Highlights

The use of saline solutions (0.8-5% NaCl) increased ultrasound-assisted SC-CO_2 *E. coli* inactivation by 24.8% compared to only SC-CO_2

Fat content in dry-cured ham was reduced in 46-% after the SC-CO_2+HPU treatment along with a ham softening (26.5-%)

Physico-chemical characteristics (moisture content, pH and texture) of ham were unaltered during refrigerated storage (30 days - 4 ºC) after the SC-CO_2+HPU treatment

Use of high-power ultrasound combined with supercritical fluids for microbial inactivation in dry-cured ham

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Abstract

The aim of this study was to analyze the application of supercritical carbon dioxide combined with high-power ultrasound (SC-CO_2+HPU) and the use of a saline solution (SS; 0.85 % NaCl) on the microbial inactivation and the quality of dry-cured ham. The effect of temperature, pressure and treatment time was studied using RSM. Physicochemical analyses were carried out after the treatments and during refrigerated storage (30 days / 4 ºC). The most significant inactivation of *Escherichia coli* (3.62 ± 0.20-log CFU/g) was obtained using the SC-CO_2+HPU+SS (25 MPa, 46-ºC and 10-min), with temperature being the most important process variable. Fat content showed a significant ($p<0.05$) reduction (46 %) after the SC-CO_2+HPU treatment. The breakage of the muscle fibers, the disorganization in the myofibrils, as well as the enlargement of the interfibrillar spaces led to the ham softening (avg 26.5 %). No significant ($p>0.05$) changes in color, texture or pH were found during storage. Thus, ultrasonic-assisted SC-CO_2 could be used, in combination or not with SS, to improve the shelf of dry-cured ham.

Industrial relevance: Supercritical carbon dioxide (SC-CO_2) inactivation technology has been shown to be highly efficient at reducing different bacteria in liquid media with minimum effect on food quality. This technology is barely applied to solid products and its use is limited by the long processing times and reduced inactivation capacity. The application of high-power ultrasound (HPU) leads to a shorter process time. This technology is useful for the inactivation of ham microbiota and inoculated *E.coli*. A liquid medium surrounding the treated solid can enhance microbial inactivation for the purposes of improving the effect of ultrasound cavitation, while only minimally affecting the quality of the samples (color, texture, fat and moisture contents).

Keywords: non-thermal technology, supercritical fluids, microbial inactivation, dry-cured ham, quality attributes

1. Introduction

In some European countries, dry-cured ham is a traditional food that has attained a high degree of consumer acceptance (Toldrá &Aristoy, 2010). As part of their processing, hams are salted in order to develop their characteristic flavor and to prevent the growth of spoilage microorganisms (Toldrá Flores& Sanz, 1997). The current market trend is to reduce the salt content, which could compromise the safety and stability of the ham. *E. coli* is among the microorganisms that may be present in dry-cured ham (García et al., 2004). Considering that *E. coli* may cause severe human diseases, it should be removed or significantly reduced from samples intended to be marketed. In this regard, the application of supercritical carbon dioxide (SC-CO_2), an environmentally-friendly and non-thermal inactivation technique, has shown itself to be an efficient means of reducing pathogenic and food spoilage microorganisms, with minimal impact on the nutritional and organoleptic properties of food (Amaral et al., 2017, Morbiato et al., 2019). The main disadvantage of SC-CO_2 inactivation treatments is that long processing times are necessary to achieve similar inactivation levels to those found for thermal processes (Meurehg, 2006; Choi et al., 2009a; Choi et al., 2009b; Silva et al., 2018). To solve this limitation, several authors have combined SC-CO_2 with other non-thermal technologies, such as high-power ultrasound (Ortuño et al., 2013; Cappelleti et al., 2014; Paniagua et al.,2016, 2018a, 2018b). This combined technique (SC-CO_2+HPU) has succeeded in reducing treatment times, achieving complete microbial inactivation in liquid food matrices. However, there are few studies into the viability of its application in solid food matrices (Ferrentino & Spilimbergo, 2015, 2016; Morbiato et al., 2019) and particularly in dry-cured ham (Spilimbergo et al., 2014). Spilimbergo et al. (2014) studied the simultaneous application of SC-CO_2+HPU in solid foods, finding an enhancement in the inactivation of *L. monocytogenes* in dry-cured ham cubes, compared to the inactivation observed using only

SC-CO_2. The quality attributes, such as color, pH and acidity, were studied, and no differences were detected between the untreated dry-cured ham and samples treated with SC-CO_2+HPU (Spilimbergo et al., 2014). An alternative means of enhancing the effect of the SC-CO_2+HPU treatment may be the use of a liquid medium (such as saline solutions) surrounding the treated solids, since SC-CO_2+HPU inactivation treatment shave proven themselves to be highly efficient in liquids (Paniagua et al., 2016, 2018a, 2018b). In this regard, no literature has been found that studies the effect of a surrounding liquid on the microbial inactivation in solids using SC-CO_2+HPU. Moreover, the effect of the combined treatment (SC-CO_2+HPU) on the texture and microstructure of the treated solids has not been covered elsewhere.

Therefore, the objective of this study was to analyze the influence of an ultrasound-assisted supercritical fluid treatment and the use of saline solutions surrounding the solid samples on the microbial inactivation of *E. coli* in dry-cured ham samples. Furthermore, the quality and microstructural properties of dry-cured ham after the treatment and during refrigerated storage were also assessed.

2. Materials and methods

2.1. Preparation of the microorganism suspension and sample inoculation

The microorganism used in this study was *Escherichia coli* DH1(chromosomal genotype:*endA1 gyr A9, thi-1, hsdR179* (rk-, mk+), *supE44, rel A1*). Cells were maintained at 4°C in Luria Bertani Broth (LB Broth, Sigma-Aldrich, USA). A single colony of *E. coli* was inoculated in 50-mL of Luria Bertani Broth (LB Broth) and allowed to grow overnight at 37-°C, using an incubation chamber (J.P. SELECTA, Model 3000957, Barcelona, Spain)with agitation (120 rpm, J.P. SELECTA, Rotabit Model 3000974, Barcelona, Spain). For each test, a subculture was prepared with an aliquot of 100-µL of the starter and inoculated into 50-mL of sterilized LB Broth and incubated with agitation at 37-°C for 18-h to obtain cells in the

early stationary phase. Fifteen mL of the cell suspension were centrifuged at 2600-g for 5-min. The supernatant was discarded. Each pellet was resuspended in 50-mL of sterilized isotonic saline solution (SI, 0.85-% w/w) to a cell concentration of 10^8-cfu/mL.

Dry-cured ham slices were purchased from a local market (Valencia, Spain). Cylindrical samples of 3.3-cm diameter and 1-cm thickness were obtained from the slices and superficially inoculated with 100-µL of SI (10^8-cfu/mL). Samples were left to dry for 10-min inside a laminar flow cabinet before their subsequent treatment (Telstar technologies, Model AH-100, Terrassa, Spain). The initial *E. coli* concentration of meat was 10^6-cfu/g. For each experiment, control samples were prepared. These samples were inoculated in the same way as previously described for treated samples,but they were not SC-CO_2treated.

2.2. Apparatus

Inactivation treatments with SC-CO_2 and HPU were carried out in a SC-CO_2 pilot-scale plant, described by Ortuño et al. (2013). The plant (Figure 1) included a CO_2-tank and a N_2-tank, which were kept at room temperature; a cooler reservoir stored at -18 ± 3-°C, which was made of stainless-steel type 316; a dual-syringe pump system and a thermostatic bath to keep the inactivation vessel at a temperature ranging from 36 to 51-°C. The vessel (internal diameter of 30.5-cm and 500-mL volume) as well as the different connections and valves in contact with SC-CO_2 were made of stainless-steel type 316. The inactivation vessel featured a leaf spring manometer (5, Figure 1) submerged in glycerin (PTG Pressure Technology, Gesellschaft fur Hochdrucktechnik mbH, Model EN 837-1, Germany) as well as a temperature probeto monitor and control the pressure and temperatureinside the inactivation vessel. The dry-cured ham sample holder was made of polypropene. The ultrasound unit mainly consisted of a transducer(>1-W/cm^2) attached to the treatment vessel lid, with two commercial ceramics (35-mm external diameter;12.5-mm internal diameter; 5-mm thickness; resonance frequency of 30-kHz) and one sonotrode, which was specially constructed to

concentrate the highest amount of acoustic energy on the application point. The transducer was powered with constant energy by the power generator unit (7, Figure 1).

2.3. Supercritical fluid processing

The inactivation treatment by SC-CO_2+HPU required five operating stages: 1. cleaning and disinfection of the inactivation vessel, 2. heating of the thermostatic water bath until a homogeneous temperature is reached in the whole system, 3. sample positioning 4. pressurization and 5. connection of the HPU system.

Dry-cured ham samples were placed into the inactivation vessel on the sample holder (12, Figure 1) and the vessel was sealed. For treatments with saline solution (SS), 90-mL of saline solution (0.9-% NaCl w/v) was added to the vessel before pressurization. The pump injected supercritical carbon dioxide into the inactivation vessel until the desired pressure was reached (1 to 4-min to operate between 100 and 350-bar, respectively). It was considered to be time zero for the SC-CO_2 + HPU treatments when the desired pressure and temperature in the inactivation vessel were reached and the HPU system was connected. The power applied during the SC-CO_2+HPU experiments was 40 ±5-W (I = 250±10-mA; U=220±5-V) measured using a Digital Power Meter (Yokogawa, Model WT210).

At the end of the treatment, the inactivation vessel was depressurized by opening the valve (11, Figure 1,). Samples were collected using sterilized stainless-steel tweezers. Subsequently, samples were placed in a sterile bag for its further microbiological, quality and microstructural analysis.

2.4. Experimental design for microbial inactivation

Response surface methodology (RSM) was employed to study the effect of the process variables (pressure, temperature and time) on the *E. coli* inactivation in dry-cured ham samples using SC-CO_2+HPU and SC-CO_2+HPU+SStreatments.

Experiments were carried out according to a Box-Benkhen design (Table 1) determined using Statgraphics Centurion 17.2.04 (2018). The experimental design consisted of 15 different treatments, which were run in triplicate (Table 1).The experimental values were: pressure (p, 150, 250 and 350-bar), temperature (T, 41, 46 and 51-°C), and treatment time (t, 5, 10 and 15-min).

The response (inactivation level of *E. coli*) was calculated according to the quadratic polynomial regression model Eq.(1), where *E. coli* inactivation (CFU/g) was represented by Eq. (2).

$$y = \beta_0 + \beta_1 p + \beta_2 T + \beta_3 t + \beta_{11} p^2 + \beta_{12} pT + \beta_{13} pt + \beta_{22} T^2 + \beta_{23} Tt + \beta_{33} t^2 \qquad (1)$$

$$y = ln\left(\frac{N}{N_0}\right) \qquad (2)$$

Whereβ_0, β_1,β_2,$\beta_3$$\beta_{11}$,$\beta_{22}$, β_{33},β_{12}, β_{13}, $\beta_{23}$are the model constant coefficients and $N_0$and N correspond to the initial number of microorganisms and the number of microorganisms after a certain treatment time, respectively.

The same model was fitted separately to the two sets of experimental results: the *E. coli* inactivation using SC-CO_2+HPU and SC-CO_2+HPU+SS.

The model coefficient assessment and generation of response surfaces were performed using MATLAB R2011b version 7.13.0.564 (Mathworks Inc., Massachusetts, USA).

2.5. Microbiological analysis

The control and treated samples were placed in a sterile bag provided with a filter (WWR No. 129-0733) and 90-mL of sterilized isotonic saline solution (SI, 0.85% NaCl) were added to the sterile bag. The mixture was homogenized for 4-min using Stomacher equipment(IUL INSTRUMENTS, model 1000475/2510, Barcelona, España).

The viability of *E. coli* in the samples was determined by the plate count method before and after every treatment. Samples were serially diluted, and 100-mL of the appropriate dilutions

were plated on Luria-Bertani Agar (LB Agar, Sigma-Aldrich, USA) in triplicate. The plates were incubated at 37-ºC for 24-h before counting. The experimental results shown are the arithmetic mean and the standard deviation of log (N/N_0) for at least three plates, where N_0 is the initial number of cells in the control sample and N is the number of cells in the sample after the different treatment times.

2.6. Quality attributes after the treatments and during the shelflife

In order to evaluate the effect of the treatments using SC-CO_2 +HPU and SC-CO_2 + HPU+ SS on the quality of dry-cured ham samples, experiments at 250-bar, 46-°C and 10-min (central point of the RSM design), were carried out on non-inoculated samples.

The treatments were run in triplicate and the physico-chemical analyses, including color, texture, pH, moisture content and microstructure, were carried out both ontreated and control meat samples.

A shelf-life study was conducted for 30-days using non-inoculated samples treated by SC-CO_2+HPU (250-bar, 46-ºC and 10-min). After the treatment, the control (without treatment) and treated samples were packed in sterile polypropylene bags and stored in a refrigerator (Liebherr, Model GKv 5730, Barcelona, Spain) at 4-°C. The samples (control and treated) were analyzed after 10, 20 and 30-days of storage. Time zero for the storage time is considered to be immediately after treatment. The physico-chemical properties (color, texture, pH, moisture content) of the treated meat samples were compared with the control (untreated) samples. Moreover, the microbiota (mesophilic bacteria, psychrophilic bacteria, fungi and yeasts, lactic acid bacteria and coliforms) were studied during refrigerated storage, following the methodology described in the microbial analysis section and using selective media and particular growth conditions depending on the target microorganism (Table 2).

200 **2.6.1. Color**

201 The color analysis was carried out at room temperature (25 ±1-°C) on the surface of dry-cured
202 ham samples, both control and treated, after SC-CO_2+HPU and SC-CO_2 + HPU+ SS
203 treatments and for 30-days of storage at 4-°C. The analyses were performed using a
204 reflectance colorimeter (CM-2500d, KONICA – MINOLTA, Japan). The equipment was
205 calibrated using white standard for the angle of observation and illuminant C. For the color
206 measurements, the colorimeter was placed at a 10° angle with respect to the samples. The
207 colorimeter displayed the L*, a*, b* coordinates directly. Five measurements were taken in
208 both the control and treated samples. Eq. (3) was used to evaluate the color difference (ΔE)
209 for the purposes of determining the effect of the treatments on the meat samples. .

210 $$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (3)$$

211 Where L*, a* y b* are the color coordinates of the sample after the treatment and L_0*, a_0* y
212 b_0* correspond to the color coordinates of the sample before the treatment.

213 **2.6.2. Texture**

214 The texture of the samples was measured by performing a compression/puncture test using a
215 texture analyzer (TA-XT2i, Stable Micro Systems; Surrey, England), connected to a computer
216 for data collection and processing. A stainless-steel cylindrical probe with a 2-mm diameter
217 flat bottom was used. The probe compressed the sample by 5-mm at 1-mm/s. A minimum of
218 16 punctures were performed in the samples, immediately after the treatments. Subsequently,
219 similar analyses were performed in both samples, control and treated, during the storage time
220 considered. The force-deformation curves obtained with the Texture Expert Software (Stable
221 micro Systems. Ltd Version 1.22) permitted measurement of the sample hardness, defined as
222 the maximum peak force (F_{max}; N). The hardness values were calculated as the average of the
223 three analyzed dry-cured ham samples.

224

2.6.3. Microstructure

Scanning Electron Microscopy (SEM) was used to analyze the microstructure of the dry-cured ham samples. The working protocol included preparation, fixation, metallization, and, finally, observation of the samples. Sample preparation consisted of cutting parallelepipeds of 2-3-mm^3 with a stainless-steel blade. Samples were packed and frozen at -28-°C. Subsequently, they were lyophilized in a lyophilizer (Telstar Lioalfa-6, Barcelona, Spain)for 24-48-h. Lyophilized samples were placed on a metal base using double-sided graphite tape or an epoxy adhesive. The samples were sputter-coated with gold and scanned on a JEOL-JSM6300 scanning electron microscope (JEOL Ltd., Tokyo, Japan) at 15-kV. Representative photomicrographs were taken at different magnifications and image analysis was carried out with ImageJ (v.1.44, National Institutes of Health, Bethesda, MD, USA).

2.6.4Chemical analysis

The fat content was determined using the AOAC Method 991.36 (AOAC, 1997) by Soxhlet extraction. The results were expressed in grams of fat per gram of dry matter (g fat/g of dm).The moisture of the meat samples was determined by drying to a constant weight following AOAC Method 934.06 (AOAC, 1990).

The pH of the samples was measured by means of a pH meter for solid foods (pH meter FG-Five GTM, Mettler Toledo, Switzerland-USA) which was inserted into the center of the meat samples. Prior to analysis, the device was calibrated at room temperature using buffer solutions of pH 4.0 and 7.0.

2.7. Statistical Analysis

The experimental data for *E. coli* inactivation was analyzed by ANOVA to determine the significance of the experimental factors, along with the response surfaces, to observe the effect of interactions. In the case of the analysis of quality parameters, the ANOVA test was followed by multiple comparisons between means using the Duncan test. All the analyses

considered a level of significance of 95% ($p<0.05$) and were carried out using Statgraphics Centurion 17.2.04 (2018).

3. Results and discussion

3.1 Microbial inactivation in dry-cured ham

The microbial reductions obtained for the different treatments in the selected process conditions are reported in Table 1. Up to now, no studies have been conducted to determine the effect of immersing solid samples in a saline solution for the purposes of improving microbial inactivation in solids matrices using SC-CO_2 and HPU. The results revealed that, on average, the samples treated with SC-CO_2+HPU+SS achieved a significantly ($p<0.05$) higher inactivation rate (2.49± 0.20-log-cycles reduction) than the SC-CO_2+HPU treated samples (1.87± 0.20-log-cycles reduction). This result may suggest that the saline solution intensifies the effect that HPU has on microbial cells, probably due to the more intense agitation of the medium surrounding the meat samples and to the enhancement of the cavitation phenomenon. Cavitation refers to the formation, growth, and implosion of tiny gas bubbles in a liquid when ultrasound travels through it. The cavitation may produce contraction and rarefaction movements or high intensity micro-currents in the medium which might enhance the solubilization of the CO_2 in the SS and its transport inside microbial cells (Ortuño et al. 2013), and also cause the disruption of cell walls. The disruption of the microorganism's walls could contribute to the extraction of intracellular compounds, accelerating the death of microbial cells. The acoustic cavitation is affected by the physical properties of the solvent, such as vapor pressure, surface tension and viscosity (Panda & Manickam, 2019). In this regard, the higher surface tension of the saline solution could lead to the bubble collapsing with greater force and, consequently, to a stronger cavitation effect than that of the SC-CO_2 medium (Martínez-Ramos et al. 2020).

Moreover, the water activity of the treated medium, which greatly increases when the SS is added, is another important factor determining the inactivation capacity of SC-CO_2 (García-Gonzalez et al., 2007). The reason could be the increased CO_2 solubility, which reduces the pH of the suspending medium. In addition, water could lead to swollen cell walls, making them more prone to CO_2 penetration and the extraction of vital components (Lin et al., 1993).

As previously stated, the maximum inactivation obtained in this study was 3.66-log-cycles; this can be considered a moderate microbial reduction compared with levels attained in liquid foods (LB Broth, apple, orange, pineapple juice) (Ortuño et al., 2013; Paniagua et al., 2018a, 2018b), where 6-8 log reductions were reported. In addition to the effect of the water activity and the influence of the medium on cavitation, the difference between the microbial inactivation using SC-CO_2+HPU (using or not SS) in solid and liquid matrices could also be explained by the greater protection that solid surfaces provide to microorganisms against the action of both SC-CO_2 and ultrasound (Spilimbergo & Bertuco, 2003).

The response surface methodology was used to estimate the relationship between the controllable experimental factors (temperature, pressure and time) and the observed inactivation level of *E. coli*. Table 3 shows the regression coefficients *(β)* and their corresponding p-values calculated by adjusting the quadratic polynomial regression model Eq.(1), to the experimental data (Table 1) using MATLAB, for SC-CO_2+HPU and SC-CO_2+HPU+SS treatments.

The multiple regression model for predicting the *E. coli* inactivation by SC-CO_2+HPU could only explain 58-% of the observed variation. Due to the poor explained variance of the model, results were used to study the effect (positive or negative) of model parameters on the inactivation of *E. coli* rather than to predict the inactivation of *E. coli* by the SC-CO_2+HPU treatment. Table 3 shows that, only temperature had a significant effect ($p<0.05$) on *E. coli* inactivation for the SC-CO_2+HPU treatment. The negative correlation observed ($\beta_2 =$

299 −1.38) indicates that a rise in the temperature of the process increases the level of
300 inactivation of *E coli* in the samples of dry-cured ham treated at different pressure values
301 (150, 250 and 350-bar) and times (5, 10 and 15-min).
302 As regards the SC-CO_2+HPU+SS treatment, the multiple regression model could explain 88-
303 % of the observed variation. In consequence, the proposed model could be used for prediction
304 purposes. Table 3 reveals a significant ($p<0.05$) linear (β_2= - 1.47) and quadratic effect (β_{22} =
305 1.85) of temperature on the inactivation of *E. coli*. In this case, the rise in temperature also
306 brought about an increase in microbial inactivation. On the other hand, the linear effect of
307 pressure (β_1= 0.15) and treatment time (β_3= -0.07) showed no significant influence ($p>0.05$)
308 on the *E. coli* inactivation.
309 The effect of the SC-CO_2+HPU+SS treatment parameters on the inactivation of *E. coli* is
310 depicted in Figure 3. The linear and quadratic components of the relationships between
311 temperature and the inactivation of *E. coli* may be observed in this figure, as well as the
312 significant ($p<0.05$) quadratic effect of pressure, time and the interaction between factors
313 shown in Table 3. Finally, the optimal treatment conditions for the SC-CO_2+HPU +SS
314 treatment, considered as those that maximized the microbial inactivation, were obtained by
315 RSM at 243.3-bar, 48.4-°C and 12.2-min, leading to a reduction of 3.88-log-cycles. For the
316 SC-CO_2+HPU treatment, the optimal conditions were 350-bar, 51-°C and 5-min, leading to a
317 reduction of 3.2-log-cycles.
318 Therefore, the combination of supercritical carbon dioxide and ultrasound allows a significant
319 microbial reduction in dry-cured ham, reduction that can be intensified when a saline solution
320 is added.
321 **3.2. Quality parameters of dry-cured ham**
322 **3.2.1. Color**

323 The chromatic coordinates, a* and b*,showed a significant increase ($p<0.05$) with both the
324 SC-CO_2+HPU and SC-CO_2+HPU+SS treatments, compared to the average value obtained for
325 the control samples; however, the brightness parameter (L*) did not significantly ($p>0.05$)
326 change after any treatment (Table 4).
327 According to Mokrzycky's scale (Mokrzycki & Tatol, 2012), the global color difference (ΔE)
328 value shows that the differences are visible to the human eye when ΔE>2; therefore,
329 noticeable changes in color are found with both treatments. The highest ΔE value was
330 observed in the SC-CO_2+HPU+SS treated samples (ΔE=7.46); in contrast, ΔE decreased to
331 4.50 when SS was not used.
332 This marked color change produced in the dry-cured ham samples submerged in saline
333 solution compared to those treated only with SC-CO_2could have been caused by the washing
334 or degradation of pigments, due to the vigorous cavitation generated in the SS, which also led
335 to a greater microbial inactivation.
336 Most of the literature has evaluated the effect of non-thermal treatments on the color change
337 indry-cured ham samples when applying high pressure treatment (HPP) (Hugas et al., 2002;
338 Andrés et al., 2006; Cava et al., 2009; Fuentes et al., 2010; De Alba et al., 2013). However,
339 there are few studies into the effect of the SC-CO_2 process on the color of the meat samples.
340 Ferrentino et al. (2013a) studied the effect of SC-CO_2 and SC-CO_2+HPU on samples of dry-
341 cured ham. As happened in the present study, these authors reported that the dry-cured ham
342 samples treated by SC-CO_2 (120-bar, 45-°C, 30-min) showed color variations visible to the
343 human eye (ΔE>4). On the other hand, Spilimbergo et al. (2014) observed that no visible
344 changes were observed in dry-cured ham samples after 5-min treatment with SC-CO_2+HPU
345 (120-bar, 35-°C, 5-min). The ΔE values of the dry-cured ham samples obtained in the present
346 study are higher than 4, similar to the results obtained by Ferrentino et al. (2013a) but higher
347 than the values observed by Spilimbergo et al. (2014). These differences could be attributed to

348 the fact that the process conditions used in the present study (250-bar, 46-°C and 10-min)
349 were similar to those used by Ferrentino et al. (2013a), and harsher (mainly higher
350 temperatures and longer times) than those used by Spilimbergo et al. (2014).
351 Although the color changed during the treatments, the values obtained were within the range
352 found in commercial dry-cured ham. In fact, dry-cured ham is a product that presents a wide
353 color variability due to the differences in the raw material and the processing conditions.
354
355 **3.2.2 Texture**
356 Samples of dry-cured ham treated by SC-CO_2+HPU and SC-CO_2+HPU+SS revealed a
357 significant decrease ($p<0.05$) in hardness compared to the average hardness of control
358 samples. However, the use of SS did not significantly ($p>0.05$) affect the treatment (Table 4).
359 This tenderization effect on dry-cured meat could be attributed to two processes. On the one
360 hand, to the compression and decompression processes induced by the HPU treatment
361 (sponge effect) as well as to the rapid exit of CO_2 after depressurization (Zhou et al., 2015).
362 On the other hand, the decrease in hardness may be due to the high capacity of SC-CO_2 to
363 penetrate and dissolve into the meat matrix due to its favorable low viscosity and high density
364 properties. The dilution of CO_2 in the matrix brings about a modification in the protein
365 structure, reaching the isoelectric point and, finally, causing the softening of the meat (Yan et
366 al., 2018).
367 Ferrentino et al. (2013a) reported that the dry-cured ham samples treated by SC-CO_2 (120-bar,
368 45-°C, 30-min) were harder than the untreated samples. However, Spilimbergo et al. (2014)
369 observed that dry-cured ham samples treated by SC-CO_2+HPU (120-bar, 35-°C, 5-min, 10-
370 W) showed higher hardness values compared to the control sample. The fact that this
371 behavior is different from that seen in the present study may be attributed to treatment

parameters, such as the pressure, temperature and HPU; in the case of the present study, these are higher than those found by Spilimbergo et al. (2014).

3.2.3. Moisture and fat content

As regards moisture content, although no significant ($p>0.05$) differences were found, results revealed that average values for treated samples were lower (Table 4).

On the other hand, Table 4 shows that the SC-CO_2+HPU treatment significantly ($p<0.05$) reduced the average fat content of treated samples compared to control samples. This was expected, as it is well known thatSC-CO_2 is a good solvent for extracting fat or lipophilic substances (Rodríguez et al., 2014).

Although the effect of the SC-CO_2+HPU treatment on the fat content of dry-cured ham has not been previously studied, its use for extracting fat from food matrices has been reported (Riera et al., 2010; Rodríguez et al.,2014).

In the present study, the results also showed that when the samples were immersed in a saline solution the fat loss was minimized (Table 4). This may be attributed to the lack of affinity between the water present in the saline solution and the fat, which hinders the ability of CO_2 to extract the lipophilic compounds.

3.2.4. Microstructure

Figure 3 shows the microstructure of cross sections of dry-cured ham. An analysis of the microphotographs revealed that the average diameter of fibers in the control sample was 47.3±5.3-µm. In this sample, the structure of dry-cured ham consists of highly compacted bundles of muscle fibers. The cells are surrounded by endomisial connective tissue, which is barely noticeable (Figure 3). The myofibrillar proteins in dry-cured ham have the characteristic appearance of a protein gel, which is caused by proteolysis during the latter

397 stages of the preparation of dry-cured ham (Larrea et al., 2007). This process involves water
398 loss and, consequently, the shrinkage of the structure and less interfibrillar space for water
399 (Vestergaard et al., 2005).
400 In general, the degree of compaction in the SC-CO_2+HPU samples (Figure 3B) is lower than
401 that observed in the control samples (Figure 3A). The muscle fibers seem more swollen and a
402 larger interfibrillar space is observed (Figure 3B). Accordingly, the average diameter of fibers
403 increased significantly (56.3±1.03 μm, $p<0.05$) compared to the average diameter observed in
404 the control sample (Zhou et al., 2015).
405 As for the SC-CO_2+HPU+SS samples, bundles of muscle fibers are also expanded (53.8±5.3-
406 μm, Figure 3C), thus showing a diameter similar to that observed in the SC-CO_2+HPU
407 samples This may suggest that the HPU treatment (compression and decompression) and the
408 expansion produced by the evaporation of CO_2 after the treatment affects the structure of the
409 muscle tissue, resulting in the aforementioned decrease in hardness (Table 4). In this sense,
410 Ozuna et al. (2014) also observed an increase in the diameter of cod fibers subjected to 180-
411 min of desalting combined with the HPU treatment (40-kHz, 750-W). The authors suggested
412 that the increase in diameter was generated by the mechanical stress induced by the HPU
413 treatment on connective tissue, leading to a lower degree of hardening.
414 **3.3 Storage of treated samples: Quality analyses**
415 **3.3.1. Color**
416 Figure 4 shows the color parameters of both the control samples of dry-cured ham and the
417 SC-CO_2+HPU treated ones (250 bar, 46 °C, 10 min) just after the treatment and at different
418 stages during storage (30 days - 4°C).
419 Although the brightness (L *) of neither sample changed significantly during storage time
420 (Figure 4-A), the a * and b* parameters of both treated and control samples significantly
421 ($p<0.05$) decreased after 30 days of storage (Figure 4-B, 4-C), which led to an overall color

422 change as depicted in Figure 4D. The decrease in the reddish hue of dry-cured ham samples
423 observed during storage could be attributed to the oxidation of ferrous groups of myoglobin
424 and to an increase in the content of methemoglobin (Carlez et al., 1995; De Alba et al., 2013;
425 Yan et al., 2018). The pressurization and the refrigerated storage of meat have also been
426 shown to enhance the decrease in the reddish hue of meat (Cava et al., 2009).
427 To our knowledge, only one study on the changes in the quality parameters of meat treated by
428 SC-CO_2 and HPU during refrigerated storage has been carried out (Spilimbergo et al., 2014).
429 In this regard, Spilimbergo et al. (2014) studied the effect of combining SC-CO_2 and HPU
430 treatments on the quality attributes of dry-cured ham. They found that the total color
431 difference of samples after 30 days of storage using 120-bar, 35-°C, 5-min, 10-W was
432 ΔE=4.4, which is lower than the average value registered in the present study (after 30 days of
433 storage). This difference may be attributed to the process parameters used by Spilimbergo et
434 al. (2014), which are milder than the ones employed in the present study (250-bar, 46-°C, 10-
435 min, 42 ±5-W).
436 **3.3.2 Moisture content, texture and pH values**
437 The moisture content, pH and texture did not significantly ($p>0.05$) change during
438 refrigerated storage. These results are coherent with those found by Spilimbergo et al. (2014),
439 who also observed that the hardness and pH of dry-cured ham samples treated using SC-
440 CO_2+HPU did not change significantly during storage (4 weeks).These results confirm that,
441 although the treatment affects the ham texture (Table 4), this characteristic is not further
442 affected during refrigerated storage.
443 **3.3.3 Microbial analysis**
444 Figure 5 shows the evolution of the microbiota present both in the control samples of dry-
445 cured ham and those treated by SC-CO_2 + HPU (250-bar, 46-°C, 10-min). The microbiota of
446 the control dry-cured ham at the beginning of storage were made up of 2.5, 1.8, 2.6, 3.0, and

1.2 logs (CFU/g) of mesophiles, psychrophiles, lactic acid bacteria, fungi and yeasts, and coliforms respectively. The plate count technique at zero storage time (just after the treatment) did not show the presence of microorganisms in treated dry-cured ham samples. This result shows the effectivity in the reduction of the ham microbiota by the proposed novel technology.

Specifically, coliforms and fungi and yeasts were not detected in the treated samples during the whole storage period. As concerns the other microorganisms tested, levels of only 2.2, 3.2 and 2.9 logs (CFU/g), were found for mesophilic, psychrophilic and lactic-acid bacteria, respectively, after 20 days of storage; these are within the permissible levels as indicated by the standard for the commercialization and consumption of meat products (BOE 2007).These results suggest that the inactivation attained initially was maintained for at least 20-days and a partial recovery took place after 30-days of storage.

4. Conclusions

SC-CO_2+HPU treatments were effective for the microbial inactivation of *E. coli* and microbiota in dry-cured ham. When the treatment was combined with the use of a saline solution (SS), the stronger cavitation taking place in the liquid improved the inactivation. Cavitation may damage microbial cells and accelerate the mass transfer phenomena, reducing the processing time.

As concerns the effect of the SC-CO_2+HPU treatment on the quality characteristics of dry-cured ham, changes in color, fat content and hardness were observed. The reduction in fat content could be used to obtain samples with improved stability and a better nutritional profile. On the other hand, ham softening was linked to the swelling of muscle fibers and the enlargement of the interfibrillar spaces observed in the microstructural analysis.

The microbiota was completely inactivated after treatments and only after 20-days of storage are the microorganisms able to recover and grow. Therefore, SC-CO_2+HPU treatments could be used to extend the shelf of dry-cured ham with minimal impact on the product quality.

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Figure 1. SC-CO_2 treatment system. 1. N_2 tank; 2. CO_2 tank; 3. Chiller reservoir; 4. Pump; 5. Manometer; 6. Thermocouple; 7. Ultrasonic generator; 8. Recirculation pump; 9. Temperature controlled bath; 10. Inactivation vessel; 11. Pressure release valve; 12. Cured ham sample; V. Valve

Figure 2. Modeled (Eq. (1)) *E. coli* inactivation $y = ln\left(\frac{N}{N_0}\right)$ in cured ham using SC-CO_2 + HPU+SS treatments.

Figure 3. Scanning electronic microscopy of a longitudinal section of cured ham surface samples. A. 200x, control sample; B. 150x, treated sample with SC-CO_2+HPU (250 bar, 46 °C, 10 min) and C. 200x, treated sample with SC-CO_2+HPU+SS (250 bar, 46 °C, 10 min). f. Muscular fiber.

Figure 4. Color parameters during storage time of control and SC-CO_2+HPU treated samples of cured ham (250 bar, 46 °C, 10 min; 42 W ± 5 W, 4°C). A. L* values; B. a* values; C. b* values and D. Total color difference (ΔE).

Figure 5. Microbiota analyzed in control and SC-CO_2+HPU treated samples of cured ham (250 bar, 46 °C, 10 min; 42 W ± 5 W, 4°C) during refrigerated storage at 4ºC.

Figure 1

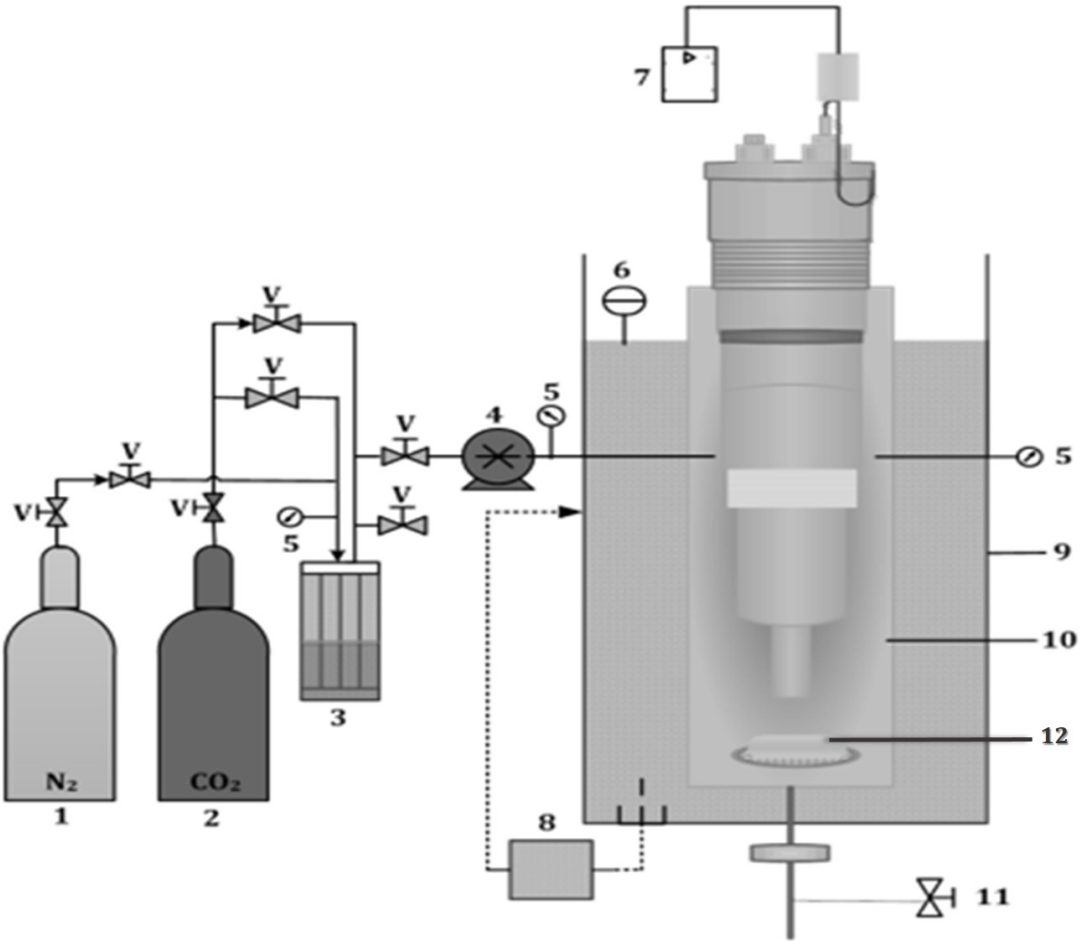

Figure 2

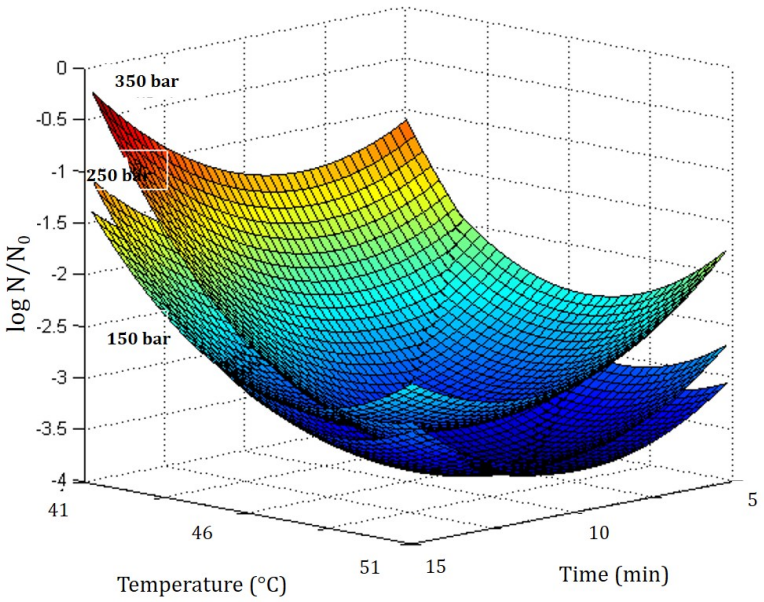
350 bar
250 bar
150 bar
log N/N0
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
41
46
51
Temperature (°C)
15
10
5
Time (min)

Figure 3

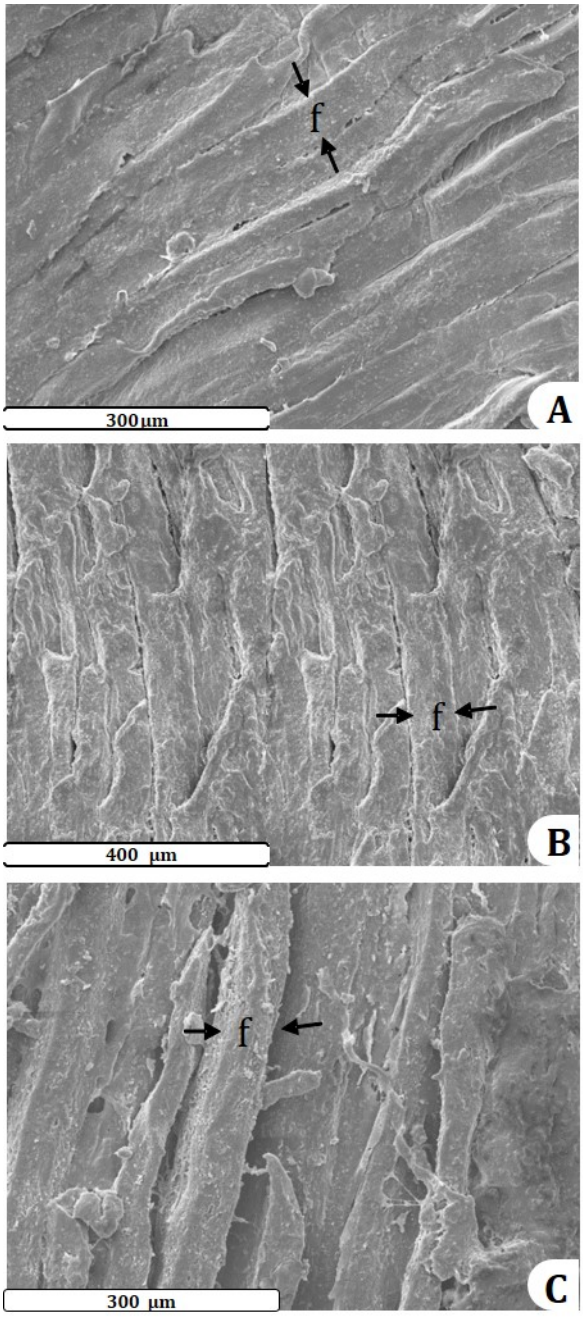

Figure 4

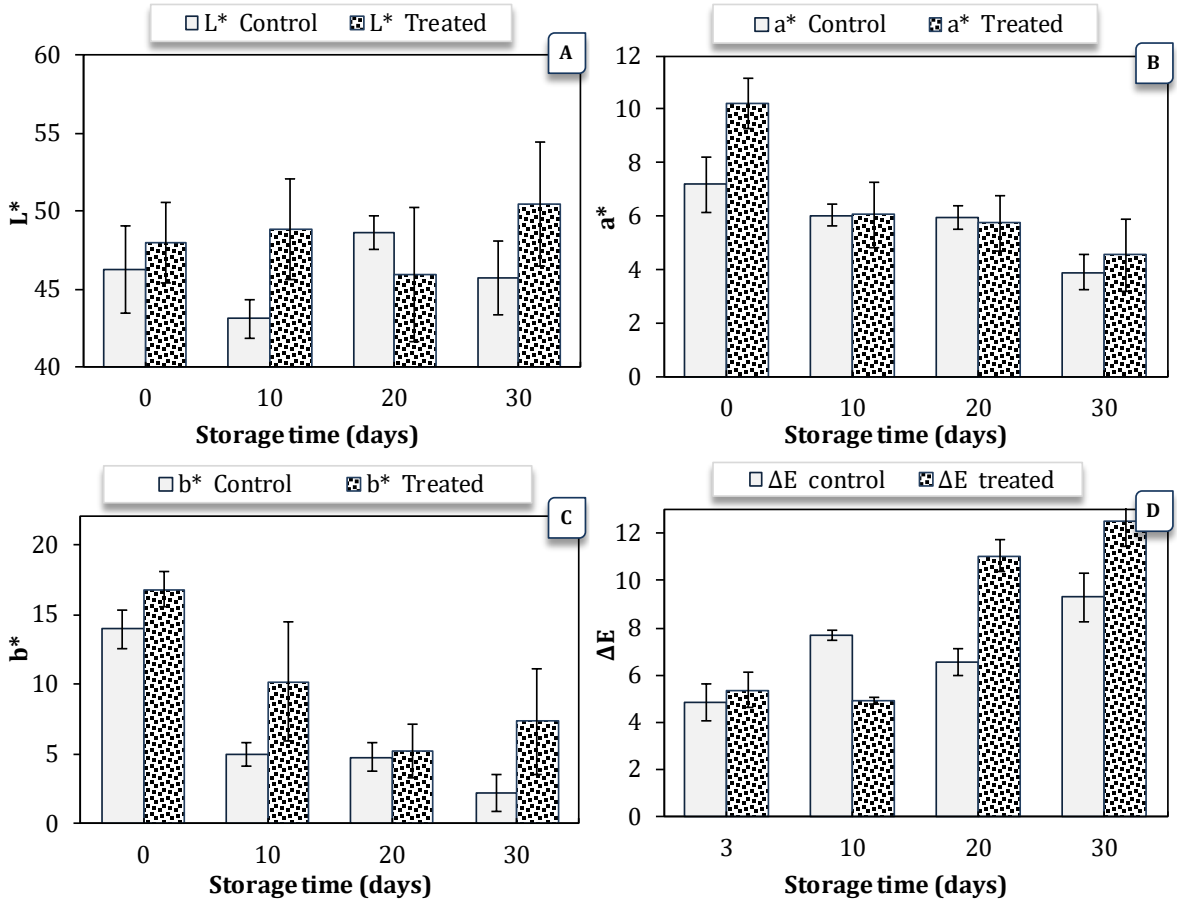
L* Control
L* Treated
A
L*
Storage time (days)
a* Control
a* Treated
B
a*
Storage time (days)
b* Control
b* Treated
C
b*
Storage time (days)
ΔE control
ΔE treated
D
ΔE
Storage time (days)

Figure 5

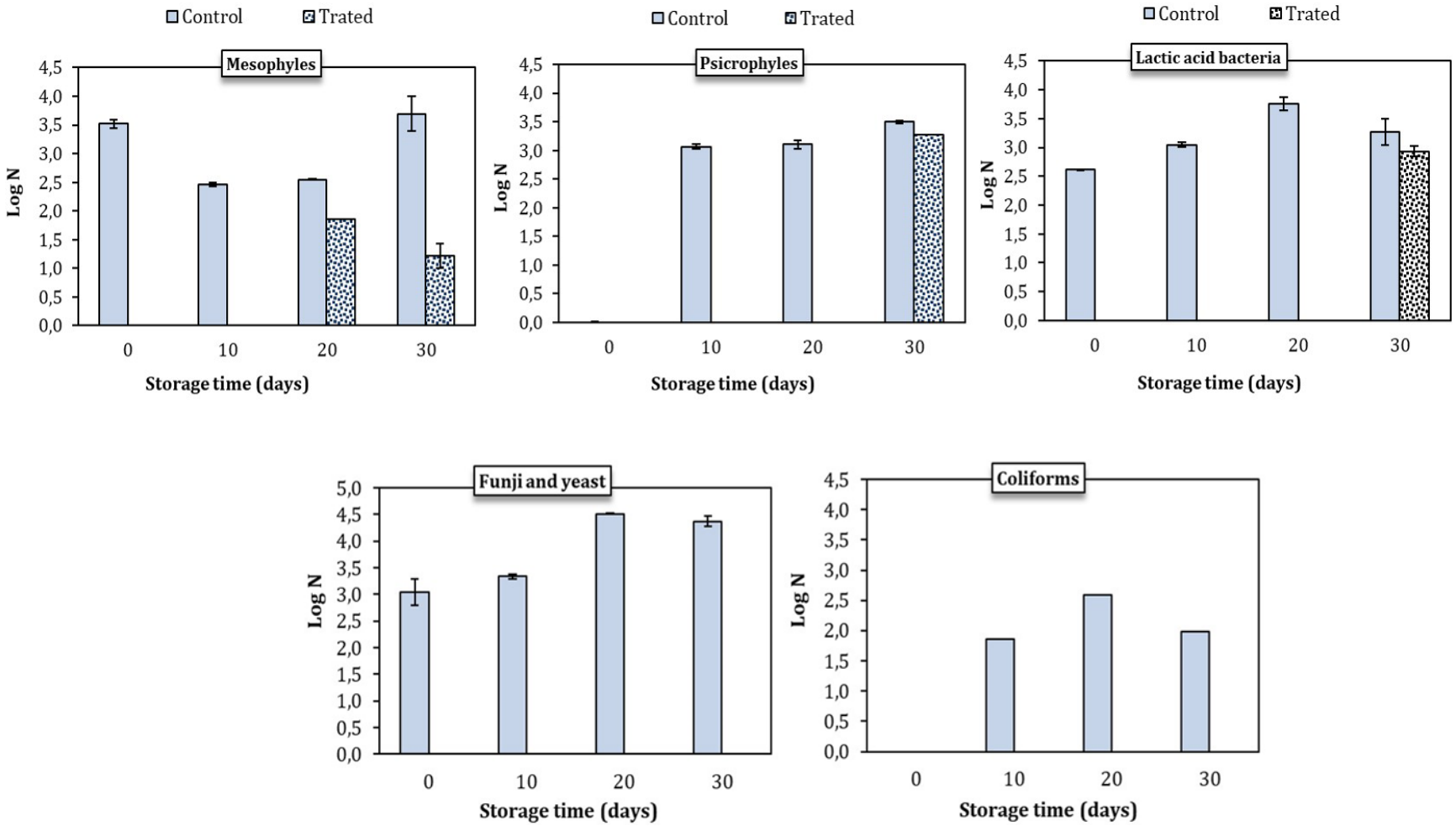
Control
Trated
Mesophyles
Psicrophyles
Lactic acid bacteria
Funji and yeast
Coliforms
Log N
Storage time (days)
0
10
20
30
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
5,0

Table 1. Results of the inactivation of E. coli by the combination of supercritical fluid treatment and high-power ultrasound on cured ham samples with and without saline solution (SS).

	Parameters			$SC\text{-}CO_2$ + HPU	$SC\text{-}CO_2$ + HPU + SS
T	P (bar)	T (°C)	t (min)	Experimental log (N/N_0)	Experimental log (N/N_0)
1	150	51	10	-1.63 ± 0.13	-2.62 ± 0.11
2	150	46	5	-1.79 ± 0.18	-2.49 ± 0.12
3	150	41	10	-0.33 ± 0.03	-1.02 ± 0.18
4	150	46	15	-2.86 ± 0.53	-3.45 ± 0.26
5	250	46	10	-1.67 ± 0.01	-3.60 ± 0.12
6	250	41	5	-1.36 ± 0.39	-2.50 ± 0.01
7	250	51	5	-2.77 ± 0.09	-2.71 ± 0.38
8	250	46	10	-2.30 ± 0.02	-3.61 ± 0.36
9	250	51	15	-3.07 ± 0.77	-3.14 ± 0.05
10	250	41	15	-1.90 ± 0.18	-1.35 ± 0.04
11	250	46	10	-1.76 ± 0.07	-3.66 ± 0.12
12	350	41	10	-1.85 ± 0.31	-1.03 ± 0.21
13	350	46	5	-1.62 ± 0.08	-2.31 ± 0.10
14	350	46	15	-1.52 ± 0.10	-2.34 ± 0.29
15	350	51	10	-3.49 ± 0.17	-3.32 ± 0.05

Table 2. Culture media and conditions for the plate count of the different microorganisms.

Microorganism	Type of streaking	Culture media*	Growth conditions
Mesophilic bacteria	Superficial	PCA	35 °C – 24 h
Psychrophilic bacteria	Superficial	PCA	4 °C – 7 d
Fungi and yeasts	Superficial	YGC	25 °C – 5 d
Lactic acid bacteria	Deep	MRS	35 °C – 3 d
Coliforms	Deep	VRB	35 °C – 24 h

* PCA (Plate Count Agar), YGC (Yeast Extract Glucose Chloramphenicol Agar Fil-IDF), MRS (Lactobacillus Agar acc to de Man Rogosa and Sharpe) and VRB (Violet Red Bile). All culture media were purchased from Sigma-Aldrich (USA).

Table 3. Regression coefficients (β) and their corresponding p-values calculated by adjusting the quadratic polynomial regression model which describes the inactivation of E. coli in cured ham using SC-CO2+HPU and SC-CO2+HPU+SS treatments.

	SC-CO_2+HPU			SC-CO_2+HPU+SS	
Parameter	Coefficient value	p-value	Parameter	Coefficient value	p-value
β_0	-1.91*	<0.01	β_0	-3.62*	0.01
β_1	-0.47	0.06	β_1	0.15	0.34
β_2	-1.38*	<0.01	β_2	-1.47*	<0.01
β_3	-0.45	0.06	β_3	-0.07	0.66
β_{11}	0.41	0.25	β_{11}	1.40*	<0.01
β_{12}	-0.17	0.61	β_{12}	-0.34	0.13
β_{13}	0.585	0.09	β_{13}	0.46*	0.04
β_{22}	-0.24	0.50	β_{22}	1.85*	<0.01
β_{23}	0.12	0.72	β_{23}	-0.80*	<0.01
β_{33}	-0.49	0.18	β_{33}	0.55*	0.02

* Significant values ($p<0.05$)

Table 4. Quality parameters of control and cured ham samples treated using SC-CO2+HPU and SC-CO2+HPU+SS treatments (250 bar, 46 °C, 10 min; 42 W ± 5 W, 4°C).

Treatment	L*	a*	b*	ΔE	Texture (N)	Moisture content (% w.b.)	Fat content (% w.b.)
Control	46.25±2.80[a]	7.18±1.05[a]	13.96±1.38[a]		4.97±0.24[b]	47.36±1.22[a]	9.89±3.66[b]
SC-CO_2+HPU	47.95±2.55[a]	10.22±0.96[b]	16.80±1.26[b]	4.50[a]	3.78±0.24[a]	44.03±1.46[a]	5.30±1.92[a]
SC-CO_2+HPU+SS	48.56±2.55[a]	11.45±2.96[b]	19.63±1.26[b]	7.46[b]	3.52±0.24[a]	45.25±1.46[a]	8.32±1.74[b]

The superscripts a and b, show homogeneous groups defined by the LSD (least significance difference) intervals for $p<0.05$.
ΔE Corresponds to the overall colour difference.