

Acceptability and Shelf-Life of Fresh and Pasteurized Crab Meat Stored Under Different
Environmental Conditions

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Abstract

Crab meat is important to the economy of coastal Virginia. The objectives of this study were to complete a shelf-life study on two different packaging styles of fresh crab meat and to test the inhibition capabilities of *Carnobacterium piscicola* against the pathogen, *Listeria monocytogenes*. In a shelf-life study, a 12 ounce food grade polyethylene traditional snap-lid container of fresh crab meat was compared to an 8 ounce SimpleStep® trays with Cryovac™ film of equally fresh crab meat sealed with 10,000 cc/m²/24hr oxygen transmission rate (OTR) film. Eleven g samples were used for the microbial shelf-life study conducted at 4°C for 12 days. Aerobic plate counts of crab meat indicated microbial growth from the SimpleStep® trays with Cryovac™ film in 10,000 cc/m²/24hr OTR versus the polyethylene snap-lid was not significant (P>0.05). In objective two, 25 g samples of fresh and pasteurized blue crab (*Callinectes sapidus*) meat were inoculated with 0.1ml of each, *C. piscicola* and *L. monocytogenes*. Three different concentrations of the inoculation levels were studied on select days at both 4°C and 10°C. Microbial spoilage was defined as 10⁷ CFU/g. In fresh crab meat, at both 4°C and 10°C, crab meat spoilage occurred at 7 days or less. In the pasteurized crab meat, at 4°C and 10°C, spoilage did not occur prior to 26 days, and studies were terminated at 28 days of storage. The growth of the two organisms in fresh crab meat was found to be significant for the differing concentration levels and sampling days (P<0.05). The growth of the two organisms in pasteurized crab meat was significant for different concentration levels, sampling days and temperature (P<0.05). In both fresh and pasteurized crab meat, regardless of the inoculation ratios, the *L. monocytogenes* and *C. piscicola* followed similar growth trends, but *L. monocytogenes* was higher in the 2:2 CFU/g concentration and lower at the 6:2 CFU/g concentration level. Although *C. piscicola* did not

completely inhibit *L. monocytogenes* growth at any concentration ratio, some inhibition was observed.

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Introduction

Crab meat from blue crabs (*Callinectes sapidus*) is available to consumers in fresh or pasteurized form. Fresh crab meat, is a fully cooked product, but has no additional steps to kill harmful bacteria. Refrigeration, Good Manufacturing Practices (GMPs), and Good Employee Hygienic Practices are needed to maintain quality and safety. Pasteurization, on the other hand, includes an additional thermal process that, when performed properly, destroys vegetative bacterial cells including *Clostridium botulinum* Type E and a reduction of non-proteolytic *C. botulinum* Type B. Refrigerated pasteurized crab meat has a shelf-life of 12 months or more.

New packaging options are needed for the domestic blue crab processing industry, that provides not only convenience, but also ensures safe, high quality crab meat (16). Two different packaging types for fresh crab meat were examined in this study. Traditional polyethylene or polypropylene plastic cups/tubs were compared to SimpleStep® trays with Cryovac™ film with an oxygen transmission rate (OTR) of 10,000 cc/m²/24hrs. Shelf-life and microbial counts of the crab meat in two different package types were evaluated at 4°C along with container head-space gases.

Crab meat is a ready-to-eat product (RTE) product. If *L. monocytogenes* is detected in RTE seafood, the product is considered adulterated. Although *L. monocytogenes* in blue crab meat has never been directly involved in known illnesses or outbreaks, (17) the presence of *L. monocytogenes* in crab meat is a safety concern in RTE seafood products (26). *Listeria monocytogenes* and *Carnobacterium piscicola* were inoculated into both fresh and pasteurized crab meat. The effect of the bacteriocin produced by *C. piscicola* was studied to determine its inhibitory effect on the growth of *L. monocytogenes*. Bacteriocins have been

studied in fish and shrimp products, but there is little to no information on the use of bacteriocins in crab meat. The interaction of bacteriocin producing *C. piscicola* and *L. monocytogenes* was examined at 4 and 10°C.

Literature Review

General Information

Crabs, along with shrimp and crayfish, are crustaceans called macro-invertebrates. Blue crabs (*Callinectes sapidus*) bury themselves in the mud in the winter months and emerge when temperatures increase during the warmer months. Blue crabs are scavengers that locate their food through a combination of chemoreceptors and touch. Blue crabs are known for their cannibalistic habits. Predators also eat many young crabs, causing the population to fluctuate from year to year. The maximum age for most mid-Atlantic blue crabs is 3 years old (55).

Blue crabs range from Nova Scotia, Canada, along the eastern coastline of North America, down into the Gulf of Mexico and Caribbean and along the coast of South America and Argentina. Blue crabs are not typically found in cooler water, but their range may change from year to year depending on the water temperature (55).

Crab meat is sold as lump or jumbo lump, backfin, special and claw meat. Lump meat is removed from the swimming or back legs of the crab sold in large white pieces with very few shell pieces mixed in with the meat. Backfin and special are collected from the legs and body of the crab. The meat pieces are typically smaller than lump meat and can contain pieces of shells. Claw meat is a reddish-brownish meat used in recipes, in which taste is more important than appearance (5, 47).

Imported Crab Meat

In the US, competition from crab meat imports has impacted the traditional local industry. The Virginia Marine Resources Commission has estimated that Virginia's yearly

crab harvest has decreased 32% since 1995 (52). Several contributing factors have influenced the decline of the blue crab in the Chesapeake Bay area, including a decrease in the quantity of blue crabs available in the Chesapeake Bay, a decline in the ecological health of the Chesapeake Bay, and a dramatic rise in the importation of crab meat from Asia (36).

Imported Asian crab meat directly competes with domestic crab meat and is sold to local crab restaurants at cheaper and more predictable prices (52). The combination of abundant supply, low labor cost, and growing demand for crab meat have all contributed to the popularity of imports, forcing a number of large domestic producers out of business (25). Consumers benefit from the large quantity of imports that are available at affordable prices without seasonal boundaries (25). Although the FDA has posted import alerts on imported crab meat due to insect, rodent, bird, cat and/or other means of filth (20). Despite this, imports exceed 3,300 tons of crab meat yearly (25).

Harvesting and Processing of Crab Meat

Blue crabs, harvested from estuarine and coastal waters, are susceptible to environmental factors that can increase the microbial flora of the crabs (10). Crab meat is a highly perishable food product (30, 53). The bacterial flora reflects the environment from which they were harvested (10) and is a reflection of the conditions under which they were processed. This bacterial flora changes from season to season with the water quality, water temperature and harvest location (10).

Blue crabs are largely harvested using baited crab pots and trot lines. During the colder months, bottom dredges are used to remove the dormant crabs from the sand. Watermen deliver their catch to the crab processing plants, and the crabs are then placed in

crates, cooked, cooled for 3-4 hours and refrigerated until the following day when they will be picked (10).

The cooking process occurs regardless of whether the crab meat will be sold as a fresh or pasteurized product. Cooking facilitates the picking process and it is accomplished by steam under pressure in the mid-Atlantic region or sometimes in the Gulf of Mexico region this is accomplished by boiling (47). Steam under pressure crabs have a longer storage life compared with boiled crabs, but boiled crabs have a higher moisture content that resulting in higher yields, which in turn provide conditions that favor bacterial growth (10). Sanitary processing facilities and refrigerated storage temperatures are critical in helping to achieve low bacterial counts in the final products (10).

The majority of crab meat is extracted from the body cavity of the crab and during processing cross contamination of microorganisms can occur (10). The blue crab is used to produce fresh and pasteurized crab meat that is sold to grocery stores or restaurants. When processors pasteurize crab meat, it increases the shelf-life, expanding the processors' market radius and boosting their economic growth (30, 53).

Crab meat is picked from the carapace of the crab by hand or less commonly, by machine. After the crabs have been picked, the crab meat is delivered to the packing room every hour or as soon as they pick 3-5 pounds, whichever comes first. In preparation for packing, the crab meat is weighed, placed in containers, lids applied, and the containers are iced and refrigerated. Deviations from these procedures results in loss of quality (10).

Fresh crab meat is removed from the crab's body or claws, placed into containers and then held on ice or refrigerated without further processing (22). Pasteurization equipment is designed to ensure that every unit of product receives the minimally required

process (19). Properly pasteurized crab meat kills nonproteolytic *Clostridium botulinum* Type E and *Listeria monocytogenes* (19). Pasteurization does not result in a commercially sterile product (10, 47). Rapid cooling followed by refrigeration is required to maintain product quality and safety (44). Rapid reduction of the internal food temperature is needed to reduce the growth of surviving microorganisms (44).

Pasteurization, as defined by the FDA, is a mild or moderate heat treatment typically performed on products in the finished container (18, 19). It is designed to eliminate targeted pathogens and extended refrigerated shelf-life of foods (18, 19). Processors develop Hazard Analysis and Critical Control Point (HACCP) plans to control post-contamination, which generally occurs through container seal integrity. Sanitation standard operating procedure's (SOPs) and/or other programs such as Code of Federal Regulation (CFR), Title 9, Part 430 are also implemented to prevent contamination of a finished product (4, 19). The CFR sets forth the regulation requirements for Ready-To-Eat (RTE) foods that have been exposed to the environment after lethality treatments such as cooking or drying (4). HACCP can also be incorporated when slight temperature abuse either during handling, distribution and/or storage occurs that increases the growth rate of psychrotrophic and non-psychrotrophic pathogens and spoilage microorganisms (41).

Microflora

Fresh crab meat is a perishable product (30, 53) that can lose its typical odor, flavor and appearance within 10-14 days or less even under the best of storage conditions (53). The degradation of seafood after harvesting is initiated by bacterial and enzymatic reactions (9). Decomposition of macromolecules such as protein, glycogen and nucleic acids into small

molecular substances, provide available nutrients for microbial growth, further decomposing the food (9). Only live crabs are recommended for use in processing since dead crabs may deteriorate to an unsafe level for human consumption (16).

Fresh crab meat is picked after the initial cooking stage with no further processing (22). The Maryland Crabmeat Quality Assurance Program (MCQAP) asserts that the maximum allowable bacterial count for fresh crab meat is 1.0×10^5 CFU/g (45). Whereas pasteurized crab meat increases the shelf life of the crab meat by decreasing the bacterial growth (53), the maximum allowable bacterial count for pasteurized crab meat, according to the MCQAP is 2.5×10^4 CFU/g (45).

Predominant aerobic microorganisms isolated from fresh crab meat in a 31 day study included both Gram positive and Gram negative organisms: *Acinetobacter*, *Carnobacterium piscicola*, *Exiguobacterium*, and *Pseudomonas*, with less predominant ones including: *Aeromonas*, *Arthrobacter*, *Bacillus*, *Brevibacillus*, *Brevibacterium*, *Chryseobacterium*, *Marcococcus*, *Providencia*, and *Staphylococcus* (48). Although *Carnobacterium piscicola* is a facultative anaerobe, no obligate anaerobes were isolated from the study (48). It is theorized by the authors, that throughout the study the amount of oxygen in the package was reduced due to the growth of the aerobic organisms until the environment inside the package became microaerophilic or anaerobic, favoring the growth of the anaerobic or facultative organism (48).

Adams et al suggests that only a small percentage of the bacteria present in the crab meat are spoilage bacteria, and therefore unlikely that all the bacteria isolated are active in spoilage (2). The inactive spoilage bacteria may perhaps have a role in the synergism of spoilage, but not a direct role (2). Taking into consideration environmental factors that

influence bacterial growth such as temperature, packaging and duration of storage, under aerobic refrigerated conditions, the spoilage of fish and other seafood typically occurs because of growth of psychrotrophic bacteria such as *Pseudomonas*, spp and *Achromobacter*, which can account for almost 100% of the bacteria present (2, 48).

Ray et al states that information on the levels and types of bacteria typically found during various stages of processing blue crab is important for recommendations on good manufacturing practices and sanitary practices (42). The presence of coliforms (including *Escherichia coli*) in fresh crab meat may indicate unsanitary handling and the need for improvement in hand washing and plant cleaning and sanitizing procedures (45). Maryland has implemented extensive safety programs that coordinate with the Departments of Health and Mental Hygiene, Natural Resources, Environment and Agriculture (7). Together these agencies perform a number of tasks that boast high quality seafood with one of the most extensive seafood safety systems in the country by monitoring and testing the Chesapeake Bay waters and the fish and shellfish that come from those waters, as well as seafood processing plants (7).

Factors Affecting Crab Meat Shelf-life

Intrinsic Factors

Due to the nature of fresh crab meat and the minimal heat treatment that it receives, processors need to be concerned with a number of potential sources of contamination, which may limit the crab meat's shelf-life (16). Growth of microorganisms is a major cause of shelf-life reduction. Microorganisms have distinct phases of growth: lag phase, logarithmic phase, stationary phase, and then cell death. An organism in lag phase is adjusting to new

conditions, such as a temperature change, prior to cell multiplication (39). Lowering of temperatures can increase lag phase of microorganisms, and therefore have a direct effect on the product's shelf-life. Frozen cells have lengthy lag times (39). These times reflect the period necessary for cellular adjustment prior to resuming growth (39). Cold storage has been shown to slow down undesirable changes to the food product (flavor, color, odor, etc) and perhaps prolong shelf-life. However, *L. monocytogenes*, a psychotrophic organism, can grow during refrigerated storage (34, 38, 41).

The rate of degradation of crab meat is temperature dependent (9). If kept, under constant refrigeration, fresh crab meat can last from 4-10 days. Refrigerated pasteurized crab meat, in its original, unopened package, can last up to 18 months. High crab meat quality and long shelf-life have been linked to storage at a constant temperature of 4°C or less (46). Robson et al found that crabs chilled at 4°C had a longer shelf lives compared with crabs stored on ice (46). Bacteria tends to be repressed in crabs stored at 4°C compared to those stored on ice (46). When crab meat is kept at refrigerated storage temperatures, other deteriorative changes can occur, such as an increase in the pH (54).

Extrinsic Factors

Environmental factors, such as product temperature, facility sanitation conditions, the cumulative time the crab meat is left unrefrigerated during the picking process influence microbial counts (42). The shelf-life of crab meat is influenced by atmospheric oxygen and the growth of spoilage microorganisms (38). Good sanitation practices prior to packaging of the crab meat are critical in maintaining low initial microbial populations (42, 47). Spoilage organisms and possible pathogens cross contaminate the crab meat during hand picking and

microbial build-ups occur during machine picking which can contaminate subsequent batches (47).

The crab industry's concerns with packaging systems are primarily fueled by economic issues. The introduction of tamper-resistant packaging prevented switching of container lids identifying the crab meat type (22). Also, packaging prevents consumers from opening and reopening containers to examine the contents before purchasing (22). Processors must choose appropriate packaging keeping in mind price, packaging quality, market appearance and production capability (22). Prior to the introduction of a new package style, research studies should be conducted to evaluate container head-space gases, microbial growth and shelf-life, chemical decomposition, sensory quality and possible toxin production by *Clostridium botulinum* (22). Fresh blue crab meat in the Chesapeake Bay area and the Virginia coast area is sold in traditional plastic snap-lids of 8oz, 12oz or 16oz. Pasteurized crab meat is sold in metal cans or 8oz, 12oz or 16oz plastic snap-lids sealed with metal pop top lids. Plastic and aluminum, commonly used to package crab meat, give longer shelf lives and better sensory and microbial qualities than crab meat packaged in steel cans (23). It has been found that vacuum skinned packaging can improve sensory qualities of freshly cooked and picked crab meat (23).

In order for domestic blue crab meat to have competitive pricing against the imported crab meat, processors are continually looking for new packaging options to reduce their costs. New packaging has the potential to improve quality and safety of the crab meat while providing smaller portion sizes for a broader consumer base (23). Gates et al compared packaging material cost for commercial packaging of both fresh and pasteurized crab meat finding the cost of packaging material for the pasteurized crab meat from the most

expensive to least expensive were steel cans, plastic cups, aluminum cans, barrier pouches and non-barrier pouches, respectively (23). He concluded that smaller packages or pouches could be pasteurized in less time, ultimately saving the processor money and energy (23).

Given time, product quality may deteriorate regardless of package type due to increased microbial growth and biochemical changes (22). The moderate to mild heat treatment during the pasteurization process (18, 19) extends the refrigerated shelf-life of the crab meat (43) with minimal changes to color, texture and flavor characteristics (43). Although, Gates et al found that the color and the pH of pasteurized crab meat changed depending container type and length of storage (23).

Listeria monocytogenes

Listeria monocytogenes

There are six species within the genus, *Listeria* (31). The species within this genus are non-spore forming and catalase positive (50). Of the six species, *Listeria monocytogenes* is the major pathogen (31). *Listeria monocytogenes* is a Gram positive, rod shaped bacterium, which are sometimes arranged in short chains (50). Flagella can be produced at room temperature, but not at its optimal growing temperature of 37°C (50).

Listeria monocytogenes is a psychrotroph capable of growth at low temperatures (50). It is therefore problematic because of its ability to grow in contaminated foods held at refrigerated temperatures (50). This pathogen is often exposed to high salt and alkaline pH environments during food processing and food preservation. It is tolerant to salt and alkaline stresses (21).

It is well documented that *L. monocytogenes* is a pathogen that can contaminate a wide variety of foods (34). It has been found in a variety of raw foods as well as in processed food contaminated after processing (8). The majority of cases of listeriosis are food-borne, however McLauchlin et al has discovered multiple complex routes of infection (32). *Listeria monocytogenes* is widespread in nature and it can be isolated from soil, foliage and feces of humans and animals (34).

Crab meat is not normally filled into containers using a continuous filling operation (hot filling), so, the possibility of recontamination from the cooking step to the filling step is maximized (18). The presence of any *Listeria* species in food may be an indication of poor hygiene from the cooking to the filling step, faulty container seals that allow the introduction of bacteria into the food product, and, or contaminated cooling water after the pasteurization step (18, 19, 31). *Listeria monocytogenes* is found in a wide variety of fish and shellfish products and is isolated from many different water sources (35). The FDA and USDA conducted a risk assessment of *Listeria* and placed smoked seafood, cooked RTE crustaceans and raw seafood/preserved fish into high, moderate and low risk categories, respectively (35). Although the Food and Agriculture Organization Code of Conduct for Responsible Fisheries called for continued liberalization of fish and shellfish trade, the policy of zero-tolerance regarding *L. monocytogenes*, still continues in the United States (51).

Listeriosis

The disease that *L. monocytogenes* causes, listeriosis, is an important public health problem in the United States (8). Because *L. monocytogenes* is ubiquitous in nature, it can easily enter the food chain (34). Listeriosis is most commonly association with refrigerated

products such as milk, fresh cheeses, meats or vegetables held for long periods of time (50). Seafood such as shrimp, mussels, artificial crab meat and cold-smoked rainbow trout have been involved in listeriosis outbreaks (35).

Dose relationship (32) and infection mechanism of *L. monocytogenes* are not clearly understood (34). Not all strains of *L. monocytogenes* cause disease (34). Certain pathogenic strains of *L. monocytogenes* initiate two types of health responses (34). The first is a severe clinical manifestation of listeriosis is meningitis and the second is sepsis (50). Through confirmed epidemiological data, high risk groups have been identified (34) including the elderly, adults with weakened immune systems (such as cancer, diabetes or kidney disease), newborns and unborn babies (8). However, people outside of these risk groups are often infected as well (8). A healthy adult can experience fever, muscle aches and sometimes gastrointestinal symptoms such as nausea and/or diarrhea. Pregnant women experience head-aches and a mild flu-like illness, however and infection during pregnancy could cause a miscarriage, stillbirth, premature delivery or infection of the newborn child (8). When listeriosis occurs, antibiotics are administered. If an infection occurs during pregnancy, medication is promptly given to the woman to prevent infection to the fetus or newborn. Even with this prompt course of action, some infections inevitably result in death (8).

Listeria monocytogenes Relationship with Crab

According to data compiled from the FDA Enforcement Reports and adapted from Jinneman et al there have been at least 126 Class I recalls since 1987 issued in the U.S. for domestic and domestic/imported ready-to-eat (RTE) seafood products (26). Recalls are

issued when *L. monocytogenes* is found in RTE seafood or seafood products receiving minimal or no additional heat treatment by the consumer prior to consumption (26). Concurrent with the information gathered, crabs account for 46 of the 126 recalls issued (26). Although FDA action was taken, there were no known illnesses or outbreaks from *L. monocytogenes* from seafood products (17). The detection of *L. monocytogenes* in crab meat and other seafood products is a concern (26). Since the initial recall of crab meat in 1987, several programs and advisory committees have been formed to conduct microbiological analysis on minimally processed or raw seafood and establish microbiological criteria for crab meat (26). As Jinneman reports, during 1991-1996, 1,886 RTE crab samples were analyzed for *Listeria*, spp. and 142 (7.5%) tested positive for *L. monocytogenes* (26). Subsequent testing during October 1999-February 2003 showed that the incidence for RTE crab fell to 2.37% (15 of 632 samples) (26).

The ability for *Listeria*, spp. to persist in the marine environment, results in the fact that raw fishery products can be naturally contaminated with this pathogen (26). Data from Fuad et al reports the ability of *L. monocytogenes* to survive in estuarine waters may fluctuate. Chitin, found in particular seafood products (ex: shrimp, crab and lobster) assists in the growth and survival of *Listerial* cells (14, 26). It is believed that there is an association of the growth of *Listeria innocua* and *L. monocytogenes*, and a suggested suppression of the latter by the former (40). Rawles et al states that *L. innocua* has been found in blue crab more frequently than *L. monocytogenes* in processing plants, yet their study found no difference in growth ($P>0.05$) of either microorganism at any inoculation level or temperature (40). Consequently, a lack of control over *L. innocua* may also indicate a lack of control over *L. monocytogenes* (40). Often only a few strains become established in the

processing facility and recovered in the final products (26). Proper sanitation in the processing facility is critical for the control of *L. monocytogenes* in the final product (26). The use of risk assessment to quantitatively and epidemiologically determine the hazards associated with pathogens (17) may prevent their product from becoming contaminated.

Control Methods of *Listeria monocytogenes* in Crabs

Ongoing Research

Listeria monocytogenes is a pathogen that can contaminate RTE foods and cause illness (8). The presence of *L. monocytogenes* in fish and seafood and product recalls prompted several scientist to investigate the incidence of *Listeria*, spp. in a variety of products harvested from many geographical locations (26). The current trend is food products with little or no preservatives and little processing (12, 15, 41). As an alternative to thermal treatments, some researchers have used high hydrostatic pressure (HHP) to process crab meat. Results from HHP indicate an inactivation of spoilage and pathogenic microorganisms, shelf life extension and the preservation of the food's nutritional value (48).

Suklim et al reported that fresh crab meat subjected to medium (200-300 MPa) and high pressures (400-550 MPa), from a HHP machine resulted in a reduction of approximately 1-2 log units in aerobic microorganism, respectively (48). Although the highest pressure treatment inactivated many of the microorganisms, they concluded that this process had a limited effect on aerobic and facultatively anaerobic bacteria (48). Moreover, the high pressure may have caused a lag period in which the multiplication of organisms was hindered possibly due to cell injury or initial adaptation of the cells to a new environment (48). Cell growth after the recovery from a prolonged lag phase can be dramatic (48).

Pressure treatment can inactivate many microorganisms and ultimately change the flora of the fresh crab meat, yet there may be pressure-resistant organisms, such as spores, that can survive (48). Resistant spores remain dormant with no detectable metabolism (49). Spores are ubiquitous in nature with long term survival rates, due to their ability to resist environmental stresses, such as physical (heat, radiation and drying) and chemical agents (hydrogen peroxide) even with low nutrient availability (49). It has been documented that *Bacillus* spp. spores have the capability to germinate after HHP and that germination produces spores that are less resistant to subsequent processes or treatments (49).

The use of antimicrobials from lactic acid bacterium (LAB) fermentation and food grade washes on *Callinectes sapidus*, to inhibit *L. monocytogenes* was studied by Degnan et al (13). Crab meat stored at 4°C for 6 days, and washed with Perlac 1911 or Micro-Gard in 10,000-20,000 AU initially decreased the number of *L. monocytogenes* colonies (0.5-1 log ut/g), but they recovered to their original levels by the last day of the study. Crab meat washed with 10,000-20,000 AU of Alta 2341, enterocin 1083 or Nisin, initially decreased the *L. monocytogenes* population by 1.5-2.7 log ut/g, but again, by the end of the study, *L. monocytogenes* counts increased by .5-1.6 log ut/g. When Degnan et al used food grade chemical washes, such as sodium acetate (4 M), sodium diacetate (0.5-1 M), sodium lactate (1 M) or sodium nitrite (1.5 M), there was only a modest reduction in the *L. monocytogenes* population (0.4-0.8 log CFU/g) (13). With the increase of sodium diacetate to 2 M, *L. monocytogenes* decreased to 2.6 log CFU/g by the end of the 6 day study period. Trisodium phosphate in 1 M concentration reduced *L. monocytogenes* counts from 1.7 - >4.6 log CFU/g by the end of the study (13). Due to the remaining *L. monocytogenes* present in the crab

meat, no sensory evaluation was performed to detect any off-flavors or odors from the LAB fermentation products or the food grade chemical products.

Food companies may benefit from the use of a bacterium to control undesirable bacteria in a food-grade and natural manner (12). Addressing public demand, food companies in over 48 countries have turned to natural food isolates to avoid undergoing extensive processing or chemical preservatives in their foods (12, 15, 41). Nisin and other LAB byproducts are of particular importance to the food industry since they are generally regarded as safe (3, 6, 12, 37). Nisin, produced by *Lactococcus lactis*, is to date the only commercially used bacteriocin, although it is not currently approved for use in fishery products. It is marketed commercially as Nisaplin® and Novasin™ (3, 12). Danisco, a company who distributes both Nisaplin® and Novasin™, is currently developing and patenting a value added Niaplin® that is heat protected and therefore capable of being incorporated into bakery items (3). Danisco also boasts that with the addition of Nisin, not only is the benefit of food safety and food preservation advantageous, but the incorporation of the Nisin into the food product may reduce heat treatment and processing times and therefore increase productivity with more consistent product results (3).

Lactic Acid Bacteria

Lactic acid bacteria (LAB) are considered aerotolerant, Gram-positive rods or cocci (37). The bacteria is capable of producing an antimicrobial compound (bacteriocin) that tends to show inhibitory characteristics towards other bacteria (12). The lactic acid production, supplemented by hydrogen peroxide and organic acid production decreases the pH of the food (12, 37). It is this lower pH that enables fermenting lactic acid to survive in

the food (24) and interact with the pathogenic bacterial cell by either killing the susceptible bacteria through membrane lysis or inhibition of essential cell processes (protein synthesis or DNA synthesis) (41).

There are numerous strains of LAB with broad spectrums of inhibition due to different receptors on different bacteriocins (1, 41). This variability within the strains determines their antagonistic capabilities, their modes of action and their biochemical properties (1, 41). They are slow growers and do not produce offensive spoilage effects until their numbers are very high (38). This bacterial group is often isolated from chilled vacuum or modified atmosphere packed meat and seafood (28). *Carnobacterium*, a genus of lactic acid bacteria, was reclassified from an atypical *Lactobacillus* species (33). This genus, which contains eight species, are heterofermentative, rod-shaped lactic acid bacteria that produce L-lactic acid from glucose (33). *C. piscicola*, formerly known as *Lactobacillus piscicola*, is able to ferment lactose, starch, and mannitol (33, 37).

Optimal production of bacteriocin occurs between a pH of 6-7. Khouiti et al saw maximum bacteriocin productivity in their supernatant occurring at pH 7 (27), concluding that a controlled pH increased the volumetric activity of the bacteriocin. Temperature also had an affect on the production of bacteriocin (27). They observed that optimal production occurred between 25-30°C (27). Although there are different inhibitory effects of bacteriocins to *L. monocytogenes* (11), other factors affect bacteriocin production. The growth environment in the food dictates bacteriocin production levels. Nutrient availability, oxygen level, and the presence of other inhibitory metabolites in the food matrix, good sanitary practices, particle size and lipid content of the food substance may enhance or restrict bacteriocin growth (41).

Bacteriocin use to control pathogens is an attractive approach (37) that companies may consider implementing. The production of an antimicrobial substance by the bacteriocin may help reduce the use of antibiotics that are used in human applications and animal feed (15). Bacteriocins are active at a nanomolar range and have no toxic effects on humans (37). No isolates of LAB are currently applied to commercial foods for protective purposes against *L. monocytogenes* (29), although it has potential application for improving food quality, safety and market access in chilled, vacuum packaged foods.

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CHAPTER 2

Characterization of Package Types on the Quality and Shelf-Life of Fresh Crab Meat

Abstract

Packaging is a visual stimulus that can either deter or attract consumers to a particular product. In this study, a 12 ounce food grade polyethylene traditional snap-lid container of fresh crab meat was compared during a shelf-life study to an 8 ounce SimpleStep® tray with Cryovac™ film of equally fresh crab meat sealed with 10,000 cc/m²/24hr oxygen transmission rate (OTR) film. During the twelve day shelf-life study, 11 g samples from snap-lid tub containers and SimpleStep® trays were compared. Aerobic plate counts were conducted on days 0, 4, 6, 8, 10 and 12. Anaerobic plate counts were conducted on days 0, 5 and 12. During storage at 4°C there were no differences in shelf-life due to packaging types. Aerobic plate counts of crab meat showed no difference between the SimpleStep® tray with Cryovac™ film in 10,000 cc/m²/24hr oxygen transmission rate (OTR) and the polyethylene snap-lid container on shelf-life (P>0.05). Anaerobic analysis of microbial growth indicates that sampling days were significant (P<0.05), but container type or style was not significant (P>0.05). Oxygen and CO₂ analyses along with coliform and fecal coliform most probable number (MPN) were also evaluated. Oxygen and CO₂ in the package headspace was significantly different between container types (P<0.05). Gas concentration between sampling days were not significant (P>0.05). Coliform numbers throughout storage fluctuated, but neither container type nor sampling day were significant (P>0.05). The results of this microbial shelf-life study suggest that there were no significant microbial differences between either the SimpleStep® trays with Cryovac™ 10,000 OTR film or the polyethylene snap-lid cups packaging (P>0.05).

Introduction

Blue crabs (*Callinectes sapidus*) are scavengers, harvested from estuarine and coastal waters, and are susceptible to environmental factors that increase microbial flora of the crabs (5). The bacterial flora present on the crab reflects the environment from which they were harvested (5). Additionally, the flora is influenced by environmental factors such as temperature, packaging and duration of storage (5). This bacterial flora may change from season to season depending on the water quality, water temperature and harvest location (5). The presence of coliforms and fecal coliforms in fresh picked crab meat indicate unsanitary handling and the need for improved employee hand washing practices and better plant cleaning and sanitizing procedures (16).

Under refrigeration, the spoilage of fish and other seafood occurs due to growth of psychrotrophic bacteria such as *Pseudomonas*, spp (17) and *Achromobacter* (2). Fresh crab meat is a perishable product that loses its typical odor, flavor and appearance within 10-14 days or sooner under the best storage conditions (19). Fresh crab meat is usually hand picked with no further processing, contributing to higher bacterial numbers (19). A storage temperature of 4.4°C or lower is recommended for refrigerated, microbiologically sensitive products. The shelf-life of crab meat depends on several contributing factors including the initial microbial counts and container integrity (15).

Due to changing consumer buying habits and the increasing number of crab meat imports into United States, the crab processing industry in Virginia is motivated to identify new packaging that is innovative and more appealing to consumers. Benefits of new packaging include innovative convenience features, such as microwavable, easy opening, reusable and resealable (15). New packaging also has the potential to maintain quality and

safety of crab meat while providing smaller portion sizes for a broader consumer base (8). Research shows that smaller, thinner packages or pouches, boil in bags and molded trays and cups can significantly increase the heating and cooling rates of their contents, saving the processor money and energy (8, 16). Consumers have indicated that packaging influences their decision in regards to purchasing products (11). In this study, package performance characteristics with an emphasis on quality, shelf-life and safety of the crab meat was evaluated.

Objective

1. Compare shelf-life of fresh crab meat in the new SimpleStep® trays sealed with Cryovac™ film with the traditional polyethylene cups with snap lids.

Materials and Methods

Evaluation of Shelf-life

The evaluation of shelf-life of fresh crab meat stored at 4°C included testing for aerobes and anaerobes for 12 days. Oxygen and CO₂ analyses as well as coliform and fecal coliform MPN testing was also conducted. The entire study was conducted in triplicate. All testing was conducted at the Food Science and Technology (FST) building at the Virginia Tech (VT), Blacksburg campus. The first two replications were performed with crab harvested in Fall 2007. The third replication was performed with crab harvested in Fall 2008.

Isolation of Aerobes in Fresh Crab Meat

Fresh, handpicked crab meat was obtained from a commercial processor in Cambridge, MD. On the day of purchase, Mr. Tom Rippen at the University of Maryland Eastern Shore (UMES) transferred the crab meat from 8oz snap-lid tubs into SimpleStep® trays and sealed the trays with Cryovac™ 10,000 OTR film. Twenty one SimpleStep® trays (8oz) and 21 snap-lid tubs (16oz) were sent overnight to the Virginia Tech Food Science and Technology building. They were packed in styrofoam ice chests with ice packs to preserve the crab meat. Upon arrival, the crab meat was placed in a refrigerator at 4°C. Testing days

were designated from the time that the crab meat arrived at the FST building. Aerobic testing was performed on 3 SimpleStep® trays and 3 snap-lid tubs on days 0, 4, 6, 8, 10, 12. Anaerobic testing on the same amount of crab meat samples as mentioned above occurred on days 0, 5, and 12.

An 11 g sample of crab meat from each container was aseptically removed using a sterile spatula and placed in a sterile 52oz stomacher bag (Nasco, Ft. Atkinson, WI) with 99 ml of 0.1% peptone (Oxoid, Basingstoke, Hampshire, England). The samples were homogenized in a Stomacher Lab Blender 400 (Tekmar Co., Cincinnati, OH) for 30 seconds. Using a sterile, prepackaged pipette (1 ml in 1/100 ml, FisherScientific, Pittsburg, PA), subsequent dilutions of the homogenate were made using 9 ml peptone dilution blanks. Dilutions of the homogenate were taken and spread plated onto trypticase soy agar (TSA), which is a mixture of trypticase soy broth (TSB; BBL, Sparks, MD and MP Biomedicals, LLC, Solon, Ohio) and granulated agar (FisherBioReagents, Fisher-Scientific, Fair Lawn, NJ). After sample usage, the crab meat was not reused. Swabbed TSA plates were incubated at 35°C and enumerated after 48 hours incubation.

Cellular Fatty Acid Identification Preparation for Aerobes

After colonies were counted, predominant, well-isolated colonies were picked and streaked onto TSA plates and incubated for an additional 24 hours at 35°C. For mixed cultures, the microorganisms were repeatedly restreaked until a pure culture was obtained. When a pure culture was obtained, using an inoculating loop (10µl, Fisher Scientific, Pittsburg, PA), the colonies were transferred into a clean (12x100), Teflon-lined screw capped tube, labeled and placed in a commercial freezer until future identification.

Isolation of Anaerobes in Fresh Crab Meat

Anaerobic testing was performed according to methods outlined by Holdeman and Moore (10). Crab meat was obtained, packaged and prepared as outlined in the previous section. On each testing day 0, 5, 12, three SimpleStep® trays and 3 snap-lid tubs were evaluated.

Crab meat samples were prepared as described above, though under anaerobic conditions. Aliquots of 1ml dilutions of the homogenate were placed in a glass anaerobe roll tube containing Brain Heart Infusion agar (BHI; BBL, Sparks, MD). After the tubes were inoculated, they were placed on a horizontal spinner (Bellco, Houston, TX) until the medium solidified. Roll tubes were incubated at 30°C for 5 days. After 5 days, the colonies were examined under a dissecting microscope.

Cellular Fatty Acid Identification Preparation for Anaerobes

After colonies were counted for an overall plate count; each different well-isolated colony type, was selected for identification. Under a constant stream of anaerobe grade CO₂, the colonies were pierced with a sterile needle and a sample of the colony was placed in a small anaerobic roll tube of cooked meat broth (CM, Difco, Sparks, MD) and placed in a 30°C incubator for 24 hours. Results from preliminary studies demonstrated that no strict anaerobic microorganisms were present. All cultures in the anaerobic CM were grown on TSA plates for identification purposes.

After 24 hours, the broth tubes were checked and viewed for gas production and microbial growth. Under the constant stream of anaerobe grade CO₂ gas, a 9" Pasteur pipette

(FisherScientific, Pittsburg, PA) was used to dispense 6 drops of the cooked meat broth into a 12x100 glass tube with rubber stopper of peptone-yeast extract basal medium broth (PYG), a custom made solution (10). The inoculated PYG solution was incubated for 24 hours at 30°C.

After 24 hours, the PYG was spun in a centrifuge (Sorvall, GLC-1, Newtown, CT) at 3000 RPM for 10 minutes. The supernatant was removed and the remaining pellet subjected to cellular fatty acid identification. Preliminary studies showed no strict anaerobes were present in the anaerobic roll tubes. All cultures in the anaerobic chopped meat broth were streaked onto TSA plates for supplementary identification.

Cellular Fatty Acid Identification for Aerobes and Anaerobes

All aerobic and anaerobic identification was performed using the Sherlock Microbial Identification System software (MIS, Microbial ID Inc., Newark, DE) in which the cellular fatty acid profile was used to identify the microorganisms. The procedure for cell sample preparation was modified from the MIS protocol in which four reagents were used to saponify, esterify, extract and base wash the fatty acid extract following MIS protocol (12).

In step one, 1 ml of reagent 1 (45 g of Sodium hydroxide, 150 ml of Methanol, and 150ml of deionized distilled water) was added to the screw capped tubes containing the colony type to be tested. The tubes were then vortexed for 5-10 seconds, and placed in a 100°C water bath for 5 m. After 5 minutes, tubes were vortexed for an additional 5-10 m and placed back into the 100°C water bath for 25 minutes. Step 2 is the methylation step. For aerobes, 1 ml of reagent 2A is used (325 ml of 6.00N Hydrochloric acid and 275ml of Methanol). In anaerobic preparation, both 1 ml of reagent 2A and 1 ml of reagent 2B (162.5

ml of H₂SO₄ added to 162.5 ml of deionized water, plus 275 ml of methanol) were added to each tube. In both cases, the tubes were vortexed for 5-10 seconds and placed in an 80°C water bath for 10 minutes. Methylated components were extracted by adding 0.625 ml of reagent 3 (200 ml of Hexane and 200 ml of methyl tert-butyl ether) to the tubes turning them end over end for 10 minutes. Once the phase differentiation was formed, the bottom phase was removed and the top phase remained in the tube. The washing step consisted of adding 3 ml of the final reagent (10.8g of sodium hydroxide and 900 ml of deionized distilled water, saturated with NaCl pellets). The tubes were turned end over end once again for 5 minutes.

Approximately 100 µl of the washed extract was removed from the screw capped tubes with a sterile glass 9" Pasteur pipete. The extract was placed into 100 µl glass inserts (Agilent, Newark, DE). The glass inserts were housed in an Ultra 2 column of 25m x 0.2mm ID x 0.3 µm film thickness of phenyl methyl silicone glass vials (Hewlett-Packard Co., Palo Alto, CA). Eleven mm crimp tops (Agilent, Newark, DE) were securely fastened to the top of the vials to prevent evaporation of the bacterial cellular fatty acid.

Standards and blanks were placed in the HP 5890A gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) to standardize the equipment prior to the injection of the unknown samples. The chromatograph is equipped with a model HP 6763 autosampler (Hewlett-Packard), a flame ionization detector and a model HP-3392A integrator (Hewlett-Packard). The air gas flow rate through the chromatograph was 400ml/min, 30 ml/min for hydrogen, and 30 ml/min for nitrogen. The temperature used in the injection port was 250°C and 300°C for the detector. After injection, the oven temperature of the apparatus was ramped from 170°C to 270°C at a rate of 5°C/min followed by an additional increase from

270°C to 310°C at a rate of 30°C/min. This end temperature was held for 2 min before returning to 170°C prior to the injection of the subsequent sample.

The MIS software was used to calculate the percentage of area for each compound in its library, comparing it with the total area of the compound detected. Compounds were identified by using the Aerobic TSBA Version 4.0 Library and the 3.9 version for anaerobes.

Gas Analyzer

The ratio of gas present in both the SimpleStep® trays and the snap-lid tubs was analyzed using the CheckPoint O₂/CO₂ (PBI Dansensor America, Glenrock, NJ). Testing was conducted on days 0, 4, 6, 8, 10, 12 using 25 gauge 1 ½ sterile needles (Becton Dickinson, Franklin Lakes, NJ) and 13mm filters (FisherScientific, Pittsburgh, PA). Tabs of weather-stripping were placed on the Cryovac film and the snap-lid tops to protect the integrity of the package prior to the insertion of the needle.

Most Probable Number (MPN) Test for Coliforms and Fecal Coliforms

Coliform testing procedure was adopted from the FDA MPN 5-tube shellfish protocol (7). Each container of crab meat on each testing day was evaluated. Four dilutions from each container of crab meat were made and two ml from each dilution was added to five separate tubes of 10 ml of Lauryl Tryptose Broth (LST; BBL, Sparks, MD) with inverted Durham tubes (VWR, West Chester, PA). Tubes were incubated at 35°C for 48 hours. The production of gas in the Durham tube or bubbles rising from the bottom of the tube was considered positive for coliforms.

In the event of an MPN gas positive LST tube as mentioned above, one 10 µl loopful of broth from that tube would be transferred to 10 ml of *E. coli* broth (EC broth medium, Difco, Sparks, MD) containing an inverted Durham tube (VWR, West Chester, PA). Tubes were incubated in a covered circulating water bath at 44.5°C for 24 hours. After 24 hours, the tubes were observed for gas production. Gas production in the Durham tubes or bubbles rising from the bottom of the EC broth was considered positive for fecal coliforms.

Statistical Analysis

The data was analyzed using a completely randomized design. All statistical analyses were conducted using SAS, version 9.1 (SAS Institute, Cary, NC). The mean log survival of aerobic and anaerobic bacterial growth from standard plate counts on TSA and BHI agar (respectively) were analyzed using the general linear model (GLM) and a model mean of the data was compared using the least squares means (LSD) for effect. The data readings from the O₂ and CO₂ gas analysis output and coliform MPN were also analyzed using the GLM and the model means of the data were compared using LSD.

Results

Microbial Spoilage in the Shelf-life Study

Preliminary studies were performed to identify appropriate sampling days for aerobic plate counts and anaerobic counts, and to compare the shelf-life of fresh crab meat in traditional polyethylene snap-lids versus SimpleStep® tray with Cryovac™ film with an OTR of 10,000 cc/m²/24hr. A storage temperature of 4°C was chosen for the shelf-life study.

Twelve days was chosen as the study period and spoilage was defined as microbial numbers at or above 10^7 CFU/g.

Aerobic Growth

At 4°C, the packaged fresh crab meat reached 7.0 log CFU/g (microbially spoiled) on or at 12 days of storage (Figure 1 A). Aerobic plate count on day 0 for the SimpleStep® trays was 5.12 log CFU/g and 4.97 log CFU/g for the traditional snap-lids. On day 12, aerobic plate count for the SimpleStep® trays was 7.50 log CFU/g and 7.53 log CFU/g for the traditional snap-lids. Aerobic plate count of crab meat held at 4°C, showed that the effect of Cryovac™ film in 10,000 cc/m²/24hr OTR versus the polyethylene snap-lid was not significant at $P>0.05$, but was significant at the $P<0.10$.

Anaerobic Growth

At 4°C, the packaged fresh crab meat reached 7.0 log CFU/g (microbially spoiled) at 12 days or less (Figure 1 B). Anaerobic microbial growth on day 0 for the SimpleStep® trays was 4.57 log CFU/g and 4.23 log CFU/g for traditional snap-lids. The anaerobic plate count on day 12 for the SimpleStep® trays was 7.13 log CFU/g and 7.33 log CFU/g for the traditional snap-lids. Anaerobic plate counts for the two different containers were statistically significant ($P<0.05$).

Gas Analysis

The rate of O₂ and CO₂ gas remained consistent for both types of packaging during the first 8 days of sampling in the first two trials. On day 10 for trial 1, the concentration of CO₂ in the polyethylene snap-lid tubs increased slightly, and O₂ levels decreased. At day 12, the CO₂ in the snap-lid tubs was still slightly higher, but O₂ increased to normal levels. Both CO₂ and O₂ remained constant throughout the second repetition. In the third repetition, CO₂ increased on day 6 and remained elevated until day 12. The O₂ levels dropped on days 4-10, but recovered on day 12. There were differences in O₂ levels within replications (P<0.05). Carbon dioxide concentrations in the snap-lid containers were higher compared with the SimpleStep® trays (P<0.05). Sample days were not statistically significant (P>0.05) (Table 2.1).

Coliform Count

The fresh crab meat was assayed for presumptive coliforms and fecal coliforms (Table 2). Fecal coliforms were detected only in the third replication. Based on analysis, neither container nor day influenced the fecal coliform count (P>0.05) (Table 2.2).

Discussion and Conclusion

Microbial Spoilage in the Shelf-life Study

There were no differences between the industry standard polyethylene snap-lid cups and the SimpleStep® trays with Cryovac™ 10,000 OTR film (P>0.05). Gates et al compared fresh crab meat in traditional cups with non barrier pouches (8). In his study, between oxygen barrier and non barrier pouches he found that barrier pouch packaging produced lower quality and shorter shelf-life crab meat (8).

Microbially spoiled meat was determined as bacterial counts at or above 10^7 CFU/g. No strict anaerobes were detected in any of the replications, which agrees with a fresh crab meat study performed by Suklim et al (17). Additionally, Ward et al noted that when anaerobic colonies were examined, the organisms isolated were identified as facultative lactobacilli (19), further indicating that no strict anaerobes were present in the sampled crab meat. Neither package type had differences in shelf-life indicating that package types had no effect on microbial shelf-life.

Before deciding if a new packaging material should be used, it is necessary to know what will cause product deterioration and the effects of commercial shipping and handling on package failure rate. Ideally, the expectation of new packaging through advanced technologies is to extend the shelf-life of perishable food products. The results of this microbial shelf-life study suggest that there were no differences in microbial concentrations between the SimpleStep® trays with Cryovac™ 10,000 OTR film or the polyethylene snap-lid cups that can be attributed to package type ($P>0.05$).

Gas Analysis

The difference in the concentration of CO₂ in the SimpleStep® trays with Cryovac™ 10,000 cc/m²/24hr OTR compared with the polyethylene snap-lid tubs on days 10 and 12 (first repetition) and day 4 through 12 of the third replication may be due to the production of CO₂ gas and from fermented lactose or the consumption of O₂ by the aerobic microorganisms (13, 14). CO₂ can inhibit the growth of spoilage microorganisms, increasing the shelf-life of certain food products (1, 3). An increase in shelf-life was not observed in this study perhaps due to the package type and the level of fat present in the crab meat (3).

No consistent trends in CO₂ levels were observed in any repetition, making it difficult to identify a cause for the CO₂ gas fluctuation. Both CO₂ increases in replications one and three occurred in the polyethylene or polypropylene snap-lid tubs suggesting that the tubs may be less efficient in releasing CO₂ into the outside environment compared with the Cryovac™ 10,000 cc/m²/24hr OTR film.

Coliform Count

The absence of coliforms and fecal coliforms in the first two trials of fresh crab meat samples indicate hygienic handling during packaging. The handling technique for the third trial was different compared with the first two trials. The SimpleStep™ trays in the first two trials were of a heterogeneous batch of crab meat, packaged in a lab at UMES. The trays for the third trial were packaged at a crab meat processing facility in Cambridge, MD. Also, a third party participant helped remove the crab meat from the polyethylene tubs and re-pack it into the SimpleStep™ trays.

Coliforms are facultatively anaerobic bacteria, which may explain how they were able to survive and persist in the CO₂ environment provided by the polyethylene tubs in the third repetition (4, 6). When the coliforms metabolized the available nutrients, CO₂ and acid were released in the form of gaseous bubbles, indicative of sanitary conditions at the food-processing environment (6, 13). There are several factors that influence the increase of coliforms (4). Water temperatures, which typically influence the increase of coliforms, were warmest in the first repetition, in which no coliforms were isolated (18). Biofilms can be resistant to disinfectants (9) and harbor coliforms. Perhaps the working surface in the processing plant contained a biofilm. Coliforms can encapsulate themselves to survive harsh

environments and cross-contaminate the crab meat. Although, *Aeromonas* spp. is not included in the coliform group, but it is a common isolate of crab meat, which can generate false positive total coliform reactions due to its ability to ferment lactose (4). In conclusion, the difference in the occurrence of coliforms in the SimpleStep™ trays versus the traditional snap-lid tubs in the third trial may be due to cross contamination of the crab meat during packaging at the crab processing plant.

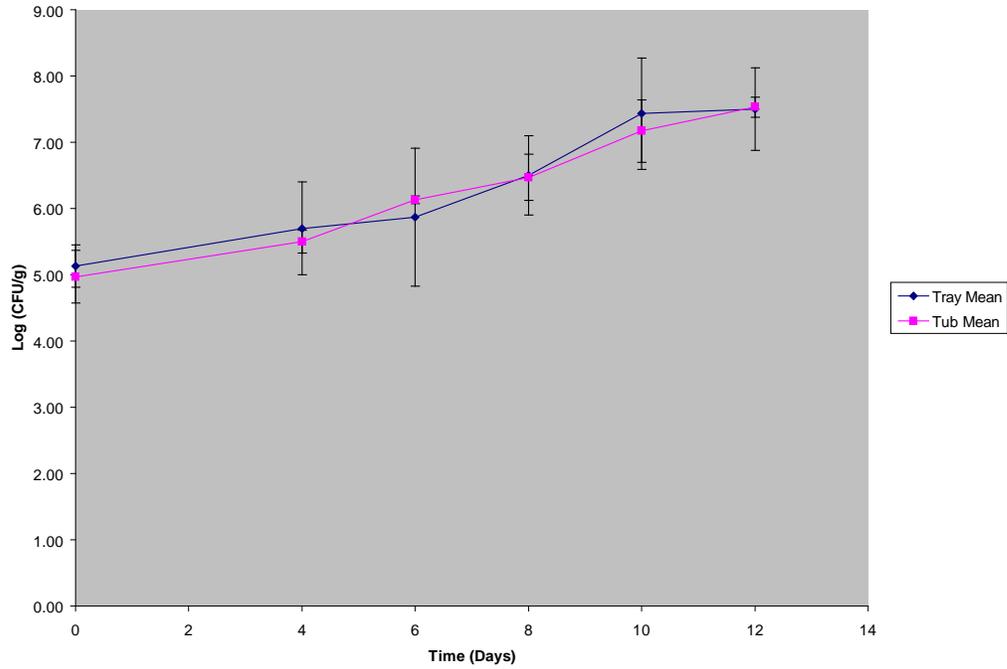
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Figure 2.1. Standard Aerobic (A) and Anaerobic (B) Plate Count Method of Fresh Crab Meat Stored at 4°C.

(A) Aerobic Trays and Tubs



(B) Anaerobic Trays and Tubs

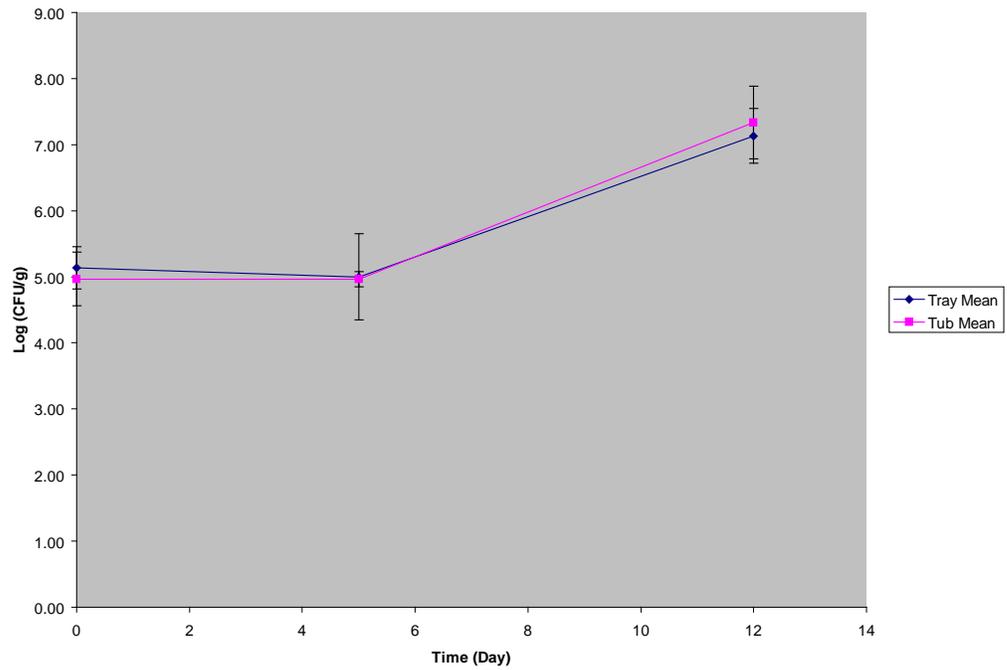


Table 2.1. Headspace gas analysis of SimpleStep® trays with Cryovac™ 10,000 cc/m²/24hr oxygen transmission rate (OTR) film and the traditional polyethylene snap-lid tubs for fresh crab meat stored at 4°C.

Gas Composition (%)

Days of Storage	Trial 1 ^A		Trial 1 ^B		Trial 2 ^A		Trial 2 ^B		Trial 3 ^A		Trial 3 ^B	
	CO ₂	O ₂										
0	0.1	21.0	0.0	20.9	0.2	20.7	0.2	20.7	0.0	20.7	0.0	20.8
4	0.1	20.8	0.1	20.6	0.0	21.1	0.0	21.0	0.1	20.7	1.1	3.9
6	0.1	20.9	0.1	20.9	0.0	20.8	0.0	20.7	0.1	20.6	8.6	0.8
8	0.1	20.9	0.1	20.9	0.0	20.9	0.0	21.1	0.0	20.8	12.5	2.7
10	0.1	21.1	0.2	0.9	0.0	20.7	0.0	20.6	0.1	20.6	12.8	0.5
12	0.1	20.8	1.1	20.8	0.0	20.7	0.0	20.8	0.1	20.6	0.1	16.5

^A denotes SimpleStep® trays with Cryovac™ 10,000 cc/m²/24hr OTR film

^B denotes traditional polyethylene snap-lid tubs

¹ Packaging procedure for trial 3 was performed at a different location than trial 1 and trial 2.

Table 2.2. Presumptive coliforms and fecal coliform (Log 10) for the shelf-life study between SimpleStep® trays with Cryovac™ 10,000 cc/m²/24hr oxygen transmission rate (OTR) film and traditional polyethylene snap-lid tubs. Three trials were performed on fresh crabmeat stored at 4°C.

Coliforms and Fecal Coliforms

Days of Storage	Trial 1 ^A		Trial 1 ^B		Trial 2 ^A		Trial 2 ^B		Trial 3 ^A		Trial 3 ^B	
	C	FC	C	FC								
0	<.255	-	<.255	-	<.255	-	<.255	-	.44	.255	.272	<.255
4	<.255	-	<.255	-	<.255	-	<.255	-	1.16	<.255	.272	<.255
6	<.255	-	<.255	-	<.255	-	<.255	-	.272	<.255	1.27	<.255
8	<.255	-	<.255	-	<.255	-	<.255	-	.580	<.255	2.92	<.255
10	<.255	-	<.255	-	<.255	-	<.255	-	<.255	<.255	2.92	<.255
12	<.255	-	<.255	-	<.255	-	<.255	-	.837	<.255	3.49	<.255

^A denotes SimpleStep® trays with Cryovac™ 10,000 cc/m²/24hr OTR film

^B denotes traditional polyethylene snap-lid tubs

¹ 10 grams sample size of crab meat used.

² packaging procedure for trial 3 was performed at a different location than trial 1 and trial 2.

³ C represents coliforms

⁴ FC represents fecal coliforms

⁵ Results reported in log form

Table 2.3. Microorganisms isolated from fresh crab meat stored at 4°C in SimpleStep® trays with Cryovac™ 10,000 cc/m²/24hr oxygen transmission rate (OTR) film and traditional polyethylene snap-lid tubs.

	SimpleStep™ trays	Polyethylene snap-lid tubs
Microorganisms		
<i>Myroides odoratus</i>	•	•
<i>Bacillus sphaericus</i>		•
<i>Staphylococcus xylosum</i>	•	•
<i>Staphylococcus cohnii</i>	•	•
<i>Staphylococcus gallinarum</i>	•	
<i>Kirithia gibsonii</i>		•
<i>Bacillus marinus</i>	•	
<i>Micrococcus luteus</i>		•
<i>Cellulomonas fimi</i>		•
<i>Kocuria varians</i>	•	•
<i>Staphylococcus hominis</i>		•
<i>Staphylococcus warneri</i>	•	
<i>Staphylococcus arlettae</i>		•
<i>Staphylococcus kloosii</i>	•	•
<i>Exiguobacterium acetylicum</i>	•	•
<i>Schwanella putrefaciens</i>	•	•
<i>Acinetobacter johnsonii</i>	•	•
<i>Aeromonas caviae</i>	•	
<i>Erwinia carotovora</i>	•	
<i>Neisseria</i>	•	
<i>Alcaligenes</i>	•	
<i>Pseudomonas putida</i>	•	

<i>Chromobacterium, spp.</i>	•	
<i>Enterococcus faecalis</i>	•	
<i>Pseudomonas nautica</i>		•
<i>Aerococcus viridans</i>	•	•
<i>Carnobacterium piscicola</i>	•	•
<i>Lactococcus plantarum</i>		•
<i>Corynebacterium ammoniage</i>		•
<i>Staphylococcus caseolyticus</i>		•
<i>Streptococcus bovis</i>	•	•
<i>Pseudomonas putida</i>	•	
<i>Acinetobacter calcoace</i>		•
<i>Staphylococcus chromogenes</i>		•
<i>Staphylococcus sanguis</i>		•
<i>Bacillus cereus</i>	•	
<i>Streptococcus mutans</i>	•	

• indicates that bacteria was found

CHAPTER 3

Inhibition of *Listeria monocytogenes* by *Carnobacterium piscicola* in Fresh and Pasteurized Crab Meat

Abstract

The effect of temperature, storage time and ability of *Carnobacterium piscicola* to inhibit *Listeria monocytogenes* at different concentration levels was examined. Twenty-five g samples of fresh and pasteurized blue crab meat (*Callinectes sapidus*) were inoculated with 0.1ml *C. piscicola* and 0.1ml *L. monocytogenes*. *C. piscicola* was administered at a 2 log CFU/g, 4 log CFU/g or 6 log CFU/g concentration and *L. monocytogenes* was administered at a constant concentration (2 log CFU/g). Three different concentration levels (2:2 log CFU/g, 4:2 log CFU/g and 6:2 log CFU/g) were evaluated on selected days at 4°C and 10°C. The inoculated crab meat was plated on modified *Lactobacillus* deMan Rogosa Sharpe (mMRS, pH 7) and modified oxford agar (MOX). The negative control was plated on trypticase soy agar (TSA) and the positive control on MOX. Microbial spoilage of the crab meat was defined as 10^7 CFU/g. For fresh crab meat, at 4°C and 10°C, spoilage occurred at 7 days or less. For pasteurized crab meat, at 4°C and 10°C, storage life was 26 days or more. Due to nominal TSA growth in pasteurized crab meat, studies were terminated after 28 days of storage. The growth of *L. monocytogenes* in fresh crab meat was significant for the different concentrations ratios ($P < 0.05$) and days ($P < 0.05$), but not for temperature ($P > 0.05$). The growth of *C. piscicola* in fresh crab meat was significant for the different concentration ratios ($P < 0.05$), days ($P < 0.05$) and temperature ($P < 0.05$). The growth of *L. monocytogenes* in pasteurized crab meat was significant for the different concentrations ratios ($P < 0.05$) and days ($P < 0.05$) and temperature ($P < 0.05$). The growth of *C. piscicola* in pasteurized crab meat was significant for the different concentration ratios ($P < 0.05$), days ($P < 0.05$) and temperature ($P < 0.05$). In both fresh and pasteurized crab meat, regardless of the inoculation ratios, the *L. monocytogenes* and *C. piscicola* appeared to follow the similar growth trends,

but the level of *L. monocytogenes* was higher in the 2:2 CFU/g concentration and lower at the 6:2 CFU/g concentration level. *Carnobacterium piscicola* did not completely inhibit *L. monocytogenes* growth at any level, some degree of inhibition was observed.

Introduction

Crab processors are aware of the possible contamination of crabmeat with pathogens. Crab meat's ready-to-eat (RTE) status makes the control of *L. monocytogenes* a high priority. *Listeria monocytogenes* is a psychrotroph capable of growth at refrigeration temperatures (20). The pathogen can be found in a wide variety of fish and shellfish products and can be isolated from different water sources (15). The ability for *Listeria*, spp. to persist in the marine environment and fresh water streams, indicates that fish and other aquatic animals can be contaminated in their natural habitat (12). Fuad et al evaluated the ability of *L. monocytogenes* to survive in estuarine waters and fresh seafood. They concluded that there is a higher incidence of *Listeria* present in chitinous seafood including shrimp, crab and lobster (6, 12).

According to the Food and Drug Administration's (FDA) Enforcement Reports and Jinneman et al, there were approximately 126 Class I recalls since 1987 issued in the U.S. for domestic and domestic/imported ready-to-eat (RTE) seafood products (12). Crabs accounted for 46 of the 126 recalls (12). In most cases there were no known illnesses or outbreaks associated with *L. monocytogenes* in the food (9).

Lactic acid bacteria (LAB) have the ability to produce antimicrobial compounds (bacteriocins) that have inhibitory characteristics towards other bacteria (5). There are numerous strains of LAB containing different receptors with broad spectrums of inhibition towards pathogens (1, 17). Lactic acid production is augmented by the production of hydrogen peroxide and organic acid, reducing the pH of the food (5, 16). The lower pH assists in the survival of fermenting lactic acid in the food (10) and interact and kill susceptible pathogenic bacteria through membrane lysis or inhibiting essential cell processes

(protein synthesis or DNA synthesis) (17). Bacteriocins inhibit Gram-positive bacteria, making it an attractive option (1, 16, 17) for companies to consider using in order to improve food safety.

Americans want safe, wholesome products with minimal processing or food additives (5, 7, 17). Biopreservation is the use of non-pathogenic microorganisms, and/or metabolites to ensure food safety and extend shelf-life of food (1, 4, 5, 7, 17). Bacteriocins, are safe additives, and a natural alternative to preservation methods (11, 16) and other food preservation methods. The prospective of using a bacteriocin from a LAB producing strain to improve food safety and food quality is a novel approach (18). Its use in hurdle technology to restrict the growth of pathogenic bacteria is innovative and practical (18). Bacteriocins have the ability to inhibit the growth of pathogenic microorganisms, while maintaining the nutritive quality of food and extending shelf life (1, 5, 7). Food companies may benefit by using a bacteria to control undesirable bacteria in a natural manner, appealing to more consumers (5). Bacteriocins have been used in fishery products, but there is little to no information on bacteriocin use in crab meat products (2, 8, 14, 18, 21).

Objective

Evaluate the effectiveness of *C. piscicola* bacteriocin to inhibit growth of *L. monocytogenes* in fresh and pasteurized crab meat.

Materials and Methods

Bacteriocin Confirmation

Bacteriocin activity was monitored using a spot on lawn technique (14). *Carnobacterium piscicola* was grown in 100ml of modified *Lactobacillus* deMan Rogosa Sharpe broth (mMRS broth, Acumedia, Lansing, MI) for 24 hours at 28°C. The mMRS broth with *C. piscicola* growth was shaken well and mMRS plates were stab inoculated in a particular pattern and incubated at 28°C for up to 72 hours under anaerobic conditions. Once growth was visible, the plates were overlaid with approximately 6ml of Brain Heart Infusion agar (BHI; BBL, Sparks, MD) seeded with approximately 2 log (CFU/g) of *L. monocytogenes*, and again incubated at 28°C for 24 hours. The plates were examined for zones of inhibition of growth of *L. monocytogenes*. Measurements were taken (ruler #15-100-100, Manostat Corp, Switzerland) from the edge to edge of the inhibited zone. The diameter of the zones was measured in millimeters. An additional carbohydrate metabolism test, API 50 CH (Biomérieux, Marcy l'Etoile, France), was performed to confirm the strain.

Culture Preparation and Maintenance of *Carnobacterium piscicola*

A *C. piscicola* strain suspected of producing a bacteriocin was isolated from cellular fatty acid identification of fresh crab meat conducted at the Food Science and Technology (FST) in Blacksburg, VA in 2007. The strain was subjected to an API CH 50 *Lactobacillus*

test for strain confirm.. Once verification of the strain was confirmed, it was placed in 100ml of mMRS broth. The culture was allowed to grow at 28°C for 24 hours. MRS was modified by combining powdered MRS medium, with 1 N NaOH to bring the pH to 6.8 ± 0.2 . Comparison growth tests of the *C. piscicola* were performed with tryptic soy agar (TSA, BBL, Sparks, MD), MRS agar (Difco, Sparks, MD) and modified MRS agar (mMRS) to confirm that optimal growth of bacteriocin was pH 7.0 (13). This also confirmed the hypothesis that the best medium to use was modified MRS. After growth at 28°C, the *C. piscicola* strain was streaked in two successive transfers onto mMRS, incubated at 28°C. The strain was divided into cryo-vials containing 20% glycerol and 80% mMRS broth medium and stored at -70°C.

Culture Preparation and Maintenance for *Listeria monocytogenes*

A *L. monocytogenes* strain isolated from raw crabs from a Maryland crab meat processing plant was used in this study. It was shipped overnight on a TSA slant to the FST department in Blacksburg, VA. Upon arrival, the strain was transferred into 10ml of tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; BBL, Sparks, MD) and incubated at 10°C for 60-72 hours. The culture was streaked onto multiple modified oxford agar plates (MOX; Difco, Sparks, MD) supplemented with antimicrobial agent (Difco, Sparks, MD) and incubated at 35°C for 48 hours. Positive colonies were confirmed using *Listeria* API strips (Biomerieux, Marcy l'Etoile, France). After positive identification, 10ml of the TSBYE was transferred into a solution of 60% nutrient broth (Difco, Sparks, MD) with 40% glycerol (MP Biomedicals, LLC, Solon, OH) and stored at -70°C.

Concentration Ratio Preparation

For each repetition, one cryo-vial from the -70°C freezer of *L. monocytogenes* and one cryo-vial of *C. piscicola* were thawed to room temperature. From each respective cryo-vial, a 10µl loopful (Fisher Scientific, Pittsburg, PA) was transferred into 10ml of broth. From the *L. monocytogenes* cryo-vial, 10µl was transferred into TSBYE and incubated at 10°C for 60-72 hours. A 10µl loopful from the *C. piscicola* cryo-vial was transferred into 10ml of mMRS broth medium and incubated for 24 hours at 28°C.

After incubation, each respective broth containing bacteria was dispensed into a sterile 15ml polypropylene centrifuge tube (Becton Dickinson, Franklin Lakes, NJ). The tubes were centrifuged at 3000 RPM for 10 min in an IEC HN-S centrifuge (Damon/IEC Division, Needham Hts, MA). After 10 min, the supernatant was removed and the remaining pellet was subjected to a wash of 10ml of 0.1% peptone, and re-centrifuged for an additional 10 minutes. This peptone wash was performed 3 times. At the end of the third wash, the centrifuge tube was shaken to suspend the bacterial cells and subsequent dilutions were made. A working suspension of *C. piscicola* using serial dilutions of 0.1% peptone solution to achieve target inoculation levels of 2 logs, 4 logs and 6 logs (CFU/g) was performed. A working suspension of 2 log (CFU/g) of the *L. monocytogenes* strain was prepared from the TSBYE washes for inoculation into the crab meat.

Product Preparation for Fresh and Pasteurized Crab Meat

Fresh and pasteurized crab meat was obtained from a commercial processor and shipped overnight to the FST department in Blacksburg, VA. Fresh crab meat arrived in FDA approved prime-grade polypropylene plastic tubs with snap lids. Pasteurized crab meat

arrived in 16 oz metal cans. In both cases, the crab meat was taken out of original packaging and 25g was placed into smaller, sterile 2oz plastic soufflé containers (Monogram, Dixie, Atlanta, Georgia) and immediately refrigerated. Two oz soufflé containers were sterilized prior to use by placing them under UV light for 10 minutes. After all of the crab meat from the original containers was placed into the soufflé containers, they were removed from the refrigerator and placed on the lab bench in no particular order. The soufflé containers were then arbitrarily labeled coinciding with the concentration ratios of *L. monocytogenes* and *C. piscicola* that they would receive and randomly assigned either 4°C or 10°C refrigerator. Each refrigerator (4°C and 10°C) had a random array of where the tubs were placed. This was also done to minimize variation within the product due to possible temperature difference within the refrigerator.

Product Inoculation

Five different log (CFU/g) concentrations of the *Carnobacterium: Listeria* ratio were tested (0:0, 0:2, 2:2, 4:2, and 6:2). Inoculation of the two bacterial microorganisms was performed separately. The concentration of *Listeria monocytogenes* inoculated into the crab was held constant at 2 log CFU/g (with an exception to the negative control) and *C. piscicola* varied. The negative control was uninoculated crab meat and the positive control was inoculated with *L. monocytogenes* and received no *C. piscicola* inoculum. A total inoculum volume of 0.1ml of each bacterium was dispensed directly over the 25 g portions of crab meat in several different places and mixed thoroughly using a sterile instrument. Each individual container of crab meat was treated, covered and placed into the refrigerator (either 4°C or 10°C).

Product Incubation and Sampling

Testing days were designated from the time that the crab meat arrived at Virginia Tech's FST building. Due to the quick spoilage of the fresh crab meat and the lack of bacterial growth in the pasteurized crab meat in preliminary studies, sampling days were slightly different for the fresh than for the pasteurized crab meat. Fresh crab meat held at 4°C, testing days were 0, 2, 4, 6, 8, and 10 or until spoilage (10^7 CFU/g). Fresh crab meat held at 10°C, testing was performed on days 0, 1, 3, 5, 7, and 9 or until spoilage (10^7 CFU/g). Fresh crab meat was sampled more frequently than the pasteurized crab meat due to its shorter shelf-life. Pasteurized crab meat held at 4°C, testing days were 0, 6, 10, 14, 18, 22, 26 and 28 or until spoilage (10^7 CFU/g). Pasteurized crab meat held at 10°C, testing was performed on days 0, 6, 10, 12, 14, 18, 22 and 24 or until spoilage (10^7 CFU/g). Three samples of each concentration level at each temperature (4°C and 10°C) were evaluated on each sampling day. Each 25 g sample of the crab meat was placed into a sterile 52oz stomacher bag (Nasco, Ft. Atkinson, WI) with 225 ml of 0.1% peptone. The sample was homogenized in a Stomacher Lab Blender 400 (Tekmar Co., Cincinnati, OH) for 30 s. Using a sterile, prepackaged pipette (1 ml in 1/100 ml, FisherScientific, Pittsburg, PA), subsequent dilutions were made using 9 ml of 0.1% peptone dilution blanks. Dilutions of the homogenate were taken and spread plated onto corresponding media. MOX was used for *L. monocytogenes*, TSA used for the total plate count and modified MRS agar was used for *C. piscicola*. MOX and TSA were incubated at 35°C and mMRS was incubated at 28°C. Randomly, colonies from the mMRS were subjected to API CH 50 tests and colonies from the MOX plates were subjected to Listeria API tests. The pH (Accumet model 15, Denver

Instrument Co., Denver, CO) of uninoculated fresh and pasteurized crab meat was taken on the day of arrival to VT's FST building. The electrode was submerged into one 25 g sample every sampling day per type of crab meat.

Statistical Analysis

Statistical analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC). This study was analyzed using a general linear model procedure with *C. piscicola* and *L. monocytogenes* as independent variables. The mean log survival of *L. monocytogenes* and *C. piscicola* were determined using standard plate counts on MOX and mMRS plates, respectively. A paired comparison was conducted using a Least Squares Means (LSD) test to determine differences between the means.

Results

Product Inoculation

Fresh Crab Meat

The mean inoculation level of *L. monocytogenes* in fresh crab meat at 4°C on day 0 was 2.4 log (CFU/g). The mean inoculation level of *L. monocytogenes* in fresh crab meat at 10°C on day 0 was 2.5 log (CFU/g). The inoculation level for the positive control in fresh crab meat at 4°C and 10°C was 2.3 log CFU/g and 2.5 log CFU/g, respectively. The range of *L. monocytogenes* survival in control treatments throughout the study in the fresh crab meat at 4°C was 2.3 log (CFU/g) – 4.3 log (CFU/g). The range of *L. monocytogenes* survival in control treatments throughout the study in the fresh crab meat at 10°C was 2.5 log (CFU/g) – 4.7 log (CFU/g). (Figure 1)

Pasteurized Crab Meat

To confirm this inoculation level of *L. monocytogenes* in crab meat, it was sampled immediately after inoculation. The mean inoculation level of *L. monocytogenes* in pasteurized crab meat at 4°C on day 0 was 2.6 log (CFU/g). The mean inoculation level of *L. monocytogenes* in pasteurized crab meat at 10°C on day 0 was 2.3 log (CFU/g). Throughout the study, the *L. monocytogenes* concentration in pasteurized crab meat controls at 4°C ranged from 2.7 log (CFU/g)-9.0 log (CFU/g). *Listeria monocytogenes* survival in control treatments throughout the study in pasteurized crab meat at 10°C ranged from 2.3 log (CFU/g)-9.1 (CFU/g). (Figure 2)

Reduction of *L. monocytogenes* in treatments

Fresh Crab Meat

L. monocytogenes

Listeria monocytogenes growth in fresh crab meat was significant with the varying concentrations of *C. piscicola* (P<0.05), the sampling days (P<0.05) and the interaction between the two (P<0.05). Temperature was not significant (P>0.05). Using LSD to compare overall means of the concentration ratios, 2:2 was not significantly different from 4:2 (P>0.05). Concentration 4:2 was not significantly different from 6:2 (P>0.05), but 2:2 was significantly different from 6:2 (P<0.05). Overall pair-wise comparison of different sampling days showed significance (P<0.05). Bacterial concentration (CFU/g), day and temperature are shown in Table 1.

C. piscicola

Carnobacterium piscicola growth in fresh crab meat was significant within the two different temperatures (4°C and 10°C) (P<0.05), the different concentration levels (P<0.05) and the sampling days (P<0.05). The interaction between these factors were not significant (P>0.05). Concentrations of 2:2 and 4:2 were not significant (P>0.05), but were both significant at 6:2 concentration (P<0.05). Overall pair-wise comparison of different sampling days revealed that days were significantly different from each other (P<0.05). Also, 4°C and 10°C were found to be significantly different from each other (P<0.05). Means of bacterial concentration (CFU/g), day and temperature are shown in Table 1.

Pasteurized Crab Meat

L. monocytogenes

The growth of *L. monocytogenes* in pasteurized crab meat, was found to be significant within varying concentration levels of *C. piscicola* (P<0.05), sampling days (P<0.05) and temperatures (P<0.05). All concentrations levels of *C. piscicola* to *L. monocytogenes* were significant from each other (P<0.05). Pair-wise comparison of the different sampling days indicated that days 6, 7 and 8 were not different from each other (P>0.05). The remaining sampling days were significantly different from days 6, 7 and 8 and each other (P<0.05). The bacterial concentrations (CFU/g) by day and temperature are shown in Table 2.

C. piscicola

Growth of *C. piscicola* in pasteurized crab meat was found to be significant within the different concentration levels of *C. piscicola* to *L. monocytogenes* (P<0.05), sampling days (P<0.05) and temperatures (P<0.05). Sampling days 1, 2, 4 and 5 were significantly

different from each other and significantly different from the remaining sampling days ($P < 0.05$). Days 3 and 8 were not significantly different from each other ($P > 0.05$), but were different from the other sampling days ($P < 0.05$). Day 8 was not different from day 6 ($P > 0.05$), but both were different from the other sampling days ($P < 0.05$). Day 6 and 7 were not different from each other ($P > 0.05$), but were different from the other testing days ($P < 0.05$). Means of bacterial concentration (CFU/g), day and temperature are shown in Table 2.

pH

Differences in crab meat pH were not expected since the crab meat originated from the same processor. A pH of 7.0 is optimum for bacteriocin production (13), yet the crabmeat was not altered if a pH of 7.0 was not attained. The range of pH for fresh crab meat was 7.74 - 7.89, with the mean being 7.79. The range of pH for pasteurized crab meat was 7.73-7.80, with the mean being 7.70. Throughout the experiment, the pH of MRS broth and MRS agar was modified using 1 N NaOH to stimulate bacteriocin production.

Discussion and Conclusion

The bacteriocin was found to be less effective in pasteurized crab meat where background microflora was reduced by thermal processing. Pasteurized crab meat is not a sterile product (3, 19). At storage temperatures above 4.4°C, aerobic sporulation of bacilli organisms, which are not destroyed during the pasteurization process, continue to grow (3).

In both fresh and pasteurized crab meat, regardless of the inoculation ratios, the *C. piscicola* and *L. monocytogenes* followed the similar growth trends in their respective study.

Although the *C. piscicola* did not completely inhibit *L. monocytogenes* growth at any concentration level, a degree of inhibition was observed. In fresh crab meat at 4°C, the ratio of 2:2 log CFU/g and 4:2 log CFU/g, the *L. monocytogenes* grew at a rate similar to the positive control. The 6:2 log CFU/g ratio showed the most difference between *L. monocytogenes* and the positive control, with less than 1 log CFU/g difference. In fresh crab meat at 10°C a similar trend to the 4°C occurred, but the increase in growth of *L. monocytogenes* in the positive control was greater by the end of the study. At spoilage, the mean log CFU/g of *L. monocytogenes* in the 6:2 ratio of *C. piscicola* to *L. monocytogenes* was compared to the positive control, revealing less than 1 log CFU/g difference.

In pasteurized crab meat at 4°C, all ratio concentrations had similar growth trends of *C. piscicola* to *L. monocytogenes*. By day 14, all concentration ratios had lower levels (log CFU/g) of *L. monocytogenes* compared with the control. This trend continued to the end of the study. By day 28, the mean log CFU/g of *L. monocytogenes* in the 6:2 log CFU/g ratio was approximately 2.5 log CFU/g less than the control. Pasteurized crab meat stored at 10°C, had similar trends as the pasteurized crab meat stored at 4°C. By day 6, *L. monocytogenes* in all concentration ratios were the same or lower than the control (log CFU/g). By day 24, the mean log CFU/g of *L. monocytogenes* in the 6:2 log CFU/g ratio was 2.4 log CFU/g less than the control.

Yamazaki et al found that in cold smoked salmon at 4°C, *C. piscicola* did indeed inhibit *L. monocytogenes* within 21 days, but the inhibition of *L. monocytogenes* depended on the initial inoculum level of *C. piscicola* (21). Duffes et al reports a bacteriocidal effect on *L. monocytogenes* by *C. piscicola* at 4°C in vacuum-packed cold smoked salmon and a delayed

growth of the pathogen at 8°C (8). Schobitz et al had similar results of a bacteriostatic effect on *L. monocytogenes* by *C. piscicola* on vacuum packaged salmon at 4°C after 15 days (18).

When considering the use of a bacteriocin in a product, sanitary conditions are important. If unsanitary or abusive handling and storage conditions occur, bacteriocins may not work (17). The decision to use bacteriocins as a hurdle in food products is an appealing option in minimally processed, refrigerated foods (18). Future work is needed in this area, both for fresh and pasteurized crab meat. Adjusting the pH of the crab meat may also help stimulate bacteriocin activity, resulting in better control of *L. monocytogenes*.

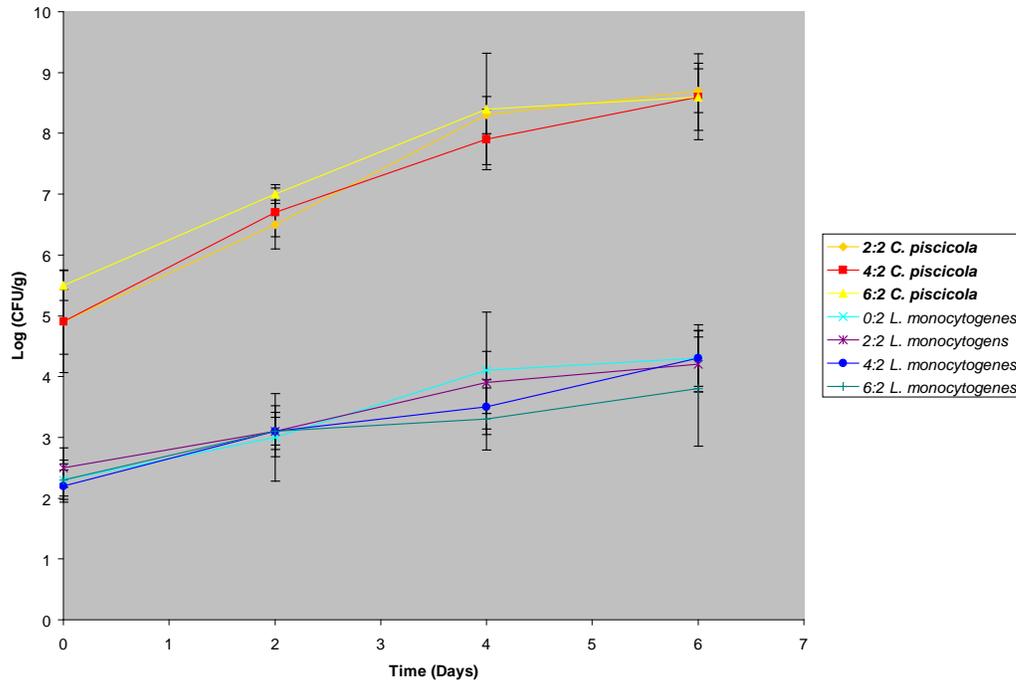
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Figure 3.1.

(A) The Growth of *Listeria monocytogenes* and *Carnobacterium piscicola* in Fresh Crab Meat Stored at 4°C.



(B) The Growth of *Listeria monocytogenes* and *Carnobacterium piscicola* in Fresh Crab Meat Stored at 10°C

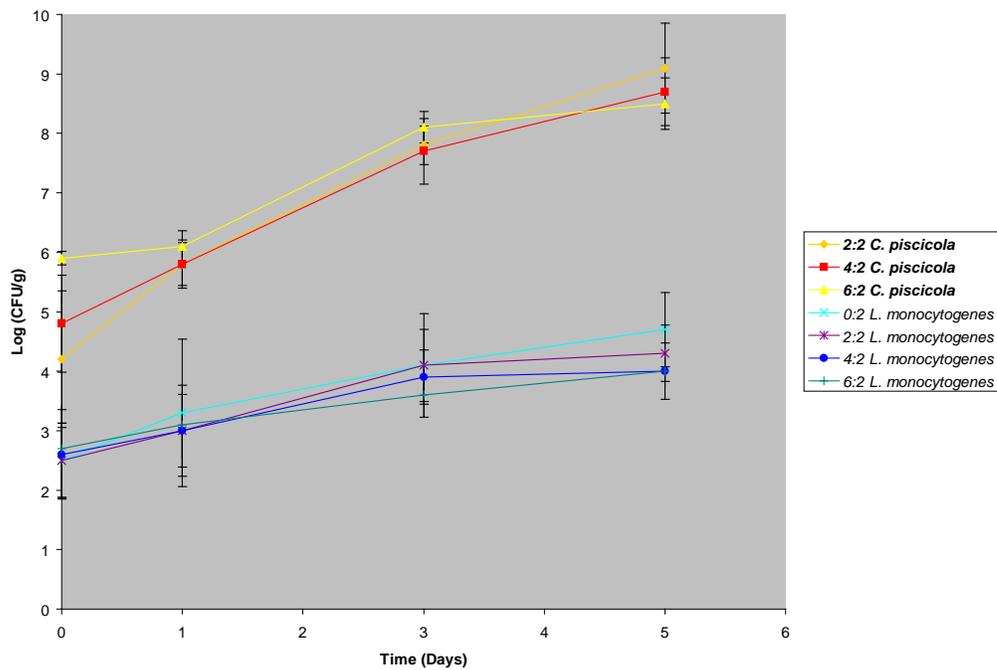
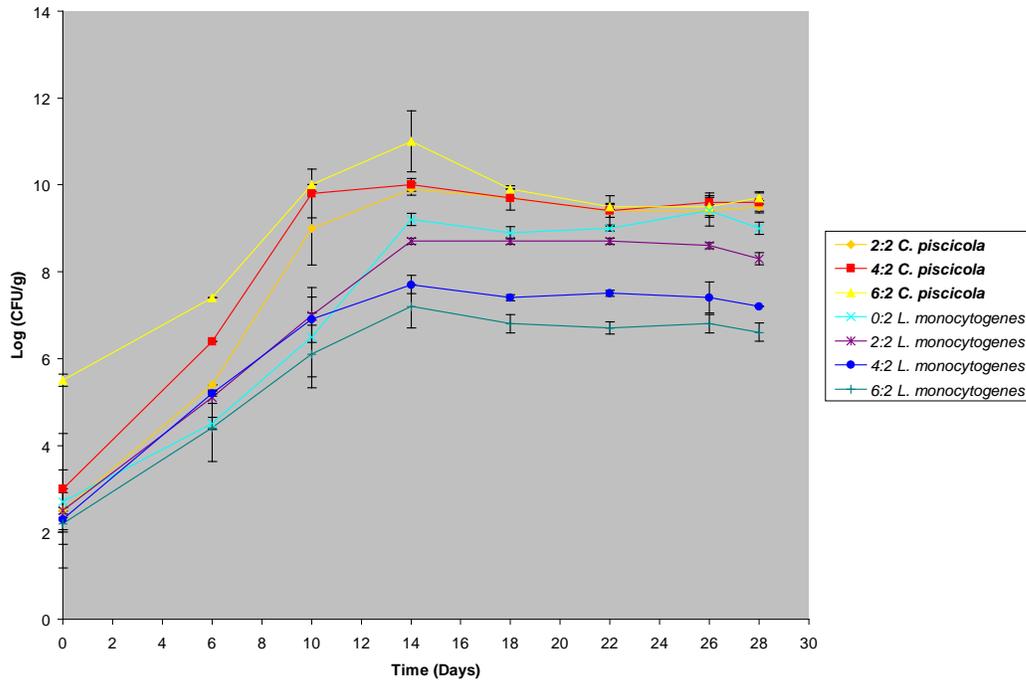


Figure 3.2.

(A) The Growth of *Listeria monocytogenes* and *Carnobacterium piscicola* in Pasteurized Crab Meat Stored at 4°C.



(B) The Growth of *Listeria monocytogenes* and *Carnobacterium piscicola* in Pasteurized Crab Meat Stored at 10°C.

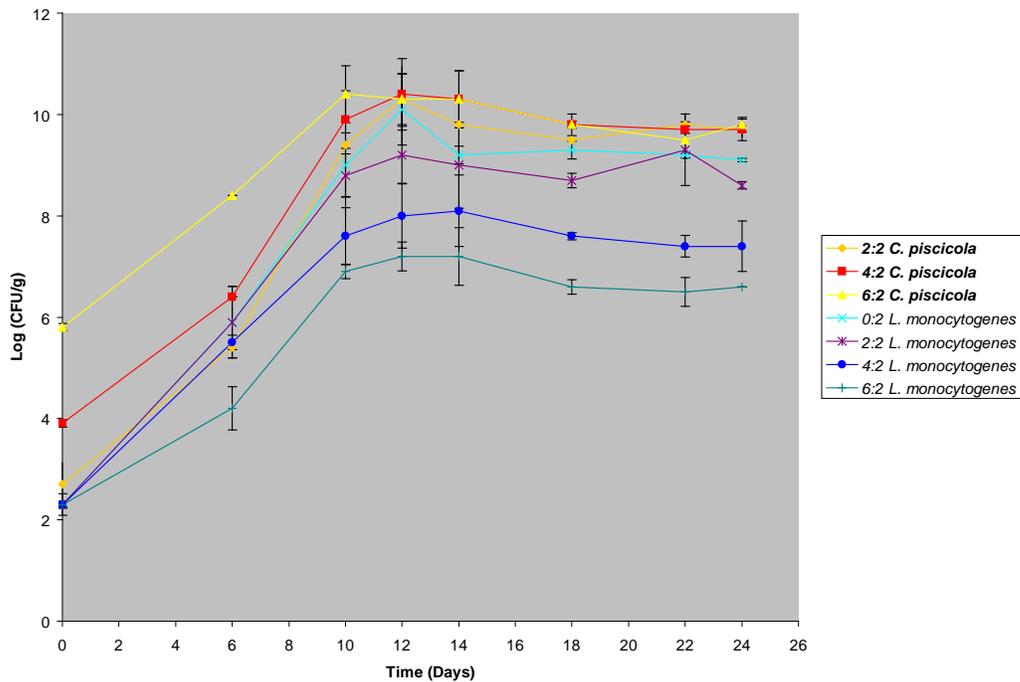


Table 3.1. The effect of individual bacterial growth in the changing ratio of *Carnobacterium piscicola* and *Listeria monocytogenes* in fresh crab meat stored at 4°C (A) and 10°C (B).

(A)

Days	Mean growth of <i>C. piscicola</i> (CFU/g) ^{2,3}			Mean growth of <i>L. monocytogenes</i> (CFU/g)			
	2:2 ¹	4:2	6:2	0:2	2:2	4:2	6:2
0	4.79 ^a +0.76	4.89 ^a +0.49	5.57 ^a +0.26	2.19 ^a +0.38	2.41 ^a +0.33	2.18 ^a +0.25	2.27 ^a +0.24
2	6.50 ^a +0.34	6.61 ^a +0.42	6.98 ^a +0.14	2.95 ^a +0.61	3.04 ^a +0.35	3.08 ^a +0.30	3.11 ^a +0.20
4	8.20 ^a +0.25	7.81 ^a +0.47	8.16 ^a +0.85	3.86 ^a +0.74	3.86 ^a +0.44	3.48 ^{ab} +0.37	3.20 ^b +0.52
6	8.63 ^a +0.36	8.44 ^a +0.53	8.47 ^a +0.64	4.22 ^a +0.41	4.15 ^a +0.38	4.18 ^a +0.53	3.55 ^a +0.77

(B)

Days	Mean growth of <i>C. piscicola</i> (CFU/g) ^{2,3}			Mean growth of <i>L. monocytogenes</i> (CFU/g)			
	2:2 ¹	4:2	6:2	0:2	2:2	4:2	6:2
0	4.27 ^a +1.1	4.69 ^{ab} +0.67	5.84 ^b +0.17	2.34 ^a +0.51	2.36 ^a +0.51	2.51 ^a +0.62	2.58 ^a +0.59
1	5.82 ^a +0.38	5.68 ^a +0.47	6.12 ^a +0.25	3.07 ^a +1.0	2.82 ^a +0.71	2.87 ^a +0.53	2.95 ^a +0.48
3	7.66 ^a +0.43	7.69 ^a +0.52	8.04 ^a +0.43	3.94 ^a +0.73	4.02 ^a +0.52	3.82 ^a +0.41	3.55 ^a +0.33
5	8.91 ^a +0.74	8.66 ^{ab} +0.51	8.46 ^b +0.38	4.41 ^a +0.71	4.15 ^a +0.41	4.01 ^a +0.44	3.96 ^a +0.24

¹ Ratio of *C. piscicola*: *L. monocytogenes* (CFU/g)

² Letters within the same row of each organism were compared

³ Means with the same letter are not significantly different. Means were of three replications.

Table 3.2. The effect of individual bacterial growth in the changing ratio of *Carnobacterium piscicola* and *Listeria monocytogenes* in pasteurized crab meat stored at 4°C (A) and 10°C (B).

(A)

Days	Mean growth of <i>C. piscicola</i> (CFU/g) ^{2,3}			Mean growth of <i>L. monocytogenes</i> (CFU/g)			
	2:2 ¹	4:2	6:2	0:2	2:2	4:2	6:2
0	2.11 ^a ±0.45	3.68 ^b ±0.26	5.54 ^c ±0.11	2.13 ^a ±0.14	2.02 ^a ±0.37	2.16 ^a ±0.23	2.17 ^a ±0.16
6	5.40 ^a ±0.0	6.40 ^b ±0.0	7.40 ^c ±0.0	4.51 ^a ±0.13	5.07 ^a ±0.15	5.13 ^a ±0.18	4.33 ^a ±0.65
10	8.98 ^a ±0.64	9.84 ^a ±0.44	10.0 ^a ±0.09	6.42 ^a ±0.75	6.62 ^a ±1.1	6.83 ^a ±0.33	5.94 ^a ±0.59
14	9.93 ^a ±0.17	10.0 ^a ±0.14	10.6 ^a ±0.48	9.16 ^a ±0.18	8.63 ^{ab} ±0.11	7.63 ^{bc} ±0.24	7.12 ^c ±0.41
18	9.70 ^a ±0.09	9.77 ^a ±0.15	9.87 ^a ±0.08	8.90 ^a ±0.18	8.64 ^a ±0.15	7.34 ^b ±0.16	6.77 ^c ±0.17
22	9.37 ^a ±0.13	9.33 ^a ±0.26	9.43 ^a ±0.12	8.94 ^a ±0.11	8.62 ^b ±0.07	7.48 ^c ±0.11	6.39 ^d ±0.19
26	9.43 ^a ±0.13	9.56 ^a ±0.18	9.43 ^a ±0.16	9.04 ^a ±0.13	8.54 ^a ±0.14	7.35 ^b ±0.32	6.75 ^b ±0.22
28	9.51 ^a ±0.10	9.53 ^a ±0.16	9.67 ^a ±0.12	9.02 ^a ±0.13	8.30 ^b ±0.11	7.14 ^c ±0.17	6.53 ^d ±0.18

(B)

Days	Mean growth of <i>C. piscicola</i> (CFU/g) ^{2,3}			Mean growth of <i>L. monocytogenes</i> (CFU/g)			
	2:2 ¹	4:2	6:2	0:2	2:2	4:2	6:2
0	2.67 ^a ±0.42	3.85 ^a ±0.11	5.74 ^b ±0.06	2.23 ^a ±0.14	2.24 ^a ±0.12	2.06 ^a ±0.40	2.25 ^a ±0.22
6	5.40 ^a ±0.0	6.40 ^b ±0.0	8.40 ^c ±0.0	5.90 ^a ±0.55	5.90 ^a ±0.55	5.33 ^a ±0.44	4.20 ^a ±0.39
10	9.40 ^a ±1.1	9.92 ^a ±0.35	10.4 ^a ±0.45	8.95 ^a ±0.50	8.80 ^a ±0.34	7.56 ^b ±0.44	6.70 ^b ±0.67
12	10.3 ^a ±0.52	10.4 ^a ±0.53	10.2 ^a ±0.38	10.1 ^a ±0.55	9.20 ^b ±0.43	7.95 ^c ±0.53	7.17 ^d ±0.30
14	9.78 ^a ±0.47	10.3 ^b ±0.47	10.3 ^b ±0.44	9.13 ^a ±0.14	9.04 ^{ab} ±0.65	8.11 ^{bc} ±0.59	7.18 ^c ±0.42
18	9.45 ^a ±0.12	9.72 ^a ±0.23	9.76 ^a ±0.11	9.20 ^a ±0.20	8.68 ^b ±0.15	7.53 ^c ±0.17	6.62 ^d ±0.14
22	9.76 ^a ±0.14	9.63 ^a ±0.09	9.21 ^a ±0.98	9.14 ^a ±0.14	8.81 ^a ±0.14	7.16 ^b ±0.46	6.50 ^c ±0.25
24	9.70 ^a ±0.15	9.67 ^a ±0.16	9.79 ^a ±0.14	9.02 ^a ±0.10	8.56 ^a ±0.09	7.44 ^b ±0.29	6.62 ^c ±0.05

¹ Ratio of *C. piscicola*: *L. monocytogenes* (CFU/g)

² Letters within the same row of each organism were compared

³ Means with the same letter are not significantly different. Means were of two replications