

## Article

# Short Wave Ultraviolet Light (UV-C) Effectiveness in the Inactivation of Bacterial Spores Inoculated in Turbid Suspensions and in Cloudy Apple Juice

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**Abstract:** Liquid foods might present interferences in their optical properties that can reduce the effectiveness of short-wave ultraviolet radiation (UV-C) treatments used for sterilization purposes. The effect of turbidity as UV-C interference factor against the inactivation of bacterial spores was analysed by using phosphate-buffered saline solutions (PBS) of different turbidity values (2000, 2500, and 3000 NTU) which were adjusted with the addition of apple fibre. These suspensions were inoculated with spores of *Bacillus subtilis* and *Alicyclobacillus acidoterrestris*. While higher UV-C doses increased the inactivation rates of spores, these were reduced when turbidity values increased; a dose of 28.7 J/mL allowed inactivation rates of *B. subtilis* spores of 3.96 Log in a 2000-NTU suspension compared with 2.81 Log achieved in the 3000-NTU one. Spores of *B. subtilis* were more UV-C-resistant than *A. acidoterrestris*. Cloudy apple juice inoculated with *A. acidoterrestris* spores was processed by UV-C at different doses in a single pass and with recirculation of the matrix through the reactor. Inactivation increased significantly with recirculation, surpassing 5 Log after 125 J/mL compared with 0.13 Log inactivation after a single-pass treatment at the same UV-C dose. UV-C treatments with recirculation affected the optical properties (absorption coefficient at 254 nm and turbidity) of juice and increased browning as UV-C doses became higher.

**Keywords:** short-wave ultraviolet radiation; sterilization; apple juice; turbidity; *Alicyclobacillus acidoterrestris*



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## 1. Introduction

In recent years short-wave ultraviolet light (UV-C) has been tested as an alternative to thermal treatments in food industry, not only for the microbiological decontamination of surfaces, but also for the reduction of the microbial load in liquid foods [1]. Many of the currently sold UV-C machines for the pasteurisation of these products assure to meet the FDA requirements for the elimination of *E. coli*, but among the microorganisms that are potentially present in these foods, bacterial spores are the ones which oppose the highest resistance to thermal treatments commonly used for pasteurisation processes and to pressure-based technologies such as high-hydrostatic pressure and ultra-high-pressure homogenisation [2–4]. Once these spores contaminate foods and survive pasteurisation treatments, they germinate and grow even at low pH spoiling the final product meant to be shelf-stable [5]. That has been described in apple juice batches contaminated with spores of *Alicyclobacillus acidoterrestris* [6]. This bacterium might not produce any visible signs of deterioration, like gas or higher turbidity before reaching an excessive cell concentration, but although it is harmless to humans and represents no health hazard it spoils the product

with the production of odd flavours and odours because of the synthesis of guaiacol making it not suitable for consumption. For that reason, it is considered a target microorganism for novel technologies for fruit juice processing [7,8].

UV-C, which usually is emitted by lamps at a wavelength of 253.7 nm [9], has been proved to successfully inactivate microorganisms in their vegetative or spore state by penetrating through their membranes and being absorbed by their DNA which has a maximum absorption peak in the wavelength range between 254 and 264 nm, forming pyrimidine dimers which blocks DNA transcription and replication processes leading to cell death [10]. Experiments performed in phosphate-buffered saline solutions (PBS), which opposes no interferences against UV-C (254 nm), demonstrate that a wide range of bacterial and fungal spores are susceptible to be inactivated by this technology [3,11]. Moreover, UV-C technology has been estimated to be cheaper than thermal pasteurisation, not only at the initial investment, but also at the energy required for processing foods that could approximately represent a 39% of the total energy spent in thermally pasteurised fruit drinks like pineapple juice [12]. The main problem that UV-C has to face when treating foods is the interferences that are naturally present in them which will not allow light to penetrate through the matrix and reach microorganisms to inactivate them. The most important interferences found in foods are the absorption coefficient at the same wavelength as UV-C because it would not let it reach deep into the matrix, and the other one is the turbidity due to suspended particles which act as a shield to microorganisms and block the pass of UV-C light. To address these problems, many continuous-flow UV-C reactors have been designed to date. One of these approaches are the thin-film reactors that shorten the distance in which UV-C has to travel through, increasing the probability to reach as many cells as possible in order to inactivate them [13]. The purpose of this work was to evaluate the effectiveness of a concentric-ring thin-film UV-C reactor in inactivating bacterial spores inoculated in suspensions of different turbidity values as the main interference in order to know the degree in which those values can oppose to the effectiveness of UV-C to inactivate bacterial spores. For that purpose spores of *Bacillus subtilis* and of *A. acidoterrestris* were chosen because the first one has been commonly used as a model microorganism for the study of the characteristics of endospores [14], and the second one for being a target microorganism in the fruit juice industry due to their resistance to many types of stresses and for growing at low pH values [7]. The inactivation of *A. acidoterrestris* spores in cloudy apple juice with ascorbic acid, a real food matrix of high turbidity and absorption coefficient at 254 nm, was studied by applying UV-C in a single pass or in recirculation. The inactivation kinetics of *A. acidoterrestris* following the later approach was also elucidated.

## 2. Materials and Methods

### 2.1. Preparation of the Spore Suspensions

*Bacillus subtilis* CECT 4002 and *Alicyclobacillus acidoterrestris* CECT 7094 were supplied from the Spanish Type Culture Collection (CECT, University of Valencia, Valencia, Spain). *B. subtilis* spores were obtained with a modified procedure based on the UNE EN ISO 13704:2002 as explained by [15]. The lyophile was rehydrated in glucose-tryptone broth consisting in 2.5 g of yeast extract (Oxoid, Basingstoke, UK), 5 g of tryptone (Oxoid) and 1 g of glucose (Sigma-Aldrich, St. Louis, MO, USA) per litre of distilled water, its pH was adjusted to 7.2. It was incubated at 30 °C for 24 h, 2 mL of this stock culture was transferred into Roux bottles containing yeast extract agar (MYA) which consisted in 10 g of meat extract (Oxoid), 2 g of yeast extract (Oxoid), 15 g of agar (Oxoid), and 0.04 g of MnSO<sub>4</sub>·H<sub>2</sub>O (Merck, Darmstadt, Germany) per litre of distilled water. These bottles were incubated at 30 °C for a maximum of 30 days to induce sporulation. Spores were collected by pouring 20 mL of sterile distilled water into each bottle and the surface was scraped with a Digralsky stick, the suspension was collected inside 50-mL Falcon tubes and centrifuged at 10,000× g for 20 min at 4 °C using a Sigma 4K15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), the supernatant was discarded and the resulting

pellet was washed with 15 mL of sterile water and centrifuged with the same settings, this procedure was done four times to be finally resuspended in 30 mL of sterile distilled water. The suspensions were heated at 75 °C for 10 min in order to inactivate vegetative cells.

*A. acidoterrestris* spores were recovered in K broth as explained by [7] by incubating them at 43 °C for 48 h. The stock culture was refrigerated at 4 °C. Sporulation was induced by transferring 1 mL of the stock culture to 250-mL Roux bottles containing 50 mL of potato dextrose agar (PDA) (Oxoid, Basingstoke, UK), and incubated at 43 °C from 7 to 15 days until reaching 80% sporulation or higher. Spores were collected by pouring 30 mL of sterile distilled water into each bottle and the surface was scraped with a Digrafsky stick, the suspension was collected inside 50-mL Falcon tubes and centrifuged at 10,000 × g for 20 min at 4 °C, the supernatant was discarded and the resulting pellet was washed with 30 mL of sterile water and centrifuged with the same settings, this procedure was done four times to be finally resuspended in 40 mL of sterile distilled water and heated at 80 °C for 20 min in order to inactivate vegetative cells.

Spore preparations were placed in an ice bath after heat treatment for 10 min and stored at 4 °C until used.

## 2.2. Matrices Preparation

Phosphate-buffered saline (PBS) (Panreac Química S. L. U., Castellar del Vallès, Spain) with a pH of 7.4 was added with apple fibre (PBS-fibre) (The Hut Group, Manchester, UK) until reaching the turbidity values of 2000, 2500 and 3000 NTU (Nephelometric Turbidity Unit). Apple juice (Golden delicious var.) was obtained from a local producer. The cold-pressed apple juice was added with 300 ppm of ascorbic acid at the moment of its extraction according to the producer. The matrices were inoculated with *B. subtilis* spores (final concentration ~5.5 Log CFU/mL) and *A. acidoterrestris* spores (final concentration ~4.5 to 5.3 Log CFU/mL) right before UV-C treatments.

## 2.3. UV-C Treatments

These treatments were done in a thin-film concentric-ring type UV-C reactor [16] (UV-Therm, Ypsicon S.L., Barcelona, Spain) of 70-mL capacity, with dimensions of 76.5-cm long and 1-mm width. This reactor has a feeding tank connected to a UV-C lamp (55 W) (LAB81055 NNI 150/76 XL, UV-Consulting Peschl España, Geldo, Spain) coated with quartz, and the lamp temperature is controlled with an air-cooling system. The lamp's irradiance was measured by actinometry with a potassium iodide/iodate solution as explained by the method of [17]. PBS suspensions were irradiated in a single pass through the reactor with the doses of 14.3, 21.5, and 28.7 J/mL. Apple juice was irradiated with the doses of 25, 50, 75, 100, and 125 J/mL for microbiological analysis in a single pass and in recirculation through the reactor at a pumping speed of 0.708 m/s. In the same way, samples were irradiated at the doses of 50, 100, 150, and 200 J/mL for physicochemical analysis. The time needed for an irradiation and the number of entrances was calculated as explained by [15] and that information can be found in Table 1. Samples destined for microbiological analysis were refrigerated at 4 °C, and samples destined for physicochemical analysis were deposited in 50-mL Falcon tubes and stored at −80 °C.

**Table 1.** Calculated treatment time in minutes and seconds, and the number of entrances per litre of apple juice through the UV-C reactor at the different doses. The pumping speed for all doses was 0.708 m/s.

UV-C Dose (J/mL)	Treatment Time Per L of Juice	Number of Entrances
25	14'52"	60
50	29'43"	120
75	44'35"	180
100	59'26"	240
125	74'18"	300
150	89'10"	360
200	118'53"	480

#### Reynolds Number Calculation in UV-C Treatments

Reynolds number ( $Re$ ) was calculated with Equation (1):

$$Re = \rho VD/\mu. \quad (1)$$

where  $V$  is fluid's velocity in m/s,  $D$  is the equivalent diameter of 0.001632 m [18]. Dynamic viscosity ( $\mu$ ) was measured at 20 °C with a Haake RheoStress1 rheometer (Thermo Fisher Scientific, Inc., Karlsruhe, Germany) with a value for apple juice of  $4.13 \pm 0.07 \times 10^{-3} \text{ Pa} \cdot \text{s}$ . Density ( $\rho$ ) at 20 °C was obtained with a density hydrometer (HYDR-100-001) (Labbox, Vilassar de Dalt, Spain) being of 1.052 g/mL (1052 Kg/m<sup>3</sup>) in apple juice.

#### 2.4. Microbiological Analysis

Serial dilutions of the samples were done in sterile peptone water (Oxoid) and plated on petri dishes. Samples with *B. subtilis* were plated on tryptone-soya agar (Oxoid) using a double layer in order to prevent excessive colony growth. Plates were incubated at 30 °C for 2 days. *A. acidoterrestris* samples were plated on SK agar [19] and they were incubated at 43 °C for 3 days. Spore inactivation was calculated as the difference between the logarithmic counts of the control and the treated sample ( $\text{Log}_{10}N_0 - \text{Log}_{10}N$ ).

#### 2.5. Physicochemical Analysis

##### 2.5.1. Optical Properties

Turbidity was measured with a turbidimeter Hach 2100Q (Hach Lange Spain, l'Hospitalet de Llobregat, Spain). Absorption coefficient at 254 nanometres wavelength ( $\alpha_{254}$ ) was measured by using a spectrophotometer NanoPhotometer Pearl (IMPLEN GmbH, München, Germany) and a quartz cuvette with a 1 cm pathlength. Samples were read at 20 °C.

##### 2.5.2. Browning Index

It was determined following the method explained by [20]. Samples were filtered with syringe filters of 0.45 µm pore diameter (Labbox) and mixed with ethanol in equal proportions. The absorbance of samples was read at 420 nm ( $A_{420}$ ) with a spectrophotometer and results were expressed as  $A_{420}$ .

#### 2.6. Statistical Analysis

Statistics and analysis of variance (ANOVA) were done by using the linear models (GLM) via software (Statistica ver 7, StatSoft, Inc, Tulsa, OK, USA). Tukey test was used for making comparisons between samples means. Differences were considered as significative when  $p < 0.05$ .

#### 2.7. Inactivation Kinetics

Survival data of *A. acidoterrestris* spores in apple juice was adjusted to a non-linear model by using GInaFiT software (version 1.7, MeBioS, Leuven, Belgium) add-in for

Microsoft® Excel. The most suitable model was chosen according to the root mean square error and the  $R^2$  coefficient. The inactivation model that better adjusted to that data was Geeraerd model with shoulder and tail [21] which is represented in Equation (2):

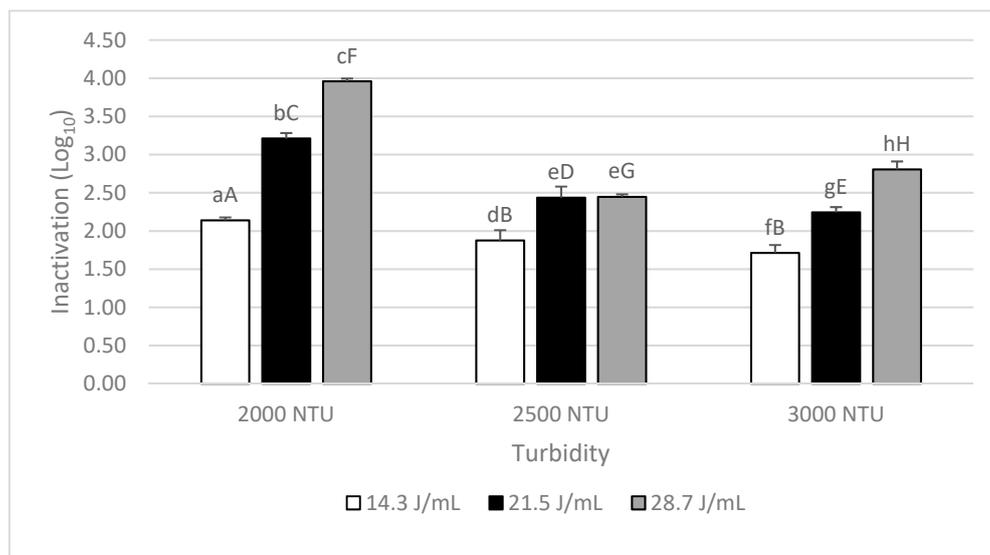
$$\text{Log}_{10}(N) = \text{Log}_{10} \left[ \left( 10^{\text{Log}_{10}(N_0) - 10^{\text{Log}_{10}(N_{res})}} \right) * e^{(-k_{max} * t)} * e^{(k_{max} * SI)} \right] / \left[ 1 + \left( e^{(k_{max} * SI)} - 1 \right) * e^{(-k_{max} * t)} \right] + 10^{\text{Log}_{10}(N_{res})} \quad (2)$$

where  $N_0$  is the starting concentration of spores,  $N_{res}$  is the number of survival subpopulations of resistant bacteria,  $t$  means the time (in this adaptation it would be UV-C dose with recirculation),  $k_{max}$  is the inactivation rate of  $0.15 \pm 0.01$  [1/time unit], and  $SI$  [time unit] is the shoulder length of  $15.64 \pm 3.64$ .

### 3. Results

#### 3.1. UV-C Effectiveness in the Different Turbidity Suspensions

As it can be observed in Figure 1, the inactivation of *B. subtilis* spores was inversely proportional to the turbidity value of the PBS-fibre suspensions, and within the same turbidity value, the inactivation of spores increased with higher UV-C doses. The highest inactivation (3.96 Log) was obtained in the suspension of the lowest assayed turbidity (2000 NTU) and processed with the highest assayed UV-C dose (28.7 J/mL). *A. acidoterrestris* was not detected after UV-C treatments, therefore that data was omitted from figures, and the highest inactivation rate for these experiments in PBS-fibre was of  $4.58 \pm 0.09$  Log.

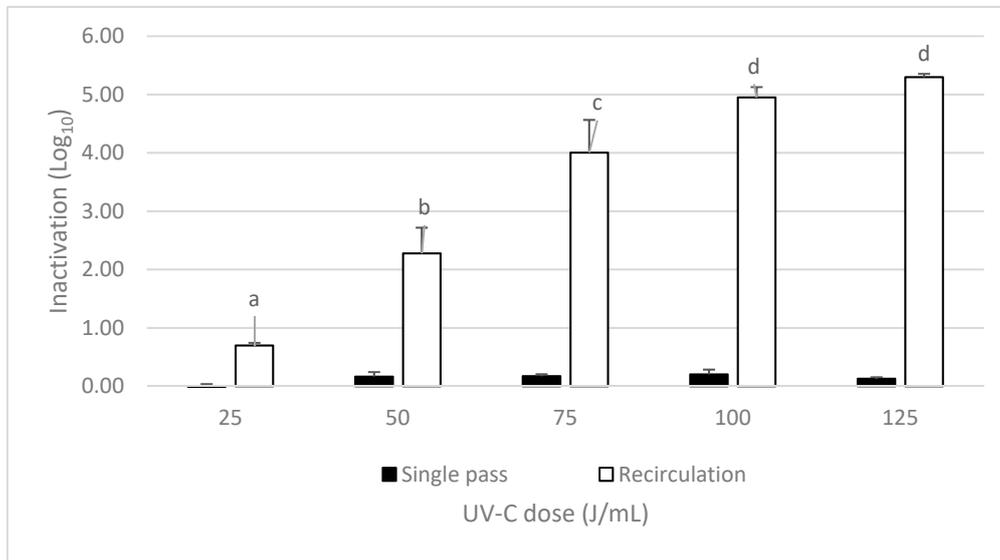


**Figure 1.** Inactivation of *B. subtilis* spores in PBS-fibre with different turbidity values modified with apple fibre. These suspensions were treated by UV-C at the doses of 14.3, 21.5, and 28.7 J/mL. Different lowercase letters represent significant differences ( $p < 0.05$ ) between samples means of the same turbidity value but a different UV-C dose; capital letters represent significant differences between sample means of those ones treated with the same UV-C dose but with a different turbidity value.

#### 3.2. *A. acidoterrestris* Inactivation in Cloudy Apple Juice with Ascorbic Acid

Cloudy apple juice with ascorbic acid presented high values of interferences coming from turbidity and  $\alpha_{254}$ . As an attempt to increase the probability of particles to get into a closer proximity to the UV-C source, the matrix was re-circulated setting the peristaltic pump at 500 rpm (pumping speed of 0.708 m/s), that produces a flow rate of 64.4 mL/s [15] which yields a dose of 0.42 J/mL every pass. The number of passes and retention time for every given dose can be found in Table 1. The result from these experiments can be seen in Figure 2, where the different doses were applied in a single pass and recirculating the sample until reaching the desired dose. Results of *A. acidoterrestris* lethality showed that this equipment is ineffective in a matrix with these interference characteristics if treated

by a single pass and no significant differences were observed when UV-C dose increased. Nevertheless, different results were observed in treatments with recirculation where an inactivation of more than 5 Log was observed in the treatment at 125 J/mL, in which no surviving cells were detected on culture plates.

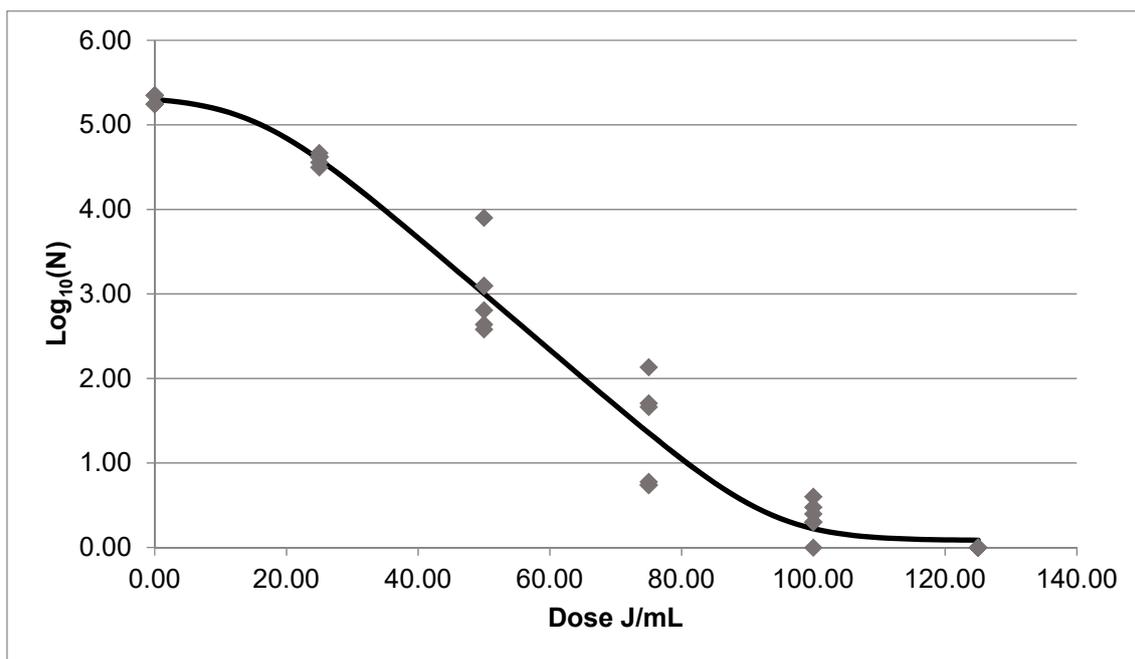


**Figure 2.** Inactivation of *A. acidoterrestris* inoculated in cloudy apple juice with ascorbic acid by UV-C at different doses (25, 50, 75, 100, and 125 J/mL) in a single pass (black bars) or in recirculation (white bars). Error bars mark the standard deviation, and letters represent significant differences ( $p < 0.05$ ) between sample means of the same treatment. No letters mean that there were no significant differences found between doses.

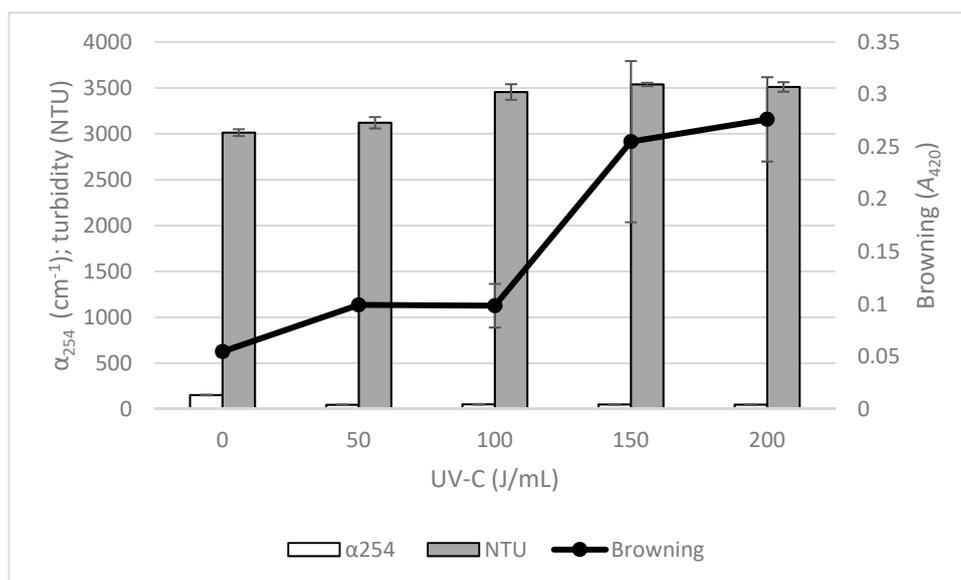
The data gathered from UV-C experiments with recirculation were utilized for adjusting to an inactivation model using GInaFiT software. Several inactivation models were assayed, from which it was selected the one with the highest  $R^2$  (0.9772) and the lowest RMSE (0.3293), being that the Geeraerd shoulder tail model [21] (Figure 3), with an equation (Equation (2)). This was used to calculate the inactivation kinetics of *A. acidoterrestris* spores in this type of apple juice. The analysis of the inactivation kinetics allowed to obtain the 4D value of 76.25 J/mL, that was the estimated dose required to inactivate 4 Log of *A. acidoterrestris* spores in apple juice under these treatment conditions.

### 3.3. Physicochemical Changes from the Application of UV-C Treatments with Recirculation

Apple juice was irradiated with higher doses than those ones used to inactivate *A. acidoterrestris* spores in order to elucidate the changes that the continuous application of UV-C with recirculation can cause on some of the physicochemical properties of the apple juice with added ascorbic acid. The first observable change was an increase of browning of juice after being irradiated from 100 J/mL onwards, as shown in Figure 4. There was also an augment of the turbidity value of the UV-C-treated samples and that became higher with higher doses. Contrary to that,  $\alpha_{254}$  value of samples decreased with higher UV-C doses.



**Figure 3.** Inactivation kinetics of *A. acidoterrestris* in cloudy apple juice treated by UV-C with recirculation according to Geeraerd shoulder tail model obtained by using GInaFIT plug-in.



**Figure 4.** Physicochemical changes in apple juice with added ascorbic acid after UV-C treatments applied with recirculation at different doses (50, 100, 150, and 200 J/mL).  $\alpha_{254}$  (cm<sup>-1</sup>) is represented by white bars, turbidity (NTU) is represented in grey bars, and browning (unitless) is represented by the plot line. Error bars mark the standard deviation between samples mean.

#### 4. Discussion

*B. subtilis* spores became harder to inactivate as turbidity value increased in fibre suspensions (Figure 1). A similar behaviour was observed in a previous experiment [3] where caramel was added to PBS to modify its absorbance coefficient, showing that even slight  $\alpha_{254}$  increments can drastically reduce the inactivation of *B. subtilis* spores by UV-C. As bacterial spores, vulnerability of fungal ascospores to UV-C is also higher when there are no interferences in the matrix, as it was also observed in a previous experiment with plain PBS [11]. In assays with PBS-fibre and *A. acidoterrestris*, spores could not be detected

after most of UV-C treatments at any of the assayed turbidity values and comparing it with the results obtained from the same experiments with *B. subtilis* spores, it was found that *B. subtilis* spores were more UV-C-resistant than those of *A. acidoterrestris*. These results can also be contrasted with those of previous research [3] in which *B. subtilis* spores show a higher UV-C-tolerance compared with other bacterial spores including *A. acidoterrestris* in plain PBS.

Previous experiments with cloudy apple juice [4] measured its  $\alpha_{254}$  at  $11.54 \text{ cm}^{-1}$ , while in our experiments with the same type of apple juice but with added ascorbic acid set the value of  $\alpha_{254}$  at  $152.1 \text{ cm}^{-1}$  (Figure 4). It is known that ascorbic acid has its absorption peak within the deep UV spectra between 243.5 and 265.5 nm [22], therefore it is reasonable to think that the addition of ascorbic acid, which is a common practice in fruit juice production, would represent an immediate interference against UV-C. The approach of a thin-film UV-C reactor has the intention of reducing the depth in which radiation has to travel through and to reach as many cells as possible to inactivate them, being most likely affected the closest ones to the UV-C source. These reactors work under laminar flow conditions, which means that liquids travel throughout the system in a layered way. If the properties of the matrix do not allow UV-C to reach deep into the matrix the microorganisms present in the farther layers of liquid from the UV-C radiation source will not be reached and affected. Similar results to those shown in Figure 2 were observed in a previous research [15] in which *B. subtilis* spores were treated by using the same recirculation approach on whole and skim milk reporting higher inactivation rates as the re-circulation and doses augmented surpassing 4-Log inactivation after a  $100 \text{ J/mL}$  treatment, which is significantly higher than the results with the same dose in a single pass. That could have happened because recirculation allowed the sample to mix before re-entering the circuit, increasing the chances for the spores present in the matrix to get into a closer distance from the UV-C source and to be affected by it. The Reynolds number inside the UV-C reactor was considerably higher in samples treated with recirculation ( $Re = 290.8$ ), but although that type of flow still corresponded to the range of laminar flow ( $Re < 2100$ ) it might have propitiated a better exposure of the whole matrix as the fluid layers closer to the UV-C source were different at each pass. Another factor that could have contributed to a better inactivation of *A. acidoterrestris* spores could be that higher UV-C doses decreased the  $\alpha_{254}$  value of the samples (Figure 4) which also might have decreased the matrix absorption of UV light. Comparing results between the experiments with PBS-fibre and juice, it could be presumed that the most limiting factor of the matrix in a UV-C treatment could be the  $\alpha_{254}$  value, even more than the interference that turbidity could represent. The “Geeraerd model with shoulder and tail” inactivation model obtained for *A. acidoterrestris* in this type of apple juice [21] (Figure 4) showed that there was a slow spore inactivation at the beginning of the treatment followed up by a later exponential increase. Similar experiments on *B. subtilis* spores inoculated into milk and treated by UV-C [15] working under the same settings showed a better adjustment to a “Weibull with tail” inactivation model [23]. A tail in the model represents a slow-down or a less exponential inactivation of spores after reaching certain dose in a treatment.

The changes observed in UV-C-treated apple juice on its  $\alpha_{254}$  value could have been the product of a prolonged exposure to UV-C that can degrade ascorbic acid into dehydroascorbic acid and to 2,3-diketogulonic acid [24,25], compounds that have different absorbance peaks [26]. The addition of ascorbic acid was the main responsible for the increase of  $\alpha_{254}$  in apple juice, and also for the inhibition of the polyphenol oxidase (PPO) activity at the moment of the juice extraction. Therefore, when lower concentrations of ascorbic acid were available as a consequence of photodegradation, it might have not been able to inhibit the activity of that enzyme [27]. Although some investigation show that UV radiation can also inhibit PPO, those treatments have not been able to achieve a total inactivation of the enzyme [28]. In our experiments a possible degradation of the ascorbic acid in apple juice, as well as a higher contact with oxygen caused by recirculation, might have enabled PPO to oxidise phenolic compounds into the pigmented ones (*o*-quinones) [9].

Turbidity might have been affected as well by the degradation of ascorbic acid due to the oxidation of polyphenols that would lead to polymerise and react with proteins, and consequently increasing the formation of larger size particles [29].

## 5. Conclusions

The presence of spoiling bacteria in juices, not only in their vegetative state, but also in the state of spores, needs to be addressed by the emerging technologies intended for these products' pasteurisation or sterilization, especially resistant spores, like those of *A. acidoterrestris*, that could cause big losses of product and money, deserve special attention. Fruit matrices themselves can oppose different types and levels of interference depending on their singular properties and it is important to understand at what degree those interferences can affect the efficiency of UV-C treatments. The equipment features also have a great influence on the performance of the treatments. Cloudy apple juice presents a high turbidity value, but addition of ascorbic acid at the moment of extraction, which is a common practice in fruit juice industry, also increases absorption coefficient at 254 nm what should not be ignored when research about the effects of UV-C treatments in this type of product is performed. The results from the experiments hereby suggest that, although the degree of turbidity can affect the effectiveness of UV-C in inactivating resistant spores, that type of interference might not be as determinant as it is the absorption coefficient. There are other factors to take also into consideration that could affect the quality of the final product like potential browning reactions. The findings derived from this research could help in the development of new designs of UV-C reactors suitable to treat a wider spectrum of fruit juices as well as other liquid foods, and also explore their potential combination with other technologies that could increase the inactivation, not only inactivating microorganisms, but also enzymes like PPO, which in turn could reduce the intensity at which UV-C treatments would need to be applied to foods.

**Author Contributions:** Conceived and designed the experiments: J.N.S.-G., M.M.-G., M.M.H.-H., R.G., and A.X.R.-S.; performed the experiments: J.N.S.-G.; analysed the data: J.N.S.-G., R.G., and A.X.R.-S.; wrote the paper: J.N.S.-G., R.G., and A.X.R.-S. All authors have read and agreed to the published version of the manuscript.

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