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The combined application of ultrasound and UVC have the potential to control mature *Listeria monocytogenes* and *Bacillus subtilis* biofilms

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ABSTRACT

The present study aims to determine the bactericidal and disintegrating effect of ultraviolet type C treatments combined with ultrasound against biofilms of *Listeria monocytogenes* and *Bacillus subtilis* formed at different incubation periods (*i.e.* 24, 48, and 72 h). Treatments applied are UVC doses ranging from 60 to 360 mJ/cm^2 (5–30 s) and US doses of 5–15 min, depending on the maturity of biofilms and the microorganism. Initially, we investigated the effects of UVC on planktonic cells, revealing a significantly higher lethality in *L. monocytogenes* compared to *B. subtilis* ($P < 0.05$). Moreover, *B. subtilis* demonstrated greater resistance in both its spore and vegetative forms ($P < 0.05$). The application of UVC on biofilms demonstrates that, as the more structured and robust the biofilm is, the more difficult it is for UVC to penetrate the biofilm structure and reach the cells that conform them, which is why higher lethality is observed in the shortest formation period (*i.e.* 24 h). In addition, as the UVC dose increases, microorganisms show a higher resistance to the treatment, thus showing a greater reduction in the microbial population at lower doses. The single application of ultrasound (US) manages to reduce the cell load between 1 and 4 log CFU/cm^2, depending on the microorganism and exposure time. The results obtained after the combination of both technologies achieve higher lethality, 5.2 log reductions at the lowest doses (*i.e.* applying 5 min of US and 5 s of UVC). Furthermore, the maximum reduction possible for these experiments ($\geq$5.6 log) is achieved in any treatment applying a 15-s UVC exposure time. Significant differences are observed ($P < 0.05$) as the maturity stage increases ([+Let] $24^a > 48^b > 72^c$ hours). The combination of these two technologies demonstrates highly promising outcomes across all examined bacterial structures, including spores and vegetative biofilms.

1. Introduction

Nowadays, significant resources are allocated in the food industry to remove pathogenic and spoilage microorganisms adhered in industrial surfaces due to their relation to cross-contamination phenomena and the derived economic and public health problems. However, despite all efforts, some microbial cells can resist the applied treatments, ultimately persisting and conforming what is known as resident microbiota (Hascoët et al., 2019). This microbiota is constantly in contact with other transient microorganisms, potentially colonizing, developing, and forming biofilms which can harbor pathogenic or spoilage species within them (Agustín & Brugnoni, 2018). Biofilms, described as sessile microbial communities adhered irreversibly to a substrate or interface and embedded in a self-produced matrix of extracellular polymeric substances (Donlan & Costerton, 2002), present a challenge for the food sector due to their difficulty in control (González-Rivas et al., 2018). Although continuous measures are taken to limit the persistence of such communities (*i.e.* cleaning and disinfection procedures), these are not intended to eliminate all microorganisms conforming the structures but rather to reduce them to safe levels (Fratamico et al., 2009; Hascoët et al., 2019). Therefore, the control of the residual microbiota is completely crucial to avoid cross-contamination to final products and minimize safety and spoilage problems.

Among the persistent microorganisms found in industrial settings, *Listeria monocytogenes* is pointed out due to its high risk to public health in terms of incidence and case severity (EFSA, 2021). It has been indicated that the persistence of this pathogen could be related to subinhibitory exposure to disinfectants used in the food industry for microbial

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that force by *L. monocytogenes* to acquire resistance mechanisms control (Mazaheri et al., 2021). Moreover, this phenomenon could also be explained by the formation of biofilms that act as protected microenvironments and the existence of niches or reservoirs in the environment not reached by disinfectants (Ripolles-Avila et al., 2019). One of the main industries affected by the persistence of *L. monocytogenes* biofilms is the meat industry. The persistence of *L. monocytogenes* in the meat industry is favored by several factors closely related to biofilm formation and disinfectant resistance. These factors include sublethal exposure to disinfectants, biofilm formation, and the presence of niches or reservoirs in the environment. Sublethal exposure to disinfectants commonly used in the food industry can inadvertently induce the selection of resistant *L. monocytogenes* strains (Mazaheri et al., 2021). Additionally, *L. monocytogenes* has a remarkable ability to form biofilms, creating a protective environment that shields cells from the action of disinfectants (Ripolles-Avila et al., 2019). Finally, food processing environments harbor hidden areas such as cracks or crevices in equipment where biofilms can establish and persist, evading sanitation procedures. In response to this situation, Regulation (EC) No 853/2004, which establishes specific hygiene requirements for food of animal origin, specifies that a thermal treatment in water at a temperature higher than 82 °C or any equivalent treatment must be applied to tools used during food handling/processing. However, this approach has inherent limitations, including high energy consumption, increasing operational costs, and inefficacy against established biofilms due to thermal resistance (Alonso Calleja et al., 2022). Therefore, there is a need to seek for alternatives to disinfect industrial surfaces by minimizing those problems, being a potential one the combination of ultrasounds (US) for biofilm matrix disruption and cell dispersal, and Ultraviolet-C (UVC) rays for cell elimination.

US is a sound or vibration with a frequency above the range of human hearing that can disrupt biofilms structure (Zips et al., 1990). It acts similarly to a bactericide (Bott & Tianqing, 2004) but with the advantage of not inducing microbial resistance (Pinto et al., 2020). Its mechanism of action involves the generation of sound waves that produce cavitation bubbles, which implode and produce a significant amount of kinetic energy (Erriu et al., 2014). This energy can break the cells that form a biofilm, dispersing their components and eliminating the three-dimensional structure that supports it. It has been determined that the detachment rate depends on three factors: (i) sound intensity (*i.e.* threshold 2 W/cm^2), (ii) exposure time, and (iii) the distance between the transducer and the membrane (Zips et al., 1990). This technology has been used to enhance the efficacy of treatments such as the application of antimicrobial agents, with the aim of allowing greater access of these agents to the biofilm structure (Pinto et al., 2020). In the present study, it is tested as a strategy for the disintegration of the structural matrix that supports the biofilm to facilitate the incision of UVC rays inside it, thereby enhancing their lethal effect.

On the other hand, UV are rays of light that fall within the electromagnetic spectrum in the range of 100–400 nm, which can be further subdivided into four types depending on their properties: (i) from 100 to 200 nm is called vacuum UV, as it can only be transmitted in a vacuum and is absorbed by most substances; (ii) from 200 to 280 nm is known as short-wave UV or UVC and it is the range of interest in the present study due to its potent microbial inactivation capability (Yin et al., 2015); (iii) the range from 280 to 315 nm corresponds to medium-wave UV or UVB, responsible for harmful effects on human cells; (iv) finally, the range from 320 to 400 nm corresponds to long-wave UV or UVA (Forney & Moraru, 2009). UVC lamps can emit monochromatic light continuously at a wavelength of 253.7 nm. This wavelength is very close to the maximum absorption wavelength of nucleic acids in cells, which is between 260 and 265 nm, causing DNA alterations in microorganisms (Yin et al., 2015). Their mode of action is related to the generation of hydrogen peroxide or superoxide anions that interact with the functional structures of microorganisms and form covalent bonds between different nitrogenous bases, mainly forming thymine and pyrimidine dimers. In the latter case, the bonds prevent the transcription and replication of nucleic acids, ultimately leading to the death of the affected microorganisms (Begum et al., 2009).

To evaluate the elimination capacity of the aforementioned technologies, tests were conducted against biofilms formed by *L. monocytogenes* and *Bacillus subtilis*. The latter was selected as the model microorganism in these experiments as it is used as a reference organism in UV-C treatments because of its resistance (Chang et al., 1985; FDA, 2000). The general objective was to determine the disintegrating and bactericidal effect of combined US and UVC treatments against biofilms of *L. monocytogenes* and *B. subtilis* formed at different incubation periods. With this goal in mind, specific objectives were established: (i) to establish differences in lethality between different microorganisms and their states (*i.e.* sporulated and vegetative), (ii) to determine if the UVC, at the applied doses, achieves microbial lethality for different stages of maturity in biofilms and microorganisms, establishing from what dose (*i.e.* exposure time) it could be considered as a disinfectant effect (*i.e.* reductions of $\geq$4 log), (iii) to determine if the US-based technology effectively disintegrates biofilms and ascertain whether the disintegration caused by US facilitates the bactericidal effect of UVC technology, and (iv) to observe whether the maturity state of a biofilm influences the elimination of the cells that comprise it after the application of the treatments under study.

2. Materials and methods

2.1. Microbial strains and inoculum preparation

Listeria monocytogenes CECT 4031 and *Bacillus subtilis* CECT 4002 (*i.e.* in both their vegetative and sporulated forms) were used in the present study. Both strains were obtained from the Spanish Type Culture Collection (CECT, University of Valencia, Valencia, Spain). Microbial recovery was done following the instructions of the CECT lyophilized recovery protocol (CECT, 2023) and, subsequently, a cryobank was prepared for strain preservation. Starting from a pure *L. monocytogenes* or *B. subtilis* culture obtained on Tryptone Soy Agar (TSA, Oxoid, Basingstoke, UK), an isolated colony was transferred to a tube containing porous beads (*i.e.* cryoballs) and a cryoprotective medium (Microkit, Madrid, Spain). The tube was vigorously agitated for 1 min and allowed it to stand for an additional minute. Then, the excess liquid was removed using a sterile Pasteur pipette and the cryobank vials were stored at a frozen temperature of -18 ± 2 °C until use. To recover the microorganisms from the cryobanks, the cryoballs were suspended in Tryptone Soy Broth (TSB, Oxoid) and incubated for 24 h at 37 °C, after which they were plated on TSA plates and incubated for 24 h at 37 °C. From that plate, the inoculum was prepared by suspending some colonies in 0.9% saline solution until a density of 1.0 and 1.5 on the McFarland scale was obtained for *L. monocytogenes* and *B. subtilis* (*i.e.* vegetative), respectively, using a densitometer (Densimat, Biomérieux, Crappone, France). These values corresponded to a cell concentration of approximately 10^8 CFU/mL, which, diluted until 10^6 CFU/mL was used as the inoculum for the planktonic assays.

Spores of *B. subtilis* were obtained following a protocol based on UNE EN 13704:2019 (AENOR, 2019). Briefly, the lyophilized culture was rehydrated in 10 mL of glucose and tryptone broth (TGB: 2.5 g of yeast extract (Oxoid), 5 g of tryptone (Oxoid), 1 g of glucose (Sigma-Aldrich, St. Louis, MI, USA), in 1 L of distilled water, pH adjusted to 7.2), and incubated for 24 h at 30 °C. Two milliliters of this culture were transferred to Roux bottles containing yeast extract agar [MYA: 10 g of meat extract (Oxoid), 2 g of yeast extract (Oxoid), 15 g of agar (Oxoid), and 0.04 g of $MnSO_4 \cdot H_2O$ (Merck, Darmstadt, Germany) in 1 L of distilled water], which were incubated at 30 °C for up to 30 days. The formed spores were collected by adding 20 mL of sterile distilled water to the Roux bottles and scraping the surface with a Digralsky stick. Spore suspensions were pooled and washed four times in 15 mL cold sterile water by centrifugation at 10,000×*g* for 20 min at 4 °C using a Sigma

4K15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The resulting sediment was then suspended in 30 mL of sterile distilled water and subjected to heat treatment at 75 °C for 10 min to ensure the inactivation of vegetative cells. The resulting spore suspension was stored at 4 °C until use.

2.2. Biofilm formation

Starting with a concentration of 10^8 CFU/mL for both microorganisms, series of dilutions were prepared on TSB to reach 10^6 CFU/mL. Subsequently, 30 μL of this concentration was inoculated onto stainless steel AISI 316 coupons with a diameter of 2 cm and a thickness of 3 mm to simulate the contact surfaces of tools, machinery, and installations in the food industries. Before their use, surfaces were cleaned and disinfected following the European standard UNE-EN 13697:2015 related to non-porous materials, and then sterilized by autoclaving them at 121 °C for 15 min.

Inoculated surfaces were placed in Petri dishes to maintain sterile conditions and were incubated at 30 ± 2 °C in a humid chamber to force relative humidity saturation (≥90%) (Fuster-Valls et al., 2008). The present study assessed the biofilm maturation variable with incubation times of 24, 48, and 72 h. After the incubation period, surfaces underwent a series of washes with distilled water (*i.e.* 3 mL, twice per coupon) to remove non-adherent biofilm cells (Ripolles-Avila et al., 2018). Once the washes were completed, the biofilms were subjected to various selected treatments.

2.3. UVC treatments

For the UVC treatments, a prototype of UVC reactor (UV-Consulting Peschl España SL, Geldo, Spain) was used, consisting of a metal cabinet with dimensions of 50 x 26 × 13.5 cm (width x depth x height, respectively), three levels of height to place sample, and three UVC lamps, each measuring 45 cm, distributed in parallel along the bottom (Fig. 1). Since it was a prototype, a mapping and characterization of the UVC source was conducted. The UVC intensity (mW/cm^2) was determined along the length, width, and height inside the cabinet, and the most suitable position for placing the samples and exposing them to UVC light was selected, avoiding shadows, dark areas, and critical points (*i.e.* low intensity). Finally, the intermediate height position (*i.e.* 9 cm from the lamps) and at the center of the X (*i.e.* width) and Y (*i.e.* depth) axes were selected. Once the sample position was selected, it was characterized with the intensity profile it received over time, observing that the maximum intensity of 12 mW/cm^2 was reached after 30–40 min, and it remained at least for 2 h (*i.e.* sufficient time to conduct the experiments for each assay). The UVC light intensity was measured using a UVC sensor or radiometer (UVM-CP model 90155, UV-Consulting Peschl, Mainz, Germany), with a reading range between 0 and 20 mW/cm^2.

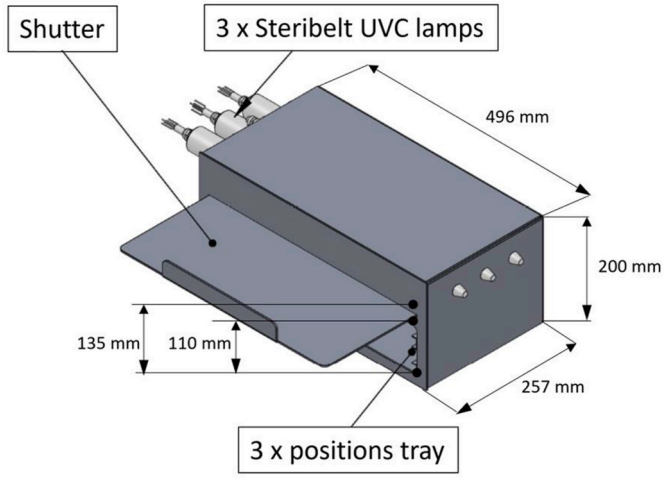

Fig. 1. Schematic illustration of the UVC reactor prototype. Source UV-Consulting Peschl España SL, Geldo, Spain (2024).

To determine the lethal dose for each target bacteria as planktonic cultures (*i.e.* without interference or protection induced by the biofilm), 30 μl of the inoculum suspension at 10^6 CFU/mL (see section 2.1) were deposited to the surface. Tests were conducted at different exposure times (*i.e.* 0, 5, 10, 15, 20, 40, and 60 s) with an intensity of 12 mW/cm^2, applying the respective irradiance doses (*i.e.* 0, 60, 120, 180, 300, 600, and 900 mJ/cm^2) of UVC. Regarding the experiments involving biofilm (see section 2.2) with the formation periods of 24, 48, and 72 h, exposure times (*i.e.* 0, 5, 10, 15, 20, 40, and 60 s) were applied for both microorganisms. For the combination with US treatments, exposure times of 0, 5, and 15 s were applied for *L. monocytogenes*, and 0, 15, and 30 s for both forms of *B. subtilis*. These exposure times were determined based on the results obtained and expressed in section 3.1, where the microorganism's resistance to UVC was determined and the doses established based on the seconds required for a 4 log reduction in CFU/cm^2 following UNE-EN 13697 standard.

2.4. Combined US-UVC treatments

The application of both technologies was conducted sequentially, consecutively (*i.e.* not simultaneously), with the US treatment being applied first followed by the UVC. The equipment used to apply US consisted of an ultrasonic bath (VEVOR, model JPS-40A, Barcelona, Spain), with a 10 L capacity, temperature control up to 90 °C, and a power of 240 W at 40 KHz ultrasonics. Surfaces with the pre-formed biofilms were placed in glass beakers containing 10 mL of physiological serum [9 g of NaCl (Panreac, Castellar del Vallès, Spain) per litre] with the biofilm always positioned in the upper part to prevent any friction between the bottom of the beaker and the surface that could affect the biofilm structure. The glass beakers were submerged in the water bath of the US equipment, and the treatment was applied at a controlled temperature (*i.e.* 25 °C). The wave transmission media in contact with the biofilms, temperature, and the material of the beaker can influence the results (Gallo et al., 2018). In this study, physiological serum was chosen as the direct medium in contact with the biofilm and for wave transmission. Exposure times of 5, 10, and 15 min were applied for both microorganisms (*i.e. L. monocytogenes* and *B. subtilis*) and biofilm maturation states (*i.e.* 24, 48, and 72 h). Due to surfaces being fully submerged in the physiological serum when the treatment was applied, some of the cells that conformed the biofilm become suspended what could contaminate other parts of the coupons. Since this could lead to overestimated results, coupons were rinsed with 6 mL of sterile saline solution and subjected to an intense UVC treatment (*i.e.* 1 min) on the side of the surface where the biofilm was not located. Subsequently, UVC doses were applied to complete all the combinations.

2.5. Microbial quantification

Treated and non-treated (*i.e.* control) were transferred to sterile containers along with 3.5 ± 0.2 g of 2 mm diameter glass beads and 10 mL of neutralizer [1 g of tryptone (BD, Madrid, Spain), 8.5 g of NaCl (Panreac), 30 g of Tween 80 (Scharlab, Barcelona, Spain), and 1000 mL of deionized water (pH 7.0 ± 0.2)]. Subsequently, containers were subjected to agitation in a vortex for 1.30 min at a frequency of 40 Hz to detach cells conforming the biofilm community (Ripolles-Avila et al., 2019). From each sample, a series of dilutions in Phosphate-Buffered Saline (PBS) solution consisting of 0.24 g of KH_2PO_4 (Panreac) in 1000 mL of distilled water with a pH adjusted to 7.4 ± 0.2. Microbial quantification was performed on specific culture media for each microorganism: ALOA (Biomérieux, Cornellà de Llobregat, Spain) for *L. monocytogenes*, and TSA medium enriched with 0.6% yeast extract (Oxoid) for the vegetative forms of *B. subtilis*. Spores count was done by

subjecting to a heat treatment of 75 °C for 10 min to ensure the elimination of vegetative cells and force the spores to germinate (UNE-EN 13704:2019). Plates were incubated at 37 °C for 24 and 48 h for the enumeration of *B. subtilis* and *L. monocytogenes*, respectively. After the corresponding incubation period, the characteristic colonies were counted on each medium.

2.6. Data processing and statistical analysis

To calculate the lethality of each treatment, **Equation 1** was used:

$$\text{Lethality} = \text{Log}_{10}\ (N_0/N)$$

Where: N_0 is the initial cell number present on the surface before the treatment, expressed in CFU/cm^2, and N is the number of colonies after subjecting the samples to the UVC or US-UVC combination treatments, expressed in CFU/cm^2. This calculation is known in the literature as the Biofilm Inactivation Factor (BIF) and was proposed by Luo et al. (2022) and Murray et al. (2015).

All experiments were performed in duplicate on three different days (n = 6). The bacterial counts obtained were converted into logarithmic decimal values to coincide with the assumption of a normal distribution. ANOVA with post hoc of Duncan was performed to compare the microorganisms used and the treatments applied (*i.e.* biofilms, US, UVC, and their combinations). The analysis was conducted using the STATISTICA data analysis system version 7 (StatSoft, Inc., 2004; California, United States). A *P*-value of <0.05 was established to consider a statistically significant level.

3. Results and discussion

3.1. Bactericidal effect of UVC treatments in planktonic cultures

To date, most studies related to UVC disinfection focus on planktonic cells, paying limited attention to the application of this technology for biofilm control (Luo et al., 2022). However, knowing the response to different doses in individual cells provides information about the treatment to be applied when bacteria are attached, since it is known that higher UVC intensities are required to inactivate them as the microbial cells become protected (Gora et al., 2019). Thus, Fig. 2 shows the lethality of *L. monocytogenes* and *B. subtilis* (*i.e.* spores and vegetative cells) planktonic cells after the application of different UVC doses. Results demonstrate a higher lethality of *L. monocytogenes* cells than *B. subtilis* cells ($P < 0.05$), the latter being significantly more resistant in its sporulated form than the vegetative state ($P < 0.05$), as also reported Martínez-García et al. (2023) and Hanlin et al. (1985). Both microorganisms were exposed to an intensity of 12 mW/cm^2 during different UVC application times that varied between 5 and 60 s. Exposure to any dose causes physical movements of electrons and destroys DNA bonds (Kim et al., 2016). UV light induces the generation of two major photoproducts due to direct photon absorption by pyrimidine and purine nucleic acid bases, named cyclobutane pyrimidine dimer (CPDs) and Pyrimidine-pyrimidone (6-4) photoproduct (6-4 PP) (Osakabe et al., 2015). These photoproducts lead to structural DNA distortion and disrupt DNA transcription and replication, ultimately leading to mutagenesis or cell death (Kim et al., 2016). In this sense, Gram-positive bacteria are generally more resistant to UV light than Gram-negative bacteria. This was demonstrated by Beauchamp and Lacroix (2012) who indicated that *L. monocytogenes* produced 35% less CPDs and 10% less 6-4 PPs than *Escherichia coli* during a UV lamp irradiation dose of 3 J/cm^2. For this reason, two Gram-positive bacteria were selected for the study, one for being considered as one of the major biofilm producers in the food industry (Ripolles-Avila et al., 2019) and the other for its ability to sporulate as a protection system in stressful situations (McKenney et al., 2013).

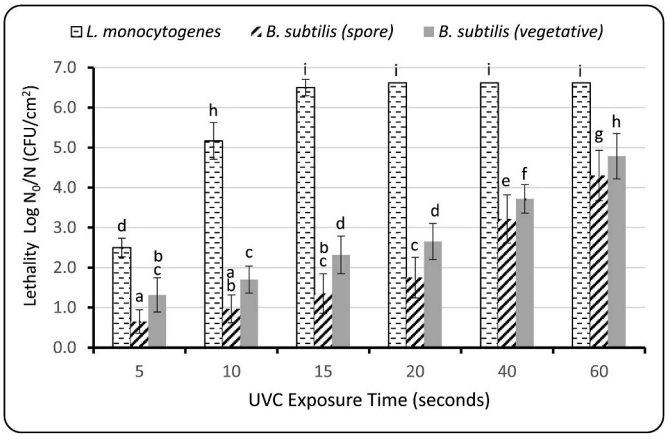

Fig. 2. *L. monocytogenes* and *B. subtilis* spores and vegetative cells lethality in a planktonic culture after the application of UV-C at different doses. Each value corresponds to the average of three repetitions with three replicates per trial (n = 9). Error bars represent the standard deviation. [a-i] The bars that, per microorganism, lack a common superscript differ significantly ($P < 0.05$).

As can be observed, greater inactivation was obtained at higher treatment doses, being maximum for *L. monocytogenes* at 15 s of exposure (*i.e.* 180 mJ/cm^2), reaching a reduction of 6.5 ± 0.2 log CFU/cm^2. At this same dose, however, the reduction observed for *B. subtilis* was 2.3 ± 0.5 log CFU/cm^2 and 1.4 ± 0.5 log CFU/cm^2 in its vegetative and sporulated form ($P < 0.05$), respectively. Moreover, maximum lethality was not achieved for *B. subtilis* at any of the UVC doses applied, with a trend of greater resistance to UVC exposure being observed in its sporulated form. This fact coincides with what has been demonstrated by other authors who indicate that *B. subtilis* spores are more resistant to UV radiation than the corresponding vegetative cells (Forney & Moraru, 2009). This greater resistance to UVC rays of the spores may be due to two different aspects according to Setlow (2001): (i) to the photochemistry of the DNA within the spores, since UVC rays generate few or no cyclobutane dimers, but a specific spore photoproduct (SP) named 5-thyminyl-5,6-dihydrothymine, with lower toxicity; or (ii) to DNA repair, in particular to *S*P-specific repair during spore germination.

Finally, it is interesting to note that, as the dose increases both bacteria resist the treatment, thus observing a greater reduction in the microbial population at lower doses and demonstrating a non-linear effect. This phenomenon, also observed by Martínez-García et al. (2023), could be related to the difficulty of UVC rays reaching the microorganisms that are most internalized in the droplet deposited as inoculum on the surface. As previously described, surfaces were inoculated with the microbial suspension and immediately treated with the UVC to observe the effect on planktonic cells. In this way, cells could put up physical resistance, generating a "shadow effect" (Robinson et al., 2000).

3.2. Effectiveness of UVC treatments for the elimination of biofilms

Biofilm consolidation on surfaces goes through different formation stages from which maturation has been highlighted for offering greater resistance for the community to be eliminated (González-Rivas et al., 2018). At this stage, biofilms are highly structured, producing a robust and solid protective matrix. It has been described that *L. monocytogenes* begins its maturity period around 72 h, being irreversible adhered and conforming microcolonies between 24 and 48 h (González-Rivas et al., 2018). For that reason, it was interesting to evaluate the effectiveness of different UVC doses for *L. monocytogenes* and *B. subtilis* biofilm elimination formed at different incubation periods (*i.e.* 24, 48 and 72 h). Both microorganisms followed similar resistance patterns to those obtained when the treatment was applied to planktonic cells, demonstrating significantly higher sensitivity of *L. monocytogenes* cells conforming biofilms to UVC (Fig. 3-A) ($P < 0.05$). However, the results for this

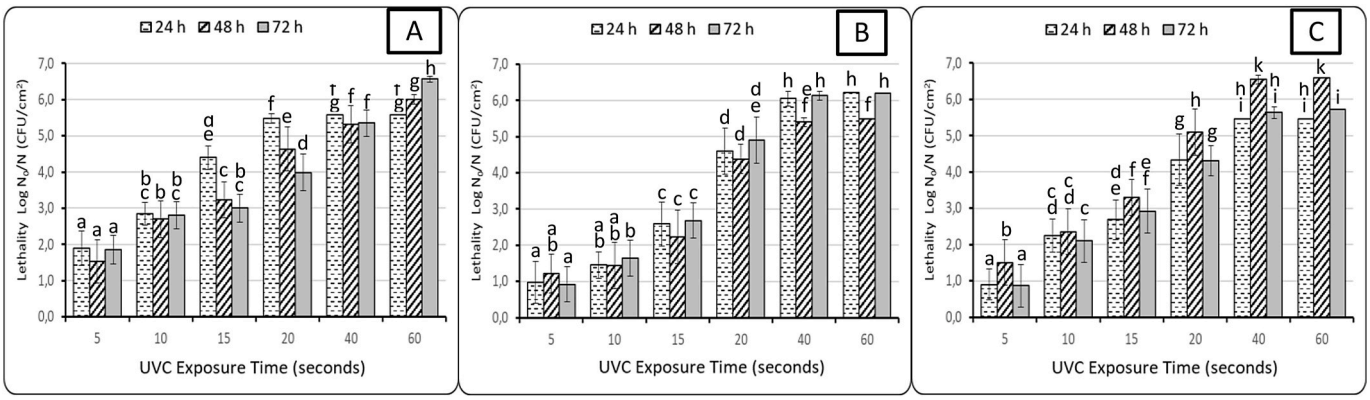

Fig. 3. Lethality of cells conforming biofilms of (A) *L. monocytogenes*, (B) *B. subtilis* spores, and (C) *B. subtilis* vegetative cells obtained after the application of different UVC doses. [a-h] The bars that lack a common superscript differ significantly ($P < 0.05$).

pathogen showed a significant decrease ($P < 0.05$) when UVC was applied in doses greater than 5 s against *L. monocytogenes* biofilms compared to when the treatment was carried out on planktonic cells. The difference in the resistance that cells exhibit when they are in the biofilm could be due to the physiological state of the cell or the degree of DNA exposure to UVC (Anast & Schmitz-Esser, 2021), as well as the presence of the matrix produced by the microbial community that serves as a physical protective barrier against UVC irradiation (Ma et al., 2022). Furthermore, it has been shown that the composition of the biofilm can vary between species of microorganisms (Allison, 2003), therefore being another variable of resistance to UVC. In this sense, *L. monocytogenes* biofilm matrix is practically proteinaceous in nature (Combrouse et al., 2013) unlike the matrix of *B. subtilis* biofilms that contain a mixture of proteins and exopolysaccharides (Branda et al., 2006). Therefore, the different composition and structure could be a reason for the difference in the lethality obtained after UVC between both microorganisms (Fig. 3-A,B,C).

The effect of UVC on the inactivation of cells conforming the biofilms depends on the direct incidence of light on the target microorganism. By applying the treatment to a surface on which a biofilm is formed, its mode of action could be considered in three different phases. Initially, UVC would directly affect the outer surface layer of the biofilm, consequently affecting those microorganisms located in this region. For this reason, at low UVC doses (*i.e.* 5 and 10 s, equivalent to 60 and 120 mJ/cm^2) no significant differences ($P > 0.05$) were obtained in *L. monocytogenes* and *B. subtilis* cell lethality in either the three incubation periods ([+Let] $48^a \approx 24^a \approx 72^a$ h). Subsequently, UVC rays reach the middle section of the biofilm, where its structure and robustness begin to gain importance. For this reason, in the application of medium doses (*i.e.* 15 and 20 s, equivalent to 180 and 300 mJ/cm^2) significant differences ($P < 0.05$) were observed in the lethality obtained between the period of 24 h and those of 48 and 72 h ([+Let] $24^a > 72^b \approx 48^b$ h) in *L. monocytogenes* biofilms. The greater the structure and robustness, a higher difficulty of the UVC rays to penetrate the biofilm structure and to reach the cells that conform the community, and therefore a greater lethality was observed in the shortest period of formation (*i.e.* 24 h). However, the application of medium doses of UVC in *B. subtilis* did not have the same effect as in *L. monocytogenes*. In this case, no significant differences were obtained ($P > 0.05$) between 24 and 72 h of formation (*i.e.* $24^a \approx 72^a$ h), which could be due to *B. subtilis* could require more time to produce a robust matrix and therefore, the formation periods studied only differed in cell load. In this sense, it should be noted that *B. subtilis* requires culture media that promotes the development of biofilms to generate a complex three-dimensional structure in 48 or 72 h, while using general media it may require more than 4 days (Gingichashvili et al., 2017). Although it can be confirmed that biofilms obtained by this microorganism were shown to reach a state of maturity, since spores were found in the structure and these are produced in biofilms when they enter the mature phase (Vlamakis et al., 2008), more time would have been necessary to produce greater amount of protective matrix. Interestingly, *B. subtilis*, both in its vegetative and sporulated forms, presented significantly greater resistance ($P < 0.05$) to UVC in planktonic cultures than when it was found conforming biofilms, results that would support the fact that there was no robust protective matrix. The greater resistance in planktonic cells could be explained by the physiological state in which they are found, since it has been shown that the sensitivity of UVC increases when the cells are in the stationary phase compared to the exponential phase (Anast & Schmitz-Esser, 2021). This observation would suggest that the physiological state of the stationary phase gives cells greater resistance to UVC.

Finally, the third phase would involve the highest doses applied (*i.e.* 40 and 60 s, equivalent to 600 and 900 mJ/cm^2), where the slope in lethality is slowed down and, with it, in proportion to the previous phases, more energy needs to be applied (*i.e.* mJ/cm^2) to eliminate the last residual microorganisms within the structure. This could be due either to the fact that the same cells of those dead microorganisms act as a barrier to those found in deeper areas of the biofilm, or, as indicated by Cheng (2020), to the existence of deep areas (*i.e.* low exposure to the treatment) and "deadly" zones (*i.e.* high exposure to the treatment) at the time the UVC is applied, so that the cells within the biofilm can be in constant movement by passive diffusion or by movement propelled by active flagella. In this case, although the passage of UVC to the deep areas is more difficult, the results of the present study show that at an irradiance of 900 mJ/cm^2 of UVC application, the lethality of the biofilms formed by both microorganisms was maximum. The difference observed between formation periods could be explained by the different starting cell concentrations, since the number of cells varied depending on whether the biofilms had been produced after 24, 48 or 72 h.

It should be noted that, according to UNE-EN 13697, a treatment and/or process can be considered bactericidal when the reduction of 4 logarithms of the load adhered to surfaces is exceeded. The results of the present study show that a minimum UVC dose of 20 s with an intensity of 12 mW/cm^2, equivalent to an irradiance of 300 mJ/cm^2, is needed for the inactivation of biofilms (*i.e.* reductions $\geq$4 log) of *L. monocytogenes* and *B. subtilis* formed at different periods (*i.e.* 24, 48 or 72 h). These results agree with BIF values reported by other authors for other microorganisms and at similar doses, as summarized in Luo et al. (2022). Interestingly, similarities are observed in the results obtained from *B. subtilis* between the sporulated form (Fig. 3-B) and the vegetative form (Fig. 3-C), and it can be considered that the treatments applied are effective processes for eliminating cellular resistance forms.

3.3. *Effectiveness of combined US and UVC treatments for the elimination of biofilms*

In the food industry, disinfection must be carried out safely and cost-

effectively, consequently involving the least possible frequency and time, with low chemical and energetic costs, producing the minimum amount of waste, and without causing damage to the equipment and food handlers (Yuan et al., 2021). Taking into consideration those objectives, the hurdle technology emerges as a highly interesting strategy for biofilm control. Recent studies have focused on the control of microbial biofilms in the food industry using combined treatments of ultrasound and chemical disinfectants (Shao et al., 2020; Sun et al., 2022; Torlak & Sert, 2013). However, there is a concern about the resistance that bacteria can generate when exposed to sublethal doses of disinfectants (Capita & Alonso-Calleja, 2013). Therefore, it is of interest to include alternative disinfection technologies such as UVC that can mitigate acquired antimicrobial resistances. For this reason, in the present study, both technologies (*i.e.* US + UVC at different doses) were combined for the removal of *L. monocytogenes* and *B. subtilis* biofilms at different incubation periods (*i.e.* 24, 48, and 72 h). The UVC treatment doses (*i.e.* 0, 5, 15 and 30 min) were selected based on those which a minimum reduction of 4 log CFU/cm^2 was achieved as therefore the treatment could be considered bactericidal according to UNE-EN 13697 standards.

Results demonstrated that a single application of US reduced the cell load by 1–4 log CFU/cm^2, depending on the microorganism and exposure time (Fig. 4). The achieved reduction could be attributed to the detachment of bacteria resulting from the breakdown of the structure, which would release the cells into the medium. It has been described that US, at certain intensities, induces cavitation phenomena, generating bubbles that implode after absorbing sufficient energy, producing shockwaves capable of breaking the matrix of a biofilm (Yu et al., 2020). Additionally, the results also show that the effectiveness of US decreases as the biofilm formation period increases (*i.e.* from 24 to 72 h), resulting in less detachment as the structure matures ($P < 0.05$). This can be explained because as the incubation period lengthens, there is a greater production of extracellular matrix (Ripolles-Avila et al., 2018). Moreover, it is also observed that as the exposure time to US increases, in some cases, there is more detachment of the structure, with significant differences ($P < 0.05$; $15^a > 10^b > 5^c$ min).

From the combination of both technologies (*i.e.* US + UVC), results demonstrated higher lethality even reaching complete elimination of the bacteria that conformed the biofilms at lower UVC doses in comparison to the lethality observed when only UVC disinfection was applied (see section 4.2 – Fig. 3). Therefore, the data show that the combination of US with UVC increases the lethality associated with bacterial cells when they are in biofilms, which is consistent with the findings reported by Char et al. (2010). By combining different treatments or complementary inactivation mechanisms, microbial cells are exposed to various stress factors, leading to considerably faster cell death (Mikš-Krajnik et al., 2017). In this case, US physically disintegrates the biofilm structure, also exerting some bactericidal effect (Yu et al., 2020), while UVC causes lethal damage to the entire cell population (Cheng et al., 2020). As can be observed, a reduction of ≥5 log CFU/cm^2 was observed in *L. monocytogenes* biofilms formed at 72 h when applying 15 s of UVC, equivalent to 180 mJ/cm^2, after a US treatment (Fig. 4-A3), whereas without the application of US (*i.e.* UVC only), reductions of only 3 log CFU/cm^2 were achieved (Fig. 3-A). In some situations, due to US treatment, the detachment of cells or parts of the biofilm structure may have been responsible for the increased lethality obtained with the tested UVC exposure times for the same biofilm. In most cases, this increase in lethality is approximately equal to the sum of the lethality

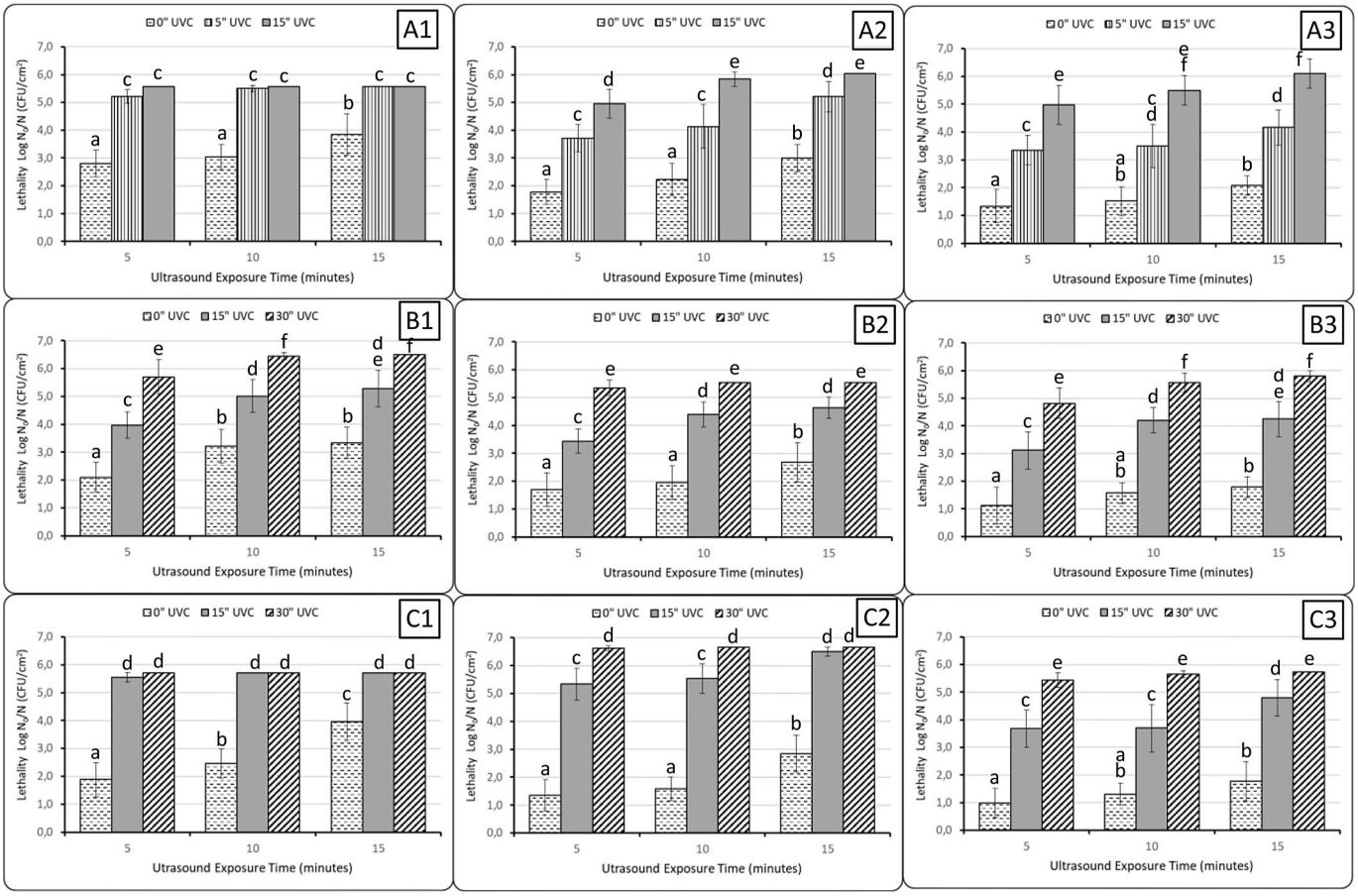

Fig. 4. Lethality of cells conforming biofilms of (A) *L. monocytogenes*, (B) *B. subtilis* spores, and (C) *B. subtilis* vegetative cells produced at (1) 24 h, (2) 48 h, and (3) 72 h after the combined application of US and UVC at different treatment doses. [a-f] The bars that lack a common superscript differ significantly ($P < 0.05$).

when only UVC was applied plus the lethality contributed by the US control (*i.e.* UVC = 0 s).

If the response to the combined action of US + UVC in biofilms formed by *L. monocytogenes* is examined, in those formed at 24 h the treatments were highly effective (Fig. 4-A1). This could be since during this period (*i.e.* 24 h), only initial stages of biofilm formation were reached (*i.e.* not matured) (Ripolles-Avila et al., 2018). In this regard, even lethality of 5.2 log CFU/cm^2 was achieved at the lowest doses of both technologies (*i.e.* applying 5 min US and 5 s UVC), and, on the other hand, the maximum possible reduction for these experiments ($\geq$5.6 log CFU/cm^2) was achieved with any treatment applying a 15 s UVC exposure. US could be interfering in the initial phase, as described in the previous section, eliminating microorganisms on the surface of the structure and consequently allowing UVC to reach the deeper regions of the biofilm more effectively. An example of this can be observed in *L. monocytogenes* biofilms formed at 72 h treated for 5 min with US followed by 15 s of UVC (Fig. 4-A3), where a reduction of 5 log CFU/cm^2 was achieved compared to those treated uniquely with UVC (Fig. 3-A), which resulted in a reduction of only 3 log CFU/cm^2. On the other hand, it is observed that higher UVC doses were required for the elimination of *B. subtilis* biofilms compared to *L. monocytogenes* ($P < 0.05$). This could be attributed to the type of matrix that *B. subtilis* forms (*i.e.* matrix composition or its distribution in the structure) and the physiological characteristics (*i.e.* genetic material composition) of the microbial species itself. It is important to emphasize that the intensity of the ultrasound (US) used is sufficient (>10 W/cm^2) for the mechanical disruption of the matrix. At inadequately low intensities (2 W/cm^2), where the biofilm structure is minimally affected, the opposite effect may occur, stimulating the metabolism of microorganisms and leading to the formation of a more resistant biofilm, as described by Erriu et al. (2014). High US intensities are primarily associated with the mechanical destruction of the complex biofilm structure, resulting in a bactericidal effect (Piyasena et al., 2003). However, low intensities enhance bacterial growth and biofilm development (Monsen et al., 2009; Pitt & Ross, 2003).

It is noteworthy that the UVC variable significantly influenced the combinations with US and biofilm times for all tested microorganisms ($P < 0.05$) ([+Let] $30^a \gg 15^b \gg 5^c \gg 0^d$ s). While for the biofilm variable, differences were also significant for *L. monocytogenes* and *B. subtilis* spores as the maturity stage increased ($P < 0.05$) ([+Let] $24^a > 48^b > 72^c$ h), for *B. subtilis* vegetative cells there were not always significant differences ($P > 0.05$) among all times ([+Let] $48^a \approx 24^a > 72^b$ h).

4. Conclusions

L. monocytogenes was the most susceptible bacteria to UVC treatments and their combination with US, followed by the vegetative forms of *B. subtilis*, with spore forms exhibiting the highest resistance. The UVC treatments, both in combination with US and alone, were highly effective at the tested intensity (*i.e.* 12 mW/cm^2) and exposure times, achieving reductions of $\geq$4 log in the studied microorganisms. Thus, reductions of $\geq$4 log were attained for all three stages of biofilm formation and all studied microorganisms with UVC alone, using exposure times of 20 s, or with 15 s of UVC combined with US. The application of disintegration technologies, in this study US, demonstrated to be effective, enhancing lethality when combined with UVC. Overall, the three biofilm maturity stages (*i.e.* 24, 48, and 72 h) showed greater resistance to treatments as the structure matured (*i.e.* 72 h). However, this resistance was not consistent and varied depending on the microorganism that formed the biofilm, suggesting the possibility of cycles with varying sensitivity to treatments, which may not follow a linear or proportional pattern.

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CRediT authorship contribution statement

G. Gervilla-Cantero: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **R. Gervilla:** Writing – review & editing, Visualization, Validation, Supervision, Software, Formal analysis, Data curation, Conceptualization. **C. Ripolles-Avila:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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