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Reducing Oyster-Associated Bacteria Levels Using Supercritical Fluid CO₂ as an Agent of Warm Pasteurization

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Abstract

An innovative approach to Post-Harvest Processing (PHP) of oysters is introduced focusing on the effects of supercritical carbon dioxide (scCO₂) on bacterial contaminants trapped in the digestive system of oysters. Oysters were exposed to scCO₂ under two conditions: (1) 100 bar and 37 °C for 30 minutes and (2) 172 bar and 60 °C for 60 minutes. Using FDA standard guidelines for food analysis, variations in the Aerobic Plate Count (APC) was assessed. It was established that exposing oysters to CO₂ at 100 bar and 37 °C for 30 minutes and at 172 bar and 60°C for 60 minutes induced 2-log and 3-log reductions in the APC respectively. The decrease in the microbial load as a result of treatment with scCO₂ was found to be significant (P=0.002). A release of adductor muscles from the shell was noted in oysters treated at 172 bar and 60 °C for 60 minutes; this was not the case for oysters treated at 100 bar and 37 °C for 30 minutes. A blind study allowing sensory analysis of treated vs. untreated oysters was also completed and no significant change in the physical appearance, smell, or texture was recorded. In this paper, we also report the effect of scCO₂ on several bacterial isolates, including a referenced ATCC strain of a non pathogenic *Vibrio* (*V. fischerii*) as well as several other bacterial isolates cultured from oyster' tissues and found to share biochemical features common to pathogenic *Vibrio* strains. A complete inactivation (minimum 7-log reduction) was achieved with these latter bacterial isolates. A 6-log reduction was observed with *V. fischerii*.

Keywords

scCO₂; *Vibrio* spp; Oysters; Post Harvest Processing

1. Introduction

As a direct consequence of filter-feeding in often heavily contaminated water, oysters can be loaded with pathogenic microorganisms. These microorganisms are absorbed concomitantly

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with nutrients and accumulate in the gastrointestinal (GI) system of oysters. Hepatitis viruses (A and E), *Salmonella*, *Shigella* spp., *Escherichia coli*, *Vibrio* spp., *Clostridium perfringens*, *C. botulinum* and *Yersinia enterocolitica* are some of the microbes that have been reported as part of this pathogenic microflora (Kamihira et al., 1987; Potasman et al., 2002; Vugia and Hadler, 2002). Since oysters are often consumed raw, these pathogens are a serious concern. These microorganisms have been associated with a wide range of mild to severe foodborne illnesses. Of pathogens that contaminate the GI system of oysters, *Vibrio* spp (especially *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv)) are of greater concern (Kamihira et al., 1987; Hu et al., 2005; Linton et al., 2003). Among immunocompromised patients, especially those with chronic liver disease, *V. vulnificus* strains have been strongly associated with severe and life-threatening conditions. *V. vulnificus* is the second leading cause of seafood-related fatality in the US (Andrews et al., 2003; Samir et al., 2005).

Pathogenic microorganisms are not the only concern associated with oyster consumption. Other risks include toxic metals, biotoxins and chemical environmental contaminants. These also accumulate in the GI system and tissues of oysters and have been associated with food poisoning outbreaks (Campàs et al., 2007).

Until recently, all attempts to reduce the incidence of oyster-related food borne illnesses were centered on consumer education. Other efforts to minimize consumer exposure to oyster-associated pathogens included natural depuration and harvesting restrictions. Despite these efforts, in 2002, Potasman et al. reported a worrisome increase in the number of outbreaks of bivalve-associated infections primarily from oysters, from 3 reports in the 1970s to 22 during the 1990s.

The emergence of innovative FDA approved Post Harvest Processing (PHP) technologies such as Individual Quick Freezing (IQF), introduced in 1989, Heat-Cool Pasteurization (HCP), introduced in 1995, and High Hydrostatic Pressure (HHP), introduced in 1999, have revolutionized the industry of seafood, particularly in regard to oysters. These technologies, which are currently commercially available, have made it possible to bring raw and healthy products to consumers. An end product of high quality (fresh taste and superior appearance) is the major advantage of Individual Quick Freezing (IQF), Heat-Cool Pasteurization (HCP) and High Hydrostatic Pressure (HHP) processing. These processing techniques also reduce the level of *V. vulnificus* to non-detectable levels (i.e., <3 MPN/g). The level of spoilage bacteria is significantly reduced as well. Unfortunately, oysters cannot survive any of the above PHP technologies (Mississippi Department of Marine Resources, 2006). In terms of shelf life, this can be an issue and thus it is mandatory to store HHP-, IQF- and HCP-treated oysters at low temperature and long term storage is expensive. Several other methods are under development including approaches using X-ray and Gamma radiation as cold pasteurizing agents.

In the present paper, we introduce an original and promising approach to post harvest processing of oysters: warm pasteurization of oysters using scCO₂ (CO₂ at a temperature and pressure above its critical point). The antimicrobial potential of dense CO₂ was first reported in 1952 by Fraser while working with *Escherichia coli*. Extensive work has since been completed and currently, the bactericidal potential of scCO₂ is well-established over a wide range of pathogenic microorganisms. ScCO₂ has been documented to effectively inactivate viruses (HIV-1, Sindbis virus, polio Sabin type I virus, and pseudorabies virus [PRV]), yeast (*Saccharomyces cerevisiae*), as well as vegetative and sporal forms of various bacteria (*Salmonella*, *S. aureus*, *listeria*, *E. coli*, *Pseudomonas fluorescens*, etc.) (Fages et al., 1998; Hemmer et al., 2006; Damar and Balaban, 2006; Garcia-Gonzalez et al., 2007). So far, however, there has been no report on the effect of scCO₂ on *Vibrio* spp.

There are also several reports of studies related to the kinetics of inactivation of bacteria in food products (e.g., milk, fruit juice, herbs, ground beef, etc.) using scCO₂ (Spilimbergo and Bertucco, 2003; Werner and Hotchkiss 2006; Damar and Balaban, 2006). However there is no reported attempt to assess whether treating oysters with scCO₂ could have a positive impact on the wide range of pathogenic bacteria which populate their GI system.

The present studies address several key questions in regards to oyster pasteurization by scCO₂. First, the effect of scCO₂ was evaluated *in vitro* on bacterial cultures of a non-pathogenic strain of *Vibrio* used here as a model for *Vibrio* spp (*Vibrio fischeri* ATCC® 7744™) and several bacterial isolates from an oyster homogenate. Second, the effect of scCO₂ on the bacterial community trapped in the oyster GI system was studied. Finally, using a panel of thirteen participants, a sensory analysis of fresh untreated oysters versus scCO₂-treated oysters was completed in a blind survey.

2. Material and Methods

2.1. Bacterial strains

Referenced bacterial strains used to assess the effect of scCO₂ on bacteria *in vitro* were *V. fischeri* (ATCC® 7744™) and *E. coli* (Migula) Castellani and Chalmers (ATCC® 25922™). *V. fischeri* was grown and maintained on either Luria Bertani Salt (LBS) medium (1% [wt/vol] bacto tryptone [DB], 0.5% [wt/vol] bacto yeast extract [BD, Franklin Lakes, NJ], 2% [wt/vol] NaCl, 0.3% [vol/vol] glycerol, 50 mM Tris-HCl [pH 7.5]) (Dunlap, 1989), or BOSS medium (3% [wt/vol] NaCl [Fisher Scientific], 0.1% [wt/vol] Glycerol [Sigma Aldrich], 1% [wt/vol] bacto peptone [DB], 0.3% [wt/vol] beef extract, [pH 7.3] (Klein et al., 1998). *E. coli* was grown and maintained in Bacto nutrient broth [Difco Laboratories, Detroit, MI], 23 g/L.

2.2. Oysters

Oysters used in these experiments were purchased from a seafood distributor in Memphis, TN, USA. These oysters were harvested on 04/13/09, properly maintained and delivered to our facility on 4/20/09. After arrival, the oysters were stored whole on ice overnight and our experiments were carried out the following day. Oysters were presumed alive unless the shell's adductor muscles were permanently released (in which case the oysters were discarded).

Concerning morphology and size, the oysters received were a mixture of “banana-shaped” and “good-quality shaped” oysters; “good-quality shaped” oysters constituted the majority. The morphology classification was performed according to procedures previously published (Lee et al., 2004). The length for these oysters varied from small (7.5cm) to large (13 cm).

2.3. Supercritical unit and Treatment of oysters using supercritical CO₂

The SFT-150 SFE system (Supercritical Fluid Technologies, Inc.) (Figure 1) was used for all scCO₂ treatments. The internal surfaces of the SFT-150's treatment chamber were sterilized with a 70% ethanol solution and allowed to dry at room temperature. The samples prepared for treatment were placed into the chamber which was then hermetically sealed. The liquid carbon dioxide source was opened filling the chamber and venting for five seconds to purge the chamber of any air. An external air source was utilized to supply the SCT-150's pneumatic compressor which increases the chamber's internal pressure. An external air pressure gauge was utilized to control the pneumatic compressor. The SCT-150's internal pressure was maintained and monitored via the fine-control valves and internal pressure displays located on the SCT-150 unit. Oysters were sampled in groups of 10–13 and placed in porous bags (Filter Specialists, Inc., [FSI] Michigan City, IN) which were placed into a 4L capacity chamber. The oysters were exposed to scCO₂ under two different conditions: 100 bar for 30 minutes at 37 °

C and 172 bar for 60 minutes at 60 °C. After treatment, the chamber was carefully vented, the oysters removed, and their microbial load (APC) was assessed alongside untreated oysters.

2.4. Microbiological analysis of treated vs. untreated oyster samples

For microbiological analysis, each group of oysters was divided into three sub-samples which were analyzed independently. Each oyster's surface was carefully cleaned with 70% ethanol solution and dried under a bacteriological hood using a paper towel. Oysters were then opened aseptically in order to separate the flesh and interstitial fluid from the shell. The flesh and interstitial fluids were macerated in a sterilized blender for about a minute in an equal volume of Butterfield's phosphate-buffer to yield an oyster homogenate (dilution 1:2). A dilution 1:10 was prepared by transferring 20 g of this homogenate into 80 mL of Butterfield's phosphate-buffered dilution water. Subsequent tenfold dilutions were then prepared. One milliliter of each dilution was then pipetted into separate, duplicate, appropriately marked Petri dishes. About 12–15 ml plate count agar (cooled to about $45 \pm 1^\circ\text{C}$) was added to each plate and the agar was allowed to solidify. The plates were then incubated promptly for $48 \pm 2\text{h}$ at 35°C .

As a positive control a group of twelve oysters was split into three subgroups, placed in self-sealing sterilization pouches, and autoclaved (30 minutes at 121°C and 18–20 psi). At the end of the sterilization process using the same procedure described above, the APC was assessed and confirmed to be zero.

2.5. Statistical analysis

All oysters' experiments were replicated twice and assessments of APC counts were run in triplicate for each replicate ($n=2 \times 3$). Mean values $\pm$ standard deviations were used to plot the corresponding graph.

The test of the hypothesis that the bacterial load in treated vs. untreated oysters was significantly different was done by application of the Mann-Whitney U test to groups of log means of each set of two within sample replicates. The difference between the set of treatment group log means and the set of untreated group log means was found to be significant when $P < 0.05$.

2.6. Biochemical characteristics of bacteria isolated from oyster tissue

Several bacteria colonies were isolated from the oysters' tissues (treated oysters); gram staining, catalase tests, oxidase tests, mobility tests and fermentation profiles were completed for each isolate. The catalase reaction was completed with 3% H_2O_2 . The ability of isolates to ferment specific carbohydrates was determined using phenol red broth (enzymatic digest of casein [1%], supplement sugar [0.5 %– 1%], sodium chloride [0.5%], phenol red [0.0018%], final pH: 7.4 ± 0.2 at 25°C) to which specific carbohydrates (glucose, sucrose, lactose or mannitol) were added. Oxidase reagent droppers [0.5 mL of a 1% aqueous solution of N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride] were used for the oxidase test. Some isolates were also cultured on the *Vibrio* selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar (yeast extract [0.5%], proteose peptone No. 3 [0.1%], sodium citrate [1%], sodium thiosulfate [1%], oxgall [0.8%], saccharose [2%], sodium chloride [1%], ferric ammonium citrate [0.1%], bromthymol blue [0.004%], thymol blue [0.004%], agar [1.5%]).

2.7. Effects of scCO_2 on bacteria isolated of oysters tissues

Four bacteria isolates possessing basic biochemical characteristics common to pathogenic *Vibrio* sp (i.e., Gram negative, catalase positive, oxidase positive and growth on TCBS agar) were cultured overnight at $35 \pm 2^\circ\text{C}$ in ISP2. Ten milliliters of each culture were pipetted into 50 mL Sterile Corning® centrifuge tubes. A hole was made in the cap of these tubes to allow the flow of scCO_2 into the tube. Dry sterilized filtered paper was used to cover the hole. Two

such culture tubes were prepared for each of the four isolates. These were placed together in the 100 mL vessel of the unit of the Supercritical Fluid Technologies, Inc., SFT-150 SFE system and treated with scCO₂ at 100 bar and 37 °C for 30 minutes. Bacterial counts were estimated by plate spread technique using ISP2 or plate count agar; serial dilutions were completed in each case and these plates were seeded with 100 µl of treated or untreated culture.

2.8. Kinetics of inactivation of two ATCC referenced Gram negative bacteria *V. fischerii* and *E. coli* by scCO₂

For each experiment, 2 mL of a freshly grown culture of *E. coli* and *V. fischerii*, contained in a 15 mL Sterile Corning® Centrifuge tube (Corning Incorporated, Big Flats, NY), were introduced in the 100 mL vessel of the unit; a hole was also generated in the plug-sealed cap of the centrifuge tube to allow the flow of scCO₂ into the tube. The experiment was completed under three different pressures (100, 150, and 200 bar) at 37 °C with the purpose of evaluating the effect of pressure variation on the sterilization potential of CO₂; exposure times were 5, 10, 15 and 20 minutes. The initial load was estimated by plate count (*E. coli*) or by using a calibrations curve (*V. fischerii*). At the end of each experiment, the number of surviving bacterial cells was counted by plate spread techniques (Collins *et al.* 1995). Briefly, a fourfold serial dilution of each test culture was made in a 96 well-plate format. A few wells were selected for the bacterial count process via spreading 25 µl of the content of the given wells or dilution on the surface of a plate containing BOSS or nutrient agar; the bacterial count per well was repeated twice and the average value was considered as the final count. The entire experiment was repeated to confirm the previously observed trend (n=4).

2.9. Sensory assessment

The sensory analysis of oysters treated by scCO₂ was assessed by a panel of 13 people. The features evaluated included physical appearance, smell, and texture of oysters; the oysters were not tasted. Because they were not trained panelists, these panelists were presented with standards (Fig. 4) that would assist them in the grading process. There were two such standards; the first was labeled as “fresh” and the second as “spoiled.” The first standard was a freshly shucked, untreated oyster while the second was an oyster that was left at room temperature for about two days (enough to allow it to develop the mild odor associated with spoiled oysters).

Each panelist was asked to comparatively examine the physical appearance, smell, and texture of these standards after what they were led to the oyster stations where five groups of oysters were aligned side by side. One of these groups included untreated oysters while the rest included oysters treated under a variety of conditions ([20 min, 100 bar, 37 °C], [50 min, 100 bar, 37 °C], [50 min, 200 bar, 37 °C], and [20 min, 200 bar 37 °C]). These oysters were labeled using simple codes: x1, x2, x3, x4 or x5 (blind study). The panelists were informed that one out of these five groups was made of standard/untreated oysters. The panelists were then asked to evaluate the physical appearance, smell, and texture of the oysters as closely resembling our standard for “fresh” or our standard for “spoiled”. A third option, “intermediate” was also offered.

A score of two (2) was allocated for the adjective “fresh” (the most desirable). A score of zero (0) was allocated for the adjective “spoiled” (the least desirable). Finally, a score of one (1) was allocated for “intermediate.” The overall “freshness score” for each given oyster was the sum of scores it received for its texture, physical appearance, and smell. This freshness score varied from 0 to 6. To be judged acceptable, an oyster must have obtained an overall score ≥ 3. Data from all the 13 panelists were pooled for each group of oysters and an overall score was assigned per the group; this was the mean value of scores achieved by individual oyster of each particular group.

3. Results

3.1. Microbiological analysis of treated and untreated oyster samples

Oysters sampled in groups of 10–13 oysters were treated with scCO₂ under the following two conditions: 100 bar, 30 min, 37 °C and 60 min, 172 bar, 60 °C. After exposure, the effect of scCO₂ on the aerobic microbial load (APC) that contaminates the GI system of these oysters was assessed using the FDA recommended approach to food analysis (n=6). The microbial load (Aerobic Plate Count) present in the GI system of untreated oyster samples was determined using this same approach and estimated to be 1.8×10^7 CFU/g of oyster meat (EAPC).

It was estimated that exposure of oysters to CO₂ (100 bar, 30 min, 37 °C) led to a significant decrease ($P=0.002$) in the load of oysters' bacterial contaminants. This treatment induced a 97.6% reduction in the microbial load that contaminated their GI tract corresponding to a 2-log reduction. A similar result was observed in previous experiments with oysters exposed to scCO₂ at 100 bar, 40 min, and 37 °C (data not shown). The residual load of bacteria in these scCO₂-treated oysters was estimated to be about 4×10^5 CFU/g (EAPC).

A greater bacteria inactivation level was observed with oysters exposed to 172 bar, 60 min and 60 °C. In this case the APC reduction was estimated to be 99.9%, which corresponds to a 3-log reduction. This translates into reducing the initial load of $\sim 1.8 \times 10^7$ CFU/g (EAPC) of oyster meat to 3.3×10^4 CFU/g.

3.2. Effects of scCO₂ on bacteria in vitro

As mentioned previously, *V. parahaemolyticus* and *V. vulnificus* are key players in quality control programs for raw oysters. We anticipate assessing the effect of scCO₂ on these microorganisms (*in vitro* and *in vivo*) in future studies. In the present document, we have evaluated the effect of scCO₂ on a non pathogenic strain, namely *V. fischerii*, which we used as a model for *Vibrio* sp. The kinetic of inactivation of *V. fischerii* by scCO₂ was studied under three different pressures conditions (100 bar, 150 bar, and 200 bar at 37°C); exposure times were 5, 10, 15 and 20 minutes. The effect of scCO₂ on oyster bacterial isolates possessing several key biochemical features common to pathogenic *Vibrio* spp (namely *V. parahaemolyticus* and *V. vulnificus*) was assessed as well.

3.2.1 Effect of scCO₂ on bacterial isolates possessing some basic biochemical characteristics common to pathogenic *Vibrio* sp (in vitro)—Ten milliliters of overnight cultures of bacterial isolates (Table 2) were exposed to scCO₂ (100 bar, 37°C) for 30 minutes. The initial microbial load was estimated to be higher than 2×10^7 CFU/mL of broth. A complete (100%) inactivation was obtained for all cultures. No growth was observed even in plates seeded with 100uL of the undiluted sample (pipetted directly from the test tube). This experiment was replicated and the number of survivals in each case was estimated in triplicate. This corresponds, at a minimum, to a 7-log reduction. Subsequent experiments help establish that a 30 minute-long exposure is not required to achieve this level of inactivation; similar results were achieved with cultures treated for only 10 minutes.

3.2.2 Comparative effects of scCO₂ on two ATCC referenced Gram-negative bacteria: *V. fischerii* and *E. coli* - kinetic of bacterial inactivation—*Vibrio fischerii* was reduced to a non-detectable level (6-log reduction) after only 5 min of exposure to scCO₂; this occurred independently of pressure. *Vibrio fischerii* appeared far more sensitive to the effect of scCO₂ when compared to *E. coli*, for instance. *E. coli* is considered one of the most sensitive bacteria to this treatment (Damar and Balaban, 2006). The complete inactivation of *E. coli* (8-log reduction), under these same conditions, was achieved only after 20 minutes of exposure to scCO₂. Dillow et al. (1999) and also Smelt and Rijke (1992) reported similar

results. Along with this decrease in bacterial viability came a decrease in the pH of the culture. This decrease in pH can suggest a possible mechanism for the bacterial inactivation.

3.3. Sensory assessment

Treated and untreated oysters were evaluated side by side by a panel of thirteen (13) volunteers in a blind study. A score was assigned to oysters individually and as a group. To be judged acceptable, an oyster or a group of oysters must have achieved an overall score ≥ 3 .

From the data collected, it was concluded that oysters remain acceptable, as far as their physical appearance, texture and smell, after exposure to CO₂ ([100 bar for 20 minutes at 37 °C], [100 bar for 50 minutes at 37 °C], [200 bar for 50 minutes at 37 °C] and [200 bar for 20 minutes at 37 °C]). The group of treated oysters that received the best rating were those treated at 100 bar for 20 minutes at 37 °C (score = 4.4). Ratings for the other groups were also ≥ 3 . The group of untreated oysters achieved a score of 4.9.

4. Discussion

Herein, we report that the level of bacterial inactivation (Aerobic Plate Count) achieved with scCO₂ (100bar for 30 minutes at 37 °C or 172bar for 60 minutes at 60 °C), is comparable to that achieved with several FDA approved PHP for oysters, namely, HHP (High Hydrostatic Pressure) and QF (Quick Frozen). In 2009, Prapaiwong et al. reported a comparable residual amount of bacteria in HHP- and QF-treated oysters. Oysters included in their study were sampled from batches of treated oysters on their way to the market. These authors reported a Total Aerobic Plate Count (TAPC) of 1.4×10^4 CFU/g, 1.6×10^4 CFU/g, and 3.3×10^4 CFU/g in HHP-treated oysters harvested during summer, winter, and fall, respectively. They reported similar amounts of residual bacteria in QF-treated oysters. Untreated oysters harvested during these same periods reportedly contained 3.5×10^5 , 2.8×10^5 , and 1.4×10^9 CFU/g for oysters harvested during winter, fall, and summer, respectively (Prapaiwong et al., 2009).

Bacteria that survived these scCO₂ treatments were mostly Gram-negative similarly to what have been reported for HHP-treated and QF-treated oysters (Prapaiwong et al., 2009). Twenty-four out of thirty isolates were identified as catalase-positive, and eighteen as oxidase-positive. Once in culture however, these isolates (a random selection of four) appeared to be extremely sensitive to the effect of scCO₂. According to Lin et al. (1994), the complex physicochemical environment of food products especially a high fat content can increase the resistance of vegetative cells to the effect of scCO₂ by limiting the penetration of CO₂.

Each of the current three FDA approved PHP for oysters, namely HHP, HCP, and IQF, has its advantages and disadvantages. One of the major selling points of High Hydrostatic Pressure (HHP), for instance, is the fact that it induces a release of adductor muscles from oysters' shells. Whether the release of these adductor muscles is truly a desirable effect is somewhat controversial. While it makes shucking oysters afterwards easier, it is important to note that the permanent release of such muscles is a clear indication that oysters did not survive the process which in terms of shelf life could be an issue. Prapaiwong et al. (2009) studying variations in the bacterial load of HHP-treated oysters stored at 4 °C, determined that the bacterial count can go from an initial 1.6×10^4 – 3.3×10^4 CFU/g (post treatment residual load) to about 10^7 CFU/g in just 7 days.

Based on the Cold Storage Chart of the FDA, the quality of such oysters can be maintained for a longer period of time (~ up to 3–6months) providing they are stored at ~-17 °C. We were able to verify that such long term storage does not affect the quality of oysters. In fact treated and untreated oysters used for our sensory study were stored at ~ -17 °C before analysis.

The scCO₂ approach stands out in this regard because the release of adductor muscles from oysters can be monitored. Oysters in this case were treated under two conditions, one that can be considered as mild (100 bar, 30 min and 37 °C) and another that can be considered harsher (172 bar, 60 min and 60 °C). Contrary to what was observed at milder conditions (100 bar, 30 min and 37 °C), treating oysters at 172 bar for 60 min at 60 °C, caused a release of adductor muscles from the shell. Oysters exposed to the milder condition however did not release their adductor muscles and retained their fresh-like appearance closely resembling the untreated ones (Fig. 4). On the other hand, the oysters treated at 172 bar, for 60 minutes at 60 °C though still watery appeared slightly cooked or steamed—certainly a direct consequence of the high temperature. This effect was most pronounced in another group of oysters treated at this same condition and left in the scCO₂ unit afterwards for about 45 minutes; this set of oysters was excluded from our analysis.

After processing, oysters that remain alive are undoubtedly a better alternative. This is, for instance, the major selling point of several emerging PHP techniques, namely those using X-ray and gamma irradiation as cold pasteurizing agents (Jakabi et al., 2003). X-ray and gamma irradiation are believed to exert their bactericidal effect by damaging the bacterial DNA. Because of the high probability that oysters' DNA could sustain the same damage, concerns have been raised concerning the shelf life of X-ray and gamma irradiation-treated oysters. As mentioned previously, treating oysters at 100 bar for 30 minutes at 37 °C did not release the adductor muscles. We have good reasons to believe that oysters can survive mild scCO₂ treatments, such as 100 bar for 30 minutes at 37 °C. During trial experiments, a few oysters treated under similar pressure and temperature conditions for 40 minutes (Fig. 5) and afterwards put in a flask filled with water were actually able to open and close their shells (on contact with a hard object) for several days. This was a clear indication that they had survived the treatment. The ability of individual oyster to survive such high pressure and CO₂ concentration vary with individual health. The LD₅₀ of scCO₂ on oysters will be addressed in future studies.

The sensory experiments were conducted, and it was revealed that oysters treated at milder conditions retain their acceptability. It is important to note that of all the groups of oysters included in this analysis, none received a perfect score of 6 (not even the group of group of untreated oysters). Two oysters from this group were graded as “spoiled.” Because of current facility limitations, the only feature used to decide whether any given oyster is alive or healthy (thus qualifying for inclusion into the study) is whether or not their shell is tightly closed. While it is true that this feature is shared by live oysters when brought out of the water, it is also true that some oysters, unhealthy or even dead, could share this feature. This constitutes a source of bias and explains why two spoiled oysters were found in the group of untreated oysters. This justifies why, as stated above, at this point, it is difficult to clearly assess the LD₅₀ of scCO₂ on oysters. Another difficulty and possibly a source of bias, especially when it comes to sensory analysis studies, is the way our scCO₂ unit is built; this instrument was designed for scCO₂ extraction; the lack of uniformity in the treatment chamber could result in some oysters being overexposed.

Although the exact mechanism of the bactericidal action of CO₂ is still unknown, it has been proposed that the bacterial inactivation might occur as a result of acidification of the medium or product being sterilized. This acidification occurs as a result of CO₂ interaction with water, an interaction which ultimately results in the generation of H₂CO₃. H₂CO₃ exists in equilibrium with HCO₃⁻; this equilibrium is responsible for the release of protons in the bacterial environment, thus decreasing pH.

Low pH can then induce structural changes in the membrane of bacterial cells. These changes, in turn, will increase the permeability of the membrane to CO₂, which then can freely diffuse and accumulate in the bacterial cell, interfering with the normal metabolism of these living cells and ultimately resulting in the death of bacteria (Dillow et al., 1999, Spilimbergo and Bertucco, 2003). There have been several other proposed mechanisms of bacterial inactivation by scCO₂, such as removal of vital constituent from cell membrane, induction of intracellular electrolytes imbalance, and direct effect of CO₂ on bacterial metabolism, among others (Damar and Balaban, 2006; Garcia-Gonzalez et al., 2007).

In conclusion, the scCO₂ based approach to warm pasteurization of oysters is a promising PHP technique. In the present document, it is established that scCO₂ is as effective as several current FDA approved standard approaches to PHP of oysters (e.g., HHP and QF) aimed at reducing the microbial load (aerobic flora) present in the GI system of oysters. We have strong reasons to believe that the scCO₂ approach will also be as effective in reducing the load of most problematic oysters-associated bacteria, namely, *V. parahaemolyticus* and *V. vulnificus*. The bacteria used here as a model for *Vibrio* sp., namely *V. fischerii*, showed an extremely high sensitivity to scCO₂ and bacterial isolates cultured from oysters' tissues and sharing common biochemical characteristics of *V. parahaemolyticus* and *V. vulnificus* showed an extremely high sensitivity to scCO₂.

As a PHP approach, the scCO₂ based approach provides quite a number of advantages over other emerging oyster PHP technologies. Large scale scCO₂ based technologies for food processing are already available commercially and used industrially for processes such as coffee decaffeination. This represents a serious advantage over gamma and/or Xray based PHP techniques. The use of scCO₂ in food industry is well- established and safe; it can thus be predicted that acceptance by the public will be easier. We have also completed a sensory analysis, and no significant change was noted as a result of oysters having been treated with scCO₂. Finally, as mentioned in our introduction, microbial contaminants are not the only health hazard associated with oysters. Other risks include biotoxins, toxic metal, and chemical environmental contaminants. None of the currently proposed PHP treatments (e.g., HHP, IQF, HCP, x-ray based approach, and gamma-based approach) can reduce the load of toxic metal, biotoxins, and chemical environmental contaminants in oysters. Though it is beyond the scope of this paper, it is important to note that the ability of scCO₂ to remove such contaminants from various biological substrates is well-established (Quan et al., 2004; Brooks and Uden, 1995).

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Fig. 1. Standard Bench-Top SFT-150 (bottom) and Schematic of supercritical sterilization apparatus (top)

(1) Filter. (2) Bulkhead. (3) Relieving Regulator. (4) CO₂ Pre-chiller. (5) Liquid CO₂ pump. (6) Preheater. (7) Vessel. (8) Sterilization unit.

Fig. 2. Inactivating effect of sc CO₂ on bacterial contaminant of oyster

Oysters were thoroughly cleaned and treated at 100 bar, 30 min and 37 °C. The oysters were separate in 3 groups shucked in aseptic environment; the amount of APC was then estimated using FDA recommended approach to food analysis. This whole experiment was repeated twice for an “n=6 (that is 2*3). Untreated Oysters were processed in a similar manner. The APC was estimated twice also for an n=6 that is (2*3)

*** Statistically significant difference

Fig. 3. Comparative Effects of scCO₂ on *V. fischerii* and another Gram negative bacterium (*E. coli*) *in vitro*

The kinetic of inactivation of two Gram negative bacteria by scCO₂ was studied. Cultures of (A) *V. fischerii* (3×10^6 CFU/mL) and (B) *E. coli* (5×10^8 CFU/mL) were exposed to sc CO₂ at 100 bar, 150 bar and 200 bar for 5, 10, 15 and 20 minutes.

Fig. 4. Standards used for the sensory analysis of untreated vs. treated oysters

A sensory analysis of untreated vs. scCO_2 - treated oysters was completed. To grade the physical appearance, smell, and texture of oysters, a panel of 13 people used two standards; the first standard was that for “fresh” oysters and the second standard was that for “spoiled” oysters. (A) Standard for “fresh” (B) Standard for “spoiled”

Fig. 5. Appearance of oysters before and after a 40 minutes exposure to scCO₂

The effect scCO₂ on the physical appearance of oysters is presented. Oysters were sampled in small groups and introduced in a supercritical unit (SFT-150 SFE system -Supercritical Fluid Technologies, Inc.) where they were exposed to scCO₂ for 40 minutes. They were then shucked and photographed. Temperature and pressure conditions were 37 °C and 100 bar.

Table 1

Guide used in grading oysters (treated and untreated): blind study

Score	Physical appearance	Smell	Texture
2 ^a	Fresh	Fresh	Fresh
1	Intermediate ^c	Intermediate ^c	Intermediate ^c
0 ^b	Spoiled	Spoiled	Spoiled

^aExtremely desirable.^bExtremely undesirable.^cNot Fresh but definitely not spoiled

Table 2
Few biochemical features of bacterial isolates obtained from treated oyster tissues

Isolate code	Gram type	Catalase reaction	Oxidase reaction	Growth	On TCBS Colonies	Colonies' Color conversion	Mobility
Isolate #13 [*]	-	+	+	+	Green centered colonies	-	+
Isolate #18 [*]	-	+	+	+	Green	+ ^y	+ [*]
Isolate #30 [*]	-	+	+	+	Green	+ ^d	+ [*]
Isolate #36 [*]	-	+	+	+	Green	+ ^d	+ [*]

^y conversion to yellowish color;
^d conversion to darker (blackish) colonies;
+: Positive;-: Negative;
^{*} did not ferment glucose, lactose or mannitol
+^{*} poor growth, AF: anaerobic facultative

Table 3

Effect of
scCO₂ on bacterial isolates possessing some basic biochemical characteristics common to pathogenic *Vibrio*
sp (bacteria count > 2E +07 CFU/mL^a)

Bacteria	Condition of treatment	Level of inactivation
Isolate #13	100bar, 37°C, 30 minutes	Complete inactivation ^b
Isolate #18	100bar, 37°C, 30 minutes	Complete inactivation ^b
Isolate #30	100bar, 37°C, 30 minutes	Complete inactivation ^b
Isolate #36	100bar, 37°C, 30 minutes	Complete inactivation ^b

^a estimation according to ASTM Standard (Sutton, 2006)

^b no growth from plating 100uL of undiluted sample