Effect of supercritical carbon dioxide processing on *Vibrio parahaemolyticus* in nutrient broth and in oysters (*Crassostrea gigas*)

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Abstract This study aimed to evaluate the technical feasibility of supercritical carbon dioxide (sc-CO₂) treatment for *Vibrio parahaemolyticus* inactivation in oysters (*Crassostrea gigas*) and in nutrient broth. For this purpose, a variable-volume reactor was used as experimental system and a 2³ factorial design was adopted considering the mass ratio between carbon dioxide and the product, pressurization and depressurization rate and pressurization cycles. Through statistical analysis of the experimental data, the mass ratio of 1:0.8 (product:carbon dioxide), depressurization rate of 10.0 MPa/min and one cycle of pressurization was determined as the best process condition to eliminate *V. parahaemolyticus*, and this was the condition used for the inactivation kinetic analysis. Comparison between the inactivation kinetics of *V. parahaemolyticus* showed that the behavior of this microorganism inactivation depends on the environment in which it operates and its initial count. The results confirm that the supercritical carbon dioxide is effective in inactivating microorganisms in oysters, including pathogenic *V. parahaemolyticus*, demonstrating the potential of this technology in the food industry.

Keywords Oysters · *Vibrio parahaemolyticus* · Supercritical carbon dioxide · Inactivation

Introduction

Oysters can be considered gourmet food and have been greatly appreciated by consumers worldwide. They can be served while still alive, raw or lightly cooked in one half of the shell. Unfortunately, raw or undercooked oysters pose a health risk to consumers due to their association with dangerous foodborne bacterial pathogens (Ye et al. 2012). As a consequence from filter feeding in contaminated water, oysters can be loaded with pathogenic microorganisms and these microorganisms are absorbed concomitantly with nutrients and accumulate in the gastrointestinal system of oysters. The literature reports a list of pathogenic microorganisms associated with oysters, such as Hepatitis viruses, *Salmonella*, *Shigella* spp., *Escherichia coli*, *Vibrio* spp., *Clostridium perfringens*, *Clostridium botulinum* and *Yersinia enterocolitica* (Potasman et al. 2002; Lee et al. 2008). Among the pathogenic microorganisms, *V. parahaemolyticus* has been indicated as a major cause of disease outbreaks related to the consumption of bivalve mollusks in the world (Teplitski et al. 2009).

Vibrio parahaemolyticus are indigenous, naturally occurring organisms that thrive in the marine environment where the bivalves are grown. These bacteria are found to be in higher numbers during the warmer months of the year when there are a higher number of reported molluscan-borne illnesses. *Vibrio* bacteria reside in the shellfish and in the environment. This pathogen can cause acute gastroenteritis through consumption of raw shellfish, particularly oysters (Jay 2000; Jones 2009). Outbreaks caused by *V. parahaemolyticus* are linked to the consumption of contaminated raw oysters in South America according to Raszl et al. (2016).

Most food safety problems associated with raw oysters would be controlled if they were harvested from clean

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waters followed by post harvesting processing. This would enhance the product safety for consumers.

In this context, the use of non-thermal process in oysters is an alternative to conventional thermal treatment in order to improve the microbiological quality of the product thus preserving its fresh sensorial characteristics and enhancing its shelf life. It would also facilitate its distribution and marketing. Treatment with pressurized carbon dioxide has been suggested as a non-thermal, promising technique that can be applied to food pasteurization, mainly due to the potential to inactivate microorganisms and to preserve food from the negative effects of heat, such as nutritional and sensorial modifications (Galvanin et al. 2014). Although supercritical CO₂ technology has not been applied on a commercial scale yet, the effectiveness bacterial inactivation has been proven in a number of applications involving both liquid (Lin et al. 1994; Erkmen 1997; Garcia-Gonzalez et al. 2009; Yuk et al. 2010; Ortuño et al. 2012; Ceni et al. 2016) and solid food matrices (Calvo and Torres 2010; Park et al. 2012; Ji et al. 2012; Ferrentino et al. 2013; Sikin et al. 2016).

In the present study, the main objective was to investigate the technical feasibility of supercritical carbon dioxide treatment for *V. parahaemolyticus* inactivation in oysters (*Crassostrea gigas*) and in nutrient broth, using a high-pressure variable-volume reactor, which enables the application of cycles of pressurization and depressurization without carbon dioxide releasing.

Materials and methods

Apparatus and experimental procedure

All the inactivation experiments were carried out employing the static-synthetic method in a high-pressure variable-volume view cell, schematically presented by Silva et al. (2013) and Soares et al. (2013). Briefly, the experimental set-up consists of a variable-volume view cell, with a maximum internal volume of 27 mL, an absolute pressure transducer (Smar LD 301), with a precision of 0.03 MPa, a portable programmer (Smar, HT 201) for pressure data acquisition and a syringe pump (ISCO 260D) to charge CO₂. The inactivation cell contains a movable piston, which permits the pressure control inside the cell. Initially, an amount of the sample was loaded into the inactivation cell through a sterile syringe. The sample mass and CO₂ were based on the experimental design. Then, the cell was kept at continuous agitation using a magnetic stirrer and a Teflon-coated stirring bar.

All the experiments were conducted at 8.0 MPa as the initial working pressure and 20 MPa as the final working pressure. The processing time for all runs was always 2 h

(except the kinetic runs) and at a fixed temperature of 33 °C. Each run consisted of a pressurization step (at a fixed rate of 10.0 MPa/min) up to 20.0 MPa, holding the system pressure at 20.0 MPa for a given time and a depressurization step down to 8.0 MPa. The processing cycles were performed according to the experimental design (Table 1).

Oysters under supercritical carbon dioxide processing

Raw material

Fresh oysters (*C. gigas*) cultivated in Florianópolis (South Brazil) were obtained at a local market. Samples were placed in an isothermal box containing ice packs and immediately transported to the laboratory. A total of twenty-four units of fresh oysters were used for each experiment. Oysters whose shells were not tightly closed were discarded. Samples were washed in running water and a brush was used to remove dirt adhered to the shells. After cleaning, oysters were sanitized with 70% alcohol, and the meat was removed manually, using sterile forceps and scissors. The oyster meat and its internal liquid were placed in a previously sanitized beaker. The meat and the liquid were then disintegrated in Turrax equipment (TE Turatec-102), for about 4 min using 18,000 rpm. After that, 10 g of the homogenized sample was added to the cell with the aid of a disposable syringe and the cell was then coupled in the system.

Experimental design

The effects of the product on CO₂ mass ratio, depressurization rate and the number of pressure cycles were evaluated by means of a factorial design with triplicate at central point. The levels of each independent variable are shown in Table 1. All results were analyzed using Statistica® 8.0 (Statsoft Inc., Tulsa, OK, USA) considering a significance level of 95% ($p < 0.05$).

Inactivation kinetics of *V. parahaemolyticus* naturally occurring in oysters

The effect of treatment time on the inactivation of *V. parahaemolyticus* naturally in fresh oyster, by use of sc-CO₂, was performed through inactivation kinetics with 0.25, 0.50, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 and 6.0 h (processing time), with one pressure cycle, product/CO₂ mass ratio of 1:0.8, pressurization and depressurization rates of 10.0 MPa/min (best experimental condition obtained). Before and after each treatment, the *V. parahaemolyticus* count in thiosulfate citrate bile salts sucrose (TCBS media)

Table 1 Matrix of the experimental results obtained in the 2^3 factorial design with triplicate at the central point to evaluate the inactivation of *V. parahaemolyticus* in oysters using sc- CO_2

Run	O: CO_2	R (MPa/min)	Pressure cycles	Log (N/N_0)	($N - N_0$)/ N (%)
1	− 1 (1:0.2)	− 1 (1.0)	− 1 (1)	0.10	20.82
2	+1 (1:0.8)	− 1 (1.0)	− 1 (1)	0.41	61.94
3	− 1 (1:0.2)	+1 (10.0)	− 1 (1)	0.74	82.29
4	+1 (1:0.8)	+1 (10.0)	− 1 (1)	1.30	95.04
5	− 1 (1:0.2)	− 1 (1.0)	+1 (5)	0.91	87.92
6	+1 (1:0.8)	− 1 (1.0)	+1 (5)	0.79	84.07
7	− 1 (1:0.2)	+1 (10.0)	+1 (5)	0.60	75.00
8	+1 (1:0.8)	+1 (10.0)	+1 (5)	1.35	95.65
9	0 (1:0.5)	0 (5.5)	0 (3)	0.27	47.82
10	0 (1:0.5)	0 (5.5)	0 (3)	0.23	42.85
11	0 (1:0.5)	0 (5.5)	0 (3)	0.22	40.91

was determined by standard plating techniques according to APHA (2001). Microbiological analyses were performed in duplicate.

Inactivation kinetics of V. parahaemolyticus intentionally added to oysters

In order to evaluate the effect of the treatment time on the inactivation of *V. parahaemolyticus* when its initial count was high, oysters were intentionally contaminated with the microorganism provided by the biological collections of Oswaldo Cruz Foundation (Fiocruz). For each experiment, *V. parahaemolyticus* ATCC 17802 was sub-cultured in 50 mL of Luria-Bertani broth (triptone 10.0 g/L; yeast extract 5.0 g/L; NaCl 5.0 g/L) at 37 °C for 24 h. Then, 15 mL of the inoculum was transferred to 150 mL of oysters that were prepared as aforementioned. The sample was stored at room temperature (~ 22 °C) for 12 h in order for the *V. parahaemolyticus* to become adapted to the environment. The initial count of *V. parahaemolyticus* before each experiment was around 10^8 and 10^9 CFU/mL. The inactivation kinetics was carried out with 0.50, 1.0, 2.0, 3.0 and 4.0 h (processing time), with one pressure cycle, product: CO_2 mass ratio of 1:0.8, pressurization and depressurization rates of 10.0 MPa/min (best experimental condition obtained). After each treatment, the *V. parahaemolyticus* count (TCBS media) was determined by standard plating techniques according to APHA (2001). Microbiological analyses were performed in duplicate.

Inoculum of V. parahaemolyticus under sc- CO_2 processing

To evaluate the effect of treatment time on the inactivation of *V. parahaemolyticus* inoculum, the microorganism *V. parahaemolyticus* ATCC 17802 was sub-cultured in 50 mL of Luria-Bertani broth (triptone 10.0 g/L; yeast extract 5.0 g/L; NaCl 5.0 g/L) at 37 °C for 24 h. The cultures used in all experiments were freshly prepared using the same

procedure. 10 g of the homogenized sample was weighed with the aid of a disposable syringe and then injected in the experimental apparatus described in 2.1. The initial count of *V. parahaemolyticus* was 2.9×10^6 CFU/mL. The inactivation kinetics was carried out at 5.0, 15, 30, 45, 60 and 90 min (processing time), with one pressure cycle, inoculum to CO_2 mass ratio of 1:0.8, pressurization and depressurization rates of 10.0 MPa/min (best experimental condition). After each treatment, the *V. parahaemolyticus* count (TCBS media) was determined by standard plating techniques according to APHA (2001). Microbiological analyses were performed in duplicate.

Modeling of the inactivation kinetics

Survival curves of *V. parahaemolyticus* inactivated by sc- CO_2 were fitted by the following Weibull equation proposed by Mafart et al. (2002):

$$\log\left(\frac{N(t)}{N_0}\right) = -\left(\frac{t}{\Delta}\right)^p \quad (1)$$

where $N(t)$ and N_0 are the number of survivors after a processing time (t) and initial number of microorganisms (CFU/mL), respectively. The model has two parameters: Δ is called the first decimal reduction time, i.e., time needed to reduce the initial population, N_0 to $N_0/10$; and p is called the shape parameter. This model presents the main advantage of being very simple and sufficiently robust to describe both monotonic downward concave (shoulder) survival curves ($p > 1$) and monotonic upward concave (tailing) survival curves ($p < 1$). The traditional first-order model is then a special case ($p = 1$) of the Weibull model (Eq. 1). Survival curves were fitted using the Matlab software (MathWorks Inc, Natick, USA) and the statistical parameter correlation coefficient (R^2) and the mean square error (MSE) were used to evaluate the goodness of the fit.

$$MSE = \sum_{i=1}^n \frac{(\text{predicted}_i - \text{observed}_i)^2}{n} \quad (2)$$

where n is the number of observations - the smaller the MSE values, the better the model fits the data.

Results and discussion

Effect of process variables on *V. parahaemolyticus* inactivation in oysters

The results of *V. parahaemolyticus* log-reduction obtained from the experimental design to evaluate the effects of product to CO₂ mass ratio, depressurization rate and the number of pressure cycles are presented in Table 1. It can be noticed that an increase in mass ratio produced better results for all depressurization rates and pressure cycle conditions. At the same mass ratio of product to CO₂, increasing the depressurization rate also increased the log-reduction. Additionally, pressure cycles increase also benefited all runs, except for run 7.

Statistical analysis of the experimental data showed that the number of cycles also presented significant effect on *V. parahaemolyticus* reduction, indicating that steps of pressurization and depressurization may have contributed to causing microorganism stress and, consequently, to its death. Spilimbergo et al. (2002) and Silva et al. (2013) also found that pressure cycles were beneficial to inoculum inactivation of *Pseudomonas aeruginosa* and *B. subtilis*, and *E. coli*, respectively. The mass product to CO₂ mass ratio also presented significant effect on *V. parahaemolyticus* reduction. The same effect was observed by Gunes et al. (2005; 2006), when they studied the inactivation of yeasts and *E. coli*, respectively. However, Soares et al. (2013) did not observe the significant positive effect of the inoculum mass to CO₂ ratio for *L. monocytogenes* inactivation.

Vibrio parahaemolyticus inactivation results presented in Table 1 were analyzed in order to select the best experimental condition. Although run 8 provided the highest microbial inactivation (1.35 log-reduction), this result was much closer to the reduction obtained from run 4 (1.30 log-reduction). The difference between these two runs is the number of pressure cycles. Run 4 had only one cycle while run 8 had five pressurization and depressurization cycles. Processing with a single cycle of pressurization and depressurization has an operational feasibility and uses less CO₂ in comparison to treatments with more pressure cycles. For these reasons, run 4 was selected as the best condition to perform the kinetic investigation.

Inactivation kinetics of *V. parahaemolyticus*

All inactivation kinetic experiments were performed using sc-CO₂ at the best processing condition obtained from the experimental design, i.e., with mass of product to CO₂ ratio equal to 1:0.8, pressurization and depressurization rates at 10.0 MPa/min and one pressure cycle. The initial and final working pressure was 8.0 and 20.0 MPa, respectively, and temperature was fixed at 33 °C. Firstly, all the *V. parahaemolyticus* kinetic data will be presented in tables: the initial and final *V. parahaemolyticus* counts, $(N - N_0)/N$, which shows the percentage reduction after the sc-CO₂ treatment, and $\text{Log}(N_0/N)$, which shows the reduction after processing with sc-CO₂ treatment. All curves will then be presented together with the parameters from Weibull model.

Inactivation kinetics of V. parahaemolyticus naturally occurring in oysters

Supercritical carbon dioxide treatment was shown to be efficient in the reduction of the initial count of *V. parahaemolyticus* naturally present in oysters. The inactivation kinetics data presented in Table 2 shows that increasing the process time in sc-CO₂ will reduce the counting of this microorganism. In the beginning of the process, the microorganism reduction was lower than one log-reduction. After 1 h of treatment, it was possible to reach higher reductions than one log-reduction. The number of reductions varied between 0.61 (lowest log-reduction at 0.25 h processing) and 2.18 (after 6 h processing). However, from 3 h of sc-CO₂ treatment on it was possible to eliminate the initial count of *V. parahaemolyticus*, corresponding to a 2-log reduction (Fig. 1).

Some microbial inactivation kinetic experimental data using pressurized carbon dioxide have been reported in the literature as a first-order reaction (Erkmen and Karaman 2001; Calvo and Torres 2010; Silva et al. 2013; Soares et al. 2013). However, in this study we found that the inactivation of *V. parahaemolyticus* naturally present in oysters did not follow a first-order reaction. For this reason, the Weibull model was chosen to describe the inactivation of this microorganism. Figure 1 shows an upward concave curve ($p < 1$), indicating that the *V. parahaemolyticus* initial count has cells that are inactivated easily. Nevertheless, the survivors are more resistant and require more processing time to be eliminated.

Table 3 shows the parameters of the Weibull model along with R^2 and MSE values, indicating the satisfactory representation of the experimental data. The same behavior was reported by Checinska et al. (2011) for *Bacillus pumilus* spores inactivation using a mixture of sc-CO₂, water and hydrogen peroxide. Their results for the

Table 2 Kinetic inactivation data of *V. parahaemolyticus* using sc-CO₂

Naturally present			Intentionally added			Inoculum (ATCC 17802)		
Time (h)	Log (N ₀ /N)	(N – N ₀)/N (%)	Time (h)	Log (N ₀ /N)	(N – N ₀)/N (%)	Time (h)	Log (N ₀ /N)	(N – N ₀)/N (%)
0	*	0	0	*	0	0.00	*	0
0.25	0.61	12.63	1.00	1.41	15.07	0.08	0.44	6.81
0.50	0.82	30.37	2.00	2.97	31.73	0.25	1.37	21.21
1.00	0.94	27.27	2.50	5.00	62.19	0.50	1.84	28.49
1.50	1.18	43.71	3.00	5.89	59.32	0.75	3.56	55.11
2.00	1.37	41.98	3.50	8.04	100	1.00	6.46	100
3.00	2.00	100	–	–	–	1.50	6.46	100

*At time zero (0) the initial and end counts are equal

Fig. 1 Inactivation of *V. parahaemolyticus* naturally occurring in oysters processed with supercritical carbon dioxide. Solid line (—) represents Weibull model fitted to experimental data **Fig. 2** Inactivation of *V. parahaemolyticus* intentionally added to oysters processed with supercritical carbon dioxide. Solid continuous line (—) represents Weibull model fitted to experimental data

inactivation kinetics showed a two-stage trend of a fast-slow mechanism. The slowing rate of sterilization as processing time progresses suggests that some spores are more resistant than others, or spores deposited in deeper layers are protected by killed spores on the top that form passive

Table 3 Statistical values and parameters of Weibull model for the inactivation of *V. parahaemolyticus* using sc-CO₂

<i>V. parahaemolyticus</i>	Δ (h ⁻¹)	<i>P</i>	MSE	R ²
Naturally present	0.938	0.527	0.016	0.965
Intentionally added	0.928	1.556	0.104	0.990
Inoculum (ATCC 17802)	0.4	2.032	0.239	0.958

or active barriers through which the mixture of sc-CO₂ must diffuse.

Inactivation kinetic of V. parahaemolyticus intentionally added to oysters

Table 2 shows the reduction of the initial count of *V. parahaemolyticus* intentionally added to oysters. Log-reductions varied from 1.41 up to 8.04, corresponding to the lowest and the highest inactivation level. Results confirm that the sc-CO₂ was an efficient treatment to reduce or eliminate *V. parahaemolyticus* in oysters even when its initial count is high (10⁸–10⁹ CFU/mL).

As can be seen in Fig. 2, the inactivation of *V. parahaemolyticus* intentionally added to oysters also did not follow a first-order reaction. For this reason, the Weibull model was chosen to fit the experimental data. The parameter *p* was higher than 1, so the survival curve presented a downward concave. This behavior is clear evidence that damage accumulation weakens the survivors and hence takes progressively shorter time to cause their destruction (Aragão et al. 2007). The Weibull model fitted the experimental data with very good agreement, as R² was 0.9906 and MSE value equal to 0.1042, as shown in Table 3.

Considering that Brazilian legislation at this stage does not present established criterion for *V. parahaemolyticus* in

Fig. 3 Inactivation of *V. parahaemolyticus* inoculum (ATCC 17802) processed with supercritical carbon dioxide. Solid line (■) represents Weibull model fitted to experimental data

fresh oysters, results obtained in this study were compared with those from the *National Shellfish Sanitation Program* (NSSP), from *United States Food and Drug Administration* (FDA 2009). According to NSSP, a post-harvest processing to control the *V. parahaemolyticus* must assure that the process can reduce the level of the microorganism in shellfish by 3.52 logs and levels < 30 MPN/g. According to this criteria, results from this study indicate that the sc-CO₂ can be used as post-harvest processing in oysters to control *V. parahaemolyticus*, as the experimental conditions tested were able to achieve a 5-log-reduction in 2.5 h of processing (Table 2).

Inactivation kinetic of *V. parahaemolyticus* inoculum

Inactivation results of *V. parahaemolyticus* inoculum presented in Table 2 show log-reductions from 0.44 up to 6.46. The initial count of the microorganism was eliminated with 1 h of sc-CO₂ processing, indicating 6.46 log-reductions. Considering that the inactivation of *V. parahaemolyticus* is non-linear, the Weibull model was chosen again to describe the inactivation behavior of the *V. parahaemolyticus* inoculum, as depicted in Fig. 3.

In this case, the survival curve also presented a downward concave ($p > 1$), indicating that the time to inactivate the inoculum progressively decreased. The statistical parameters (MSE = 0.2399 and $R^2 = 0.9583$) again showed that the Weibull model can describe the inactivation of *V. parahaemolyticus* inoculum in the processing conditions evaluated in this study (Table 3).

As can be observed, the inactivation of the *V. parahaemolyticus* inoculum presented similar behavior to the case of *V. parahaemolyticus* intentionally added to oysters (Fig. 3). Lin et al. (1992), Erkmen (1997, 2000), Erkmen and Karaman (2001), when studying the inactivation of inoculum of *S. typhimurium*, *Brochothrix thermosphacta*, *S. aureus* and *Saccharomyces cerevisiae*, respectively, also

Fig. 4 Comparison of Weibull Model fitting for *V. parahaemolyticus* naturally present in oysters (●●●●●), *V. parahaemolyticus* intentionally added to oysters (—), and *V. parahaemolyticus* inoculum (■) processed with supercritical CO₂ at 1:0.8 ratio (product:CO₂), 10.0 MPa/min as pressurization and depressurization rate, one cycle of pressurization and depressurization, at 33°C

observed the same behavior. Soares et al. (2013) investigated the inactivation of *L. monocytogenes* inoculum using exactly the same equipment and processing conditions (33 °C, pressurization and depressurization rate of 10.0 MPa/min, one pressurization cycle, starting at 8.0 MPa up to 20.0 MPa). The initial count of *L. monocytogenes* was totally reduced at 3 h of processing, which was higher than the required time to inactivate the *V. parahaemolyticus* inoculum. One reason for this difference could be the amount of CO₂, as they used a mass of inoculum to CO₂ ratio lower (1:0.2) than the ratio tested in this study (1:0.8). Another explanation could be the different microbial sensitivity of species to high pressure CO₂ treatments. In general, Gram-positive bacteria (like *L. monocytogenes*) are expected to be more resistant than Gram-negatives (*V. parahaemolyticus*) due to the composition of their (thicker) cell wall (Garcia-Gonzalez et al. 2007).

Silva et al. (2013) studied the effect of pressure on the inactivation kinetics of *E. coli*, using the same equipment used in this work. However, those authors chose the log linear model to fit their experimental data and obtained D values of 5.35 min (at 8.0 MPa) and 1.03 min (at 16.0 MPa), with R^2 value around 0.96.

Meujo et al. (2010) evaluated the inactivation kinetics of two Gram-negative bacteria inoculum (*Vibrio fischeri* and *E. coli*), using sc-CO₂ at pressure levels of 10.0, 15.0 and 20.0 MPa and temperature of 37°C, with exposure time of 5, 10, 15 and 20 min. The initial count of *V. fischeri* was totally inactivated after 5 min of processing time for all pressure values. On the other hand, *E. coli* required 20 min of sc-CO₂ treatment to be eliminated.

Comparison of the *V. parahaemolyticus* inactivation kinetics

The inactivation rate is strongly affected by the constituents of the suspending media and nature of foods during sc-CO₂ treatment. As can be noticed from Fig. 4, when *V. parahaemolyticus* was suspended in a simple solution media, the microbial load reduction was faster than the case of being suspended in a complex environment of a food system. The complete inactivation of *V. parahaemolyticus* initial load using sc-CO₂ requires a processing time three times higher to eliminate the bacteria presented in oysters in comparison with when *V. parahaemolyticus* is presented in a culture medium. This difference may be due to the fact those fats, proteins and other food contents (Lin et al. 1994), as in oysters, may have protected the cells from penetration of sc-CO₂ and, consequently, interfered in the inactivation rate.

Erkmen (2001) also observed that the *E. coli* inactivation in whole milk was not as efficient as the inactivation of the bacteria suspended in the nutrient broth. Similar behavior was also observed for the reduction of *Brochothrix thermosphacta* in whole milk, skimmed milk and meat. Results from Ortuño et al. (2012) indicated the influence of culture mixtures and the use of food matrices, which might contain materials that may interfere in the microbial inactivation.

A comparison between the required time to achieve 2 and 3.52 log-reduction of *V. parahaemolyticus*, as calculated using the Weibull parameters presented previously, shows that 0.56 and 0.74 were the values found for *V. parahaemolyticus*—inoculum, while 3.49 and 10.19 (model extrapolation) were observed for *V. parahaemolyticus*—naturally present in oysters. Finally 1.45 and 2.08 were obtained for *V. parahaemolyticus*—intentionally added to oysters.

If we consider the required time to inactivate 3.52 log-reduction (criterion for a post-harvest processing of oysters, from the NSSP - Guide for the Control of Molluscan Shellfish—USA) (FDA 2009), it can be seen that the case of *V. parahaemolyticus* naturally present in oysters demanded a longer processing time than the other cases. This behavior is depicted in Fig. 4, where it can be observed that the cases of *V. parahaemolyticus* intentionally added to oysters and the inoculum showed a similar inactivation dynamic. A possible reason for the similar behavior may be because that in these two cases the same *V. parahaemolyticus* strain was used. This means that oysters were intentionally contaminated using the same microbial strain of the kinetics studies of *V. parahaemolyticus* inoculum. Despite the similarity in behavior, it can be noticed that the constituents of the suspending media can interfere in the inactivation performance, as

indicated in the literature. When the bacteria was present in oysters (which is considered a complex medium), the required time to reach a 2-log-reduction was higher than when *V. parahaemolyticus* was suspended in a nutrient broth (which is considered a less complex medium). Therefore, in this case the oyster constituents, such as the fat content, may make the CO₂ penetration into the microbial cell more difficult and, consequently delay the inactivation.

Inactivation of *V. parahaemolyticus* naturally occurring in oysters presented the opposite behavior to the other two cases, showing an asymptotic profile. A possible reason for this difference might be related to the strains. It is possible that the strain of *V. parahaemolyticus* naturally present in oysters may be different from the other cases. Another reason could be related to the bacteria adaptation to the medium. It is likely that the *V. parahaemolyticus* strains naturally present in oysters were well adapted to the marine environment and the inactivation process was therefore more difficult than in other cases. Another possible reason influencing microbial inactivation is the stage of cell growth. *V. parahaemolyticus* naturally present in oysters might be at the stationary phase and, therefore, the strains were more pressure resistant.

Literature indicates that the initial count (N_0) plays an important role in the microbial inactivation using high pressure carbon dioxide (Garcia-Gonzalez et al. 2009). When *V. parahaemolyticus* initial count was higher (inoculum and the intentionally added cases), log reductions were minimal at the beginning of the process (Fig. 4) in comparison with the case of *V. parahaemolyticus* naturally present in oysters, where the initial load was lower than the other cases. So, it is possible that when the initial load is low, the sc-CO₂ can access the microbial cells easier than when the initial count is high. This could also be the explanation for the faster inactivation rate at the beginning of the process that happened for the oysters contaminated naturally. It is important to remember that the same ratio of product and CO₂ was used for the oyster cases. Then, it is reasonable to suppose that when the initial bacterial load is high (oyster intentionally contaminated), the CO₂ molecules have more difficulty accessing the microbial cell. For this reason, the log-reductions are minimal during the beginning of the sc-CO₂ process.

Conclusion

This study demonstrated that combining the variables product to carbon dioxide mass ratio, depressurization rate and pressure cycles in sc-CO₂ treatment was effective to inactivate *V. parahaemolyticus* in oysters and also in nutrient broth. The results confirmed that sc-CO₂ is a

promising technology towards *V. parahaemolyticus* inactivation in oysters, hence demonstrating the potential for possible industrial large-scale processing, enhancing food shelf life and preserving its sensorial and nutritional values. Comparison of three-kinetics suggests that the suspending medium, the initial bacterial concentration and the amount of supercritical carbon dioxide influence the inactivation behavior of *V. parahaemolyticus*. Although the Weibull model was able to satisfactorily represent the experimental data, other characteristics should be investigated in order to provide a better understanding of the real inactivation mechanism.

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