1-1-2018

Effects of X-ray irradiation on Quality and Shelf Life of Seafood Products

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By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Food Science and Technology
in the Department of Food Science, Nutrition and Health Promotion

Mississippi State, Mississippi

May 2018

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Comparing the protein compositions of three fishes, grass carp exhibited lower band intensity at 47.9 KDa, β-tropomyosin (36.5 KDa), and missed the band at 15.9 KDa myosin light chain. Bigmouth buffalo had a darker tropomodulin (38.8 KDa) band and smaller α-tropomyosin (33-37 KDa) than silver and grass carp. The breaking force (611.8 g) and deformation (11.7 mm) of silver carp cooked gel were significantly higher than the other two fish products. The addition of starches at 2, 4, and 6% to the grass carp paste lowered the breaking force of the cooked gel in a dose-responsive manner compared to the control ($P<0.05$), but no differences were found in bigmouth buffalo.

The bio-accumulated Murine Norovirus-1 (MNV-1) was found to maintain infectivity during storage of live oysters at 5°C for 15 days while the inoculated MNV-1 kept infectious for 20 days in cooked surimi and salmon fillet. Treatments with 4.0 kGy X-ray achieved the reductions of 3.7 log PFU mL^{-1} in pure culture or 2.7, 2.2, and 2.0 log PFU g^{-1} in half-shell oyster, salmon sushi and tuna salad, respectively. X-ray significantly reduced the population of internalized MNV-1 in live oysters from 4.3 ± 0.4 log PFU g^{-1} to 3.6 ± 0.5, 3.2 ± 0.2, 2.8 ± 0.2, and 2.5 ± 0.1 log PFU g^{-1}, by 1.0, 2.0, 3.0, and 4.0 kGy X-ray, respectively. The population of MNV-1 was reduced to less than 2.0 log PFU g^{-1}

at 5.0 kGy X-ray. The survivability of live oysters was not significantly affected by treatment with 5.0 kGy X-ray, in comparison with the control, for up to 10 days, respectively, during storage at 5°C.

Fish sauce was fermented from the by-products of silver carp. The total nitrogen content of fish sauce made in April, and November were 9.86±0.9 and 9.71±4.5 g/l, respectively, which was significantly ($p<0.05$) higher than the sample of February (8.45± 0.25 g/l), reflecting seasonal effect. The total nitrogen, amino acid nitrogen, pH, and sodium chloride of fish sauce made from silver carp by-products met the international fish sauce standard code of CODEX STAN 302-2011.

DEDICATION

I will be dedicating this thesis to my father and hope he stays healthy.

ACKNOWLEDGEMENTS

I want to express my deepest appreciation to my advisor, Dr. Sam Chang for his valuable and patient guidance throughout my project. Without his help, I could not complete my study. My previous advisor Dr. Barakat S.M. Mahmoud guided me in research and writing. I appreciate his support and experience sharing.

I would like to thank my committee members, Dr. Ramakrishna Nannapaneni, Dr. Din-Pow Ma, and Dr. Peter Bechtel for their constructive criticism and suggestions.

I would like to thank Dr. Herbert W. Virgin IV (the Department of Pathology and Immunology at the Washington University School of Medicine) for the generous gifts of MNV-1 and BV-2 cells.

Special thanks also to Yuqing Tan (the Department of Food Science, Nutrition and Health Promotion at Mississippi State University) for operation of protein electrophoresis.

Funding is provided through USDA-ARS sca agreement no. 58-6402-2729 and state CRIS project No MIS 501170 for MS Center for Food Safety and Postharvest Technology.

Sincere thanks must go to our awesome research group which I am proud.

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CHAPTER I

INTRODUCTION

Estimates of foodborne illness revealed that human norovirus (HuNoV), an acute gastroenteritis pathogen, causes more than 58% of the U.S. foodborne illnesses, 11% foodborne deaths and 25% hospitalizations, annually (Scallan, Hoekstra, Angulo, Tauxe, Widdowson, Roy, Jones, & Griffin, 2011). HuNoV is highly contagious with an infectious dose as low as 10 particles (D'Souza, Sair, Williams, Papafragkou, Jean, Moore, & Jaykus, 2006). Symptoms of HuNoV infection, including diarrhea, vomiting, nausea, abdominal cramps, headache, and fever, usually last for 24-48 h in healthy individuals, but may be prolonged in the immunocompromised patients, children and the elderly (Iwamoto, Ayers, Mahon, & Swerdlow, 2010). HuNoV could be transmitted by person-to-person spread through the fecal-oral route or through contaminated food or water with low dose and high attack rates. Because HuNoV can persist in the environment, seafood such as oysters harvested from sewage-contaminated waters has caused outbreaks of gastroenteritis. The control measures of seafood include monitoring harvest waters for fecal coliforms and encouraging adequate cooking. However, continued outbreaks of illness have brought renewed attention to the risks associated with HuNoV gastroenteritis.

Researchers have to rely on proper surrogates to study the survival of HuNoV because HuNoV is not cultivable (Ye, Lingham, Huang, Ozbay, Ji, Karwe, & Chen,

2015). Compared with feline calicivirus (FCV) and Tulane virus, Murine norovirus (MNV-1) was chosen in this research because it is a closer genetic relative of HuNoV in the norovirus genus, and had been used in treatment with UV irradiation, gamma irradiation and electron beam (Richards, 2012). MNV-1 was also shown to be propagated and assayed in an efficient microglial cell line murine BV-2 cells (Cox, Cao, & Lu, 2009).

Several post-harvest techniques have been proposed to reduce HuNoV including cold and high temperature, UV treatment, and high-hydrostatic pressure. Ionizing irradiation is a non-thermal process which has been used to inactivate pathogenic and spoilage microorganisms and extend shelf life of food products (Thakur & Singh, 1994). Gamma ray, electron beam and X-ray are the three kinds of ionizing irradiation that were approved to treat shellfish up to a dose of 5.5 kGy (USFDA, 2015). The biological effects of ionizing irradiation were reported mainly linked to the DNA damage by free radicals during the irradiation process and the ionizing radiation-generated reactive oxygen species (Ahn, Kim, & Lee, 2013). In the last decade, Experimental Seafood Processing Lab (ESPL) of Mississippi State University, has extensively studied the use of X-ray to inactivate different pathogens in different food products. Scientists at ESPL irradiated gulf coast oysters to kill the *Vibrio vulnificus* and *Vibrio parahaemolyticus* on the oysters without killing the oysters (Wagner, 2010). The ESPL installed an RS 2400 x-ray irradiator in the summer of 2007. Researchers at the ESPL accomplished many projects related to pathogen inactivation by X-ray. Mahmoud (2012b) reported that X-ray significantly ($p < 0.05$) reduced the initial level of artificially inoculated *Listeria monocytogenes* on smoked salmon from 3.7 log CFU g^{-1} to 2.0 log CFU g^{-1} after

treatment with 0.5 kGy X-ray, while exposure to 1.0 kGy X-ray reduced *L. monocytogenes* to below the detection limit (<1.0 log $CFUg^{-1}$). Mahmoud (2009b) reported that greater than a 6-log reduction of *V. vulnificus* was achieved with 3.0 kGy in whole-shell oysters. Treatment with 3.0 kGy X-ray did not affect the survivability of live oysters.

Ionizing irradiation had not been considered as a feasible process for inactivating viruses. Harewood, Rippey, & Montesalvo (1994) stated that the viral indicator F-coliphage was not significantly affected by gamma ray with high a D_{10} value (dose for 90% reduction on population, 13.50 kGy) in hard-shell clams (*Mercenaria mercenaria*). Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai (2013) reported that 4 kGy electron beam only reduced MNV-1 by 1 log $PFUg^{-1}$ on whole-shell oysters. In this study, X-ray was the first time used as a potential antiviral agent against HuNoV surrogate MNV-1 in food products including half-shell oysters, salmon sushi and tuna salad. Plaque assays were also used to reveal the X-ray reduction of the internalized MNV-1 in live oysters.

In 2016, oyster landings in United States reached to 34.7 million pounds and valued $225.6 million. Among them, the Mexican Gulf coast region produced approximately 16 million pounds of oyster meats, over 46 percent of the national total (https://www.st.nmfs.noaa.gov/pls/webpls/MF_ANNUAL_LANDINGS.RESULTS). Once known as the "Seafood Capital of the World", Biloxi represents the abundant aqua-resource in Mississippi state (Boudreaux, 2011). Seafood harvested from the Mississippi coast supports a long linage of fishing families of the residents.

(https://www.gulfcoast.org/articles/post/mississippi-opens-oyster-season/). Not only the emigrants moving here to fish, many of their children and grandchildren (and beyond) are still in the fish-related business. The fishermen here are truly dedicated to the area and their craft. Despite the severe damage caused by Hurricane Katrina, the Department of Marine Resources (DMR) is expediting the natural process of oyster production (https://www.undercurrentnews.com/2017/06/16/mississippi-aims-for-big-increase-in-oyster-production/). Their goal for off-bottom oyster aquaculture is to produce 1 million sacks a year by 2025. Whether it is history or prospects, oyster research is still an important issue.

There were serious concerns about side-effects of irradiation treatment. The changes of color, sensory characteristics and oxidation of lipid are among the major concerns in irradiating meat (Kim, Nam, & Ahn, 2002). Treatment with X-ray kept the mesophilic and psychotropic counts significantly lower than the control during storage. Bacteria are the cause of high total volatile basic nitrogen (TVB-N) during seafood spoilage due to production of amines, ammonia and reduced pH by proteolytic and saccharolytic process (Wu, Chang, Nannapaneni, Zhang, Coker, & Mahmoud, 2017). The aim of this study was to investigate the effects of X-ray treatments on MNV-1 and quality of seafood during storage at their normal storage condition.

The objectives of this study were:

1. Effects of starches on the textural properties of cooked surimi gel of wild grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and bigmouth buffalo (*Ictiobus cyprinellus*).

2. The effects of cold storage on the murine norovirus-1 (MNV-1), inherent microbiota, weight loss and total volatile base nitrogen (TVB-N) in whole shell oyster, salmon fillet and silver carp surimi.
3. Effects of X-ray irradiation on the murine norovirus-1 (MNV-1) in pure culture, half-shell oyster, salmon sushi, and tuna salad.
4. The effects of X-ray treatments on bioaccumulated murine norovirus-1 (MNV-1) and survivability, inherent microbiota, color, and firmness of Atlantic oysters (*Crassostrea virginica*) during storage at 5 ℃ for 20 days.
5. Manufacturing fish sauce compliant with CODEX standard from the by-products of wild silver carp (*Hypophthalmichths molitrix*).

CHAPTER II

LITERATURE REVIEW

2.1 Asian carp and surimi made of Asian carp meat

To improve water quality of aquaculture ponds in the Mississippi river area, Asian carp were imported from China in 1970s (Everett, Jasim, Oh, Rychtář, & Smith, 2015). Because they could eat 5-20% of their body weight each day, the fish can grow incredibly quickly and can weigh up to 150 pounds and reach an average size of about 30-40 inches. The population of Asian carp grew exponentially since no natural predators threatens their growth. The Asian carp causes a decrease of the population of native fish quickly in the upper Mississippi river system because of diet overlaps (Oh, 2014).

Surimi is a fish paste that can be used to make imitation crab meat such as crab sticks, fishcakes and other foods (Park, 2013). The surimi industry demands mainly white fish because of the requirements in the whiteness and textural properties. The growing volume of surimi requirement created growing interest on alternative meat sources such as silver carp (Liu, Yin, Zhang, Li, & Ma, 2008).

Silver carp belongs to fatty fish and have much larger proportion of dark muscle. BuCHtoVá & Ježek (2011) ranked the silver carp among high-fat fish because the fatter ventral sections contain lipid up to 158.14 ± 11.28 g/kg. There are some quality issues for using fatty fish meat in surimi such as poor thermal stability, low gel forming ability and pink color in its surimi products (Hultin & Kelleher, 2000). Because of its higher lipid

contents, less stable proteins, greater concentrations of heme proteins, lower ultimate pH values, higher proteolytic activities, and higher concentrations of sarcoplasmic proteins, the fatty fish is not suitable in making high-quality surimi from raw material with high contents of dark muscle (Martín-Sánchez, Navarro, Pérez-Álvarez, & Kuri, 2009). The quality in odor, thermal stability, and gel strength of surimi might be improved by excluding dark muscle of fatty fishes (Kelleher, Hultin, & Wilhelm, 1994). However, adjustment of gel properties by additives such as starch is necessary for stable quality since gel-forming properties of wild silver carp surimi vary by seasons (Yuan, Fukuda, Kaneniwa, Chen, Cheng, Wang, & Konno, 2005).

Starch is used to control wetness, stickiness, gel strength, storage stability and thermal stability. It mimics the chewy, elastic texture for seafood imitation because of its high capacity to swell and retain water (Hunt, Getty, & Park, 2009; Ramírez, Uresti, Velazquez, & Vázquez, 2011). Tapioca starch is widely used in food applications, such as thickening and gelling because of its abundance and low price (Desmond, Troy, & Buckley, 1998). The functions of tapioca starch have been improved by chemical/physical modification because the properties of native starch are not optimal in many applications (Agudelo, Varela, Sanz, & Fiszman, 2014). Although modified starch has extensive industrial applications, few studies reported the effects of modified starches and the interaction mechanisms with proteins in surimi (Kong, Zhang, Feng, Xue, Wang, Li, Yang, & Xue, 2016). Modified starch is starch derivatives with altered physico-chemical properties for specific applications (Wongsagonsup, Pujchakarn, Jitrakbumrung, Chaiwat, Fuongfuchat, Varavinit, Dangtip, & Suphantharika, 2014). Cross-linking and stabilization are the most important chemical modification in the starch

industry (Eliasson, 2004). Cross-linked starches with a phosphate bridge improves resistance to high temperature, low pH and reduces syneresis. Stabilization prevents retrogradation and enhance tolerance to temperature fluctuations by substituting interacting the protein's bulky groups with the starch.

2.2 Oyster

Generally, the term "seafood" refers to animals consisting not only of fish (habitats in freshwater, estuarine, or marine), but also of shellfish which include crustacea and mollusks (Venugopal, 2005). Seafood is considered nutrient-rich, and healthful because seafood consumption is associated with potential health benefits, including neurologic development during gestation and infancy and reduced risk of heart disease (Gould, Smithers, & Makrides, 2013; Simmer, 2016). Global per capita consumption of fish in 2013 was estimated at 19.7 kg. Fish accounts for 17 percent of the global population's intake of animal proteins and 6.6 percent of all proteins consumed. Fish provides more than 3.1 billion people with almost 20 percent of their average per capita intake of animal proteins (http://www.who.int/nutrition/topics/3_foodconsumption/en/index5.html). Preliminary estimates for 2014 indicated a further growth in per capita consumption to about 20 kg, with the share of aquaculture production in total food supply overtaking that of capture fisheries for the first time.

Among the seafood, the safety of products varies considerably and is influenced by many factors such as origin, microbiological ecology of the product, handling and processing practices and traditional preparations before consumption. Taking most of these aspects into consideration, seafood can conveniently be grouped according to risk

factors (Huss, Reilly, & Embarek, 2000). Among them, oysters eaten without any cooking were ranked risk category 1 because of the high risk of contamination.

Oysters can be processed for various products (Venugopal, 2005). According to the preservation treatments, oysters can be classified as live (or fresh), frozen, smoked, canned, and other meat conditions. Based on the shell condition, there are shell-on, half-shell, and shucked meat. Oysters, clams, mussels, and scallops have been shucked at commercial scale, particularly in the case of oysters. Traditionally, the adductor muscle in oyster is cut by hand with a sharp knife, which is a time-consuming job, although machines are now available for the purpose. Microwave heating may be used to open oyster shells, but it gives a heat treatment that changes the sensory characteristics. Live oysters frozen by spreading single layers in an air blast freezer causes the shells to open, and makes subsequent shucking easier (Stroud, 1981). Oysters can also be frozen in the half shell by laying on trays in an air blast freezer. However, many oysters were sold as shell-on ones (Venugopal, 2005). Because oysters survive out of water for weeks under cool and humid environment, it could be distributed and sold alive (Stroud, 1981).

A stock of marketable Pacific oyster (*Crassostrea gigas*) was stored during 22 days in non-immersed conditions at 3°C with 100% humidity (Buzin, Baudon, Cardinal, Barillé, & Haure, 2011). At the end of the experiment, the oyster mortality rate remained less than 3.5%. The sensory attributes, odor, appearance, texture and flavor did not show significant change. However, the oyster total weight and fresh flesh weight exhibited losses of 10.5% and a 16.6% after 15 days of storage. These results suggested that the cold storage represents a partial solution to sustain the commercialization of bivalves during closures of 15 days.

During transportation, live oysters should be protected from mechanical damage by the package and should be kept between 1 and 10°C at all times (Stroud, 1981). Because the damaged edge of the shell could lead to losing liquor, they should reach the wholesaler within 3 days after harvest to be in the best condition. A simple barrel, box or sack could be used for commercial packaging. Special designed container with separate compartments for individual oysters or higher degree of sophistication can be applied depending on the value of the product, the journey time, and the market for which it is destined. A healthy live oyster feels heavy, sounds solid when tapped, and either remains tightly closed or closes quickly when it is handled. Live oysters stored in a shop or restaurant should be inspected daily, and be removed if dead.

A wide range of parameters has been used in the literature to assess bivalve quality (Buzin, Baudon, Cardinal, Barillé, & Haure, 2011). Chemical parameters often used for fish have been applied to bivalves as well. Among them, the quantification of TVB-N results from the degradation of nitrogenous compounds by microbial activity or post-mortem nucleotide catabolism. Spoilage bacteria and tissue degradation are strongly correlated with flavor changes (Buzin, Baudon, Cardinal, Barillé, & Haure, 2011). End-product quality can be evaluated through sensory assessment to determine the maximum storage time compatible with food safety for consumers.

As suspension feeders, bivalves interact significantly with living and non-living particles in the seston, including bacteria, as they filter large quantities of water per unit time (Pierce, Ward, Holohan, Zhao, & Hicks, 2016). The accumulation of microorganisms from their environment directly related the microbiological safety to the waterways in which they are cultivated (Son & Fleet 1980). Outbreaks of typhoid,

cholera, dysentery, hepatitis, and various forms of gastroenteritis have been linked to the consumption of oysters. Pathogenic bacteria and virus have been implicated in seafood-borne diseases, for which oysters are considered as carriers (Venugopal, 2005). These include *Vibrio cholera*, *V. parahaemolyticus*, *V. vulnificus*, and HuNoV. The microbial communities of oysters were influenced by season (Pierce, Ward, Holohan, Zhao & Hicks, 2016). Water temperature has a significant relationship with microbial community.

Consumption of raw oysters frequently causes outbreaks of viral gastroenteritis (Nappier, Graczyk, & Schwab 2008), but little is understood about the rate of uptake and retention of viruses. Viral pathogens can accumulate in oysters when they are adsorbed to food or suspended particles and transported into the oysters' digestive system during feeding. Rates of bioaccumulation can be affected by environmental factors, such as temperature and salinity, or oyster physiological factors, such as size and species. Human norovirus (HuNoV) and hepatitis A virus (HAV) contribute to many cases of oyster-associated enteric diseases (Nappier, Graczyk, & Schwab, 2008). However, unlike laboratory strains of HAV, HuNoV is not easily replicated in cultured cells, and few studies have assessed HuNoV in oyster bioaccumulation and depuration trials.

Because molluscan shellfish such as oysters, clams, and mussels are filter feeders and accumulate microorganisms and other particulate matter in the gut from the surrounding water (Venugopal, 2005), one of the important preprocessing step for bivalves is depuration. In depuration, the shellfish are stored in tanks of potable water usually for 36-48 h and allowed to decontaminate naturally.

Gamma irradiation of the hard-shelled clam (*Mercenaria mercenaria*), and the Atlantic oyster (*Crassostrea virginica*) significantly reduced virus carriage numbers without unduly affecting desirable sensory qualities (Venugopal, 2005). It was suggested that ionizing radiation can provide an extra, highly effective safeguard of shellfish sanitary quality when combined with traditional depuration treatment. Cobalt-60 irradiation of the hard-shell clam and oyster significantly reduced virus carriage numbers without unduly affecting the sensory qualities. A D_{10} value of 2 kGy was found to inactivate HAV in clams and oysters. It was concluded that radiation in conjunction with depuration could improve the safety of the shellfish.

2.3 Seafood-associated illnesses

The USDA Economic Research Service estimated the annual cost of foodborne illnesses for HuNoV, HAV, *V. parahaemolyticus*, *V. vulnificus*, *L. monocytogenes*, *Escherichia coli*, and Salmonella species, in the U.S (USDA, 2017) and concluded that the total cost is the sum of productivity loss, visiting physician, hospitalization, and death. The total cost of norovirus was estimated to be about $2.255 billion annually. For *V. parahaemolyticus*, *V. vulnificus*, *L. monocytogenes*, *E. coli*, and *Salmonella,* the total cost is approximately $40.7, $320, $2,834, $271 and 3,666 million, respectively.

Because seafood is susceptible to surface or tissue contamination originating from the marine environment, seafood-associated infections can be caused by a variety of bacteria, viruses, and parasites (Iwamoto, Ayers, Mahon, & Swerdlow, 2010). There are various clinical syndromes resulting from the diverse seafood pathogens. Among them, bivalve mollusks are in the high-risk group of contamination because these organisms are fed by filtering large volumes of seawater (Bedford, Williams, & Bellamy, 1978). The

process of filtration accumulates and concentrates pathogenic microorganisms of the water body. Contamination of seafood by pathogens can occur when growing areas are contaminated with human sewage. Outbreaks of seafood-associated illness linked to polluted waters have been reported by *Vibrio* species, *Salmonella, Shigella species,* parasites, HAV, and HuNoV (Iwamoto, Ayers, Mahon, & Swerdlow, 2010).

HuNoV and HAV are considered the principal viral pathogen threats to shellfish consumers (Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai, 2013). It is estimated that HuNoV are responsible for more than half of all reported outbreaks of gastroenteritis. Bivalves, such as oysters, filter large volumes of water and bioaccumulate HuNoV and HAV, as well as a variety of bacterial pathogens. Because of the highly infectious capacity, HuNoV and HAV often cause widespread outbreaks (Koopmans & Duizer, 2004). The symptoms of HuNoV infection include diarrhea, vomiting, nausea, abdominal cramps, headache, and fever (Iwamoto, Ayers, Mahon, & Swerdlow, 2010). HuNoV is a highly stable and contagious virus, less than 10 virus particles can cause an infection. Introduction of HuNoV in a community or population (a seeding event) may be followed by additional spread. HAV is an important problem in developing countries. The symptoms of HAV may include nausea, vomiting, diarrhea, jaundice, fever, and abdominal pain.

V. parahaemolyticus and V. vulnificus are bacterial foodborne pathogens that can cause illnesses in humans after ingestion or exposure to contaminated seafood or coastal waters (Banerjee & Farber, 2017). *V. parahaemolyticus* is a halophilic bacterium, which causes illness associated with the consumption of oysters, including gastroenteritis, vomiting, diarrhea, headache, and nausea (Mahmoud & Burrage, 2009). It exists naturally

in the marine environment water, where the oysters are harvested. *V. parahaemolyticus* can cause death to individuals with underlying diseases, especially liver disease. *V. vulnificus* is a gram-negative bacterium, occurring naturally in warm environments, such as the Gulf Coast water (Mahmoud, 2009b). *V. vulnificus* occurs in warm estuarine environments, such as the Gulf Coast water where most of the US oysters are harvested. *V. vulnificus* is the leading cause of foodborne illness associated with the consumption of raw oysters in the United States and has the highest fatality rate (40-50%) among food-borne pathogens in the United States.

Salmonella is a facultatively anaerobic, nonsporulating, Gram negative bacterium; most strains are motile by means of flagella. They are mesophilic, with optimum growth temperature between 35 and 37 °C, with a growth range of 5 to 46 °C (Amagliani, Brandi, & Schiavano, 2012). The presence of Salmonella in seafood may derive from contamination occurring in the natural aquatic environment, in aquaculture or during processing. In addition, the isolation of Salmonella serovars that are resistant and multi-resistant to antibiotics continues to raise concerns.

Helminths are large, worm-like parasites such as nematodes, trematodes, and cestodes (Hayunga, 2013). Their infections are associated with the consumption of raw seafood. Clinical manifestations associated with helminthic infections range from no symptoms to mild, chronic gastrointestinal symptoms, allergic reactions, and, rarely, intestinal perforation and invasive disease.

2.4 The food safety concern related to histamine formed by bacteria

Visciano, Schirone, Tofalo and Suzzi (2012), in reporting a study conducted by Zhai, Yang, Li, Xia, Cen, Huang, and Hao (2012), highlight the presence of bio-amine

(BA) compounds in seafood. They are low molecular weight organic bases with biological activity that are formed in foods by microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes and ketones by amino acid transaminases. The study of Zarei, Najafzadeh, Enayati, and Pashmforoush, (2011, as cited in Visciano, Schirone, Tofalo and Suzzi 2012) highlights that the most important BAs, including histamine, tyramine, tryptamine, putrescine, and cadaverine, are formed from free amino acids namely histidine, tyrosine, tryptophan, ornithine and lysine, respectively.

Visciano, Schirone, Tofalo and Suzzi (2012) stated that the consumption of high amount of BAs, as described above, can result in food borne poisoning which is a worldwide problem. According to European Regulation, the maximum limits for histamine ranges from 100 to 200 mg/kg. The most common form of toxicity caused by the ingestion of fish throughout the world and is perhaps histamine poisoning (Visciano, Schirone, Tofalo and Suzzi, 2012).

Zarei, Najafzadeh, Enayati, and Pashmforoush (2011, as cited in Visciano, Schirone, Tofalo and Suzzi, 2012) highlighted that BA accumulation in foods requires the presence of microorganisms with amino acid decarboxylases and favorable conditions for their growth and decarboxylation activity.

It is well-known that histidine decarboxylase (HDC) is the enzyme that catalyzes the reaction that produces histamine from histidine. Histidine decarboxylase catalyzes the decarboxylation of histidine to histamine and CO_2. There are two groups of bacterial histidine decarboxylases. One group uses pyridoxal-P as the essential coenzyme and the other uses a covalently bound pyruvoyl residue at the active site. Histidine

decarboxylases from Gram-positive bacteria are pyruvoyl-dependent enzymes, those from Gram-negative bacteria are pyridoxyal-P-dependent enzymes (Kimura, Takahashi, Hokimoto, Tanaka, Fujii, 2009).

The clinical syndrome of Scombroid-fish poisoning (scombrotooxin) is due to the ingestion of spoiled fish, usually of the families Scombridae and Scomberesocidae. Scombroid-fish poisoning is the most common cause of toxicosis worldwide (Morrow, Margolies, Rowland, Roberts, 1991). Symptom of scombroid-fish poisoning can occur within one hour after the ingestion of spoiled fish and may last for several hours. The symptoms include flushing, sweating, nausea, vomiting, diarrhea, headache, palpitations, rash, and occasionally swelling of the face and tongue. Morrow, Margolies, Rowland, Roberts (1991) stated that the highest morbidity worldwide from fish poisoning is from the ingestion of spoiled scombroid fish, such as tuna and mackerel. Morrow, Margolies, Rowland, Roberts (1991) stated histamine could be responsible, because spoiled scombroid fish contain large quantities of histamine. To answer whether histamine is the causative toxin or not, Morrow, Margolies, Rowland, Roberts (1991) looked for more supporting evidences and investigated whether histamine homeostasis is altered in poisoned people.

The urinary excretion of histamine and its metabolite, N-methylhistamine, was measured in three persons who had scombroid-fish poisoning after the ingestion of marlin. In urine samples collected one to four hours after fish ingestion, the levels of histamine and N-methylhistamine were 9 to 20 and 15 to 20 times higher than the normal mean values, respectively. During the subsequent 24 hours, the levels fell to 4 to 15 times and 4 to 11 times higher than the normal values. Levels of both were normal 14 days

later. Scombroid-fish poisoning is associated with urinary excretion of histamine in quantities far exceeding those required to produce toxicity. The histamine is most likely derived from the spoiled fish. Morrow, Margolies, Rowland, Roberts (1991) stated that these results identified histamine as the toxin responsible for scombroid-fish poisoning.

The environmental conditions decide the type of habitat microorganisms in fish for human consumption. The microflora of fish consists of primarily psychrotrophic Gram-negative bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella*, and *Flavobacterium*. Members of the *Vibrionaceae* (*Vibrio* and *Photobacterium*) and the *Aeromonadaceae (Aeromonasspp*.) families are also common aquatic bacteria and typical of the fish flora (Visciano, Schirone, Tofalo and Suzzi, 2012). Furthermore, Visciano, Schirone, Tofalo and Suzzi (2012) highlighted the wide range of environmental habitats (freshwater to saltwater, tropical waters to arctic waters, pelagic swimmers to bottom dwellers, and degree of pollution) and the variety of processing practices (iced fish products to canned products) are all important factors in determining the initial contamination of fish and fish products. In the saltwater environment, the types of histamine-production-associated bacteria are commonly present. On the gills, external surfaces, and in the gut of live fish, these bacteria naturally exist with no harm to the saltwater fish. Upon death, the defense mechanisms of the fish no longer inhibit bacterial growth in the muscle tissue, and histamine-forming bacteria may start to grow, resulting in the production of BA. Therefore, controlling the temperature of fresh fish is extremely important to prevent histamine production due to microbial growth. Food irradiation may offer an approach to reduce total bacterial counts.

2.5 Norovirus

HuNoV belongs to the Caliciviridae family, a group of nonenveloped, icosahedral viruses with a single-stranded, positive sense RNA genome (Park & Ha, 2014). HuNoV is the most common cause of epidemic gastroenteritis following consumption of bivalve shellfish contaminated with fecal matter (Campos & Lees, 2014). HuNoV is proven to be stable in the environmental water body such as river, lake and sea. Invertebrates like oysters could accumulate HuNoV through filtration of sea water. Most outbreaks have been associated with shellfish harvested from waters affected by untreated sewage. The abundance and distribution of HuNoV in shellfish waters causes outbreak of this disease. For example, a recent norovirus outbreak with a total of 289 illnesses has been reported from eating raw or under-cooked oysters in northern pacific coast region (https://globalnews.ca/news/3296579/raw-and-undercooked-b-c-oysters-linked-to-289-cases-of-illness-across-3-provinces/). The federal government closed oyster farming at seven diverse locations in the Southern coast of British Columbia waters on March 22, 2017. Several other commercial growers have also voluntarily stopped selling. According to the personal communication with Mississippi Department of Marine Resources, the cost to recall the contaminated oysters from the customers nationwide is much higher than the price of the products itself. Therefore, a reliable intervention to reduce the infection risk of HuNoV is quite important.

Viral survival and inactivation by intervention is assessed through infectivity assays, including the plaque assay and molecular techniques (Hirneisen & Kniel, 2013). Molecular methods cannot distinguish between infectious and noninfectious virus. RT-qPCR always underestimated reductions in surrogate virus titer when compared to

infectivity (Knight, Haines, Stals, Li, Uyttendaele, Knight, & Jaykus, 2016). Because no reproducible model can be used in assessing human norovirus infectivity, current methods have been based on viral surrogates such as MNV, feline calicivirus (FCV), and the bacteriophage MS2**.**

2.6 Ionizing irradiation

Ionizing radiations are emitted by unstable nuclei in process of the atomic decay, X-ray machines, cyclotrons and other devices (Khaled & Held, 2012), with high energy to create ions or free radicals from atoms. Accelerated electrons (E-beam), gamma-rays, and x-rays are used as sources for ionizing radiation (Ahn, Kim, & Lee, 2013). Because of a high penetration power and can treat bulk foods on shipping pallets, gamma irradiation is a common radiation source in food industry. E-beam irradiation is generated from a linear accelerator. The stream of high-energy electrons can penetrate only a few centimeters into the food and is limited to treat thin layers of a particular food product such as grains or packages of fish fillets with less than 8 to 10 cm thickness. X-ray is like gamma rays in physical properties and their effects on matter, differing only in their origin. X-ray is produced by machines, while gamma rays come from radioactive isotopes (Thakur & Singh, 1994).

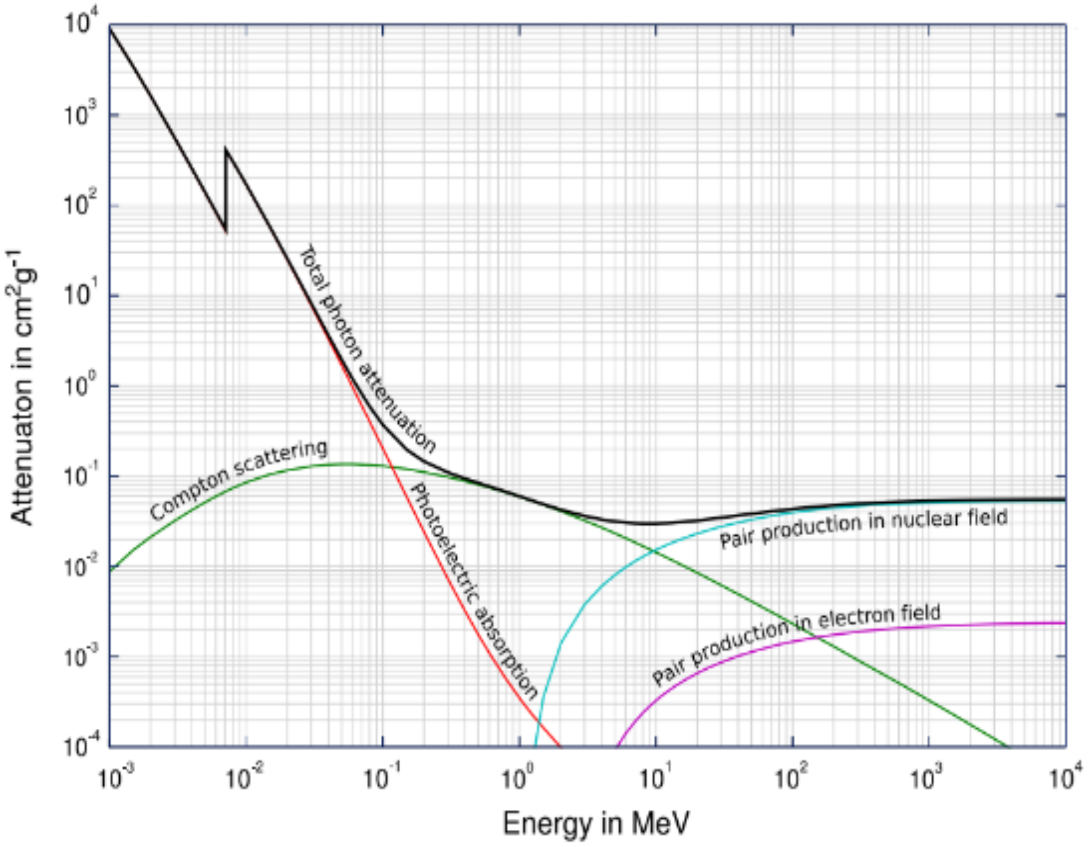

Figure 2.1 The total absorption coefficient versus gamma energy, and the contributions of aluminum (https://en.wikipedia.org/wiki/Gamma ray and Krane, 1988).

Ionizing radiations deposit energy gradually traverse through matter along the length of their path. When an ionizing irradiation passes through matter, the probability for absorption is proportional to the thickness of the layer, the density of the material, and the absorption cross section of the material (Urbain, 2012). As it passes through matter, ionization taken via three processes: the photoelectric effect (1), Compton scattering (2), and pair production (3). Figure 2.1 shows the total absorption coefficient versus gamma energy, and the contributions of aluminum (atomic number 13) by the three effects. The photoelectric effect is largest at low energies, Compton scattering dominates at intermediate energies, and pair production dominates at high energies.

Photoelectric effect (1) causes the ejection of that electron from the atom when an ionizing irradiation photon interacts with and transfers its energy to an atomic electron. The photoelectric effect is the dominant energy transfer mechanism for X-ray with energies below 50 keV (thousand electron volts). Compton scattering (2) is an interaction in which an ionizing irradiation photon loses enough energy to an atomic electron to cause its ejection, with the remainder of the original photon's energy emitted as a new, lower energy photon whose emission direction is different from that of the incident gamma photon, hence the term "scattering". Compton scattering is thought to be the principal absorption mechanism for gamma rays or electron beam in the intermediate energy range 100 keV to 10 MeV. Pair production (3) becomes possible with irradiation energies exceeding 1.02 MeV, and becomes important as an absorption mechanism at energies over 5 MeV (see illustration at right, for lead). By interaction with the electric field of a nucleus, the energy of the incident photon is converted into the mass of an electron-positron pair.

For an absorber, the rate of energy transfer depends on the energy and type of radiation as well as the density of the material (Khaled & Held, 2012). The density of energy deposition in a material is called the Linear Energy Transfer (LET) of the radiation. The definition is the average energy deposited per unit length of track of radiation and the unit is keV/μm. LET essentially indicates the quality of radiation because the biological effect of a radiation (its relative biological effectiveness, RBE) depends on its average LET (Hunter & Muirhead, 2009). Base on the considerable evidence of *in vitro* cellular radiobiology, the RBE of high energy low-LET radiation (gamma radiation, electrons) is less than that of low energy low-LET radiation (x-rays,

betas). Low-LET radiations appear to have different RBEs, depending on the energy. For ^{60}Co gamma rays, the LET is 0.2 KeV/μm, while it reaches to 2.0 KeV/μm when 250 kVp X rays is applied. Literature review shows that LET and RBE have not been considered in the food safety research for the applications of food irradiation to inactivate pathogens and for food preservation.

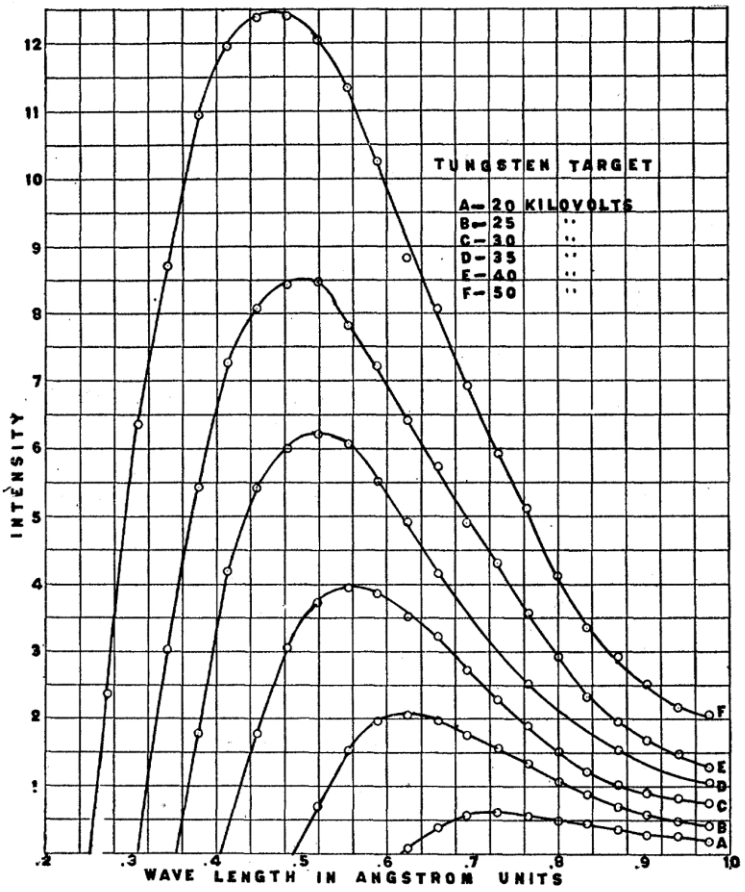

Figure 2.2 The energy-wavelength distribution curves are determined by the accelerating voltage (from the classic work of Ulrey at the Columbia University in New York in 1918).

The quality of radiation is important to biological killing effect (Bomford, Miller, Kunkler, Sherriff, Bomford, & Kunkler, 1993). The factors, which influence the quality

are penetrating ability, scattering by irradiated materials and the doses delivered to the surface. When X-ray is used for the study, the quality of radiation could be adjusted by accelerating voltage, inherent filtration and the added filtration. The accelerating voltage range of the x-ray tube can be adjusted in a continuous range voltage and current. Exposure rate in the X-ray machine is a function of the accelerating voltage (Wagner, Dillon, Blythe, & Ford, 2009.). As shown in the Figure 2.2, the energy-wave length distribution curves could be determined by the accelerating voltage (Ulrey, 1918). The research on radiation quality has been applied on the medical treatment and researches of cell gene expression for decades. To the author's best knowledge, the research and application of radiation quality has not been performed on food treatments for killing pathogens.

2.7 Inactivation of pathogen by irradiation

Farkas (1998) stated that irradiation is a good hygienic practice to reduce the level of contamination of pathogens, which cannot be eliminated by primary processing. Previous research work using ionizing irradiation for food treatment, such as gamma ray, X-ray and electron beam has been summarized in the literatures. Ionizing radiation is considered a safe, efficient, environmentally clean and energy efficient process for food decontamination, and is particularly valuable as an end products procedure for HACCP management and effectively eliminate pathogens including *Salmonella*, *L. monocytogenes* and *E. coli* O157:H7 without affecting sensory, nutritional and technical qualities.

Ahn, Kim, & Lee (2013) summarized that ionizing irradiation has been proven to be effective to reduce the contamination of bacterial pathogens. However, it is not

accepted as adequate decontamination method for virus (Urbain, 2012). Intervention strategies inducing microbial inactivation are required to achieve a 3-log reduction of the level of viruses (Baert, Debevere, & Uyttendaele, 2009) while decontamination of fresh produce by gamma ray could only reduce viruses by 2-log. Application of E-beam on live bivalves is not adequate to prevent viral outbreaks (Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai, 2013).

For a long time, radiobiologists have noticed that most organisms are sensitive to X-ray and gamma rays (Daly, 2009). Although some bacteria from the *Deinococcus-Thermus* group can survive doses of more than 12 kGy, exposure of most vertebrate animals at just 10 Gy is lethal. Most bacteria cannot survive 200 Gy. However, invertebrate animals can survive 500 Gy. Jakabi, Gelli, Torre, Rodas, Franco, Destro, & Landgraf (2003) exposed the oysters to gamma radiation (^{60}Co) in doses ranging from 0.5 to 3.0 kGy. A dose of 3.0 kGy is generally sufficient to reduce the level of Salmonella serotypes by 5 to 6 log units. A dose of 1.0 kGy was sufficient to produce a 6-log reduction in the level of *V. parahaemolyticus*. The highest irradiation dose (3.0 kGy) did not kill the oysters or affect their sensory attributes. Research conducted in ESPL at the Mississippi State University reported that greater than a 6-log reduction of *V. vulnificus* was achieved with 3.0 kGy in whole shell oysters without killing the oysters (Mahmoud, 2009b). It can be concluded from the above research, the risk of bacterial infection could be greatly reduced by ionizing irradiation while keeping whole shell oyster alive. The retailers could sell live oysters treated after cold sterilization by irradiation.

Despite of the successful examples, the US Food and Drug Administration (USFDA) currently restricts the maximum irradiation level for fresh fruits and vegetables

to 1.0 kGy, a low level of irradiation. E-beam could only reduce the inherent bacteria of fresh-cut cantaloupe about 1 log $CFUg^{-1}$ even combined with chlorinated water (Boynton, Welt, Sims, Balaban, Brecht, & Marshall, 2006). Mahmoud (2012a) reported 1 log $CFUg^{-1}$ reduction of the inherent bacteria of cantaloupe could be achieved by only 0.1 kGy X-ray. Both gamma and X-ray are ionizing irradiation and have better penetrating ability compared with electron beam. This might be the reason why X-ray has higher inherent reduction than electron beam.

Extensive work has been performed to calculate D_{10} -values for most pathogens in broth culture and different food matrices. Researches reported from different group reported D_{10} values of the same non-spore forming bacteria differed greatly (Farkas, & Mohácsi-Farkas, 2011). For non-frozen food, D_{10} -values of *Vibrio spp*. ranged from 0.02 to 0.14 (kGy), a difference of 7 times. For frozen food, D_{10} -values ranged from 0.04 to 0.44 (kGy) which represents a 10-fold difference when comparing the maximum to minimum value. The author could not find the explanations to these differences. For different research groups, the penetration ability, and intensity of the radiation are not the same or not disclosed clearly in their publications. In addition to the dose of energy absorption, the radiation quality might have played an important role in pathogen inactivation, however no related information has been published according to our best knowledge.

2.8 Impact of irradiation on quality of food

Color of food might be the most important thing for marketing (Mancini & Hunt, 2005). Consumers always associate color and firmness with product freshness and quality (He, Liu, Nirasawa, Zheng, & Liu2013). Consumers rely mainly on fresh meat color as

an indicator of freshness during purchasing, just the same behavior that evaluates cooked color as an indicator of doneness at the point of consumption. The bad color of meat lead to product rejected by customers and the loss of turnover and profit. It is well known that the protein myoglobin is responsible for the red color. Myoglobin doesn't circulate in the blood but exists in the muscle and is purplish in color. When the oxygen concentration of muscle is high, it becomes oxymyoglobin and produces a bright red color. It is also well known that meat color is mainly influenced by the concentration and chemical states of heme-pigments of individual animals (Nam & Ahn, 2002). Besides, the stability of meat color is closely related to the inherent oxygen consumption rates, oxidation-reduction potential, metmyoglobin reducing capacity, and metmyoglobin reductase activity of muscles (Mancini & Hunt, 2005). As to the irradiated meat, Ahn, Kim, & Lee, (2013) reported that carbon monoxide myoglobin is responsible for the development of pink color in irradiated poultry meat based on the results of carbon monoxide and color measurement. They found that irradiation decreased the oxidation-reduction potential and produced carbon monoxide (CO) in turkey breast meat (Nam & Ahn, 2002). Among the instrument measurement of oxidative stress from irradiation, using myoglobin's redox state to quantify discoloration is more time consuming and more difficult than estimating pigment oxidation using a loss of red (decrease in a*), reflectance spectra, which has greater diagnostic benefits for cause and effect (Mancini & Hunt, 2005). It is very common to use instrument to measure color.

Quantifying the color and firmness of tuna during storage at 5, 10, and 25 ℃ for 25, 15 and 5 days, Mahmoud, Chang, Nannapaneni, Wu, & Coker, (2016) reported a significant effect of 0.6 kGy X-ray treatment on tuna color after treatment (day 0). At this

dosage, mesophilic bacterial counts were reduced to under detectable level and the values L* (lightness), a* (redness-greenness) and b* (yellowness-blueness) changed significantly. However, no significant differences ($p > 0.05$) in color or firmness of control and treated samples were observed after day 0. Mahmoud (2012a) also found that no significant differences were observed ($p > 0.05$) in color or firmness of untreated or treated cantaloupe with 2.0 kGy X-ray.

There are many reports concluded that treatment with ionizing radiation did not affect the quality of treated food. Suklim, Flick, & Vichitphan (2014) reported that no significant effects on the texture of crabmeat were observed by irradiation doses of 0, 2, 4 and 6 kGy gamma rays. Abreu, Zapata, Figueiredo, Garruti, Freitas, Pereira, & Braga (2009) found no change to the quality in fresh shrimp irradiated up to 6 kGy gamma rays. Kanatt, Chawla, Chander & Sharma (2006) stated that no significant changes were observed in the textural and sensory qualities of the ready-to-eat shrimps treated with 2.5 kGy gamma rays. A dose of 3.0 kGy did not change the odor, favor, or appearance of treated oysters (Jakabi, Gelli, Torre, Rodas, Franco, Destro, & Landgraf, 2003). The treatment with up to 4.0 kGy did not change the color of tuna skin (Nei, Kawasaki, Inatsu, Yamamoto, & Satomi, 2012). Sensory quality is very important as it has been reported that when exposing to 1.0 and 2.5 kGy gamma rays, had no significant differences between the acceptance of irradiated and non-irradiated watermelon (Martins, Aragon-Alegro, Behrens, Souza, Vizeu, Hutzler, & Landgraf, 2008).

2.9 Impact of irradiation on shelf life of food

Impact of irradiation on food could not only reduce the microbial population in plant tissue but also delay the process of senescence (Thakur & Singh, 1994). Irradiation

of strawberries extended their shelf-life by more than 9 days. Irradiation of mushrooms with 2 kGy increased shelf-life by approximately 4 days. Gamma-irradiation could delay senescence of carrot tissues. Usually, doses of 0.2-0.5 kGy could induce delay in ripening of fruits, some temperate fruits, such as apple and pears, however require up to 1 kGy doses for effective inhibition.

In addition to the technologies that reduce the microbial loads of fresh produce after harvest such as the applications of chlorinated water or distribution under ensured controlled refrigeration, many other physical methods can be applied on these products, including the application of E-beam on fresh-cut fruits (Ma, Zhang, Bhandari, & Gao, 2017). At the exposure of 0.7 and 1.5 kGy, E-beam treatment resulted in a significant decrease in *Salmonella enterica* serotype inoculated on fresh cut cantaloupe compared with the control (non-irradiated) both right after irradiation and after 21 days of storage. E-beam could induce a lower rate of respiration in fresh-cut cantaloupe for 20 days than non-irradiated samples (Boynton, Welt, Sims, Balaban, Brecht, & Marshall, 2006). Lower bacterial counts were reported in irradiated samples. Color and texture remained stable as measured by instruments and trained sensory panels. Low-dose E-beam of fresh-cut cantaloupe with modified atmosphere packaging (MAP, 4% oxygen, 10% carbon dioxide) offers promise as a method of extending shelf life.

Seafoods are dynamic systems with continuous changes of pH, atmosphere, nutrient composition and microflora. Among them, microorganisms are the major cause of spoilage (Gram & Dalgaard 2002). Each seafood product has its own unique microflora because of different raw materials, processing parameters and storage conditions. Fish has a high content of free amino acid, the spoilage metabolites can be

used as quality indices by the measurements of TVB-N. TVB-N increases with storage time and believed to be caused by bacterial and endogenous proteolytic enzymatic activities (Songsaeng, Sophanodora, Kaewsrithong, & Ohshima, 2010). Therefore, TVB-N is an important index for shelf life.

2.10 Application of irradiated foods

According to the estimation of Kume, Furuta, Todoriki, Uenoyama, & Kobayashi (2009), the quantity of irradiated foods in the world in 2005 was 405,000 tons, which included 186,000 tons of spices and dry vegetables, 82,000 tons of grains and fruits, 32,000 tons of meat and fish, 88,000 tons of garlic and potato for sprout inhibition, and 17,000 tons of other food items that included health foods, mushroom, honey, and others. The potential of food irradiation is not limited to these applications. Due to rapid development, China has been ahead in its use of irradiation for several foods and currently the biggest in the world. Their total amount is estimated to be more than 500,000 tons (Prakash, 2016). In United States, life oysters and blanched shellfish from the Gulf of Mexico are irradiated to reduce the risk of contamination by virulent microorganisms, particularly *Vibrio spp* (Ehlermann, 2016). These products are successfully processed in USA by Gateway America located in Gulfport Mississippi, and the products sold under the CrystalSeas brand. According to our personal communication with Gateway America, more types of food are under treatment and several fresh produce products are under trial for improving quality, shelf life and food safety.

Figure 2.3 Storage pool of irradiation source. Photo were taken in an USDA/APHIS certified phytosanitary treatment facility located in Gulfport, MS (Gateway America).

There were 103,000 pounds of food stuffs irradiated in the US in 2010 with nearly 8,000 pounds attributed to meat and poultry (Chamberlain, 2015). Gateway America has installed a pool type irradiator for food phytosanitation at the Gulfport international airport (Figure 2.3). This is the third one installed in the United States. The primary foodstuff utilizing this radioactive process of sanitation in Gulfport, MS is Gulf oysters with 53,251 sacks of oysters (roughly 20-pound sacks) harvested in Mississippi in 2012-2013 season. Currently, Gateway America has expanded the application of irradiation treatment to frozen meat, crab meat, fruits and vegetables. The company is building two new facilities, one in Texas and the other in New Jersey due to the business expansion.

2.11 Effects of X-ray irradiation on quality and shelf life of seafood products

The ESPL received RS 2400 irradiator in the summer of 2007. The technical notes of the irradiator states that it can deliver up to 45 Gy per minute (Wagner, 2010). Researchers at ESPL have extensively studied the effect of X-ray on food safety, including inactivating food borne pathogenic bacteria, virus, and its impact on food quality and shelf life. Mahmoud (2012b) reported that X-ray significantly ($p < 0.05$) reduced the initial level of inoculated *L. monocytogenes* on smoked salmon from 3.7 log CFU g^{-1} to 2.0 log CFU g^{-1} after treatment with 0.5 kGy X-ray, while exposure to 1.0 kGy X-ray reduced *L. monocytogenes* to below the detection limit (<1.0 log g^{-1}). Mahmoud (2009b) reported that greater than a 6-log reduction of *V. vulnificus* was achieved with 3.0 kGy in whole shell oysters.

Treatment with 1.5 kGy X-ray significantly reduced the initial inherent microbiota on parsley leaves, and inherent levels were significantly ($p < 0.05$) lower than the control sample throughout refrigerated storage for 30 days (Mahmoud, 2012c). Approximately 5.8, 3.1, 5.7, and 5.2 log CFU g^{-1} reductions of *E. coli* O157:H7, *L. monocytogenes*, *S. enterica*, and *Shigella flexneri* were achieved by treatment with 1.0 kGy X-ray, respectively. Furthermore, the populations of *E. coli* O157:H7, *L. monocytogenes*, *S. enterica*, and *Shigella flexneri* were reduced to less than the detectable limit (1.0 log CFU g^{-1}) by treatment with 1.5 kGy X-ray.

A more than 5 log CFU g^{-1} reduction of inoculated *E. coli* O157:H7 (CT-SMAC agar), *L. monocytogenes* (MOA), and *S. enterica* and *S. flexneri* (XLD) on cantaloupe was reported by 2.0 kGy X-ray (Mahmoud, 2012a). No significant effects of X-ray treatment on color or firmness were detected. Furthermore, X-ray treatment significantly

reduced the initial inherent microflora level on the whole cantaloupes and the levels remained significantly ($p<0.05$) lower than the control sample throughout storage for 20 days.

CHAPTER III

EFFECTS OF STARCHES ON THE TEXTURAL PROPERTIES OF COOKED SURIMI GEL OF WILD GRASS CARP, SILVER CARP AND BIGMOUTH BUFFALO

3.1 Introduction

Invasive Asian carps, such as the silver carp (*Hypophthalmichthys molitrix*) and grass carp (*Ctenopharyngodon idella*), have proliferated rapidly in the water basin of Mississippi river and have been threating the native fish species since 1980s (Sampson, Chick, & Pegg, 2009; DeGrandchamp, Garvey, & Colombo, 2008; Chapman, Davis, Jenkins, JKocovsky, Miner, Farver, & Jackson, 2013). Although Asian carp is generally accepted for direct human consumption in many countries, it is not popular in the United States (Banerjee & Farber 2017). Following the rapid expansion of Asian carp populations, several companies have been encouraged and established to utilize freshwater fish species as food in the Mississippi River Basin. This management strategies aimed at controlling their movement and reducing their density.

In the state of Kentucky, Blue Shore Fisheries (Farmington, KY) invested more than $1.3 million to produce food products from Asian Carp including surimi. Two Rivers Fisheries (Wickliffe, KY) was established in 2012, and the company has processed and exported all varieties of carps, including silver, and grass carp as well as bigmouth buffalo (*Ictiobus cyprinellus*) and all other freshwater fish. In the state of

Mississippi, Moon River Foods, Inc (Indianola, MS, Figure 3.1) was established in 2014 to process wild-harvested Asian carps for exporting to foreign market. Moon River installed an advanced surimi production line at the scale of mass production aiming for feasible utilization of wild Asian carp for global marketing. Practically, all the companies above must process a variety of the fish species captured by the fisherman, including Asian carp and native freshwater fish.

Figure 3.1 Removing fish scale by machine in Moon River Foods Inc.

(a) grass carp

(b) bigmouth buffalo

Figure 3.2 Picture of (a) grass carp, (b) bigmouth buffalo and (c) silver carp obtained from Two Rivers Fisheries.

(c) silver carp

Figure 3.2 (continued)

Grass carp (Figure 3.2a) and Silver carp (Figure 3.2c) were widely introduced from Asia to the world and reported to have adverse ecological impact after introduction in Mississippi river basin (Banerjee & Farber, 2017, Koel, Irons, & Ratcliff, 2000). As a native species, Bigmouth buffalo (Figure 3.2c) inhabits in North America and has an overlap diet with Asian carp (Sampson, Chick, & Pegg, 2009). According to our communication with the freshwater fishery companies, all the fish species mentions above are common catch in Mississippi river basin and the quantity fluctuated with seasons. By conducting dynamic mechanical analysis, Tao, Kobayashi, Fukushima, & Watabe, (2007) reported the seasonal effects on thermal gelation of grass carp myosin through the identification on the changes of dynamic viscoelastic parameters including storage modulus (G′), loss modulus (G″) and loss factor (tan δ) upon heating. Yuan,

Fukuda, Kaneniwa, Chen, Cheng, Wang, & Konno, (2005) stated that the gel-forming properties of silver carp surimi were also dependent on the season. To achieve the high profit business such as surimi production, the business needs to fill the gap of un-stable quantity and quality of wild freshwater fish.

Starches are the most common additives in surimi, because it can reduce the cost by decreasing the meat ratio, and stabilize the quality by improving the gel strength of gels as well as the appearance and taste of products (Hunt, Getty, & Park, 2009). The application of starch and modified starches on surimi made of grass carp and silver carp was reported by two groups (Liu, Nie, & Chen, 2014; Sun, Huang, Hu, Xiong, & Zhao, 2014). However, the approach of these researches was not eligible for application in manufacturing and comparison with previous research because meat ratio was not reduced in line with added starch concentration as industrial practice and some previous studies (Yang & Park, 1998).

In this study, the tapioca cassava native starch, tapioca derived starch acetate (E1420), tapioca derived distarch phosphates (E1412), Novation 1900 (comprise of corn, waxy corn, potato, tapioca and rice), Firmtex™ (modified waxy corn, E1442 (hydroxypropyl distarch phosphate)) and N-Hance 59 (native potato starch) were used as additive at the level of 0, 2, 4 and 6 g/100 g for preparing surimi paste and gel because the range of concentration had higher quality improvement on surimi gel (Hunt, Getty, & Park, 2009). The starch gelatinization profiles were determined by a rapid visco-analyzer (RVA) which analyzed the pasting properties of starch during heating and cooling processes which might affect the quality of final starch-based products (Chantaro & Pongsawatmanit 2010). Rheological assay was used for gathering information on the

textural characteristics of surimi by reading the storage modulus (G′), loss modulus (G″), and loss factor (tan δ) during thermal gelation (Jafarpour & Gorczyca 2009). Texture property was measured in breaking force (g) and deformation (mm) of surimi gel. Yang & Park (1998) reported the decrease of gel strength of Alaska pollock (*Theragra chalcogramma*) surimi when concentration of potato starch, modified potato starch or corn starch was higher than 60 g/kg. The starch concentration was ranged from 0 to 6% to compare the effects of the species of fish and starch.

3.2 Materials and Methods

3.2.1 Fish, surimi and starch

Fresh-harvested wild carp fishes (grass carp (*Ctenopharyngodon idella*), bigmouth buffalo (*Ictiobus cyprinellus*) and silver carp (*Hypophthalmichthys molitrix*)) were obtained from Asian carp processors (Two Rivers Fisheries), and transported by a cooler with cubed ice to the walk-in cooler of pilot plant in Department of Food Science, Nutrition and Health. Fish meat was cut into 3 mm cubes and washed 3 times at the ratio of 1:4 (mince: ice water) as described by Jafarpour & Gorczyca (2008). The mince was dewatered by wrapping and squeezing with cheesecloth and then ground with a Lem grinder (Big Bite, West Chester, OH) at 4°C. Cryoprotectants (4 g/100 g sucrose, 4 g/100 g sorbitol, and 0.3 g/100 g sodium tri-polyphosphate) were incorporated into the paste and homogenization by Hobart mixer (Troy, Ohio) at lowest speed (speed one) for 2 min, then increase to speed two for 2 min and then to highest speed (speed three) for 20 min. The resulting surimi was placed into a bag, formed into a block of about one pound, and frozen at -70 °C until use.

Native starch, distarch phosphate and starch acetate produced by Vedan Vietnam Enterprise Corp., Ltd (Dong Nai, Vietnam) were then applied on surimi paste to determine their effects. Native starch was made of tapioca cassava root harvested at the area of Binh Phuoc province in Vietnam. Distarch phosphate and starch acetate were made from native starch by chemical modifications. Novation 1900 is a trade name of the clean label starches of Ingredion Incorporate (Bridgewater, NJ) which comprise of corn, waxy corn, potato, tapioca and rice. Firmtex^{TM} is made from modified waxy corn. N-Hance 59 is a native starch made of potato.

3.2.1 SDS-PAGE analysis of the three species of carp

Fish meat were grounded and mixed with phosphate buffer solution (pH 8.5, 1:10 w/v) and shake for 1 hour with an orbital shaker at 4°C, and then centrifuge at 5000 rpm for 10 min to obtain the supernatant. The protein content of supernatant was determined by Bradford assay (Bradford, 1976). The protein content was diluted to 2 mg/mL with distilled water, and 0.5 mL of diluted samples was mixed with 0.5 mL SDS sample buffer. After boiling for 10 min, 8 µL of the cooled solution containing equivalent of 8 µg proteins were loaded onto a gradient gel (8-16%). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) based on the procedure of Laemmli (1970). At the end of electrophoresis, gels were stained with Coomassie Brilliant Blue R-250. The SDS-PAGE gels were scanned by Molecular Imager (Bio-Rad ChemidocTM XRS+, Hercules, California).

3.2.2 Preparation of surimi gels

Sample preparation was based on test formulation in Table 3.1 and homogenized for further 3 min. Dynamic rheological measurements were performed immediately after homogenization. For preparing cooked surimi gel, the paste was stuffed into sealed polypropylene casing (inner diameter, 3 cm; length, 10 cm), heated in a water bath at 40 °C for 15 min, and then followed by heating at 90 °C for 30 min. The heated surimi gel was cooled in ice-cold water for 1 h and stored at 4 °C overnight.

Table 3.1 Experimental formula for making surimi.

Ingredients (g)	A	B	C
Surimi, raw	850	700	550
Water/ice	120	240	360
Salt	30	30	30
Test starch	0	30	60
Total	1000	1000	1000

Each formula was based on equal moisture (80%), and salt (3%).

3.2.3 Dynamic rheological measurements

To compare the dynamic rheological properties of fish meat (silver carp, grass carp, and bigmouth buffalo), surimi paste was prepared according to formula A in Table 3.1. The dynamic rheological measurements were performed with an MCR101 rheometer (Anton Paar Ltd., Austria). Temperature sweep tests were performed for dynamic thermomechanical analysis from 10 to 90 °C at a heating rate of 1°C/min. Frequency (10 Hz) and strain γ= 0.1% (within the linear viscoelastic region) were fixed. The storage modulus (G') and loss factor (tan δ) of dynamic viscoelastic parameters were measured

using a parallel plate of 25 mm diameter setting. To prevent moisture loss during measurement, silicone oil was gently applied to the edge of parallel plate.

3.2.4 Starch gelatinization profiles

Pasting properties of 6% (w/w) starch were determined using a rapid visco-analyser (RVA, RVA 4500, Perten Instruments, Hägersten Sweden). Starch and water were added to achieve a total weight of 25 g containing 6 % (w/w) starch. The dispersions were kept at room temperature for a further 30 min to hydrate the starch. The pasting profile of the sample and agitation speeds of the paddle were monitored during a thermal treatment as follows: increasing the temperature from 35℃ to 95℃ at a heating rate of 1.5℃/min, holding the temperature at 95℃ for 10 min, decreasing the temperature to 50℃ at 1.5℃/min. Agitation speed of paddle was started at 960 rpm for the first 10 s and kept constant at 160 rpm until the end of the experiment (Chantaro & Pongsawatmanit, 2010). Pasting profiles were evaluated in triplicate for the average values of pasting parameters.

3.2.5 Swelling power test

The method of swelling power test is modified from the methods of Rahman, Appels, Stoddard, & McMaster (2001). Starch (0.8 g, accurately weighed and calculated the dry weight (*S*)) was put into a 50 mL centrifuge tube. The sample was well mixed with 40 mL of water (shaken by hand) and then kept for 30 min in a 90 °C water bath. The samples were shaken by hand for every at 10, and 20 minutes during incubation. The samples were equilibrated to room temperature for 1 h and then centrifuged for 15 min at

3000 *g*. The supernatant was removed by suction and the weight of the residue (*B*) used to calculate the total starch swelling power.

Starch swelling power = weight of residue (*B*) / dry weight of starch (*S*)

3.2.6 Measurement of texture properties

The gel texture of cooked surimi was measured according to Meng, Chang, Gillen & Zhang (2016) with minor modifications. Gels were equilibrated to room temperature for 1 h and then cut into cylinders (25 mm height). The puncture test was performed with a texture analyzer (TA-XT2i, Stable Micro Systems, UK). Determination of the breaking force (g) and the deformation distance (mm) was conducted with a probe (8 mm diameter) traveling from the surface of the gel to the point of breakage at the speed 60 of mm/min. Both the breaking force and deformation are reported for the first force peak (Kong, Zhang, Feng, Xue, Wang, Li, Yang & Xue 2016). The gel strength was calculated by

Gel strength = Breaking force × Deformation

3.2.7 Color measurement

The gel samples with the same dimension for texture tests were equilibrated to room temperature for 1 h. Instrumental color was obtained using a colorimeter (Model CR-400, Minolta Camera Co Ltd, Osaka, Japan) and expressed as L* (lightness), a* (redness "+" or greenness "–") and b* (yellowness "+" or blueness "–").

3.2.8 Statistical Analysis

For measurements of texture, color and cooking loss were determined in three replications. Data were subject to analysis of variance using XLStat (2015, Addinsoft USA, New York, NY). Significant differences among means were made with Tukey honestly significant difference (HSD) analysis using probability level of 0.05.

3.3 Results and Discussion

3.3.1 Protein profile of grass carp, silver carp, and bigmouth buffalo

The protein distribution patterns of three species of fish are shown in Figure 3.3. Proteins were extracted from fish meat and surimi paste to investigate the processing effect on protein compositions. No significant processing effect was observed. For all three kinds of fish, myosin heavy chain (MHC), actin, tropomodulin, α-tropomyosin, β-tropomyosin and three myosin light chain (18.5, 16.8 and 13.5 KDa) were all found in the protein profile. However, the protein distribution patterns of three species of fish were different. Grass carp exhibited lower band intensity at 47.9 KDa as well as β-tropomyosin (36.5 KDa) (Carne, van de Ven, Bekhit, & Hopkins, 2015), and the 15.9 KDa myosin light chain protein band of grass carp was absent compared with other 2 species (Crockford & Jonhston, 1990). Most of the bands of bigmouth buffalo were similar to that of grass carp and silver carp, except the tropomodulin (38.8 KDa) band was darker (Wang, Xu, Kang, Shen, & Zhang, 2016) and tropomyosin α (33-37 KDa) (Carne, van de Ven, Bekhit, & Hopkins, 2015; Tao, Kobayashi, Fukushima, & Watabe, 2007) was in different position in buffalo carp.

To my best knowledge, no muscle protein profile of bigmouth buffalo was ever reported. Recently, MHC (200 KDa), actin (45 KDa), troponin-T (35 KDa), myosin light chain (21 KDa), tropomyosin (40 KDa) were identified in protein recovered from silver carp (Abdollahi, Rezaei, Jafarpour & Undeland, 2017, Zhang, Xiong, SYou, Hu, Liu, & Yin, 2017). As to grass carp, MHC (approximately 200 KDa), three myosin light chain, tropomyosin β and tropomyosin α (33-37 KDa) were reported by Tao, Kobayashi, Fukushima, & Watabe (2007) and Yang, Wang, Wang, & Ye (2014). No literature has been reported to compare the differences among carp species in terms of protein compositions. This is the first time to report the different protein compositions of bigmouth buffalo, silver and grass carp.

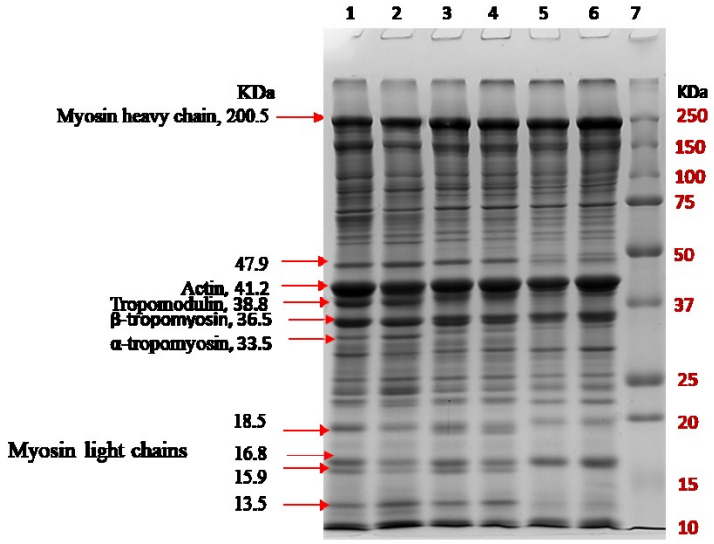

Figure 3.3 SDS-PAGE analysis of the three species of carp.

Lane 1: bigmouth buffalo meat; Lane 2: bigmouth buffalo surimi paste; Lane 3: silver carp meat; Lane 4: silver carp surimi paste; Lane 5: grass carp meat; Lane 6: grass carp surimi paste; Lane 7: molecular mass marker.

3.3.2 Dynamic rheological properties

Sol-gel transition thermograph of three kinds of surimi paste is shown in Figure 3.4 to describe their thermal transition behavior. All the three kinds of surimi showed two peaks of G' during the heating process from 10 °C to 90 °C, however, the temperature range of silver carp peak 1 (34.7°C) was significant lower than that of grass carp (46.0°C) and bigmouth buffalo (48.0°C) as shown in Table 3.2. No significant difference was found between the peak 2 temperature of three fishes.

Zhang, Fang, Wang, Shi, Chang, Yang, & Cui, (2013) suggested the first peak of G' profile reflected the cross-linking effect between proteins molecules through hydrogen bonds, and the drop of G' value after the first peak was due to endogenous proteolytic enzymes, disaggregation of actin-myosin network structure, denaturation of myosin tail and the heat-rupture of hydrogen bonds. Kong, Zhang, Feng, Xue, Wang, Li, Yang, & Xue (2016) further summarized the formation of surimi gel during heating into three-phase processes. The temperatures ranging from 0 to 40 °C is corresponding to the myosin heavy chain (MHC) denatures, unfolds, and polymerization without the formation of non-sulfide covalent crosslinks. The temperatures ranging from 40-70 °C is related to the subunits dissociation and further unfolds of myosin light chain. At a temperature above 70 °C involve intermolecular disulfide bonds and intermolecular hydrophobic attractions. Because the first G' peak of thermograph seems to depend on the fish species, Jafarpour & Gorczyca (2009) summarized G' first peaks at 43, 44 and 52 °C for flying fish myosin, carp myosin and Alaska pollock surimi, respectively. Abdollahi, Rezaei, Jafarpour, & Undeland (2017) reported the first peak temperature of the silver carp myosin existed at 48 °C. Tao, Kobayashi, Fukushima & Watabe (2007) reported the first

peak of grass carp myosin in spring and summer ranged from 38 to 44°C. The first peak of myosin from grass carp in autumn and winter ranged from 28 to 33°C. This evidence suggests that the first peak temperature could be influenced by the culture environment.

The silver carp surimi had a significant higher G' value at peak 2 and final when compared with the meat of the other two fishes. In contrast, bigmouth buffalo had a significant higher G' value at peak 1.

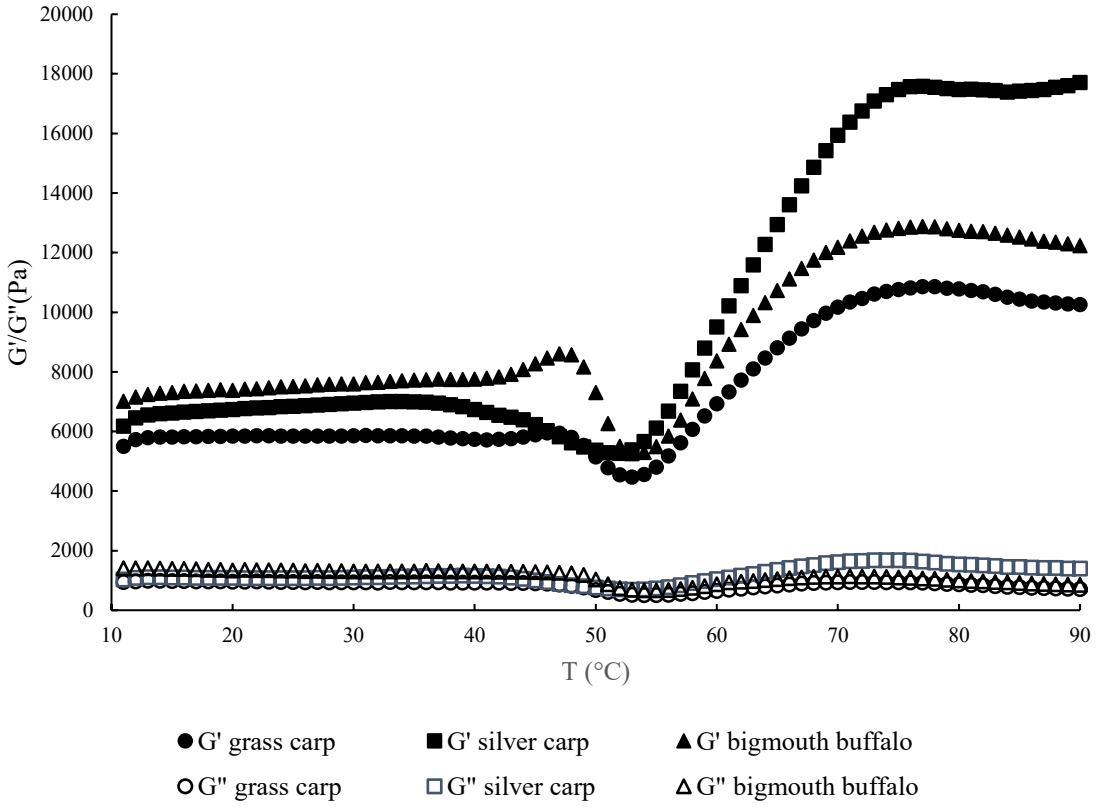

Figure 3.4 Storage modulus (G') and loss modulus (G") of surimi paste made of three kinds of fish (grass carp, silver carp and bigmouth buffalo).

Table 3.2 Storage modulus (G') peaks and loss factor (tan δ) of grass carp, silver carp, and bigmouth buffalo measured by rheometer.

	temperature range (°C)		G' [Pa]			Loss Factor (tanδ)
	peak 1	peak 2	peak 1	peak 2	final	final
grass carp	46.0±0.5 a	78.4±0.7 a	6613±939 a	12748±2671 b	10279±33 b	0.074±0.008 a
silver carp	34.7±1.2 b	77.0±1.7 a	6785±735 a	17764±2516 a	17784±2778 a	0.082±0.003 a
bigmouth buffalo	48.0±1.0 a	77.0±1.0 a	8680±729 b	13825±957 ab	13283±928 b	0.073±0.004 a

Different letters in the same column indicated significant difference between starch at $P<0.05$.

Because G'>G'' (loss modulus) in the whole temperature range, the surimi showed the consistency as a solid (Mezger, 2006). The loss factor (tan δ , the ratio of the viscous to the elastic portion) of grass carp, silver carp and bigmouth buffalo carp meat surimi, declined after the first G' peak and decrease the values continuously with the increase of temperature. This phenomenon indicated more cross-linked covalent were formed during this range of thermal treatment.

3.3.3 RVA pasting properties

Figure 3.5 presents the gelatinization properties for native starch (tapioca), starch acetate (tapioca), distarch phosphate (tapioca), Novation 1900, Firmtex™ and N-Hance 59 measured by RVA, and the parameters of gelatinization temperature, gelatinization maximum, setback, breakdown, and final viscosity are summarized in Table 3.3 as previously described (Agudelo, Varela, Sanz, & Fiszman 2014). After starch dispersions were gelatinized, the samples were subjected to both thermal and shear stresses at the holding temperature (95 °C). The viscosity at the peak of the RVA profile (gelatinization maximum) is considered as the equilibrium point between swelling and rupture of starch granules during heating (Smanalieva, Salieva, Borkoev, Windhab, & Fischer, 2015; Damodaran, Parkin, & Fennema, 2007). Further disruption of starch granules and leaching out of starch molecules caused a decrease in viscosity. The resultant drop in viscosity from peak to a holding strength (minimum viscosity after the peak, occurring around the beginning of RVA cooling stage) was determined and defined as breakdown. Therefore, the breakdown measures the susceptibility of gelatinized starch to disintegration due to the loss of starch granule integrity and subsequent disruption,

leading to a reduction of the paste viscosity (Chantaro & Pongsawatmanit, 2010). The RVA setback obtained from the measurement occurs not only due to the degree of re-association of gelatinized starch (particularly amylose) molecules during cooling, but also due to the simple kinetic effect of cooling on viscosity. The setback value indicates short-term retrogradation of starch. The final viscosity is the viscosity after cooling at the end of RVA experiment.

The viscosity (cP) of native starch (tapioca) and starch acetate (tapioca) increases quickly and reached a peak of the gelatinization to 705.3±0.3 and 908.7±0.1 respectively. During holding at 95°C for 10 min, the viscosity of native starch (tapioca) and starch acetate (tapioca) decreased, while distarch phosphate (tapioca) was still increasing. The breakdown in viscosity was the reduction of viscosity from gelatinization maximum to the end of 95°C holding. Such breakdown has been correlated to the gel stability during cooking of starch (Ragaee & Abdel-Aal, 2006). With few or no breakdown in viscosity, starch acetate and distarch phosphate showed a resistance to thermal treatment. Starch acetate had the highest final viscosity of tapioca starch due to stabilization for retrogradation. In contrast, Novation 1900, Firmtex™ and N-Hance 59 have much higher gelatinization maximum due to use the raw material like waxy corn and potato starches. However, Firmtex™ was similar to distarch phosphate (tapioca) which has higher setback viscosity.

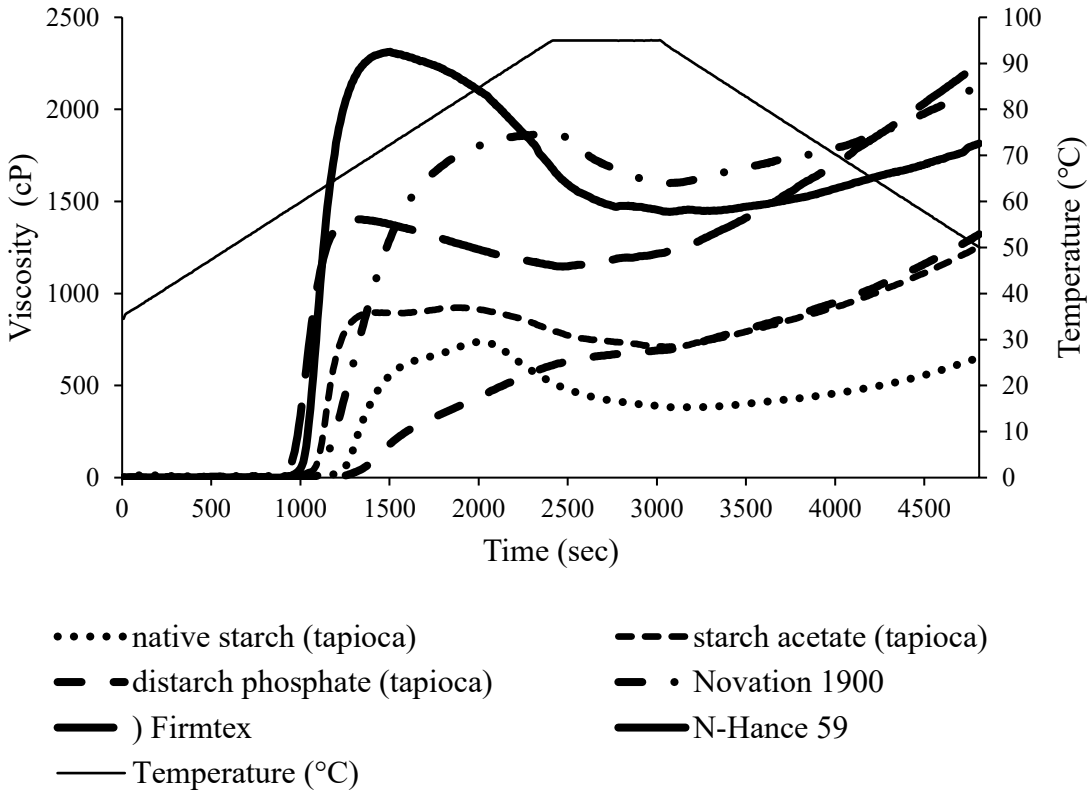

Figure 3.5 Gelatinization properties of (a) native starch (tapioca), (b) starch acetate (tapioca), (c) distarch phosphate (tapioca), (d) Novation 1900, (e) Firmtex™ and (f) N-Hance 59 measured by RVA.

Table 3.3 Gelatinization parameters of native starch (tapioca), starch acetate (tapioca), Distarch phosphate (tapioca), Novation 1900, FirmtexTM and N-Hance 59 measured by RVA.

	Visc(cP)				
	Sample Gelatinization temperature (^{O}C)	Gelatinization maximum	Breakdown viscosity	Setback viscosity	Final viscosity
native starch (tapioca)	64.5±0.3b	705.3±25.4a	336.7±9.7b	259.7±19.7a	628.3±32.8a
starch acetate (tapioca)	60.9±0.1c	908.7±19.6a	236.7±18.2ab	505.0±26.4b	1177.0±55.9b
Distarch phosphate (tapioca)	66.6±0.2a			718.0±63.2c	1515.7±138.9c
Novation 1900	60.1±0.1d	1815.3±125.1c	222.0±40.0a	529.7±55.9b	2123.0±96.3e
Firmtex™	57.2±0.1f	1465.3±93.8b	258.3±55.9ab	1014.3±26.5d	2221.3±82.7e
N-Hance 59	59.0±0.2e	2274.7±27.8d	901.0±29.6c	431.7±57.2b	1805.3±41.8d

Different letters in each column indicated significant difference between starch at P<0.05.

3.3.4 Swelling power of starches

The swelling power of native starch (tapioca), starch acetate (tapioca), distarch phosphate (tapioca), Novation 1900, Firmtex™ and N-Hance 59 were 8.81, 9.88, 8.53, 19.76, 16.69 and 22.16 respectively (Table 3.4). There was no significant difference between three tapioca derived starch in swelling power. The swelling power of Firmtex™, Novation 1900 and N-Hance 59 were significantly higher than all tapioca derived starch. N-Hance 59 had the highest swelling power among all starch samples. Sasaki & Matsuki (1998) stated that the swelling power of starch depends on the water-holding capacity of starch molecules by hydrogen bonding. Besides three kinds of tapioca starches, Novation 1900 was the mixture of corn, waxy corn, potato, tapioca and rice, Firmtex™ was modified waxy corn, and N-Hance 59 was native potato starch. Swinkels (1985) reported the native potato starch had a higher swelling power than native starch made of tapioca and waxy corn. Our results were in consistence with previous reports.

Table 3.4 The swelling power of starches.

	swelling power
native starch (tapioca)	8.66±0.37 a
starch acetate (tapioca)	9.69±0.26 a
Distarch phosphate (tapioca)	8.28±0.21 a
Novation 1900	19.51±0.70 c
Firmtex™	16.02±0.63 b
N-Hance 59	21.66±1.62 d

Different letters indicated significant difference between starch at $P<0.05$

3.3.5 Effects of different starches and fish meat on the textural properties of cooked surimi gel

The results of breaking force (g) of grass carp surimi, silver carp surimi, and bigmouth buffalo are shown in Figure 3.6. The breaking force (611.8 g) of silver carp

surimi were significantly higher than those of grass carp surimi (323.7 g) and bigmouth buffalo (420.4). The results of textural properties were in line with the G' value of peak 2 and final in dynamic rheological assay where the G' of silver carp surimi was significantly higher than those of carp surimi and bigmouth buffalo (Table 3.2). To our best knowledge, there were no reports on the textural and dynamic rheological properties of bigmouth buffalo. Summarized the results from several research groups, the breaking force of silver carp surimi ranged from 300-560 g (Abdollahi, Rezaei, Jafarpour, & Undeland, 2017; Cao, An, Xiong, Li, & Liu, 2016, Luo, Kuwahara, Kaneniwa, Murata, & Yokoyama, 2001; Luo, Shen, Pan, & Bu, 2008, Zhang, Xiong, You, Hu, Liu, & Yin, 2017). As to grass carp surimi, the breaking force was reported 297-502 g (Luo, Shen, & Pan, 2006; Xiong, Cheng, Ye, Du, Zhou, Lin, R., ... & Cai, 2009). Our results were in line with these previous reports. The addition of starches in the grass carp surimi had a significantly destructive effect on the textural properties of the breaking force values compared to the control ($P<0.05$). The breaking force of grass carp surimi with starch added decreased significantly compared with the grass carp surimi without starch ($P<0.05$). Meanwhile, there were significant decrease in the breaking force with Novation 1900 and Firmtex^{TM} when starch levels further increase from 2% to 6% ($P<0.05$). At the level of 6 % added starch, the breaking force of grass carp surimi with Novation 1900 (109.7 g) and Firmtex^{TM} (118.0 g) were significantly lower than those with tapioca derived starch including native starch (151.6 g), distarch phosphate (155.0 g) and starch acetate (181.1 g). In silver carp surimi, the breaking force decreased as the starch concentration increased. At the same additive level, difference existed between different kinds of starch. Different from grass carp surimi, at the same starch level silver carp

surimi with added tapioca derived starch did not have higher breaking force than the others. In silver carp surimi, the breaking force decreased as the tapioca native starch, tapioca acetate starch, or Novation 1900 concentration increased. For all the bigmouth buffalo surimi with addition of starches, the breaking force was significantly lower than the control ($P<0.05$). No difference was found between the bigmouth buffalo surimi with the same starch species at the level 2, 4 and 6% except the samples with Firmtex™.

(a) grass carp

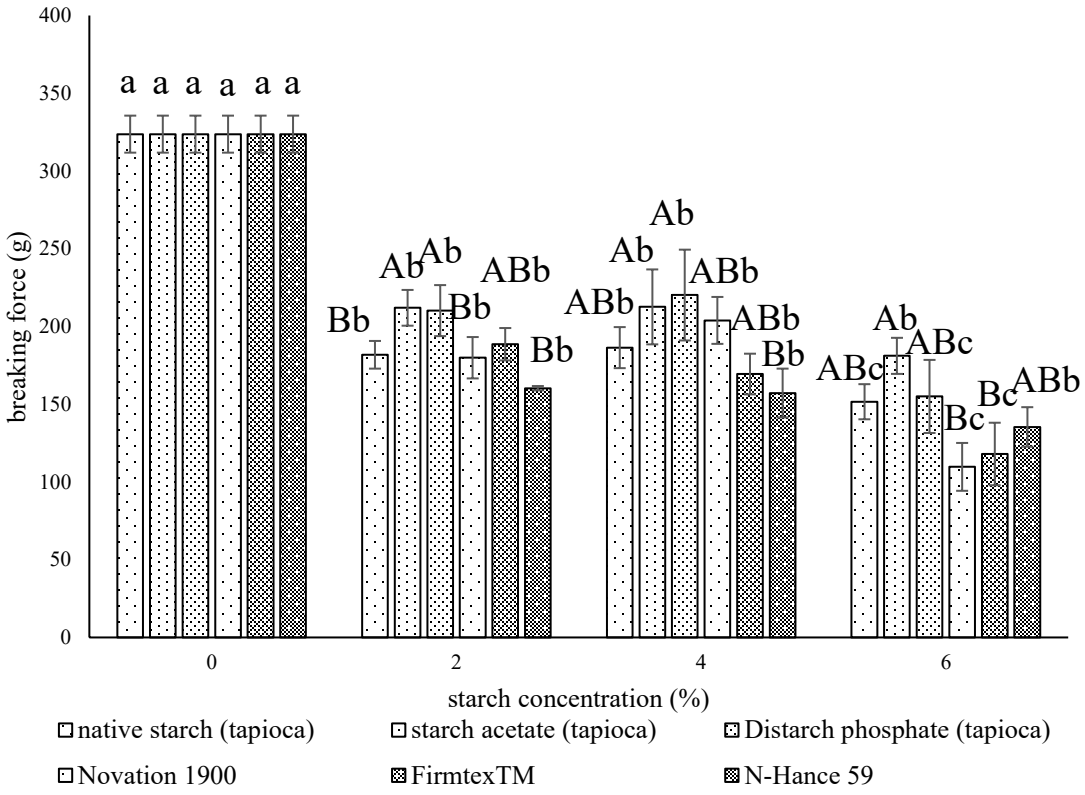

Figure 3.6 Breaking force of cooked surimi gel made of the combination of three kinds of meat ((a) grass carp, (b) silver carp, and (c)bigmouth buffalo) and six kinds of starches (native starch (tapioca), starch acetate (tapioca), distarch phosphate (tapioca), Novation 1900, Firmtex^{TM} and N-Hance 59).

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

(b) silver carp

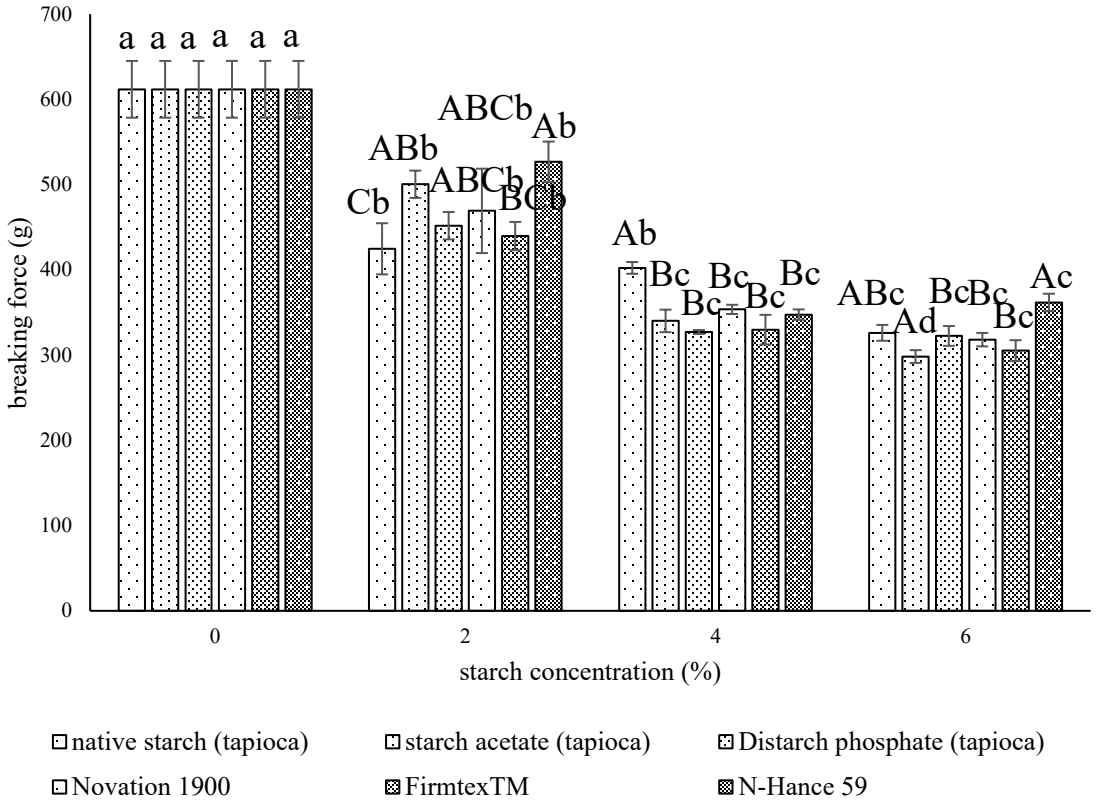

Figure 3.6 (continued)

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

(c) bigmouth buffalo

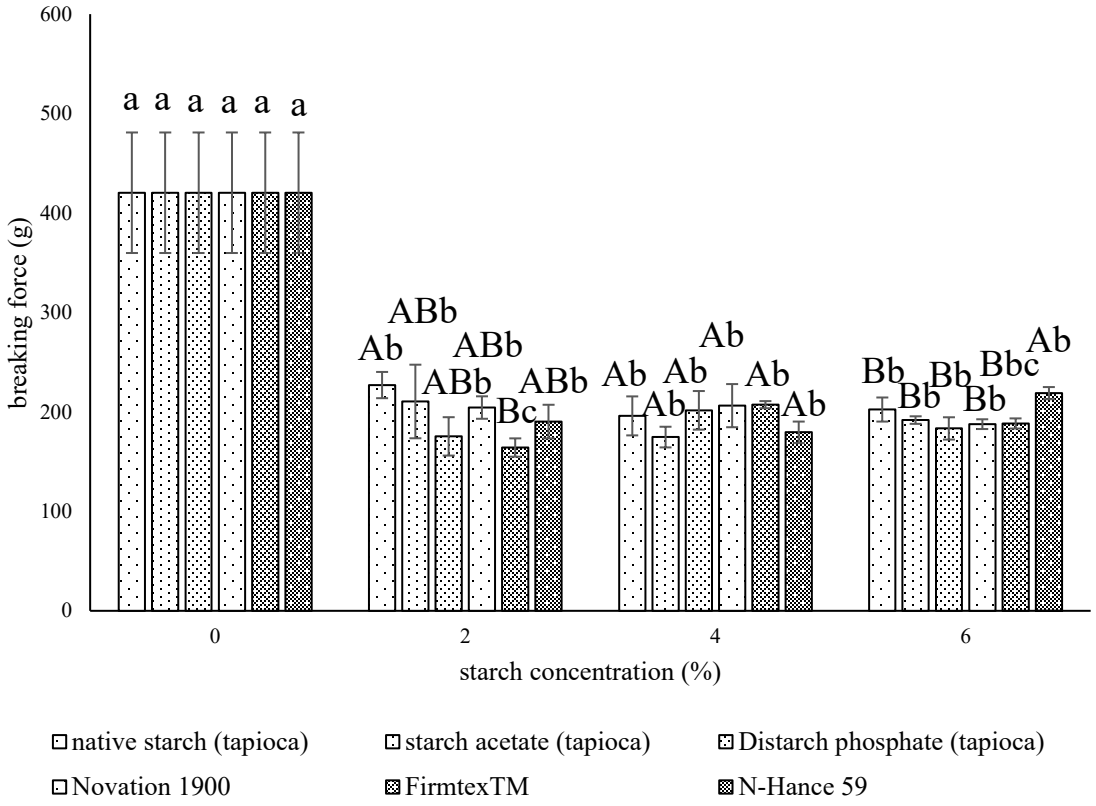

Figure 3.6 (continued)

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

The results of deformation (mm) of grass carp surimi, silver carp surimi, and bigmouth buffalo are shown in Figure 3.7. The deformation (11.7 mm) of silver carp surimi were significantly higher than those of grass carp surimi (9.6 mm) and bigmouth buffalo (8.3 mm). Summarized the results from several research groups, the deformation of silver carp surimi was from 9.0-14.3 mm (Abdollahi, Rezaei, Jafarpour, & Undeland, 2017; Cao, An, Xiong, Li, & Liu, 2016, Luo, Kuwahara, Kaneniwa, Murata, &

Yokoyama, 2001; Luo, Shen, Pan, & Bu, 2008, Zhang, Xiong, You, Hu, Liu, & Yin, 2017). As to grass carp surimi, the deformation was reported 6.7-10.0 mm (Luo, Shen, & Pan, 2006; Xiong, Cheng, Ye, Du, Zhou, Lin, R., ... & Cai, 2009). Our results were in line with these previous reports (9.6 mm). The deformation of all grass carp surimi with starch were significantly lower than control ($P<0.05$) except those with 4% distarch phosphate and Novation 1900 (9.4 mm). At the level of 6 % added starch, the deformation of grass carp surimi with Novation 1900 (6.6 mm), Firmtex™ (6.6 mm) and N-Hance 59 (7.7 mm) were significantly lower than those with tapioca derived starch including native starch (8.4 mm), distarch phosphate (8.9 mm) and starch acetate (9.4 mm). No significant difference of deformation was found between silver carp surimi with 0, 2, 4 and 6% tapioca distarch phosphate. The deformation of silver carp with 2% N-Hance 59 (12.9 mm) was higher than control (11.7 mm). On the contrary, the deformation of bigmouth buffalo surimi was increased by the addition of starch except N-Hance 59.

(a) grass carp

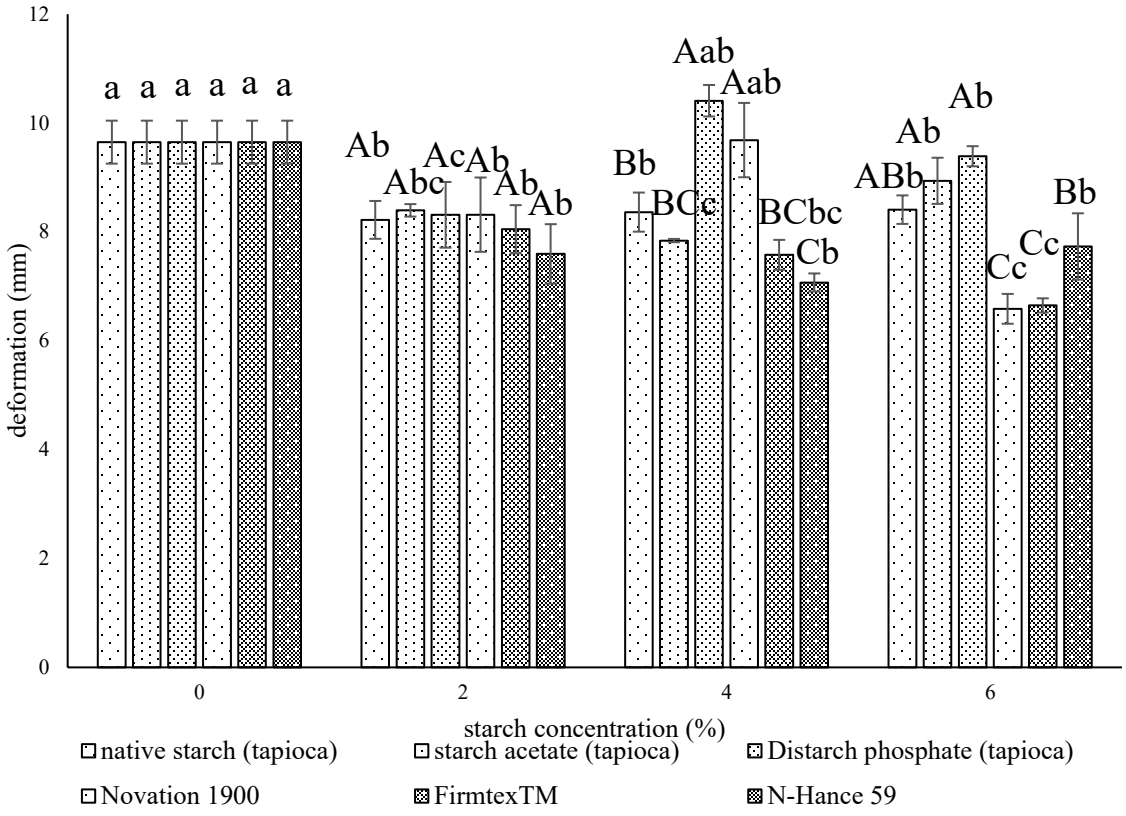

Figure 3.7 Deformation of cooked surimi gel made of the combination of three kinds of meat ((a) grass carp, (b) silver carp, and (c)bigmouth buffalo) and six kinds of starches (native starch (tapioca), starch acetate (tapioca), distarch phosphate (tapioca), Novation 1900, Firmtex^{TM} and N-Hance 59).

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

(b) silver carp

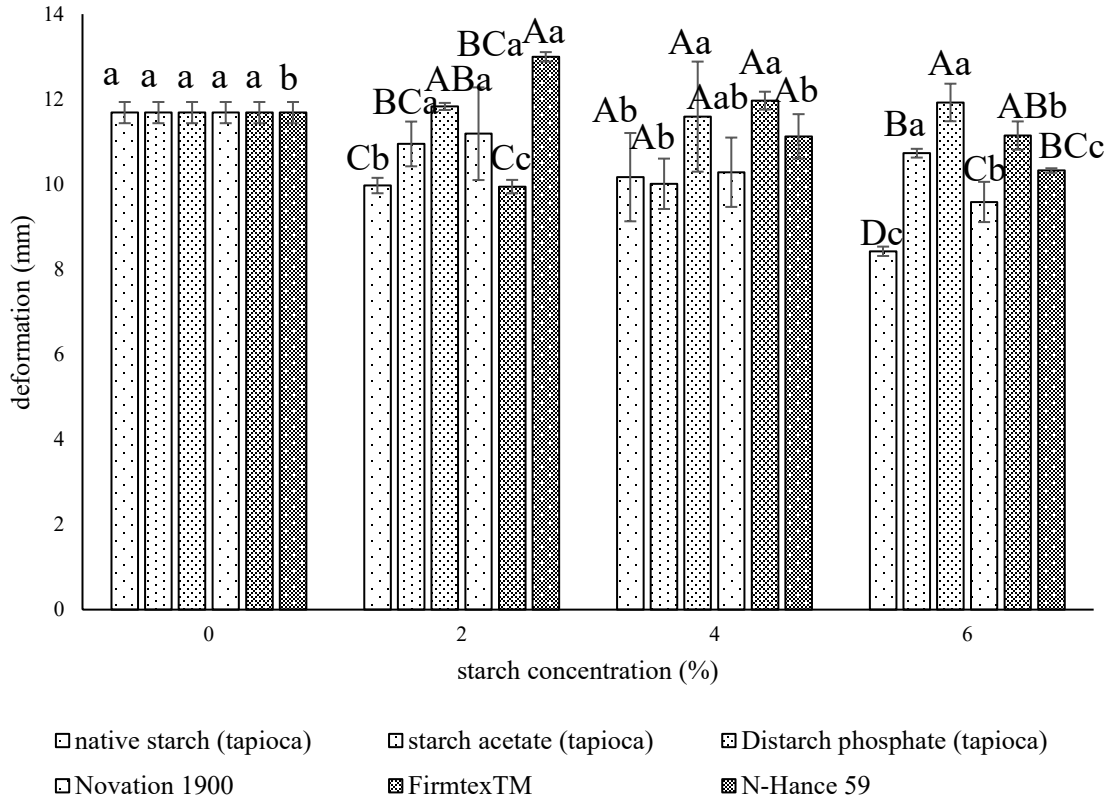

Figure 3.7 (continued)

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

(c) bigmouth buffalo

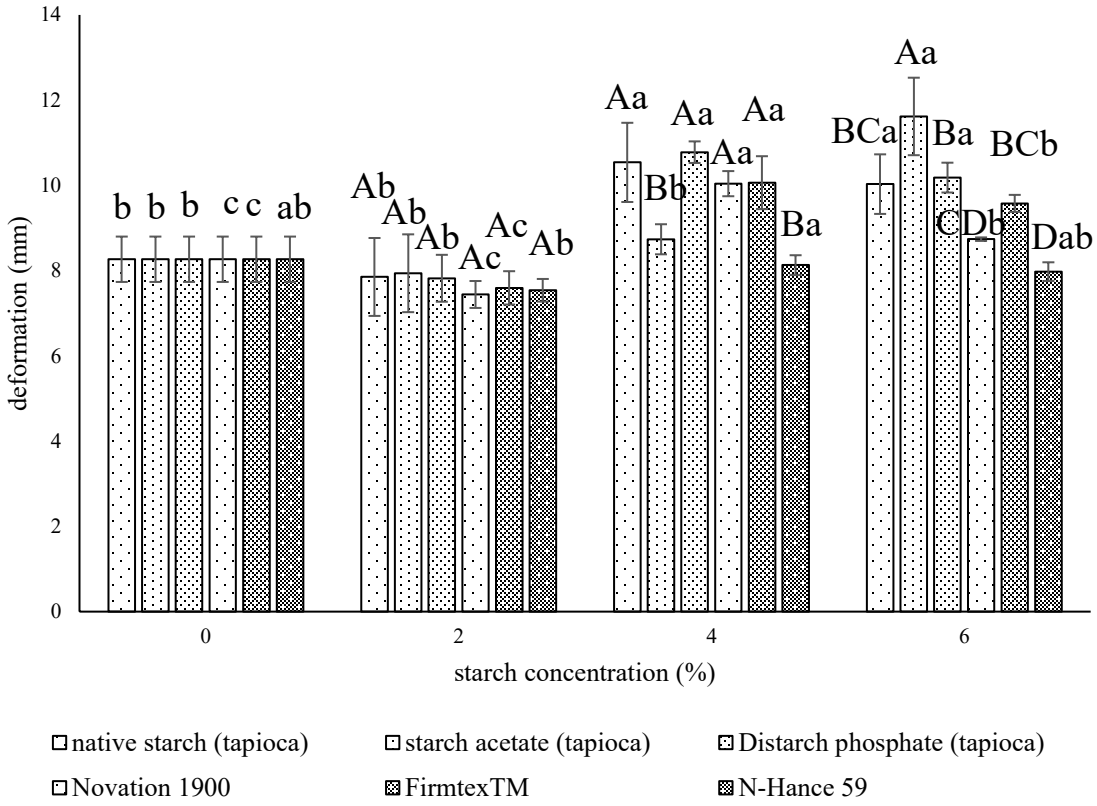

Figure 3.7 (continued)

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

The results of gel strength (g × mm) of grass carp surimi, silver carp surimi, and bigmouth buffalo are shown in Figure 3.8. The impact of the addition of starch or modified starch on strength (g × mm) were different among grass carp, silver carp and bigmouth buffalo as the values of breaking force and deformation mentioned above. The formation of the three-dimensional network by protein during heating is generally thought to be due to the denaturation of the protein molecules and interaction of the

denatured molecules to form cross-linkages (Park, 2013). In the mixture of surimi, the starch gelatinized during heating through granule swelling, disruption of crystalline regions, loss of birefringence, increase of viscosity, fragmentation of the granules. Although the relationships between starch and surimi protein in fish-meat gels have not been fully elucidated, it had been proposed that that the starch granules bound in the surimi proteins had a "packing effect" on the surimi protein due to the internal pressure (Kong, Ogawa, & Iso, 1999). The starch granules embedded in the surimi not only competed the water bound in the surimi to swell and gelatinize, but also exerted pressure against the surimi which had already started gelatinization. In contrast, the growth of the starch granules may be limited by the gelatinized surimi and the shortage of water.

Yang & Park (1998) investigated the effects of starch and thermal-processing conditions on texture of high-grade Alaska pollock (*Theragra chalcogramma*) surimi by measuring shear stress. By application of native potato starch, potato starch acetate, native corn starch and two modified waxy corn starch, Yang & Park (1998) generalized that the more amylopectin in test starches, the stronger the surimi-starch gels became. The effects and mechanism of modified starches on the gel properties were studied on myofibrillar protein from grass carp (Sun, Huang, Hu, Xiong, & Zhao, 2014). Modified starch such as hydroxypropylated cassava starch and cross-linked hydroxypropylated cassava starch facilitated the formation of compact and homogeneous network. Sun, Huang, Hu, Xiong, & Zhao, (2014) stated the addition of modified starch promoted the heat-induced conformational transition from the α-helix companied with β-turn to β -sheet, leading to the myofibrillar molecules stretching out. Therefore, properties of starch interact with the proteins and affects the gel texture of surimi.

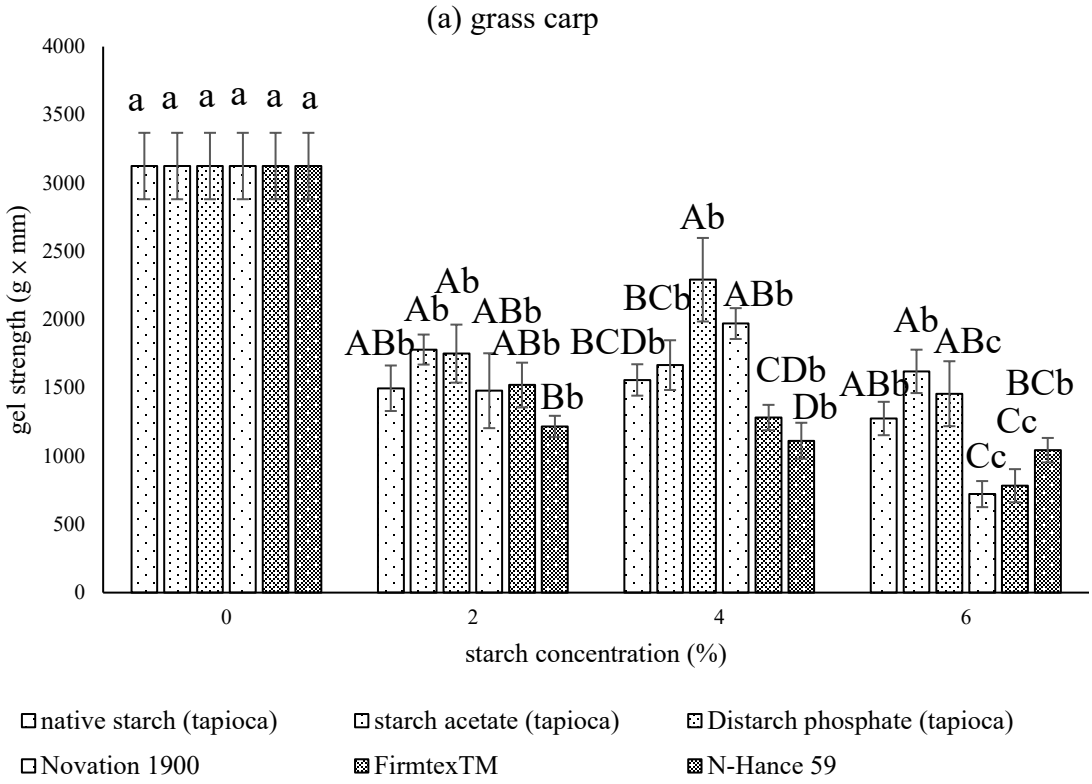

Figure 3.8 Gel strength (c) of cooked surimi gel made of the combination of three kinds of meat ((a) grass carp, (b) silver carp, and (c) bigmouth buffalo) and six kinds of starches (native starch (tapioca), starch acetate (tapioca), distarch phosphate (tapioca), Novation 1900, Firmtex^{TM} and N-Hance 59).

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

(b) silver carp

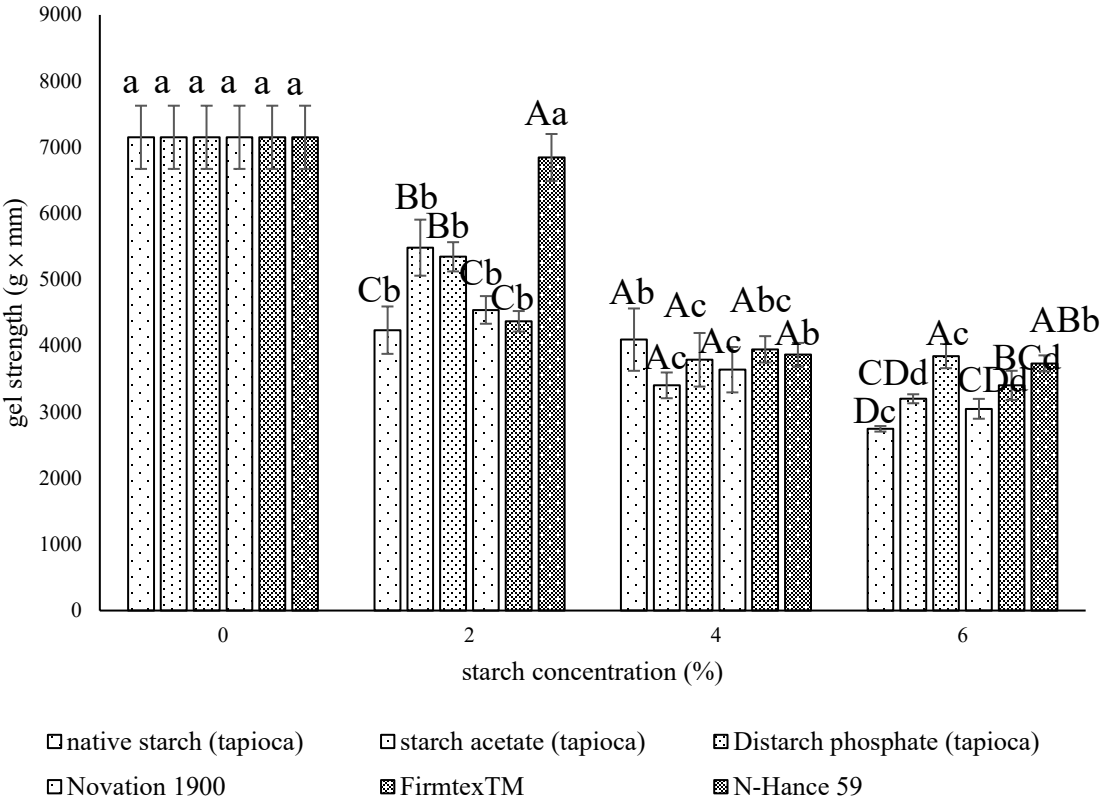

Figure 3.8 (continued)

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

(c) bigmouth buffalo

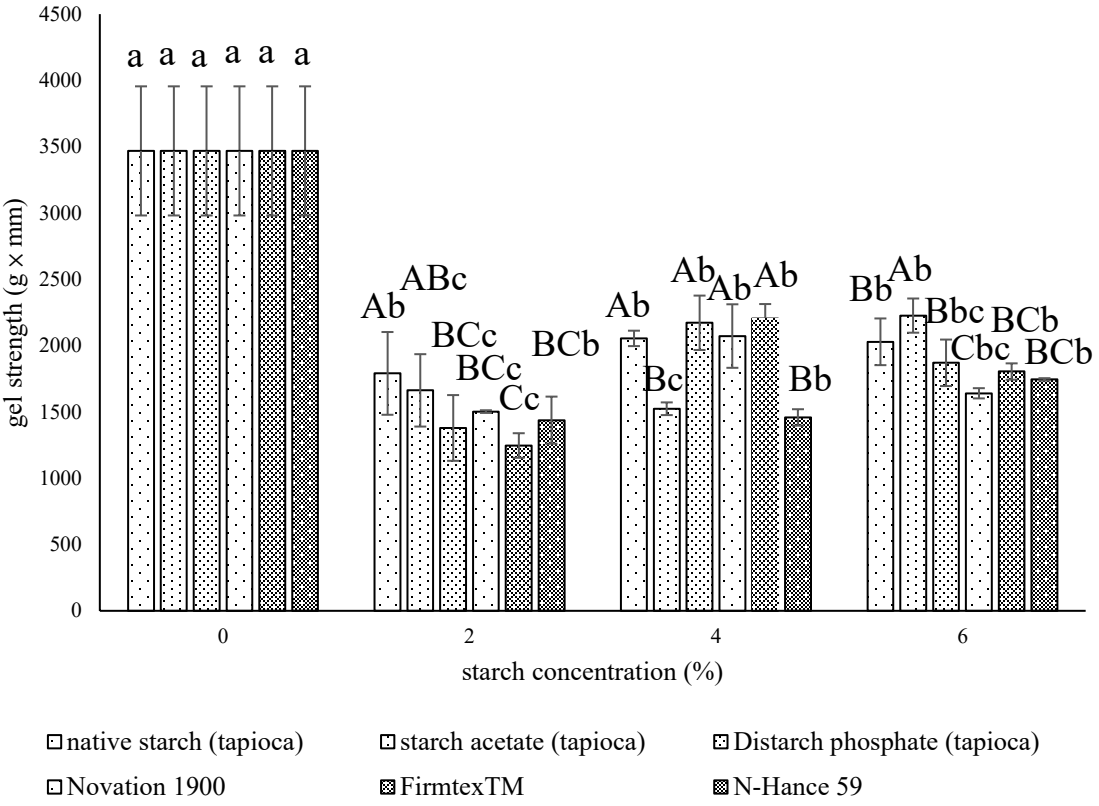

Figure 3.8 (continued)

Different lower-case letters indicated differences ($p < 0.05$) among levels of one kind of starch addition ($p < 0.05$). Different capital letters indicated differences ($p < 0.05$) among six kind of starch addition of same starch concentration.

3.3.6 Effects of different starches and fish meat on the color of cooked surimi gel

For all three kinds of freshwater fish, L* and b* values (Table 3.5) significant by increased by the concentration of all six kinds of starch compared with the control. The a* value was decreased significantly except the silver carp surimi with acetate starch or bigmouth buffalo surimi with Novation 1900, and Firmtex^{TM}. Yang & Park (1998) reported the color (L* and b*) of surimi-starch gels depended on concentration and

properties of starch. The more the granule swollen, the lower the L* value and the b* value of gels. Our results were different from their findings. The addition of starch increased the lightness of cooked surimi gel in our experiments.

The preferred color for surimi is white. Hur, Choi, Choi, Kim, & Jin (2011) reported the L* of freshly made imitation crab sticks ranging from 77.1 to 79.4. For this reason, Jafarpour & Gorczyca (2008) reported an effort on improvement of color of surimi by hydrogen peroxide. However, hydrogen peroxide decreased the water holding capacity of the fillets.

Table 3.5 The color (Hunter parameters, L*, a*, b*, Saturation and Hue) of cooked surimi gel made of the combination of three kinds of meat (silver carp, grass carp, and bigmouth buffalo) and six kinds of starches (native starch (tapioca), starch acetate (tapioca), distarch phosphate (tapioca), Novation 1900, Firmtex™ and N-Hance 59).

(L*)

fish	starch	concentration (%)			
		0	2	4	6
grass carp	native starch (tapioca)	45.79±1.49 c	48.12±0.72 b	49.16±1.36 ab	51.09±0.27 a
grass carp	starch acetate (tapioca)	45.79±1.49 b	46.74±3.75 ab	49.59±1.21 ab	50.72±0.29 a
grass carp	distarch phosphate (tapioca)	45.79±1.49 b	46.42±1.04 b	49.08±0.39 a	50.57±1.01 a
grass carp	Novation 1900	45.79±1.49 c	48.07±1.39 bc	50.35±1.56 ab	51.42±0.78 a
grass carp	Firmtex™	45.79±1.49 c	46.55±0.56 c	48.82±0.50 b	52.29±1.70 a
grass carp	N-Hance 59	45.79±1.49 c	46.62±1.13 bc	49.11±1.29 b	52.31±1.68 a
silver carp	native starch (tapioca)	43.07±0.58 b	43.18±0.22 b	48.41±3.09 a	48.33±0.94 a
silver carp	starch acetate (tapioca)	43.07±0.58 b	44.53±0.40 b	46.09±0.46 a	47.16±0.92 a
silver carp	distarch phosphate (tapioca)	43.07±0.58 b	44.91±0.27 b	44.61±0.11 b	46.97±1.51 a
silver carp	Novation 1900	43.07±0.58 c	44.01±0.27 bc	46.43±1.43 ab	48.46±1.83 a
silver carp	Firmtex™	43.07±0.58 b	45.97±0.41 a	45.18±1.24 ab	46.13±0.96 a
silver carp	N-Hance 59	43.07±0.58 b	44.72±1.01 b	45.38±10.5 b	48.19±1.23 a
bigmouth buffalo	native starch (tapioca)	46.07±0.69 b	45.97±0.69 b	48.22±0.83 a	49.68±0.75 a
bigmouth buffalo	starch acetate (tapioca)	46.07±0.69 b	45.66±0.58 b	49.37±0.73 a	50.14±0.44 a
bigmouth buffalo	distarch phosphate (tapioca)	46.07±0.69 c	46.20±0.07 c	49.24±0.22 b	52.04±1.79 a
bigmouth buffalo	Novation 1900	46.07±0.69 b	46.32±0.81 b	48.44±0.79 a	48.62±0.92 a
bigmouth buffalo	Firmtex™	46.07±0.69 c	46.59±0.53 bc	49.23±0.47 a	48.33±1.80 ab
bigmouth buffalo	N-Hance 59	46.07±0.69 c	47.31±1.42 bc	49.02±0.51 ab	50.04±0.73 a

Mean values with different lower case letters in same row are significantly different ($p < 0.05$).

Table 3.5 (continued)

(a*)

fish	starch	concentration (%)			
		0	2	4	6
grass carp	native starch (tapioca)	4.77±0.52 a	4.27±0.13 ab	3.83±0.51 bc	3.24±0.12 c
grass carp	starch acetate (tapioca)	4.77±0.52 a	4.63±1.23 a	3.89±0.36 ab	2.89±0.65 b
grass carp	distarch phosphate (tapioca)	4.77±0.52 ab	4.87±0.41 a	4.01±0.29 b	2.89±.041 c
grass carp	Novation 1900	4.77±0.52 a	4.13±0.40 a	3.77±0.85 ab	2.83±0.41 b
grass carp	Firmtex™	4.77±0.52 a	4.68±0.18 a	4.33±0.25 a	2.62±0.63 b
grass carp	N-Hance 59	4.77±0.52 a	4.71±0.81 a	3.99±0.45 a	2.39±0.52 b
silver carp	native starch (tapioca)	3.93±0.08 ab	4.45±0.49 a	4.13±0.58 ab	3.33±0.55 b
silver carp	starch acetate (tapioca)	3.93±0.08 a	4.40±0.15 a	3.80±0.54 a	4.14±0.09 a
silver carp	distarch phosphate (tapioca)	3.93±0.08 b	4.67±0.19 b	4.06±0.29 a	4.58±0.09 b
silver carp	Novation 1900	3.93±0.08 ab	3.65±0.77 a	4.08±0.38 b	4.49±0.28 ab
silver carp	Firmtex™	3.93±0.08 a	4.29±0.50 a	4.68±0.28 a	4.35±0.13 a
silver carp	N-Hance 59	3.93±0.08 ab	4.35±0.13 ab	5.23±1.19 a	3.60±0.49 b
bigmouth buffalo	native starch (tapioca)	4.23±0.17 a	4.24±0.42 a	3.76±0.28 ab	3.33±0.63 b
bigmouth buffalo	starch acetate (tapioca)	4.23±0.17 a	4.28±0.26 a	3.59±0.28 a	2.72±0.64 b
bigmouth buffalo	distarch phosphate (tapioca)	4.23±0.17 a	4.21±0.12 a	3.59±0.74 a	2.46±0.24 b
bigmouth buffalo	Novation 1900	4.23±0.17 a	3.95±0.21 a	3.64±0.75 a	3.72±0.24 a
bigmouth buffalo	Firmtex™	4.23±0.17 a	4.57±0.31 a	3.66±0.39 a	3.79±0.77 a
bigmouth buffalo	N-Hance 59	4.23±0.17 a	3.93±0.34 a	3.99±0.23 a	3.12±0.10 b

Mean values with different lower case letters in same row are significantly different ($p < 0.05$).

Table 3.5 (continued)

(b*)

fish	starch	concentration (%) 0	2	4	6
grass carp	native starch (tapioca)	19.42±1.22 b	20.29±0.02 b	20.60±0.39 b	22.58±0.64 a
grass carp	starch acetate (tapioca)	19.42±1.22 a	19.45±0.33 a	21.03±0.29 a	21.10±1.33 a
grass carp	distarch phosphate (tapioca)	19.42±1.22 a	20.12±0.75 a	20.69±0.59 a	20.48±1.33 a
grass carp	Novation 1900	19.42±1.22 b	19.16±0.81 b	21.44±0.97 a	19.45±0.97 b
grass carp	Firmtex™	19.42±1.22 b	19.69±0.53 b	21.84±0.69 a	20.05±0.89 b
grass carp	N-Hance 59	19.42±1.22 ab	19.75±0.32 ab	20.95±0.21 a	18.95±0.99 b
silver carp	native starch (tapioca)	15.67±0.21 b	16.35±0.66 b	18.40±0.69 a	18.72±0.40 a
silver carp	starch acetate (tapioca)	15.67±0.21 d	16.76±0.41 c	18.29±0.16 b	19.39±0.31 a
silver carp	distarch phosphate (tapioca)	15.67±0.21 d	16.56±0.35 c	18.07±0.33 b	19.14±0.62 a
silver carp	Novation 1900	15.67±0.21 b	17.18±0.64 ab	17.73±1.03 a	18.79±1.29 a
silver carp	Firmtex™	15.67±0.21 b	17.78±1.05 a	18.09±0.83 a	18.80±0.25 a
silver carp	N-Hance 59	15.67±0.21 c	17.40±0.36 b	18.03±0.54 ab	19.24±1.02 a
bigmouth buffalo	native starch (tapioca)	18.28±0.73 b	18.37±0.25 b	19.81±1.00 a	20.39±7.73 a
bigmouth buffalo	starch acetate (tapioca)	18.28±0.73 b	18.33±0.24 b	19.87±0.28 a	20.10±1.00 a
bigmouth buffalo	distarch phosphate (tapioca)	18.28±0.73 b	18.29±0.24 b	19.51±0.32 a	20.05±0.41 a
bigmouth buffalo	Novation 1900	18.28±0.73 b	18.19±0.46 b	19.93±1.09 a	19.26±0.33 ab
bigmouth buffalo	Firmtex™	18.28±0.73 c	18.78±0.72 bc	19.77±0.28 ab	20.06±0.92 a
bigmouth buffalo	N-Hance 59	18.28±0.73 b	18.66±0.23 b	21.12±1.94 a	19.51±0.17 ab

Mean values with different lower case letters in same row are significantly different ($p < 0.05$).

3.4 Conclusion

The SDS-PAGE analysis, puncture, and dynamic rheological assay revealed the differences between the surimi made of the meat of silver carp, grass carp, and bigmouth buffalo and explained the highest gel strength of silver carp surimi. Novation 1900, Firmtex^{TM} and N-Hance 59 had higher viscosity and swelling power compared with native starch (tapioca), starch acetate (tapioca), distarch phosphate (tapioca). The result of the puncture test is in line with the temperature sweep test data, confirming the higher plasticity of silver carp than that of either bigmouth buffalo surimi or grass carp surimi. The effects of the added starch or modified starch to breaking force and deformation were different between the surimi made of grass carp, silver carp and bigmouth buffalo. The use of starch and modified starch improved the color in L* and b* values. It needs more studies to make the white surimi products such as imitation crab sticks.

CHAPTER IV

THE EFFECTS OF COLD STORAGE ON THE MURINE NOROVIRUS-1 (MNV-1), INHERENT MICROBIOTA, WEITGH LOSS AND TOTAL VOLATILE BASE NITROGEN (TVB-N) IN WHOLE SHELL OYSTER, SALMON FILLET AND SILVER CARP SURIMI

4.1 Introduction

HuNoV are the primary cause of epidemic gastroenteritis worldwide and are estimated to cause 218,000 deaths and 1.1 million hospitalizations among children in developing countries each year (Seitz, Leon, Schwab, Lyon, Dowd, McDaniels, Abdulhafid, Fernandez, Lindesmith, Baric, & Moe 2011). They are nonenveloped, single stranded RNA viruses that spread primarily through the fecal-oral route (Scipioni, Mauroy, Vinje, & Thiry, 2008). HuNoV can be transmitted through environmental water body. Evidence shows that HuNoV was detectable in mineral water at 4 and 25°C up to 100 days by reverse transcription-quantitative PCR (RT-qPCR). Seeded HuNoV remained infectious in groundwater after storage at room temperature in the dark for 61 days while virus RNA within intact capsids could be detected for 1,266 days, with no significant $\log_{10}$ reduction throughout 427 days (Seitz, Leon, Schwab, Lyon, Dowd, McDaniels, Abdulhafid, Fernandez, Lindesmith, Baric, & Moe 2011).

With its ability to remain in the suspension or association with organic and inorganic matters for considerable long time, HuNoV can be accumulated by shellfish in coastal waters (Campos & Lees, 2014). Because shellfish take considerably longer to purge HuNoV than fecal indicator bacteria during purification, outbreak of this virus might happen even the products are in line with the microbiological criteria of seafood standard. Besides bio-accumulated contamination in shellfish, faecally contaminated surfaces and directly person-to-person contact can also contribute to foodborne transmission of viral disease outbreaks (D'Souza, Sair, Williams, Papafragkou, Jean, Moore, & Jaykus, 2006).

The study of survivability of HuNoV in shellfish or contaminated food can help to understand the risk of infection of these foods during storage or processing. Because HuNoV can't be cultured on artificial media, the virus therefore can't be studied using cell culture and small animal model. HuNoV surrogates are widely used by researchers to predict the behavior of HuNoV (Cannon, Papafragkou, Park, Osborne, Jaykus, & Vinjé 2006). Murine norovirus (MNV-1) is widely accepted as the surrogate and is assumed to mimic the survival and inactivation of human noroviruses (Hirneisen & Kniel, 2013). MNV was proven to remain infectious in water over 30 days at a titer of approximately 5 log PFU/mL at 4°C. MNV-1 also persisted over a wider range of pH values, and at 2 ppm of chlorine without a loss of the titer at 4°C.

Many researches reported the persistence of norovirus in various environmental conditions, artificial surface or food matrix. Lee, Yoo, Ha, & Choi (2012) found the significant reduction of norovirus surrogate (1.47 log units) with increased lactic acid

bacteria and acidity for 20 days of Dongchimi fermentation (a type of vegetable fermented in a water base). When inoculated on lettuce, there was about a 1.5 log PFU g^{-1} drop in MNV-1 infectivity for the lettuce stored 14 days at 4°C, and about a 3.0 log PFU g^{-1} drop for when stored at room temperature (Escudero, Rawsthorne, Gensel, & Jaykus 2012). When Alfalfa seeds were inoculated with MNV-1 with titers of 6.5 log PFU g^{-1}, the virus remained infectious with titers about 1.6 log PFU g^{-1}after 50 days of storage at 22°C (Wang, Hirneisen, Markland, & Kniel 2013).

In this study, the oysters were supplied by a local family-own company in Mississippi state, which could supply oysters year-round (Figure 4.1, Figure 4.2). The family has been in the oyster business for several generations. Because the family controls private oyster beds, runs oyster boats, and operates buying docks along the Mississippi and Louisiana coasts, the company can provide fresh oysters for this research. The titers of bio-accumulated MNV-1 in alive oysters, and salmon meat and silver carp surimi inoculated with MNV-1 were evaluated for the survival of HuNoV and infection risk of these food during 20 days of storage in 5°C. The weight loss, microbiological quality, and total volatile basic nitrogen (TVB-N) were also analyzed to reflect the dynamic state of food quality and the persistence of norovirus.

Figure 4.1 Automatic oyster washing in a food company of Mississippi State, Crystal Seas Oysters in Mississippi state.

Figure 4.2 IQF processing in a food company of Mississippi State, Crystal Seas Oysters in Mississippi state.

4.2 Materials and Methods

4.2.1 Atlantic oysters (*Crassostrea virginica*)

Fresh harvested Atlantic oysters were purchased from an oyster processor (Crystal Seas Seafood, Pass Christian, MS) and transported immediately (within 1 h) in a cooler with cubed ice to ESPL in Pascagoula, MS. After washing with tap water and removing mud from the shells, oysters with sizes of 7-9 cm were chosen for the experiments.

4.2.2 Murine norovirus (MNV-1)

Murine norovirus-1 (MNV-1) was generously provided by Dr. Herbert W. Virgin IV, Washington University School of Medicine, St. Louis, MO. A hematopoietic lineage

(murine BV-2) was used as the host throughout the experiments because in the its high efficiency in the detection of MNV-1 (Cox, Cao, & Lu, 2009).

For preparing viral stock, BV-2 cells were seeded into a 75 cm^2 flask with DMEM-10 [DMEM-10 is high-glucose Dulbecco's modified eagle's medium (DMEM, HyClone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), 10 mM HEPES (HyClone Laboratories, Logan, UT), 1 mM non-essential amino acid (HyClone Laboratories, Logan, UT), 2mM L-glutamine (HyClone Laboratories, Logan, UT),100 U/mL penicillin (HyClone Laboratories, Logan, UT) and 100 mg/mL streptomycin (HyClone Laboratories, Logan, UT) (Gonzalez-Hernandez, Cunha, & Wobus, 2012)] to form an approximately 80-90% confluent cell monolayer. After removing the medium, 2 mL of previously-made viral stock were added into the flask. The flasks were incubated for one hour at room temperature, and then washed twice with DMEM-0 (DMEM-0 is the same medium as DMEM-10 except FBS is reduced to 0%). After washing, 20 mL of DMEM-5 (DMEM-5 is the same medium as DMEM-10 except FBS is reduced to 5%) were added into the flasks. The flasks were then incubated until approximately 90% viral-induced cytopathic effects (CPE), including rounding of cells, loss of contact inhibition and cell death were observed. The flasks were then stored at -80 °C. After 24 h storage, the flasks were then allowed to thaw at room temperature (22 °C). Following two additional freeze-thaw cycles, the content of the flask was centrifuged at 3000 rpm for 5 min to remove all cellular debris. The supernatant was then stored at -80 °C until use.

For determining the titer of the viral stock, plaque assays were performed in triplicate according to the method of Gonzalez-Hernandez, Cunha, & Wobus (2012). BV-2 cells were seeded into 6-well plates at a ratio that allowed the formation of a confluent monolayer within 24 h. For MNV-1 infection at various dilution rates, 0.5 mL of 10-fold diluted virus samples was inoculated onto the monolayer cell grown on the 6-well plates. The plates were incubated for one hour at room temperature, and gently shaken every 15 min for even distribution of the viral inoculum. The viral inoculum was removed at the end of incubation. Two mL of overlay (1:1 mixture of 3% w/v Sea-Plaque agarose (Lonza, Allendale, NJ) and 2 X MEM media (Gibco-Invitrogen, Grand Island, NY) were slowly overlaid on the edge of each well. The overlay was allowed to solidify for 10 min at room temperature before placing plates into the tissue culture incubator. The plates were incubated for 48 h at 37 °C in 5% CO_2. The neutral red staining solution was prepared by adding 3 mL of neutral red (Sigma Chemical Co., St. Louis, MO) to 97 mL cell culture grade phosphate-buffered saline (Gibco-Invitrogen, Grand Island, NY). Two mL staining solution was added directly onto the overlay. Plaques were count by proper dilution in wells where plaques are clearly separated. The titer of the viral stock that was used in this report was approximately 4×10^7 plaque forming unit (PFU) per mL.

4.2.3 MNV-1 bioaccumulation in whole-shell oyster

Whole-shell oysters accumulated MNV-1 were prepared as previously described Araud, DiCaprio, Ma, Lou, Gao, Kingsley, et al. (2016) with a few modifications. For each batch, 18 live oysters were cultivated in a polyethylene tank, containing

recirculating 60 L of artificial seawater (1.5% sea salt, Oceanic system, Dallas, TX) at room temperature (20-22 °C). Oysters were fed using phyto-plankton (Reef Phytoplankton, Seachem, Madison, GA) according to the instruction of the manufacturer. The seawater was contaminated with MNV-1 at 10^4 PFU/mL where oysters were cultivated for 24 h before X-ray treatments (the initial level of MNV-1 in oysters was about 4.6 log PFU g^{-1}). The MNV-1 contaminated oysters were placed into sterile Whirl-Pak bags and kept at 5 °C for 20 days.

4.2.4 Fresh salmon fillet

Fresh salmon fillets were purchased from local producer (Rouses Market, Ocean Springs, MS). The raw fish products were brought to the laboratory in chilled containers. The salmon sushi was cut to the weight of 25 g, placed into sterile Whirl-Pak bags and kept at 5 °C.

4.2.5 Preparation of surimi gels

Frozen surimi was thawed at 4 °C for 12 h and then homogenized with ice water in a hand blender for 1 min to adjust moisture content. Ingredients including distarch phosphate (tapioca) were added as formula C with starch acetate in Table 3.1 and homogenized for further 3 min. The paste was stuffed into sealed polypropylene casing (3 cm inner diameter; 10 cm length), heated in a water bath at 40 °C for 15 min, and then followed by heating at 90 °C for 30 min. The cooked surimi gel was cooled and stored at 4 °C overnight. The cooked surimi was cut to the weight of 25 g, placed into sterile Whirl-Pak bags, and kept at 5 °C.

4.2.6 Inoculation of salmon fillet and cooked surimi

For salmon fillet and cooked surimi, 100 µL of MNV-1 virus stock solution were spotted (10 drops) on the samples (25 g). After air-dried for 30 min at 22 °C in biosafety cabinet, the inoculated sample was aseptically placed into a sterile Whirl-Pak bag for irradiation treatment. The packed samples were placed in an incubator at 5 °C for 20 days.

4.2.7 MNV-1 enumeration (plaque assay)

Whole shell oysters, salmon fillet and cooked surimi were sampled on 0, 5, 10, 15 and 20 d for plaque assay. Virus was extracted as described by Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai (2013) with a few modifications. For each plaque assay, three contaminated oysters were shucked and pooled in 200 mL of phosphate buffer (0.15 M Na_3PO_4, pH 9.5) and homogenized by a laboratory blender (Waring Inc., New Hartford, CT) for 3 min. The homogenized tissues were centrifuged with a benchtop centrifuge. The supernatant was neutralized with 2N HCl. Tenfold serial dilutions of neutralized supernatant were made in Earle's balanced salt solution (EBSS) (Gibco-Invitrogen, Grand Island, NY). Plaque assays were performed as described by Wu, Chang, Nannapaneni, Coker, Haque, & Mahmoud (2016). A series of 10-fold dilutions of sample were inoculated onto the 6-well plates after the formation of a confluent monolayer of BV-2, aspiration of the medium and two cell washes with DMEM-0. The inoculums were incubated for one hour at room temperature with gentle rock every 15 min for even distribution. The viral inoculum was removed at the end of incubation. To

each well, 2 mL of overlay (1:1 mixture of 3% w/v Sea-Plaque agarose (Lonza, Allendale, NJ) and 2 X MEM media (Gibco-Invitrogen, Grand Island, NY)) were slowly poured on the edge. The plates were placed into the tissue culture incubator after solidified for 10 min at room temperature.

The neutral red staining solution (3%) was prepared with neutral red (Sigma Chemical Co., St. Louis, MO) and culture grade phosphate-buffered saline (Gibco-Invitrogen, Grand Island, NY). Two mL staining solution were added directly onto the overlay. Plaques were count in wells where plaques were clearly separated.

4.2.8 Weight loss

Whole shell oysters, salmon fillet and cooked surimi gel were taken on days 0, 5, 10, 15 and 20 for measurement of weight loss. The weight loss of the samples was measured by a gravimetric method (Ercolini, Russo, Torrieri, Masi, & Villani (2006). The weight loss percentage was expressed as $\Delta g/g\%$, The difference of weight (Δg) was divided by the initial weight of the product (g). For oysters, the weight loss was calculated after the deduction of the weight of shell.

4.2.9 Determination of total volatile basic nitrogen (TVB-N)

Whole shell oysters, salmon fillet and cooked surimi gel were sampled at day 0, 5, 10, 15 and 20 for measurement of weight loss and for determination of total volatile basic nitrogen (TVB-N). TVB-N was analyzed by using Conway microdiffusion method (Kim, Paik, & Lee 2002). Samples (2 g) was immersed with 10 mL 2.2 % trichloroacetic acid (Cl3CCOOH；TCA) for 10 min. The homogenate was filtered through Whatman no. 1

filter paper, and made up to 20 mL with trichloroacetic acid (Cl_3CCOOH；TCA). In the investigation of the ammonia absorption, 1 mL of boric acid (H_3BO_3) was introduced into the inner chamber, 1 mL of potassium carbonate (K_2CO_3) was introduced into the left side of outer chamber, and 1 mL of sample extract was introduced into the right side of outer chamber. The sample and the K_2CO_3 solution were mixed gently and kept at 37 °C for 90 min. The TVB-N was titrated with 0.01N HCl.

4.2.10 Microbiological Quality

Whole shell oysters, salmon fillet and cooked surimi gel were sampled on days 0, 5, 10, 15 and 20 for assay of the inherent microbiota. Samples (25 g) were placed in sterile Whirl-Pak bags, followed by addition of 225 mL of sterile alkaline peptone water (APW). The samples were homogenized for 2 min using a Stomacher 80 Lab-blender. Serial 10-fold dilutions were prepared in APW. Mesophilic and psychrotrophic counts were performed as previously described (Wu, Chang, Nannapaneni, Zhang, Coker, & Mahmoud, 2017). For mesophilic counts, 0.1 mL of each dilution was plated onto trypticase soy agar and incubated at 37 °C for 24 h. For psychrotrophic counts, 0.1 mL of each dilution was plated onto trypticase soy agar and incubated at 5 °C for 10 days. Viable counts were expressed as log CFU g^{-1}.

4.2.11 Statistical analysis

All experiments were conducted in 3 replications. The mean values and standard deviations were determined using ANOVA tests (XLStat 2015, Addinsoft USA, New York, NY). Significant differences among means were made with Tukey honestly

significant difference (HSD) analysis using the probability level of 0.05. A first-order kinetic model (linear model) was used to determine the effect of the storage days on the reduction of MNV-1. The analyses were done using Excel software (XLStat 2015, Addinsoft USA, New York, NY).

4.3 Results and Discussion

4.3.1 MNV-1 enumeration

All samples of whole shell oysters, salmon fillet and cooked surimi gels showed reduction of MNV-1 when stored at 5°C for 20 days. The titer of MMV-1 in whole shell oysters after bio-accumulation (the initial level of MNV-1 in oysters) was about 4.6 log PFU g^{-1}. The titer (log PFU g^{-1}) of MNV-1 did not change significantly in whole shell oysters for 10 days, however, the titers decreased significantly from 4.6±0.1 to 4.1±0.1 log PFU g^{-1} after the live oysters were stored for 15 days ($P<0.05$) (Figure 4.3). On days 20, the titer (log PFU g^{-1}) of MNV-1 was lower than the detection limit (<2.0 log PFU g^{-1}).

After 20 days storage at 5°C, all the oysters were found alive before shucked. In the literature, there were no study about norovirus inactivation by storage at 5°C in shell-on oysters. Our results were the first one to show that the infectivity of norovirus in live oysters could be maintained at least 15 days after the animal was contaminated with the virus and kept in refrigeration.

Many studies had focused on factors influencing the stability of the cultivable enteric viruses, including relative humidity (RH), temperature, and the type of surface

contaminated (D'Souza, Sair, Williams, Papafragkou, Jean, Moore, & Jaykus, 2006). Persistence of MNV-1 could be affected by the food matrix. As to the shucked and processed oyster meat, Park & Ha (2014) examined the effect of different concentrations of NaCl on the survival of MNV-1 in experimentally contaminated raw oysters during 72-h storage at 10 °C. Increases in NaCl concentration and storage time stepwise decreased MNV-1 titers significantly ($p<0.05$). With the first-order reaction model, the MNV-1 decimal reduction time (D-value) was 64 h at 10% NaCl. Park & Ha (2014) suggests that NaCl concentrations of higher than (or equal to) 10% could be potentially used for inactivation of HuNoV in traditional Korean foods, including those containing raw oysters such as Eoriguljeot and fermented Jeotgals.

The MNV-1 titer of salmon fillet did not change significantly in salmon fillet for 15 days, however, the titers decreased from 5.06 to 3.43±0.63 log PFU g^{-1} after the samples were stored for day 20. (Figure 4.3). The titer (log PFU g^{-1}) of cooked surimi gel was significantly ($p < 0.05$) reduced from 5.2± 0.2 to 4.5 ± 0.1, 4.2 ± 0.3, 3.6 ± 0.6, and 3.1 ± 0.7 after storage at 5 °C for 5, 10, 15 and 20 days, respectively.

The square of the correlation (r^2) is a measure of how successfully the regression explains the response in the statistical model for simple linear regression (Moore, McCabe, & Craig, 2012). Using the values of the storage day as defining different subpopulations for the linear regression analysis on three experiments of storage inactivation, r^2 of MNV-1 titers in whole shell oysters and salmon fillet are 0.254 and 0.491, respectively, which were much lower than that of cooked surimi gel (0.846). The

results showed that the linear regression model better fit in MNV-1 titers in cooked surimi gel than in whole shell oysters and salmon fillet.

During food preparation, infected food handlers with poor personal hygiene practice may contaminate surfaces with fecal matter containing enteric viruses, including the HuNoV (D'Souza, Sair, Williams, Papafragkou, Jean, Moore, & Jaykus, 2006). HuNoV is highly contagious with an infectious dose as low as 10 particles. Because the MNV-1 titer (log PFU g^{-1}) could last longer than 15 days in whole shell oysters, salmon fillet and cooked surimi gel, norovirus contaminated seafood could maintain their infectivity during their shelf life.

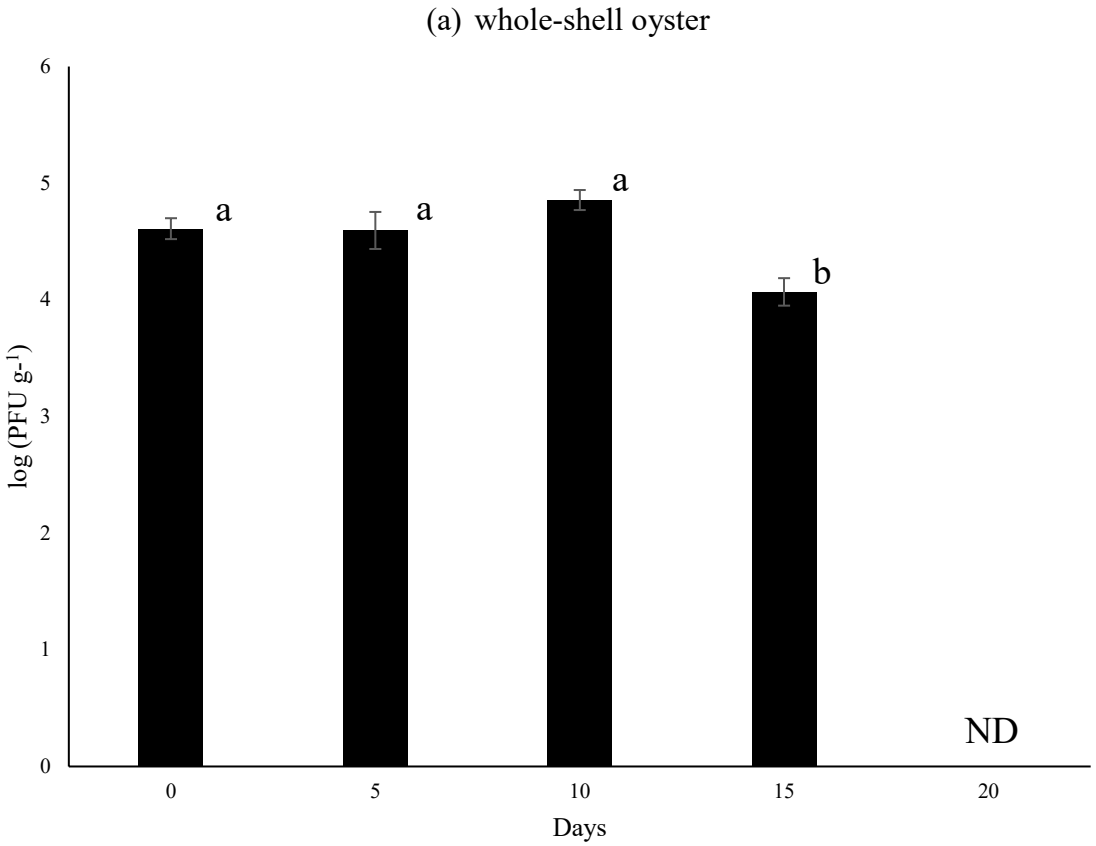

Figure 4.3 Titers of inoculated norovirus MNV-1 (log PFU mL^{-1}) on whole-shell oysters (a), salmon fillet (b) and cooked surimi (c) during storage at 5 °C for 20 days.

Different lower case letters for each treatment are significantly different ($p<0.05$). ND = not detectable (<2.0 log PFU g^{-1}).

(b) salmon fillet

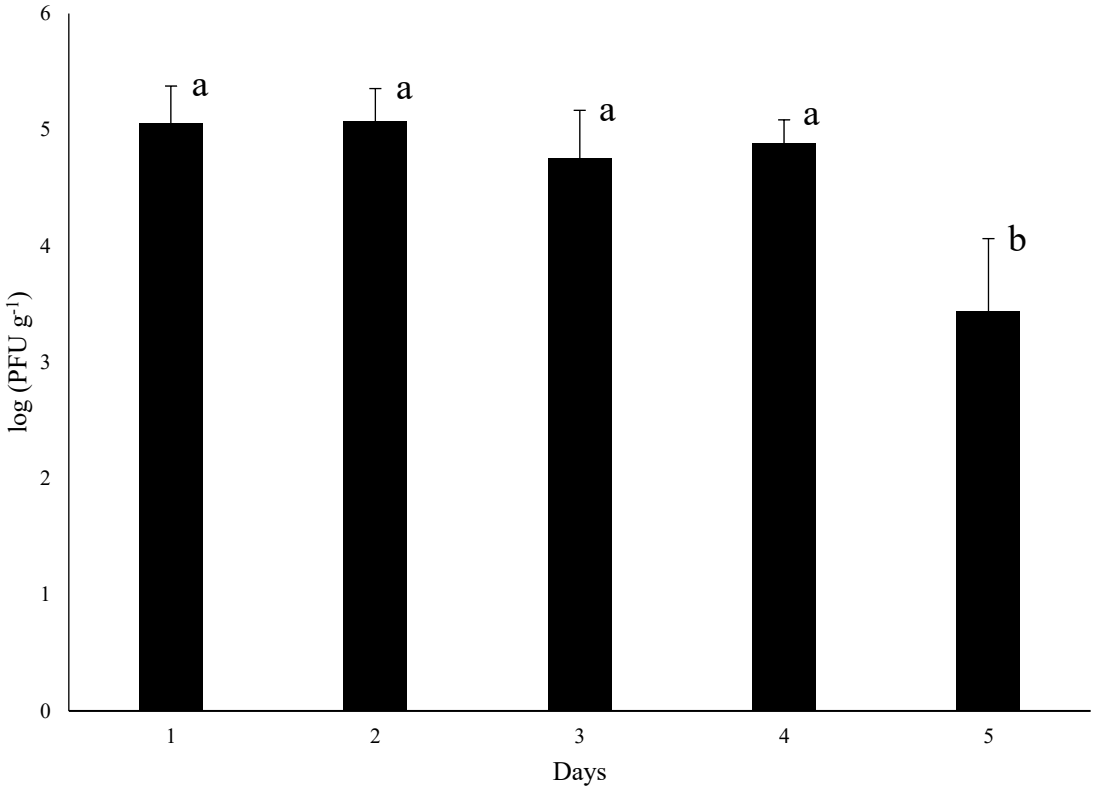

Figure 4.3 (continued)

Different lower case letters for each treatment are significantly different ($p<0.05$). ND = not detectable (<2.0 log PFU g^{-1}).

(c) cooked surimi

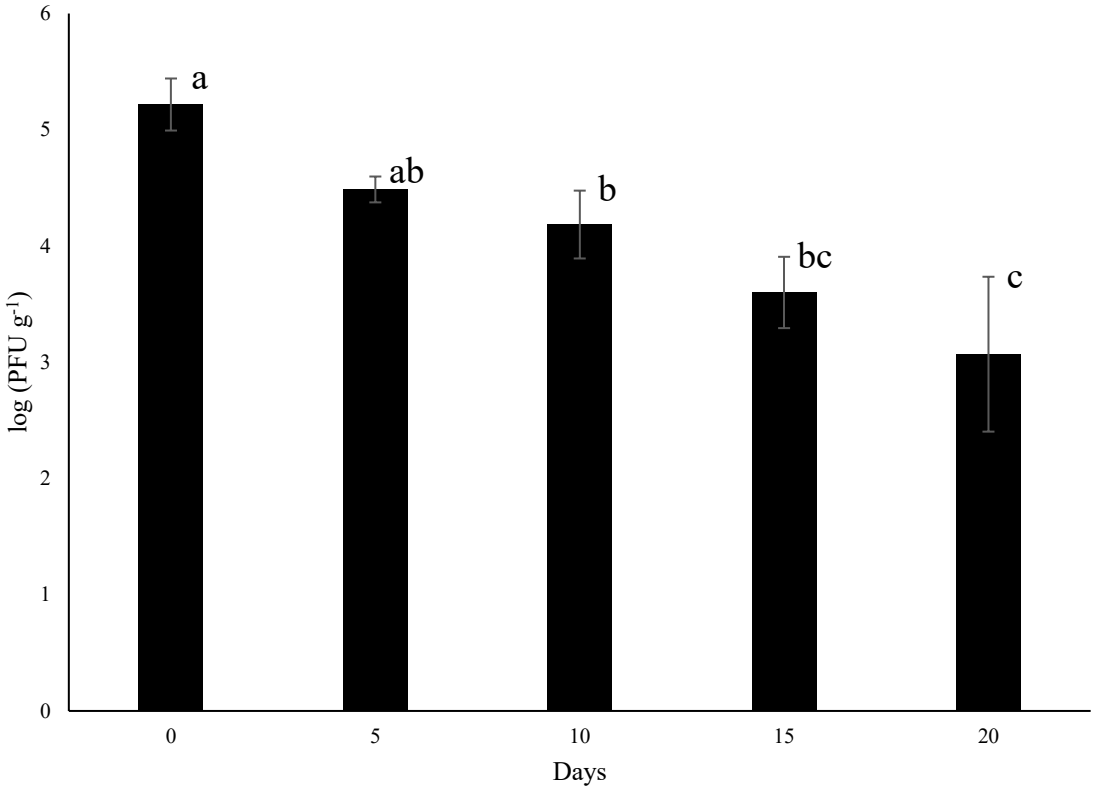

Figure 4.3 (continued)

Different lower case letters for each treatment are significantly different ($p<0.05$). ND = not detectable (<2.0 log PFU g^{-1}).

4.3.2 Weight loss

A stock of 10 Atlantic oysters was stored for 20 days at 5°C. At the end of the experiment, all oysters were found alive. Based on visual observation of the bivalves, all the oysters were classified as Level 0 of qualitative Intervalval Water Index (IWI) (Buzin, Baudon, Cardinal, Barillé, & Haure, 2011). The intervalval water was present, and flesh

was correctly moist. This indicated that the loss of water was limited due to the firmly closed molluse valves by the adductor muscle.

All the samples lost weight during storage (Table 4.1). The weight loss of whole shell oysters increased significantly to 0.7±0.6 on day 15. For salmon fillet and cooker surimi, the weight loss increased significantly to 5.4±0.8 % and 1.4±0.5 on day 5, respectively. Because of the loss of water, the concentration of substrates and metabolite in the food was considered to increase.

Table 4.1 Changes in weight (%) of whole shell oysters, salmon fillet and cooked surimi during storage at 5°C for 20 days.

	Days				
Food matrices	0	5	10	15	20
whole shell oysters	0.0±0.0 a	0.2±0.7 ab	0.5±0.3 abc	0.7±0.6 bc	1.0±0.9 c
salmon fillet	0.0±0.0 a	5.4±0.8 b	7.3±0.2 bc	9.2±0.3 dc	10.2±3.2 d
cooked surimi	0.0±0.0 a	1.4±0.5 b	2.5±0.9 bc	2.3±0.6 bc	3.2±1.9 c

Mean values with different lower case letters in same raw are significantly different ($p < 0.05$). ND = not detectable (<2.0 log CFU g^{-1}).

4.3.3 Microbiological Quality

Changes in mesophilic and psychrotrophic bacterial counts of whole shell oysters, salmon fillet and cooked surimi during storage at 5°C are shown in Table 4.2 and Table 4.3. No significant changes were found in mesophilic and psychrotrophic bacterial counts of whole shell oysters during 20 days of storage. According to the suggestion (Kim, Paik, & Lee, 2002), a total aerobic plate count of 10^7 CFU g^{-1} is an acceptable quality limit of oysters. The oysters maintained in acceptable microbiological quality through 20 days

storage. For cooked surimi, the mesophilic and psychrotrophic bacterial counts were under the detection limit (<2.0 log CFU g^{-1}). The mesophilic and psychrotrophic bacterial count of salmon fillet significantly increased from 6.74±0.17 and 6.56±0.05 to 8.09±0.68 and 8.80±0.11 CFU g^{-1}at day 5.

Table 4.2 Changes in the mesophilic bacterial counts (log CFU g^{-1}) of whole shell oysters, salmon fillet and cooked surimi during storage at 5°C for 20 days.

Food matrices	Days				
	0	5	10	15	20
whole shell oysters	5.09 ± 0.05 a	5.69 ± 0.21 a	5.83 ± 0.48 a	6.84 ± 1.08 a	6.98 ± 1.27 a
salmon fillet	6.74±0.17 a	8.09±0.68 b	8.44±0.27 b	8.48±0.26 b	7.95±0.09 b
cooked surimi	ND	ND	ND	ND	ND

Mean values with different lower case letters in same raw are significantly different ($p < 0.05$). ND = not detectable (<2.0 log CFU g^{-1}).

Table 4.3 Changes in the psychrotrophic bacterial counts (log CFU g^{-1}) of whole shell oysters, salmon fillet and cooked surimi during storage at 5°C for 20 days.

Food matrices	Days				
	0	5	10	15	20
whole shell oysters	5.70 ± 0.18 a	5.79 ± 0.40 a	6.33 ± 0.48 a	6.76 ± 0.41 a	6.90 ± 1.39 a
salmon fillet	6.56±0.05 a	8.80±0.11 b	9.15±0.24 b	9.05±0.79 b	8.60±0.33 b
cooked surimi	ND	ND	ND	ND	ND

Mean values with different lower case letters in same column are significantly different ($p < 0.05$). ND = not detectable (<2.0 log CFU g^{-1}).

4.3.4 Determination of total volatile base (TVB-N)

Bacterial and endogenous proteolytic enzymatic actions caused TVB-N to increase (Songsaeng, Sophanodora, Kaewsrithong, & Ohshima, 2010). The initial TVB-N value of whole shell oyster, salmon fillet and cooked surimi was 0.5±0.4, 2.3±0.4 and 2.3 ±0.8 mg N/100 g mg/100g meat, respectively (Table 4.4). After storage for 15 days, the whole shell oysters significantly increased to 7.5±2.1 mg N/100 g, while the salmon fillet increased significantly to 7.5±0.8 mg N/100 g on day 10. TVB-N values of all cooked surimi maintained at low level during storage of 20 days. Although these values were considered low levels, the whole shell oysters and salmon fillet showed spoilage due to the microbiological quality (Table 4.4). Songsaeng, Sophanodora, Kaewsrithong, & Ohshima (2010) stated that TVB-N values of white-scar oysters (*C. belcheri*) after storage under different conditions were 4.4-14.8 mg N/100 g. Our studies agreed with their results.

Table 4.4 Changes in TVB-N (mg/100g meat) of whole shell oysters, salmon fillet and cooked surimi during storage at 5°C for 20 days.

	Days				
Food matrices	0	5	10	15	20
whole shell oysters	0.5±0.4 a	1.9±0.8 a	3.3±0.8 ab	7.5±2.1 b	7.9±3.5 b
salmon fillet	2.3±0.4 a	5.1±0.8 ab	7.5±0.8 b	9.8±3.7 b	21.0±1.4 c
cooked surimi	2.3±0.8 a	2.3±1.6 a	3.3±0.8 a	3.7±0.8 a	3.3±0.8 a

Mean values with different lower case letters in same column are significantly different ($p < 0.05$). ND = not detectable (<2.0 log CFU g^{-1}).

4.4 Conclusion

The bio-accumulated MNV-1 was found to maintain infectivity in whole shell oysters during storage at 5°C for 15 days, while the inoculated MNV-1 on salmon fillet and cooked surimi kept infectious for 20 days. This is the first report about the survivability of MNV-1 in whole shell oyster, salmon fillet and cooked surimi. The results show that the infectivity can last long during storage of these three kinds of seafood products at 5°C.

CHAPTER V

THE EFFICACY OF X-RAY DOSES ON MURINE NOROVIRUS-1 (MNV-1) IN PURE CULTURE, HALF-SHELL OYSTER, SALMON SUSHI, AND TUNA SALAD

(published in *Food Control*, *64*, 77-80, 2016)

5.1 Introduction

Human norovirus (HuNoV) is a major foodborne pathogen which causes acute gastroenteritis (Bae & Schwab, 2008). HuNoV is a highly stable and contagious virus, and less than 10 virus particles can to cause an infection (Feng, Divers, Ma, & Li, 2011). The symptoms of HuNoV infection include diarrhea, vomiting, nausea, abdominal cramps, headache, and fever (Iwamoto, Ayers, Mahon, & Swerdlow, 2010). The CDC estimated 5,461,731 cases of illnesses, 14,663 hospitalizations and 149 deaths annually related to this pathogen (CDC, 2011).

Raw and/or ready-to-eat (RTE) seafood products such as oysters, salmon sushi, and tuna salad are popular in several parts of the world; however, these products are highly associated with HuNoV-related foodborne illness (Rippey, 1994). HuNoV can contaminate seafood during pre-harvest cultivation from surrounding water or during post-harvest processing including cutting, washing and sanitizing, packaging and storing (Iwamoto, Ayers, Mahon, & Swerdlow, 2010; Jung, Jang, & Matthews, 2014). Infected food handlers also contribute to foodborne HuNoV outbreaks by inadequate of hand

washing, inadequate cleaning and disinfection (Grove, Suriyanarayanan, Puli, Zhao, Li, Li, Schaffner, & Lee, 2015).

Finding suitable measures to reduce the risk of foodborne illness in these products is quite important. Traditional disinfecting methods such as heating, freezing and drying cannot be used with raw/fresh consumed food product because they cause chemical and physical deterioration, affecting the sensory quality. On the other hand, food irradiation is a process that is referred to as "cold" sterilization which can be applied to these kinds of food products (Crawford & Ruff, 1996). In the United States, the FDA has approved three kinds of radiation including electrons beam, gamma-ray, and X-ray (GAO, 2000). Also, irradiation of food products is allowed in 57 countries; and more than 70 commercial irradiation facilities are operating in the world (Ahn, Kim, & Lee, 2013). However, there has been no research report using X-ray to inactivate norovirus in seafood products. The aim of this study was to determine the sensitivity of MNV-1 (widely used HuNoV surrogate) to X-ray irradiation in pure culture, half-shell oyster, salmon sushi, and tuna salad.

5.2 Materials and Methods

5.2.1 Seafood product

Live, freshly harvested Atlantic oysters (*Crassostrea virginica*), with 7-9 cm length, were obtained from seafood market (Quality Poultry and Seafood, Biloxi, MS) and transported immediately in a cooler with cubed ice to the ESPL. The oysters were washed with tap water to remove mud from the shells and shucked with a sterile shucking knife. Fresh salmon nigiri sushi samples were purchased from local producer (Rouses Market, Ocean Springs, MS) which combined with uncooked salmon fillet and sushi rice.

The products were brought to the laboratory in chilled containers and kept at 4 ℃ until use (within a few hours). The salmon sushi samples were cut to 25 g. Tuna salad samples were prepared by mixing mayonnaise (The Kraft Heinz Company, Northfield, IL) and canned tuna meat in brine (Bumble Bee, San Diego, CA) at the ratio 3:1 (w/w), according to Leuschner & Hammes, 1999.

5.2.2 Inoculation of pure culture, half-shell oyster, salmon sushi, and tuna salad

For pure culture, 1 mL aliquot of MNV-1 was made up to 10 mL with cell culture grade phosphate-buffered saline. For oysters, 100 μl of MNV-1 virus stock solution were spotted (10 drops) on the meat of half-shell oyster (25 g) samples. The inoculated samples were then air-dried for 30 min at 22 ℃ in a biosafety cabinet and then placed into sterile Whirl-Pak bags for X-ray treatments. For salmon sushi,100 μl of MNV-1 virus stock solution was spotted (10 drops) on the meat of salmon sushi (25 g). The inoculated samples were then airdried for 30 min at 22 ℃ in the biosafety cabinet and then placed into sterile Whirl-Pak bags for X-ray treatments. For tuna salads, samples (25 g) were mixed with 100 μl of MNV-1 virus stock solution, evenly, and placed into sterile Whirl-Pak bags for X-ray treatments.

5.2.3 X-ray treatment

The RS 2400 (Rad Source Technologies, Inc.) is an industrial cabinet x-ray irradiator. The operating range of the x-ray tube varies from 25 kV to 150 kV and 2 mA to 45 mA, both being continuously adjustable. The x-ray tube itself consists of a tungsten filament running down the center of a 10.2 cm (4 inch) diameter stainless steel cylinder. This is housed within a larger 11.4 cm (4.5 inch) diameter stainless steel cylinder. Both

stainless steel cylinders are 0.17 cm (0.065 inches) thick. A layer of gold is plated inside the inner cylinder. As the tungsten filament is heated, electrons are released from the surface. At higher current (measured in mA), move electrons through the filament. An electric potential difference accelerating voltage (measured in kV) is applied between the filament and the inner tube, attracting the electrons toward the inner tube. A vacuum is drawn between the filament and the inner tube so the electrons do not interact with gas molecules. Specific irradiation doses (1.0, 2.0, 3.0, 4.0 and 5.0 kGy) at 22 ℃ and 55% relative humidity were generated using the RS 2400 industrial cabinet X-ray irradiator (Rad Source Technologies, Inc.) according to Mahmoud, Chang, Wu, Nannapaneni, Sharma, & Coker, (2015). Briefly, at higher currents (mA), more electrons leave the filament. The electrons gather energy, equal to the potential difference; the higher the potential difference, the more energy the electrons gather. When the electrons reach the gold target plated inside the inner tube, they interact with the gold atoms and emit photons called X-rays. The X-ray doses in the treatment chamber were determined using a dosimeter (Rad Sources Technology. Inc., GA).

5.2.4 MNV-1 enumeration (plaque assay)

After treatment with 0, 1.0, 2.0, 3.0, 4.0, and 5.0 kGy X-ray, pure culture samples (10 mL) were directly diluted 10-fold serially in DMEM-5 for plaque assay. Seafood product samples (25 g) were made up to 250 g by phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM NaH_2PO_4; pH 7.4) and homogenized for 2 min using a Stomacher 80 Lab-blender. Serial 10-fold dilutions were made in DMEM-5 and plaque assays were performed using the same method, previously described (Chapter 4), to determine the titer of the viral stock.

5.2.5 Effect of X-ray on color

The color of oyster meat was evaluated using the Hunter colorimeter values (Lab Scan XE Hunter Colorimeter; Hunter Associates Laboratory, Inc., Reston, VA) by L*(lightness), a*(redness-greenness), and b*(yellowness-blueness).

5.2.6 Statistical analysis

All experiments were replicated three times using two samples per experiment for a total of six data points per treatment. Data were pooled and the mean values and standard deviations were determined using ANOVA tests (XLStat 2015, Addinsoft USA, New York, NY). Significant differences among means were made with Tukey honestly significant difference (HSD) analysis using probability level of 0.05.

5.3 Results and Discussion

5.3.1 Inactivation of MNV-1 in pure culture

The inactivation of MNV-1 was significantly greater in pure culture than in seafood products. The log PFU mL^{-1} was significantly ($p < 0.05$) reduced from 6.3 ± 0.4 to 5.4 ± 0.2, 4.0 ± 0.4, 3.2 ± 0.4, and 2.6 ± 0.3 after treatment with 1.0, 2.0, 3.0 and 4.0 kGy X-ray, respectively, in pure culture. Treatment with 5.0 kGy X-ray achieved a 4 log PFU mL^{-1} reduction, in pure culture (Figure 5.1). Our results agree with those obtained by De-Roda-Husman, Bijkerk, Lodder, Van Den Berg, Pribil, Cabaj, et al., (2004) who reported that a 3-log virus reduction in a low protein-content solution at 0.5 kGy gamma irradiation.

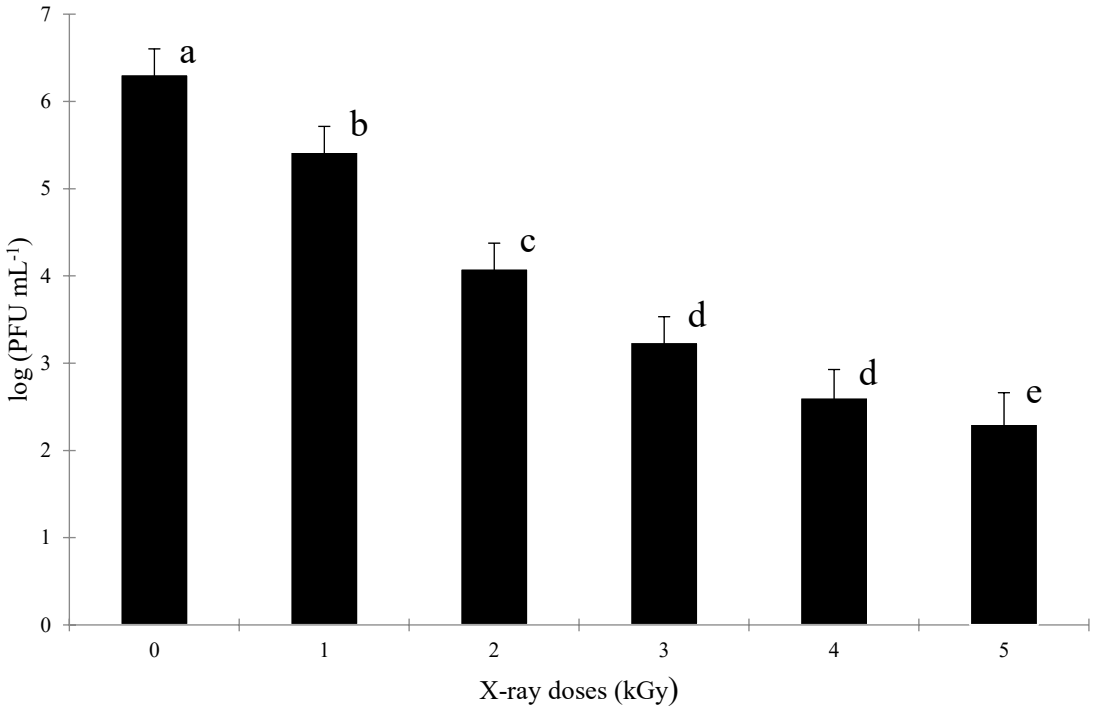

Figure 5.1 Inactivation of inoculated norovirus MNV-1 (log PFU mL-1) in PBS by X-ray. Error bars represent standard error.

Different lower case letters for each treatment are significantly different ($p<0.05$).

5.3.2 Inactivation of MNV-1 in half-shell oyster salmon sushi, and tuna salad

There were no significant differences between inactivation of MNV-1, by X-ray, in raw oysters and salmon sushi. Treatment of raw oysters with 1.0, 2.0, 3.0, and 4.0 kGy X-ray significantly reduced the log PFU g^{-1} from 5.4 ± 0.1 to 4.7 ± 0.2, 4.3 ± 0.5, 3.6 ± 0.4, and 2.7 ± 0.1, respectively, however, exposure to 5.0 kGy X-ray reduced the population of MNV-1 to less than 2.0 log PFU g^{-1} in raw oysters (Figure 5.2). The effect of X-ray treatment on the inactivation of MNV-1 on salmon sushi is shown in Figure 5.3. The log PFU g^{-1} was significantly reduced from 5.2 ± 0.2 to 4.7 ± 0.6, 4.2 ± 0.3, 3.4 ± 0.5, and 3.0 ± 0.6 after treatment with 1.0, 2.0, 3.0, and 4.0 kGy X-ray, respectively.

Treatment with 5.0 kGy X-ray reduced the population of MNV-1 to below the detection limit (<2.0 log PFU g^{-1}).

The inactivation of MNV-1 was greater on raw oyster and salmon sushi than on tuna salad. The log PFU g^{-1} was significantly reduced from 5.3 ± 0.1 to 4.8 ± 0.2, 4.6 ± 0.3, 4.2 ± 0.4, and 3.3 ± 0.4 after treatment with 1.0, 2.0, 3.0, and 4.0 kGy X-ray, respectively, on tuna salad. However, treatment with 5.0 kGy X-ray achieved approximately 2.4 log PFU g^{-1} reduction on tuna salad (Figure 5.4). The food matrix in which the virus was present does have an impact on MNV-1 sensitivity to irradiation. Organic materials were known to reduce irradiation effectiveness as they scavenge the reactive species that produced during treatment (Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai, 2013). Previous studies had shown that X-ray doses had different effect on inactivation of the same pathogens on different food products (Mahmoud, 2009a, b; Mahmoud, 2010a, b; Mahmoud, Chang, Nannapaneni, Wu, & Coker, 2016; Mahmoud, Coker, & Su, 2012; Mahmoud, Chang, Wu, Nannapaneni, Sharma, & Coker, 2015).

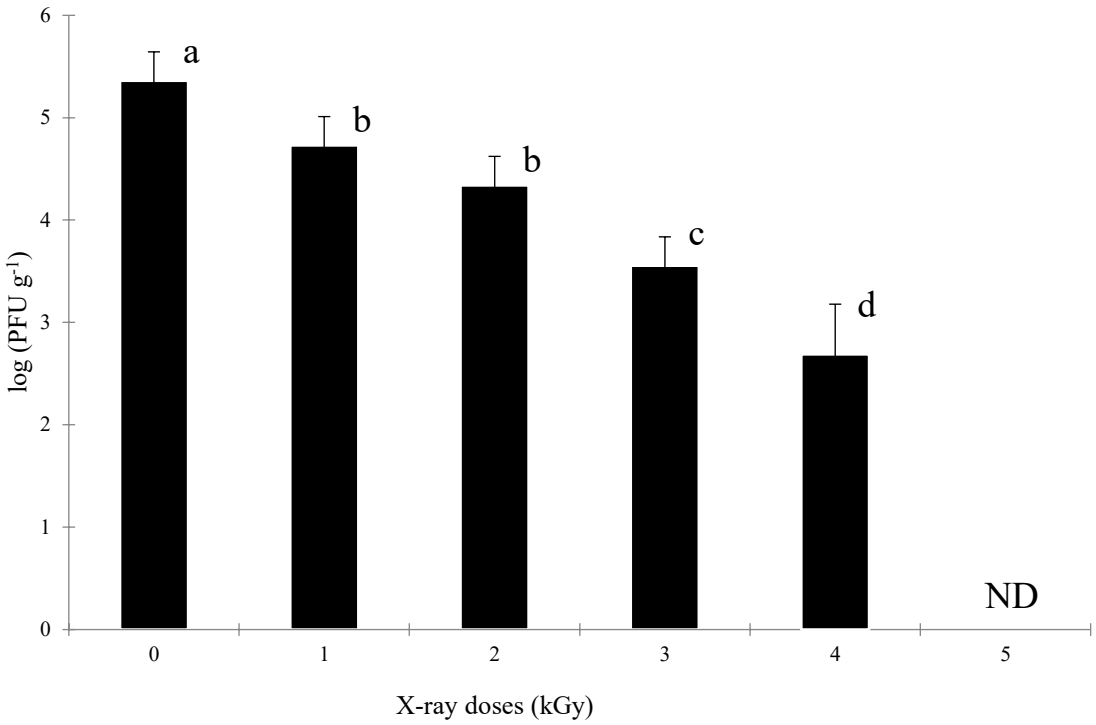

Figure 5.2 Inactivation of inoculated norovirus MNV-1 (log PFU g-1) on raw oyster by X-ray.

Different lower case letters for each treatment are significantly different ($p<0.05$). ND = not detectable (<2.0 log PFU g-1).

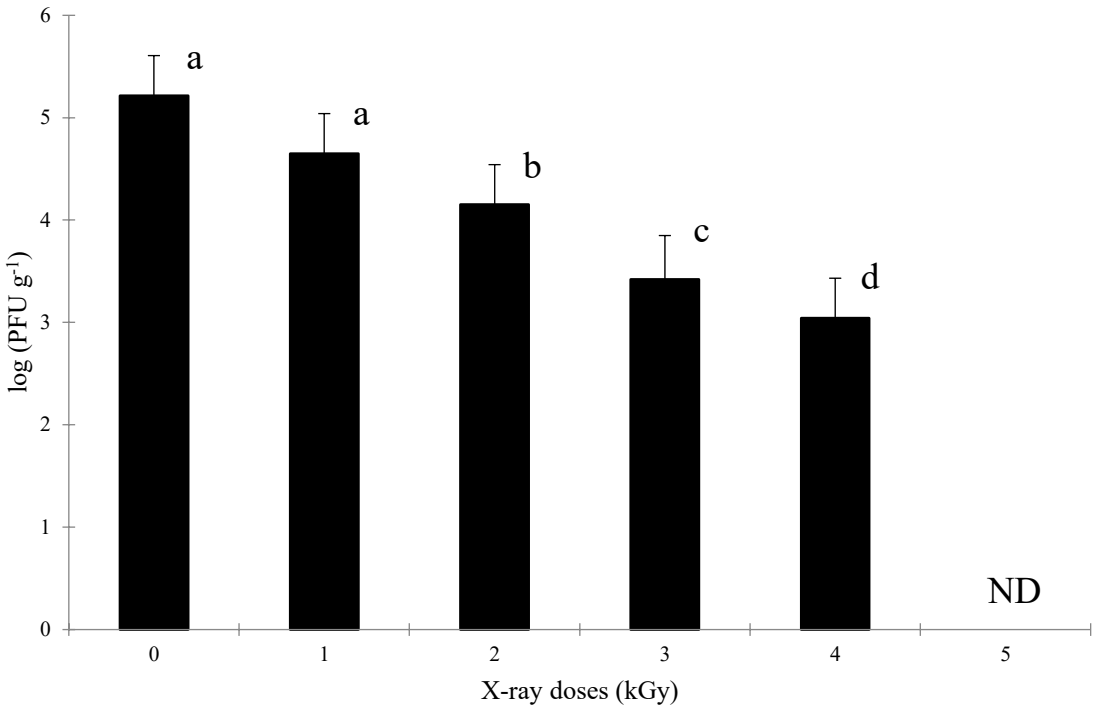

Figure 5.3 Inactivation of inoculated norovirus MNV-1 (log PFU g-1) on salmon sushi by X-ray.

Error bars represent standard error. Different lower case letters for each treatment are significantly different ($p<0.05$). ND = not detectable (<2.0 log PFU g-1).

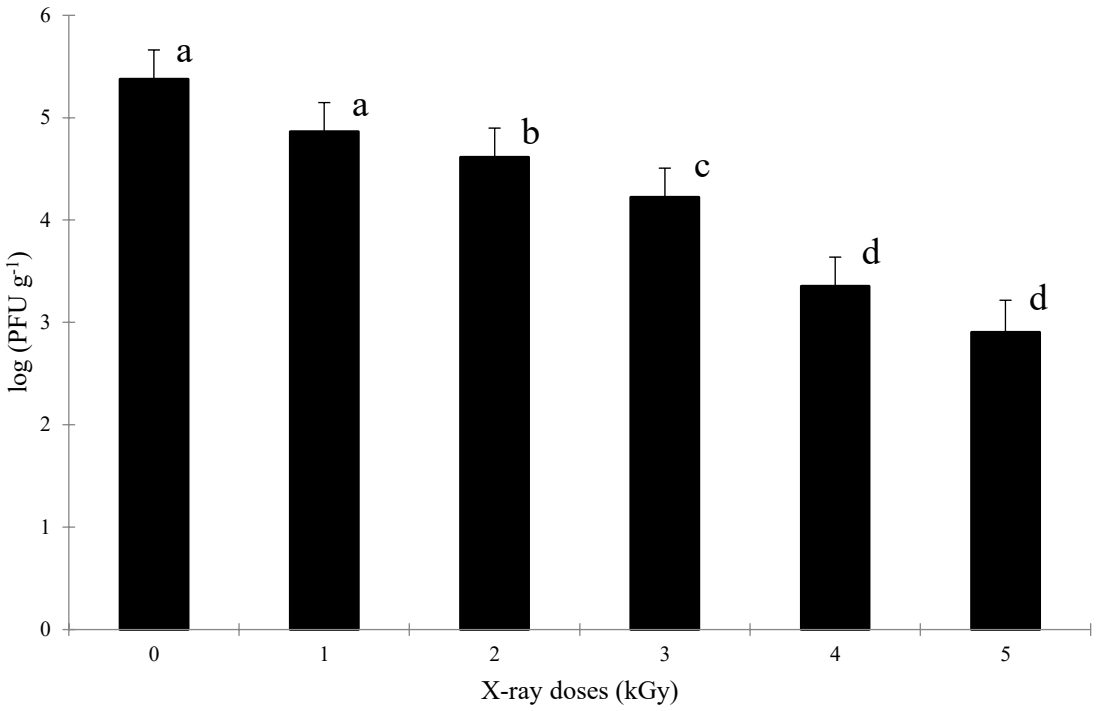

Figure 5.4 Inactivation of inoculated norovirus MNV-1 (log PFU g-1) on tuna salad by X-ray.

Error bars represent standard error. Different lower case letters for each treatment are significantly different ($p<0.05$).

Harewood, Rippey, and Montesalvo (1994) reported that virus inactivation required a higher irradiation dose than bacterial pathogens. Our group also found that bacterial pathogens were more susceptible to X-ray than norovirus in seafood products. Mahmoud (2012b) reported that the initial level of artificially inoculated *Listeria monocytogenes* in smoked salmon (control) was 3.7 log CFU g^{-1} which significantly ($p < 0.05$) reduced to 2.0 log CFU g^{-1} after treatment with 0.5 kGy X-ray. Exposure to 1.0 kGy X-ray reduced the population of *L. monocytogenes* in smoked salmon to below the detection limit (<1.0 log g^{-1}). Mahmoud (2009a) found that more than a 6 log CFU reduction of *Escherichia coli* O157: H7, *Salmonella enterica*, *Shigella flexneri* and *Vibrio*

parahaemolyticus was achieved with 2.0, 4.0, 3.0 and 3.0 kGy X-ray, respectively, on ready-to-eat shrimp. Mahmoud (2009b) reported that greater than a 6-log reduction of *Vibrio vulnificus* was achieved with 0.75, 1.0, 3.0 kGy X-ray in pure culture, half shell and whole shell oysters, respectively. However, our current results indicate that virus infection risks can be reduced on seafood products by X-ray processing at the current FDA-approved level (5.5 kGy).

Our results indicate that X-ray has stronger effect on reducing MNV-1 than E-beam or gamma ray. Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai (2013) reported that 4.0 kGy E-beam dose was required to reduce the MNV-1 by 1.0 log reduction in whole oyster. Feng, Divers, Ma, & Li (2011) reported 2.8 and 5.6 kGy gamma ray doses reduced MNV-1 by 1.0- and 2.0-log, respectively in pure culture (PBS). Also, Feng, Divers, Ma, & Li (2011) reported that treatment of 5.6 kGy of gamma irradiation yielded a 1.7 to 2.4 log reduction of MNV-1 in different types of fruit and vegetables. Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai (2013) stated that 2.6 and 4.0 kGy E-beam doses were required to reduce MNV-1 by 1.0 log in pure culture (PBS) and whole oysters, respectively. The mechanism of viral inactivation has been demonstrated with gamma irradiation and E-beam but no published study to date has evaluated if X-ray would have a similar mechanism (Feng, Divers, Ma, & Li ,2011; Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai, 2013). A study is needed to understand the mechanism of viral inactivation by X-ray which would also guide the food industry to properly use X-ray irradiation.

5.3.3 Effect of X-ray treatment on the inherent microbiota on salmon fillet

The mesophilic counts for the control samples of salmon fillet were 6.74, 8.09, 8.44, 8.48 and 7.95 log CFU g^{-1} on day 0, 5, 10, 15 and 20, respectively, during storage at 5 °C (Table 5.1). On day 0, treatment with 1 kGy X-ray significantly reduced the mesophilic count log CFU g^{-1} from 6.74 to 4.51. Furthermore, treatment with 2-5 kGy X-ray significantly reduced the mesophilic count to below the detection level (2 log CFU g^{-1}). Treatment with 5 kGy X-ray kept the mesophilic counts significantly lower than the control until day 20.

The psychotropic counts for the control samples of salmon fillet were decreased by X-ray doses, but slightly increased with time of storage at 5 °C (Table 5.2). On day 0, treatment with 1 and 2 kGy reduced the psychotropic count from 6.56 to 5.60 and 2.65 log CFU g^{-1}, respectively. Furthermore, treatment with 3-5 kGy X-ray significantly reduced the psychotropic count to below the detection level (2 log CFU g^{-1}). Treatment with 5 kGy X-ray kept the psychotropic counts significantly lower than the control up to day 20.

Table 5.1 Changes in the mesophilic bacterial counts (log CFU g^{-1}) of salmon fillet with X-ray during storage at 5 °C for 20 days.

	X-ray dose (kGy)					
Days	0	1	2	3	4	5
0	6.74±0.17aA	4.51±0.61bA	ND	ND	ND	ND
5	8.09±0.68aB	6.63±0.24bB	ND	ND	ND	ND
10	8.44±0.27aB	7.22±0.39aBC	4.47±0.73bA	2.39±2.09bA	ND	ND
15	8.48±0.26aB	7.99±0.43aC	6.52±0.95abB	3.84±0.69bcA	2.63±2.28cA	ND
20	7.95±0.09aB	7.69±0.83aC	6.57±0.74abB	4.10±1.02cA	5.37±0.25bcA	3.74±0.41cA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).
ND = not detectable (<2.0 log CFU g^{-1}).

Table 5.2 Changes in the psychrotrophic bacterial counts (log CFU g^{-1}) of salmon fillet with X-ray during storage at 5°C for 20 days.

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	6.56±0.05 aA	5.60±0.38 bA	2.65±0.27 cA	ND	ND	ND
5	8.80±0.11 aB	7.77±0.30 aB	5.66±0.46 aB	ND	ND	ND
10	9.15±0.24 aB	9.03±0.61 aC	8.20±0.35 aC	5.62±0.34 bA	5.93±0.68 bA	4.21±1.22 bA
15	9.05±0.79 aB	9.80±0.21 aC	8.53±0.47 abCD	8.20±0.24 abB	7.37±0.70 bAB	5.61±0.15 cAB
20	8.60±0.33 aB	8.98±0.25 aBC	9.63±0.40 aC	8.77±0.21 aB	8.97±0.65 aB	6.63±0.09 bB

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$). ND = not detectable (<2.0 log CFU g^{-1}).

5.3.4 Effect of X-ray treatment on color of salmon fillet

The color of salmon fillet during storage at 5 °C for 20 days is shown in Table 5.3. Right after the treatment with X-ray (day 0), no significant difference was found on L value until the X-ray dose increased to 3 kGy or more. As to a* value and hue, 2 kGy or more X-ray dose was needed to bring significant change. No impact was found on b* value and saturation even X-ray dose up to 5 kGy.

The control samples (no X-ray dose) changed L value significantly after 15 days storage while no inference was found on a*, b*, saturation and hue. Mahmoud, Chang, Nannapaneni, Wu, & Coker (2016) reported the color of tuna fillet was changed by the impact of 0.6 kGy and recovered during the storage period. In this study, our lowest dose was 1 kGy. At this dose, no impact was found for L* value on day 0 or through the storage of 20 days. During storage, the L value changed significantly on day 15 and day 5 on the samples with 2 and 3 kGy X-ray treatment, respectively. The impact of storage to a* values was found on day 15 and 5 on the sample with 3 and 4 kGy X-ray treatment, respectively. On the sample with 2 and 4 kGy X-ray treatment, the impact of storage to b* values was found on day 10. The change of saturation and Hue values were only found on day 10 when irradiated with 4 kGy X-ray.

Table 5.3 Changes in the color (Hunter parameters, L, a, b, Saturation and Hue) of salmon fillet with X-ray treatment during storage at 5 °C for 20 days.

(L)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	53.90±0.02 aA	56.44±0.50 abcA	57.36±1.52 abcA	55.19±0.91 bcA	59.64±2.87 bcA	60,76±0.46 cA
5	56.61±0.56 abAB	54.45±2.13 aA	58.17±1.03 abcAB	61.31±0.53 bcB	63.60±2.75 cA	60.46±2.32 bcA
10	57.28±1.52 abAB	55.36±3.13 aA	59.98±0.61 abcABC	61.13±0.86 bcB	63.11±0.46 cA	61.67±1.45b cA
15	57.94±1.98 aB	58.98±1.40 abA	61.13±0.45 abcBC	62.55±1.16 bcB	64.39±0.36 cA	58.65±1.76 abA
20	56.63±0.88 aAB	58.18±1.12 aA	61.81±0.93 bcC	62.85±0.94 bcB	64.81±0.67 cA	59.82±1.18 abA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

Table 5.3 (continued)

(a)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	22.17±2.15 aA	17.95±3.45 abA	14.09±2.09 bcA	13.79±0.27 bcA	7.16±0.77 cdA	5.40±2.55 dA
5	23.59±2.91 aA	20.85±4.11 aA	9.83±1.51 bcA	12.07±0.62 bAB	4.15±0.42 cB	3.01±1.87 cA
10	22.85±2.95 aA	19.13±4.99 abA	8.81±1.77 cdA	11.26±0.31 bcB	2.95±0.77 cdB	1.97±1.56 dA
15	22.71±2.60 aA	17.51±4.12 abA	8.91±1.37 cdA	10.89±0.78 bcB	3.09±0.45 dB	2.09±1.82 dA
20	25.48±0.81 aA	19.38±3.63 aA	8.88±2.22 bcA	11.07±0.66 bB	2.75±0.79 cdB	1.58±1.28 dA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

(b)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	22.04±4.43aA	25.73±3.39aA	28.30±2.45aA	26.69±4.32aA	23.13±1.51aA	19.71±2.09aA
5	22.08±4.71abA	27.31±0.98aA	22.95±2.12 abAB	22.82±1.91abA	19.89±0.29 abAB	18.78±1.57bA
10	21.79±4.69abA	27.26±1.87aA	21.58±1.87abB	22.26±1.21abA	17.30±1.63bB	18.06±0.62bA
15	21.16±4.30abA	22.91±0.19aA	21.23±2.00abB	21.86±1.66abA	19.62±1.27 abAB	15.02±1.67bA
20	24.67±1.28aA	24.80±0.95aA	21.92±1.67 aAB	21.50±1.46aA	19.46±1.23 abAB	14.75±2.68bA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

Table 5.3 (continued)

(Saturation)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	31.31±4.62 aA	31.45±4.32 aA	31.64±2.97 aA	30.11±3.86 aA	24.23±1.44 aA	20.54±2.66 aA
5	32.35±5.31 abA	34.48±3.21 aA	25.04±1.74 bcAB	25.86±1.47 abcA	20.33±0.23 cAB	19.08±1.85 cA
10	31.61±5.34 abA	33.46±4.19 aA	23.38±1.77 bcB	24.96±0.99 abcA	17.57±1.62 cB	18.22±0.78 cA
15	31.08±4.79 aA	29.03±2.44 aA	23.09±1.61 abcB	24.46±1.18 abA	19.87±1.23 bcB	15.27±1.68 cA
20	35.47±1.35 aA	31.66±1.57 aA	23.72±2.06 bB	24.21±0.98 bA	19.67±1.24 bcB	14.88±2.77 cA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

(Hue)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	44.36±3.29 aA	55.22±3.80 abA	63.63±2.23 bcdA	62.17±3.88 bcA	72.73±2.13 cdA	75.35±5.48 dA
5	42.64±2.98 aA	53.08±4.67 abA	66.67±4.49 cdA	61.96±3.07 bcA	78.20±1.28 deAB	81.34±4.79 eA
10	43.15±2.92 aA	55.55±5.61 abA	67.78±4.62 bcA	63.09±1.76 bA	80.26±2.52 cdB	83.96±4.64 dA
15	42.52±3.05 aA	53.17±6.62 aA	67.05±4.48 aA	63.35±3.35 aA	80.98±1.63 aB	21.92±78.78 aA
20	44.04±1.15 aA	52.30±6.27 abA	68.16±4.45 cA	62.64±3.04 bcA	81.98±2.27 dB	84.23±3.98 dA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

5.4 Conclusion

To my knowledge, this is the first study of the inactivation of MNV-1 on seafood products by X-ray. In this research, we investigated the potential use of X-ray radiation at doses of 1.0, 2.0, 3.0, 4.0, and 5.0 kGy to control norovirus in raw and/or RTE (ready-to-eat) seafood products was investigated. The results of this study indicate that the efficacy of X-ray irradiation against MNV-1 increased with increasing X-ray doses. Based on the fact that X-ray machine is easy to operate without excessive labor requirement, X-ray could be a suitable nonthermal processing alternative to the current disinfection techniques for seafood products. More studies are needed to understand the mechanism of viral inactivation by X-ray and to determine the effect of X-ray treatments on the quality and shelf life of treated seafood products.

CHAPTER VI

THE EFFECTS OF X-RAY TREATMENTN ON BIOACCUMULATED MURINE NOROVIRUS-1 (MNV-1) AND SURVIRABILITY, INHERENT MICROBIOLOTA, COLOR, AND FIRMNESS OF ATLANTIC OYSTERS (*CROSSOSTREA VIRGINICA*) DURING STORAGE AT 5 ℃ FOR 20 DAYS (Published in *Food Control*, *73*, 1189-1194, 2017)

6.1 Introduction

Estimates of foodborne illness revealed that human norovirus (HuNoV), an acute gastroenteritis pathogen, causes more than 58% of the U.S. foodborne illnesses, 11% foodborne 50 deaths and 25% hospitalizations, annually (Scallan, Hoekstra, Angulo, Tauxe, Widdowson, Roy, Jones, & Griffin, 2011). HuNoV transmission occurs through the fecal-oral route (person-to-person contact, contaminated food, and contaminated water) or airborne transmission of viral particles. HuNoV is highly contagious with an infectious dose as low as 10 particles (D'Souza, Sair, Williams, Papafragkou, Jean, Moore, & Jaykus, 2006). Symptoms of HuNoV infection, including diarrhea, vomiting, nausea, abdominal cramps, headache, and fever, usually last for 24-48 h in healthy individuals, but may be prolonged in the immunocompromised patients, children and the elderly (Iwamoto, Ayers, Mahon, & Swerdlow 2010; Partridge, Evans, Raza, Kudesia, & Parsons, 2012).

Shellfish are known as vectors for human pathogens, including norovirus (Campos & Lees, 2014; Le Guyader, Atmar, & Le Pendu 2012; McIntyre, Galanis, Mattison, Mykytczuk, Buenaventura, Wong, et al. (2012). Several post-harvest techniques have been proposed to reduce HuNoV in live oysters, including cold and high temperature, UV treatment, and high-hydrostatic pressure. However, most of these treatments have low log reduction on norovirus or they kill oysters, change the taste and texture of oysters (Araud, DiCaprio, Ma, Lou, Gao, Kingsley, et al. 2016; Venugopal, 2005; Ye, Lingham, Huang, Ozbay, Ji, Karwe, & Chen, 2015).

Ionizing irradiation is a non-thermal process which has been used to inactivate pathogenic and spoilage microorganisms and extend the shelf life of food products (Thakur & Singh, 1994). Gamma ray, electron beam and X-ray are the three kinds of ionizing irradiation that approved to treat shellfish to up to 5.5 kGy (USFDA, 2015). The biological effects of ionizing irradiation were reported mainly linked to the DNA damage by free radicals during the irradiation process and the ionizing radiation-generated reactive oxygen species (Ahn, Kim, & Lee, 2013). In the last decade, researchers at Mississippi State University have extensively studied the use of X-ray to inactivate different pathogens in different food products. Mahmoud (2012b) reposted that X-ray significantly ($p < 0.05$) reduce the initial level of artificially inoculated *Listeria monocytogenes* on smoked salmon from 3.7 log CFU g^{-1} to 2.0 log CFU g^{-1} after treatment with 0.5 kGy X-ray, while exposure to 1.0 kGy X-ray reduced *L. monocytogenes* to below the detection limit (<1.0 log g^{-1}). Mahmoud (2009b) reported that greater than a 6-log reduction of *Vibrio vulnificus* was achieved with 3.0 kGy in whole shell oysters. Treatment with 3.0 kGy X-ray treatment did not affect the

survivability of live oysters. Wu, Chang, Nannapaneni, Coker, Haque, & Mahmoud, (2016) stated that X-ray is a potential antiviral agent against HuNoVs surrogate murine norovirus (MNV-1) in food products, including salmon sushi and tuna salad. We have to rely on proper surrogates to study the survival of HuNoV because it is not cultivable (Ye, Lingham, Huang, Ozbay, Ji, Karwe, & Chen, 2015). Compared with feline calicivirus (FCV) and Tulane virus, we chosen Murine norovirus (MNV-1) for our research because it is a closer genetic relative of HuNoV in the norovirus genus and has been used in UV irradiation, gamma irradiation and electron beam research (Richards, 2012). MNV-1 was propagated and assayed in an efficient microbial cell line murine BV-2 cells (Cox, Cao, & Lu, 2009).

The aim of this study was to investigate the effects of X-ray treatments on bioaccumulated murine norovirus-1 (MNV-1) and survivability, inherent microflora, color, and firmness of whole shell Atlantic oysters (*Crassostrea virginica*) during storage at 5 ℃ for 20 days.

6.2 Materials and Methods

6.2.1 Murine norovirus (MNV-1)

MNV-1 has been chosen, for this study, due to its genetic similarity and environmental stability, compared with other surrogates such as feline calicivirus and Tulane virus (Richards, 2012; Ye, Lingham, Huang, Ozbay, Ji, Karwe, & Chen, 2015). Murine norovirus (MNV-1) was generously provided by Dr. Herbert W. Virgin IV, Washington University School of Medicine, St. Louis, MO. MNV-1 was propagated in murine BV-2 cells cultured in Dulbecco's modified eagle's medium (DMEM, HyClone

Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), 10 mM HEPES (HyClone Laboratories, Logan, UT), 1 mM non-essential amino acid (HyClone Laboratories, Logan, UT), 2 mM L-glutamine (HyClone Laboratories, Logan, UT), 100 U/mL penicillin (HyClone Laboratories, Logan, UT) and 100 mg/mL streptomycin (HyClone Laboratories, Logan, UT) (Cox, Cao, & Lu, 2009; Gonzalez-Hernandez, Cunha, & Wobus, 2012). The MNV-1 stocks were prepared and stored as previously described (Wu, Chang, Nannapaneni, Coker, Haque, & Mahmoud, 2016)

6.2.2 Atlantic oysters (Crassostrea virginica)

Freshly harvested Atlantic oysters were purchased from an oyster processor (Crystal Seas Seafood, Pass Christian, MS) and transported immediately (within 1 h) in a cooler with cubed ice to the MSU Experimental Seafood Processing Laboratory in Pascagoula, MS. After washing with tap water and removing mud from the shells, oysters with 7-9 cm were chosen for the experiments.

6.2.3 MNV-1 bioaccumulation in whole-shell oysters

Whole-shell oysters accumulated MNV-1 were prepared as previously described (Araud, DiCaprio, Ma, Lou, Gao, Kingsley, et al. (2016) with a few modifications. For each batch, 18 live oysters were cultivated in polyethylene tank containing recirculating 60 L of artificial seawater (1.5% sea salt, Oceanic system, Dallas, TX) at room temperature (20-22 °C). Oysters were fed using phyto-plankton (Reef Phytoplankton, Seachem, Madison, GA) according to the instruction of the manufacturer. The seawater was contaminated with MNV-1 at 10^4 PFU/mL where oysters were cultivated for 24 h

before X-ray treatments (the initial level of MNV-1 in oysters was about 4.3 log PFU g^{-1}).

6.2.4 X-ray treatment

Specific irradiation doses (0, 1.0, 2.0, 3.0, 4.0 and 5.0 kGy X-ray; at rate of 31.25 Gy/min) were generated using the RS 2400 industrial cabinet X-ray irradiator (Rad Source Technologies, Inc., Alpharetta, GA). Samples were treated with X-ray at 22 ℃ and 55-60% relative humidity and placed at same distance around the generator X-ray tube as described by Mahmoud (2009a).

For inactivation of bio-accumulated MNV-1 study, oysters (3 contaminated oysters) were treated with 0, 1.0, 2.0, 3.0, 4.0 or 5.0 kGy X-ray. For survivability study, oysters (20 uncontaminated oysters for each treatment) were subjected to 0, 1.0, 2.0, 3.0, 4.0 or 5.0 kGy X-ray treatments. For inherent microbiota, texture, and color studies, oysters (3 uncontaminated oysters) were subjected to 0.0, 1.0, 2.0, 3.0, 4.0 or 5.0 kGy X-ray.

6.2.5 MNV-1 enumeration (plaque assay)

Virus was extracted as described by Praveen, Dancho, Kingsley, Calci, MeadeMena, & Pillai (2013) with a few modifications. For each plaque assay, three contaminated oysters were shucked and pooled in 200 mL of phosphate buffer (0.15 M Na3PO4, pH 9.5) and homogenized by a laboratory blender (Waring Inc., NewHartford, CT) for 3 min. The homogenized tissues were centrifuged with a benchtop centrifuge. The supernatant was neutralized with 2N HCl. Tenfold serial dilutions of neutralized

supernatant were made in Earle's balanced salt solution (EBSS) (Gibco-Invitrogen, Grand Island, NY).

Plaque assays were performed as described by Wu, Chang, Nannapaneni, Coker, Haque, & Mahmoud (2016). A series of 10-fold dilutions of sample were inoculated onto the 6-well plates after the formation of a confluent monolayer of BV-2, aspiration of the medium and two cell washes with DMEM-0. The inoculums were incubated for one hour at room temperature with gentle rock every 15 min for even distribution. The viral inoculum was removed at the end of incubation. To each well, 2mL of overlay (1:1 mixture of 3% w/v Sea-Plaque agarose (Lonza, Allendale, NJ) and 2 X MEM media (Gibco-Invitrogen, Grand Island, NY)) were slowly poured on the edge. The plates were placed into the tissue culture incubator after solidified for 10 min at room temperature. The neutral red staining solution (3%) was prepared with neutral red (Sigma Chemical Co., St. Louis, MO) and culture grade phosphate-buffered saline (Gibco-Invitrogen, Grand Island, NY). Two mL staining solution was added directly onto the overlay. Plaques were count in wells where plaques are separated clearly.

6.2.6 The survivability of oysters

Live uncontaminated oysters were kept at the refrigerator temperature (5 ℃) for up to 20 days and their survival was determined every 5 days. The survivability of treated oysters was determined on the basis of difficulty to open the valves during storage (Mahmoud, 2009b).

6.2.7 Effect of X-ray treatment on the inherent microbiota on oysters

Uncontaminated oysters were kept at the refrigerator temperature (5 ℃) and live oysters were sampled on days 0, 5, 10, 15 and 20. Oysters were shucked, mixed and 25 g samples were placed in sterile Whirl-Pak bags, followed by addition of 225 mL of sterile Alkaline Peptone Water (APW). The samples were homogenized for 2 min using a Stomacher 80 Lab-blender. Serial 10-fold dilutions were prepared in APW. Mesophilic and psychrotrophic counts were performed as previously described (Mahmoud, 2012a). For mesophilic counts, 0.1 mL of each dilution was plated onto TSA and incubated at 37 ℃ for 24 h. For psychrotrophic counts, 0.1 mL of each dilution was plated onto TSA and incubated at 5 ℃ for 10 days. Viable counts were expressed as log CFU g^{-1}.

6.2.8 Effect of X-ray treatment on color and firmness

Uncontaminated oysters were taken from the refrigerator (5 ℃) on days 0, 5, 10, 15 and 20. The texture and color were analyzed as previously described by Mahmoud, Chang, Nannapaneni, Wu, & Coker (2016). The texture was measured by a puncture test using an Instron 4944 (Instron, Norwood, MA). Meat of oysters were punctured with a star-shape probe (5 sharp points, 10 mm diameter). Crosshead speed was set at 50 mm/min. Force-distance curves were obtained from the puncture tests and firmness was taken as the force (N) required puncturing the thickest point of the oyster samples. The color of oyster meat was evaluated using the Hunter colorimeter values (Lab Scan XE Hunter Colorimeter; Hunter Associates Laboratory, Inc., Reston, VA) by L*(lightness), a*(redness-greenness), and b*(yellowness-blueness).

6.2.9 Statistical analysis

All experiments were replicated three times. The mean values and standard deviations were determined using ANOVA tests (XLStat 2015, Addinsoft USA, New York, NY). Significant differences among means were made with Tukey honestly significant difference (HSD) analysis using the probability level of 0.05. A first-order kinetic model (linear model) was used to determine the effect of the storage days on the reduction of MNV-1. The analyses were done using Excel software (XLStat 2015, Addinsoft USA, New York, NY).

6.3 Results and Discussion

6.3.1 MNV-1 bioaccumulation in whole-shell oysters

No MNV-1 was found in the live oysters before the bioaccumulation experiments. Our results indicated that the viral (MNV-1) titers was 4.3 log PFU g^{-1} in oyster meat when oyster was harvested after accumulation of MNV-1. This result is in agreement with that of Araud, DiCaprio, Ma, Lou, Gao, Kingsley, et al. (2016) who reported that a 3-4 log $PFUg^{-1}$ of MNV-1 uptake in oyster when harvested after 24 h of accumulation.

6.3.2 MNV-1 enumeration (plaque assay)

The treatment of MNV-1 bioaccumulated whole-shell oysters with 1, 2, 3, and 4 kGy X-ray significantly reduced the log $PFUg^{-1}$ from 4.3 to 3.6, 3.2, 2.8, and 2.5, respectively, however, exposure to 5.0 kGy X-ray reduced the population of MNV-1 to less than 2.0 log PFU g^{-1} (Figure 6.1). This study demonstrated that X-ray had a strong antiviral activity against norovirus (MNV-1) compared with gamma and e-beam irradiation. Ionizing irradiation was not considered as a feasible process for inactivating

viruses in contaminated shell stock because some of the lately studies found that the radiation (gamma ray and electron beam) exposures needed to significantly reduce PFU of viruses in hard-shelled shellfish was higher than the maximum FDA-allowable dose of 5.5 kGy, which substantially increased the shellfish mortality. Harewood, Rippey, & Montesalvo (1994) stated the viral indicator F-coliphage was not significantly affected by gamma ray with a high D_{10} value (13.50 kGy) in shellfish. Praveen, Dancho, Kingsley, Calci, Meade, Mena, & Pillai (2013) reported that 4 kGy electron beam only reduced MNV-1 by 1 log PFU g^{-1} on whole-shell oysters. Further understanding of the mechanism of viral inactivation in whole-shell oyster is needed to guide the food industry to properly use the X-ray irradiation.

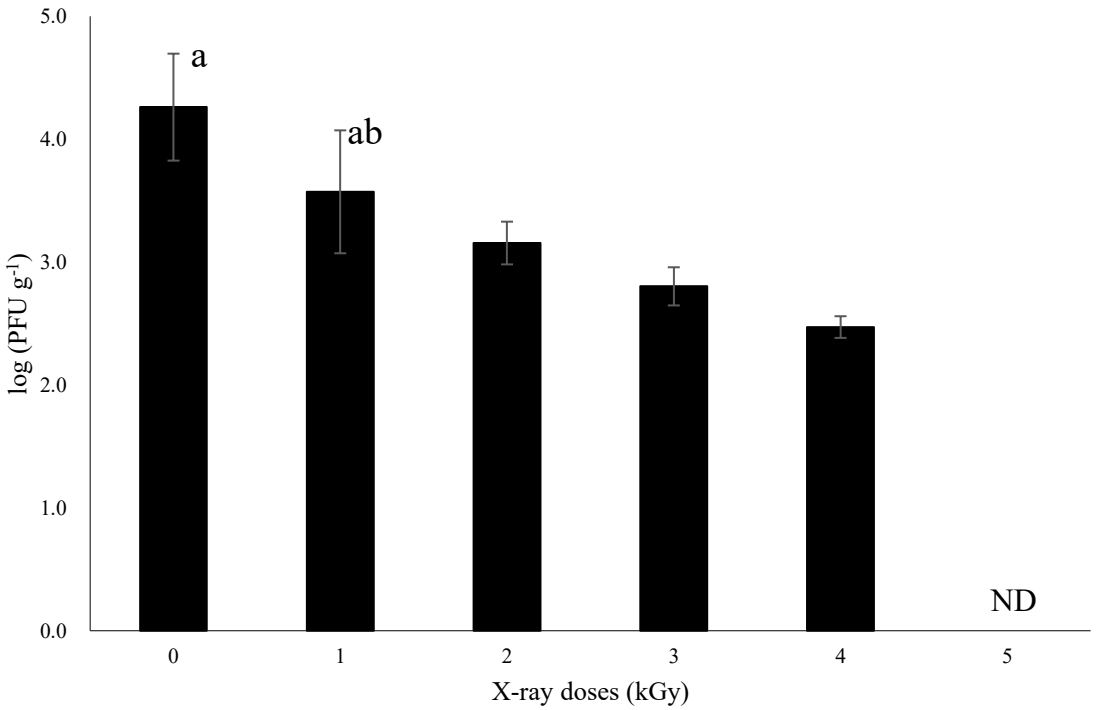

Figure 6.1 Inactivation of internalized MNV-1 (log PFU g-1) on whole shell oyster by X-ray.

Error bars represent standard error. Different lower case letters for each treatment are significantly different ($p < 0.05$). ND = not detectable (<2.0 log PFU g^{-1}).

6.3.3 The survivability of oysters

Irradiation is the only known method to pasteurize live molluscs without significantly affecting mortality (Mbarki, Nahdi, Barkallah, & Sadok, 2009). In our study, the survivability of control group was 19.7 of 20 oysters (98.5%) after storage of oysters at 5 ℃ for 20 days (Table 6.1). Our study also found that the treatment of whole-shell oysters with up to 5 kGy did not kill oysters on day 0(day 0). These findings are in agreement with those reported by Farcy, Voiseux, Robbes, Lebel, & Fievet (2011), who stated that mollusks are irradiation-resistant and can survive after exposure to doses up to 1 kGy. Jakabi, Gelli, Torre, Rodas, Franco, Destro, & Landgraf (2003) reported that the gamma ray irradiation did not kill the oysters nor affect their sensory attributes at 3.0 kGy. The average survivability decreased, during storage, with higher X-ray doses, however, there were no significant differences among all treatment doses on days 5 or 10. Furthermore, the average survivability significantly decreased with higher X-ray doses at day 15 and 20 (Table 6.1). The survivability of oysters treated with 5.0 kGy X-ray (9.3) was significantly lower than the survivability of oysters treated with 1.0 kGy X-ray (14.3) on day 20. These results were in agreement with those obtained by Mahmoud (2009b) who reported that 3.0 kGy of X-ray treatment did not affect the survivability of live oysters up to 7 days.

Table 6.1 The mean survivability of 20 oysters (*Crassostrea virginica*) in three groups. Initially, 20 oysters of each group was subjected to 0, 1, 2, 3, 4 and 5 kGy X-ray treatments and was kept at 5 °C for up to 20 days for determining the numbers of survival every five days.

	X-ray dose (kGy)					
Days	0	1	2	3	4	5
0	20.0±0.0 aA	20.0±0.0 aA	20.0±0.0 aA	20.0±0.0 aA	20.0±0.0 aA	20.0±0.0 aA
5	20.0±0.0 aA	20.0±0.0 aA	19.0±1.7 aAB	19.0±1.0 aA	19.0±1.0 aA	17.0±1.7 aAB
10	20.0±0.0 aA	19.7±0.6 aA	18.0±1.7 aAB	17.0±3.0 aA	17.3±0.6 aAB	16.3±1.5 aAB
15	19.7±0.6 aA	17.3±2.5 abAB	15.7±1.5 abcBC	11.7±2.1 cB	13.0±1.0 cB	13.7±0.6 bcB
20	19.7±0.6 aA	14.3±1.2 bB	13.3±0.6 bC	9.3±1.2 bcB	8.0±3.6 bcC	9.3±2.0 cC

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

6.3.4 Effect of X-ray treatment on the inherent microbiota on oysters

The mesophilic counts for the control samples of whole-shell oyster were 5.09, 5.69, 5.83, 6.84 and 6.98 log CFU g^{-1} on days 0, 5, 10, 15 and 20, respectively, during storage at 5 °C (Table 6.2). On day 0, treatment with 1 kGy X-ray significantly reduced the mesophilic count log CFU g^{-1} from 5.09 to 3.11. Furthermore, treatment with 2-5 kGy X-ray significantly reduced the mesophilic count to below the detection level (2 log CFU g^{-1}). Treatment with X-ray kept the mesophilic counts significantly lower than the control until day 15.

Table 6.2 Changes in the mesophilic bacterial counts (log CFU g^{-1}) of whole shell oysters with X-ray during storage at 5 °C for 20 days.

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	5.09±0.05 aA	3.11±0.05 bA	ND	ND	ND	ND
5	5.69±0.21 aA	3.49±0.57 bAB	ND	ND	ND	ND
10	5.83±0.48 aA	4.65±0.38 bBC	4.59±0.30 bcA	3.89±0.33 bcA	3.70±0.32 cA	ND
15	6.84±1.08 aA	5.99±0.35 aCD	5.58±0.92 aAB	5.84±0.09 aB	5.77±0.18 aB	5.02±0.79 aA
20	6.98±1.27 aA	7.04±0.98 aD	7.23±0.97 aB	7.23±0.89 aC	7.48±0.91 aC	6.82±0.38 aB

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).
ND = not detectable (<2.0 log CFU g^{-1}).

The psychotropic counts for the control samples of whole-shell oyster were slightly increased during storage at 5 °C (Table 6.3). On day 0, treatment with 1 and 2 kGy reduced the psychotropic count from 5.70 to 3.74 and 2.67 log CFU g^{-1}, respectively. Furthermore, treatment with 3-5 kGy X-ray significantly reduced the psychotropic count to below the detection level (2 log CFU g^{-1}). Treatment with X-ray kept the psychotropic counts significantly lower than the control until day 15. These results showed the same trend as those reported by Mahmoud & Burrage (2009) where treatment with 1.0 kGy significantly reduced the inherent microbiota in whole-shell oysters from 4.6 log CFU to undetectable limit (<1.0 log CFU g^{-1}). The microbial community structure and sensitivity in oysters can be changed throughout seasons (Pierce, Ward, Holohan, Zhao, & Hicks, 2016). Lopez-Caballero, Lopez-Caballero, Perez-Mateos, Montero, & Borderias (2000) stated that bacteria would cause the formation of high total volatile base nitrogen (TVB-N) and small amounts TMAO (Trimethylamine N-oxide) during oyster spoilage which it produces amines, ammonia and reduces the pH by proteolytic and saccharolytic process. Improving the microbial quality might alleviate biochemical changes during refrigerated storage (Mbarki, Nahdi, Barkallah, & Sadok, 2009). Buzin, Baudon, Cardinal, Barillé, & Haure (2011) reported that cold storage of Pacific oyster (*Crassostrea gigas*) caused a low mortality rate, and the sensory attributes, odor, appearance, texture and flavor did not show significant variations. A further investigation on chemical changes on live oyster by X-ray preservation is in progress.

Table 6.3 Changes in the psychrotrophic bacterial counts (log CFU g^{-1}) of whole shell oysters with X-ray during storage at 5°C for 20 days.

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	5.70±0.18 aA	3.74±0.03 bA	2.67±0.48 cA	ND	ND	ND
5	5.79±0.40 aA	4.96±0.13 abB	4.22±0.47 bAB	4.00±0.95 bA	3.97±0.49 bA	ND
10	6.33±0.48 aA	5.89±0.31 aAB	5.82±0.41 aBC	5.60±0.49 aB	5.89±0.43 aB	5.40±0.65 aA
15	6.76±0.41 aA	6.33±5.55 aBC	6.58±0.90 aC	5.72±0.38 aB	6.51±0.62 aBC	5.89±0.21 aAB
20	6.90±1.39 aA	7.11±0.61 aC	6.83±1.19 aC	6.67±0.58 aB	7.53±0.91 aC	6.29±0.20 aB

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).
ND = not detectable (<2.0 log CFU g^{-1}).

6.3.5 Effect of X-ray treatment on color and firmness of oyster meat

Enhancing food quality and food safety on the food industry must be considered simultaneously. It is critical to retain the color of seafood products when considering development of intervention methods of pathogens. For example, high pressure treatment was found to changes the color of oysters at pressures above 300 MPa due to protein denaturation, and the extent of color change increased gradually with the increase of treatment pressure (Wang, Hirneisen, Markland, & Kniel, 2013). The firmness and the color of oyster meat during storage at 5 °C for 20 days were shown in Tables 6.4 and 6.5. The results indicated that X-ray treatment did not significantly affect firmness nor a*, b* and saturation values of oyster meat on day 0 or throughout the storage for 20 days. The hue value of oyster meat on day 20 with 5 kGy X-ray treatment was significantly higher than day 0 but no significant difference compared with other X-ray doses on day 20.

Table 6.4 Changes in the firmness (Maximum load, N) of the meat of treated whole-shell oyster with X-ray during storage at 5 °C for 20 days.

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	0.24±0.22 aA	0.69±0.28 aA	0.49±0.19 aA	1.02±0.21 aA	0.97±0.41 aA	0.78±0.35 aA
5	0.65±0.11 aAB	0.55±0.33 aA	0.64±0.37 aAB	0.80±0.47 aA	0.93±0.34 aA	0.92±0.84 aA
10	0.59±0.66 aAB	1.37±0.11 aA	0.98±0.19 aAB	0.72±0.09 aA	0.89±0.08 aA	1.18±0.56 aA
15	1.39±0.29 aB	1.36±0.92 aA	1.24±0.16 aAB	1.55±0.79 aA	1.46±0.38 aA	1.60±0.33 aA
20	0.86±0.31 aAB	1.07±0.47 aA	1.35±0.49 aB	1.27±0.19 aA	1.80±0.47 aA	1.21±0.22 aA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

Table 6.5 Changes in the whole-shell oyster color (Hunter parameters, L, a, b, Saturation and Hue) of whole-shell oyster with X-ray during storage at 5 °C for 20 days.

(L)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	63.7±7.6 aA	63.4±5.5 aA	63.0±4.5 aA	50.5±18.1 aA	60.2±9.2 aA	58.9±2.9 aA
5	49.5±16.1 aA	48.5±14.7 aA	53.3±18.9 aA	61.1±3.8 aA	56.9±3.1 aA	49.2±10.0aA
10	63.6±5.3 aA	59.3±3.8 abA	66.4±0.4 aA	52.7±8.1 abA	57.9±4.6 abA	36.7±21.1 bA
15	47.5±16.2 aA	54.3±2.6 aA	45.8±13.0 aA	50.4±12.7 aA	49.6±8.9 aAB	45.9±12.5 aA
20	57.4±2.2 aA	59.9±4.9 aA	63.2±5.9 aA	54.9±1.4 aA	37.7±6.9 bB	57.9±4.5 aA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

Table 6.5 (continued)

(a)

Days	X-ray dose (kGy) 0	1	2	3	4	5
0	2.4±0.5 aA	1.6±1.4 aA	1.6±0.2 aA	3.2±1.4 aA	1.8±0.8 aA	2.1±0.8 aA
5	2.2±0.5 aA	3.6±1.6 aA	2.0±0.3 aA	2.2±0.3 aA	2.7±0.5 aA	2.9±0.5 aA
10	2.4±0.9 aA	3.2±0.5 aA	3.1±0.6 aA	2.2±0.5 aA	2.6±0.4 aA	4.2±2.8 aA
15	1.7±0.9 aA	2.4±0.4 aA	2.9±0.4 aA	2.7±0.7 aA	2.2±0.9 aA	1.9±0.4 aA
20	1.8±0.5 aA	2.3±0.6 aA	2.4±1.2 aA	3.4±0.7 aA	4.2±2.1 aA	3.2±0.4 aA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

(b)

Days	X-ray dose (kGy) 0	1	2	3	4	5
0	14.3±0.7 aA	11.1±5.4 aA	13.3±2.4 aA	14.4±1.9 aA	13.7±0.2 aA	16.0±1.3 aA
5	11.8±3.1 aA	14.0±4.2 aA	12.2±3.9 aA	14.2±0.9 aA	14.0±0.2 aA	13.9±2.5 aA
10	13.0±2.3 aA	14.3±1.3 aA	14.8±1.9 aA	12.4±2.2 aA	13.2±1.9 aA	19.1±2.6 aA
15	12.3±1.3 aA	15.0±2.4 aA	15.6±0.9 aA	15.9±1.7 aA	14.4±0.9 aA	14.2±1.1 aA
20	12.9±0.9 aA	13.4±0.6 aA	13.0±1.2 aA	15.1±0.8 aA	14.2±2.8 aA	14.7±0.9 aA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

Table 6.5 (continued)

(Saturation)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	14.9±0.7 aA	11.2±5.6 aA	13.4±2.4 aA	14.7±2.0 aA	13.9±0.1 aA	16.2±1.4 aA
5	12.0±3.1 aA	14.6±3.7 aA	12.4±3.8 aA	14.4±0.9 aA	14.3±0.2 aA	14.2±2.5 aA
10	13.2±2.5 aA	14.6±1.4 aA	15.1±2.0 aA	12.6±2.3 aA	13.4±1.9 aA	19.6±8.1 aA
15	12.4±1.2 aA	15.2±2.4 aA	15.8±0.9 aA	15.3±1.7 aA	14.6±0.9 aA	14.3±1.1 aA
20	12.9±0.9 aA	13.6±0.8 aA	13.2±1.4 aA	15.5±0.7 aA	14.9±3.1 aA	15.0±1.0 aA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

(Hue)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	80.5±1.8 aA	83.6±5.2 aA	82.9±1.8 aA	77.8±4.9 aA	82.3±3.5 aA	82.7±2.1 aA
5	79.4±2.2 aA	73.7±11.6 aA	79.7±4.0 aA	81.1±0.8 aA	79.0±2.1 aA	77.9±0.6 aAB
10	79.8±2.0 aA	77.4±0.9 aA	78.2±0.8 aA	79.9±0.6 aA	78.8±0.5 aA	78.3±3.2 aAB
15	81.8±5.4 aA	80.7±0.9 aA	79.5±1.2 aA	80.0±2.6 aA	81.6±3.4 aA	82.3±1.2 aAB
20	82.1±1.9 aA	80.1±2.2 aA	79.9±4.6 aA	77.2±2.8 aA	73.9±6.6 aA	77.7±1.1 aB

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

6.3.6 Effect of X-ray treatment on shell color of oyster

The changes in the shell color (Hunter parameters, L, a, b, saturation and Hue) of whole-shell oyster treated with X-ray during storage at 5 °C for 20 days are shown in Table 6.6. No significant difference was found in L, a and Hue of oyster shell after treatment of 1, 2, 3, 4 and 5 kGy X-ray. However, the values b and saturation were highly positively related to the X-ray dose. Using the values of the X-ray dose as defining different subpopulations for the linear regression analysis on three experiments of storage inactivation, r^2 of values b* and saturation are 0.92 and 0.93, respectively. The results showed that the linear regression model well fits b* and saturation of oyster shell.

Table 6.6 Changes in the shell color of oyster (Hunter parameters, L, a, b, Saturation and Hue) of whole-shell oyster with X-ray during storage at 5 °C for 20 days.

(L)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	81.00±8.58aA	81.88±6.87aA	72.45±6.65abA	58.01±2.51bA	65.31±4.71abA	61.81±6.20abA
5	64.06±7.34aA	66.11±7.88aA	66.36±8.14aA	62.82±5.08aA	63.17±3.99aA	58.82±5.06aA
10	67.90±3.89aA	66.37±2.48aA	64.67±3.39aA	61.58±6.76abA	64.29±6.84aA	46.65±5.88bA
15	56.99±11.22aA	68.89±7.38aA	59.89±3.62aA	64.93±10.17aA	52.22±9.76aA	51.72±6.54aA
20	66.59±5.60aA	67.37±6.09aA	656.95±8.61aA	56.23±6.74aA	56.50±9.58aA	63.19±4.11aA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

Table 6.6 (continued)

(a)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	0.11±0.44aA	0.53±0.78aA	1.49±2.18aA	1.84±1.04aA	1.81±0.74aA	2.47±0.72aAB
5	0.84±1.02aA	2.42±3.03aA	2.01±0.93aA	3.18±1.38aA	2.07±1.06aA	2.99±0.83aAB
10	1.81±0.63abA	0.75±0.39aA	1.45±1.45aA	2.83±1.32abA	1.97±0.40abA	5.42±1.96bA
15	2.31±1.35aA	0.83±0.83aA	1.19±0.36aA	1.23±0.92aA	2.26±1.54aA	1.33±0.95aB
20	1.11±1.21aA	1.28±1.06aA	1.03±1.14aA	4.05±0.84aA	3.36±1.99aA	2.95±1.34aAB

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

(b)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	-0.07±0.31aA	1.23±1.07aA	3.69±1.46abA	6.88±0.87bcA	9.56±1.67cdA	12.39±1.48dA
5	5.18±1.75aB	6.69±2.90aB	7.28±0.56aA	11.25±3.48aA	7.57±1.54aA	11.54±1.28aA
10	4.37±0.55aAB	3.89±1.29aAB	7.52±1.44aA	10.70±2.54abA	8.24±0.38aA	15.79±4.42bA
15	2.99±2.80aAB	5.01±1.30aAB	7.20±1.88abA	9.00±0.49abA	13.15±2.98abA	9.09±2.48bA
20	2.18±0.81aAB	3.69±0.67 abAB	6.50±2.63abcA	11.90±3.00bcA	11.93±1.71cA	10.16±3.99abcA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

Table 6.6 (continued)

(Saturation)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	0.53±0.16aA	1.69±0.82abA	4.73±0.61bcA	7.19±0.86cdA	9.74±1.78deA	12.64±1.55dA
5	5.29±1.88aB	7.81±2.70aB	7.63±0.29aA	11.69±3.72aA	7.89±1.67aA	11.93±1.45aA
10	4.79±0.29aB	3.99±1.24aAB	7.85±1.08aA	11.09±2.79abA	8.49±0.36aA	16.92±4.01bA
15	4.61±1.66aB	5.18±1.16aAB	7.31±1.91abA	9.12±0.58abA	13.48±2.76bA	9.21±2.59abA
20	2.84±0.12aAB	4.02±0.83aAB	6.82±2.24abA	12.60±2.99bA	12.47±2.21bA	10.59±4.19abA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

(Hue)

Days	X-ray dose (kGy)					
	0	1	2	3	4	5
0	-7.43±49.53aA	48.56±17.95aA	8.02±71.03aA	75.15±8.39aA	79.67±2.36aA	78.81±2.44aA
5	22.38±78.14aA	12.12±72.16aA	74.24±7.88aA	74.74±1.68aA	75.49±6.53aA	75.71±2.21aA
10	67.02±9.29aA	77.46±8.81aA	77.63±13.31aA	75.92±2.91aA	76.51±2.80aA	69.79±10.16aA
15	45.49±40.10aA	78.77±11.62aA	80.63±1.27aA	82.44±5.36aA	79.27±8.57aA	82.54±3.74aA
20	2.96±69.70aA	13.10±70.19aA	14.85±73.42aA	70.59±4.38aA	75.31±6.26aA	73.89±2.08aA

Mean values with different lower case letters in same row or capital letters in same column are significantly different ($p < 0.05$).

6.4 Conclusion

To our knowledge, this is the first report about the X-ray inactivation of internalized MNV-1 on whole-shell oysters. The results of this study indicate that the efficacy of X-ray irradiation against internalized MNV-1 increased with increasing X-ray doses up to 5 kGy. For application of X-ray technology in oyster industries, the shelf-life studies were performed on survivability, inherent microbiota, color, and firmness on whole-shell oyster. The results indicated that X-ray is effective in reducing spoilage microflora. The main advantage of using X-ray for reducing MNV-1 in oysters was that the X-ray treatment did not kill the oysters (at 0 day) even at the highest dose tested (5 kGy) and the survivability was not significantly affected by treatment with X-ray within 10 days of storage at 5℃. Our results suggest that X-ray irradiation is a feasible process for inactivating norovirus and inherent microbiota for the oyster industry.

CHAPTER VII

MANUFACTURING FISH SAUCE COMPLIANT WITH CODEX STANDARD FROM THE BY-PRODUCTS OF WILD SILVER CARP

7.1 Introduction

Asian carps particularly the silver carp (*Hypophthalmichthys molitrix*) have altered ecosystem structure and negatively affected commercial and recreational fisheries in the Mississippi river basin (Wittmann, Cooke, Rothlisberger, & Lodge, 2014). One of the best strategy for reducing the number of Asian carp is to consume them as food (Barrera, Ramirez, Gonzalez-Cabriales, and Vazquez, 2002). According to our private communication, companies such as Blue Shore Fisheries (Farmington, KY) and Moon River Foods, Inc (Indianola, MS, Figure 3.1) installed advanced surimi production line at the scale of mass production for several years. Because the remains of silver carp processing contribute the cost for waste treatments, Asian carp processors are looking for the technology to re-use or re-cycle the waste material to increase profit.

Fish sauce has a strong demand in South-Eastern Asian countries like Vietnam and Thailand (Lopetcharat, Choi, Park, & Daeschel, 2001). Thailand and Vietnam produce and export most of the fish sauce available in the global market. In 2011, Thailand exported a total of 43 million liters of fish sauce to 106 countries (www.fao.org/tempref/codex/Meetings/CCFFP/CCFFP32/fp32_15e.pdf). Domestically, the average fish consumption in Thailand is 9.07 g/day (conducted by a national survey

in 2003-2004). However, anchovy (the traditional raw material for fish sauce production) have been exploited beyond their natural capacity (Khemakorn, 2015). To better protect the global fish ecosystem, it would be reasonable to manufacture fish sauce from fish by-products (Gildberg, 1993).

The 34th Session of Codex Alimentarius Commission adopted the fish sauce standard as Codex Standard (CODEX STAN 302-2011) drafted by Thailand and Vietnam. This standard applies to fish sauce produced by fermentation of mixed fish, salt and other ingredients. All the three kinds of fish sauce products (pure, hydrolyzed and diluted fish sauce) must meet the requirements of this standard. Pure fish sauce is obtained from fish fermented with salt or brine (Lopetcharat, Choi, Park, & Daeschel, 2001). Hydrolyzed fish sauce can be obtained from the hydrolysates of fish or other kinds of animals, which are often treated with hydrochloric acid (HCl) or other hydrolyzing processes with enzymes or inoculated micro-organisms. Diluted fish sauce is obtained from pure fish sauce or hydrolyzed fish sauce, but is diluted using approved additives or flavoring agents.

Because the complete hydrolysis of muscle proteins is slowly progressed by the action of intestinal proteases and proteases generated from halophilic microorganisms, the harvest of pure fish sauce is carried out in two stages or more during fish sauce processing (Lopetcharat, Choi, Park, & Daeschel, 2001). The earlier harvested products are packed and sold as pure fish sauce because of high concentration of total nitrogen. On-site sampling results at Phuoc Quoc island indicated that the total nitrogen of the pure fish sauce ranged from 20-40 g/l. The later harvested liquid has only about 10 g/l or less total nitrogen content, which is mainly transported to the factories around Ho Chi Minh

city for the production of diluted fish sauce. Based on our preliminary results, the by-products of silver carp could produce fish sauce with total nitrogen around 10 g/l. In this study, our objectives were to produce fish sauce from wild silver carp by-product to meet CODEX standards, and to use the silver carp by-product fish sauce as ingredient of commercial diluted fish sauce and performed the sensory assessment by substituting the commercial diluted fish sauce with our product at different ratio.

7.2 Materials and methods

7.2.1 Meat preparation

Freshly-harvested wild silver carp was obtained from an Asian carp processor (Moon River Foods) in February, April and November of 2016, and transported in a cooler with cubed ice to the pilot plant in Department of Food Science, Nutrition and Health Promotion, Mississippi State University, MS within two hrs. Immediately after arrival, fishes were gutted. An electrical knife was used to cut the fish behind its gill, and sliced the fish meat along the bone of fish to separate the frame and fillet. The portions of visceral, pale meat, dark meat, head, skin and frame were collected and their weight recorded. All silver carp by-products were stored at -20°C before use.

7.2.2 Fish sauce fermentation

Fish sauce was produced according to the method as described previously with moderate modification (Gildberg, 2001; Sanceda, Suzuki, & Kurata, 2003). Fish sauce was produced by mixing grounded frame, skin and viscera with food-grade salt (20%w/w) in proportion as in Table 7.1, and fermented in the dark at 22±2°C. The mixture was filtered to obtain fish sauce after 6 months of fermentation, and the

remaining proteins in the residue were allowed further fermentation by mixing with a portion of 20%w/w salt brine for up to 5 months.

Table 7.1 Preparation of fish sauce fermentation samples

Material	%w/w
Frame & Skin	76
Visceral	4
NaCl	20
Total	100

7.2.3 Chemical composition

All test methods for analysis of chemical properties corresponded to Codex Standard (CODEX STAN 302-2011). Total nitrogen was determined according to AOAC 940.25. Amino acid nitrogen was analyzed by determining formaldehyde nitrogen (AOAC 2.066) and subtracting by ammoniacal nitrogen (AOAC 2.065). The pH was measured by diluting fish sauce with water to 1:10 using a pH meter (AOAC 981.12). The dilution of fish sauce is necessary because of the high ionic strength in the undiluted sauce. Sodium chloride was determined by AOAC 976.18.

7.2.4 Preparation of sensory evaluation solutions

Three fish sauce made from the silver carp by-product (February, April and November) was mixed at the ratio (1:1:1) evenly to prepare for sensory evaluation as at low, middle and high substitution ratios of a popular Vietnamese fish sauce (Nam ngu, MASAN Consumer Corporation, Ho Chi Minh City, Vietnam) as described in Table 7.2.

Table 7.2 Preparation for sensory evaluation of fish samples

	Ratio (%w/w)			
Fish sauce material	Commercial product	Low substitution	Middle substitution	High substitution
Fish sauce made from frame of silver carp	0	25	50	100
Popular Vietnamese fish sauce (Nam ngu)	100	75	50	0

7.2.5 Sensory evaluation

A sensory panel with 12 panelists were trained for descriptive analysis of sensory characteristics using a Spectrum method (Meilgaard, Civille, & Carr, 2007). The panelists had at least five years of experience in the evaluation of fish sauce products prior to this study (Codex Alimentarius Commission, 1999). The sensory evaluation was performed according to previous reports with limited modification (Costa, Conte, & Del Nobile, 2014; Hajeb & Jinap, 2015). The four fish sauce samples (commercial product Nam ngu and three substituted fished sauce as described in Table 7.2) were assessed by the trained panel.

Appropriate descriptive terms for sensory evaluation were decided during the re-training sessions. Fish sauce-like aroma, umami, saltiness, lightness of the color, and overall quality were selected as main quality attributes. Panelists were trained using previously identified standards as well as trained with fish sauce aroma of Nam ngu fish sauce, umami of 5%w/w monosodium glutamate and saltiness of 30%w/w NaCl using a 15-cm intensity line scale. 0 was considered as “not detected” and 15 was considered “extremely strong”. Fish sauce samples were served to panels for the following four descriptors of quality.

7.2.6 Statistical analysis

For chemical composition, each fish sauce was sampled three times for analysis. Data were subject to analysis of variance using XLStat (2015, Addinsoft USA, New York, NY). Significant differences among means were made with Tukey honestly significant difference (HSD) analysis using probability level of 0.05.

7.3 Results and discussion

In this study, we randomly selected six whole silver carps and weighted visceral, pale meat, dark meat, head, frame and skin separately after scaling, gutting and filleting (Table 7.3). The average total weight of these silver carp was 4621.5±570.6 g. The ratio of pale meat and dark meat are 20.0±2.0 and 7.0±1.0 g/100 g, respectively. Pale meat and dark meat could be sold directly as meat or utilized as raw material for surimi production. Because fish head could be used for a famous Chinese food “Fish head stewed with brown sauce in casserole”, the remaining parts (visceral, frame and skin) were used for the production of fish sauce.

Table 7.3 Component yields of silver carp.

	Visceral	Pale meat	Dark meat	Head	Frame & Skin	Total
Weight (g)	483.2±68.5	919.7±169.0	310.2±70.0	1690.3±267.9	1219.2±132.9	4621.5±570.6
Average %	10±2	20±2	7±1	37±3	26±2	100

The initial weight of by-product mixture was 1540 g. The first liquid obtained by filtration of the fermented by-product mixture after 6 months was kept separately. The remained sediment was continuously made up with 20% salt brine and filtered to produce the second liquid after another 5 months. The first and second liquid were mixed together. The volume of fish sauce made in February, April and November were 600, 545, and 625 mL, respectively.The total nitrogen content (g/l) of the three fish sauce samples ranged from 8.45 to 9.86 (Table 7.4). The amino acid nitrogen content of all the samples were all higher than 60%. The pH value was about 5.42-5.48 and the salt concentration was a little bit lower than 30%. According to CODEX STAN 302-2011, total nitrogen content should not be less than 10 g/l, however, competent authorities may also specify a lower level of total nitrogen if it is the preference of that country. Because CODEX STAN 302-2011 allowed to add monosodium glutamate or pure fish sauce from other source in diluted fish sauce, the total nitrogen content could be adjusted according to market requirement. It was mandatory that amino acid nitrogen content should be higher or equal to 40%. All the fish sauce made of silver carp fish sauce met this requirement. All the samples met the obligatory pH (5.0-6.5) and NaCl ($\geqq$200g/l) values.

Table 7.4 Chemical properties of three batches of fish sauce fermentation

Chemical properties	First batch (Feb.2016)	Second batch (Apr. 2016)	Third batch (Nov. 2016)
Total nitrogen content (g/l)	8.45±0.25 a	9.86±0.90 b	9.71±4.5 b
Amino acid nitrogen content (g/l)	6.49±0.18 a	6.09±0.26 a	6.33±0.12 a
Amino acid nitrogen %	76.84±4.41 a	61.84±2.57 a	65.28±2.94 a
pH	5.43	5.48	5.42
Salt (%)	29.88±0.21 a	29.83±0.17 a	29.83±0.17 a

Uchida, Ou, Chen, Yuan, Zhang, Chen, Funatsu, Kawasaki, Satomi, & Fukuda, (2005) reported the production of silver carp fish sauce and the minced meat of silver carp was used for fermentation and the total nitrogen content reached 0.74-1.07 (g/100 g). Our results were in line with theirs and proved the by-product could also produce the similar nitrogen content compared with the fish sauce made of minced meat of silver carp. Furthermore, our report is the first one to confirm that the fish sauce made of by-products of wild silver carp met the chemical composition requirement of CODEX standard. To access the feasibility of industrial application, samples were sent to Vietnam and sensory evaluation was performed on a popular Vietnamese fish sauce (Nam ngu, MASAN Consumer Corporation, Ho Chi Minh City, Vietnam) substituted with the by-product fish sauce at low, middle and high ratio of as described in Table 7.2.

As shown in Table 7.5, substitutions of high ratio of fish sauce made by silver carp by-product did not make significant difference on the attributes of umami, saltiness, lightness of color and overall qaulity between low substitution samples (25% w/w) and commercial diluted fish sauce, while increased substitution ratios significantly decreased

the score of aroma and umami. According to the product label, fish-smell flavors and umami enhancers (monosodium glutamate, disodium inosine 5'monophophate and disodium 5' guanylate) are applied on "Nam Ngu fish sauce". To further improve the quality of aroma and umami in fish sauce of wild silver carp by-products, it might be necessary to use fish-smell flavors and umami enhancers as the currently used in popular products. Because glutamic acid, was a relatively stable amino acid without any secondary decomposition during fermentation, monosodium glutamate is very suitable to be an additive for fish sauce as flavor enhancer (Mizutani, Kimizuka, Ruddle, & Ishige, 1992).

Table 7.5 Sensory score of fish sauce with five descriptors of quality: fish sauce-like aroma, umami, saltiness, lightness of the color, and overall quality.

Descriptors of quality	Commercial product	Low substitution	Score (0-15) Middle substitution	High substitution
fish sauce-like aroma	15.0±0.0 a	10.9±1.9 b	8.2±2.5 c	3.5±2.3 d
umami	11.7±1.6 a	9.6±2.7 ab	7.1±4.0 b	3.3±1.5 c
saltiness	11.1±3.3 a	8.7±3.3 a	9.2±3.1 a	10.8±3.8 a
lightness of the color	7.5±2.1 a	8.1±3.3 a	7.8±2.9 a	8.7±2.4 a
Overall quality	12.4±2.8 a	10.5±1.7 ab	8.8±1.9 b	4.5±1.7 c

Mean values with different lower case letters in same row are significantly different ($p < 0.05$).

The fish processing industry produces a large quantity of by-products and discards (Shahidi, 2006; Nurdiani, Dissanayake, Street, Donkor, Singh, & Vasiljevic, 2015). Because fish by-products are an excellent source of high quality proteins that may supply a major part of the essential amino acids that are required for a balanced nutrition, recovery of proteins from by-products may be profitable. We do not think the raw material will be limited to the by-product of wild silver carp. It is worth to expand the use of material to many kinds of fish, such as catfish by-products from the fillet processing plant.

7.4 Conclusion

By conducting this research, we will be able to offer the industry a clear image about cost, technical feasibility and the quality of fish sauce made of silver carp by-products. Restoring the river, fish sauce could promote the consumption of silver carp and help maintain ecosystem structure, and the growth of economy locally and nation-wide.

CHAPTER VIII

OVERALL PROJECT SUMMARY AND FUTURE WORK RECOMMENDATION

In this study, we investigated the HuNoV persistence characteristics in seafood and efficacy of X-ray treatment to these food by its surrogate (MNV-1) mimic the survival and inactivation. The BV-2 cell line was used for maintenance of MNV-1 stock solution. MNV-1 bio-accumulation protocol was developed to internalized MMV-1 into oysters. To accomplish this task, several seafoods were used for MNV-1 inactivation study including whole shell oysters, silver carp surimi, salmon fillet, half shell oyster, salmon sushi, and tuna salad. The cooked surimi was optimized by six kinds of starch after a series of assays of protein, starch RVA profiles, surimi rheological test, and gel puncture test. Tuna salad formula was implemented for experiments.

Three kinds of wild freshwater fish (grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and bigmouth buffalo (*Ictiobus cyprinellus*)) and six kinds of starch (tapioca cassava native starch, tapioca derived starch acetate (E1420), tapioca derived distarch phosphates (E1412), Novation 1900 (comprise of corn, waxy corn, potato, tapioca and rice), Firmtex™ (modifed waxy corn, E1442 (hydroxypropyl distarch phosphate) and N-Hance 59 (native potato starch)) were used for building of the surimi formula. The investigation of the persistence characteristics of HuNoV was performed on three kinds of food matrix (whole shell oysters, salmon fillet and silver carp surimi) under cold storage by the analysis of the murine norovirus-1 (MNV-1),

inherent microbiota, weight loss and total volatile base nitrogen (TVB-N). The effects of X-ray irradiation on the murine norovirus-1 (MNV-1) were investigated in pure culture, half-shell oyster, salmon sushi, and tuna salad at 0, 1, 2, 3, 4, and 5 kGy X-ray. The bioaccumulated MNV-1, survivability, inherent microbiota, color, and firmness of Atlantic oysters (*Crassostrea virginica*) were and analyzed right after X-ray treatments and during 20 days storage at 5 ℃. To reveal the difference between the surimi made of the meat of silver carp, grass carp, and bigmouth, the protein profile and dynamic dynamic rheological measurements (temperature sweep) were performed. Compared with native starch (tapioca), starch acetate (tapioca), and distarch phosphate (tapioca), Novation 1900, Firmtex™ and N-Hance 59 had lower, gelatinization temperature (℃), higher viscosity and swelling power. The dynamic rheological measurements were performed on the surimi made of silver carp, grass carp and bigmouth buffalo and collated with the information from protein profile, and gel strength. The result of the puncture test is in line with the temperature sweep test data, confirming the highest plasticity of silver carp compared among all the surimi samples. The effects of the added starch or modified starch to breaking force and deformation were different between the surimi made of grass carp, silver carp and bigmouth buffalo while the mechanism is not clear. The results above help to build a surimi formula for study about the HuNoV persistence characteristics.

The results of total volatile basic nitrogen (TVB-N), mesophilic and psychrotrophic bacterial count concluded that the shell life of oyster and silver carp surimi could last for 20 days, and the salmon fillet was not more than 5 days. Because the bio-accumulated MNV-1 was found to maintain infectivity during storage of whole shell

oysters at 5°C for 15 days while the inoculated MNV-1 kept infectious for 20 days in cooked surimi and salmon fillet, the infectivity can last over the shelf life of these three seafood products at 5°C.

Using X-ray radiation to control norovirus in pure culture, half oyster, salmon sushi, tuna salad, and live oyster indicated that the efficacy of X-ray irradiation against human norovirus (HuNoV) surrogate MNV-1 increased with increasing X-ray doses. These results indicated that X-ray is a promising antiviral technology. However, because the inactivation of 3 log was only found in pure culture, not in food matrix such as half-shell oyster, salmon sushi, tuna salad and whole shell oyster, further optimization of X-ray treatment will be necessary for industrial application.

As described earlier, even though studies on the application of ionizing irradiation such as electron beam, X-ray and gamma ray on food pathogens have been conducted, these results have been expressed based on absorbed dosage (kGy) without considering the "quality of radiation" in their experimental designs. In the future, ESPL will utilize the advantage of X-ray machine by adjusting power output and filters to produce various radiation quality for pathogen inactivation and food preservation. The modern benchtop X-ray machine is equipped with various power output (accelerating voltage) from 0 to 350 kV, and more than 5 kinds of filters could be used. Furthermore, the specific atmosphere conditions of the X-ray treatment chamber can be designed and achieved by using the modern X-ray instrument. X-ray technology has been widely used for medical treatment and gene expression studies. To our best knowledge, improving X-ray irradiation efficiency has not yet been researched and applied on food pathogen inactivation and food preservation.

A combination of irradiation with other preservation techniques (cold storage, heat, packaging, etc.) can decrease the adverse effects of irradiation on the quality of food products (Kirkin, Mitrevski, Gunes, & Marriott, 2014). Modified atmosphere packaging (MAP) can improve the effects of pathogen inactivation by irradiation and prolong the shelf life. MAP alters the head-space gas composition of a food product in the package to inhibit microbial growth, decrease degradation of quality, and extend shelf life. MAP could significantly decrease the side effects of irradiation such as softening, respiration, and lipid oxidation in various fresh produce (cabbage, carrot, and endive) and meat products (marinated chicken and seasoned ground beef). Future research may explore the advantage of using MAP in two phases to enhance X-ray inactivation efficiency during irradiation and preservation of food quality. MAP can be designed to maximize and to preserve food quality during post-processing storage. After we develop the best pathogen inactivation protocols of X-ray technology, we will test various MAP conditions for shelf life researchers.

Because the irradiated cell ultimately depends on the preservation of genome, most of studies have focused on how DNA is damaged and repaired upon irradiation (Daly, 2009). For a long time, DNA double-strand breaks (DSBs) was presumed to be the most lethal damage by radiations. However, recent data showed that the amount of protein damage caused during irradiation of bacteria was more related to survival than to DNA damage (Daly, 2009). Therefore, to understand the mechanism of pathogen inactivation at the molecular level by ionizing radiation, oxidative protein damage to pathogens and food quality should be analyzed in various combinations of X-ray processing and post-storage conditions.

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