Effects of high hydrostatic pressure processing on *Bacillus cereus* spores in fresh blue crab meat (*Callinectes sapidus*)

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EFFECTS OF HIGH HYDROSTATIC PRESSURE PROCESSING ON BACILLUS CEREUS SPORES IN FRESH BLUE CRAB MEAT (*Callinectes sapidus*)

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(ABSTRACT)

The Food and Drug Administration has recently expressed concern for the safety of seafood and seafood products. One of the concerns is the presence of *Bacillus cereus* in fresh blue crab meat. *Bacillus cereus* is a spore-forming pathogen whose spores survive the customary thermal treatments applied during cooking and pasteurization; therefore it could potentially present a health concern to consumers as the microorganism could increase to pathogenic levels.

The objectives of this study were to evaluate the effects of a post-processing method i.e. high hydrostatic pressure treatment on the quality of fresh crab meat and to evaluate the effectiveness of high pressures on the inactivation of *B. cereus* spores.

Fresh blue crab meat was pressurized at 300 and 550 MPa at 25° C for 5 min and stored at 4° C for 31 days to determine the pressurization effects on the microbiological, physical, and sensory quality of the meat. A pressure of 300 MPa caused a 1 log reduction in total aerobic plate count and a 3 day lag period, whereas 550 MPa inactivated 2 logs in total aerobic plate count with no evident lag phase. Physical and sensory qualities of pressurized crab meat were not statistically different from the untreated crab meat ($P>0.05$). A pressure of 300 MPa extended the shelf-life from 17 to over 24 days with the prevalence of *Carnobacterium piscicola* at the time of spoilage. Crab meat treated with 550 MPa was not rejected by sensory panels at day 31 and *Enterococcus spp*. was identified as the predominant microorganism.

High hydrostatic pressure (550 MPa at 40° C for 15 min) inactivated less than 1 log (0.66 log) of *B. cereus* spores inoculated in fresh crab meat. The meat essentially had a protective effect on pressure inactivation of the spores. During storage (31 days), surviving *B. cereus* was suppressed and outgrown by the other pressure resistant microflora at a storage temperature of

12° C. At 4° C, *B. cereus* could compete with the other pressure-resistant microflora and was isolated even at the end of the storage period (day 31); however, diarrheal toxin was not detected in any stored samples.

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CHAPTER I
INTRODUCTION

The Food and Drug Administration's Office of Seafood has recently expressed concerns over the safety of seafood and seafood products. Two of the four food safety concerns listed are associated with the presence of spore-forming pathogens: *Bacillus cereus* and *Clostridium botulinum* in processed and vacuum or modified packaged seafood. These microorganisms are of concern because their spores are able to survive the customary thermal treatments applied during cooking and pasteurization due to their high heat resistance, and their survival could lead to food spoilage and food poisoning. Processed seafood is therefore in need of a post-processing method to increase the safety of this ready-to-eat seafood.

Bacillus cereus is a Gram-positive, aerobic, facultative anaerobic sporeformer which produces endospores. This bacterium produces spores following vegetative growth when nutrients in the growing environment are depleted. Spores exhibit high resistance to various physical (heat, radiation, drying) and chemical agents, thus they are difficult to inactivate. Spores have a high resistance to their surroundings and are frequent inhabitants of soil, vegetation, dust, sediment, and water and are spreaded to food through cross-contamination.

The common food vehicles for *B. cereus* range from dairy products, rice and oriental foods, spice and dried products, to meat and meat products and seafood. Although spores have no detectable metabolic activities in their dormant state, they are considered still viable and respond to external changes. When nutrients become available they germinate and resume vegetative growth. Consumption of foods contaminated with *B. cereus* vegetative cells or with *B. cereus* toxins could result in both a diarrheal and an emetic type foodborne illness. To maintain quality and increase the safety of foods, especially processed seafood, an effective post-processing method that would inactivate spoilage and pathogenic bacteria while retaining the food quality is required.

High hydrostatic pressure processing is a new technology and has been reported to inactivate spoilage and pathogenic bacteria in a variety of food products. It is regarded as a non-thermal treatment that causes only minimal changes in foods compared to traditional thermal treatments. The objectives of this study are to investigate the use high hydrostatic pressure processes to increase the shelf-life of fresh crab meat, and to evaluate whether this technology would effectively inactivate *B. cereus* spores.

CHAPTER II
LITERATURE REVIEW

2.1 *Bacillus cereus*

Bacillus cereus is a large, Gram-positive, rod-shaped, aerobic, facultative anaerobic sporeformer which produces central to terminal ellipsoid or cylindrical spores that do not swell the sporangium (vegetative cells). The organism is a member of the *B. cereus* group consisting of *B. cereus* itself and other closely related species having highly similarities in phenotypic and genetic properties. The other members of *B. cereus* group are *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, and *Bacillus pseudomycoides*. It is reported that the similarity in the 16rRNA sequences among *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. thuringiensis* is more than 99% which complicates taxonomy of the genus *Bacillus* (Kotiranta et al., 2000).

However, there are some characteristics that differentiate members of the *B. cereus* group as shown in Table 1. Among the members of *B. cereus* group, *B. thuringiensis* cannot be differentiated from *B. cereus* using the procedures normally used to identify *B. cereus* due to the loss of plasmids harboring a gene encoding for a crystalline inclusion (Cry protein) or δ-endotoxin that distinguishes *B. thuringiensis* from *B. cereus* (Granum, 2001 and Kotiranta, et al., 2000).

Table 1: Criteria for differentiating members of the *B. cereus* group

Bacillus spp.	Colony Morphology	Motility	Hemolysis	Crystalline Inclusion	Penicillin Susceptibility
B. cereus	White	+	+	-	-
B. anthracis	White	-	-	-	+
B. thuringiensis	White/gray	+	+	+	-
B. mycoides	Rhizoid	-	(+)	-	-
B. weihenstephanensis	Differentiated from *B. cereus* based on growth at <7° C and not at 43° C; can be identified rapidly using rDNA or cspA (cold shock protein A) targeted PCR				
B. pseudomycoides	Not distinguishable from *B. mycoides* by physiological and morphological characteristics; clearly differentiated based on fatty acid composition and 16S RNA sequences				

B. cereus is widely distributed in the environment, its vegetative cells and spores are frequent inhabitants of soil, vegetation, dust, sediment and water. It then spreads to foods of plant origin and other foods through cross-contamination. The common food vehicles for *B. cereus* range from dairy products, rice and oriental foods, spices and dried products, to meat and meat products. In 1906, *B. cereus* was first associated with a food poisoning in Europe, 300 hospital patients developed gastroenteritis after consuming meatballs containing large numbers of *B. peptonificans* (later *B. cereus*). In 1950, *B. cereus* was first recognized and established as a food poisoning agent as a result of Steinar Hauge's investigation on consumption of vanilla sauce in Norway (Johnson, 1984). During 1947 to 1949, four outbreaks of food poisoning were investigated affecting 600 persons, which were traced back to vanilla sauce prepared from cornstarch contaminated with *B. cereus* spores. Steiner Hauge demonstrated that *B. cereus* was the causative agent by consuming a pure culture of the microorganisms with the symptoms of "diarrheal type" *B. cereus* food poisoning (abdominal pain and water diarrhea) which developed after 13 hr. The incidence of *B. cereus* food poisoning was reported to include at least 230 outbreaks of the diarrheal type food poisoning worldwide between 1950 and 1976. Implicated foods were meat and vegetable soups, cooked meat and poultry, raw and cooked vegetables, pasta, milk and ice cream (Shinagawa, 1990).

The first outbreak of "emetic type" of *B. cereus* foodborne illness was reported in England in 1971. This type of food poisoning is characterized by nausea and vomiting within 0.5 to 6 hr after consumption of contaminated foods. Shinagawa (1990) stated that at least 170 outbreaks of the emetic type of *B. cereus* food poisoning have been reported worldwide since 1971. More that 110 outbreaks were reported in Great Britain alone between 1971 and 1978. Cooked and fried rice were implicated in 108 of the 110 outbreaks of which 104 were related to boiled or fried rice from Chinese restaurants. Although all the emetic outbreaks are associated with consumption of cooked rice, other starchy foods such as macaroni and cheese, vanilla slices, and a product similar to cream puffs have been implicated (Johnson, 1984).

In the United States, 52 outbreaks of *B. cereus* food poisoning were reported to the Center of Disease Control and Prevention (CDC) between 1972 and 1986. During 1993 to 1997, *B. cereus* was reported as the cause of 14 outbreaks and 691 cases of foodborne illness (Doyle, 2001).

B. cereus is not a common cause for foodborne outbreaks in the United States, since it accounts for only 1.3% of the bacterial food poisoning cases reported between 1972 and 1982 (Kotiranta et al., 2000). Nonetheless, *B. cereus* is one of pathogens of concern in processed seafood listed by the Food and Drug Administration (FDA)'s Office of Seafood since its spores are considerably heat-resistant and may survive thermal treatments applied during processing. In addition, heating may activate spores which can germinate and outgrow when environmental conditions becomes favorable.

There are two distinct forms of *B. cereus* foodborne illness: diarrheal and emetic type. The characteristics of the two types of illness caused by *B. cereus* are summarized in Table 2. The diarrheal type is characterized by watery diarrhea, abdominal pain and cramps and rarely vomiting after 6 to 15 hr of consumption of contaminated food. The symptoms persist for 12 to 24 hr in most instances. The symptoms of this illness mimic the symptoms of *Clostridium perfringens* food poisoning. The emetic type of food poisoning is characterized by nausea and vomiting, occasionally abdominal cramp and diarrhea, within 0.5 to 6 hr after consumption of contaminated foods and last less than 24 hr. The symptoms of emetic type resemble those of *Staphylococcus aureus* food poisoning (FDA/CFSAN, 2005 and Johnson, 1984). The pathogenicity of *B. cereus* has been associated with ability to produce toxic metabolites during its growth.

Table 2: Characteristics of the two types of illness caused by *B. cereus*

Characteristics	Diarrheal syndrome	Emetic syndrome
Infective dose	10^5-10^7 (total)	10^5-10^8/g
Toxin production	Small intestine of host	Preformed in foods
Toxin types	Protein; enterotoxin(s)	Cyclic peptide; emetic toxin
Incubation time (h)	6-15 (periodically >24)	0.5-6
Illness duration (h)	12-24 (periodically several days)	6-24
Symptoms	Abdominal pain, watery diarrhea, occasionally nausea	Nausea, vomiting, malaise (sometimes followed by diarrhea due to production of enterotoxin)
Implicated foods	Meat products, soups, vegetables, puddings and sauces, milk and milk products	Fried and cooked rice, pasta, pastry, noodles

Turnbull (1981) extensively reviewed and elaborated the characteristics as well as pathogenicity of certain toxic metabolites of *B. cereus*. These included lethal toxin, hemolysins, phospholipases-C, loop fluid-inducing/skin test/necrotic toxin and emetic toxin. However, the literature differently characterizes the types of toxins. In general, the diarrheal type is caused by enterotoxin complexes and is sometimes referred to as diarrheal toxins, whereas the emetic type is caused by the emetic toxin. It has been shown that *B. cereus* produces at least five different proteins or enterotoxins e.g. Hemolysin bl (Hbl), Nonhemolytic enterotoxin (Nhe), Enterotoxin T (BceT), Enterotoxin FM (EntFM), and Cytotoxin (CytK). Three of these five enterotoxins (Hbl, Nhe, and CytK) are likely to be involved in food poisoning; the other two have not been recently reported.

Hemolysin bl is the first known three-component bacterial toxin and the best-characterized enterotoxin by far. The genes for all three components have been cloned and the sequences have been determined. The Hbl is composed of three components (a binding component, B [37.8 kDa], and two lytic components, L_1 and L_2 [38.5 and 43.2 kDa]). It has been shown that all three components are required for maximal toxic activities: hemolysis, cytotoxicity, enterotoxicity, necrosis in rabbit skin and vascular permeability as well as fluid accumulation in ligated rabbit ileal loops. Neither B and L_1 nor any of the components alone was hemolytic (Beecher and Macmillan, 1991). The mechanism and the interactions of Hbl components at the molecular level was studied by Beecher and Wong (1997), generally it is a binding of the protein B component to the cell surface before the L components cause cell lysis. In addition to Hbl, other hemolysins have been identified in *B. cereus* strains, but the Hbl is potent and therefore has been suggested as the primary virulence factor in diarrhea caused by *B. cereus*.

Lund and Granum (1996) characterized a Nonhemolytic enterotoxin (Nhe) complexes which is also a three-component enterotoxin complex consisting of three proteins of 39, 45, and 105 kDa. Although these three proteins are not identical to those of Hbl, the 39 kDa showed some similarity (70% identity) to the L_1 component of Hbl. Cytotoxin K (CytK), which has been recently characterized, is a one-component enterotoxin (34 kDa). The two three-component enterotoxins, Hbl and Nhe, are currently regarded as etiological causes of diarrheal food poisoning due to *B. cereus*.

The emetic toxin has been extensively studied after an investigation of using the HEp-2 cell assay showed a potential for toxin detection by Hughes et al., 1988. Prior to this assay the most reliable toxin assay was the oral challenge in primates. It was discovered that culture filtrates from an emetic type *B. cereus* isolate caused a vacuole formation in HEp-2 cells, the epithelial cell line, and correlated with the toxin activity. After the emetic toxin was purified, its structure was analyzed to reveal a ring structure, a cyclic dodecadepsipeptide, having a molecular weight of 1.2 kDa and named as cereulide by Agata et al. (1994). It is assumed that cereulide is more likely an enzymatically-synthesized peptide in the growth medium not a gene product like Hbl and Nhe which are transcribed from the *hbl* and *nhe* operon.

In addition to enterotoxin complexes and emetic toxin, phospholipase C or lecithinase is also considered to be a virulence factor of *B. cereus*. Three types of phospholipase C (phosphatidylinositol hydrolase, phophatidylcholine hydrolase and hemolytic sphingomyelinase) are produced with a different mechanism. It is suggested that phospholipase C influences the healing process by destroying the epithelium of the infected tissues and increasing the degradation of the subepithelial matrix (Kotiranta et al., 2000).

There are currently two commercial rapid methods marketed for the detection of *B. cereus* enterotoxins in food and food-related samples and enrichment cultures, Tecra® and Oxoid®. The TECRA *Bacillus* diarrheal enterotoxin (BDE) visual immunoassay (VIA) detects the NheA (45 kDa) component of the Nhe enterotoxin. The TECRA BDE VIA is an enzyme-linked immunosorbent assay (ELISA) performed in a "sandwich" configuration. If BDE antigens are present in the food sample, they are captured by the antibodies coated on the surface of microwells. The antigens are then bound to enzyme labeled antibodies (conjugate) specific for BDE. The presence of BDE is indicated when the bound conjugate converts substrate to a green color (Tecra International Pty Ltd, Australia). The BCET-RPLA toxin detection kit provided by Oxoid detects the presence of the HblC (L_2) component of the enterotoxin by reversed passive latex agglutination (RPLA). In a reversed agglutination assay, the antibody, which is attached to the particles (latex), reacts with the soluble antigen. The cross-linking of the latex particles by the specific antigen/antibody reaction results in the visible latex agglutination reaction (Oxoid Limited, United Kingdom).

In contrast to enterotoxins detection, a method to detect emetic toxin immunochemically is not yet currently available due to the low antigenicity of this small molecular weight toxin; however, bioassays by using HEp-2 cells assay have been developed, as previously mentioned. An improved quantitative analysis method to detect emetic toxin of *B. cereus* with vacuole response assay has been developed and applied to various food samples e.g. boiled rice, fried rice, noodle, pasta, bread, cake, mashed potato, egg and egg products, meat and meat products, milk and soybean curd (Mikami et al., 1994; and Agata et al., 2002).

Isolation and enumeration of *B. cereus* from food samples is normally performed by surface plating onto a selective medium such as mannitol egg-yolk polymyxin (MYP) agar. The presumptive colonies are pink, galaxy-liked colonies which do not ferment mannitol and produce lecithinase or phospholipase with a precipitation of egg yolk around its colony. The presumptive colonies are then transferred to nutrient agar slants, incubated 35° C for 24 hr, and inoculated into confirmation medium. Confirmation tests based on biochemical reactions e.g., anaerobic utilization of glucose, reduction of nitrate, production of acetylmethyl-carbinol, decomposition of lysine, resistance to lysozyme, and production of acid from mannitol are used to differentiate *B. cereus* from other *Bacillus* as shown in Table 3 (FDA/CFSAN, 2005).

Table 3. Differential characteristics of large-celled Group I *Bacillus* species

Feature	***B. cereus***	***B. thuringiensis***	***B. mycoides***	***B. anthracis***	***B. megaterium***
Gram reaction	+[(a)]	+	+	+	+
Catalase	+	+	+	+	+
Motility	+/-[(b)]	+/-	-[(c)]	-	+/-
Reduction of nitrate	+	+	+	+	-[(d)]
Tyrosine decomposed	+	+	+/-	-[(d)]	+/-
Lysozyme-resistant	+	+	+	+	-
Egg yolk reaction	+	+	+	+	-
Anaerobic utilization of glucose	+	+	+	+	-
VP reaction	+	+	+	+	-
Acid produced from mannitol	-	-	-	-	+
Hemolysis (sheep RBC)	+	+	+	-[(d)]	+
Known pathogenicity/characteristic	Produces enterotoxin	Endotoxin crystals pathogenic to insects	Rhizoidal growth	Pathogenic to animals and humans	

[a]+, 90-100% of strains positive.

[b]+/-, 50-50% of strains are positive.

[c]-, 90-100% of strains are negative.

[d]-, Most strains are negative.

2.2 High hydrostatic pressure processing

The use of high hydrostatic pressure in the field of food science and technology was first demonstrated by Hite in 1899 (Hite, 1899). He applied pressure to milk to destroy microorganisms and extend shelf-life. He demonstrated that after an application of a pressure treatment at 600 MPa for 1 hr at room temperature, milk shelf-life could be extended for 4 days. However, it took nearly a century before the first high-pressure processed food (jam) was introduced to the Japanese market in 1990 by the Meidi-ya Company. Since then, high hydrostatic pressure has been extensively researched in various aspects e.g. mechanism of microbial inactivation and enzyme inactivation to ultimately achieve high-quality food products. Examples of the commercial pressurized products currently available on the market are jam, fruit juices, yogurts, guacamole, salad dressing, ham, and seafood (Lado, 2002).

The reason high hydrostatic pressure has gained considerable interest is because of increasing demand for minimally processed, additive-free, shelf-stable products. High hydrostatic pressure has been shown to preserve the sensory and nutritional qualities of a food usually lost during traditional thermal treatments which eliminate spoilage microorganisms and pathogenic bacteria. High hydrostatic pressure is one of several existing alternatives to thermal treatments such as irradiation, pulsed electric fields, ultraviolet radiation, and high magnetic fields. It is sometime referred to as “cold-pasteurization”.

High hydrostatic pressure (HHP) also referred to as high pressure processing (HPP) or ultra high pressure (UHP) processing is defined as a process applied to liquid or solid foods, with or without packaging, with pressures between 100 and 800 MPa. Pressures used in commercial systems are usually between 400-700 MPa. Generally, pre-packaged food is placed into a cylindrical pressure vessel, which is later filled with a pressure transmitting fluid. The fluid is pressurized by an intensifier which uses liquid from the low-pressure pump to generate a high-pressure fluid for the system compression. Food is held for a desired period of time and temperature during a compression cycle. After the compression cycle is achieved, the vessel is

decompressed and the pressure released. The decompression cycle is accomplished within seconds and the food is cooled to its original temperature.

The temperature in commercial system during a pressure treatment can be programmed from below 0° C to above 100° C. The exposure time of food under pressure is limited from a millisecond to over 1200 seconds or 20 min, the practical exposure time is usually less than 20 min (Farkas and Hoover, 2001). The pressure transmitting medium is normally a low-compressibility liquid such as water rather than gases as liquid compression results only in small volume change comparing to gases, in addition, liquid posses less potential hazards when operating machines. The general schematic diagram for discontinuous equipment for high pressure processing of packaged food products is shown in Figure 1.

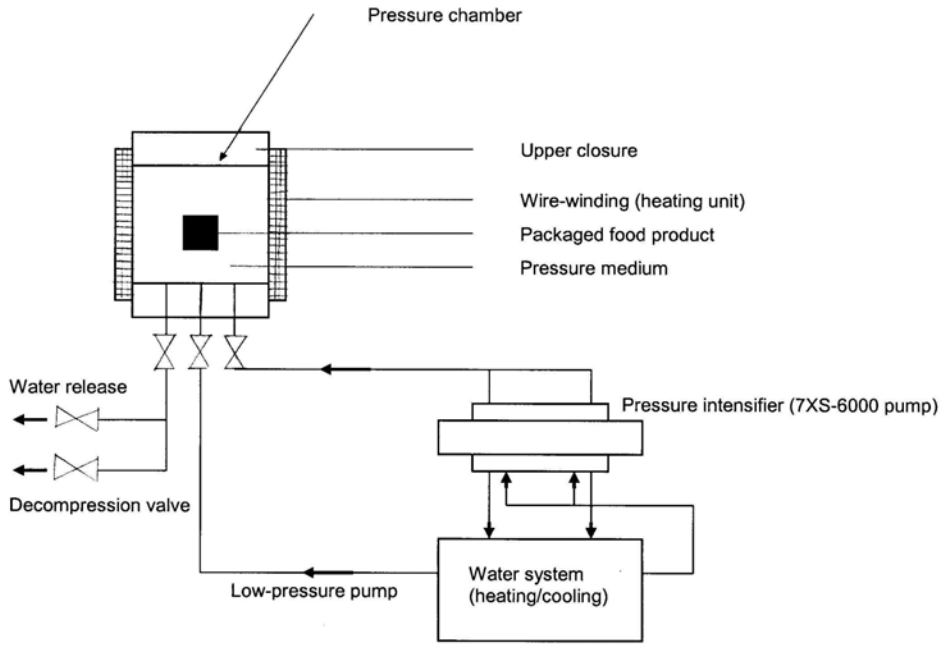

Figure 1: Schematic diagram of a discontinuous equipment for high pressure processing of packaged food products

2.2.1 Mechanisms of high hydrostatic pressure processing

The effects of high hydrostatic pressure are based on two important principles: Le Chatelier's principle and the isostatic principle. According to Le Chatelier's principle, high hydrostatic pressure affects any biochemical systems where a change in volume is involved and favors any system which results in a volume decrease (Tewari et al., 1999). Interactions (covalent bonds, hydrogen bonds, electrostatic and hydrophobic interactions) among biomolecules are modified by high pressure in one way or another depending on whether the formation of these interactions results in positive or negative changes (Yuste et al., 2001). It is generally accepted that high pressure has little effect on covalent bonds as only non-covalent interactions are disrupted. Therefore, food subjected to high pressure treatments near or at room temperature will not undergo significant chemical changes due to the pressure treatment itself (Farkas and Hoover, 2001). Low molecular weight molecules responsible for nutritional and sensory characteristics are not affected, thus, freshness and flavor do not markedly change and are retained. Also, with no breakage of covalent bonds, the development of flavors not naturally present in food products does not occur.

The other principle is the isostatic transmisssion, which indicates that the transmission of pressure is uniform and almost instantaneous independent of size, shape and composition of food. Therefore package size, shape and composition are not factors in process determination. Since high pressure processing is isostatic, food does not become deformed under such high pressure. The pressurization time is independent of sample volume in contrast to conventional thermal processing, once the pressure reaches the desired pressure the sample can be kept for an extended period of time with no additional energy required. High pressure can be applied at room temperature thus reducing the amount of thermal energy needed during conventional thermal treatment. Although high pressure processing is generally regarded as non-thermal treatment, the temperature of food will increase through adiabatic heating (an increase in temperature due to an increase in pressure) during compression. If water is the compression vehicle, an approximate 3° C per 100 MPa will occur depending on the composition of food, with a larger increase for fatty foods (Cheftel, 1995; Thakur and Nelson, 1998; Tewari et al., 1999; and Yuste et al., 2001).

2.2.2 Effects of high hydrostatic pressure on food and food components

Conventional (thermal) treatment and non-conventional (non-thermal e.g. high pressure) treatments have been implemented with an ultimate goal to achieve food safety and quality. However, high pressure processing has become increasingly more popular because it inactivates pathogenic and spoilage microorganisms in foods with minimal changes in texture, color, and flavor as compared to conventional technologies. Effects of high pressure on food and food components on microorganisms as well as its effects on modifying function have been studied and reviewed (Cheftel, 1995; Thakur and Nelson, 1998; and Knorr, 1999). Pressure effects on the composition of food i.e. water, proteins, enzymes, starch, and lipids are well described.

Physicochemical properties of water are modified under pressure e.g. a decrease in water volume, an increase in water temperature, ionic dissociation of water (a lowering in pH), and phase transition of water. Knorr (1999) stated that at about 1,000 MPa water freezes at room temperature, whereas the freezing point can be lowered to –22° C at 207.5 MPa. This allows for pressure shift freezing of foods with instant and small ice crystal formation, storage of food at subzero temperatures without ice formation, or fast thawing of frozen food by pressurization.

The effects of pressure on the structure and activity of proteins and enzymes depend on the kind of protein, processing condition, and the pressure applied. It is generally accepted that high pressure induces the breakage of ionic or salt bonds and part of hydrophobic interactions, whereas hydrogen bonds and covalent bonds are almost unaffected under pressure as mentioned earlier. Therefore the primary structure of proteins will remain intact during pressure treatments, secondary, tertiary and quarternary protein structures are significantly affected and modified as follows, pressure causes a dissociation of oligomeric structures into their subunits, a partial unfolding and denaturation of monomeric structures, protein aggregation and protein gelation. Pressures have been shown to coagulate and modify functional properties of proteins e.g. egg yolk, fish muscle proteins, rabbit meat paste, soy protein as a result of changes in their protein structures (Tewari et al., 1999).

The effects of high-pressure treatment on enzyme activity are different depending on the type of enzymes involved and other parameters of processing (the pressure level, the process time and temperature). At room temperature, pressure may cause partial, total enzyme inactivation, or enzyme activities enhancement, reversible or irreversible. Enhanced enzyme activities may be due to enzyme released from cellular components and closer contact with substrates. Inactivation of enzyme is probably due to intra-molecular structural changes by high pressure. In general, enzymes such as pectin methyl esterase, peroxidase, polyphenol oxidase or lipoxygenase are resistant to pressure and require higher pressures for inactivation.

Starches are reported to gelatinize with the use of high pressures which may increase resistance to enzyme digestion or increase susceptibility to enzymes depending on the processing condition. Lipids or triglycerides are crystallized under pressure which results in an increase in the melting temperature of lipids. Crystallization of phospholipids in cell membrane of microorganisms is thought to be one of the reasons of pressure inactivation which later will be discussed in detail (Cheftel, 1995; Thakur and Nelson, 1998; and Knorr, 1999).

2.2.3 Effects of high hydrostatic pressure on microorganisms (vegetative bacterial cells)

Resistance of microorganisms to pressure is extremely variable; in general, vegetative cells in the early growth phase or exponential phase are more sensitive than cells in stationary or dormant phase. Gram-positive bacteria are more resistant to pressure than Gram-negative bacteria, this may be linked to the rigidity of the teichoic acids in the peptidoglycan layer of the Gram-positive cell wall. Bacteria in small size and coccoid shape are generally more resistant to pressure than the larger rod-shaped ones. In most cases, at ambient temperatures it is necessary to apply pressures above 200 MPa in order to induce inactivation of vegetative microbial cells. Vegetative cells of yeast and molds are inactivated by pressure between 200 and 300 MPa. The degree of inactivation depends on several parameters such as the type of microorganisms, the pressure level, the process temperature and time, and the composition of the medium. In spite of a number of fundamental studies concerning the effects of pressure on the microstructure, the metabolism, and the genetic mechanisms of given microorganisms, the causes of microbial in activation are still poorly understood (Cheftel, 1995; and Smelt, 1998).

2.2.4 Mechanisms of microbial inactivation by high hydrostatic pressure

High hydrostatic pressure induces a number of changes in microorganisms. Hoover et al. (1989) described changes occurring in microorganisms as changes in morphology, biochemical reactions, genetic mechanisms, and cell membrane and cell wall. Morphological changes are observed at increasing pressures e.g. compression of gas vacuoles, cell lengthening, separation of cell membrane from cell wall, contraction of cell wall with formation of pores, release of intracellular constituents out of the cell. High pressure interrupts cellular functions responsible for reproduction and survival i.e. DNA replication and transcription, and protein synthesis are inhibited due to inactivation of enzymes involving in these processes and a reduction in the number of ribosomes (Lado and Yousef, 2002), however, the DNA structure is not affected because its structure is primarily hydrogen bonding.

The most important inhibitory effect of high pressure comes from cytoplasmic membrane damage. High pressure damages bacterial membranes and thus affects transport phenomena involved in nutrient uptake and disposal of cell waste material. High pressure also alters the permeability of cell membranes and makes cell membranes permeable as indicated by an increase in extracellular ATP concentration and intracellular DNA staining with propidium iodide. This damage reduces the proton gradient across the cell membrane, preventing cells from synthesizing ATP. Without energy normally supplied by ATP hydrolysis, active transport of protons out of the cell cannot take place and the cell dies due to acidification. Also, membrane-bound enzyme (F_0F_1 ATPase) is denatured or displaced with pressure, ATP synthesis and hydrolysis does not occur to support cell activities.

Membrane fluidity is believed to be involved in membrane damage as membrane lipid (lipid bilayers) undergoes phase transition under pressure from a liquid crystalline phase (conformationally disordered) to a gel phase (relatively ordered) accompanied by a change in thickness. The decrease in biomembrane fluidity as a result of phase transition may result in

breakage of the membrane and in denaturation of the membrane-bound proteins causing a functional disorder of these proteins (San Martin et al., 2002).

Lado and Yousef (2002) described structural and functional changes in microorganisms at different pressures. They stated that microbial growth is retarded at pressures in the range of 20-180 MPa, protein synthesis is also inhibited at these pressures range as a result of a reduction in the number of ribosomes. Cell viability begins to decrease at pressures about 180 MPa and the rate of inactivation increases exponentially as the pressure increases. Lethal pressure (above threshold of lethality) damages membrane integrity and denatures proteins. Above 300 MPa, proteins are denatured irreversibly and results in a leakage of cell content, at this pressure most vegetative cells and bacteriophages are inactivated.

2.2.5 Effects of high hydrostatic pressure on bacterial spores

Bacterial spores are very resistant to heat, radiation, chemical, and pressure. Bacterial spores of some species survive pressures above 1,000 MPa when the temperature does not exceed 45-75° C, whereas spores from yeasts and molds are easily inactivated at a pressure of 300 (*Aspergillus oryzae*) or 400 MPa (*Rhizopus javanicus*) at ambient temperature. The use of hydrostatic pressures above 800 MPa or heat in conjunction with pressure is required to effectively eliminate bacterial endospores (Farkas and Hoover, 2001). Cheftel (1995) also stated that significant bacterial spore inactivation cannot be obtained by pressure alone even with pressure cycling or at a temperature as low as –20° C. Although heated pressure and pressure cycling treatments have been reported to increase the effectiveness of high pressure for spore inactivation, the efficacy depends on initial temperature, adiabatic heating, pH, pressure level and processing time (Torres and Velazquez, 2005). However, it has been known that the resistance of bacterial spores is reduced when germination is induced to terminate spore dormancy. Therefore, recent studies have been primarily focused on inactivation of bacterial spores by either prior germination of spores with subsequent inactivation of all vegetative cells or with combined processes such as pressure with temperature and the use of additives (Cheftel 1995).

Spore germination is generally defined as the period beginning with the addition of a compound that can cause initiation of germination and continuing through a number of irreversible reactions e.g. cation release, dipicolinic acid (DPA) release, partial core hydration, small acid-soluble proteins (SASPs) degradation, cortex hydrolysis, further core hydration, core expansion, RNA, protein, and DNA synthesis (Paidhungat and Setlow, 2002). In the laboratory, germination is followed by; 1) analysis of a decrease in optical density at 600 nm of a spore population; 2) conversion of a dormant spore that appears white or bright in phase-contrast microscope to a phase gray or phase dark germinated spore; 3) excretion of dipicolinic acid (DPA); 4) release of spore cortex peptidoglycan fragments; 5) loss of spore resistance properties such as heat resistance; and 6) resumption of spore metabolism by monitoring the reduction of some redox dye (Paidhungat and Setlow, 2002).

Generally, spores are triggered to germinate by nutrients, a variety of small molecules will trigger spore germination, including amino acids, sugars, purine ribosides, and in some species, simple salts. The requirements for spore germination are generally species specific, but L-alanine and D-glucose are relatively general germinants. With *B. subtilis* spores, L-alanine is a good germinant, as is a mixture of L-asparagine, D-glucose, D-fructose, and potassium ions (defined as AGFK). For *B. cereus* spores, germination has an absolute requirement for glycine or a neutral L-amino acid and purine ribosides. Besides a variety of chemical stimuli such as nutrient initiators (L-alanine, purine ribosides, sugars), non-nutrient initiators (Ca-DPA), enzymatic initiators (lysozymes), pressure is also can be used as a germinant after a discovery of using high pressure to germinate *Bacillus* spores done by Clouston and Wills (1969).

Initiation of germination of spores by hydrostatic pressure was first demonstrated by Clouston and Wills (1969). When *Bacillus pumilus* spores were treated with pressure up to 170 MPa at 25° C, spores began to initiate germination when the pressure exceeded 50 MPa which was a prerequisite for inactivation by compression. The germination rate of *Bacillus spp*. spores increased with addition of some additives such as salts, amino acids primarily L-alanine, and glucose. Gould and Sale (1970) also used L-alanine in combination with pressure to study the initiation of spore germination. They stated that the germination rate of *B. cereus* and *B.*

coagulans was markedly increased from 50-64% to 95-99% when the spores were treated at a pressure of 25 MPa for 30 min at 50° C in the presence of L-alanine. From these early studies, high pressure has been shown to initiate germination of bacterial endospores at ambient temperature, thus allowing germinated spores to be inactivated by pressure, pressure in combination with heat, the presence of nisin, low pH, and oscillatory compression procedures. This concept has become widely acceptable and applied to high pressure treatments to eliminate spores of interest.

2.2.6 Mechanism of bacterial spore inactivation by high hydrostatic pressure

B. subtilis has been chosen as a surrogate for pathogenic strains of *Bacillus* to study on its resistance properties due to genetic and molecular genetic information and the genetic techniques on this bacterium. General features and factors identified as important in spore resistance are well conserved among spores of *Bacillus* species. Therefore, research is primarily performed on *B. subtilis* rather than a pathogen. The mechanisms of spores killed by heat, irradiation and chemical have been well studied and described. However, research on the mechanisms of spore killing by high pressure is still poorly understood (Setlow et al., 1997; Cabrera-Martinez et al., 2002; Melly et al., 2002; Setlow et al., 2002; and Young and Setlow, 2004). The mechanisms of spore germination induction by high pressure have been studied by several investigators (Wuytack et al., 1998; Wuytack et al., 2000; Paidunghat and Setlow, 2002; and Margosch et al., 2004. Wuytack et al. (1998) studied the germination of *B. subtilis* spores at low (100 MPa) and high pressure (600 MPa) and compared resistance properties of these pressure-germinated spores to spores germinated by nutrients in the nutrient-induced germination pathways (Ala or AGFK).

For *B. subtilis*, the two best-known germination pathways are those induced by the chemical effectors alanine (Ala) and the combination of asparagine (Asn), glucose (Glu), fructose (Fru), and potassium ions (AGFK). In both pathways, ungerminated spores have to undergo physicochemical changes i.e. hydration of spore core, release of dipicolinic acid (DPA) and Ca^{2+}, loss of optical density (OD), degradation of small acid-soluble spore protein (SASPs) and ATP synthesis corresponding to spore resistance properties i.e. an increase in temperature sensitivity,

a decrease in UV sensitivity and an increase in UV and hydrogen peroxide sensitivity respectively (Wuytack et al., 1998, and Wuytack et al., 2000).

Wuytack et al. (1998) found that germination of *B. subtilis* spores induced at low pressure (100 MPa) resulted in the loss of spore DPA, degradation of SASPs, and rapid generation of ATP. Spores germinated at high pressure (600 MPa) also caused a DPA release, however, the degradation of SASPs and ATP generation were not observed. They concluded that germinated spores induced at low pressure are more sensitive to pressure, UV light, and hydrogen peroxide than those germinated at high pressure (600 MPa) because high pressure causes an incomplete germination process. This incomplete germination process explains why inactivation of *Bacillus spp*. spores is more efficient at moderate pressures (200 to 500 MPa) than at higher (>500 MPa) pressures.

Wuytack et al. (2000) and Paidunghat and Setlow (2002) investigated the pathways of pressure-induced germination at low (100 MPa) and high pressures (500 and 600 MPa) of *B. subtilis* and their relationship to the pathways of nutrient-induced germination. They concluded that a pressure of 100 MPa induces spore germination by activating the germinant receptors. Germination at high pressures involves activation of a true physiological germination pathway by opening channels for release of dipicolinic acid from the spore core, which leads to the later steps in spore germination. Margosch et al. (2004) experimented on spores of *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* with pressures and temperatures (200 to 800 MPa and 60 to 80° C) in mashed carrots. Their results suggested that at a pressure between 600 and 800 MPa and a temperature greater than 60° C, DPA is released predominantly by a physicochemical rather than a physiological process from spores and resulted in DPA-free, phase-bright spores. These DPA-free spores are inactivated by moderate heat independent of the pressure level.

2.3 High hydrostatic pressure and *Bacillus cereus* spores

It is well known that dormant spores have no metabolic activities and exhibit considerable resistance to heat, chemical and radiation due to many intrinsic factors with the importance of any particular factor varying depending on the specific inactivating treatment. Setlow (1994)

described mechanisms which contribute to the long-term survival and resistance to chemical, radiation, and heat of spores of *Bacillus* species. In general, parameters contributing to spore resistance are as follows: 1) genetic makeup of the sporulating species; 2) sporulation conditions; 3) the thick spore coat structure; 4) the low permeability of the spore core; 5) the spore core's low water content; 6) high levels of spore mineral content; 7) DNA binding proteins, the α/β-type small, acid soluble protein (SASP); and 8) repair of DNA damage (Nicholson et al., 2000).

Spore killing by various physical and chemical treatments has been studied and the following mechanisms have been identified: 1) DNA damage; 2) alteration in the spore's inner membrane; 3) disruption of the spore's inner membrane and release of the spore's core contents damage; and 4) abolishing of germination step, however, the mechanisms vary largely depending on the treatment (Setlow and Setlow, 1993; Popham et al., 1995; Setlow and Setlow, 1995; Xue and Nicholson, 1996; and Riesenman and Nicholson, 2000).

The destruction of spores by high pressure is achieved through stimulation of spore germination, followed by the destruction of germinated spores as described earlier. Since the important step is the germination step, factors affecting spore germination by pressure as well as nutrients, Ca^{2+}-dipicolinic acid and cationic surfactants are focuses of current researches. Factors included are: 1) the level of receptors for nutrient germinant in the dormant spore's inner membrane; 2) individual nutrient germinant receptors; 3) covalent lipid addition to nutrient germinant receptors 4) the absence of lytic enzymes; 5) the absence of DPA; 6) the sporulation temperatures; 7) levels of unsaturated fatty acids in the spore's inner membrane; 8) pretreatment of spores with oxidizing agents; and 9) inhibitors of spore germination with nutrients (Setlow, 2005).

Few studies have been performed specifically upon *B. cereus* in investigating factors affecting spore inactivation with high hydrostatic pressure (Raso et al., 1998a and 1998b; Shearer et al., 2000; Prestamo et al., 2001; McClements et al., 2001; Fujii et al., 2002; Oh and Moon, 2003; and Opstal et al., 2004). Raso et al. (1998a and 1998b) studied environmental factors (sporulation temperatures, pH, a_w, l-alanine, fat concentration) affecting initiation of germination and inactivation of *B. cereus* ATCC 14579 spores by high hydrostatic pressure. Raso et al. (1998a) investigated the influence of pressure, time and temperature on the initiation of germination and

inactivation of *B. cereus* spores in phosphate buffer sporulated at different sporulation temperatures (20, 30, and 37° C). They also examined the relationship between sporulation temperatures and heat resistance and found that spores sporulated at 37° C were the most heat-resistant than spores sporulated at 20 or 30° C. To determine the relationship between sporulation temperatures and pressure resistance, spores sporulated at different temperatures were treated with high pressure at different levels (85 to 690 MPa) at different processing temperatures (25, 40, and 60° C) for 15 min.

Spore resistance to pressure did not correlate with resistance to heat, in fact, it was found that spores produced at 20° C were more resistant to germination and inactivation by high pressure than spores produced at 30 and 37° C. Spores were more sensitive to pressure at higher treatment temperatures. At 25° C, there was an optimum pressure for the initiation of germination, which was about 250 MPa for all three suspensions. However, at higher temperatures, an increase of pressure up to 690 MPa caused progressively more germination. A treatment of 690 MPa of 15 min at 60° C allowed the germination of 6 log cycles of *B. cereus* spores sporulated at 20° C and more than 8 log cycles of *B. cereus* spores sporulated at 30 and 37° C. The same treatment inactivated 4, 6, and 7 log cycles of the population of *B. cereus* sporulated at 20, 30, and 37° C, respectively.

Raso et al. (1998b) further studied other environmental factors (pH, a_w, L-alanine and fat concentration) on the initiation of germination and inactivation by high hydrostatic pressure of *B. cereus* sporulated at 20, 30 and 37° C. Based on the first study, the results showed the optimum pressure, temperature and time for initiation of germination and germination for *B. cereus* were 250 MPa at 25° C for 15 min and 690 MPa at 40° C for 2 min, therefore, in this study these conditions were used throughout. It was found that high pressure initiated the germination of *B. cereus* even at low pH. The optimum pH for germination depended on the sporulation and treatment temperatures. The optimum pH for germinating *B. cereus* sporulated at 30 and 37° C was similar to the optimum pH for germination at ambient pressure which is around neutral pH. The pH barely affected the germination of *B. cereus* sporulated at 20° C. That is, at 250 MPa

and 25° C, the extent of germination and inactivation increased with higher pH for spores sporulated at three different temperatures. At 690 MPa and 40° C, the pH affected the germination of spores sporulated at 20° C (3 log cycles), but the inactivation increased as the pH of the medium was lowered. At this treatment (690 MPa), spores sporulated at 30 and 37° C were germinated by 6-7 log cycles around neutral pH and were optimally inactivated at pH 6.

Water activity was also found to influence the initiation of germination and inactivation. At lower water activities, spores were more resistant to the initiation and germination and inactivation by high pressure, and the differences in sensitivities of the three suspensions became smaller. At a_w 0.92, no germination was detected at 250 MPa, only 1, 1, and 2 log cycles of spores sporulated at 20, 30, and 37° C were germinated respectively at 690 MPa. No inactivation at any of the treatments was observed. The presence of L-alanine in the pressurization medium increased germination at 250 MPa, but not at 690 MPa. A combination of 250 MPa at 25° C with L-alanine provided an additive response. The fat concentration of milk did not affect the initiation of germination and inactivation by high pressure i.e. *B. cereus* was not protected from high pressure by milk at any fat concentration, the extent of germination and inactivation was approximately the same as in a buffer of the same pH.

Shearer et al. (2000) evaluated the inhibitory effects of chemical preservatives (sucrose esters of fatty acids i.e. sucrose laurates, sucrose palmitate, sucrose stearates, and monoglycerides i.e. monolaurin or lauricidin) in combination with high hydrostatic pressure and mild heat on *Bacillus* spores in food model systems. The model systems under investigation were *B. subtilis* 168 in milk, *B. cereus* 14579 in beef in gravy food, *B. coagulans* 7050 in tomato juice, *Alicyclobacillus* sp. N1089 in tomato juice and *Alicyclobacillus* sp. N1098 in apple juice. It was found that *B. cereus* 14579 was less resistant to sucrose laurate than *B. subtilis* 168 and 6051. The contribution of pressure to the inhibition and inactivation of *B. cereus* was greater than that provided by sucrose laurate alone or sucrose laurate with mild heat, neither of which had an effect on spore counts. When sucrose laurate was used in conjunction with pressure and mild heat, an additional 1 log reduction was observed as compared to the use of pressure and mild heat alone. The combined treatment of sucrose laurate (< 1.0%), 392 MPa at 45° C for 10 to 15

min provided 3 to 5.5 log reductions from an initial population of 10^6 cfu/ml for *B. subtilis* 168 in milk, *B. cereus* 14579 in beef in gravy food, *B. coagulans* 7050 in tomato juice, *Alicyclobacillus* sp. N1089 in tomato juice and *Alicyclobacillus* sp. N1098 in apple juice.

Prestamo et al. (2001) studied the effect of high pressure on *B. cereus* vegetative cells and peroxidase in apples and pressures > 400 MPa, without any other combined treatment. The results showed the tailing effect of pressure when pressures of 200, 300 350 and 400 MPa were applied for 30 min at 20° C, the microbial population decreased as the pressure increased, but a pressure of 400 MPa for 30 min was insufficient to completely inactivate all *B. cereus*. When pressures of 600, 800 and 1,000 MPa were applied for 15 min, the population continued to decrease; however, the pressure of 1,000 MPa for 15 min was unable to inactivate *B. cereus*. The behavior of *B. cereus* with regard to pressure and time (400 MPa for 25, 30, and 60 min) was also analyzed. It was observed the microbial population decreased and significant differences were found on the control in comparison with the sample treated at 400 MPa for 15, 30, and 60 min. However, no significant differences were found between treated samples.

McClements et al. (2001) determined the effect of growth temperatures (8 and 30° C), growth stage (exponential and stationary phase) on pressure resistance, variation in pressure resistance between different strains of the same organism, and survival and recovery of three psychrotrophic organisms *L. monocytogenes*, *B. cereus* NCFB 578 and 1031 (BC1 and BC2) and *P. fluorescens* in UHT skimmed milk. The results indicated growth temperature had a significant effect at the two growth stages studied. Exponential cells grown at 8° C were more resistant than those grown at 30° C, but for stationary-phase cells the reverse was true. *B. cereus* stationary-phase cells grown at 30° C were found to be the most pressure-resistant vegetative cells studied with 3.44 and 2.90 log reductions observed for BC1 and BC2 respectively at 400 MPa at 30° C for 18 min. *L. monocytogenes* showed the most sublethal damage compared to *B. cereus* and *P. fluorescens* (*B. cereus* and *P. fluorescens* showed a lower degree of pressure injury with a 0.5 and 1.0 log difference observed between the counts on the nonselective and selective agars). *B. cereus* spores were more resistant to pressure than its vegetative cells. A pressure treatment at 8° C did not inactivate the spores of either BC1 or BC2. The greatest

reduction observed at a pressure treatment of 400 MPa for 25 min at 30° C was a 0.45-log inactivation. Pressure at 8° C induced significantly less spore germination than at 30° C. A process of 400 MPa for 30 min at 8° C induced 13.4 and 8.3% germination for BC1 and BC2, respectively, compared to 75.8 and 18.7% observed at 30° C for 30 min.

Fujii et al. (2002) investigated the high-pressure inactivation of *B. cereus* IAM 12605 spores in water with the presence of argon. The spore suspension containing dissolved argon was treated under high pressure of 500 and 600 MPa at 20, 30, and 40° C for 30 min. No inactivation was observed at 20° C after pressurization at 600 MPa for 30 min, but there was a 2 log reduction of spore numbers at 40° C. At the pressure of 600 MPa, addition of argon accelerated the inactivation of spores at 20° C, but had no effect on the inactivation at 40° C. That is, the argon-containing sample showed a 1 log reduction after pressurization for 30 min at 20° C, whereas both the argon-free and argon-containing samples showed the same a 2 log reduction after pressurization for 30 min at 40° C.

Oh and Moon (2003) and Moon and Oh (2001) investigated the effects of pH in sporulation medium (6, 7 and 8) and pH of suspension medium (4.5, 6, 7, and 8) on the initiation of germination and on the inactivation of *B. cereus* KCTC 1012 spores with high pressures (0.1, 150, 300, 450 and 600 MPa) at different temperatures (20, 40 and 60° C) for 15 min. They found that inactivation of *B. cereus* spores with high pressure was affected more by sporulation medium pH than by suspension medium pH. Spores of *B. cereus* obtained through sporulation at pH 6.0 appeared to be more resistant to inactivation by high pressure (≥ 300 MPa) at 20, 40, and 60° C than did those obtained through sporulation at pH 7.0 and 8.0. For *B. cereus* spores obtained through sporulation at pH of 6 and 7, reduction greater than 1.5 logs were observed at pressures greater than 300 MPa at 40 and 60 °C processing temperatures. When the processing temperature increased from 20 to 40° C, the effect of pH on the inactivation was significantly increased, apparently greatest when pressure treatment was constant at 40° C. Regardless of sporulation pH, the initiation of *B. cereus* spore germination appeared to be more sensitive to pressures around 300 MPa at 20° C. However, increasing processing temperatures (40 and 60° C) enhanced the effect of sporulation medium on the inactivation of the spores.

Opstal et al. (2004) studied the germination and inactivation of *B. cereus* spores by mild high hydrostatic pressure and heat treatments. They reported the germination of *B. cereus* LMG6910 spores treated at 0, 100, 300, and 600 MPa at 40° C for 30 min were 1.5 to 3 logs higher in milk than potassium phosphate buffer. Almost the entire spore population (6 logs) germinated irrespective of the pressure level, while in buffer; germination was incomplete but increased with pressure from 2.5 logs at 100 MPa to 4 logs at 600 MPa. Spore inactivation also occurred as a result of pressure treatment. The level of inactivation increased with the pressure applied, but were always lower than the levels of germination, indicating that not all of the germinated spores could be inactivated by pressure even at 600 MPa.

Opstal et al. (2004) also investigated germination and inactivation of *B. cereus* spores at different pressures (100, 200, 300, 400, 500 and 600 MPa) and temperatures (30, 40, 45, 50 and 60° C) for 30 min. Lower treatment pressures or temperatures resulted in considerably less germination, and higher pressures and temperatures further increased germination, but a small fraction of spores always remained ungerminated. For all pressures, the levels of germination increased from 100 to 200 MPa, but remained relatively constant at pressures >200 MPa. The germination levels increased strongly from 30 to 40° C, but much less or not at all at >40° C. All treatments at ≥ 200 MPa and ≥ 40° C induced 6 to 8 logs germination, while pressure treatments at 30° C induced only 3 to 5 logs germination. The inactivation levels increased with temperatures at all pressures. The inactivation level also increased with pressure with some exceptions. However, a ≥ 6 log inactivation was achieved by a treatment at 60° C and ≥ 400 MPa. Treatments that allow a 6 log inactivation of *B. cereus* spores in milk were identified. It was found that the mildest treatment (200 MPa at 40° C for 30 min) resulting in a 6-log germination could be achieved by inducing a 6 log germination by high pressure first, and applying a mild heat treatment to kill the germinated spores subsequently.

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CHAPTER III

MICROBIOLOGICAL, PHYSICAL AND SENSORY QUALITY OF VACUUM-PACKAGED FRESH BLUE CRAB MEAT (*Callinectes sapidus*) TREATED WITH HIGH HYDROSTATIC PRESSURE

Abstract

Vacuum-packaged fresh lump blue crab meat (*Callinectes sapidus*) was pressurized at 300 and 550 MPa for 5 min at 25° C and evaluated for changes in microbiological, physical, and sensory qualities after pressure treatments and during storage (4° C for 31 days). A pressure of 300 MPa caused a 1 log reduction in total aerobic plate count and a 3 day lag period, whereas 550 MPa inactivated 2 logs in total aerobic plate count with no evident lag phase. Physical and sensory qualities of pressurized crab meat were not statistically different from the untreated crab meat ($P>0.05$). High hydrostatic pressure treatments killed or inactivated pressure-sensitive microflora in the fresh crab meat resulting in the following surviving microorganisms: *Aerococcus viridans, Brevibacillus agri, Brevibacterium iodinum, Brevibacterium linens, Brevibacterium casei, Brevibacterium epidermidis, Enterococcus mundtii, Enterococcus avium, Enterococcus solitarius,* and *Macrococcus (Staphylococcus) caseolyticus*. Under reduced oxygen and low temperature storage condition, sensory evaluations along with the identification of predominant organisms in fresh and pressurized crab meat (550 MPa) were conducted to determine shelf-life as well as to identify dominant spoilage microorganisms. A pressure of 300 MPa extended shelf-life of fresh crab meat from 17 to over 24 days with the prevalence of *Carnobacterium piscicola*. Crab meat treated with 550 MPa was not rejected by sensory panels at day 31; *Enterococcus spp*. was identified as the predominant microorganism suggesting that this organism could have an inhibitory effect to the other microflora.

Keywords: blue crab meat, *Callinectes sapidus*, high hydrostatic pressure, microflora

1. Introduction

Blue crab (*Callinectes sapidus*) is processed and marketed in a variety of forms including frozen, canned, pasteurized, and fresh. Fresh lump crab meat, distributed under refrigerated conditions, is highly perishable with a short shelf-life of 10-14 days or sooner depending on the microbiological quality during processing and subsequent storage (Ward et al., 1977). The blue crab industry has used several post-processing procedures, for example, pasteurization, sterilization or freezing in order to extend the shelf life of fresh meat. However, it is reported these processes cause undesirable changes in flavor, color and texture during processing and storage (Henry et al., 1995a and 1995b).

Fresh and minimally processed food products have recently been in demand and are the preference of consumers who have continually driven the food industry to generate new processing and preservation techniques that would not dramatically change the sensory and nutrition attributes of the products. Among novel approaches, high hydrostatic pressure processing has gained considerable interest with its advantages over conventional thermal treatments due to its inactivation of spoilage and pathogenic microorganisms as well as a shelf-life extension while maintaining the nutritional values.

Quality and safety of several species of seafood processed with high pressure (i.e. bluefish, carp, cod, octopus, oysters, pacific mackerel, prawn, and salmon) have been investigated (Murchie et al., 2005); however, a pressure-treated fresh blue crab meat has not been sufficiently studied. With the exception pressurization processes, the microflora and microbiological safety and quality of blue crab meat derived from current commercial processes have been studied (Ward et al., 1977; Rawles et al., 1995a; Rawles et al., 1995b; Harrison et al., 1996; and Reinhard et al., 1996; and Gates et al., 1996). In general, blue crab meat does not contain significant quantities of pathogenic microorganisms if good manufacturing practices are employed. However, changes in the microflora content of hydrostatic pressure treated product have not been adequately studied to determine how safety may be affected. There is a concern that inactivation of certain normal microflora may facilitate the growth of pathogenic microorganisms thereby creating a potential health hazard.

Since minimal information is available on the microflora and quality of pressure-treated crab meat, the objectives of this study were 1) to investigate the effect of pressure treatments on the physical, and sensory properties of fresh hand-picked blue crab meat; 2) to investigate microbiological changes including identification of surviving microorganisms after pressure treatments and subsequent storage; and 3) to determine if shelf-life could be extended after the application of pressurization processes.

2. Materials and methods

2.1 Crab meat

Fresh hand-picked blue crab lump meat (*Callinectes sapidus*) in 454 g containers was obtained from a commercial facility (Graham and Rollins Inc.) in Hampton, Virginia. Within 24 hours post-processing, the meat was transferred to the Virginia Tech campus, Blacksburg, VA in insulated containers with ice packs and stored in ice in a refrigerator at 0-4° C after arrival. All samples were used within 48 hr and the meat was mixed thoroughly prior to the high pressure treatments.

2.2 High hydrostatic pressure treatment

High hydrostatic pressure treatments were performed in a commercial scale high pressure apparatus (The Quintus Food Press QFP 35L-600, Avure Technologies., Kent, WA). Pressure and temperature were controlled by a pressure transducer and a thermocouple interfaced with the pressure unit. Water was used as the pressure transmitting medium. Samples of 15 g of lump blue crab meat were double-bagged and vacuum-packaged in 3-mm nylon/polyethylene vacuum pouches (Koch, North Kansas, MO) and then subjected to 300 and 550 MPa with an end temperature of 25° C for 5 min. Decompression occurred within 2-3 s.

2.3 Product storage and sampling

After application of the pressure treatments, all samples were stored under refrigeration temperature (4° C) for 31 days and sampled on days 0, 3, 7, 12, 17, 24, and 31 for aerobic and anaerobic microorganism enumeration, isolation, and identification; sensory analyses; physical analyses; pH and color measurements.

2.4 Aerobic microorganism enumeration and isolation

A 10 g sample of crab meat was transferred to a 90 ml 0.1% peptone dilution blank and homogenized with a Stomacher (Stomacher Lab Blender 400, Tekmar Co., Cincinnati, OH). From this dilution, subsequent dilutions were made using 9 ml peptone dilution blanks. Aliquots of 0.1 ml were spread on the surface of previously prepared Trypticase Soy Agar (TSA; Becton Dickinson and Company, Sparks, MD) plates. The plates were incubated at 30° C and enumerated after 48 hr of incubation.

Predominant well-isolated colonies were picked, quadrant streaked on TSA plates, and incubated at 28° C for 24 hr. After 24 hr of incubation, the cultures on TSA plates were examined for purity under a dissecting microscope. To obtain pure cultures, a 4 mm loop was used to harvest bacterial cells from the third quadrant of the quadrant streaked plate and transformed to a clean screw-capped tube. The tube was closed with Teflon-lined screw caps, and kept frozen until identification. For mixed cultures, the microorganisms were restreaked until pure cultures were obtained.

2.5 Anaerobic microorganism enumeration and isolation

All anaerobic culture and identification methods were in accordance with procedures outlined by Holdeman et al. (1977). A 10 g sample of crab meat was transferred to a 90 ml 0.1% peptone dilution blank and homogenized using a Stomacher. From this dilution, subsequent dilutions were made using pre-reduced 9.9 ml peptone dilution blanks. Inoculations from the appropriate blanks were placed into pre-reduced Brain Heart Infusion Agar (BHIA; Becton Dickinson and

Company, Sparks, MD) tubes. All serial dilutions and inoculations were performed under a stream of oxygen-free CO_2. The BHIA tubes were then placed on horizontal spinners until the medium was solidified. The tubes were then removed and incubated at 30° C for 5 days. After incubation, the tubes were spirally marked with pen on the streaker unit of the VPI Anaerobic Culture System. Colonies were counted with the use of a dissecting microscope.

Predominant well-isolated colonies were picked from countable tubes and transferred to pre-reduced Cooked Meat (CM; Becton Dickinson and Company, Sparks, MD) Medium, which served as the maintenance medium, and grown at 30° C for 24 hr. The isolates were then anaerobically streaked onto spun out BHIA roll tubes for purity examination after 24 hr of incubation at 30° C. A preliminary study showed no strict anaerobic microorganisms were present and as a result, all cultures in anaerobic Cooked Meat Medium were grown on Trypticase Soy Agar (TSA) plates for identification purposes. Tubes with one morphological type were streaked onto TSA plates and harvested after 24 hr at 28° C into tubes and capped with Teflon-lined screw caps for subsequent identification. Tubes with more than one morphological type were restreaked until a pure culture was obtained.

2.6 Aerobic and anaerobic microorganism identification: cellular fatty acid analyses

All aerobic and anaerobic identification was performed using the Sherlock Microbial Identification System (MIS, Microbial ID Inc., Newark, DE) in which cellular fatty acid profile is used for identification. The procedure used for sample preparation was adapted from the MIS protocol. Cells that have been frozen were thawed prior to cellular fatty acid analysis. The cells were lysed and saponified with 1.0 ml of basic methanol (45 g of NaOH, 150 ml of methanol, 150 ml of deionized water), heated in a boiling water bath for 5 min, mixed with a vortex mixer, and heated in the boiling water bath for an additional 25 min. To methylate cell constituents, 1 ml of HCl-methanol (325 ml of 6.0 N HCl, 275 ml of methanol) and 1 ml of sulfuric acid-methanol (162.5 ml of H_2SO_4 added to 162.5 ml of deionized water, 275 ml of methanol) were added, and the solution was heated at 80° C for 10 min. After rapid cooling, the methylated components were extracted by adding 1.25 ml of hexane-ether (200 ml of hexane, 200 ml of methyl-tert-butyl ether) and turning the tube end over end for 10 min. Each extract was washed

once with 3 ml of a solution containing 5.4 g of NaOH in 450 ml of deionized distilled water saturated with NaCl.

A 2 μl portion of the washed extract was chromatographed on an Ultra 2 column-a 25 m×0.2 mm ID×0.33 μm film thickness phenyl methyl silicone fused silica capillary column (Agilent, Newark, DE) with a model HP-5890A gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a model HP 6763 autosampler (Hewlett-Packard), a flame ionization detector and a model HP-3392A integrator (Hewlett-Packard). The gas flow rates were 400 ml/min for air, 30 ml/min for hydrogen, and 30 ml/min for nitrogen. The temperatures used were 250° C for the injection port and 300° C for the detector. After injection, the oven temperature was ramped from 170 to 270° C at a rate of 5 C°/min and then from 270 to 310° C at a rate of 30 C°/min, held at 310° C for 2 min, and then returned to 170° C before the next sample was injected. A standard mixture containing known fatty acids was chromatographed at the beginning of each day and after each set of 10 samples.

The MIS software package was used to identify the peaks and to determine the area, the ratio of area to height, the equivalent chain length, the total area, and the total area for named or listed compounds. The MIS software package also was used to calculate the percentage of area for each named or listed compound compared with the total area of the compound detected. Compounds were identified by using the TSBA Version 4.0 Library.

2.7 Objective measurements/ physical analyses

The objective measurements of texture was obtained by analysis of resistance to shear force conducted on an Inston Universal Testing Machine Model 3365 (Instron Corp., Canton, MA) device equipped with a Kramer shear cell. The Kramer shear cell is a multi-bladed fixture, the upper part or blade holder contains ten 3.0 mm thick and 70 mm wide blades and the lower part is a sample compartment which has guides to align the blades to slots in the bottom. The shear cell was mounted to the Instron load frame with an adapter. The cell traveled at the crosshead speed of 100 mm/min for the 44 mm distance. The constant distance of 44 mm was

predetermined as the point the blades reached the bottom slots and the sample was sheared. This fixed value of distance was set as end of test and referred to as break for all samples measured.

Weighed portions (15-20 g or 4-5 lumps) of blue crab meat were mounted in the holding compartment of the cell with muscle fibers aligned perpendicular to the plane of shear plates. As the blades of the Kramer shear cell moved down, the sample was compressed, deformed and sheared. The force-deformation curve (Fig 1) between compressive load (force, N) and compressive extension (distance, mm) was generated by the Bluehill® software (Instron Corp., Canton, MA). Also, the compressive load at maximum compression load (the maximum peak force, N), compressive load at break (force at break, N), and energy at break (total energy which is area under the curve, J) was recorded. The peak force (N), force at break (N), and energy at break (J) were normalized by dividing these values by the corresponding weight, which were resulted in units of peak force per gram (N g^{-1}), force at break per gram (N g^{-1}), and energy at break per gram (J g^{-1}). Results are reported as a mean of 10 samples per replication and triplicate analyses.

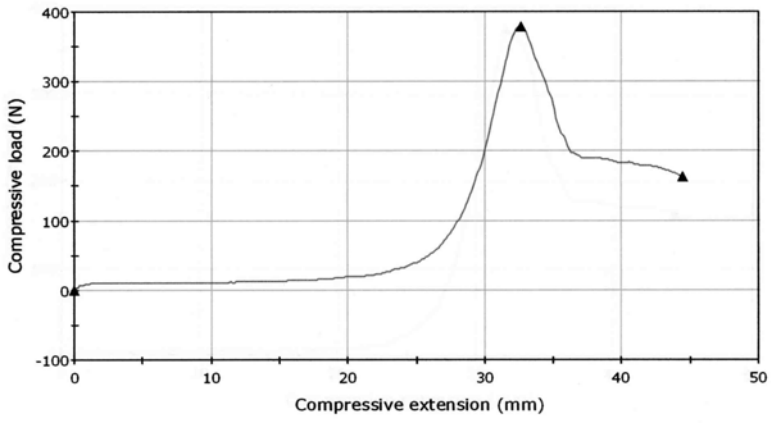


Fig 1: Force-deformation curve between compressive load and compressive extension

2.8 Subjective measurement /sensory analyses

A triangle test was selected to determine whether an overall difference existed resulted from using high hydrostatic pressure to process the crab meat. Crab meat pressure processed at 300 and 500 MPa was compared with non high pressure processed crab meat. Three sets of triangle tests were performed by each panelist, the three triangle tests were 300 MPa and control, 500 MPa and control, and 300 MPa and 500 MPa. The subjective measurements were performed by 23 experienced panel members consisting of faculty, staff, graduate and undergraduate students from the Department of Food Science and Technology at Virginia Tech.

Samples were prepared by placing 1-2 lumps (about 10 g) of crab meat into 1 oz plastic soufflé cups (Solo Cup Company, Urbana, IL) coded with randomly selected three-digit numbers. All samples were kept under refrigeration temperature (4° C) until served. Each panelist was presented with 3 sets of triangle test simultaneously; each test consisted of 3 coded samples in random order such that each panelist evaluated samples in a different order. Panelists were asked to evaluate samples in a specified order and requested to identify the odd sample.

2.9 Colorimetric measurements

Colorimetric measurements on the absolute CIE L^*, a^*, b^* values were obtained with a Minolta Chroma Meter Model CR-200 (Minolta, Ramsey, NJ) using CIE illuminant C and D65 as a light source. A 15 g crab meat sample packaged in polyethylene bags was measured with the chroma meter calibrated with a standard white plate CR-A43 (Minolta). L^*, a^*, and b^* values (L^* = lightness, a^* = redness, and b^* = yellowness) were recorded. All analyses were performed in triplicate.

2.10 Statistical analyses

Data were analyzed using the General Linear Model (GLM) procedure of SAS (V. 8.02, Statistical Analysis Systems Institute, Inc. 2002). The complete randomized factorial design was

utilized to test the effects of two variables (pressure treatments and storage days) and their interactions on color characteristics (CIE L^*, a^*, b^*) and pH. The same experimental design was also used to test the effect of pressure treatments on textural parameters (compressive load at break, compressive load at maximum compression load, and energy at break). All the tests used 10 samples for each treatment except for pH (4 samples). If significant differences were found, means were separated by a Least Significant Difference test.

3. Results

3.1 Microbiological changes

Crab meat used throughout this study was processed with a scheduled thermal process which consisted of cooking live crabs under pressure (10 min at 250° F) to facilitate the removal of meat from the bodies and claws, cooling to ambient temperature, and subsequent storing under refrigeration temperature overnight prior to hand picking. Hand-picked crab meat was then packaged into containers for retail and institutional sale and then stored packed in ice. This hand-picked meat with no subsequent processing is referred to as fresh crab meat.

Preliminary studies were performed to study the effects of different pressure levels, processing temperatures, and processing times on Aerobic Plate Counts (APC) and Anaerobic Counts. Pressures of 100 and 300 MPa were chosen for the starting temperatures of 25 and 50 ° C for 15 min with a storage temperature of 4° C for 31 days (Appendix 1 and 2). The results showed that a low pressure of 100 MPa had an insignificant effect on reducing the total aerobic and anaerobic plate counts when compared to the control at both temperatures. A pressure of 300 MPa had more pronounced effect than the 100 MPa pressure of at both processing temperatures.

The 50° C process temperature was later found to be unacceptable due to potential metal fatigue of the pressure chamber and was eliminated from the study. The processing temperature was then decreased from 50 to 40° C and the starting temperatures at different pressures was calculated to account for the adiabatic temperature rises during compression in order to achieve the same end temperatures for all treatments. Fresh crab meat was processed with two end processing temperatures (25 and 40° C) at six different pressure levels for 15 min (Appendix 3). The end temperatures did not have a significant difference in a reduction of the aerobic plate counts. Again, a low pressure of 100 MPa showed a relatively minimal effect on aerobic plate counts, whereas medium pressures (200 and 300 MPa) and high pressures (400 to 550 MPa) resulted in an approximate 1 and 2 log reduction in aerobic microorganisms respectively. These results influenced the last study on the effects of processing or holding times which is considered to be one of the important factors in high pressure processing. The processing time was varied

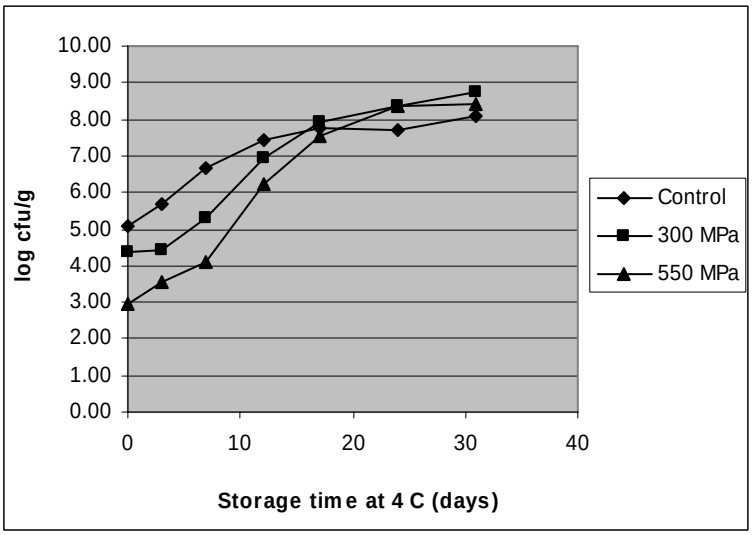
from 5 to 10 and 15 min at medium and high pressures (300 and 550 MPa) at 25° C (Appendix 4). The results showed a 5 min pressurization process was as effective as a 10 or 15 min in the reduction of aerobic organisms. From these preliminary results, pressures of 300 and 550 MPa, a processing time of 5 min with an end temperature of 25° C was used throughout the study.

Microbiological quality of fresh blue crab meat after the cooking process should approach sterility, however, bacteria are often reintroduced into the meat by subsequent processes resulting in high initial levels of aerobic organisms, in this case, 1.3×10^5 cfu/g as shown in Fig 2a. When fresh crab meat was treated with high hydrostatic pressures at 300 and 550 MPa for 15 min at 25° C, the aerobic and anaerobic plate counts on day 0 (the day of pressure treatment) decreased as shown in Figures 2a and 2b. A pressure of 300 MPa decreased the aerobic plate counts from 1.3×10^5 to 2.3×10^4 cfu/g which is approximately a 1 log reduction. A treatment of 550 MPa resulted in a reduction on aerobic plate counts from 1.3×10^5 cfu/g to 9.4×10^2 cfu/g, an approximate 2 log reduction. It was observed that at the same processing temperature, higher pressure inactivated more aerobic organisms than the lower pressure treatment.

Anaerobic counts on pressurized samples were enumerated in order to identify surviving organisms (which are to be discussed in the next session) and to determine whether the crab meat possessed anaerobic pathogens. In general, crab meat contained more aerobic organisms than anaerobic organisms as anaerobic plate counts are always lower than the aerobic plate counts. For example, in the control sample on day 0, the aerobic plate counts were 1.3×10^5 cfu/g, whereas an anaerobic plate count of 2.9×10^3 cfu/g was observed. The effect of hydrostatic pressures on anaerobic plate counts are shown in Fig 2b. Crab meat treated with 300 MPa did not show any reduction in anaerobic organisms, whereas at 550 MPa a reduction of 2 logs was observed.

After pressure treatments all samples as well as the controls were stored at 4° C for the 31 day shelf life extension study. During storage, microbiological changes in aerobic and anaerobic organisms were observed. For the control or unpressurized crab meat, the aerobic count increased from 1.3×10^5 to 8.6×10^6 cfu/g at the first 7 days of storage. From day 7 to day 17, the

a.



b.

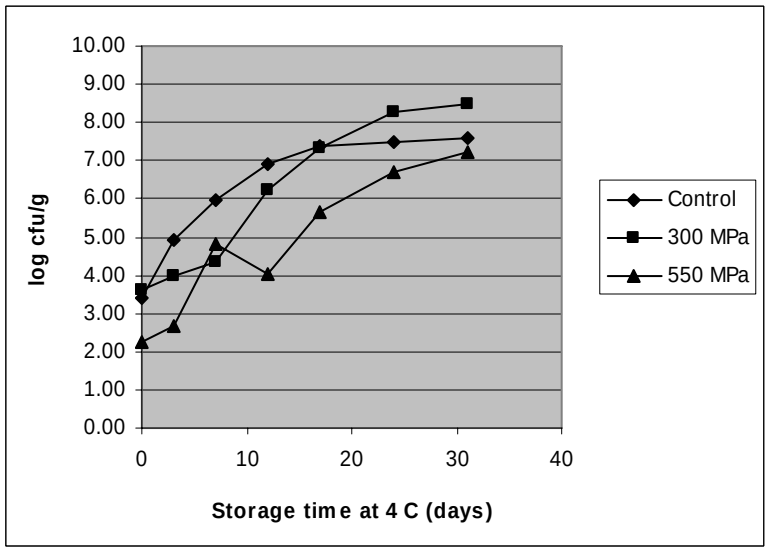


Fig 2: Aerobic plate counts (a) and anaerobic counts (b) of control and pressurized crab meat at 300 and 550 MPa at 25° C for 5 min

APC gradually increased from 8.6×10^6 to 6.4×10^7 cfu/g and remained relatively constant through the last day of storage period (day 31). At day 31, the APC was reported as 1.2×10^8 cfu/g.

In addition to a reduction in aerobic counts, high pressures caused a lag period in which the growth or a multiplication of organisms was inhibited for a certain period of time due to cell injuries or adaptation of cells to a new environment resulting in a small increase in organisms. A pressure of 300 MPa caused a 3-day lag period, after which, the growth resumed and increased dramatically from 3.1×10^4 cfu/g at day 3 to 1.4×10^8 cfu/g at day 17 and gradually increased to the final APC of 5.6×10^8 cfu/g at day 31. A lag phase was not observed in the treatment of 550 MPa as in the 300 MPa treatment. Surviving organisms could have resumed growth immediately after pressure treatments with a decrease at day 7 to the final level of 2.9×10^8 cfu/g at day 31.

With an exception to a major difference in the initial number of organisms, the behavior of anaerobic organisms in unpressurized and pressurized crab meat is similar to those of aerobic organisms. Anaerobic organisms in the control sharply increased from 2.9×10^3 to 2.6×10^7 cfu/g during the first 17 days of storage. After day 17, the organisms population remained constant until day 31. Crab meat treated with 300 MPa exhibited no reduction in anaerobic counts on day 0, however, the pressure caused a 7 day lag period. During these 7 days, the anaerobic counts slightly increased from 2.9×10^3 to 1.5×10^4 cfu/g and continually increased with a higher growth rate to 3.0×10^8 cfu/g at day 31.

In the 550 MPa samples, a reduction of 2 log cycles was observed with no evident lag period. A starting level of 4.2×10^1 cfu/g in 550 MPa-treated samples increased to 1.6×10^7 by the end of storage period. From these results, high hydrostatic pressures did not appreciably reduce the number of aerobic and anaerobic bacteria, the 300 MPa process reduced or inactivated aerobic organisms approximately 1 log and the 550 MPa treatment inactivated about 2 logs of aerobic and anaerobic organisms.

3.2 Microflora of fresh crab meat and surviving microorganisms after pressure treatments

To determine the effects of high pressure treatments on the microflora of fresh crab meat, all pressure-resistant organisms surviving the highest pressure applied in this study (550 MPa) were identified, and compared to microflora in the fresh crab meat. Observations on colony morphologies of predominant organisms under both aerobic and anaerobic conditions were performed prior to isolation.

All microorganisms isolated from fresh and 550 MPa pressurized meat were stored at 4° C for 0, 3, 7, 12, 17, 24 and 31 days as shown in Table 1. The aerobic and anaerobic predominant organisms isolated from the meat samples withdrawn from the same day (0, 3, 7, 12, 17, 24 and 31) are contained in Table 2 and 3. In this section, only surviving microorganisms after pressure treatments (day 0) will be discussed. Surviving microorganisms during storage (day 3, 7, 12, 17, 24 and 31) are discussed in section 3.3.

The microflora of fresh meat isolated from aerobic plates were *Acinetobacter johnsonii, Acinetobacter lwoffii, Arthrobacter aurescens, Brevibacillus agri, Brevibacterium iodinum, Exiguobacterium acetylicum (Brevibacterium acetylicum),* and *Staphylococcus kloosii.* The most prominent isolates were identified as *Exiguobacterium acetylicum,* and *Acinetobacter* (Table 2). Pressure treatment of 550 MPa inactivated many of the microorganisms in the fresh meat. The composition of 550 MPa pressure-tolerant organisms recovered immediately after pressurization (Day 0) of crab meat were *Aerococcus viridans, Brevibacillus agri, Brevibacterium iodinum, Brevibacterium linens, Brevibacterium casei, Brevibacterium epidermidis,* and *Macrococcus (Staphylococcus) caseolyticus* (Table 1). The predominant aerobic organism, which tolerated 550 MPa pressure, was identified as *Brevibacterium.*

Under anaerobic incubation conditions, the types of organisms isolated were different from the aerobic incubation condition as expected. *Carnobacterium piscicola* was the only organism isolated from fresh crab meat on day 0, therefore this species was the predominant anaerobic organism. On the same day, only the genus of *Enterococcus* genus with species of *mundtii* and *solitarius* was isolated from pressurized meat. It can be generally concluded that the

Table 1: Microorganisms isolated from vacuum-packaged unpressurized (control) and pressurized (550 MPa at 25° C for 5 min) crab meat stored at refrigeration temperature for 31 days

Microorganisms	Days of storage and treatment													
	Control							**550 MPa**						
	0	3	7	12	17	24	31	0	3	7	12	17	24	31
Acinetobacter														
johnsonii	•													
lwoffii	•													
Aerococcus														
viridans								•						
Aeromonas														
salmonicida							•							
Arthrobacter														
aurescens	•													
agilis			•											
Bacillus														
megaterium		•												
Brevibacillus														
agri	•							•						
Brevibacterium														
iodinum	•							•	•					
linens								•						
casei								•	•					
epidermidis								•						
Carnobacterium														
piscicola		•	•	•	•	•	•						•	
	*	*	*	*	*	*	*				*		*	
Chryseobacterium														
balustinum			•											
Corynebacterium														
auris										•	•			
Enterococcus														
mundtii									•	•	•	•		
								*	*	*		*	*	*
avium														•
								*			*	*	*	*
solitarius												•	•	•
								*			*	*	*	*
Exiguobacterium														
acetylicum	•	•		•										
Macrococcus														
caseolyticus		•						•	•					
										*				
Moraxella														
catarrhalis											•			
Providencia														
rustigianii							•							
Pseudomonas														
putida		•	•			•								
aureofaciens		•		•										
Psychrobacter														
immobilis										•	•	•		
Staphylococcus														
kloosii	•													

• = isolated from aerobic plates ∗ = isolated from anaerobic roll tubes

Table 2: Predominant microorganisms isolated from aerobic plates

Day	Control	550 MPa
0	*Exiguobacterium acetylicum* *Acinetobacter*	*Brevibacterium*
3	*Pseudomonas putida*	*Brevibacterium*
7	*Pseudomonas putida*	*Psychrobacter immobilis*
12	*Carnobacterium piscicola* *Pseudomonas putida*	*Psychrobacter immobilis*
17	*Carnobacterium piscicola*	*Enterococcus*
24	*Carnobacterium piscicola*	*Enterococcus*
31	*Carnobacterium piscicola*	*Enterococcus*

Table 3: Predominant microorganisms isolated from anaerobic roll tubes

Day	Control	550 MPa
0	*Carnobacterium piscicola*	*Enterococcus*
3	*Carnobacterium piscicola*	*Enterococcus*
7	*Carnobacterium piscicola*	*Enterococcus*
12	*Carnobacterium piscicola*	*Enterococcus*
17	*Carnobacterium piscicola*	*Enterococcus*
24	*Carnobacterium piscicola*	*Enterococcus*
31	*Carnobacterium piscicola*	*Enterococcus*

predominant genera of anaerobic organisms in pressurized crab meat is *Enterococcus spp*. Although *Carnobacterium piscicola* and *Enterococcus spp*. were isolated from anaerobic roll tubes, these two organisms are not strict anaerobes; they are in fact facultative anaerobic organisms. No obligate anaerobes were isolated in this study.

3.3 Dominant microflora during storage

The predominant microflora in fresh and 550 MPa-treated crab meat during storage (day 0 to day 31) under aerobic and anaerobic conditions were isolated and identified in a similar manner to the procedure described in section 3.2. The microflora in fresh and 550 MPa-treated samples during storage changed due to competition with other surviving organisms. Under aerobic conditions, *Acinetobacter johnsii, Acinetobacter lwoffii, Arthrobacter aurescens, Arthrobacter agilis, Bacillus megaterium, Carnobacterium piscicola, Chryseobacterium balustinum, Exiguobacterium acetylicum, Pseudomonas putida, Pseudomonas aureofaciens* and *Staphylococcus kloosii* were isolated from the control samples within the first 12 days of storage (Table 2). During storage, the prevalence of organisms changed from one species to another. On day 0, *Exiguobacterium acetylicum* and *Acinetobacter* were identified as the primary isolates which then changed to *Pseudomonas* on Day 3 and 7. On day 12, *Carnobacterium piscicola* was the predominant organisms observed along with the minor presence of *Pseudomonas spp*. After day 12, *Carnobacterium piscicola* outgrew the other organisms and became the dominant aerobic organism until day 31.

A treatment of 550 MPa resulted in a different group of aerobic surviving organisms as mentioned previously in section 3.2. For the first three days of storage, *Aerococcus viridans, Brevibacillus agri, Brevibacterium iodinum, Brevibacterium linens, Brevibacterium casei, Brevibacterium epidermidis, Enterococcus mundtii, Macrococcus caseolyticus* were isolated, with *Brevibacterium* as the predominant organism. Between day 7 and day 12, *Corynebacterium auris, Enterococcus mundtii, Moraxella catarrhalis* and *Pyschrobacter immobilis* were observed with *Psychrobacter immobilis* identified as the predominant organism. After day 17 until day 31, *Carnobacterium piscicola, Enterococcus mundtii, Enterococcus avium, Enterococcus*

solitarius, and *Psychrobacter immobilis* were isolated with *Enterococcus spp.* as the predominant organism.

During the 31-day storage period, residual oxygen in the vacuum-packaged was reduced through the growth of aerobic organisms until the environment became microaerophilic or anaerobic. The diminished oxygen had an adverse effect on aerobes but on the contrary favored the growth of anaerobic or facultative anaerobic organisms which was observed. Under anaerobic conditions that occurred during storage, there were not as many species of organisms identified as under the aerobic condition. Only *Carnobacterium piscicola* was isolated from fresh crab meat from day 0 to day 31, whereas *Carnobacterium piscicola, Enterococcus mundtii, Enterococcus avium,* and *Enterococcus solitarius* were isolated with the predominance of *Enterococcus spp.* (Table 3). However, it should be noted that *Carnobacterium piscicola* and *Enterococcus spp.* are not obligate anaerobic organisms; they are both in fact facultative anaerobic organisms. No strict anaerobe such as *Clostridium botulinum* was isolated during this study.

3.4 Sensory analyses

Sensory analyses were conducted to evaluate overall differences between the control and meat treated with pressures of 300 and 550 MPa. Overall differences between the control and pressurized samples at 300 MPa were performed with triangle tests by sensory panels were not statistically different ($P>0.05$). Pressure-treated samples at 550 MPa were also not statistically different from the control ($P>0.05$). Pressurized samples at two different pressure levels (300 and 550 MPa) did not show a statistically difference in overall difference ($P>0.05$).

When the sensory panelists were requested to judge and describe the meat characteristics, especially odor of the stored meat, a very pungent and strong odor in the control samples when the APC reached about 10^7 cfu/g (Fig 1) was observed resulting in rejection by panel members on day 17 (Table 4). On the contrary, sensory evaluations based upon the smell or odor of the pressurized samples differed from that of the fresh meat. The higher plate count level did not result in a rejection from sensory panelists, in fact, the meat was still considered acceptable. If

tested microbiologically, the meat may have been considered as spoiled. If the meat was unacceptable, the aerobic plate counts could not serve as a criterion of quality.

Panelists accepted and described the 300 MPa-treated products as fresh (Table 4) even at the time the aerobic counts reached 10^7cfu/g at day 12 (Fig 1). The 300 MPa-treated samples eventually became spoiled and unacceptable to sensory judges at day 31 when the APC reached 5.6×10^8 cfu/g. For the 550 MPa samples, the APC of pressurized crab meat reached 10^7 cfu/g at day 15 (Fig 1) and the meat was still considered acceptable. At the last day of storage day 31, when the APC was 2.9×10^8 cfu/g (Fig 1), the meat was still acceptable, however, a slight stale odor was detected.

Table 4: Sensory characteristics of unpressurized (control) and pressurized crab meat stored at 4 °C for 31 days

Day	Control	300 MPa	550 MPa
0	Fresh	Fresh	Fresh
3	Fresh	Fresh	Fresh
7	Pungent, strong odor	Fresh	Fresh
12	Pungent, strong odor	Fresh	Fresh
17	Pungent, strong odor, spoiled	Fresh	Fresh
24	Strong odor, spoiled	Stale	Fresh, acceptable
31	Strong odor, spoiled	Pungent, sweet flavor, cabbage flavor, smoky flavor, unacceptable	Little stale, acceptable

3.5 Physical analyses

3.5.1 Color measurement

L^*, a^*, and b^* color values representing lightness, redness, and yellowness of unpressurized and pressurized crab meat sampled on day 0, 3, 7, 12, 17, 24, and 31 are described in Tables 5, 6, and 7. After pressurization (day 0), the crab meat became slightly darker as indicated by lower values of lightness. There was more green ($-a^*$) color in the 550 MPa-treated meat, but less