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Inactivation of *Clostridium* Spores in Honey with Supercritical CO_2 and in Combination with Essential Oils

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Abstract: The presence of tens of *Clostridium botulinum* spores per gram of honey can cause infantile botulism. Thermal treatment is insufficient to inactivate these resistant forms. This study explored the effectiveness of supercritical CO_2 ($scCO_2$) on its own and combined with lemon (LEO), clove (CLEO), and cinnamon (CEO) essential oils on the inactivation of *Clostridium sporogenes* (CECT 553) as a surrogate of *Clostridium botulinum*. In water, the degree of inactivation at 10 MPa after 60 min increased with the increasing temperature, reducing the population by 90% at 40 °C and by 99.7% at 80 °C. In contrast, when applied to honey, $scCO_2$ did not inactivate *Clostridium* spores satisfactorily at temperatures below 70 °C, which was related to the protective effect of honey. Meanwhile, $scCO_2$ modified with CEO (<0.4% mass) improved the inactivation degree, with a 1.3-log reduction achieved at 60 °C. With this same mixture, a reduction of 3.7 logs was accomplished in a derivative with 70% moisture. Honey was very sensitive to the temperature of the applied CO_2. The obtained product could be used as a novel food, food ingredient, cosmetic, or medicine.

Keywords: high-pressure carbon dioxide; lemon essential oil; clove essential oil; cinnamon essential oil; *Clostridium sporogenes*

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1. Introduction

Clostridium species are anaerobic spore-forming gram-positive bacilli that are widely distributed in nature, both in soil and dust, in addition to sweet, salty, and residual aqueous bodies [1]. They are highly resistant to extreme temperatures, ultraviolet (UV) radiation, water scarcity, and physical and chemical agents, and, therefore, to the environment [2]. In particular, the ability of *Clostridium botulinum* to cause disease is linked to its ability to survive in adverse environmental conditions through spore formation, rapid growth, and toxin production. Thus, this bacterium can result in botulism, a disease contracted by food poisoning, due to the ingestion of its spores or toxins (type A and B). Botulism is mainly characterized by descending paralysis that leads to death by cardiorespiratory failure [3].

Honey is known as a tasty and nutritious sweetener; it is usually considered a natural, healthy, and clean product. The low pH (typically 3.9), low water activity (a_w 0.5–0.6), and high sugar concentration of honey prevent the growth and survival of many types of bacteria. In addition to its osmolarity, the antimicrobial effect of honey is mainly due to the generation of hydrogen peroxide from the oxidation of glucose by glucose oxidase [4]. Thus, the European Commission (EC) Regulation No. 2073/2005 [5] on microbiological criteria for foodstuffs considers honey a ready-to-eat food during its marketed shelf-life, in which no limits are described for any microorganism, toxin, or metabolites other than *Listeria monocytogenes*, and only if honey is intended for infants and under special medical purposes. However, honey may be a natural reservoir of *Clostridium* species [6].

Grabowski and Klein [7] reported that *C. botulinum* spores were identified in up to 62% of honey samples although spore counts varied widely, from 36 to 60 spores per gram in the most contaminated samples to less than 1 spore per gram in the others. Primary

sources of this biocontamination in honey may be pollen, the bee itself, soil, water, air, or dust, which are natural sources that are very difficult to control [7]. Secondary sources are closely connected with the hygiene of processing, handling, and storage of honey [8]. Because of this, honey has been identified as a possible cause of infantile botulism [7].

Clostridial spores are ingested and then germinate and multiplicate to start toxic genesis in babies because of their poorly developed (anaerobic) intestinal flora. Accordingly, health authorities in several countries have ordered or advised the use of labels on honey packaging alerting to the danger of feeding infants under one year of age. Related to known outbreaks of infant botulism, the number of *Clostridium* spores is usually in the order of 1 to 80 spores per gram [7].

Conventional processing of commercial honey involves indirect heating around 60 °C–65 °C for about 25 min–30 min to destroy yeasts that are responsible for honey fermentation when the moisture content is high and storage temperature is favourable. This treatment is also intended to dissolve crystals of glucose as it exists in a supersaturated state, to avoid the total or partial crystallization of honey in storage, allowing it to remain liquid for longer [9]. Inconveniently, *Clostridium* spores are not inactivated and certain starch-digesting enzymes (invertase, amylase, glucose oxidase) are destroyed [10]. Heating also causes the formation of hydroxymethylfurfural (HMF), a substance characteristic of heated or old honey that can even have detrimental effects (mutagenic, genotoxic, organotoxic, and enzyme inhibition) [11]. Moreover, thermal processing reduces the aromatic richness, as volatile components are lost at high temperatures. Similarly, if not carried out appropriately, this processing results in a loss of diversity in flavours, as it produces a darkening in the tonality of the honey, a situation that is attributed to a partial caramelization of the sugars [12].

Other preservation methods are sought to produce honey with superior quality and free from *Clostridium* spores. Al-Ghamdi et al. [13] studied the effect of high-pressure thermal treatments on the inactivation of nonproteolytic spores type E *Clostridium botulinum*. High pressures (300 MPa to 600 MPa) and elevated temperatures (80 °C to 100 °C) were tested in four low-acid foods. In the said work, processing at 90 °C and 600 MPa resulted in inactivation below the detection limit after 5 min. Traditional thermal processing of spores at 90 °C for 10 min, on the other hand, did not result in an estimated 6-log reduction [13]. According to these studies, high-energy treatments are required to eliminate *Clostridium* spores, which is a difficult task to achieve without damaging the sensitive quality of the honey.

Meanwhile, the inactivation of *Clostridium sporogenes* spores in honey was studied with the application of ultraviolet-C (UV-C) [14]. In the aforementioned work, a maximum reduction of 2.5 Log_{10} colony-forming units g^{-1} (CFU g^{-1}) was observed after an 18 J mL^{-1} treatment. The effect of UV-C on some quality parameters of honey, such as HMF, pH, and colour, was also assessed by the authors, who claimed that UV-C light induced changes in most of these parameters.

Another option is the application of supercritical carbon dioxide ($scCO_2$) [15]. The $scCO_2$ conservation method presents some fundamental advantages related to the mild conditions employed, particularly because it allows processing to be carried out at a much lower temperature than those used in conventional pasteurization and sterilization techniques [16]. In the $scCO_2$ technique, food is exposed to pressurized carbon dioxide (CO_2) for a certain amount of time in a batch, semi-batch, or continuous manner [17]. In addition, CO_2 is inert, non-toxic, accessible, and affordable. Under ambient conditions, it is a gas, so it leaves no residue on the treated product and it is a Generally Recognized as Safe (GRAS) solvent [18].

The $scCO_2$ treatment has been mainly explored for the inactivation of pathogens in liquid food samples [15,17] and less so on viscous or solid foodstuffs. To the best of our knowledge, the reported cases of the use of $scCO_2$ to inactivate bacterial spores in these latter products are the inactivation of natural biocontamination of mesophilic and thermophilic spores in cocoa powder [19] and the bioburden, mostly *Bacillus cereus* spores, in paprika [20], *Alicyclobacillus acidoterrestris* in apple cream [21], and *Geobacillus*

stearothermophilus in cheese [22]. In this previous research, the inactivation efficiency of the $scCO_2$ treatment was limited by the high resistance of these forms in media of low a_w. More recently, Zambon et al. [23,24] explored the feasibility to apply $scCO_2$ to dry and increase the microbial safety of strawberries, finding that the process was efficient against different strains of pathogens (*Escherichia coli*, *Salmonella* spp., and *L. monocytogenes*). Studies have also established that essential oils (EOs) have sporicidal effects in the range of 1% to 3% in mass fraction [25]. Our research group successfully used oregano EO in combination with $scCO_2$ to inactivate *Bacillus cereus* spores in paprika [26]. González-Alonso et al. [27] also inactivated *E. coli* inoculated in raw poultry meat after treatment with herbal EOs in combination with $scCO_2$. Other works reporting the application of $scCO_2$ to improve food shelf-life and safety by inactivating spores can be found in a recent review [28].

With the growing number of consumers seeking natural products from medicinal plants for the well-being of people, new initiatives are already trying to boost all the benefits of honey with different plants, fruits, and EOs. Latin American entrepreneurs are already commercializing 100% pure honey with EOs [29]. According to the companies themselves, EOs are added to honey to boost the benefits that it already has. To this end, EOs of mint, eucalyptus, ginger, cinnamon, and lemon have been added. This type of initiative increases the spectrum of the possible final market in which the product could be purchased, since besides food use (honey with EOs is mainly used for infusions), the final product can be used as a cosmetic and/or ancestral medicine [30].

Therefore, the current study aimed to evaluate the use of pure $scCO_2$, and $scCO_2$ in combination with several EOs to reduce the count of *Clostridium* spores in honey. Because of its morphological and genetic similarity and its nontoxigenicity, *C. sporogenes* was used as a surrogate for *C. botulinum* [31]. Moreover, it has previously been considered an adequate substitute since *C. sporogenes* spores are more resistant than those of *C. botulinum* to heat [32]. The impact of the most relevant operational variables of the technology was investigated according to previous studies in solid or low a_w matrices and within the range of the technology itself [33]. A minimum reduction of one order of magnitude in the *Clostridium* count was sought based on the contamination typically found in honey. In addition, this treatment's impact on the quality of the honey was analysed. This is the first time that the inactivation of *Clostridium* spores by high-pressure CO_2 has been reported both in aqueous media and in complex food matrices such as honey.

2. Materials and Methods

2.1. Raw Materials

Lemon EO (LEO; CAS No. 8008-56-8; Ref. W262528, lot No. 13503DE), clove EO, *Eugenia* spp. (CLEO; CAS No. 8000-34-8; Ref. C8392, lot No. 018K1137), and cinnamon EO, Ceylon type (CEO; CAS No. 8015-91-6; Ref. W229105, lot No. 05211MA), all from Sigma-Aldrich (Spain) were stored in a refrigerator until assayed. Commercial honey (Granja San Francisco, Spain) was also used. The honey label indicated that it was thousand-flower honey from a mixture of both native and non-native European honey. Its containers were opened in a laminar-flow chamber to ensure they were kept sterile and free of enzyme activity. After opening, the containers were carefully closed and refrigerated. The CO_2 (purity >99.95%, <7 vpm H_2, <10 vpm O_2, <5 vpm THC, <2 vpm CO, <25 vpm N_2) was supplied by Carburos Metálicos (Spain). All reagents and culture media were supplied by Sigma-Aldrich (Spain) and used as received.

2.2. Bacteria

C. sporogenes was used as a surrogate for *C. botulinum* [31]. The strain used was the CECT 553 (NCIMB 8053; ATCC 7955) from the Spanish Type Culture Collection (Universidad de Valencia, Spain).

2.3. Culture Medium

The reinforced clostridial medium (RCM) specific culture recommended by the strain supplier was used. A volume of 1 L of RCM was composed of 10 g of meat extract, 10 g of peptone, 5 g of glucose, 5 g of NaCl, 3 g of yeast extract, 3 g of sodium acetate, 1 g of raffinose, and 0.5 g of cysteine. The optimal pH was 7.1 and Na_2CO_3 (0.66 M) was used for its correction.

The lyophilized strain was dissolved in approximately 1 mL of fresh culture medium and then transferred to a small volume of fresh medium that was incubated anaerobically at 37 °C for 24 h to recover the bacteria. Once recovered, the revived strain was transferred to 100 mL of fresh medium and allowed to grow for 48 h at 37 °C.

2.4. Generation and Concentration of Spores

Aliquots were extracted from the live culture in a growing medium after 48 h of incubation. The aliquots were inoculated in the sporulation medium and impoverished at a volumetric ratio of 1 to 10. The composition of 1 L of sporulation medium was 50 g of peptone trypticase, 5 g of peptone, and 1 g of sodium thioglycolate. The inoculated sporulation medium was then incubated for 7 days at 30 °C. After this time, the presence of spores was confirmed by differential staining and subsequent microscopic observation. The spores were centrifuged at 5000× *g* for 15 min and washed with sterile water. The supernatant was discarded. The sediment was washed with distilled water and the process was repeated until it was centrifuged three times. The final sediment was diluted in 50 mL of distilled water and stored at 4 °C.

The order of magnitude of the spore suspension was measured by counting the CFU in Petri dishes per deep seeding in solid RCM. To verify that the plate count did not include vegetative forms and that only spores had been sown, a parallel count was carried out in which the dilutions were introduced to a water bath at 80 °C for 15 min. The initial spore concentration in the suspension was of the order of 10^6 CFU mL^{-1}.

2.5. Sample Preparation

For inactivation tests of spores in honey, samples had to be previously contaminated. Since the resulting contamination was intended to be maximal, but not capable of altering the properties of the honey, a small amount of the spore water suspension in high concentration was added. Thus, for each 50 g of honey, 5 mL of suspension was added, and the sample was shaken until a homogeneous mixture was observed. The obtained concentrations ranged from 10^4 CFU mL^{-1} to 10^5 CFU mL^{-1}. These concentrations were much higher than those of *Clostridium* spores usually found in honey, but they ensured precision in counts and analyses. The resulting honey was made more fluid by the addition of water; thus, if the water content was initially between 18% and 20% in mass fraction, the newly prepared samples had a water content between 25% and 27% in mass fraction. To prepare diluted honey samples, more water was added to the already contaminated honey to increase moisture levels until the desired moisture content was determined, as determined by weight.

2.6. Experimental Installation for the $scCO_2$ Treatment

CO_2 was supplied in liquid form. It passed through a thermally controlled bath (Selecta, Frigiterm-30, Spain) and was cooled to −10 °C before it reached the diaphragm pump (Milroyal D; Dosapro Milton Roy, Spain). The cooling of CO_2 prevented cavitation during pressurization. The pressurized CO_2 was preheated in a spiral inside of a heating jacket before entering a 50 mL capacity 316 ss vessel (Autoclave Engineers, MicroClave™, Series 401A-8067, USA). The temperature in the vessel was controlled with an external heating jacket and read by a type K thermocouple (±1 °C) located inside the vessel, and the pressure was read at the outlet of the vessel with a Bourdon gauge with an accuracy of ±0.2 MPa. Stirring was achieved by an impeller with a speed that could vary between 50 rpm and 500 rpm. A pre-vessel was located upstream from the main vessel for tests in which the CO_2 was mixed with the EO. The CO_2 pressure and flow rate were controlled by

the combined action of a back-pressure regulator (BPR, 26-1761-24-161, TESCOM Europe, Germany) and a pump flow regulator, respectively. The installation had a rupture disk at 38 MPa to avoid overpressure. The total mass and flow rate of CO_2 were measured using a mass flow meter (M-10 SLPM-D, Alicat Scientific, Tucson, AZ, USA) connected to the outlet. The scheme of the $scCO_2$ apparatus is shown in a previous work of our research group [34].

2.7. Method for the Inactivation of Clostridium Spores by $scCO_2$

To begin a $scCO_2$ treatment, the vessel was loaded with the raw material. For the tests with pure water, 15 mL of suspension was introduced. For assays with honey and diluted honey, 15 g of the sample was loaded. After the closures were adjusted, the heating jacket was connected and heated to the operating temperature. Then, the CO_2 container was opened, and the pump was turned on to reach the working pressure. When the EO was used, a piece of cotton soaked with about 0.5 g was placed in the pre-vessel. When the CO_2 passed through the pre-vessel, the EO was solubilized by the CO_2, and the mixture passed into the vessel containing the sample. Then, the BPR valve was opened to provide a continuous flow of CO_2 over the sample at approximately 1 g min^{-1}. The CO_2 (or its mixture with the EO) entered from the bottom of the vessel and passed over the sample. The stirrer in the vessel was set to the desired rotation (300 rpm for spore suspension; 60 rpm for honey and diluted honey samples). The mixing was carried out both to facilitate the contact of the sample with the CO_2 stream inside the container and to guarantee a homogeneous mixture. There was no honey carry-over during processing. When the end of the operating time was reached, the pump was turned off, the CO_2 supply was cut off, and depressurization began very slowly to avoid dragging or freezing the sample. Typically, the time required for the operating temperature and pressure to be reached was 5 min, and the depressurization time was 12 min. Once depressurized and separated from the equipment, the vessel containing the sample was taken to the sterile laminar-flow chamber for further analyses.

2.8. Thermal Treatments

To study the separate effect of temperature on spore inactivation, several non-CO_2 tests were carried out. In these experiments, the operating procedure was the same, except that there was no CO_2 flow in the vessel. Instead, the spore suspension or the contaminated honey was kept inside the vessel at the desired temperature for the same time as in the comparative tests with CO_2.

2.9. Microbial Analysis

In a laminar-flow chamber, 1 mL of spore suspension or 1 g of honey or diluted honey was taken to prepare serial dilutions in the RCM to obtain the concentration of spores in the sample by counting the CFU in Petri dishes. The degree of inactivation of the *Clostridium* spores was expressed as logarithm (log) reduction, which is the logarithm of the count after treatment (N) divided by the initial count (N_0) before each test.

2.10. Quality Analysis of the Honey

The impact of the $scCO_2$ treatment on honey quality variables such as HMF content, diastase index, and pH was analysed.

2.10.1. Determination of Hydroxymethylfurfural (HMF)

The increase of HMF in honey and diluted honey samples was determined according to the method proposed by the International Honey Commission [35]. This method is based on the absorbance of HMF at 284 nm using a UV-Vis spectrophotometer (MRC, model UV-1800, Tel-Aviv, Israel). To avoid the interference of other components at this wavelength, the difference between the absorbances of a clear aqueous honey solution and the same

solution after the addition of bisulphite was determined. The HMF content was calculated after subtraction of the background absorbance at 336 nm as shown in Equation (1):

$$\text{HMF}\left(\text{mg kg honey}^{-1}\right) = \frac{(A_{284} - A_{336}) \times 149.7 \times 5 \times D}{W} \quad (1)$$

where A_{284} and A_{336} are absorbances at 284 nm and 336 nm, respectively; D is the final volume of the sample solution divided by 10; 5 is a theoretical nominal sample weight; W is the weight in g of the honey sample; and 149.7 is a constant [35].

2.10.2. Determination of the Diastase Activity

The diastase activity determination was conducted by an enzymatic-spectrophotometric method according to the International Honey Commission [35]. The principle of the method is based on the fact that the enzymes in the sample act on a starch standard solution that is capable of developing colour, with iodine, in a defined range of intensity, under standard conditions. The decrease in the blue colour was measured photometrically at 660 nm. A plot of absorbance against time was used to determine the time (t_x) required to reach the specified absorbance, 0.235. The results were expressed as a diastase number (DN, also known as Schade or Goethe units), which was calculated as 300 divided by t_x. DN is defined as the amount of enzyme which converts 10 mg of starch to the prescribed endpoint in one hour at 40 °C under the conditions of the test.

2.10.3. Measurement of pH

The pH was measured during the CO_2 treatment using reactive strips (McolorpHast, sensitivity of 0.3 pH unit, Merck, Spain) introduced into the high-pressure vessel along with the sample, where the pH was in two ranges: 2.5−4.5 and 4.0−7.0.

2.11. Data Analysis

The experiments were replicated three times. Microbial analyses were performed in duplicate for each replicate ($n = 3 \times 2$). Means and standard deviations were computed for all data. The maximum standard deviation in the readings of the *Clostridium* degree of inactivation was 0.3-log cycles. The statistical significance of the factors on the *Clostridium* inactivation degree was analysed using the ANOVA General Linear Models tool of STATGRAPHICS XVIII at a 5% significance level ($p \leq 0.05$). For this purpose, the following factors were used as categorical variables: (a) the type of treatment: thermal, $scCO_2$, or $scCO_2$ plus CEO; and (b) the matrix: water, honey, or derivatives. Temperature (data from Figures 1–3) and water content (data from Figure 4) were included as quantitative factors.

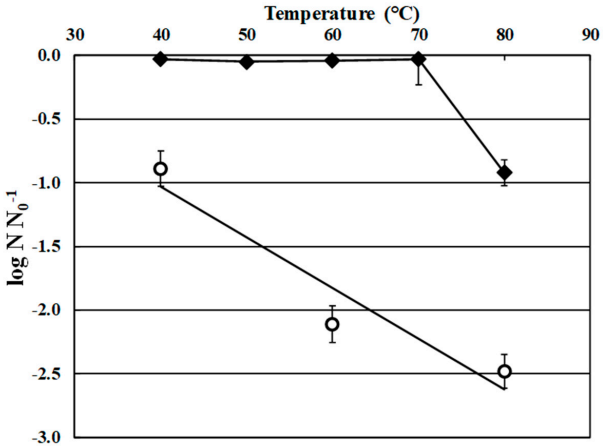

Figure 1. Inactivation of *Clostridium* spores suspended in water using $scCO_2$ (○) and comparison with heat at atmospheric pressure (♦). Conditions: P = 10 MPa; Q = 1 g min^{-1}; operation time = 60 min; stirring rate = 300 rpm. The solid line is the linear fitting.

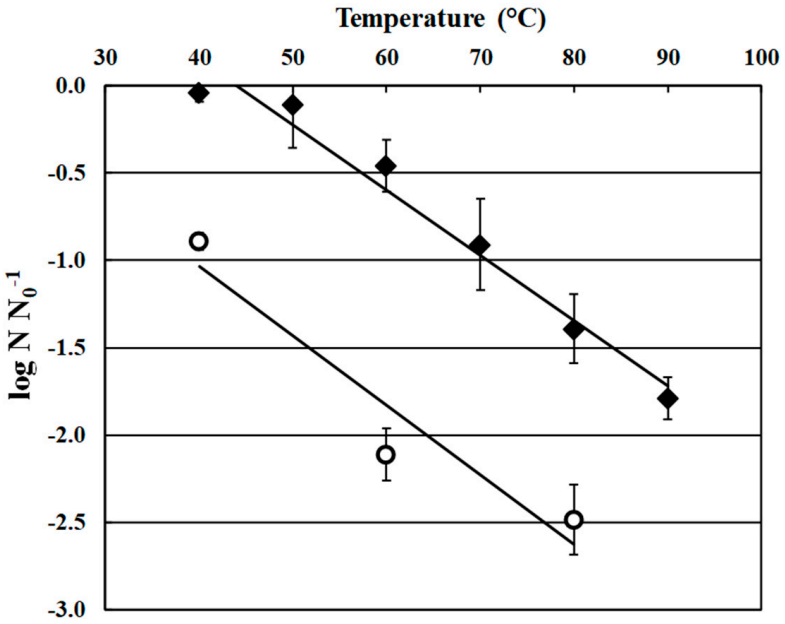

Figure 2. Effect of temperature on the inactivation of *Clostridium* spores using $scCO_2$ in aqueous suspension at 300 rpm (○) and biocontaminated honey at 60 rpm (♦). Conditions: P = 10 MPa; Q = 1 g min^{-1}; operation time = 60 min. The solid line is the linear fitting.

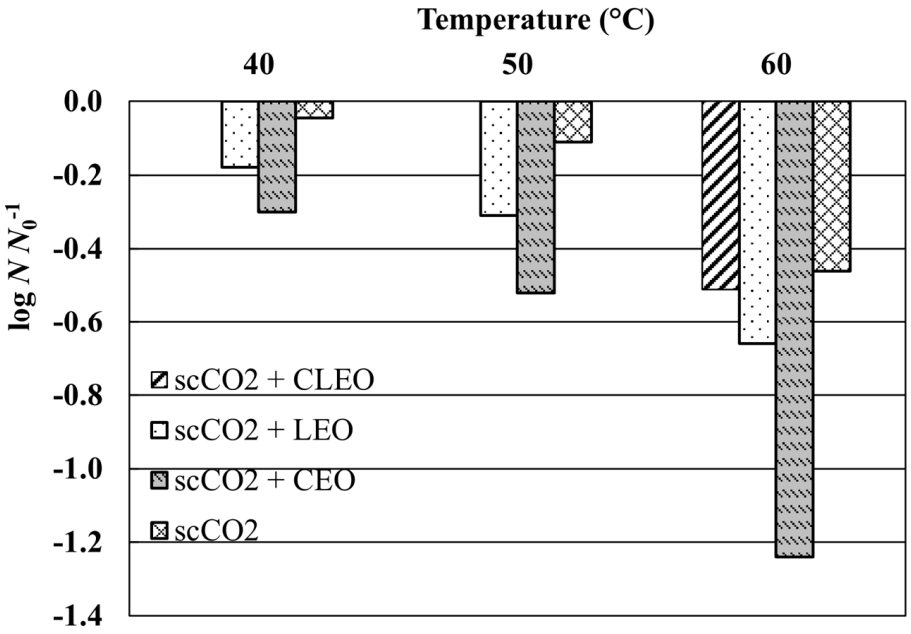

Figure 3. Inactivation of *Clostridium* spores in honey using $scCO_2$ alone and $scCO_2$ combined with lemon essential oil (LEO), clove essential oil (CLEO), and cinnamon essential oil (CEO). Conditions: P = 10 MPa; Q = 1 g min^{-1}; stirring rate = 60 rpm; operation time = 60 min.

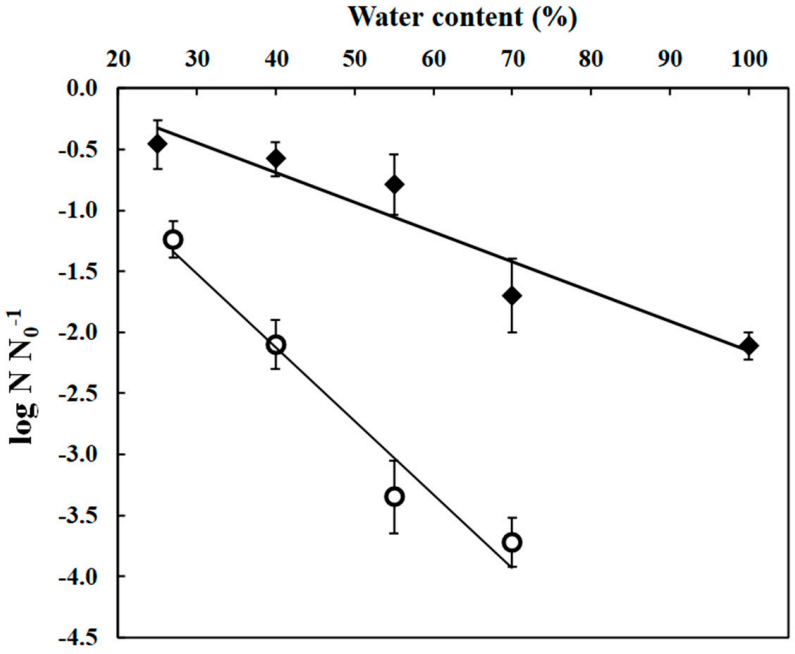

Figure 4. Effect of the initial water content on the inactivation of *Clostridium* spores in honey using $scCO_2$ (♦) and $scCO_2$ saturated with CEO (○). Conditions: P = 10 MPa; T = 60 °C; Q = 1 g min^{-1}; stirring rate = 60 rpm; operation time = 60 min. The solid line is the linear fitting.

3. Results and Discussions

First, the efficacy of the $scCO_2$ method on the inactivation of *Clostridium* spores suspended in water was analysed. Subsequently, the inactivation of these spores in honey and diluted honey samples was studied. The previous study on the water made it possible to establish the possible effects of the food matrix on the effectiveness of the treatment. The main variables that affected the operation and the influence that honey had on the degree of inactivation were studied. The pressure varied between 10 MPa and 30 MPa. The temperature ranged between 40 °C and 80 °C. Up to 60 °C, the treatment could be considered a low-temperature conservation method, but higher values were explored to reach a superior degree of inactivation and to be able to compare with the conventional heat treatment. Agitation at different speeds and increased CO_2 flow rates were explored to promote the transfer of CO_2 to water and viscous honey. Treatment times in the order of several hours were studied to evaluate their impact on the clostridial spores' survival. Furthermore, the use of $scCO_2$ saturated with EOs was investigated. Both CO_2 and EOs do not present a risk of toxicity and their combination could lead to a non-thermal treatment of honey for the elimination of its microbial contamination. The impact of the increasing amounts of water in the honey was intended to prove the importance of the a_w of the food in the inactivation of the bacterial spores. In the treated honey, the quality parameters were analysed to determine the feasibility of the implementation of this technology.

3.1. Inactivation of Clostridium Spores in Aqueous Suspension by $scCO_2$ and Comparison with Heating at Atmospheric Pressure

Figure 1 shows the results of the inactivation of *Clostridium* spores in an aqueous solution using $scCO_2$ and its comparison with heat at atmospheric pressure after 60 min of treatment. The pressure was set at 10 MPa and the solution was stirred at 300 rpm. With the $scCO_2$ process, at 40 °C, a significant ($p \leq 0.05$) degree of inactivation was observed; about 90% of the spore population with respect to the initial population was inactivated.

As the temperature increased (see Figure 1), the degree of inactivation was higher, reducing the population by 99.7% at 80 °C, which corresponded to a 2.5-log reduction. In contrast, in tests performed with heat at atmospheric pressure, without the passage of CO_2, only a small decrease in the total population was observed, less than 1-log reduction

at 80 °C. Therefore, it appeared that CO_2 had sporicidal effects on its own. This fact was previously published by other authors [36–38]. They reported that $scCO_2$ had a lethal effect because in media with high a_w it could promote the activation and germination of spores, reducing their tolerance to heat [39].

3.2. Effect of Operation Conditions on the Inactivation of Clostridium Spores in Honey by $scCO_2$

For these assays, biocontaminated honey with *Clostridium* spores was used as a raw material to determine the buffering effects of the food itself on the process. The pressure was set at 10 MPa and the treatment time at 60 min, as in the water tests, unless otherwise specified. However, the stirring speed was reduced to 60 rpm because it was observed that a high stirring speed produced a dense foam.

3.2.1. Effect of Temperature

Similar to the aqueous suspension, the degree of inactivation of *Clostridium* spores increased with the temperature, as can be seen in Figure 2. However, clear differences could be observed compared with the evolution observed in the aqueous suspension. The lethal effect of $scCO_2$ was much lower in honey than in the aqueous suspension, as the degree of inactivation did not achieve a 2-log reduction, even after reaching 80 °C. This was probably due to the protective effect of honey.

A previous study [40] reported that the presence of salts and sugars in the media reduced the CO_2 effectiveness on the inactivation of *G. stearothermophilus* spores. This negative impact was proportional to their concentration, and therefore to a lower a_w. Additionally, on the inactivation of *A. acidoterrestris* in apple cream, the effectiveness of the $scCO_2$ in this medium was lower than that obtained in liquid juice [21]. The apple cream of the study was much more viscous than the juice and this resulted in a less effective level of contact with CO_2.

3.2.2. Effect of Pressure

The effect of the $scCO_2$ treatment was studied using a pressure of 30 MPa to compare with the results already obtained at 10 MPa. However, no important differences were obtained. A reduction of less than 0.05-log cycles was found in both cases at 40 °C. This was in line with other results obtained on the inactivation of spores in viscous [21] or solid [19,20] food products. It was widely demonstrated that a hydrostatic pressure of the range used had no effect on spores. Specifically, a recent paper reviewed all of the research undertaken on the inactivation of *Clostridium* spores in low-acid foods under high-pressure conditions in the range of 345 MPa–900 MPa [41]. Modest results were reported (<3-log reduction) even in combination with temperatures of the order of 80 °C–86 °C after 15 min–16 min of treatment. Therefore, it is unlikely that there was any significant effect of pressure related to the $scCO_2$ treatment in honey. Since no significant difference was found between 10 MPa and 30 MPa ($p > 0.05$), for economic and safety reasons, the lowest pressure (10 MPa) was used for the following tests.

3.2.3. Effect of the CO_2 Flow Rate

The effectiveness of increasing the CO_2 flow rate was tested to improve the contact between the CO_2 and honey, which had a high viscosity. These tests had good results in the inactivation of *A. acidoterrestris* in apple cream [21], which was also very viscous. However, no differences ($p > 0.05$) were found at 10 MPa and 60 °C between the resulting inactivation using 1 g min^{-1} or 4 g min^{-1} after 60 min (not shown). On the contrary, the introduction of CO_2 at a higher flow rate produced a greater amount of foam, and therefore, it was necessary to spend more rest time after the treatment to allow the CO_2 to escape from the honey. Specifically, by using 1 g min^{-1}, a rest time close to 2 h was needed for a partial recovery (only one layer of foam remained on the surface), and more than 3 h for its total recovery. When using 4 g min^{-1}, times longer than 6 h were needed for the partial recovery, and around 24 h to return to the original state. Therefore, since no significant difference was

found between the highest and the lowest CO_2 flow rate, to reduce the CO_2 consumption and the pumping costs, 1 g min^{-1} was set for the rest of the tests.

3.2.4. Effect of Treatment Time

Operating at 10 MPa, 60 °C, 60 rpm, and 1 g min^{-1}, it was attempted to increase the treatment time to 4 h and consequently the contact with the $scCO_2$. However, this method did not have any significant benefit ($p > 0.05$) on the inactivation of *Clostridium* spores in honey in comparison with the results obtained from a treatment time of 60 min. Specifically, at 60 min, the inactivation degree reached -0.5 ± 0.2 logs, while after 240 min, it was -0.4 ± 0.1 logs.

3.3. Inactivation of Clostridium Spores via $scCO_2$ + EOs

Since the treatment with $scCO_2$ was not effective enough to achieve a 1-log reduction of spores at a mild temperature, the addition of EOs was tested.

In a previous study by our research group [26], it was demonstrated that it was possible to reduce *B. cereus* spores in paprika to 3-log cycles using $scCO_2$ mixed with oregano EO. For this reason, a similar treatment was considered for honey. Three EOs were chosen from the list of EOs that are effective as antimicrobials [42] and whose organoleptic properties were more compatible with honey: lemon (LEO), clove (CLEO), and cinnamon (CEO). For these treatments, the CO_2 was passed through the pre-vessel where the EO was placed before contact with the samples. Conditions were set in the pre-vessel at 10 MPa and 40 °C to improve the solubilization of the EO in the CO_2 because the solubility of EO is better at higher supercritical solvent densities. Under these conditions, the EO solubility in CO_2 was in the order of 0.3–0.4% in mass fraction [43]. Figure 3 compares the results obtained after honey treatment with $scCO_2$ combined with the mentioned EOs and with $scCO_2$ on its own at temperatures between 40 °C and 60 °C.

As can be seen in Figure 3, the CEO was the only EO in which significant ($p \leq 0.05$) improvement was found. The explanation for the different behaviour of the three EOs could lie in the different composition and richness of the main constituents, as demonstrated by Bagheri et al. [44]. In this same work, CEO (Cassia-Aliksir) is also one of the best against *C. tyrobutiricum*. The degree of efficacy achieved with this EO was better than that obtained with $scCO_2$ for the three explored temperatures. Thus, by using $scCO_2$ with CEO at 60 °C, a reduction of more than 1.3-log cycles (94%) in the *Clostridium* spore count in honey was achieved. A previous study discussed the mechanism of action of supercritical CO_2 on spores. With flow cytometry analysis, Rao et al. [45] probed that the permeability of the inner membrane and the cortex was increased. Moreover, the electron microscopy images showed clear evidence of damages to the external structure and morphology changes. Consequently, the pyridine-2,6-dicarboxylic acid (DPA) of the CO_2-treated spores was released to the medium. The presence of moisture would increase the fluidity of the membranes, facilitating the attack of CO_2. In parallel, scanning electron microscope (SEM) observations showed that exposure to high concentrations of EO resulted in damage to the spore coat of *Bacillus subtilis* [25]. Thus, it is logical to find that both agents combined cause a greater sporicidal effect.

Finally, it is highly likely that under these conditions in which *Clostridium* spores were greatly inactivated, other pathogens in the vegetative form were also inactivated due to their lower resistance to the $scCO_2$ treatment [46,47].

3.4. Impact of Initial Product Moisture

As indicated above, honey is a product with a low a_w, which may protect the spores and make $scCO_2$ inactivation difficult. Previous research on the microbial inactivation of solid foods, such as herbs and spices [48], cocoa [19], and paprika [20], have shown that CO_2 under pressure was sporicidal only in the presence of some water. These results were directly related to the role of water in the germination of spores in vegetative forms that are much less resistant [49].

For this reason, the effect of increasing the proportion of water in honey on the inactivation of *Clostridium* spores was analysed. Honey samples with different water content were treated with $scCO_2$ on their own and modified with the CEO. The percentages of moisture investigated were between 25% in mass fraction with respect to the sample (the minimal amount reached after *Clostridium* spore contamination) and 100% in mass fraction with respect to the sample (corresponding to the suspension of spores in water). Honey mead typically contains around 70% water. Please note that mead also contains alcohol and other components that could change these results.

As can be seen in Figure 4, the degree of inactivation of *Clostridium* spores was progressively augmented as the moisture of the samples was increased ($p \leq 0.05$), while operating at 60 °C. A higher degree of inactivation in the treatment with $scCO_2$ + CEO was obtained than in the treatment with $scCO_2$ alone ($p \leq 0.05$). For example, by using the CO_2 mixed with the CEO, a 1.2 ± 0.2-log reduction was reached when the water content in honey was 27%; meanwhile, 2.1 ± 0.2-log cycles (equivalent to a 99% reduction in the total population of spores) was found with an increase of the water content up to 40%. The linear adjustment shown in Figure 4 with the solid line predicts one log reduction in honey with 20% water, i.e., native. During the inactivation of *Clostridium* spores in the honey with 70% water, using $scCO_2$ modified with the CEO, a 3.7 ± 0.2-log reduction was reached, which would be equivalent in practice to obtaining a sterile product.

3.5. Effect of $scCO_2$ Treatment on Honey Quality

The increase in HMF content in honey and the decrease in the diastase activity are parallel processes to the degradation of vitamins, proteins, enzymes, and flavour of this product [11]. HMF is generated by the decomposition of fructose in acid conditions. It occurs naturally in most honey and increases rapidly with heat treatment. Therefore, it can be used as an indicator of heating and storage time [50]. The diastase activity is a measurement of the enzyme content in honey, so it is used as a quality parameter because of the sensitivity of enzymes to heat [9]. For this reason, the European legislation [5] sets a minimum DN of 8 for diastase activity and a maximum HMF content value of 40 mg HMF kg honey^{-1}, excluding honey produced in tropical areas, for which the highest level of HMF allowed is 80 mg HMF kg honey^{-1}.

This part of the study aimed to examine the HMF and diastase contents in honey samples with and without $scCO_2$ treatment to obtain objective information regarding its quality, and to study how the $scCO_2$ process affected these variables. The values of these parameters were initially determined in honey as purchased and are shown in Table 1.

Table 1. Hydroxymethylfurfural and diastase content in untreated honey and honey subjected to the $scCO_2$ treatment at 10 MPa and 60 min at increasing temperatures.

Treatment	Diastase Number (DN)	mg HMF kg Honey^{-1}
Untreated honey	13.4 ± 1.1 ^{a}	30.5 ± 2.5 ^{a}
$scCO_2$ at 45 °C	10.9 ± 0.2 ^{b}	33.1 ± 2.0 ^{a}
$scCO_2$ at 60 °C	7.2 ± 0.1 ^{c}	40.7 ± 1.9 ^{b}

Different lowercase letters within the same column represent statistically significant differences at a 5% significance.

Values of 13.4 for DN and 30.5 mg HMF kg honey^{-1} were found in the starting honey. These values were already low for DN and high for HMF, which showed that the honey had already been treated with heat. Table 1 also shows HMF values and DN in honey samples treated with $scCO_2$. The HMF content in honey samples treated with $scCO_2$ at 45 °C had no statistically significant differences ($p > 0.05$) with the HMF content of untreated honey samples. However, a significant ($p \leq 0.05$) change in the HMF content in samples treated by $scCO_2$ at 60 °C was found, with an increase of 33% with respect to the initial value. This also resulted in a darkening of the honey, which was noticeable to the naked eye at temperatures higher than 60 °C.

In contrast, the diastase index was more affected by the $scCO_2$ treatment. For example, the DN of honey subjected to $scCO_2$ at 45 °C was reduced by 18% of its initial value, and by 46% using $scCO_2$ at 60 °C, with statistically significant differences ($p \leq 0.05$) among all groups. This was because $scCO_2$ had a damaging impact on enzymes. The application of $scCO_2$ has been previously explored as an effective non-thermal technique to inactivate harmful enzymes in liquid and solid food systems. Structural, morphological, and electrophoretic behaviour changes in the enzymes have been detected after $scCO_2$ contact [51].

The dissolution of CO_2 in water generates carbonic acid (H_2CO_3), which reduces the pH. This reduction in acidity has synergistic effects on the inactivation of spores through the use of $scCO_2$. Haas et al. [48] claimed that the efficacy of $scCO_2$ for the inactivation of *C. sporogenes* in thioglycolate broth (pH = 5.5) at 5.5 MPa and 70 °C was substantially greater (7-log) if the medium was acidified to pH 2.5–3.0. In the same way, Casas and Calvo [52] showed that the inactivation of *B. cereus* spores in a phosphate-buffered solution (pH = 7) was null in contrast to that achieved in pure water, where at 30 MPa and 70 °C, the spore population was reduced by four orders of magnitude. The pH of the water under these conditions was found to be 3.9 inside the vessel.

Table 2 shows the variation of the pH in the vessel due to the $scCO_2$ treatment in honey, diluted honey, and the spore suspension. In the aqueous suspension, the pH was significantly ($p \leq 0.05$) lowered after $scCO_2$ treatment due to the better solubilization of CO_2. In contrast, the pH in pure honey was not significantly ($p > 0.05$) altered by the $scCO_2$ treatment since it was already low and the free water (a_w) for CO_2 dissolution was low. The naturally low pH of the honey could be one of the causes of the lower inactivation of *Clostridium* spores in this medium, along with the other protecting factors earlier mentioned. In contrast, in honey samples with added water, the pH was slightly reduced ($p \leq 0.05$).

Table 2. Variation of pH due to $scCO_2$ treatment.

Sample	Initial pH	Final pH
Honey	3.9 [a]	4.1 [a]
Diluted honey (40% water)	4.1 [a]	3.8 [b]
Diluted honey (55% water)	4.7 [a]	4.2 [b]
Diluted honey (70% water)	4.8 [a]	4.3 [b]
Spore suspension	5.5 [a]	4.3 [b]

Different lowercase letters within the same row represent statistically significant differences at a 5% significance. The average standard deviation in the readings was ±0.1.

The honey aroma is rich in volatile compounds such as alcohols, ketones, aldehydes, acids, terpenes, hydrocarbons, benzene, and furan derivatives [53]; many studies showed good solubility of flavours and fragrances in $scCO_2$ [54] although the pressure was usually higher than that used by us. Still, we did not observe any extract in the vessel placed after the BPR where depressurization to room conditions occurred, even after many runs.

By adding increasing quantities of the EO to the honey, our research group identified that the level of smell that was perceptible but pleasant was of the order of 200 ppm, while it was excessive at or above 400 ppm and even unpleasant when it was higher than 800 ppm. However, given the relatively high solubility of the EO in $scCO_2$ [43], it would be possible to remove all or part of the EO with an extra passage of pure CO_2. This was done successfully in an earlier study to eliminate the remaining odour of oregano EO from paprika [26]. In the aforementioned work, 20 min of continuous CO_2 passage was enough to render the odour in the paprika imperceptible to a panel of six trained people. Nevertheless, more experiments are needed to confirm this hypothesis.

4. Conclusions

Among several parameters, temperature and initial moisture content of the honey derivatives were variables with the greatest impact on the efficacy of the *Clostridium* spores' inactivation. Pressure and/or treatment time were less influential. The smallest

reduction of *Clostridium* spore count (i.e., 1-log reduction) was obtained in an aqueous suspension with $scCO_2$ at 10 MPa and 40 °C after 60 min. In comparison, this minimum inactivation degree in pure honey was not achieved until 70 °C due to its low water content, naturally low pH, other protective effects associated with the presence of nutrients (sugar), and its high viscosity. However, adding CEO to the CO_2 (0.3–0.4% in mass fraction) significantly increased the effectiveness of inactivation, so the temperature to achieve a 1-log reduction was about 55 °C. Therefore, this combined method could be an alternative to traditional thermal treatments for the inactivation of honey microflora causing infant botulism. However, this method should be validated with *C. botulinum* spores before its implementation.

This treatment, if carried out at temperatures below 60 °C, did not cause significant increases in the HMF content of the honey, nor changes in its pH or colour. However, it significantly reduced the enzymatic activity of the honey due to the inactivation capacity of free enzymes caused by the $scCO_2$. The sensory impact of the addition of EOs may be acceptable for consumers based on flavour compatibility, but it could also be controlled with an extraction step of pure CO_2.

Better clostridial spore inactivation degrees were found in honey with a high-water content, as it could be mead. $scCO_2$ combined with the addition of CEO caused an inactivation degree of nearly 4-log cycles of the *Clostridium* spores at 60 °C, which opens the possibility of applying the process for the sterilization of other thermolabile liquid food products or those with high moisture content. In liquid products, this could be done by pumping them simultaneously with the $scCO_2$, providing contact time in a holding tube. This operating method would allow continuous treatment with higher capacity and lower operating costs [34].

The introduction of the CEO in combination with the $scCO_2$ left a noticeable odour in the honey. However, recipes for flavouring honey with herbs, edible flowers, and spices can be easily found on the Internet and are already marketed. Thus, it is highly possible that consumers would readily accept the aroma and flavour imparted by the CEO. Indeed, all the research team members of our group found the aroma and flavour to be very pleasant. Therefore, the product obtained in this work could have a wide range of uses, including as a food ingredient (e.g., for infusions and tea); in cosmetics (mainly for use on the skin); and in traditional medicine, since honey with CEO is used ancestrally in Latin America against colds, joint pains, indigestion, and other ailments [30]. Furthermore, its possible use as a novel food (since it includes an EO) should be authorized according to the EU regulation.

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