µL of the starter culture were transferred to 50 mL of nutrient both medium and incubated (for 14h) until reaching the stationary phase; this period of time was established from the growth curves by Gomez-Gomez et al. (2020).

A. niger was cultured on Potato Dextrose Agar (Scharlab, Spain) at 25ºC 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 4ºC until use.

B. pumilus cells were sporulated following the methodology of Mafart et al. (2002), with modifications. A single colony of the bacteria was grown in nutrient broth (Scharlab, Spain) at 30ºC until the stationary phase was reached, according to bibliography (around 24 h) (Han et al., 2017; Liu et al., 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 30ºC 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 (8000x 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 4ºC 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.

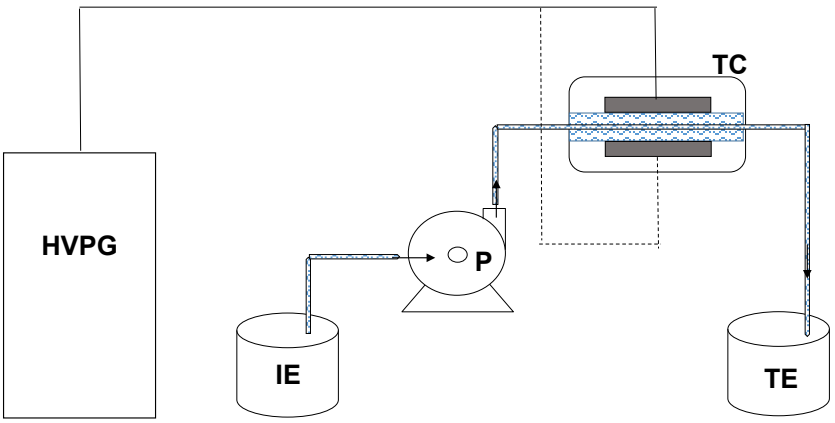


Fig. 1. Schematic diagram of the PEF system. HVPG: high voltage pulse generator. P: pump, TC: treatment chamber, IE: inoculated emulsion, TE: treated emulsion.

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 40ºC for *E. coli* and *A. niger* to reach a final temperature lower than that for thermal inactivation (known for each microorganism though the thermal treatment kinetics); and at 85ºC 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

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 25ºC).

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.

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 10000 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 60ºC for *E. coli* and *A. niger;* and at 85, 90ºC and 95ºC 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 37ºC for 24 h, 30ºC for 24 h and 25ºC 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 (25ºC). 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 25ºC).

The application of an electric field of 30 kV/cm increased the 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 et al., (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 et al. (2014) when treating *E. coli* in buffer solution at 40 kJ/L and an input temperature of 22ºC, compared to an inactivation of 1.2 log-cycles at 41.5 kJ/kg and 25ºC 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 25ºC. 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 40ºC compared to 1.2 log-cycles at 30ºC. The maximum temperature reached in the present study during the PEF treatment was 50ºC (30 kV/cm, 170 µs and an input temperature of 25ºC), 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.

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 15ºC of inlet temperature (log-cycles)	Microbial reduction at 25ºC 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.

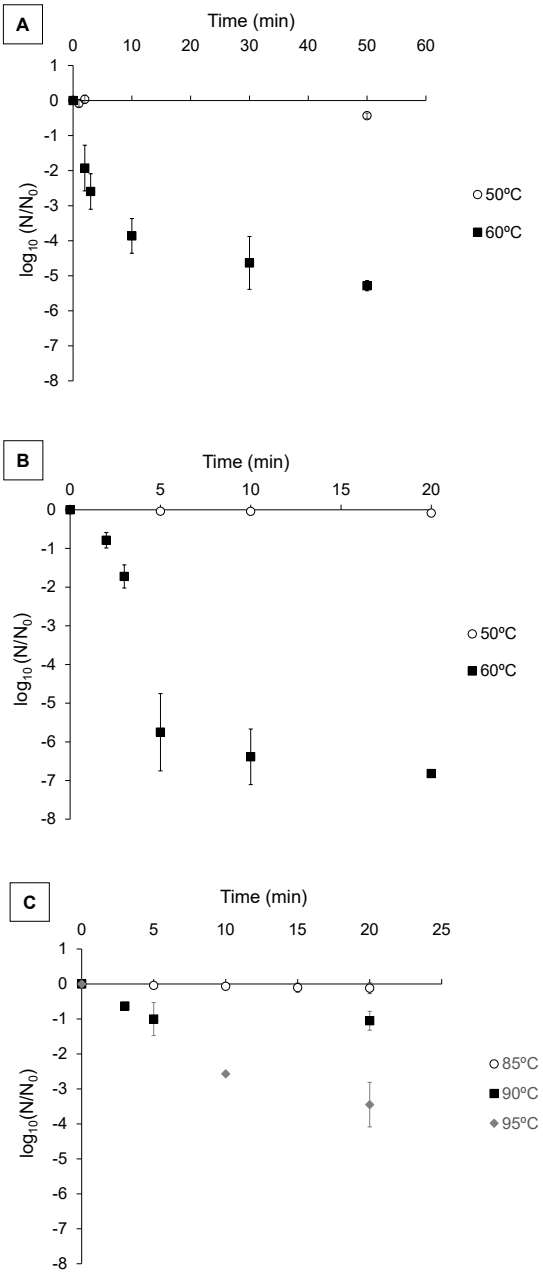


Fig. 2. Inactivation kinetics for the thermal treatment of *E. coli* (**A**), *A. niger* spores **(B)** and *B. pumilus* spores (**C)** in oil-in-water emulsion.

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 25ºC). 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 60ºC, respectively. The level of inactivation achieved by the HPU2 treatment was equal to the level reached in the conventional thermal treatment at 60ºC (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 extended to 3 min, a greater degree of inactivation was reached than in the thermal treatment at 60ºC: 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 synergistic effect between ultrasound cavitation and heat. Ince & 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, 170μs and 25ºC 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 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 et al., 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.

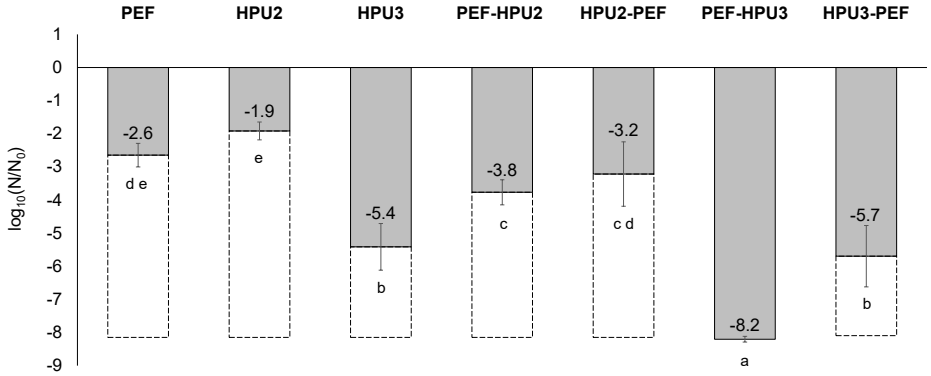


Fig. 3. Inactivation of *E. coli* in oil-in-water emulsion treated by PEF at 30 kV/cm, 170 μs of treatment time, 5 μs of pulse width, 50 Hz and 25ºC of inlet temperature (176.3 kJ/kg), HPU for 2 (HPU2) and 3 min (HPU3) and its combination. Dashed lines indicate complete inactivation. Letters show homogeneous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

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 5μs and an inlet temperature of 25ºC (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 60ºC (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 et al., 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.

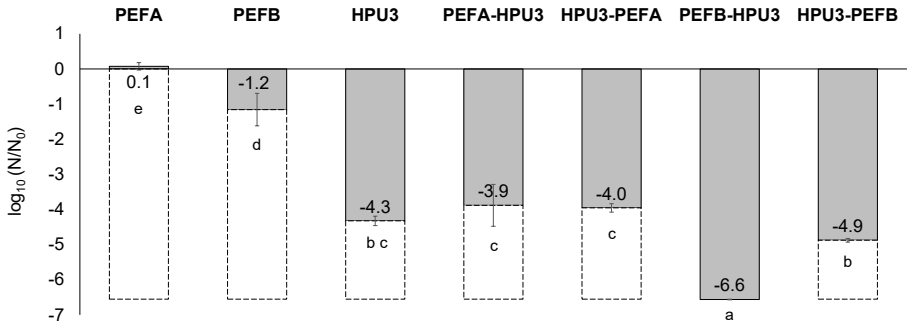


Fig. 4. Inactivation of *A. niger* spores in oil-in-water emulsion treated by PEF at 50Hz and 25ºC of inlet temperature (PEFA: 30 kV/cm, 170 µs of treatment time, 5 µs of pulse width and 76.3 kJ/kg of total energy and PEFB: 32.3 kV/cm, 340 µs of treatment time, 10 µs of pulse width and 176.3 kJ/kg of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

3.5. *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 et al., 2004; Heinz et al., 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 90ºC was reached, led to a reduction of 0.3 log-cycles. However, the 3 min thermal treatment at 90ºC 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 80ºC to achieve a 2.4 log-cycle inactivation of *B. subtilis* spores and Evelyn & Silva (2018) needed 60 min at 0.33 W/g

and 75ºC 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 et al., 2012; Ishihara et al., 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*. 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.

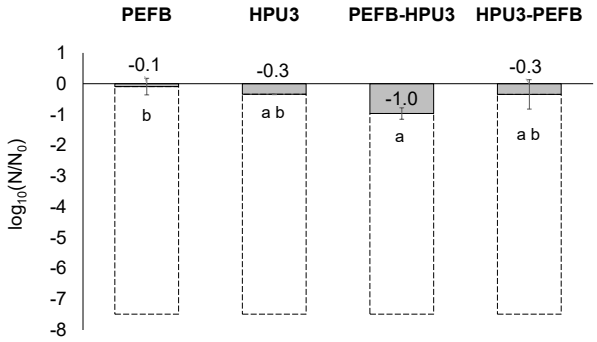


Fig. 5. Inactivation of *B. pumilus* spores in oil-in-water emulsion treated by PEF at 50 Hz and 25ºC of inlet temperature, 32.3 kV/cm, 340 µs of treatment time, 10 µs of pulse width and 152.3 kJ/kg of total energy (PEFB), HPU for 3 min (HPU3) and its combination. Dashed lines indicate complete inactivation.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

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.

Acknowledgements

The authors acknowledge the financial support from Fresenius Kabi.

References

Aadil, R. M., Zeng, X. A., Han, Z., Sahar, A., Khalil, A. A., Rahman, U. U., Khan, M., & Mehmood, T. (2018). Combined effects of pulsed electric field and ultrasound on bioactive compounds and microbial quality of grapefruit juice. Journal of Food Processing and Preservation, 42(2). https://doi.org/10.1111/jfpp.13507

Atalar, I., Gul, O., Saricaoglu, F. T., Besir, A., Gul, L. B., & Yazici, F. (2019). Influence of thermosonication (TS) process on the quality parameters of high pressure homogenized hazelnut milk from hazelnut oil by-products. Journal of Food Science and Technology, 56(3), 1405–1415. https://doi.org/10.1007/s13197-019-03619-7

Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., Saraiva, J. A., Raso, J., Martin-Belloso, O., Witrowa-Rajchert, D., Lebovka, N., & Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. Food Research International, 77, 773–798. https://doi.org/10.1016/j.foodres.2015.09.015

Barbosa-Cánovas, G. V., Tapia, M. S., & Cano, M. P. (2005). Novel food processing technologies. CRC Press, Boca Raton.

Barsotti, L., Dumay, E., Mu, T. H., Fernandez Diaz, M. D., & Cheftel, J. C. (2001). Effects of high voltage electric pulses on protein-based food constituents and structures. Trends in Food Science and Technology, 12(3–4), 136–144. https://doi.org/10.1016/S0924-2244(01)00065-6

Bi, X., Wang, X., Chen, Y., Chen, L., Xing, Y., & Che, Z. (2020). Effects of combination treatments of lysozyme and high power ultrasound on the Salmonella typhimurium inactivation and quality of liquid whole egg. Ultrasonics Sonochemistry, 60, 104763. https://doi.org/10.1016/J.ULTSONCH.2019.104763

Black, E. P., Setlow, P., Hocking, A. D., Stewart, C. M., Kelly, A. L., & Hoover, D. G. (2007). Response of spores to high-pressure processing. In Comprehensive reviews in food science and food safety: Vol. 6 (4) (pp. 103–119).

Cárcel, J. A., García-Pérez, J. V., Benedito, J., & Mulet, A. (2012). Food process innovation through new technologies: Use of ultrasound. Journal of Food Engineering, 110(2), 200–207. https://doi.org/10.1016/J.JFOODENG.2011.05.038

Contreras, M., Benedito, J., Bon, J., & Garcia-Perez, J. V. (2018). Accelerated mild heating of dry-cured ham by applying power ultrasound in a liquid medium. Innovative Food Science and Emerging Technologies, 50(September), 94–101. https://doi.org/10.1016/j.ifset.2018.10.010

Devlieghere, F., Vermeiren, L., & Debevere, J. (2004). New preservation technologies: Possibilities and limitations. International Dairy Journal, 14(4), 273–285. https://doi.org/10.1016/j.idairyj.2003.07.002

Dunn, J. (1996). Pulsed light and pulsed electric field for foods and eggs. Poultry Science, 75(9), 1133–1136. https://doi.org/10.3382/ps.0751133

Erkmen, O., & Doğan, C. (2004). Kinetic analysis of Escherichia coli inactivation by high hydrostatic pressure in broth and foods. Food Microbiology, 21(2), 181–185. https://doi.org/10.1016/S0740-0020(03)00055-8

Evelyn, Kim, H. J., & Silva, F. V. M. (2016). Modeling the inactivation of Neosartorya fischeri ascospores in apple juice by high pressure, power ultrasound and thermal processing. Food Control, 59, 530–537. https://doi.org/10.1016/J.FOODCONT.2015.06.033

Evelyn, & Silva, F. V. M. (2018). Differences in the resistance of microbial spores to thermosonication, high pressure thermal processing and thermal treatment alone. Journal of Food Engineering, 222, 292–297. https://doi.org/10.1016/J.JFOODENG.2017.11.037

Fan, L., Ismail, B. B., Hou, F., Muhammad, A. I., Zou, M., Ding, T., & Liu, D. (2019). Thermosonication damages the inner membrane of Bacillus subtilis spores and

impels their inactivation. Food Research International, 125(July), 108514. https://doi.org/10.1016/j.foodres.2019.108514

Feofilova, E. P., Ivashechkin, A. A., Alekhin, A. I., & Sergeeva, Y. E. (2012). Fungal spores: Dormancy, germination, chemical composition, and role in biotechnology (review). Applied Biochemistry and Microbiology, 48(1), 1–11. https://doi.org/10.1134/S0003683812010048

From, C., Hormazabal, V., & Granum, P. E. (2007). Food poisoning associated with pumilacidin-producing Bacillus pumilus in rice. International Journal of Food Microbiology, 115(3), 319–324. https://doi.org/10.1016/j.ijfoodmicro.2006.11.005

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2020). Non-thermal pasteurization of lipid emulsions by combined supercritical carbon dioxide and high-power ultrasound treatment. Ultrasonics Sonochemistry, 67, 105–138. https://doi.org/10.1016/J.ULTSONCH.2020.105138

Gow, N. A. R., Latge, J.-P., & Munro, C. A. (2017). The fungal cell wall: structure, biosynthesis, and function. American Society for Microbiology, 267–292. https://doi.org/10.1128/microbiolspec.funk-0035-2016

Griffiths, S., MacLean, M., Anderson, J. G., MacGregor, S. J., & Helen Grant, M. (2012). Inactivation of microorganisms within collagen gel biomatrices using pulsed electric field treatment. Journal of Materials Science: Materials in Medicine, 23(2), 507–515. https://doi.org/10.1007/s10856-011-4526-x

Halpin, R. M., Cregenzán-Alberti, O., Whyte, P., Lyng, J. G., & Noci, F. (2013). Combined treatment with mild heat, manothermosonication and pulsed electric fields reduces microbial growth in milk. Food Control, 34(2), 364–371. https://doi.org/10.1016/j.foodcont.2013.05.008

Han, L. L., Shao, H. H., Liu, Y. C., Liu, G., Xie, C. Y., Cheng, X. J., Wang, H. Y., Tan, X. M., & Feng, H. (2017). Transcriptome profiling analysis reveals metabolic

changes across various growth phases in Bacillus pumilus BA06. BMC Microbiology, 17(1), 1–13. https://doi.org/10.1186/s12866-017-1066-7

Heinz, V., Alvarez, I., Angersbach, A., & Knorr, D. (2001). Preservation of liquid foods by high intensity pulsed electric fields—basic concepts for process design. Trends in Food Science & Technology, 12(3–4), 103–111. https://doi.org/10.1016/S0924-2244(01)00064-4

Hodgins, A. M., Mittal, G. S., & Griffiths, M. W. (2002). Pasteurization of fresh orange juice using low-energy pulsed electrical field. Journal of Food Science, 67(6), 2294–2299. https://doi.org/10.1111/j.1365-2621.2002.tb09543.x

Huang, E., Mittal, G. S., & Griffiths, M. W. (2006). Inactivation of Salmonella enteritidis in liquid whole egg using combination treatments of pulsed electric field, high pressure and ultrasound. Biosystems Engineering, 94(3), 403–413. https://doi.org/10.1016/j.biosystemseng.2006.03.008

Ince, N. H., & Belen, R. (2001). Aqueous phase disinfection with power ultrasound: Process kinetics and effect of solid catalysts. Environmental Science and Technology, 35(9), 1885–1888. https://doi.org/10.1021/es000157r

Ishihara, Y., Saito, H., & Takano, J. (1999). Differences in the surface membranes and water content between the vegetative cells and spores of Bacillus subtilis. Cell Biochemistry and Function, 17(1), 9–13. https://doi.org/10.1002/(SICI)1099-0844(199903)17:1<9::AID-CBF803>3.0.CO;2-9

Iurlina, M. O., Saiz, A. I., Fuselli, S. R., & Fritz, R. (2006). Prevalence of Bacillus spp. in different food products collected in Argentina. LWT - Food Science and Technology, 39(2), 105–110. https://doi.org/10.1016/j.lwt.2005.01.006

Jambrak, A. R. (2018). Non-thermal and innovative processing technologies. Encyclopedia of Food Security and Sustainability, 1, 477–483. https://doi.org/10.1016/B978-0-08-100596-5.22285-3

Kernou, O. N., Belbahi, A., Amir, A., Bedjaoui, K., Kerdouche, K., Dairi, S., Aoun, O., & Madani, K. (2021). Effect of sonication on microwave inactivation of Escherichia

coli in an orange juice beverage. Journal of Food Process Engineering, February, 1–9. https://doi.org/10.1111/jfpe.13664

Khanal, S. N., Anand, S., & Muthukumarappan, K. (2014). Evaluation of high-intensity ultrasonication for the inactivation of endospores of 3 bacillus species in nonfat milk. Journal of Dairy Science, 97(10), 5952–5963. https://doi.org/10.3168/jds.2014-7950

Liao, X., Muhammad, A. I., Chen, S., Hu, Y., Ye, X., Liu, D., & Ding, T. (2019). Bacterial spore inactivation induced by cold plasma. Critical Reviews in Food Science and Nutrition, 59(16), 2562–2572. https://doi.org/10.1080/10408398.2018.1460797

Liu, R. F., Huang, C. L., & Feng, H. (2015). Salt stress represses production of extracellular proteases in Bacillus pumilus. Genetics and Molecular Research, 14(2), 4939–4948. https://doi.org/10.4238/2015.May.11.27

López-Malo, A., Palou, E., Jiménez-Fernández, M., Alzamora, S. M., & Guerrero, S. (2005). Multifactorial fungal inactivation combining thermosonication and antimicrobials. Journal of Food Engineering, 67(1–2), 87–93. https://doi.org/10.1016/j.jfoodeng.2004.05.072

Lyu, C., Huang, K., Yang, N., Wang, H., & Wang, J. (2016). Combination of thermosonication and pulsed electric fields treatments for controlling Saccharomyces cerevisiae in Chinese rice wine. Food and Bioprocess Technology, 9(11), 1854–1864. https://doi.org/10.1007/s11947-016-1769-z

Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. International Journal of Food Microbiology, 72(1–2), 107–113. https://doi.org/10.1016/S0168-1605(01)00624-9

Mañas, P., & Pagán, R. (2005). Microbial inactivation by new technologies of food preservation. Journal of Applied Microbiology, 98(6), 1387–1399. https://doi.org/10.1111/j.1365-2672.2005.02561.x

Michalac, S., Alvarez, V., Ji, T., & Zhang, Q. H. (2003). Inactivation of selected microorganisms and properties of pulsed electric field processed milk. Journal of

Food Processing and Preservation, 27(2), 137–151. https://doi.org/10.1111/j.1745-4549.2003.tb00507.x

Mosqueda-Melgar, J., Elez-Martínez, P., Raybaudi-Massilia, R. M., & Martín-Belloso, O. (2008). Effects of pulsed electric fields on pathogenic microorganisms of major concern in fluid foods: A review. Critical Reviews in Food Science and Nutrition, 48(8), 747–759. https://doi.org/10.1080/10408390701691000

Muriel Mundo, J. L., Zhou, H., Tan, Y., Liu, J., & McClements, D. J. (2020). Stabilization of soybean oil-in-water emulsions using polypeptide multilayers: Cationic polylysine and anionic polyglutamic acid. Food Research International, 137(May), 109304. https://doi.org/10.1016/j.foodres.2020.109304

Nadumane, V. K., Venkatachalam, P., & Gajaraj, B. (2016). Aspergillus applications in cancer research. In New and Future Developments in Microbial Biotechnology and Bioengineering: Aspergillus System Properties and Applications. Elsevier B.V. https://doi.org/10.1016/B978-0-444-63505-1.00020-8

Noci, F., Walkling-Ribeiro, M., Cronin, D. A., Morgan, D. J., & Lyng, J. G. (2009). Effect of thermosonication, pulsed electric field and their combination on inactivation of Listeria innocua in milk. International Dairy Journal, 19(1), 30–35. https://doi.org/10.1016/j.idairyj.2008.07.002

Odriozola-Serrano, I., Bendicho-Porta, S., & Martín-Belloso, O. (2006). Comparative study on shelf life of whole milk processed by high-intensity pulsed electric field or heat treatment. Journal of Dairy Science, 89(3), 905–911. https://doi.org/10.3168/jds.S0022-0302(06)72155-5

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012). Supercritical carbon dioxide inactivation of Escherichia coli and Saccharomyces cerevisiae in different growth stages. The Journal of Supercritical Fluids, 63, 8–15. https://doi.org/10.1016/J.SUPFLU.2011.12.022

Palgan, I., Muñoz, A., Noci, F., Whyte, P., Morgan, D. J., Cronin, D. A., & Lyng, J. G. (2012). Effectiveness of combined pulsed electric field (PEF) and manothermosonication (MTS) for the control of Listeria innocua in a smoothie

type beverage. Food Control, 25(2), 621–625. https://doi.org/10.1016/j.foodcont.2011.11.009

Pataro, G., De Lisi, M., Donsì, G., & Ferrari, G. (2014). Microbial inactivation of E. coli cells by a combined PEF-HPCD treatment in a continuous flow system. Innovative Food Science and Emerging Technologies, 22, 102–109. https://doi.org/10.1016/j.ifset.2013.12.009

Pataro, G., Ferrentino, G., Ricciardi, C., & Ferrari, G. (2010). Pulsed electric fields assisted microbial inactivation of S. cerevisiae cells by high pressure carbon dioxide. Journal of Supercritical Fluids, 54(1), 120–128. https://doi.org/10.1016/j.supflu.2010.04.003

Pillet, F., Formosa-Dague, C., Baaziz, H., Dague, E., & Rols, M. P. (2016). Cell wall as a target for bacteria inactivation by pulsed electric fields. Scientific Reports, 6(February), 1–8. https://doi.org/10.1038/srep19778

Piyasena, P., Mohareb, E., & McKellar, R. . (2003). Inactivation of microbes using ultrasound: a review. International Journal of Food Microbiology, 87(3), 207–216. https://doi.org/10.1016/S0168-1605(03)00075-8

Pyatkovskyy, T. I., Shynkaryk, M. V., Mohamed, H. M., Yousef, A. E., & Sastry, S. K. (2018). Effects of combined high pressure (HPP), pulsed electric field (PEF) and sonication treatments on inactivation of Listeria innocua. Journal of Food Engineering, 233, 49–56. https://doi.org/10.1016/j.jfoodeng.2018.04.002

Raso, J., Frey, W., Ferrari, G., Pataro, G., Knorr, D., Teissie, J., & Miklavčič, D. (2016). Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes. Innovative Food Science and Emerging Technologies. https://doi.org/10.1016/j.ifset.2016.08.003

Raso, J., Palop, A., Pagán, R., & Condón, S. (1998). Inactivation of Bacillus subtilis spores by combining ultrasonic waves under pressure and mild heat treatment. Journal of Applied Microbiology, 85(5), 849–854. https://doi.org/10.1046/j.1365-2672.1998.00593.x

Reineke, K., & Mathys, A. (2020). Endospore inactivation by emerging technologies: a review of target structures and inactivation mechanisms. Annual Review of Food Science and Technology, 11, 255–274. https://doi.org/10.1146/annurev-food-032519-051632

Salve, A. R., Pegu, K., & Arya, S. S. (2019). Comparative assessment of high-intensity ultrasound and hydrodynamic cavitation processing on physico-chemical properties and microbial inactivation of peanut milk. Ultrasonics Sonochemistry, 59(August), 104728. https://doi.org/10.1016/j.ultsonch.2019.104728

Scudino, H., Silva, E. K., Gomes, A., Guimarães, J. T., Cunha, R. L., Sant'Ana, A. S., Meireles, M. A. A., & Cruz, A. G. (2020). Ultrasound stabilization of raw milk: Microbial and enzymatic inactivation, physicochemical properties and kinetic stability. Ultrasonics Sonochemistry, 67, 105185. https://doi.org/10.1016/j.ultsonch.2020.105185

Spilimbergo, S., Cappelletti, M., Tamburini, S., Ferrentino, G., & Foladori, P. (2014). Partial permeabilisation and depolarization of Salmonella enterica Typhimurium cells after treatment with pulsed electric fields and high pressure carbon dioxide. Process Biochemistry, 49(12), 2055–2062. https://doi.org/10.1016/j.procbio.2014.10.003

Spilimbergo, S., Dehghani, F., Bertucco, A., & Foster, N. R. (2003). Inactivation of bacteria and spores by pulse electric field and high pressure CO2 at low temperature. Biotechnology and Bioengineering, 82(1), 118–125. https://doi.org/10.1002/bit.10554

Timmermans, R. A. H., Nierop Groot, M. N., Nederhoff, A. L., van Boekel, M. A. J. S., Matser, A. M., & Mastwijk, H. C. (2014). Pulsed electric field processing of different fruit juices: Impact of pH and temperature on inactivation of spoilage and pathogenic micro-organisms. International Journal of Food Microbiology, 173, 105–111. https://doi.org/10.1016/j.ijfoodmicro.2013.12.022

Tischler, B. Y., & Hohl, T. M. (2019). Menacing mold: recent advances in Aspergillus pathogenesis and host defense. Journal of Molecular Biology, 431(21), 4229–4246. https://doi.org/10.1016/j.jmb.2019.03.027

Walkling-Ribeiro, M., Noci, F., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2009). Shelf life and sensory evaluation of orange juice after exposure to thermosonication and pulsed electric fields. Food and Bioproducts Processing, 87(2), 102–107. https://doi.org/10.1016/j.fbp.2008.08.001

Walkling-Ribeiro, M., Noci, F., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2010). Shelf life and sensory attributes of a fruit smoothie-type beverage processed with moderate heat and pulsed electric fields. LWT - Food Science and Technology, 43(7), 1067–1073. https://doi.org/10.1016/j.lwt.2010.02.010

Walkling-Ribeiro, M., Noci, F., Riener, J., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2009). The impact of thermosonication and pulsed electric fields on Staphylococcus aureus inactivation and selected quality parameters in orange juice. Food and Bioprocess Technology, 2(4), 422–430. https://doi.org/10.1007/s11947-007-0045-7

Yan, Z., Yin, L., Hao, C., Liu, K., & Qiu, J. (2021). Synergistic effect of pulsed electric fields and temperature on the inactivation of microorganisms. AMB Express, 11(1). https://doi.org/10.1186/s13568-021-01206-8

Yeom, H. W., Streaker, C. B., Howard Zhang, Q., & Min, D. B. (2000). Effects of pulsed electric fields on the quality of orange juice and comparison with heat pasteurization. Journal of Agricultural and Food Chemistry, 48(10), 4597–4605. https://doi.org/10.1021/jf000306p

Yildiz, S., Pokhrel, P. R., Unluturk, S., & Barbosa-Cánovas, G. V. (2019). Identification of equivalent processing conditions for pasteurization of strawberry juice by high pressure, ultrasound, and pulsed electric fields processing. Innovative Food Science and Emerging Technologies, 57(April), 102195. https://doi.org/10.1016/j.ifset.2019.102195

Yildiz, S., Pokhrel, P. R., Unluturk, S., & Barbosa-Cánovas, G. V. (2020). Changes in quality characteristics of strawberry juice after equivalent high pressure, ultrasound, and pulsed electric fields processes. Food Engineering Reviews. https://doi.org/10.1007/s12393-020-09250-z

5. GENERAL DISCUSSION

Microbial safety of emulsions represents a relevant issue in the food and pharmaceutical industry. In particular, spores contamination is a major risk since they are the most resistant microbial form against inactivation due to their complex cell structure. Consumers also demand high-quality products and, in this sense, non-thermal preservation technologies are emerging since they could affect the physicochemical and nutritional properties of the treated products to a lower extent than conventional thermal treatments. Supercritical carbon dioxide (SC-CO_2), high power ultrasound (HPU) and pulsed electric fields (PEF) are emerging methods for pasteurization, but there is still room for improvement in terms of the inactivation rate and product quality. The combination of several non-thermal technologies could enhance the inactivation performance and therefore, milder conditions or shorter processing times could be used, obtaining a better quality of the treated products and less costly processes.

Different aspects can affect the sensitivity of microorganisms, including the process conditions (pressure, temperature, time…), the use of combined treatments, the composition of the medium and the type of microorganism. All these issues have been addressed in the present PhD Thesis and will be discussed as a whole in this section.

SC-CO_2 + HPU microbial inactivation (CHAPTER 1)

Supercritical carbon dioxide treatments

The effectiveness of SC-CO_2 inactivation and conventional thermal treatments was compared for vegetative bacteria (*E. coli* and *B. diminuta*) and fungal (*A. niger*) and bacterial spores (*C. butyricum* and *B. subtilis*), achieving higher inactivation in SC-CO_2. As an example, in the oil-in-water emulsions thermally treated (50ºC), only a reduction of 0.4 log-cycles of *E. coli* was achieved in 50 min, while when emulsions were SC-CO_2–treated at 100 and 350 bar (50ºC), on average, a reduction of 7.1 log-cycles was achieved at the same time. However, SC-CO_2 could be considered as a highly time-consuming process, which could seriously restrict its industrial application. In fact, to achieve the complete inactivation for vegetative bacteria (*E. coli* and

B. diminuta), which was the most sensitive type of microorganism studied in this PhD Thesis, more than 40-50 min were required. Consequently, the application of HPU to the SC-CO_2 treatment for different microorganisms and media was investigated.

Effect of the application of HPU to the SC-CO_2 treatments

The implementation of HPU drastically enhanced the SC-CO_2 inactivation for vegetative bacteria (*E. coli* and *B. diminuta*) in both water and emulsions. In fact, it shortened the complete inactivation time of both bacteria by approximately 1 order of magnitude. HPU also noticeably improved the SC-CO_2 inactivation of *A. niger* spores in water. This fact was in accordance with the damage observed by microscopy techniques for the microbial cells (*E. coli, B. diminuta* and *A. niger* spores) inactivated by SC-CO_2 + HPU in water. The inactivated cells lost their shape and their cell walls appeared partially disintegrated. In addition, in the intracellular space, the cytoplasm was observed unevenly distributed, with extensive empty regions, indicating that an important amount of cytoplasmic content was released and with aggregated cytoplasmic content in some areas. However, no effect of HPU was found on the inactivation of *A. niger* spores in the emulsion, which could indicate that the oil-in-water dispersion protected the *A. niger* spores, in particular, from cavitation. Therefore, the effect exerted by HPU on SC-CO_2 treatments may depend on both the microorganism and the composition of the medium.

As for the inactivation of bacterial spores in emulsions, in general terms, an enhanced inactivation was found in the SC-CO_2 + HPU treatments. For example, the time required for the complete SC-CO_2 + HPU inactivation of *C. butyricum* spores was shortened from 10 to 3 min at 550 bar and 85ºC compared to the individual SC-CO_2 treatment. In addition, for the inactivation of *B. subtilis* spores at 20 min, 350 bar and 85ºC, an inactivation of 5.4 log-cycles was achieved when using SC-CO_2 + HPU, compared to 3.1 log-cycles in SC-CO_2. Nevertheless, SC-CO_2 + HPU treatments at 95ºC did not significantly ($p>0.05$) enhance the inactivation of *G. stearothermophilus* spores, regardless the pressure applied (350 or 550 bar), compared to the thermal treatment at the same temperature (95ºC) and time (20 min). Watanabe et al. (2003)

also studied the SC-CO_2 inactivation of *G. stearothermophilus* spores at 95ºC and 300 bar, although in water, and achieved an inactivation of 5.0 log-cycles in 120 min without the use of HPU. Probably, longer treatment times than the ones used in our experiments (20 min) are required to achieve a noticeable reduction of these spores, regardless of the use of HPU. However, treatments longer than 20 min could be considered unfeasible for industrial implementation and, consequently, the inactivation of *G. stearothermophilus* spores with SC-CO_2 + HPU could not be considered at industrial level.

Several authors (Ortuño et al., 2012, 2013; Paniagua-Martínez et al., 2016) found that the SC-CO_2 inactivation of vegetative cells drastically improved with the implementation of HPU. In this sense, Ortuño et al., (2012) demonstrated that the effect of the combined SC-CO + HPU treatment was synergistic. HPU is known, on the one hand, to induce cavitation which affects the microbial cells by causing fractures on the cell walls, facilitating the penetration of SC-CO_2 inside the cell and the extraction of internal vital compounds (Ortuño et al., 2013). On the other hand, HPU has demonstrated to increase the mass and heat transfer in SC-CO_2 (Cárcel et al., 2012; Gao et al., 2009) and, consequently, to enhance the solubilisation of CO_2 into the medium and its contact with the cells. Thus, the penetration of SC-CO_2 into the microbial cells is facilitated. However, the effects of SC-CO_2 + HPU treatments on the inactivation of inoculated fungal and bacterial spores had not been previously investigated. Generally, fungal spores have shown a larger resistance to HPU than vegetative cells (López-Malo et al., 2005) probably because of their more resistant cell wall structure. In addition, *A. niger* spores in particular, might be more resistant to cavitation due to the larger rigidity of their cell wall provided by melanin (Tischler & Hohl, 2019). In fact, although in the present PhD Thesis, HPU affected the inactivation of *A. niger* in water, the effect was of less magnitude than on *E. coli* and *B. diminuta*.

In bacterial spores, HPU could act damaging its highly dehydrated and resistant structure and triggering the hydration of the spore (Barbosa-Cánovas et al., 2005). Thereby, HPU makes the cell more sensitive to SC-CO_2 treatment since CO_2 can dissolve and penetrate more easily inside the bacterial spore, as may occur for *C. butyricum, B. subtilis* and *B. pumilus* spores. However, the structure of

G. stearothermophilus spores was probably stronger and more resistant to cavitation effects than the spores from the *Clostridium* and *Bacillus* genus.

Effect of pressure and temperature

The results found in the present PhD Thesis illustrated that, in general terms, the higher the pressure and the temperature in the SC-CO_2 and SC-CO_2 + HPU treatments, the more intense the microbial inactivation.

The increase in the pressure and temperature led to an improvement in the inactivation of *B. diminuta,* regardless the treatment (SC-CO_2 or SC-CO_2 +HPU) or the medium (water or 20% oil-in-water emulsion). The same effects were found for *E. coli* in the 20% oil-in-water emulsion while only the temperature had a significant ($p<0.05$) effect on the inactivation of *E. coli* in water in both SC-CO_2 and SC-CO_2 + HPU treatments.

As regards the fungal spores (*A. niger*), the higher the pressure and the temperature, the higher the inactivation in both media (water and 20% oil-in-water emulsions) and treatments (SC-CO_2 and SC-CO_2 + HPU). As for bacterial spores, the increase in the temperature also led to a higher inactivation of *C. butyricum* and *B. subtilis*, regardless the treatment (e.g. the inactivation level of *B. subtilis* spores increased from 0.1 to 2.7 log-cycles, from 3.1 to 6.2 log-cycles and from 5.4 to 6.7 log-cycles when temperature was raised from 85 to 95ºC, in 20 min of thermal, SC-CO_2 and SC-CO_2 + HPU treatment, respectively) and the pressure also affected the inactivation, being of larger magnitude as pressure was increased but up to a limit (350 bar) from which an increase in pressure did not result into a larger inactivation of *C. butyricum* or *G. stearothermophilus* spores. Therefore, the effect of pressure was probably limited by the CO_2 solubility, which may increase weakly from 350 to 550 bar. Therefore, in general terms, pressure seems to have a lower effect than temperature in the ranges considered in this PhD Thesis for both parameters.

Contrary to the results presented in this PhD Thesis, Ortuño et al. (2012, 2013, 2014) did not reported so far a noticeable effect of the pressure and temperature in SC-CO_2 + HPU inactivation kinetics. This fact could be explained by the less resistant

microorganisms analyzed by these authors since they studied only vegetative cells in which the strong effect of HPU leads to very fast inactivation kinetics, which could mask the influence of other variables, such as pressure and temperature.

High pressure increases the solubility of CO_2 in the medium, therefore, the contact between CO_2 and the microbial cell is higher and the CO_2 penetration into the cells could be improved (Hong et al., 1997; Liao et al., 2007). However, the effect of the CO_2 pressure on inactivation is limited by the saturation solubility of CO_2 in the suspending medium (Garcia-Gonzalez et al., 2007). As mentioned, this saturation was reflected in our inactivation kinetics when the pressure was increased more than 350 bar. In addition, high temperatures are known to increase CO_2 diffusivity and to make vegetative cell membranes more fluid, facilitating the penetration of CO_2 (Ferrentino & Spilimbergo, 2015; Hong et al., 1999). Regarding fungal and bacterial spores, these mechanisms may not be applicable due to their highly resistant and dehydrated structure. Thus, two hypotheses for the SC-CO_2 inactivation of bacterial spores were found in the literature, having in common that it is necessary to reach a threshold temperature, which was dependent on the bacterial spore specie, to reduce the extreme resistance of the spores to the treatment and to obtain lethal effects. In this sense,

- Spilimbergo et al. (2003) suggested that the effect of CO_2 acidification along with a certain temperature was able to initiate the activation and germination of the bacterial spores. Thus, the spore structure is rehydrated becoming more sensitive to SC-CO_2 treatment. However, the length of the inactivation kinetics in the present PhD Thesis is too short to believe that this mechanism could be taking place.
- Rao et al. (2015) proposed that in SC-CO_2 treatments, a certain temperature, which was dependent on the bacterial spore, is necessary to exert direct damage on the external structure of the spores and make them more prone to hydration and more permeable to SC-CO_2. This hypothesis could be more plausible, considering the short treatment times applied on this PhD Thesis.

Regardless these hypotheses, the exact mechanisms for SC-CO_2 inactivation of spores have not been elucidated on this PhD Thesis and its understanding could be considered matter of relevant future research.

Effect of the type of microorganism

As for the vegetative bacteria studied in the present PhD Thesis (*E. coli* and *B. diminuta*), in general terms, very slight differences in terms of resistance to SC-CO_2 and SC-CO_2 + HPU treatments were found. However, *A. niger* spores was significantly ($p<0.05$) more resistant compared to both vegetative bacteria, regardless of the type of treatment (thermal, SC-CO_2 or SC-CO_2+ HPU) or the medium. For example, for the SC-CO_2 treatment at 50ºC and 350 bar in water, 55 min were required to achieve the complete inactivation of *A. niger* (6.8 log-cycles), while around 10 min were needed for *E. coli* or *B. diminuta* (8 log-cycles). Other studies already reported that fungal spores are more resistant to SC-CO_2 treatments than vegetative bacteria, due to their different and more resistant structure (Soares et al., 2019). Specifically, in *A. niger* spores, the multi layered wall, which contains melanin, may confer high rigidity to the cell wall (Tischler & Hohl, 2019). Among all the types of microorganisms studied, bacterial spores were found to be the most resistant to thermal, SC-CO_2 and SC-CO_2 + HPU treatments. Bacterial spores own a very resistant and dehydrated structure, which could hinder CO_2 to dissolve and to penetrate thought the cell. Among the bacterial spores studied, the anaerobic one (*C. butyricum*) was the most sensitive, compared to those aerobic (*B. subtilis*, *B. pumilus* and *G. stearothermophilus*). No difference was found between the resistances of spores from the genus *Bacillus* (*B. subtilis* and *B. pumilus*), which were completely inactivated with a SC-CO_2 + HPU treatment at 350 bar, 95ºC and 20 min. On the contrary, the spores of *G. stearothermophilus* were the most resistant, since, even with the most intense treatment employed (SC-CO_2 + HPU at 550 bar, 95ºC and 20 min), the inactivation obtained was negligible (0.5 log-cycles). *G. stearothermophilus* spores are often used for the validation of sterilization processes where temperature, steam and pressure are involved due to its extreme resistance to external stresses. Therefore, as mentioned before, the proposed inactivation

technologies were not useful for the inactivation of *G. stearothermophilus* spores due to its extremely strong resistance.

Effect of the treating medium

Most of the authors previously addressing microbial inactivation observed protective effects against external stresses on microbial cells in complex physicochemical systems, compared to more simple media. The content of certain compounds such as water, fat, sugar and salt or the pH of the suspending medium has demonstrated to affect the microbial sensitivity to SC-CO_2 inactivation (Garcia-Gonzalez et al., 2007). In the present PhD Thesis, SC-CO_2 and SC-CO_2 + HPU treatments were carried out in different media (water and oil-in-water emulsions with different oil content). For both vegetative bacteria, *E. coli* and *B. diminuta*, the inactivation obtained by SC-CO_2 were significantly ($p<0.05$) higher in water than in the emulsions. Moreover, in overall terms, the higher the oil content in the emulsion (10, 20 or 30%), the slower the bacterial inactivation. Therefore, it seems that the presence of oil in the medium protected the microbial cells against SC-CO_2 inactivation. Some hypotheses explaining the reduced effectiveness of the SC-CO_2 inactivation in the emulsions, compared to water, are suggested:

- The different components in the emulsions could provide certain buffering capacity, which could be not present in a simple medium, such as water. Thus, the inactivation performance of SC-CO_2 could be limited since one of the assumed mechanisms for SC-CO_2 inactivation is the decrease of pH in the suspending medium.
- The contact between SC-CO_2 and microbial cells could be reduced by the presence of oil in the suspending medium. Lipid substances could act as a barrier protecting the lipid bilayer of the membranes and hindering the SC-CO_2 penetration into the cells.
- The SC-CO_2 could be partly dissolved in the oil-phase of the suspending medium and, consequently, less CO_2 could be available to be dissolved in the water phase of the medium or solubilized in the lipid bilayer of the cell membrane. Thus,

the decrease of the pH of the medium and the increase of permeability of the microbial cell membranes could be restrained.

When HPU was applied to the SC-CO_2 treatments, similar inactivation levels of *E. coli* and *B. diminuta* were obtained, regardless of the oil content in the emulsion (10, 20 or 30%). Probably, the intense turbulence caused by cavitation lead to a faster diffusion and penetration into the microbial cells of SC-CO_2. Thus, the protective effect of the oil was masked.

On the contrary, the inactivation capacity of both SC-CO_2 and SC-CO_2 + HPU treatments was not significantly ($p>0.05$) affected by the suspending medium for *A. niger* spores (on average, 4.4 and 4.3 log-cycles of reduction were obtained for the treatments in water and in the 20% oil emulsion). In this regard, Noman et al. (2018) found a slightly higher inactivation level in *A. niger* spores treated with SC-CO_2 in water (6.0 log-cycles), compared to seawater or saline solutions (5.5 log-cycles). Therefore, it seems that the resistance of *A. niger* spores can be affected by the content of salts in the suspending medium. However, as shown in the present PhD Thesis, the presence of oil in the suspending medium did not exert any protective effect for this microorganism.

Effect of the SC-CO_2 + HPU treatments on the physicochemical properties of the emulsions

In order to determine a SC-CO_2 + HPU treatment able to inactivate spores while minimizing the physicochemical changes in the emulsions, different combinations of pressure (100, 350 and 600 bar), temperature (55, 75 and 95ºC) and time (5, 12.5 and 20 min) were considered and the most relevant quality parameters of the emulsions were measured. In general terms, only a mild effect of the process variables (temperature, pressure and time) on the quality of the emulsions was found. Treated emulsions showed, in general, minimal changes in density and good electrostatic stability (measured by the zeta potential). However, the pH decreased and the droplet size increased.

Depressurization and/or the application of HPU for a long time (over-processing) could have broken the oil-water interface of some droplets, leading to coalescence and the appearance of larger droplets. In fact, a small fraction of the emulsion (~2 mL) with visible oil separation was found in the last fraction extracted from the vessel. Consequently, a better appearance and a lower mean droplet size could be obtained by removing the separated oil fraction of the processed emulsion.

The basic pH of the untreated emulsions (8.4) could enhance the dissociation of the carbonic acid formed by the dissolution of the CO_2 in the water phase of the emulsion, leading to a decrease of pH. However, the reduction in the pH did not affect the repulsive forces between droplets, as shown by the zeta potential. Despite these slight physicochemical changes, suitable conditions of pressure, temperature and time (e.g. 600 bar, 95ºC and 12.5 min) permitted the quality and stability of the treated emulsions to be maintained while achieving a satisfactory microbial inactivation, except for *G. stearothermophilus* spores.

In short, the combined SC-CO_2 + HPU technology was feasible for the processing of oil-in-water emulsions in order to achieve microbial and physicochemical stability. The presence of oil in the medium was not a limitation for SC-CO_2 + HPU inactivation, which was completely achieved for all the microorganisms studied, including bacterial spores, except for *G. stearothermophilus* spores. SC-CO_2 + HPU treatments at 350 bar and around 10 min were enough to completely inactivate vegetative bacteria at 50ºC and *A. niger* spores at 60ºC. Moreover, 350 bar and 20 min were sufficient to achieve the complete inactivation of *C. butyricum* at 80ºC and *Bacillus* spores at 95ºC.

PEF and HPU microbial inactivation (CHAPTER 2)

The impact of individual and combined pulsed electric field (PEF) and high power ultrasound (HPU) treatments on the inactivation of different types of microorganisms (a vegetative bacteria, a fungal spore and a bacterial spore) in oil-in-water emulsions was studied for the first time.

Effect of the treatment conditions

In the PEF treatments, it was observed that the higher the field strength, time and input temperature in the PEF treatments, the larger the inactivation level of *E. coli*. Thus, the highest level of *E. coli* inactivation by PEF was achieved at the highest total energy delivered (176.3 kJ/kg) at 25ºC input temperature. The fluidity of the membranes in vegetative cells could increase at high temperatures , making cells more prone to electroporation (Mosqueda-Melgar et al., 2008). The total energy applied in the PEF treatment was also significant ($p<0.05$) on the inactivation of *A. niger* spores (no inactivation at 76.3 kJ/kg and 1.2 log-cycles of inactivation at 176.3 kJ/kg at 25ºC).

Regarding HPU, the longer the treatment, the greater the *E. coli* inactivation (the inactivation achieved increased from 1.9 to 5.4 log-cycles from using 2 to 3 min). In fact, the inactivation level reached after 2 min of HPU treatment equalled that of the thermal treatment at the same temperature. Therefore, probably, the inactivation achieved up to 2 min was mainly linked to only the heating effect. On the contrary, when the HPU treatment was extended to 3 min, the inactivation level reached was higher than that of the thermal treatment at the same temperature and, consequently, it could be related to cavitation effects. Nevertheless, the maximum inactivation levels achieved by PEF and HPU were of 2.6 and 5.4 log-cycles, respectively, for *E. coli* (out of 8.1 log-cycles, corresponding to the complete inactivation), 1.2 and 4.3 log-cycles, respectively, for *A. niger* spores (out of 6.6 log-cycles) and 0.1 and 0.3 log-cycles, respectively, for *B. pumilus* spores (out of 7.5 log-cycles). Therefore, in order to increase the microbial inactivation, the combination of both technologies applied sequentially was investigated.

Effect of the combined treatments

The sequence in which the combined PEF and HPU treatments was applied was found to be more effective if PEF was conducted as the first hurdle. The combination of PEF (152.3-176.3 kJ/kg) followed by HPU (3 min) led to a higher inactivation than that of the sum of the individual treatments for all the studied microorganisms, leading to synergistic effects for *A. niger* and *B. pumilus* spores. On

the contrary, when HPU (3 min) was followed by PEF, the inactivation level was lower than the sum of the inactivation levels for the individual treatments, regardless the microorganism. There are two hypotheses that could explain these results:

- As some authors suggested, cavitation effect on inactivation could be an “all or nothing” treatment with no sublethal effects on the microbial cells (Barbosa-Cánovas et al., 2005; Walkling-Ribeiro et al., 2009). Consequently, synergistic effects should not be expected when HPU is applied prior to an additional inactivation treatment. Thus, when HPU is applied first, the most sensitive cells may be inactivated by cavitation, leaving the most resistant cells intact to be inactivated by the subsequent PEF treatment. On the contrary, PEF treatment has demonstrated to exert lethal and sublethal effects in microbial cells, leaving the non-inactivated cells more sensitive for the subsequent HPU treatment.
- PEF treatment involves accumulation of electrical charges at both sides of the cell membrane, which shows a non-conductive behaviour (Delso et al., 2020). Thus, when the transmembrane potential increases above a critical value, electroporation occurs. However, the fractures in the cell membrane caused by HPU are conductive structures and, consequently the transmembrane potential exerted by the further PEF treatment could be diminished and it does not lead to the electroporation. Thus, if the microbial cells are initially damaged or cracked by the HPU treatment, the effect of the further PEF is limited than the one found in the intact cell. On the contrary, HPU may similarly affect both intact and electroporated cells, being possible to lead to synergistic effects.

Effect of the type of microorganism

PEF treatments at the same temperature (25ºC) and similar energies (152.3-176.3 kJ/kg) reached reductions of 2.6, 1.2 and 0.1 log-cycles for *E. coli, A. niger* and *B. pumilus*, respectively. HPU treatment applied for 3 min led to 5.4, 4.3 and 0.3 log-cycles reduction for *E. coli, A. niger* and *B. pumilus,* respectively. The microorganism with the highest resistance to both treatments, PEF and HPU, was the bacterial spore (*B. pumilus*), followed by the fungal spore (*A. niger*) and lastly, by the vegetative

bacteria (*E. coli*). When PEF is applied, charges located at both sides of the cell membranes exert electrocompressive forces, which are equilibrated by elastic deformation of the membranes until a critical field strength where the electric breakdown of the membrane occurs and cells are electroporated. However, the complex and rigid structure of bacterial and fungal spores is not easily deformed and extremely high field strengths are probably required for achieving lethal effects on spores. In addition, it is known that the internal electrical conductivities of spores was lower than in the vegetative cells, due to the highly dehydrated structure and the low mobility of ions in the spore structure (Deeth & Datta, 2018; Mastwijk & Bartels, 2007), which could also explain the lower effect of PEF on spores.

The inactivation levels achieved with the combination of PEF and HPU showed the same order in terms of microbial resistance than in the case of the treatments individually applied, being the inactivation levels 8.2, 6.6 and 1 log-cycles for *E. coli*, *A. niger* and *B. pumilus*, respectively, after the PEF (152.3-176.3 kJ/kg)-HPU (3 min) treatment and 5.7, 4.9 and 0.3 log-cycles for *E. coli*, *A. niger* and *B. pumilus*, respectively, after the HPU (3 min)-PEF (152.3-176.3 kJ/kg) treatment.

In summary, the combined PEF-HPU treatment is a promising hurdle technology to inactivate vegetative bacteria or fungal spores in emulsions. However, the effect on the inactivation of bacterial spores is limited.

Comparison of combined technologies

Both combined technologies (SC-CO_2 + HPU and PEF-HPU) were feasible alternatives to the thermal pasteurization of lipid emulsions, which were greatly affected by the process variables. The comparison of the combined technologies (SC-CO_2 + HPU and PEF-HPU) in terms of inactivation effectiveness is difficult since usually, different temperatures and times were employed. The peak temperature reached in the PEF-HPU treatment to inactivate *E. coli* was of 60ºC while the highest temperature used in the SC-CO_2 + HPU treatments was 50ºC. Consequently, a longer time was required in the SC-CO_2 + HPU treatment (at least 8 min) to achieve the complete *E. coli* inactivation, compared to the PEF-HPU treatment (around 3 min). The SC-CO_2 + HPU

treatment at 60ºC completely inactivated *A. niger* spores in 10-25 min, depending on the pressure, while approximately 3 min of the PEF-HPU treatment was enough to achieve the same inactivation level at the same temperature. Therefore, the PEF-HPU treatment was more effective for *A. niger* spores. The inactivation of *Bacillus* spores with SC-CO_2 + HPU at 85ºC was approximately of 1.4 log-cycles in 3 min, which was a higher inactivation than the one obtained after the PEF-HPU treatment (1 log-cycle) at a higher temperature (90ºC) and approximately the same time. Hence, it seems that the SC-CO_2 + HPU treatment was more effective for *Bacillus* spores. Therefore, since the inactivation mechanisms of each technology are different, the effectiveness of the treatment depends on the type of microorganism and its particular resistance to those mechanisms.

The comparison of both combined technologies in terms of industrial implementation is not easy since several aspects should be taken into consideration. The use of high pressures in SC-CO_2 + HPU treatments implies a higher initial investment than in the case of the implementation of the PEF technology. However, a wider variety of products can be treated by SC-CO_2 + HPU while a more limited number are appropriate to be treated by PEF, due to the conductivity characteristics needed for its optimum application. Notwithstanding, nowadays, there are very few companies in Europe manufacturing SC-CO_2 or PEF equipment to treat products at industrial levels, which makes these technologies rare in the industry.

6. CONCLUSIONS

Based on the obtained experimental results of the present thesis, the main conclusions are listed and divided according to the two chapters of the Results and Discussion section. Moreover, a general conclusion is outlined at the end of this section.

6.1. SC-CO_2 + HPU microbial inactivation (CHAPTER 1)

6.1.1. Supercritical carbon dioxide treatments

- For all the microorganisms studied (*E. coli*, *B. diminuta*, *A. niger* spores, *C. butyricum* spores and *B. subtilis* spores), higher inactivation levels were achieved by the SC-CO_2 treatments compared to the thermal ones.
- The microbial inactivation in oil-in-water emulsions using SC-CO_2 was highly time-consuming, which could seriously restrict its industrial application.

6.1.2. Effect of the application of HPU in the SC-CO_2 treatments

- HPU improved the SC-CO_2 inactivation of vegetative bacteria (*E. coli* and *B. diminuta*) in both water and emulsions. Therefore, shorter processing times than in the treatments using only SC-CO_2 can be applied.
- HPU also enhanced the SC-CO_2 inactivation of *A. niger* spores in water. However, no effect of HPU was found on the inactivation of *A. niger* spores in the oil-in-water emulsion, which could indicate that the effect of ultrasonic cavitation on SC-CO_2 treatments may depend both on the type of microorganism and the composition of the medium.
- Microscopy analyses revealed important morphological changes on vegetative bacteria and fungal spores treated with SC-CO_2 + HPU in water, including disintegrated wall cells and loss of cytoplasmic content.
- The combined SC-CO_2 + HPU enhanced the inactivation of *C. butyricum* and *B. subtilis* spores in the oil-in water emulsions compared to the individual SC-CO_2 treatment.

- The SC-CO_2 + HPU treatment (95ºC and 350 or 550 bar) had no effect on the inactivation of *G. stearothermophilus* spores.

6.1.3. Effect of pressure and temperature

- The higher the pressure and temperature, the greater the SC-CO_2 and SC-CO_2 + HPU inactivation of *B. diminuta* and *A. niger* spores in water. However, only the temperature affected the inactivation of *E. coli* in water for both the SC-CO_2 and SC-CO_2 + HPU treatments.
- The higher the pressure and the temperature, the greater the SC-CO_2 and SC-CO_2 + HPU inactivation of *E.coli*, *B. diminuta* and *A. niger* in the oil-in-water emulsion.
- The higher the temperature, the larger the SC-CO_2 + HPU inactivation of the bacterial spores (*C. butyricum* and *B. subtilis*).
- The pressure increase improved SC-CO_2 + HPU inactivation of the bacterial spores (*C. butyricum* and *G. stearothermophilus*) until a threshold was reached (350 bar).

6.1.4. Effect of the type of microorganism

- In general terms, no differences in the resistance to the SC-CO_2 and SC-CO_2 + HPU treatments were found between the vegetative bacteria (*E. coli* and *B. diminuta*).
- *A. niger* spores were found to be more resistant than the vegetative bacteria (*E. coli* and *B. diminuta*), regardless of the type of treatment (thermal, SC-CO_2 or SC-CO_2+ HPU) or the treatment medium.
- *C. butyricum* spores were more resistant to the inactivation treatments (thermal, SC-CO_2 and SC-CO_2 + HPU) than *A. niger* spores.
- *B. subtilis* spores were more resistant to the inactivation treatments (thermal, SC-CO_2 and SC-CO_2 + HPU) than *C. butyricum.* No difference was found between

the resistance of spores from the genus *Bacillus* (*B. subtilis* and *B. pumilus*) to the inactivation treatments applied (thermal and SC-CO_2 + HPU).

- *G. stearothermophilus* spores were the most resistant microorganism since even with the most intense treatment applied (SC-CO_2 + HPU at 550 bar, 95ºC and 20 min) the inactivation achieved was below 0.5 log-cycles reduction.

6.1.5. Effect of the treatment media

- Oil content in the treatment media protected the vegetative bacteria from SC-CO_2 inactivation. The higher the oil content in the medium (0, 10, 20 and 30%), the lower the effectiveness in the SC-CO_2 inactivation.
- The application of HPU reduced the protective effect of the oil on the vegetative bacteria inactivation.
- The treatment media (water or oil-in-water emulsions) did not have a significant effect on the resistance of *A. niger* spores to SC-CO_2 or SC-CO_2 + HPU treatments.
- The presence of oil in the emulsions did not affect the SC-CO_2 + HPU inactivation, which was completely achieved for all the microorganisms, including bacterial spores, except for *G. stearothermophilus*.

6.1.6. Effect of the SC-CO_2 + HPU treatments on the physicochemical properties of the emulsions

- In general terms, only a mild effect of the process variables (temperature, pressure and time) on the quality of the emulsions was found.
- Treated emulsions had good electrostatic stability (ζ-potential ≤ -30 mV) and changed density minimally. However, in general terms, they presented lower pH and higher droplet size.

- The selection of adequate conditions of pressure, temperature and treatment time preserved the quality and stability of the emulsions, while achieving a satisfactory microbial inactivation, except for *G. stearothermophilus* spores.

6.2. PEF and HPU microbial inactivation (CHAPTER 2)

6.2.1. Effect of the treatment conditions

- The higher the field strength, time and input temperature in the PEF treatments, the greater the inactivation of *E. coli*.
- The effect of the total energy applied in the PEF treatment was also noticeable on the inactivation of *A. niger* spores.
- The longer the HPU treatment, the greater the inactivation of *E.coli*.
- The inactivation levels achieved by the individual PEF and HPU treatments were limited and the complete inactivation was not reached for any of the microorganisms studied (*E. coli, A. niger* spores and *B. pumilus* spores).

6.2.2. Effect of the combined treatments

- The combined PEF-HPU sequence was more effective than the reverse one (HPU-PEF).
- Synergistic effects on inactivation were found with the combined PEF-HPU treatment for all the studied microorganisms.
- PEF-HPU was able to completely inactivate vegetative bacteria and fungal spores. However, it seems not to be effective inactivating bacterial spores since only 1 log-cycle of *B. pumilus* spores was reduced.

6.2.3. Effect of the type of microorganism

- *E. coli* was more sensitive to PEF and HPU treatments than *A. niger* and *B. pumilus* spores, being *B. pumilus* the most resistant microorganism.

6.3. General conclusion

It can be concluded that the simultaneous application of HPU and SC-CO_2 enhanced the solubilization of CO_2 and mass and heat transfer processes, which facilitates the SC-CO_2 inactivation mechanisms. In addition, HPU could also damage cell membranes, increasing its permeability. This was evidenced by the observation of the SC-CO_2 + HPU-inactivated vegetative bacteria and fungal spores, since important membrane and cytoplasmic alterations were shown. Moreover, the combined SC-CO_2 + HPU technology was feasible for the microbial inactivation in oil-in-water emulsions while maintaining their physicochemical stability.

The sequential application of HPU after PEF treatments led to synergistic effects on microbial inactivation, compared to the individual treatments. After the PEF treatment, the remaining surviving cells were probably sublethally damaged, leading to incremented sensitivity to the HPU treatment.

Therefore, the application of HPU in non-thermal treatments (SC-CO_2 and PEF) could lead to short the processing time and/or to apply milder process conditions to achieve a target microbial inactivation. Thus, the cost of the processes could be reduced and higher product quality could be obtained, which is very relevant for industrial applications. In addition, the protective effect that some components of the media, such as oil, exert on microbial inactivation, could be minimized by the strong effect of HPU. Therefore, these emerging technologies, applied in combination, could be an alternative to thermal treatments of oil-in-water emulsions used in the food and pharmaceutical industries, minimizing quality changes on the treated products.

7. RECOMMENDATIONS

According to the results obtained in the present PhD Thesis and in order to improve the knowledge related to the different emerging non-thermal technologies studied (SC-CO_2 , PEF and HPU), applied in individual and combined form, further research on the following topics is recommended:

Microorganisms

- To evaluate combined treatments (SC-CO_2 + HPU and PEF-HPU) on the inactivation of other types of microorganisms, different from those studied in the present PhD Thesis, such as viruses and other bacteria and from different provenance, in particular, on pathogen and spoilage microorganisms naturally present in food and pharmaceutical products.

- To study the effect of the size, shape, type of cell wall and concentration of the microorganisms present in the treatment medium on the microbial inactivation.

Inactivation mechanisms

- To deep on the inactivation mechanisms involved in the combined treatments, especially those related to bacterial spores inactivation, since these mechanisms have not been fully elucidated in the present work. For that purpose, several analyses could be performed, such as the study of the external and internal cell structure by microscopy techniques and the identification of the possible increase of the membrane permeability using staining agents, changes in the enzymatic activity and sublethally injured cells by the use of selective and non-selective growing media,.

- To elucidate how the order of the sequence of the combined PEF and HPU treatments affects the inactivation mechanisms involved.

Composition of treatment medium

- To measure the effect of the oil content of the emulsions studied in the present Thesis on the inactivation of the individual and combined PEF and HPU treatments.

- To study the individual effect of other properties, different from the oil content, such as pH, viscosity, conductivity and protein content in model simple media, on the inactivation achieved by the SC-CO_2 + HPU and the PEF-HPU treatments. In addition, the study could be extended to other food and pharmaceutical products, including other commercial emulsions.

Quality of the treated products

- To extend the study of the SC-CO_2 + HPU treatment effects on other additional properties of the oil-in-water emulsions, such as lipid oxidation, viscosity or color changes, among others.

- To study the effect of the combined PEF and HPU technologies on the physicochemical properties of the emulsions

- To determine the effect of the combined treatments on the quality of other treated media, different from the oil-in-water emulsions investigated in the present PhD Thesis, such as water-in-oil emulsions or fruit and vegetable juices.

Industrial implementation

- To improve technical aspects of the inactivation systems:

  - To adapt both batch systems studied in the present Thesis (SC-CO_2 + HPU and PEF-HPU), to be applied in a continuous mode, in order to make these technologies more suitable for industrial implementation.
  - To design a flexible ultrasonic system that allows to adjust the supplied power and frequency and to study the effect of these variables on microbial inactivation.
  - To design and build a device capable of simultaneously applying the PEF and HPU fields and study its effectiveness on the inactivation.
  - To decrease the electrolysis and corrosion phenomena related to the PEF technology by the design of titanium electrodes or the application of bipolar squared pulses.

- To evaluate possible approaches to increase inactivation effectiveness, such as the recirculation of the sample, or the use of several chambers in order to increase the treatment time in both combined treatments (SC-CO_2 + HPU and PEF + HPU).

-To evaluate the shelf-life of the treated products in terms of microbial and physicochemical stability during storage.

- To assess the technical and economic feasibility of the different combined treatments to be industrially implemented and to select optimum conditions to find a compromise between inactivation effectiveness, changes in the physicochemical properties of the products and economic cost.

-To compare both combined technologies in terms of inactivation of target microorganisms, preservation of the product quality, cost of the treatment and environmental impact.

8. SCIENTIFIC CONTRIBUTION

Research papers

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., Quiles A. & Benedito, J. (2021). Microbial inactivation by means of ultrasonic assisted supercritical CO2. Effect on cell ultrastructure.-Submitted

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2020). Non-thermal pasteurization of lipid emulsions by combined supercritical carbon dioxide and high-power ultrasound treatment. Ultrasonics Sonochemistry, 67, 105–138. https://doi.org/10.1016/J.ULTSONCH.2020.105138

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2021). Combination of supercritical CO2 and high-power ultrasound for the inactivation of fungal and bacterial spores in lipid emulsions- Submitted.

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2021). Ultrasonic-assisted supercritical CO2 inactivation of bacterial spores and effect on the physicochemical properties of oil-in-water emulsions. The Journal of Supercritical Fluids, 174(March), 105246. https://doi.org/10.1016/j.supflu.2021.105246

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2021). Combined pulsed electric field and high power ultrasound treatments for the microbial inactivation of oil-in-water emulsions- Submitted.

Contributions to congresses

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2019). Pasteurización de emulsiones lipídicas con CO_2 supercrítico asistida mediante ultrasonidos de potencia. X Congreso Nacional de Ciencia y Tecnología de los Alimentos (CyTA/CESIA). León (Spain).

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2019). Microbial inactivation in lipid emulsions using supercritical CO_2 assisted by high power ultrasound. International Congress on Ultrasonics (ICU). Bruges (Belgium).

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2020). Effect of pressure and treating media on the supercritical CO_2 inactivation of *Brevundiomonas diminuta*. VII International Student Congress of Food Science and Technology. Valencia, Spain.

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2020). Inactivation of *E.coli* in lipid emulsions with pulsed electric fields. VII International Student Congress of Food Science and Technology. Valencia (Spain).

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2020). Ultrasonic assisted supercritical fluid treatment for the inactivation of *Clostridium butyricum* spores in lipid emulsions. VII International Student Congress of Food Science and Technology. Valencia (Spain).

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2021). Inactivation of *Bacillus subtilis* spores in lipid emulsions by supercritical CO_2 combined with high power ultrasound. 18 th European Meeting on Supercritical Fluids (EMSF). Bordeaux (France).

Gomez-Gomez, A., Brito-de la Fuente, E., Gallegos, C., Garcia-Perez, J. V., & Benedito, J. (2021). Effect of temperature, oil content and high power ultrasound on the supercritical CO_2 pasteurization of lipid emulsions. 18 th European Meeting on Supercritical Fluids (EMSF). Bordeaux (France).

9. REFERENCES

The references used in the Results and Discussion section of the present PhD Thesis are included in each of the parts into which the aforementioned section is divided.

Aadil, R. M., Zeng, X. A., Han, Z., Sahar, A., Khalil, A. A., Rahman, U. U., Khan, M., & Mehmood, T. (2018). Combined effects of pulsed electric field and ultrasound on bioactive compounds and microbial quality of grapefruit juice. *Journal of Food Processing and Preservation*, *42*(2). https://doi.org/10.1111/jfpp.13507

Ahmad, T., Butt, M. Z., Aadil, R. M., Inam-ur-Raheem, M., Abdullah, Bekhit, A. E. D., Guimarães, J. T., Balthazar, C. F., Rocha, R. S., Esmerino, E. A., Freitas, M. Q., Silva, M. C., Sameen, A., & Cruz, A. G. (2019). Impact of nonthermal processing on different milk enzymes. *International Journal of Dairy Technology*, *72*(4), 481–495. https://doi.org/10.1111/1471-0307.12622

Amaral, G. V., Silva, E. K., Cavalcanti, R. N., Cappato, L. P., Guimaraes, J. T., Alvarenga, V. O., Esmerino, E. A., Portela, J. B., Sant' Ana, A. S., Freitas, M. Q., Silva, M. C., Raices, R. S. L., Meireles, M. A. A., & Cruz, A. G. (2017). Dairy processing using supercritical carbon dioxide technology: Theoretical fundamentals, quality and safety aspects. *Trends in Food Science and Technology*, *64*, 94–101. https://doi.org/10.1016/j.tifs.2017.04.004

Amran, M. H. H., Zulfakar, M. H., Danik, M. F., Abdullah, M. S. P., & Shamsuddin, A. F. (2019). A new alternative for intravenous lipid emulsion 20% w/w from superolein oil and its effect on lipid and liver profiles in an animal model. *DARU, Journal of Pharmaceutical Sciences*, *27*(1), 191–201. https://doi.org/10.1007/s40199-019-00262-7

Anton, M., Chapleau, N., Beaumal, V., Delépine, S., & De Lamballerie-Anton, M. (2001). Effect of high-pressure treatment on rheology of oil-in-water emulsions prepared with hen egg yolk. *Innovative Food Science and Emerging Technologies*, *2*(1), 9–21. https://doi.org/10.1016/S1466-8564(00)00036-9

Anton, N., Benoit, J.-P., & Saulnier, P. (2008). Design and production of nanoparticles formulated from nano-emulsion templates—A review. *Journal of Controlled*

Release, *128*(3), 185–199. https://doi.org/10.1016/J.JCONREL.2008.02.007

Arreola, A. G., Balaban, M. O., Marshall, M. R., Pepelow, A. J., Wei, C. I., & Cornell, J. A. (1991). Supercritical Carbon Dioxide Effects on Some Quality Attributes of Single Strength Orange Juice. *Journal of Food Science*, *56*(4), 1030–1033. https://doi.org/10.1111/j.1365-2621.1991.tb14634.x

Atalar, I., Gul, O., Saricaoglu, F. T., Besir, A., Gul, L. B., & Yazici, F. (2019). Influence of thermosonication (TS) process on the quality parameters of high pressure homogenized hazelnut milk from hazelnut oil by-products. *Journal of Food Science and Technology*, *56*(3), 1405–1415. https://doi.org/10.1007/s13197-019-03619-7

Ávila, M., Gómez-Torres, N., Hernández, M., & Garde, S. (2014). Inhibitory activity of reuterin, nisin, lysozyme and nitrite against vegetative cells and spores of dairy-related Clostridium species. *International Journal of Food Microbiology*, *172*, 70–75. https://doi.org/10.1016/j.ijfoodmicro.2013.12.002

Awad, T. S., Moharram, H. A., Shaltout, O. E., Asker, D., & Youssef, M. M. (2012). Applications of ultrasound in analysis, processing and quality control of food: A review. *Food Research International*, *48*(2), 410–427. https://doi.org/10.1016/j.foodres.2012.05.004

Azmi, N. A. N., Elgharbawy, A. A. M., Motlagh, S. R., Samsudin, N., & Salleh, H. M. (2019). Nanoemulsions: Factory for food, pharmaceutical and cosmetics. *Processes*, *7*(9). https://doi.org/10.3390/pr7090617

Balakrishna, A. K., Abdul Wazed, M., & Farid, M. (2020). A review on the effect of high pressure processing (HPP) on gelatinization and infusion of nutrients. *Molecules*, *25*(10), 1–19. https://doi.org/10.3390/molecules25102369

Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., Saraiva, J. A., Raso, J., Martín-Belloso, O., Witrowa-Rajchert, D., Lebovka, N., & Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. *Food Research International*, *77*, 773–798. https://doi.org/10.1016/j.foodres.2015.09.015

Barbosa-Cánovas, G. V., Tapia, M. S., & Cano, M. P. (2005). *Novel food processing technologies*. CRC Press, Boca Raton.

Barsotti, L., Dumay, E., Mu, T. H., Fernandez Diaz, M. D., & Cheftel, J. C. (2001). Effects of high voltage electric pulses on protein-based food constituents and structures. *Trends in Food Science and Technology*, *12*(3–4), 136–144. https://doi.org/10.1016/S0924-2244(01)00065-6

Bermúdez-Aguirre, D., & Barbosa-Cánovas, G. V. (2011). An update on high hydrostatic pressure, from the laboratory to industrial applications. *Food Engineering Reviews*, *3*(1), 44–61. https://doi.org/10.1007/s12393-010-9030-4

Bermúdez-Aguirre, D., Corradini, M. G., Mawson, R., & Barbosa-Cánovas, G. V. (2009). Modeling the inactivation of Listeria innocua in raw whole milk treated under thermo-sonication. *Innovative Food Science and Emerging Technologies*, *10*(2), 172–178. https://doi.org/10.1016/j.ifset.2008.11.005

Bonnaillie, L. M., & Tomasula, P. M. (2015). Carbon dioxide: An alternative processing method for milk. *Emerging Dairy Processing Technologies: Opportunities for the Dairy Industry*, 205–250. https://doi.org/10.1002/9781118560471.ch8

Brewer, M. S. (2009). Irradiation effects on meat flavor: A review. *Meat Science*, *81*(1), 1–14. https://doi.org/10.1016/j.meatsci.2008.07.011

Buzrul, S. (2012). High hydrostatic pressure treatment of beer and wine: A review. *Innovative Food Science and Emerging Technologies*, *13*(JANUARY), 1–12. https://doi.org/10.1016/j.ifset.2011.10.001

Cameron, M., McMaster, L. D., & Britz, T. J. (2009). Impact of ultrasound on dairy spoilage microbes and milk components. *Dairy Science and Technology*, *89*, 83–98. https://doi.org/10.1051/dst/2010003

Cappelletti, M., Ferrentino, G., & Spilimbergo, S. (2014). Supercritical carbon dioxide combined with high power ultrasound: An effective method for the pasteurization of coconut water. *The Journal of Supercritical Fluids*, *92*, 257–263. https://doi.org/10.1016/J.SUPFLU.2014.06.010

Cárcel, J. A., García-Pérez, J. V., Benedito, J., & Mulet, A. (2012). Food process

innovation through new technologies: Use of ultrasound. *Journal of Food Engineering*, *110*(2), 200–207. https://doi.org/10.1016/J.JFOODENG.2011.05.038

Chansiri, G., Lyons, R. T., Patel, M. V., & Hem, S. L. (1999). Effect of surface charge on the stability of oil/water emulsions during steam sterilization. *Journal of Pharmaceutical Sciences*, *88*(4), 454–458. https://doi.org/10.1021/js980293i

Chaturvedi, P., Patel, N., & Lodhi, S. (1992). Effect of terminal heat sterilization on the stability of phospholipid-stabilized submicron emulsions. *Acta Pharmaceutica Nordica*, *4*(1), 51–55.

Chemat, F., Grondin, I., Sing, A. S. C., & Smadja, J. (2004). Deterioration of edible oils during food processing by ultrasound. *Ultrasonics Sonochemistry*, *11*(1), 13–15. https://doi.org/10.1016/S1350-4177(03)00127-5

Chen, H., & Hoover, D. G. (2004). Use of Weibull model to describe and predict pressure inactivation of Listeria monocytogenes Scott A in whole milk. *Innovative Food Science and Emerging Technologies*, *5*(3), 269–276. https://doi.org/10.1016/j.ifset.2004.03.002

Damar, S., & Balaban, M. O. (2006). Review of dense phase CO2 technology: microbial and enzyme inactivation, and effects on food quality. *Journal of Food Science*, *71*(1), 1–11.

de Oliveira Silva, A. C., de Oliveira, L. A. T., de Jesus, E. F. O., Cortez, M. A. S., Alves, C. C. C., Monteiro, M. L. G., & Conte Junior, C. A. (2015). Effect of gamma irradiation on the bacteriological and sensory analysis of raw whole milk under refrigeration. *Journal of Food Processing and Preservation*, *39*, 2404–2411. https://doi.org/10.1111/jfpp.12490

Deeth, H. C., & Datta, N. (2018). Non-thermal technologies: Pulsed electric field technology and ultrasonication. In *Reference Module in Food Science*. Elsevier. https://doi.org/10.1016/b978-0-08-100596-5.22608-5

Delso, C., Martínez, J. M., Cebrián, G., Álvarez, I., & Raso, J. (2020). Understanding the occurrence of tailing in survival curves of Salmonella Typhimurium treated by

pulsed electric fields. *Bioelectrochemistry*, *135*, 107580. https://doi.org/10.1016/j.bioelechem.2020.107580

Desrumaux, A., & Marcand, J. (2002). Formation of sunflower oil emulsions stabilized by whey proteins with high-pressure homogenization (up to 350 MPa): Effect of pressure on emulsion characteristics. *International Journal of Food Science and Technology*, *37*(3), 263–269. https://doi.org/10.1046/j.1365-2621.2002.00565.x

Diels, A. M. J., & Michiels, C. W. (2006). High-pressure homogenization as a non-thermal technique for the inactivation of microorganisms. *Critical Reviews in Microbiology*, *32*(4), 201–216. https://doi.org/10.1080/10408410601023516

Dong, P., Georget, E., Aganovic, K., Heinz, V., & Mathys, A. (2016). Inactivation of Bacillus amyloliquefaciens spores by continuous high-pressure-assisted thermal sterilization in an oil-in-water (o/w) emulsion with 10 % soybean oil. *European Food Research and Technology*, *242*(6), 935–942. https://doi.org/10.1007/s00217-015-2600-1

Driscoll, D. F. (2006). Lipid injectable emulsions: 2006. *Nutrition in Clinical Practice*, *21*(4), 381–386. https://doi.org/10.1177/0115426506021004381

Dunn, J. (1996). Pulsed light and pulsed electric field for foods and eggs. *Poultry Science*, *75*(9), 1133–1136. https://doi.org/10.3382/ps.0751133

Elias, M. I., Madureira, J., Santos, P. M. P., Carolino, M. M., Margaça, F. M. A., & Cabo Verde, S. (2020). Preservation treatment of fresh raspberries by e-beam irradiation. *Innovative Food Science and Emerging Technologies*, *66*(July), 102487. https://doi.org/10.1016/j.ifset.2020.102487

European Medicines Agency. (2016). *Guideline on sterilisation of the medicinal product, active substance, excipient and primary container.*

Evrendilek, G. A., Li, S., Dantzer, W. R., & Zhang, Q. H. (2004). Puelsed electric field processing of beer: Microbial , sensory , and quality analyses. *Journal of Food Science*, *69*, M228–M232.

Fan, L., Hou, F., Muhammad, A. I., Ruiling, L. V., Watharkar, R. B., Guo, M., Ding, T., & Liu, D. (2019). Synergistic inactivation and mechanism of thermal and

ultrasound treatments against Bacillus subtilis spores. *Food Research International*, *116*(September 2018), 1094–1102. https://doi.org/10.1016/j.foodres.2018.09.052

Ferrentino, G., Plaza, M. L., Ramirez-Rodrigues, M., Ferrari, G., & Balaban, M. O. (2009). Effects of dense phase carbon dioxide pasteurization on the physical and quality attributes of a red grapefruit juice. *Journal of Food Science*, *74*(6). https://doi.org/10.1111/j.1750-3841.2009.01250.x

Ferrentino, G., & Spilimbergo, S. (2015). High pressure carbon dioxide combined with high power ultrasound pasteurization of fresh cut carrot. *The Journal of Supercritical Fluids*, *105*, 170–178. https://doi.org/10.1016/J.SUPFLU.2014.12.014

Ferrentino, G., & Spilimbergo, S. (2016). A combined high pressure carbon dioxide and high power ultrasound treatment for the microbial stabilization of cooked ham. *Journal of Food Engineering*, *174*, 47–55. https://doi.org/10.1016/J.JFOODENG.2015.11.013

Ferrentino, G., & Spilimbergo, S. (2017). Non-thermal pasteurization of apples in syrup with dense phase carbon dioxide. *Journal of Food Engineering*, *207*, 18–23. https://doi.org/10.1016/j.jfoodeng.2017.03.014

Floyd, A. G. (1999). Top ten considerations in the development of parenteral emulsions. *Pharmaceutical Science and Technology Today*, *2*(4), 134–143. https://doi.org/10.1016/S1461-5347(99)00141-8

Gallegos, C., Valencia, C., Partal, P., Franco, J. M., Maglio, O., Abrahamsson, M., & Brito-De La Fuente, E. (2012). Droplet-size distribution and stability of commercial injectable lipid emulsions containing fish oil. *American Journal of Health-System Pharmacy*, *69*(15), 1332–1335. https://doi.org/10.2146/ajhp110520

Gao, Y., Nagy, B., Liu, X., Simándi, B., & Wang, Q. (2009). Supercritical CO2 extraction of lutein esters from marigold (Tagetes erecta L.) enhanced by ultrasound. *The Journal of Supercritical Fluids*, *49*(3), 345–350. https://doi.org/10.1016/J.SUPFLU.2009.02.006

Garcia-Gonzalez, L., Geeraerd, A. H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Van Impe, J. F., & Devlieghere, F. (2007). High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. In *International Journal of Food Microbiology* (Vol. 117, Issue 1, pp. 1–28). https://doi.org/10.1016/j.ijfoodmicro.2007.02.018

Gasperi, F., Aprea, E., Biasioli, F., Carlin, S., Endrizzi, I., Pirretti, G., & Spilimbergo, S. (2009). Effects of supercritical CO2 and N2O pasteurisation on the quality of fresh apple juice. *Food Chemistry*, *115*(1), 129–136. https://doi.org/10.1016/j.foodchem.2008.11.078

Gharibzahedi, S. M. T., Hernández-Ortega, C., Welti-Chanes, J., Putnik, P., Barba, F. J., Mallikarjunan, K., Escobedo-Avellaneda, Z., & Roohinejad, S. (2019). High pressure processing of food-grade emulsion systems: Antimicrobial activity, and effect on the physicochemical properties. *Food Hydrocolloids*, *87*(March 2018), 307–320. https://doi.org/10.1016/j.foodhyd.2018.08.012

Gondrexon, N., Cheze, L., Jin, Y., Legay, M., Tissot, Q., Hengl, N., Baup, S., Boldo, P., Pignon, F., & Talansier, E. (2015). Intensification of heat and mass transfer by ultrasound: Application to heat exchangers and membrane separation processes. *Ultrasonics Sonochemistry*, *25*(1), 40–50. https://doi.org/10.1016/j.ultsonch.2014.08.010

Grandison, A. S. (2012). Irradiation. In *Food Processing Handbook.* (pp. 153–177).

Haas, G. J., Prescott, H. E., Dudley, E., Dik, R., Hintlian, C., & Keane, L. (1989). Inactivation of Microorganisms By Carbon Dioxide Under Pressure. *Journal of Food Safety*, *9*(4), 253–265. https://doi.org/10.1111/j.1745-4565.1989.tb00525.x

Heinz, V., Alvarez, I., Angersbach, A., & Knorr, D. (2001). Preservation of liquid foods by high intensity pulsed electric fields—basic concepts for process design. *Trends in Food Science & Technology*, *12*(3–4), 103–111. https://doi.org/10.1016/S0924-2244(01)00064-4

Hippalgaonkar, K., Majumdar, S., & Kansara, V. (2010). Injectable lipid emulsions-advancements, opportunities and challenges. *AAPS PharmSciTech*, *11*(4),

1526–1540. https://doi.org/10.1208/s12249-010-9526-5

Hodgins, A. M., Mittal, G. S., & Griffiths, M. W. (2002). Pasteurization of fresh orange juice using low-energy pulsed electrical field. *Journal of Food Science*, *67*(6), 2294–2299. https://doi.org/10.1111/j.1365-2621.2002.tb09543.x

Hong, S. I., Park, W. S., & Pyun, Y. R. (1997). Inactivation of Lactobacillus sp. from Kimchi by high pressure carbon dioxide. *LWT - Food Science and Technology*, *30*(7), 681–685. https://doi.org/10.1006/FSTL.1997.0250

Hong, S. I., Park, W. S., & Pyun, Y. R. (1999). Non-thermal inactivation of Lactobacillus plantarum as influenced by pressure and temperature of pressurized carbon dioxide. *International Journal of Food Science and Technology*, *34*(2), 125–130. https://doi.org/10.1046/j.1365-2621.1999.00241.x

Huang, E., Mittal, G. S., & Griffiths, M. W. (2006). Inactivation of Salmonella enteritidis in liquid whole egg using combination treatments of pulsed electric field, high pressure and ultrasound. *Biosystems Engineering*, *94*(3), 403–413. https://doi.org/10.1016/j.biosystemseng.2006.03.008

Huang, H.-W., Lung, H.-M., Yang, B. B., & Wang, C. Y. (2014). Responses of microorganisms to high hydrostatic pressure processing. *Food Control*, *40*(1), 250–259. https://doi.org/10.1016/j.foodcont.2013.12.007

Jeyamkondan, S., Jayas, D. S., & Holley, R. A. (1999). Pulsed electric field processing of foods: a review. *Journal of Food Protection*, *62*(9), 1088–1096. https://doi.org/10.1007/s13197-015-2061-3

Jo, C., & Ahn, D. U. (2000). Production of volatile compounds from irradiated oil emulsion containing amino acids of proteins. *Journal of Food Science*, *65*(4), 612–616. https://doi.org/10.1111/j.1365-2621.2000.tb16059.x

Kamat, A., Warke, R., Kamat, M., & Thomas, P. (2000). Low-dose irradiation as a measure to improve microbial quality of ice cream. *International Journal of Food Microbiology*, *62*(1–2), 27–35. https://doi.org/10.1016/S0168-1605(00)00251-8

Kincal, D., Hill, W. S., Balaban, M., Portier, K. M., Sims, C. A., Wei, C. I., & Marshall, M. R. (2006). A continuous high-pressure carbon dioxide system for cloud and

quality retention in orange juice. *Journal of Food Science*, *71*(6). https://doi.org/10.1111/j.1750-3841.2006.00087.x

Knorr, D., Zenker, M., Heinz, V., & Lee, D. U. (2004). Applications and potential of ultrasonics in food processing. *Trends in Food Science and Technology*, *15*(5), 261–266. https://doi.org/10.1016/j.tifs.2003.12.001

Larrea-Wachtendorff, D., Sousa, I., & Ferrari, G. (2020). Starch-Based Hydrogels Produced by High-Pressure Processing (HPP): Effect of the Starch Source and Processing Time. *Food Engineering Reviews*. https://doi.org/10.1007/s12393-020-09264-7

Lett, A. M., Yeomans, M. R., Norton, I. T., & Norton, J. E. (2015). Enhancing expected food intake behaviour, hedonics and sensory characteristics of oil-in-water emulsion systems through microstructural properties, oil droplet size and flavour. *Food Quality and Preference*.

Lewis, S. J., Velasquez, A., Cuppett, S. L., & McKee. (2002). Effect of electron beam irradiation on poultry meat safety and quality. *Poultry Science*, *81*(6), 896–903. https://doi.org/https://doi.org/10.1093/ps/81.6.896

Li, X., & Farid, M. (2016). A review on recent development in non-conventional food sterilization technologies. *Journal of Food Engineering*, *182*, 33–45. https://doi.org/10.1016/J.JFOODENG.2016.02.026

Li, Y.-Q., Chen, Q., Liu, X. H., & Chen, Z.-X. (2008). Inactivation of soybean lipoxygenase in soymilk by pulsed electric fields. *Food Chemistry*, *109*(2), 408–414. https://doi.org/10.1016/j.foodchem.2008.01.001

Liao, H., Hu, X., Liao, X., Chen, F., & Wu, J. (2007). Inactivation of Escherichia coli inoculated into cloudy apple juice exposed to dense phase carbon dioxide. *International Journal of Food Microbiology*, *118*(2), 126–131. https://doi.org/10.1016/J.IJFOODMICRO.2007.06.018

Lidgate, D. M., Tarttner, T., Shultz, R. M., & Maskiewicz, R. (1992). Sterile filtration of a parenteral emulsion. *Pharmaceutical Research*, *9*(7).

Liu, G., Carøe, C., Qin, Z., Munk, D. M. E., Crafack, M., Petersen, M. A., & Ahrné, L.

(2020). Comparative study on quality of whole milk processed by high hydrostatic pressure or thermal pasteurization treatment. *Lwt*, *127*(April), 109370. https://doi.org/10.1016/j.lwt.2020.109370

Liu, J., Kharat, M., Tan, Y., Zhou, H., Muriel Mundo, J. L., & McClements, D. J. (2020). Impact of fat crystallization on the resistance of W/O/W emulsions to osmotic stress: Potential for temperature-triggered release. *Food Research International*, *134*(March), 109273. https://doi.org/10.1016/j.foodres.2020.109273

Llavata, B., García-Pérez, J. V., Simal, S., & Cárcel, J. A. (2020). Innovative pre-treatments to enhance food drying: a current review. *Current Opinion in Food Science*, *35*, 20–26. https://doi.org/10.1016/j.cofs.2019.12.001

López-Malo, A., Palou, E., Jiménez-Fernández, M., Alzamora, S. M., & Guerrero, S. (2005). Multifactorial fungal inactivation combining thermosonication and antimicrobials. *Journal of Food Engineering*, *67*(1–2), 87–93. https://doi.org/10.1016/j.jfoodeng.2004.05.072

Lu, G. W., & Gao, P. (2010). Emulsions and microemulsions for topical and transdermal drug delivery. In *Handbook of Non-Invasive Drug Delivery Systems* (pp. 59–94). https://doi.org/10.1016/b978-0-8155-2025-2.10003-4

Lung, H. M., Cheng, Y. C., Chang, Y. H., Huang, H. W., Yang, B. B., & Wang, C. Y. (2015). Microbial decontamination of food by electron beam irradiation. *Trends in Food Science and Technology*, *44*(1), 66–78. https://doi.org/10.1016/j.tifs.2015.03.005

Lyu, C., Huang, K., Yang, N., Wang, H., & Wang, J. (2016). Combination of thermosonication and pulsed electric fields treatments for controlling Saccharomyces cerevisiae in Chinese rice wine. *Food and Bioprocess Technology*, *9*(11), 1854–1864. https://doi.org/10.1007/s11947-016-1769-z

Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, *72*(1–2), 107–113. https://doi.org/10.1016/S0168-1605(01)00624-9

Martín-Belloso, O., & Sobrino-López, A. (2011). Combination of pulsed electric fields with other preservation techniques. *Food and Bioprocess Technology*, *4*(6), 954–968. https://doi.org/10.1007/s11947-011-0512-z

Mastwijk, H. C., & Bartels, P. V. (2007). Future potential of pulsed electric field treatment: Treatment of bacterial spores, emulsions and packed products. In *Food Preservation by Pulsed Electric Fields: From Research to Application* (Issue 1997). Woodhead Publishing Limited. https://doi.org/10.1533/9781845693831.3.307

McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition*, *47*(7), 611–649. https://doi.org/10.1080/10408390701289292

McClements, D. J. (2015). *Food emulsions. Principles, practices and techniques*. CRC press.

Mendes, K. F., Mendes, K. F., Guedes, S. F., Silva, L. C. A. S., & Arthur, V. (2020). Evaluation of physicochemical characteristics in cherry tomatoes irradiated with 60Co gamma-rays on post-harvest conservation. *Radiation Physics and Chemistry*, *177*(June), 109139. https://doi.org/10.1016/j.radphyschem.2020.109139

Michalac, S., Alvarez, V., Ji, T., & Zhang, Q. H. (2003). Inactivation of selected microorganisms and properties of pulsed electric field processed milk. *Journal of Food Processing and Preservation*, *27*(2), 137–151. https://doi.org/10.1111/j.1745-4549.2003.tb00507.x

Mikkonen, K. S. (2020). Strategies for structuring diverse emulsion systems by using wood lignocellulose-derived stabilizers. *Green Chemistry*, *22*(4), 1019–1037. https://doi.org/10.1039/c9gc04457d

Monfort, S., Ramos, S., Meneses, N., Knorr, D., Raso, J., & Álvarez, I. (2012). Design and evaluation of a high hydrostatic pressure combined process for pasteurization of liquid whole egg. *Innovative Food Science & Emerging Technologies*, *14*, 1–10. https://doi.org/10.1016/J.IFSET.2012.01.004

Montejo, O., Cardona, D., Sanchez, F., Rigueira, A. I., Coll, P., & Bonal, J. (2000). Microbiological quality control study of “all-in-one” total parenteral nutrition admixtures. *Journal of Parenteral and Enteral Nutrition*, *24*(3), 183–186. https://doi.org/10.1177/014860710002400318

Mosqueda-Melgar, J., Elez-Martínez, P., Raybaudi-Massilia, R. M., & Martín-Belloso, O. (2008). Effects of pulsed electric fields on pathogenic microorganisms of major concern in fluid foods: A review. *Critical Reviews in Food Science and Nutrition*, *48*(8), 747–759. https://doi.org/10.1080/10408390701691000

Muriel Mundo, J. L., Zhou, H., Tan, Y., Liu, J., & McClements, D. J. (2020). Stabilization of soybean oil-in-water emulsions using polypeptide multilayers: Cationic polylysine and anionic polyglutamic acid. *Food Research International*, *137*(May), 109304. https://doi.org/10.1016/j.foodres.2020.109304

Noci, F., Walkling-Ribeiro, M., Cronin, D. A., Morgan, D. J., & Lyng, J. G. (2009). Effect of thermosonication, pulsed electric field and their combination on inactivation of Listeria innocua in milk. *International Dairy Journal*, *19*(1), 30–35. https://doi.org/10.1016/j.idairyj.2008.07.002

Noman, E., Norulaini Nik Ab Rahman, N., Al-Gheethi, A., Nagao, H., Talip, B. A., & Ab. Kadir, O. (2018). Selection of inactivation medium for fungal spores in clinical wastes by supercritical carbon dioxide. *Environmental Science and Pollution Research*, *25*(22), 21682–21692. https://doi.org/10.1007/s11356-018-2335-1

Odriozola-Serrano, I., Bendicho-Porta, S., & Martín-Belloso, O. (2006). Comparative study on shelf life of whole milk processed by high-intensity pulsed electric field or heat treatment. *Journal of Dairy Science*, *89*(3), 905–911. https://doi.org/10.3168/jds.S0022-0302(06)72155-5

Oliveira, F. A. de, Neto, O. C., Santos, L. M. R. dos, Ferreira, E. H. R., & Rosenthal, A. (2017). Effect of high pressure on fish meat quality – A review. *Trends in Food Science and Technology*, *66*, 1–19. https://doi.org/10.1016/j.tifs.2017.04.014

Ortuño, C., Duong, T., Balaban, M., & Benedito, J. (2013). Combined high hydrostatic pressure and carbon dioxide inactivation of pectin methylesterase, polyphenol

oxidase and peroxidase in feijoa puree. *Journal of Supercritical Fluids*, *82*, 56–62. https://doi.org/10.1016/j.supflu.2013.06.005

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012a). Supercritical carbon dioxide inactivation of Escherichia coli and Saccharomyces cerevisiae in different growth stages. *The Journal of Supercritical Fluids*, *63*, 8–15. https://doi.org/10.1016/J.SUPFLU.2011.12.022

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2012b). An ultrasound-enhanced system for microbial inactivation using supercritical carbon dioxide. *Innovative Food Science & Emerging Technologies*, *15*, 31–37. https://doi.org/10.1016/J.IFSET.2012.02.006

Ortuño, C., Martínez-Pastor, M. T., Mulet, A., & Benedito, J. (2013). Application of high power ultrasound in the supercritical carbon dioxide inactivation of Saccharomyces cerevisiae. *Food Research International*, *51*(2), 474–481. https://doi.org/10.1016/J.FOODRES.2013.01.041

Ortuño, C., Quiles, A., & Benedito, J. (2014). Inactivation kinetics and cell morphology of E. coli and S. cerevisiae treated with ultrasound-assisted supercritical CO2. *Food Research International*, *62*, 955–964. https://doi.org/10.1016/J.FOODRES.2014.05.012

Palgan, I., Muñoz, A., Noci, F., Whyte, P., Morgan, D. J., Cronin, D. A., & Lyng, J. G. (2012). Effectiveness of combined pulsed electric field (PEF) and manothermosonication (MTS) for the control of Listeria innocua in a smoothie type beverage. *Food Control*, *25*(2), 621–625. https://doi.org/10.1016/j.foodcont.2011.11.009

Pan, L., Xing, J., Zhang, H., Luo, X., & Chen, Z.-X. (2020). Electron beam irradiation as a tool for rice grain storage and its effects on the physicochemical properties of rice starch. *International Journal of Biological Macromolecules*, *164*, 2915–2921. https://doi.org/10.1016/j.ijbiomac.2020.07.211

Paniagua-Martínez, I., Mulet, A., García-Alvarado, M. A., & Benedito, J. (2016). Ultrasound-assisted supercritical CO2 treatment in continuous regime:

Application in Saccharomyces cerevisiae inactivation. *Journal of Food Engineering*, *181*, 42–49. https://doi.org/10.1016/J.JFOODENG.2016.02.024

Paniagua-Martínez, Ingrid, Ramírez-Martínez, A., Serment-Moreno, V., Rodrigues, S., & Ozuna, C. (2018). Non-thermal Technologies as Alternative Methods for Saccharomyces cerevisiae Inactivation in Liquid Media: a Review. *Food and Bioprocess Technology*, *11*(3), 487–510. https://doi.org/10.1007/s11947-018-2066-9

Paniwnyk, L. (2017). Applications of ultrasound in processing of liquid foods: A review. *Ultrasonics Sonochemistry*, *38*, 794–806. https://doi.org/10.1016/j.ultsonch.2016.12.025

Park, S. J., Lee, J. I., & Park, J. (2002). Effects of a combined process of high-pressure carbon dioxide and high hydrostatic pressure on the quality of carrot juice. *Journal of Food Science*, *67*(5), 1827–1834. https://doi.org/10.1111/j.1365-2621.2002.tb08730.x

Pataro, G., De Lisi, M., Donsì, G., & Ferrari, G. (2014). Microbial inactivation of E. coli cells by a combined PEF-HPCD treatment in a continuous flow system. *Innovative Food Science and Emerging Technologies*, *22*, 102–109. https://doi.org/10.1016/j.ifset.2013.12.009

Pataro, G., Ferrentino, G., Ricciardi, C., & Ferrari, G. (2010). Pulsed electric fields assisted microbial inactivation of S. cerevisiae cells by high pressure carbon dioxide. *Journal of Supercritical Fluids*, *54*(1), 120–128. https://doi.org/10.1016/j.supflu.2010.04.003

Peleg, M. (2006). *Models for predicting growth and inactivation. Advanced quantitative microbiology for foods and biosystems*.

Perrut, M. (2012). Sterilization and virus inactivation by supercritical fluids (a review). *The Journal of Supercritical Fluids*, *66*, 359–371. https://doi.org/10.1016/J.SUPFLU.2011.07.007

Pertkiewicz, M., Cosslett, A., Mühlebach, S., & Dudrick, S. J. (2009). Basics in clinical nutrition: Stability of parenteral nutrition admixtures. *E-SPEN*, *4*(3), 117–119.

https://doi.org/10.1016/j.eclnm.2009.01.010

Piyasena, P., Mohareb, E., & McKellar, R. . (2003). Inactivation of microbes using ultrasound: a review. *International Journal of Food Microbiology*, *87*(3), 207–216. https://doi.org/10.1016/S0168-1605(03)00075-8

Rahman, U. ur, Sahar, A., Ishaq, A., Aadil, R. M., Zahoor, T., & Ahmad, M. H. (2018). Advanced meat preservation methods: A mini review. *Journal of Food Safety*, *38*(4). https://doi.org/10.1111/jfs.12467

Rao, L., Xu, Z., Wang, Y., Zhao, F., Hu, X., & Liao, X. (2015). Inactivation of Bacillus subtilis spores by high pressure CO2 with high temperature. *International Journal of Food Microbiology*, *205*, 73–80. https://doi.org/10.1016/J.IJFOODMICRO.2015.04.012

Raso, J., Frey, W., Ferrari, G., Pataro, G., Knorr, D., Teissie, J., & Miklavčič, D. (2016). Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes. *Innovative Food Science and Emerging Technologies*. https://doi.org/10.1016/j.ifset.2016.08.003

Reverter-Carrión, L., Sauceda-Gálvez, J. N., Codina-Torrella, I., Hernández-Herrero, M. M., Gervilla, R., & Roig-Sagués, A. X. (2018). Inactivation study of Bacillus subtilis, Geobacillus stearothermophilus, Alicyclobacillus acidoterrestris and Aspergillus niger spores under ultra-high pressure homogenization, UV-C light and their combination. *Innovative Food Science and Emerging Technologies*, *48*(June 2017), 258–264. https://doi.org/10.1016/j.ifset.2018.06.011

Ribeiro, N., Soares, G. C., Santos-Rosales, V., Concheiro, A., Alvarez-Lorenzo, C., García-González, C. A., & Oliveira, A. L. (2020). A new era for sterilization based on supercritical CO2 technology. *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, *108*(2), 399–428. https://doi.org/10.1002/jbm.b.34398

Riera, P., Garrido-Alejos, G., Cardenete, J., Moliner, E., Zapico-Muñiz, E., Cardona, D., & Garin, N. (2018). Physicochemical stability and sterility of standard parenteral nutrition solutions and simulated Y-Site admixtures for neonates.

Nutrition in Clinical Practice, *33*(5), 694–700. https://doi.org/10.1002/ncp.10013

Rzoska, S., Musial, F., Rutkowska, Fonberg-Broczek, M., Sokolowska, B., Drozd-Rzoska, A., & Nowakowska, J. (2015). Simultaneous impact of high pressures and pulsed electric field on Saccharomyces cerevisiae model system. *Journal of Food Processing & Technology*, *6*(12). https://doi.org/10.4172/2157-7110.1000518

Sahena, F., Zaidul, I. S. M., Jinap, S., Karim, A. A., Abbas, K. A., Norulaini, N. A. N., & Omar, A. K. M. (2009). Application of supercritical CO2 in lipid extraction - A review. *Journal of Food Engineering*, *95*(2), 240–253. https://doi.org/10.1016/j.jfoodeng.2009.06.026

Salve, A. R., Pegu, K., & Arya, S. S. (2019). Comparative assessment of high-intensity ultrasound and hydrodynamic cavitation processing on physico-chemical properties and microbial inactivation of peanut milk. *Ultrasonics Sonochemistry*, *59*(August), 104728. https://doi.org/10.1016/j.ultsonch.2019.104728

Santacatalina, J. V., Contreras, M., Simal, S., Cárcel, J. A., & Garcia-Perez, J. V. (2016). Impact of applied ultrasonic power on the low temperature drying of apple. *Ultrasonics Sonochemistry*, *28*, 100–109. https://doi.org/10.1016/j.ultsonch.2015.06.027

Sasagawa, A., Hoshino, J., Fujii, T., Yamazaki, A., Ohshima, T., Yamada, A., Kobayashi, A., & Sato, M. (2006). Inactivation of Bacillus subtilis spores by a combination of hydrostatic high-pressure and pulsed electric field treatments. *Review of High Pressure Science and Technology/Koatsuryoku No Kagaku To Gijutsu*, *16*(1), 45–53. https://doi.org/10.4131/jshpreview.16.45

Sihvonen, M., Järvenpää, E., Hietaniemi, V., & Huopalahti, R. (1999). Advances in supercritical carbon dioxide technologies. *Trends in Food Science and Technology*, *10*(6–7), 217–222. https://doi.org/10.1016/S0924-2244(99)00049-7

Soares, G. C., Learmonth, D. A., Vallejo, M. C., Davila, S. P., González, P., Sousa, R. A., & Oliveira, A. L. (2019). Supercritical CO2 technology: The next standard sterilization technique? *Materials Science and Engineering: C*, *99*, 520–540.

https://doi.org/10.1016/J.MSEC.2019.01.121

Spilimbergo, S., Bertucco, A., Lauro, F. M., & Bertoloni, G. (2003). Inactivation of Bacillus subtilis spores by supercritical CO2 treatment. *Innovative Food Science and Emerging Technologies*, *4*(2), 161–165. https://doi.org/10.1016/S1466-8564(02)00089-9

Spilimbergo, S., Cappelletti, M., Tamburini, S., Ferrentino, G., & Foladori, P. (2014). Partial permeabilisation and depolarization of Salmonella enterica Typhimurium cells after treatment with pulsed electric fields and high pressure carbon dioxide. *Process Biochemistry*, *49*(12), 2055–2062. https://doi.org/10.1016/j.procbio.2014.10.003

Sulaiman, A., Farid, M., & Silva, F. V. M. (2017). Quality stability and sensory attributes of apple juice processed by thermosonication, pulsed electric field and thermal processing. *Food Science and Technology International*, *23*(3), 265–276. https://doi.org/10.1177/1082013216685484

Tahergorabi, R., Matak, K. E., & Jaczynski, J. (2012). Application of electron beam to inactivate Salmonella in food: Recent developments. *Food Research International*, *45*(2), 685–694. https://doi.org/10.1016/j.foodres.2011.02.003

Takagi, J., Khalidi, N., Wolk, R. A., Tjolsen, E., de Leon, R., & Wesley, J. R. (1989). Sterility of total parenteral nutrient solutions stored at room temperature for seven days. *American Journal of Hospital Pharmacy*, *46*(5), 973–977. https://doi.org/10.1093/ajhp/46.5.973

Tischler, B. Y., & Hohl, T. M. (2019). Menacing mold: recent advances in Aspergillus pathogenesis and host defense. *Journal of Molecular Biology*, *431*(21), 4229–4246. https://doi.org/10.1016/j.jmb.2019.03.027

Tiwari, B. K., O'Donnell, C. P., & Cullen, P. J. (2009). Effect of non thermal processing technologies on the anthocyanin content of fruit juices. *Trends in Food Science and Technology*, *20*(3–4), 137–145. https://doi.org/10.1016/j.tifs.2009.01.058

Trujillo, A. J. (2002). Applications of high-hydrostatic pressure on milk and dairy products. *High Pressure Research*, *22*(3–4), 619–626.

https://doi.org/10.1080/08957950212449

van Boekel, M., Fogliano, V., Pellegrini, N., Stanton, C., Scholz, G., Lalljie, S., Somoza, V., Knorr, D., Jasti, P. R., & Eisenbrand, G. (2010). A review on the beneficial aspects of food processing. *Molecular Nutrition and Food Research*, *54*(9), 1215–1247. https://doi.org/10.1002/mnfr.200900608

Van Impe, J., Smet, C., Tiwari, B., Greiner, R., Ojha, S., Stulić, V., Vukušić, T., & Režek Jambrak, A. (2018). State of the art of nonthermal and thermal processing for inactivation of micro-organisms. *Journal of Applied Microbiology*, *125*(1), 16–35. https://doi.org/10.1111/jam.13751

Villamiel, M., & de Jong, P. (2000). Influence of high-intensity ultrasound and heat treatment in continuous flow on fat, proteins, and native enzymes of milk. *Journal of Agricultural and Food Chemistry*, *48*(2), 472–478. https://doi.org/10.1021/jf990181s

Walkling-Ribeiro, M., Noci, F., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2009). Shelf life and sensory evaluation of orange juice after exposure to thermosonication and pulsed electric fields. *Food and Bioproducts Processing*, *87*(2), 102–107. https://doi.org/10.1016/j.fbp.2008.08.001

Walkling-Ribeiro, M., Noci, F., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2010). Shelf life and sensory attributes of a fruit smoothie-type beverage processed with moderate heat and pulsed electric fields. *LWT - Food Science and Technology*, *43*(7), 1067–1073. https://doi.org/10.1016/j.lwt.2010.02.010

Walkling-Ribeiro, M., Noci, F., Riener, J., Cronin, D. A., Lyng, J. G., & Morgan, D. J. (2009). The impact of thermosonication and pulsed electric fields on Staphylococcus aureus inactivation and selected quality parameters in orange juice. *Food and Bioprocess Technology*, *2*(4), 422–430. https://doi.org/10.1007/s11947-007-0045-7

Wanten, G. J. A. (2015). Parenteral lipids: Safety aspects and toxicity. *World Review of Nutrition and Dietetics*, *112*, 63–70. https://doi.org/10.1159/000365434

Watanabe, T., Furukawa, S., Hirata, J., Koyama, T., Orihara, H., & Yamasaki, M.

(2003). Inactivation of Geobacillus stearothermophilus spores by high-pressure carbon dioxide treatment. *Cambridge University Press*, *69*(12), 7124–7129. https://doi.org/10.1128/AEM.69.12.7124

Werner, B. G., & Hotchkiss, J. H. (2006). Continuous flow nonthermal CO2 processing: The lethal effects of subcritical and supercritical CO2 on total microbial populations and bacterial spores in raw milk. *Journal of Dairy Science*, *89*(3), 872–881. https://doi.org/10.3168/jds.S0022-0302(06)72151-8

World Health Organization. (1994). Safety and nutritional adequacy of irradiated food. In *World Health Organization*. https://doi.org/10.1016/s0969-806x(96)90041-9

Xiang, B. Y., Simpson, M. V., Ngadi, M. O., & Simpson, B. K. (2011). Flow behaviour and viscosity of reconstituted skimmed milk treated with pulsed electric field. *Biosystems Engineering*, *109*(3), 228–234. https://doi.org/10.1016/j.biosystemseng.2011.04.004

Yang, N., Huang, K., Lyu, C., & Wang, J. (2016). Pulsed electric field technology in the manufacturing processes of wine, beer, and rice wine: A review. *Food Control*, *61*, 28–38. https://doi.org/10.1016/j.foodcont.2015.09.022

Yeom, H. W., Streaker, C. B., Howard Zhang, Q., & Min, D. B. (2000). Effects of pulsed electric fields on the quality of orange juice and comparison with heat pasteurization. *Journal of Agricultural and Food Chemistry*, *48*(10), 4597–4605. https://doi.org/10.1021/jf000306p

Yildiz, S., Pokhrel, P. R., Unluturk, S., & Barbosa-Cánovas, G. V. (2020). Changes in quality characteristics of strawberry juice after equivalent high pressure, ultrasound, and pulsed electric fields processes. *Food Engineering Reviews*. https://doi.org/10.1007/s12393-020-09250-z