DEVELOPMENT OF FISH GELATIN FILMS WITH ENHANCED PHYSICAL, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES

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Nageshwar Tammineni

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Major Professor: Gülhan Ünlü, Ph.D.

AUTHORIZATION TO SUBMIT DISSERTATION

This dissertation of Nageshwar Tammineni, submitted for the degree of Doctor of Philosophy with a major in Food Science and titled "Development of fish gelatin films with enhanced physical, antioxidant and antimicrobial properties" has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the college of Graduate Studies for approval.

Major Professor: _____Date:_____
Gülhan Ünlü, Ph.D.

Committee Members: _____Date:_____
Barbara Rasco, Ph.D.

_____Date:_____
Gregory Möller, Ph.D.

_____Date:_____
Joseph R. Powers, Ph.D.

Department Administrator: _____Date:_____
Barbara Rasco, Ph.D.

Discipline's College Dean: _____Date:_____
Larry D. Makus, Ph.D.

Final Approval and Acceptance

Dean of the College of Graduate Studies: _____Date:_____
Jie Chen, Ph.D.

Abstract

Fish processing byproducts account for a large percent of the weight of total catch. These byproducts can be used for a variety of purposes including gelatin extraction. Gelatin has numerous food and pharmaceutical applications. However, bovine gelatin has traditionally been used for most applications due to its superior properties. Fish gelatin is being reexamined as gelatin source for food applications due to concerns (prion contamination and cultural restrictions) associated with bovine gelatin. This research is focused on improving fish gelatin properties and demonstrating additional uses for fish gelatins by developing antioxidant and antimicrobial films.

First objective involved the development of trout (*Oncorhynchus mykiss*) skin gelatin films with epigallocatechin gallate (EGCG) (50 and 250 ppm) and green tea powder (1% and 20% wt/wt of gelatin). Films were tested for physical properties, *in vitro* antioxidant activity and effect on stabilizing cod liver oil held at 40^{o}C for 20 days. Antioxidant activity was retained in films containing green tea powder, but was reduced ($P < 0.05$) in EGCG films. Physical properties varied with antioxidant incorporation. There was significant increase in the oxidation parameters of uncovered oil compared to treatments.

In the second objective, fish gelatin (6.75% wt/wt) films were prepared at a gelatin:tannic acid ratio (wt/wt) of 1:0.05, 1:0.10 and 1:0.15. Films were evaluated for antioxidant, physical, water permeability and water solubility properties. Comparative data with bovine gelatin was collected. Treatments effect on oxidative stability of salmon fillets was examined at 4 and 10°C. Tensile strength and water solubility of bovine gelatin films varied significantly compared to fish gelatin films. Films with tannic acid possessed antioxidant

activity. Treatments lowered oxidation (TBARS values) in gelatin coated refrigerated salmon held for 12 days under refrigerated temperatures.

Trout skin gelatin films were developed with nisin (18 μg nisin/cm^2 film) in the third objective. Trout fillets were challenged with 2 log CFU *L. monocytogenes*/g before or after coating with nisin-containing films. Growth of *L. monocytogenes* was monitored for 30 days at 4 and 10°C. Films with nisin reduced *L. monocytogenes* counts below the detection limit (1 log CFU/g) at 4°C. The effectiveness of treatments varied with storage time and temperature.

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Table of contents

List of figures

Chapter 2

Chapter 3

Chapter 4

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 1

Literature review

Justification

The food industry encompasses farming, processing, retail and consumption. Food safety and quality needs to be monitored at every step from farming to consumption. In the United States, regulatory agencies such as the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA) and several state authorities have regulatory guidelines in place to ensure food safety and quality. However, despite these regulations, about 9.4 million foodborne illnesses with approximately 1,300 deaths are still reported annually in the United States (Scallan et al., 2011). An estimated $23 billion is spent every year in the United States to deal with costs associated with foodborne illnesses (Mead et al., 1999). Intrinsic factors (e.g., pH, water activity, oxidation-reduction potential) and extrinsic factors (e.g., temperature, gas composition, and RH at which the food is held) associated with foods largely influence the microbial growth, chemical and/or enzymatic reactions in foods. These properties, in turn, dictate the safety and quality of foods. Several processing/preservation aids and technologies are in use to reduce microbial growth and/or biochemical reactions, which deteriorate quality in food systems. However, consumer demand for natural food ingredients and the need to reduce packaging waste is on the rise. Bioactive packaging can make use of natural bioactive ingredients and can reduce packaging waste. This research has focused on developing bioactive packaging materials from fish processing byproducts to extend microbiological and oxidative stability of seafood products.

The Unites States was the second highest consumer of seafood in the world in 2011. Americans spent approximately $85 billion on seafood products in 2011. About 84% of the seafood consumed in the United States is imported (NOAA, 2007). Consumption of seafood is on the rise as there are several health benefits attributed to seafood. Seafood is rich in polyunsaturated fats and low in saturated fats (IOM, 2006). However, seafood also accounts for about 10-19% of the foodborne illnesses every year in the United States. About half of these illnesses are caused by bacteria (Butt et al., 2004). With increasing demand for seafood, quality and safety of seafood need to be monitored closely.

Fish processing byproducts can account for up to 75% of the weight of the total catch (Gomez-Guillen et al., 2002). About 1.7 million metric tons of processing byproducts per year are generated in Alaska alone. Catching unrelated species and returning them back into the sea, dumping waste generated onboard (when processing fish on a fishing vessel) into the sea and wastage on land (due to elaborate processing) are some of the factors which increase under utilization of sea resources. Fish processing byproducts, used for a variety of purposes, are sourced from various parts of the fish. Extraction of collagen, pigments and mucous from the skin, chitosan from heads, enzymes, oil and silage from the viscera, lectins from gonads, fish meal and oil from other remains are some of the byproducts that are extracted. Recently, several bioactive compounds and antifreeze proteins are also being extracted from fish tissues (Blanco et al., 2007).

Fish processing companies have regulatory issues and costs associated with disposing their waste in sea or on land. Recently, strict regulations have been imposed on dumping fish processing waste into sea. In the recent past, onshore processors could dispose of waste in

the sea according to the United States Environmental Protection Agency (EPA) regulations and thus have an advantage over offshore processors, but this is no longer the case unless certain precautions are taken regarding particle size, composition and volume of the waste. Often, offshore processors send processing waste to composting factories free of charge (grounded waste) or at a cost of $0.01/lb (ungrounded waste) (Mathies et al., 2002) but, as regions become more urbanized and pressure placed upon land fill operations by residential communities increase, these actions are reduced. Current practices that make use of fish processing byproducts involve converting fish processing waste into fish meal, fish oils, direct feeding of certain types of wastes to other animals, production of phosphate fertilizer, composting and silage. Other waste reduction strategies include removal of more flesh from each fish (requiring change in equipment), avoidance in catching of unwanted species or undersized fish (by improving net designs), production of other byproducts such as surimi and flaked fish, and transportation of processing waste to other locations (if byproduct generation is not locally available) (PPRC, 1993).

All these aforementioned waste management practices may be effective, but they also have costs associated with them. A cheaper alternative may be to extract gelatin from fish processing waste. Gelatin has numerous applications in food and pharmaceutical industries. However, animal sources rather than fish are preferred for gelatin extraction due to their superior functional and rheological properties (Yang et al., 2007). Worldwide production of gelatin is nearly 326,000 tons. Out of this 46% is from pigskin, 29.4% is from bovine hides, 23% is from bovine bones and the remaining 1.5% is from other sources (Hong et al., 2010). Terrestrial animal gelatins often do not comply with Halal and Kosher requirements, and there has been concern that they may be contaminated with prions (Gimenez et al., 2009).

Therefore, alternative sources of gelatin, such as fish, are being sought. Fish gelatin is shown to stabilize emulsions (reasonably stable to creaming and droplet aggregation even with changes in temperature and pH), and allows better release of aroma and lower melting point compared to other gelatins, facilitating applications in food product development (Blanco et al., 2007). Developing edible packaging using fish gelatins and enhancing their physical and antioxidant properties by the use of plant phenolics was investigated in this study.

General aspects of edible film packaging

Edible films were in use in the ancient world in the form of waxes to preserve the quality of fruits and vegetables. Food applications of edible films became prominent in 1960s. Although the terms films and coating are used interchangeably, films refer to preformed stand-alone materials while coatings refer to materials that are applied and formed on the surface itself. Annual revenue of edible film applications exceeds about $100 million (Pavlath and Orts, 2009). Commercial uses of edible films are diverse in the food industry. Some of the applications described in Pavlath and Orts (2009) include the use of sucrose esters, calcium ascorbate, calcium pectinate and calcium acetate to avoid water loss and preserve freshness of several fruits and vegetables; corn protein to extend quality of different nuts; and candies coated with shellac to increase shelf life. When frying battered meat products, oil uptake can be reduced by coating the meat first with cellulose derivatives. Use of sucrose esters can prevent hydration of snack products. Edible films can be used to prevent moisture migration from toppings to crust in pies and pizza to ensure proper texture of the crust. Coating meat products with polysaccharides and gelatin to maintain desired

moisture level was patented as early as in 1876 (Pavlath and Orts, 2009). With such diverse applications edible films have a great role to play in our foods.

Different food ingredients, antioxidants, antimicrobials, vitamins and nutritive substances can be incorporated into packaging. Packaging materials that interact with foods are referred to as active packaging. The primary purpose of packaging is to offer mechanical support and hold foods, but importance is now being placed on how that packaging interacts with food materials (Cha et al., 2004). Packaging plays an important role in determining the shelf life of foods as it controls the migration of gases and moisture (Ko et al., 2001). For minimally processed foods, water loss and respiration are the major factors responsible for limited shelf life. These can be controlled by the use of packaging materials that interact with food systems. Low temperature storage is the most commonly used method to preserve food quality. However, in certain cases, chill damage occurs in some products and not all the undesirable chemical reactions are hindered at low temperatures. Several factors such as food storage conditions, type of bioactive agents being used, and the matrix of packaging material determines the effectiveness of bioactive packaging (Quintavalla et al., 2002). Several synthetic and natural matrices are being investigated for use in bioactive packaging.

Edible films can be prepared from polysaccharide, protein and lipid-based materials. In general, lipids reduce water transmission, proteins impart mechanical strength, and polysaccharides control gas transmission (Pavlath and Orts, 2009). Some of the polysaccharide-based materials include starch, alginates, carrageenan, chitosan, agar, cellulose and cellulose derivatives. Protein-based materials include corn zein, soy proteins, milk proteins, peanut proteins, wheat gluten and collagen. Several waxes and glycerides

comprise lipid based materials. Polysaccharide-based films are good barriers to oxygen, lipid and flavor, but are poor barriers to moisture. Lipid-based films have good moisture barrier properties but are poor barriers to gas, lipid and flavor. Multilayer and emulsion films are developed using both polysaccharide and lipid materials to produce films that are good barriers to the properties listed above. Multilayer film formation can be complex; layers might separate over time and is not cost effective. Emulsion-based films have more practical applications and are feasible.

Edible films can be formed in several ways including spraying or brushing the film forming solution on food products, dipping food products in film forming solutions and forming stand alone films. Stand-alone films can be formed by evaporating film-forming solutions or by thermo-formation, the latter being used rarely. Films prepared by different methods can have different properties (Pavlath and Orts, 2009). The accelerated drying of film forming solutions can lead to untimely immobilization of structure. Depending on how the films are formed, crystalline or amorphous films can be obtained. The process of making water insoluble protein-lipid emulsion-based films was developed by Krochta et al. (1996). Films developed from an emulsion of water soluble proteins and lipids are water insoluble and are barriers to gas, lipids, flavors and moisture. Mechanical properties and film flexibility can be improved by incorporating plasticizers into film structure. Glycerol and several other polyols are commonly used plasticizers. Emulsion films are prepared by heating an emulsion of protein and lipid and casting the solution into films. Heating denatures proteins, resulting in the formation of new intermolecular and intramolecular disulfide cross linkages. Thiol oxidation (sulfhydryl group converted to disulfide) and thiol disulfide exchange (exchange of thiolate and disulfide) reactions occur. In the process, several bonds are formed, broken,

and/or rearranged between various amino acids of the proteins. Later the emulsion is homogenized and dried to form films. Incorporation of lipid into films changes the film properties considerably. Change in film properties depends on several factors such as rate of homogenization of emulsion, extent of heat denaturation, lipid chain length, melting point and concentration of lipid incorporated. Since lipids are hydrophobic, they tend to decrease the free volume in the film structure, reducing water solubility.

Several bioactive components including antimicrobials and antioxidants can be incorporated into edible films. Interactions among bioactive components and their interactions with the film matrix determine the effectiveness of the films. When two or more antimicrobials are incorporated into edible films, there can be an enhanced or reduced antimicrobial effect depending on the interactions. For instance, Gram-positive bacteria are often more susceptible to the bacteriocin nisin, but the combination of nisin with organic acids have antimicrobial activity against both Gram-positive and Gram-negative bacteria (Eswaranandam et al., 2004). Synergistic effects are highly dependent on the components of antimicrobial agents, microbial contaminants and food systems used.

Use of edible films with bioactive components can improve food safety and quality. Bioactive components can be made available on the surface of foods for prolonged time periods by using edible films. Edible films slow the diffusion of these components onto food surfaces. Spraying antimicrobials and antioxidants onto the surface of a food is usually not as effective due to the rapid diffusion of these components into the bulk of the food and limited contact time with food surface (Yudi et al., 2004). Most edible films change the texture and appearance of food. Consumer acceptance of these changes is important.

Economic factors and the film's barrier and strength properties play an important role in the commercial feasibility of edible film technology.

Gelatin

Gelatin is obtained by partial hydrolysis of collagen found in the bones and skins of animals (Gomez et al., 2002). It is the denatured form of collagen and has numerous applications. The structure of collagen consists of three polypeptide chains, which form a three dimensional helix, stabilized by hydrogen bonding (Djabourov, 1988). The amino acid composition of the polypeptide chain of collagen contains glycine as every third amino acid (33% of collagen). Proline and hydroxyproline account for 22% of the composition. Glycine and alanine, amino acids with simple side groups, orient themselves in the center of the helix while proline and hydroxyproline orient away from the chain (Djabourov, 1988). Glycine and alanine help the chains to stay close while proline and hydroxyproline impart rigidity to the chain. The triple helix rods are arranged in parallel and are stabilized by covalent bonds formed at the end of the rods.

To form gelatin from collagen, hydrogen and covalent bonds (which stabilize the structure) need to be broken. This can be done by acid and/or alkali pretreatments followed by extraction in water at 45-50^{o}C. Pretreatment with acid or alkali can remove non-collagenous protein material, degrade covalent bonds, and inactivate native proteases (Zhou et al., 2005). However, if the treatments are not severe, the triple helical structure of collagen is usually retained. To convert collagen into an extractable form (i.e., solubilization of collagen rod), a sufficient number of cross linkages must be broken. In this process, peptide bonds between the amino acids are also broken; however, excessive bond breakage is not desirable. Severe

pretreatment will produce hydrolyzed products with molecular weights less than that of native α chains. This results in the production of gelatins with lower gel strength (Hong et al., 2010). Gelatin with more α chains has more gel strength. Pretreatment with acid and alkali results in sufficient breakage of covalent cross linkages without excessive peptide bond breakage (Zohu et al., 2005). Collagen rod is solubilized without alterations to the triple helix, resulting in gelatin with desirable properties. After the pretreatment, gelatin is extracted in hot water. During this extraction process, hydrogen bonds and covalent bonds are cleaved and the triple helical structure of collagen destabilizes. Transition of helix to coil occurs and gelatin is obtained (Hong et al., 2010).

In general, gelatin exists as random coil in solutions at temperatures of 35^{o}C and above (Djabourov, 1988). If the temperature of this solution is reduced, the conformation of gelatin changes from coil to helix. A three dimensional structure of gelatin network is formed if the concentration of the solution exceeds 0.5% (wt/wt) (Djabourov, 1988). The stable form of gelatin solution at temperatures below 30^{o}C is said to be the re-natured helix form. Formation of helix is facilitated by nucleation of proline-rich regions in the chain. Triple helical regions formed due to proline and hydroxyproline help retain water and facilitate gel formation. These thermo-reversible gels have a melting temperature close to body temperatures (Hong et al., 2010).

Quality of gelatin is affected by several parameters such as source of gelatin and severity of extraction process (Fernandez et al., 2003). Functionality of gelatin, as described by several parameters such as gel strength, melting temperature and viscosity, depends on amino acid composition of gelatin (Hong et al., 2010). The amino acid composition in turn depends on

the source of gelatin. For instance, the imino acid content (proline and hydroxyproline) is higher in warm-blooded animals and fish living in warm waters compared to fish living in cold waters. Gelatin obtained from fish residing in warm and cold waters are different (Yang et al., 2007). Amount of imino acids in bovine and porcine gelatins are 23% and 22%, respectively. Presence of more imino acids results in gelatins with better rheological properties comparatively, as they are involved in the formation of helical structures that hold water. Yield of gelatin varies depending on the species of fish. Composition of skins, amount of collagen and soluble components also vary depending on the age of fish. All these factors contribute to different yields of gelatin from different sources. Gelatin has numerous applications in food and pharmaceutical industries due to its gelling, water holding capacity, melting temperature and film forming ability. Development of fish gelatin-based bioactive edible films is one such application and is of interest in this research.

Polyphenol-protein interaction

Tannins are oligomeric compounds with several structural units containing free phenolic groups. Molecular weights of tannins range from 500 to greater than 20,000. All tannins are formed via the phenylpropionanoid pathway (Cannas 2014). They bind with proteins and form soluble and insoluble protein complexes. Tannins are generally soluble in water with the exception of a few compounds. Tannins can complex with metals and form metal tannates, which are insoluble and can precipitate. Tannins are broadly classified as condensed and hydrolysable tannins. Hydrolysable tannins are compounds with a central polyol core where several hydroxyl groups in the polyol are esterified with phenolic groups. Hydrolysable tannins can be hydrolyzed into carbohydrates and phenolic acids. Condensed

tannins are also called proanthocyanidins. These are polymers of flavonoid units and are not susceptible to hydrolysis. They have complex structures with about 2 to 50 flavanoid units and varying degrees of substitution.

Tannin-protein interaction is widely studied and has numerous applications in food industry. Four kinds of interactions can be involved in protein polyphenol systems: hydrogen bonding, hydrophobic interactions, ionic interactions, and covalent interactions. Of these interactions, covalent bonds are the strongest and thermally stable (Zhang et al., 2010a). Hydrogen bonding and hydrophobic interactions are more common in protein polyphenol interaction compared to covalent bonding (Cannas 2014). Covalent bonding occurs only in oxidized conditions. Hydrophobic interactions are stronger at high temperatures and in solutions of high tannin and protein concentration. Phenolic group in tannin is an excellent hydrogen donor which bonds with the carboxylic group of the protein (Cannas 2014). Exact mechanism of protein polyphenol interactions are complex and are not clearly known. It is postulated that the polyphenols undergo an initial oxidation (consuming several reactive sites) to form quinone intermediates (Zhang et al., 2010a). However, enough reactive sites on the polyphenols would be left to react with proteins. Quinone intermediates can react with nucleophiles from reactive amino acid groups in the proteins to crosslink.

Bioavailability of polyphenols largely depends on their interactions with proteins (Hagerman 2002). Polyphenols are shown to interact irreversibly with dietary proteins and enzymes in the digestive tract and are transported *in vivo*. Specifically, salivary proteins are proline rich and can complex with polyphenols. Large molecular weight and structural mobility of tannins promotes more binding. Tannin protein binding can affect the

digestibility of some proteins as bound proteins are resistant to enzymatic activity. If the digestive enzymes do not hydrolyze the bound tannin, then the proteins may not be nutritionally available. Salivary proteins are proline rich. This is advantageous as the animal can use this to bind tannins, reducing the binding of vital proteins in the diet to tannins and making them available nutritionally. There are several adverse affects reported due to excess tannin in ruminant diets. However, moderate tannin in the diet has been shown to enhance nitrogen retention and protect dietary proteins (as they form complex with tannins) from microbial enzyme activity in the rumen. These complexes can later be digested in the intestine.

Zhang et al. (2010a) crosslinked gelatin with tannic and caffeic acids to study the reaction mechanisms. Labeled caffeic acid and gelatin were successfully crosslinked and the behavior of the crosslinkers in the systems was studied using NMR. All these reactions were done in alkaline medium and the pH was maintained around 9-10 by addition of NaOH. A model system consisting of amino acid lysine and dimethyl phenol was used to study the product structures by NMR. It is not feasible to study the reaction mechanism when crosslinked networks are formed. NMR studies revealed that there is C-N covalent bond formation when lysine is crosslinked with dimethyl phenol. When tannic acid and caffeic acid was reacted with gelatin at different pH values, the gel time decreased significantly with increasing pH. Gel formation time also varied with concentration and was not influenced by the type of crosslinker. Possible reason might be the formation of caffeic acid oligomers, which could react similarly to tannic acid. These findings show that crosslinking can be controlled by varying the concentration, pH and type of crosslinkers. The hydrogels formed did not melt after heating to 100^{o}C. Gelatin gels formed when cooling a gelatin

solution is different than the hydrogels described above. Gels formed during cooling tend to melt at much lower temperatures. The difference in the molecular interactions of the gels was shown by measuring the hydrogen atom relaxation time, which is dependent on the molecular mobility in the system. If there are covalent linkages in the system, relaxation time would decrease due to restricted molecular motion. This is observed in case of gelatin-tannic acid and gelatin-caffeic acid systems. For the reactions systems without oxidation, relaxation times tend to be higher showing that there is no covalent crosslinking involved.

Nature of protein primarily determines the mechanism of interaction of protein with tannins. Tannin structure and flexibility influence the stoichiometry and energetics of the reaction, but are not the primary determining factor. Tannins bind to conformationally different proteins in different ways. Tannins have poor affinity to small globular proteins and bind selectively to proline rich proteins. Isothermal microcalorimetry revealed interaction mechanisms of tannins with different kinds of proteins (Frazier et al., 2003). Tannins interact with globular proteins in a nonspecific manner. With random coil protein like gelatin, polyphenols interact in a two-step mechanism. First stage involves cooperative binding of tannins with proteins followed by saturation of binding sites. Second stage of interaction involves nonspecific binding leading to aggregation.

Protein molecules are held by tannin compounds (non-covalent interactions), which act as bridges (form hazes). Proteins with proline form haze in proportion to the molar concentration of proline in the protein. Proteins without proline do not produce haze. Gelatin and tannic acid are known haze-active components. Different concentrations of tannic acid and gelatin are combined and haze formation was studied (Siebert et al., 1996). It

was observed that for a fixed concentration of gelatin or tannic acid and varying concentration of other, haze formation increased to a certain extent and decreased with further increase in concentration. This phenomenon was explained by the assumption that proteins and tannins have fixed number of binding sites. When there is more tannin than protein, all the binding sites on proteins would be occupied and bridges between proteins cannot be formed. If the concentration of protein is greater than tannin, it is likely for a protein to find a free binding site on tannin and form bridges with other proteins.

Proline is crucial for proteins to be able to form hazes. It was shown that proteins without proline do not form hazes. However, other amino acids can influence the haze formation ability by hindering or exposing the polyphenol binding site on a protein. Hydroxyproline does not produce haze but can aid in haze development by creation of large polyphenol binding sites. Different polyphenols bind to different extents to the same proteins. Structural orientation, accessibility of binding sites and the density of hydroxyl groups on the tannins are likely the reasons. Hydroxyproline is expected to be more active in hydrogen bonding compared to proline. It was reported that there is no haze formation due to hydroxyproline and increased haze formation upon heating proteins (leading to hydrophobic region exposure) (Siebert et al., 1996). It was concluded that hydrophobic interactions play an important role in protein polyphenol interactions than hydrogen bonding. When protein polyphenol system is heated, more hydrophobic regions (polyphenol binding sites) of the protein may be exposed. Tannin and protein interaction is initially due to hydrophobic interactions while they are further stabilized by hydrogen bonding (Frazier et al., 2003).

Cross-linking of gelatin

Substances that have at least two reactive groups, which are reactive towards numerous functional groups like amines, carbonyls, sulfhydryls and carboxylic acids, are called crosslinkers (GBioSciences, 2013; Pierce Biotechnology, 2006). Proteins can be easily crosslinked as these groups are common in proteins. The process of chemically joining two or more molecules is termed as cross-linking. Cross-linking has applications in determining protein structure and several other applications in immunology. Crosslinkers are classified as homo-bifunctional and hetero-bifunctional crosslinkers depending on if they have two similar or different groups, respectively. Different crosslinkers with varying arm lengths, reactive groups, degree of conjugation and water solubility are available and are chosen depending on the application of interest.

Use of fish gelatin coatings and films to extend shelf life of fish products are limited as most studies deal with bovine or porcine gelatin. Inferior fish gelatin properties and high water sensitivity might be the contributing factors. However, attempts were made to enhance the water solubility and strength properties of fish gelatins through cross-linking. From the scope of literature review, no attempts were made to show the effect of cross-linking on the antioxidant efficacy of these coatings. The water solubility and water uptake of gelatin coatings can disrupt the integrity of coatings, which affects the permeability properties. Antioxidant activity is usually a combination of film permeability and the presence of antioxidants. To our knowledge, no studies correlate different degree of cross-linking of gelatins and the antioxidant activity of the resulting films and thus were subject to our study.

Cross-linking of gelatins using a variety of cross-linking agents has been reported. Most of these studies have been intended to reduce the solubility of gelatins and enhance the strength properties. Piotrowska et al. (2007) cross-linked fish gelatin with transglutaminase (tgase). Enzymatic cross-linking with tgase resulted in formation of cross-linked proteins due to a reaction between glutamine (carboxy amide group) and lysine (amino group) residues. Treatment with a reducing agent, such as cysteine, further increased the formation of disulfide linkages. Enzymatic treatment of film forming solution reduced the solubility of the films from 100% to 32% and 34% at pH 3 and 6, respectively, during exposure to water at 25^{o}C for 24 hours. When cysteine was included in the treatments at 2.5 and 5mM concentrations, the solubility further reduced to 22%, 23% and 14%, 18% at pH 3 and 6, respectively. The water vapor permeability of the films was not changed due to these treatments. Similar results were obtained using EDC (1-ethyl-3-(3-dimethylaminopropyl)) and DTT (Dithiothreitol), but they cannot be used in foods due to their toxicity. Kolodziejska et al. (2006, 2007) reported similar results with cross-linked fish gelatin-chitosan composite films.

Bovine gelatin was cross-linked with tannic and caffeic acid under alkaline conditions (Zhang et al., 2010a). The NMR studies revealed that the reactive sites on phenolic compounds and the amino groups in the gelatin form a C-N cross-linking covalent bond. The DMA temperature ramps showed that cross-linked films did not show glass transition or melting phenomenon. Solubility of the films was not studied. In another study, bovine gelatin films were prepared by cross-linking with tannic acid (Zhang et al., 2010b). Film solubility and equilibrium moisture uptake of films was measured. Solubility of the films was reduced (by about 80%). Equilibrium moisture uptake of cross-linked films (700%) was

lower than that of the control films (1000%). However, cross-linking did not limit the water uptake of the films. This might be due to lower cross-linking density of the cross-linker. With increasing tannic acid concentration (up to 6% wt/wt), both the tensile strength and the % elongation of films increased. Normally with cross-linked films, a reduction in % elongation would be observed with increasing tensile strength. This suggests that tannic acid acts both as a cross-linker and as a plasticizer.

White grape juice and coffee were used as sources of crosslinkers for food grade gelatins (Strauss et al., 2004). For a given polyphenol/NH_2 mole ratio, gels produced by cross-linking with coffee and grape juice had lower gel strength compared to caffeic acid gels. This might be due to the lower concentration of phenolics in grape juice and coffee in comparison to pure caffeic acid. Lim et al. (1999) cross-linked bovine gelatin with tgase and studied different properties under different temperature and RH conditions. They found that the oxygen permeability of films was low when films were dry, but increased with increasing RH. This can be a limitation when using gelatin coatings to delay oxidation on fish fillets. The antioxidant property of the gelatin films depends to a greater extent on the oxygen barrier property of the films. Coatings should be modified according to the end use conditions.

Oxygenated (30 min, 60^{o}C) seaweed extract was added to the gelatin (extracted from big eye snapper skins) film forming solution, stirred for an hour at room temperature and casted into films (Rattaya et al., 2009). There was reduction in solubility of the films due to treatment with seaweed extract; however, the magnitude of the change was not large. By using different solvents to determine film solubility, these researchers were able to study the

interactions in the film matrix. Cross-linking was governed in part by hydrogen bonding and hydrophobic interactions. Covalent cross-linking was observed between phenolic compounds and the amino and sulfhydryl side chains. Disulfide bonds were not present in the matrix as the cysteine content in gelatin was very low. However, addition of cysteine could lead to formation of disulfide linkages and strengthen the matrix further (Piotrowska et al., 2007).

Cross-linking food grade gelatin-polyacrylamide composite film with phenolic extract (from Acacia bark) resulted in a decrease in the degree of swelling of films in phosphate buffer saline solution (Haroun et al., 2010). Films with 2% and 10% (wt/wt) phenolic extract had a degree of swelling of 4250% and 2250%, respectively. In a study by Bigi et al. (2002), films were prepared by drying 10% porcine gelatin solution. Later, the films were placed in 10 ml buffer solution with genipin (0.07 to 0.2%) for 24 h at room temperature. Films were repeatedly washed with water. Extent of swelling of films and the degree of cross-linking were measured. The concentration of genipin (0.15%) used was able to cross link 60% of amino groups. Genipin was not able to cross-link all the amino groups even at higher concentration (as opposed to glutaraldehyde which can cross-link all the amino groups). The maximum degree of cross-linking observed was about 85% at the highest concentration of genipin tried. Cross-linking with 1% genipin resulted in a reduction of degree of swelling from 700% to 150%. So, the degree of cross-linking of a crosslinker should be considered while producing a cross-linked system.

Lipid oxidation in seafood products

Fish oils and fish muscle contain polyunsaturated fatty acids (He et al., 1997). Fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are attributed to several health benefits (Barrett et al., 2009). However, they are susceptible to oxidation and need to be stabilized. Lipid oxidation is a deteriorative reaction in many types of foods, but is prominent in marine foods because of high concentration of polyunsaturated fatty acids. Due to neutral pH, high water activity, presence of autolytic enzymes and relatively high concentration of free amino acids, fish can be spoiled easily (Duna et al., 2010). Fish oils are usually stabilized by encapsulation or addition of antioxidants such as BHA (butylatedhydroxyanisole), BHT (butylatedhydroxytoluene) and tocopherols (Wanasundara et al., 1998). Lipid oxidation can lead to off-odor production, loss of muscle color due to oxidation of oxymyoglobin and drip accumulation (liquid oozing out of stored muscle), leading to deterioration in appearance (Antionewski et al., 2007). Off-odor is a result of production of rancid volatiles due to oxidation. For instance, in modified atmosphere packaging (MAP) of muscle foods, the microbial counts were well within the limit for 3 weeks. However, deterioration of muscle color occurred after 12 days. This limits the shelf life of muscle foods (Antionewski et al., 2007). If the oxidative reactions in meat and seafood products can be limited, the shelf life of foods can be extended.

Lipid oxidation process is described in detail by McClements and Decker (2008) and Ladikos and Lougovois (1990). The substrates for oxidation in seafood are lipids and molecular oxygen. It is initiated by hydrogen abstraction from the polyunsaturated fatty acids and formation of alkyl radicals. The exact mechanism of hydrogen abstraction is not

known, but metal ions, UV light and singlet oxygen are known to be involved (McClements and Decker, 2008). These alkyl radicals further react with triplet oxygen and form high energy peroxyl radicals. Peroxyl radicals are involved in propagating the reaction by reacting with other polyunsaturated fatty acids molecules and producing more free radicals. Eventually, hydroperoxides are formed, which in turn can produce several other radicals, resulting in exponential increase in oxidation reaction. Hydroperoxides lead to formation of alkoxyl radicals, which form rancid compounds via the β-scission reaction.

Important prooxidant in foods is the singlet oxygen. Singlet oxygen can attack carbons on either side of double bonds in polyunsaturated fatty acids. Hence several kinds of hydroperoxides are formed, resulting in a variety of decomposition compounds. Singlet oxygen can be produced in foods by photosensitization (energy absorption by photosensitizers present in foods), chemical or enzymatic means (McClements and Decker, 2008). Other prooxidant is lipoxygenase, which undergoes a series of oxidation and reduction reactions leading to lipid oxidation. Transition metals also act as prooxidants. Especially, iron (associated with heme proteins and other metabolites) is an activator of molecular oxygen, part of prooxidant enzyme active sites and is also involved in decomposition of hydroperoxides resulting in formation of several rancid end products.

Lipid oxidation can be controlled by using antioxidants that scavenge free radicals. Antioxidants react with high energy free radicals and form less reactive radicals, which cannot readily catalyze oxidation reaction. This leads to termination of oxidation reaction. Oxidation can also be reduced by controlling prooxidants. Transition metals can be removed by use of metal chelators, metal binding proteins and other sequestering agents.

Lipoxygenase can be heat inactivated and singlet oxygen can be quenched by physical or chemical means. However, consumer expectation results in limitations as to what treatments can be used on foods.

Lipid oxidation can be monitored by measuring the primary or secondary oxidation products. Low levels of oxidation can be detected by measuring formation of hydroperoxides and change in oxygen uptake. Other changes, including the formation of hydrocarbons and carbonyls, can be monitored for oxidation. However, the most popular method for monitoring lipid oxidation in muscle foods is the Thio-Barbutiric Acid Reactive Substances (TBARS) method, which measures the extent of malonaldehyde formed.

Use of natural antioxidants to preserve seafood quality

Use of synthetic antioxidants such as BHT and BHA is common in foods. Several natural antioxidant systems were investigated to meet customer demands and reduce the use of synthetic additives in foods (He et al., 1997). Rate of oxidation in trout fillets dipped in rosemary extracts (Ozogul et al., 2010), bonito fillets dipped in grape seed and green tea extracts, salmon fillets dipped in isoeugenol solution (Tuckey et al., 2009), and lingcod fillets dipped in solution of chitosan and vitamin E (Duna et al., 2010) were investigated. All of these antioxidant systems reduced oxidation to a certain extent.

Recently, bioactive components such as plant phenolics gained popularity due to their health benefits. Green tea is a plant phenolic that contains catechins. Studies on animal models showed that green tea also possesses anti-mutagenic, anti-diabetic, anti-inflammatory, antibacterial, antiviral and anticancer properties. Other health benefits such as promoting

oral health, anti-fibrotic and neuroprotective properties are attributed to green tea (Cabrera et al., 2006). Tea catechins can delay oxidation by inhibiting the formation of free radicals and hindering free radical propagation (Chen et al., 1996). Ethanol extracts of green tea provided greater stabilization than that of BHT for oxidation of canola oil (Chen et al., 1996) and marine oils (Wanasundara et al., 1998). Epigallocatechingallate (EGCG) is an important catechin present (about 59% of all catechins) in green tea (Cabrera et al., 2006). He et al. (1997) showed that EGCG was a more effective antioxidant compared to other tea catechins in cooked mackerel muscle during storage. Several research groups investigated the direct addition of tea polyphenols to reduce oxidation in fish products. Addition of green tea extracts to minced mackerel muscle (He et al., 1997; Tang et al., 2001) and minced carp (Dembele et al., 2010) was studied. Oxidation was studied in blue sprat dipped in polyphenol solution (Seto et al., 2005) and frozen mackerel fillets treated with instant green tea powder (Alghazeer et al., 2008). These treatments were effective in retarding oxidation to varying degrees.

Edible films to extend oxidative stability of meat and seafood

While the incorporation of antioxidants into foods is challenging, use of edible matrices such as gelatin films as a carrier for antioxidants is actively being investigated. Edible films and coatings can slow the oxidative reactions in muscle foods. Application of edible films is of particular interest in fresh or unprocessed foods. Edible films can act as barriers to oxygen and water and hence limit lipid oxidation and purge. Gelatin can be applied to meat products in several forms. Meat products can be wrapped in a preformed gelatin film, or

dipped in or sprayed with gelatin film forming solution. However, dipping the meat in gelatin solutions would be more practical on an industrial scale.

Several studies have investigated the use of gelatin to extend shelf life of fish and meat products by delaying oxidation. Fish patties were dipped in chitosan-megrim skin gelatin solution and stored at 2^{o}C for 14 days (Caballero et al., 2005) and analyzed for Total Volatile Basic Nitrogen (TVBN) and TBARS. Treatments were able to reduce TVBN, but the TBARS values were inconclusive due to lower values encountered in the samples. Ou et al. (2001) dipped tilapia fillets in gelatin-benzoic acid solution and stored for 7 days at 4^{o}C. Samples were analyzed for TVBN. After 7 days of storage, significant difference was observed in the TVBN values for treated and untreated samples. Herring et al. (2010) studied the oxidative stability of gelatin-coated pork. Pork loin samples were treated with aqueous pork gelatin solutions, stored for 7 days at 4^{o}C and analyzed for oxidative stability. By the end of storage time, the TBARS values increased from 1 mg MDA/Kg sample to 10 and 4.2 mg MDA/Kg sample for untreated and gelatin treated samples, respectively. Coatings were also able to reduce the moisture loss significantly. Muscle food samples (beef tenderloins, pork loins, salmon fillets and chicken breasts) were spray dried with bovine gelatin solution, air-dried, packed with 20% CO_2 and 80% O_2, and stored at 4^{o}C for 2 weeks (Antoniewski et al., 2007). Samples were analyzed for color change and lipid oxidation. Lipid oxidation was not affected as a result of gelatin coating. Gelatin extracted from catfish was used to prepare edible films by incorporating chitosan nanoparticles containing tea polyphenols to study oxidation of fish oil (Bao et al., 2009). Treatments were able to reduce fish oil oxidation compared to controls. Tuna fish skin gelatin films with murta extracts (Gomez-Guillen et al., 2007) and chitosan edible films with green tea extracts (Siripatrawan

et al., 2010) were developed. These films possessed antioxidant activity, however, they were not tested on fish or meat products.

Edible films with various materials have been investigated for antioxidant activity. In a study by Kilincceker et al. (2009), trout fillets were coated with three different coatings (made of different kinds of flours and food gums), fried in oil and analyzed for TVBN and TBARS during 7-month storage at -18^{o}C. Spoilage was delayed; the TBARS and TVBN values were well within the acceptable limit. Sensory analysis revealed that coated samples had superior sensory profiles than the untreated ones. Lin et al. (2008) prepared antioxidant film forming solution with zein and BHT, BHA or propylene glycol. Fish balls were dipped in the above solutions and stored at 4^{o}C for 15 days and analyzed for PV and TBARS. Different treatments reduced oxidation to different degrees, the most effective treatment being the propylene glucol-zein sample. Cod fillets were dipped in chitosan solution containing fish oil and vitamin E (Duan et al., 2010). The fillets were stored at 2^{o}C and -20^{o}C for 3 weeks and 3 months, respectively. Samples were analyzed for TBARS and drip loss. The TBARS values for untreated samples and treated samples were around 5 and 3 mg MDA/Kg fish, respectively, for samples stored at 2^{o}C. Treatments did not show any effect on color of samples. Fish oil in the chitosan solution increased the omega-3 fatty acid content of the fillets.

Use of synthetic films with antioxidant systems to retard oxidation in fish and meat products was investigated. Abreu et al. (2010) prepared antioxidant low-density polyethylene films using antioxidant extracts from barley husks to extend shelf life of salmon fillets. These films were used to cover salmon fillets stored at -20^{o}C for 12 months. Film coatings were

able to reduce the PV and TBARS of samples; however, decomposition of peroxides was slower in samples coated with antioxidant film. Microcapsules containing horseradish extract were prepared using chitosan and coated onto ethylene vinyl acetate film using corn dextrin as adhesive (Jung et al., 2009). Pork and Spanish mackerel were wrapped with this film, stored at 5^{o}C for 9 days and analyzed for PV and TBARS. The PV and TBARS values were 11.5, 8.3 meq/Kg-fish and 8.3, 7 mg-MDA/Kg-fish for fish wrapped in control and microencapsulated film, respectively, by end of storage. Significant oxidation was not detected in pork.

Listeria monocytogenes and **seafood**

Several factors, including salt content, temperature of water, handling and storage, nutritional content and water activity of seafood, determine the type of microflora on seafood. Bacteria present in seafood can be categorized as indigenous, non-indigenous (fecal contaminants) and bacteria present due to cross contamination (Feldhusen, 2000). Several pathogens including *Listeria monocytogenes, Campylobacter, Escherichia coli* O157:H7, *Salmonella,* and *Clostridium botulinum* type E were isolated from seafood (Dillon et al., 1994, Embarek 1994; Ayulo et al., 1994*,* Kumar et al., 2003, 2004; Lyver et al., 1998).

L. monocytogenes is a Gram-positive bacterial pathogen and is known to cause listeriosis in humans, especially the susceptible populations. People with compromised immunity, pregnant women, neonates and elderly are at greater risk of listeriosis. Symptoms of listeriosis include still births and fetal abortion in pregnant women. Meningitis and septicemia occur in people with compromised immunity. Once this bacterium invades the gastrointestinal epithelium, it becomes blood borne and invades other parts of body like

brain. In healthy adults listeriosis causes nausea and influenza like conditions (FDA Bad Bug Book, 2013). Infectious dose in humans is not clearly known as studies with human subjects are not possible. However, it is believed that in susceptible populations fewer than 1000 cells can cause the disease (FDA, 2013).

In the mid 1980s, about 48 foodborne illness-related deaths were due to consumption of cheese contaminated with *L. monocytogenes* in Southern California (Gombas et al., 2003; Uhlich et al., 2006). This caused FDA to recognize *L. monocytogenes* as a severe foodborne pathogen and establish policies. Since then, *L. monocytogenes* caused several foodborne illnesses in the meat and seafood industry. Seafood was implicated with *L. monocytogenes* contamination in several instances (Bretta et al., 1998; Ericsson et al., 1997; Farber et al., 2000). Between 1987 and 1988, there were 112 Class I recalls for the presence of *L. monocytogenes* in domestic and imported seafood products (Elliot and Kvenberg, 2000).

In a study by Gudbjörnsdóttir et al. (2004), it was shown that the incidence of *L. monocytogenes* in seafood processing plants, raw seafood and RTE seafood was between 6 to 22%, 39% and 4.8%, respectively. Of the samples tested positive for *Listeria* species, 91% were reported to be *L. monocytogenes*. Improper cleaning procedures were implicated for the presence of *Listeria* in processing facilities. Due to its ubiquitous nature, *L. monocytogenes* can contaminate food products in several different ways including raw materials, environment and/or cross contamination from other surfaces. Presence of *L. monocytogenes* in seafood can vary from 0 to 50% and raw fish can be contaminated from 0 to 30%. In a study by Dimitrijevic et al. (2011), *L. monocytogenes* was isolated from 6% of processing equipment surface swabs at two different cold-smoked trout processing facilities

in Serbia. Same study reported the presence of *L. monocytogenes* in 13% fish samples collected during different stages of processing.

There are few options to control *L. monocytogenes* in seafood apart from designing effective sanitation programs and prevention of growth in Ready-To-Eat (RTE) products (Huss et al., 2000). Processing steps involved in cold smoking fish cannot eliminate *L. monocytogenes* (Aution et al, 1999). Routes and common areas of contamination are not well known in cold-smoked fish processing facilities due to the ubiquitous nature of *L. monocytogenes*. It was also reported that identical pulsotypes (indistinguishable bands in pulsed field gel electrophoresis) of *Listeria* were recovered from both the processed and raw products (Aution et al., 2005). Raw fish can be a major source of contamination of *L. monocytogenes* in processed fish (Aution et al., 2005). It is important that the raw fish not be contaminated with *L. monocytogenes*. It is clear that adherence to strict hygienic and process standard is crucial to minimize contamination.

L. monocytogenes is ubiquitous and has the ability to survive refrigeration temperatures, grow in a wide temperature range (2 to 45^{o}C) and can tolerate extremes of osmotic stress and pH (Gandhi et al., 2007), making it a difficult bacteria to control in foods. When *L. monocytogenes* is exposed to cold temperatures, change in lipid membrane composition occurs, facilitating membrane fluidity even at lower temperatures. This helps to maintain normal cell activity. Other mechanisms involve the production of cryoprotectants, cold shock proteins and proteases capable of degrading unusual peptides. Acid adaptation is the result of induction of proteins such as ATP synthases, use of glutamate decarboxylase system and pH homeostasis. Mechanisms which are responsible for acid adaptation can also

provide cross resistance against heat and osmotic shock. Cross resistance is alarming as several foods normally undergo acidic treatments.

Approaches to inhibit bacterial growth in meat and seafood

Different approaches have been studied to inhibit the growth of pathogens in seafood. Most common ones include the use of active packaging materials and direct additives to seafood either alone or in combination with other processing technologies. A novel packaging method that employs the use of MAP to prevent bacterial growth on salmon was studied by Schirmer et al. (2009). Salmon was packed with 100% CO_2 (in the headspace) in brine solution with citric acid, acetic acid and cinnamaldehyde in different combinations. The treatments were able to inhibit lactic acid bacteria, sulphur-reducing bacteria and the members of the family *Enterobacteriacae*. Mixed extracts of oregano and cranberry in combination with lactic acid were tested against *Vibrio parahaemolyticus* on cod fish fillets and shrimp. These extracts, either alone or in combination with lactic acid, showed antimicrobial activity against the bacterium tested (Lin et al., 2005). Juices of *Averrhoa bilimbi* and *Tamarindus indica* were used to control growth of *L. monocytogenes* and *S. typhimurium* on raw shrimp for 7 days (Norhana et al., 2009). The antimicrobial activity of these extracts is primarily due to the organic acids present in them. Treatments were able to reduce growth of *S. typhimurium* to some extent while the growth of *L. monocytogenes* increased slightly due to its psychrotrophic nature. Monolaurin and lactic acid solutions were also tested to inhibit *L. monocytogenes* on catfish fillets. Monolaurin showed no antimicrobial activity against *L. monocytogenes* while lactic acid did (Verhaegh et al., 1996). Adding different organic acids can reduce elasticity (Nykanen et al., 2000) and

sensory characteristics of seafood. Therefore in-depth sensory evaluations and consumer acceptance surveys are needed to validate this technology.

Post-process contamination is a major safety concern in processing RTE meat and seafood products. Coating meat and seafood products with antimicrobial edible films can help reduce the risk of cross contamination. Antimicrobial edible films can be used in combination with other preservation methods (hurdle technology) to control microbial growth. Severity of the processing steps can be reduced when multiple hurdles are present. Foods can thus be treated with milder processes while preserving desirable sensory and nutritional characteristics (McCormick et al. 2005). Concentrations of active agents required to attain the desired reduction in microbial growth are always higher in food systems than in microbiological growth media. Minimum Inhibitory Concentrations (MIC) are generally higher when edible films are used when compared to the MICs determined in microbiological broth media due to diffusion limitations (Sivarooban et al., 2006). Antimicrobial activity of the bioactive agent can sometimes be determined by the nature of the film matrix. A bacteriocin like inhibitory substance (BLIS) from *Pediococcus parvulus* was incorporated into corn zein and whey protein isolate films (Baciliza et al., 2005). Higher antimicrobial activity was reported against *L. innocua* in case of corn zein films than whey protein films. Weak interaction between antimicrobial agent and the corn zein film matrix facilitates easy release of the antimicrobial.

Several researchers developed antimicrobial plastic packaging materials with bacteriocins and other antimicrobials and tested them on seafood and meat products. Nisin was incorporated into low-density polyethylene (Neetoo et al., 2008) and tested against *L.*

monocytogenes inoculated onto cold-smoked salmon stored at 4 and 10^{o}C for 49 and 56 days, respectively. Films with nisin (2000 IU/cm^{2}) led to a 3.9 log reduction compared to control films. Edible films were prepared by combining solutions of hydroxypropylmethyl cellulose and chitosan (Ye et al., 2008). Nisin, sodium lactate, sodium diacetate, potassium sorbate and sodium benzoate were incorporated into films. These films were tested against *L. monocytogenes* on cold-smoked salmon stored at 4 and 20^{o}C. Films with sodium lactate showed greater listerial inhibition than other films. Growth of *L. monocytogenes* in smoked salmon was reduced by using whey protein films with incorporated lactoperoxidase system. Lactoperoxidase catalyzes oxidation of thiocyanate and produces oxidation products (hypothiocyanite and hypothiocyanous acid), which oxidize sulfhydryl (SH) groups in microbial proteins and enzymes, leading to cell death (Min et al., 2005). Differences in antimicrobial activity can be due to the nature of the antimicrobial, interaction among antimicrobials and/or the interaction between the antimicrobial and film matrix.

Bacteriocins: Nisin and its application through incorporation into edible films

Bacteriocins are synthesized proteinaceous compounds. They have narrow spectrum of activity against closely related bacterial species. Bacteriocins differ from antibiotics as antibiotics are secondary metabolites and have broad spectrum of activity. Bacteriocins are produced only by certain strains of bacteria. Both Gram-positive and Gram-negative bacteria can produce bacteriocins, but they vary greatly in their structure and functionality. Bacteriocins of Gram-positive bacteria are classified as class 1, 2 and 3 (Holtsmark et al., 2007). Class 1 bacteriocins are called lantibiotics and contain post-translationally modified

peptides. Different kinds of lantibiotics include type A (linear flexible cationic peptides), type B (globular rigid peptides) and type C (two component bacteriocins). Class 2 bacteriocins are diverse group of native peptides. Mode of action for Class 2 bacteriocins is similar to class 1 bacteriocins, but the receptors for class 2 bacteriocins are lipids and not proteins as for type 1 (Chen and Hoover, 2003). Class 3 bacteriocins are heat labile proteins and are characterized according to their ability to lyse cells. Bacteriocins from Gram-negative bacteria include colicins, pyocins, microcins and trifolitoxins (Holtsmark et al. 2007). Colicins are produced by *E. coli* and have narrow specificity acting on *E. coli* and related species. Pyocins act on the target cell by self-assembly into particles, which resemble bacteriophage tails. Bacteriocins produced by *Pseudomonas aeruginosa* come under this class. Microcins are antimicrobial peptides produced by *Enterobacteriaceae* species. These include post-translationally modified and non-modified compounds. Trifolitoxins are post - translationally modified and are produced by *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*.

Most of the bacteriocins that have applications in the food industry belong to class I and class II. Lactococcal bacteriocins are grouped under class I and class II bacteriocins. Lactococcal bacteriocins like lacticin 3147, lacticin 481, nisin and lactococcin ABM have potential applications in the food industry (Guinane et al., 2004). Bacteriocins can be applied to foods in several ways, including addition of purified bacteriocins to foods, addition of ingredients previously fermented by bacteriocin producers and inoculation of food with a bacteriocin producing culture (Jones et al., 2005). Some bacteriocins are often used with thermal processing, as they are heat resistant.

Nisin is the most popular bacteriocin approved for commercial use. Nisin was added to EU food additives in 1983. Bacteriocin nisin has GRAS (generally recognized as safe) status and is approved in more than 40 countries to inhibit *Clostridium* species in cheese and canned foods. The natural ingredient designation of nisin, relatively broad spectrum of activity compared to other bacteriocins, its sensitivity to α-chymotrypsin and stability at high temperature and low pH makes it the most used bacteriocin. Global food additives market is valued at approximately $22 billion and bacteriocins such as nisin are major part of this (Jones et al, 2005). Currently, Nisaplin™ by Danisco (Copenhagen, Denmark) is the most popular commercial form of nisin.

Nisin belongs to a group of bacteriocins called lantibiotics that are produced by several Gram-positive bacteria and have antimicrobial activity against several related species. Nisin is produced by *Lactococcus lactis* subsp. l*actis*. Nisin is active against select foodborne pathogenic microorganisms. Activity of nisin was reported against *L. monocytogenes*, *Staphylococcus aureus* and *Lactobacillus bulgaricus.* Nisin also prevents the spore outgrowth of *Bacillus* and *Clostridium* species (Harris et al. 1992). Sensitivity of Gram-negative bacteria towards nisin can be increased in the presence of chelating agents, such as EDTA, as they weaken the outer cell membrane.

Nisin has the unusual amino acids dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionine and methyllanthionine. Dehydration of serine and threonine yields Dha and Dhb. Condensation of Dha and Dhb with cysteine yields lanthionine and methyllanthionine (Harris et al., 1992). Most amino acids in nisin are basic giving it a net positive charge. Structure of nisin consists of 34 amino acids and can form dimers and oligomers. Nisin does

not have a secondary structure, but can form a rigid three dimensional structure. At high pH, hydroxyl groups and nucleophilic R groups can react with Dha and Dhb, reducing the solubility and stability of nisin. It was shown that the solubility of nisin reduces from 57 mg/ml (pH 2) to 0.25 mg/ml (pH 8 to 12). Other properties of nisin include heat stability (121^{o}C) at pH 2, resistant to trypsin, elastase, carboxypeptidase A, pepsin and sensitivity to chymotrypsin (Chen and Hoover, 2003).

Nisin attaches to the surface of vegetative cells and becomes incorporated into the cytoplasmic membrane. This results in the formation of pores or channels, leading to efflux of ATP and amino acids. This effect is more prominent in Gram-positive cells; however, in Gram-negative cells the outer membrane should be weakened to see this effect. Nisin disrupts the pH and membrane potential gradient as a negative potential gradient and an alkaline pH inside the cell membrane is required for insertion of nisin into the membrane (Eswaranandam et al., 2004). Other inhibitory mechanisms of nisin, including partial inhibition of peptidoglycan synthesis and cellular lysis, are reported. Nisin inhibits spore germination and spore development. Dehydro groups of nisin react with membrane sulphydryl groups present in newly germinated spores, preventing spore development.

Development of resistance against bacteriocins is an issue that is yet to be addressed. The development of resistance against each bacteriocin is different. The frequencies at which bacteriocin-resistant mutants develop vary greatly with the bacteriocin. In case of *L. monocytogenes,* the frequency with which resistance against nisin develops is influenced by environmental stress such as low pH, low temperature and presence of sodium chloride. Resistance to nisin is not stable. When nisin-resistant cells were subjected to heat treatment,

they again became susceptible to nisin (Chen and Hoover 2003). Major challenge for use of nisin as antimicrobial is the occurrence of nisin-resistant strains and the natural variability in sensitivity of strains to nisin. Development of resistance to nisin in presence of sublethal concentrations of nisin was reported in *L. monocytogenes*, *C. butyricum*, *S. aureus*, *Streptococcus agalactiae* and *P. pentosaceus*. Nisin resistance was linked to plasmid DNA in select cases. Resistance to nisin can also be attributed to production of enzyme nisinase. Nisinase production was reported in several genera such as *Bacillus*, *Lactobacillus* and *Streptococcus*.

Incorporation of nisin into edible films can lead to better antimicrobial activity than direct addition of nisin to foods. Use of edible films can partially prevent inactivation of nisin (Sanjurjo et al., 2006). Several studies investigated antimicrobial activity of nisin incorporated edible films on growth media. Additional studies investigating the use of edible films with nisin on muscle foods are needed.

Casein-based edible films were developed with different antimicrobials including nisin, propolis, zeolite and potassium sorbate (Anean et al., 2013). Antimicrobial activity of the films was examined against *L. monocytogenes* using agar well diffusion method. Films with nisin possessed more antilisterial activity compared to other films. Antimicrobial activity increased as the concentration of nisin in the films increased. Nisin and potassium sorbate are incorporated individually and in combination into tapioca starch-hydroxypropyl methylcellulose films (Basch et al., 2013). Antimicrobial activity of the films was examined against *L. innocua* and *Zygosaccharomyces bailii* using agar diffusion method. It was shown

that antimicrobials used in combination are more effective against the target microorganisms than when used individually.

Pintado et al. (2009) developed whey protein isolate films with different organic acids (lactic, citric and malic acids) at 3% (wt/vol) concentration in combination with nisin (50 IU/ml). Films with nisin and malic acid were most active against *L. monocytogenes* (examined using agar diffusion method) compared to other treatments. Edible films were developed from whey protein isolate, soy protein isolate, wheat gluten and egg albumin protein (Ko et al., 2001). Different film forming proteins were chosen as they have different hydrophobicities. Nisin was incorporated into the film forming solutions at different concentrations and at different pH ranging from 2 to 8. It was shown that nisin interacts with different proteins in diverse ways due to their hydrophobicity. Activity of these films was tested against *L. monocytogenes* using microbiological growth media. Of all the treatments, films made with whey protein concentrate and nisin in acidic environments were most active against *L. monocytogenes*. Whey protein concentrate was shown to have high hydrophobicity (due to exposure of hydrophobic groups) in comparison to other film forming materials in this study.

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Chapter 2

Trout skin gelatin-based edible films containing phenolic antioxidants: Effect on physical properties and oxidative stability of cod liver oil model food

Abstract

Trout skin (*Oncorhynchus mykiss*) gelatin-based films containing antioxidants (epigallocatechin gallate EGCG, 50 and 250 ppm wt/wt) and green tea powder (1% and 20% wt/wt of gelatin) were tested for tensile strength, elastic modulus and elongation, and oxygen and water vapor transmission rates, *in vitro* antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and effect on stabilizing cod liver oil held under mild thermal abuse conditions. Cod liver oil overlaid with films was stored at 40^{o}C for 20 days and analyzed for peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). Antioxidant activity was retained in films containing green tea powder, but was reduced ($P < 0.05$) in EGCG films (20 days, 23^{o}C). Water vapor transmission rate of the films incorporated with antioxidants did not change significantly ($P > 0.05$), but the oxygen transmission rate for films with 50 ppm EGCG and 20% green tea powder was significant ($P < 0.05$). Other physical properties varied with antioxidant incorporation. The TBARS and PV of control oil increased from 0.05±0.01 to 4.71±0.30 g MDA/kg oil and from 3.6±0.2 to 178.3±24.5 millieq peroxides/kg oil, respectively, after 20 days. For cod liver oil covered with control or antioxidant-containing films, TBARS remained below 0.37 g MDA/kg oil and PV below 7 millieq peroxides/kg oil. Incorporation of antioxidants to the films did not reduce oil oxidation ($P > 0.05$) at the levels tested and this was confirmed by activation

energy calculations. The rate of oil oxidation was more dependent upon the inherent oxygen barrier property of the films than the presence of antioxidants.

Introduction

Consumption of fish oils in various forms is popular due to the presence of high concentration of polyunsaturated fatty acids. Fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) promote anti-inflammatory eicosanoid synthesis and provide other health benefits (Barrett and others 2009). One major drawback is that these fatty acids are susceptible to oxidation and there is a need to stabilize them. Fish oils are usually stabilized by encapsulation or addition of antioxidants such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and tocopherols (Wanasundara and Shahidi 1998). Consumer rejection of synthetic additives in foods is becoming more common (He and Shahidi 1997) and as a result, alternative natural antioxidants are being investigated.

Recently, green tea has gained popularity due to its health benefits. Green tea contains catechins - polyphenolic compounds that possess anti-carcinogenic, anti-mutagenic and anti-allergenic properties (Tang and others 2001). Tea catechins can delay oxidation by inhibiting the formation of free radicals and hindering free radical propagation (Chen and others 1996). Ethanol extracts of green tea provided greater stabilization than BHT for oxidation of canola oil (Chen and others 1996) and marine oils (Wanasundara 1998). Epigallocatechin gallate (EGCG) is an important catechin (about 59%) present in green tea (Cabrera and others 2006). He and Sahidi (1997) showed that EGCG was more effective antioxidant compared to other tea catechins in cooked mackerel muscle during storage.

While the incorporation of antioxidants into foods is challenging, the use of edible matrices such as gelatin films as a carrier for bioactive agents is actively being investigated. Edible films with incorporated active components can result in prolonged activity of the active components in food system than direct application (dipping or spraying) due to the diffusion of the active agent from the film to the surface of the food (Yudi and others 2004). Most gelatins are from bovine and porcine sources although fish gelatins (currently only 1.5% of total gelatin being used) are becoming more popular (See and others 2010). Terrestrial animal gelatins often do not comply with Halal and Kosher requirements, and, there has been a concern that these may be contaminated with prions (Gimenez and others 2009).

Fish processing waste can account for up to 75% of the weight, and about 30% of this waste is skin and bones containing collagen (Gomez-Guillen and others 2002). Annually, about 72% of trout grown in USA is from Idaho (Idaho State Department of Agriculture 2010). The byproducts of trout processing resulting from filleting and producing minced fish are of interest to the aquaculture industry due to potential to generate value-added products. Therefore, trout skin was chosen as the raw material for gelatin extraction in this study. The objectives were to develop edible films from trout skin gelatin by incorporation of antioxidants, investigate the antioxidant activity and physical properties (film thickness, tensile and gas barrier properties) of the films, and assess the antioxidant activity of the films on cod liver oil as a model food system.

Materials and methods

Gelatin extraction and preparation of antioxidant edible films

Gelatin was extracted from trout skins following the method of Chiou and others (2006). Film forming solution (FFS) was formulated to contain 6.75% gelatin and 20% glycerol (wt/wt of gelatin). The EGCG (50 and 250 ppm in FFS) (Sigma, St. Louis, MO) and Green Tea Powder (GTP) (1 and 20% wt/wt of gelatin) (Tazo Tea, Portland, OR) were incorporated into the films. For preparation of films with GTP, the powder (20% of total weight) was added to water and the solution was stirred under low heat then gelatin was added in small increments while stirring (approximately 70^{o}C). The remaining GTP was added incrementally with continuous stirring for 30 min at 70^{o}C, followed by heating in a 90^{o}C water bath for 30 min. After cooling to room temperature, glycerol (Sigma, St. Louis, MO) was added (Gelatin: glycerol ratio, 1:0.2) and the solution was vacuum degassed. The amount of dry solids in each film was kept constant (3 g) to maintain uniform film thickness. The FFS was poured into Teflon plates as described by Krishna and others (2012) then left to dry in chamber at 25^{o}C and 30% RH. The dried films were stored at 50% RH until use. Films with EGCG were prepared similarly except that EGCG was added to the FFS after the 90^{o}C heating step, followed by stirring for 30 min at 70^{o}C.

Antioxidant activity of films

Antioxidant activity of the films was tested by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The protocol from Bao and others (2009) was followed using 0.072 mM/L DPPH reagent in ethanol. Films were stored for 20 days (50% RH, 22±2^{o}C) with sampling on day 0 and 20.

Results were expressed as a percentage of DPPH quenched (% Radical scavenging activity = [1-(AS-AO)/AC)]*100); where AS, AC, AO are the absorbance values of sample, control, and solution of 5.5ml ethanol and 500µl sample, respectively).

Total phenolic content of films

Total phenolics present in the film were measured using Folin-Ciocalteu method following the protocol of Siripatrawan and Harte (2010). Calibration curve was constructed using gallic acid (Sigma, St. Louis, MO) and results were expressed as gallic acid equivalents (GAE).

Tensile properties of films

Tensile strength (TS), elastic modulus (EM) and percent elongation at break (%E) were measured using standard method D 882-01 (ASTM 2001). Films were conditioned for 2 days at 22±2^{o}C and 50% RH using saturated solution of magnesium nitrate (Fisher Scientific, Pittsburgh, PA) before analysis. Films were cut to 50 mm (long) and 8 mm (wide) to facilitate gripping to the clamps of texture analyzer (TA-XT2, Stable Micro System Ltd., Surrey, UK). Crosshead speed of 50 mm/min and a load cell of 25 kg were used.

Gas barrier properties of films

The water vapor transmission rate (WVTR) was measured by Gravimetric Modified Cup method based on ASTM standard E 96-92 (McHugh and others 1993). The WVTR was calculated as described by Krishna and others (2012). Oxygen transmission rate (OTR) of

the films was measured using Oxtran 2/21 (Modern Controls Inc., Minneapolis, MN). The OTR was determined at 23°C and 50±1% RH using ASTM method D 3985 (ASTM 1995).

Thermal properties of films

Thermal transitions of the films were measured using a Modulated Differential Scanning Calorimeter (MDSC) (DSC Q200, TA Instruments, New Castle, DE). Experiments were performed at a heating rate of 10°C/min and heat flow data collected to determine the glass transition temperature (Tg) and enthalpy change (J/g film) associated with the event. Instrument software, Universal Analysis 2000, v. 4.3, (TA Instruments, New Castle, DE) was used to calculate the enthalpy by multiplying the step change in heat flow (W/g) by the event duration (s) from onset to endpoint.

Oxidation of cod liver oil

To demonstrate the effectiveness of trout skin gelatin films in retarding oxidation, cod liver oil without antioxidants (Twinlab Inc., American Forks, UT) was chosen. The oil was covered with films containing antioxidants and stored at 40°C (35±1% RH) for up to 20 days. Samples were analyzed every fifth day during storage. Oil samples (13 ml) were placed in specially fabricated cylindrical wells made from a 2.54 cm thick acrylic plate (Figure 1). Each well with oil was covered with a circular film containing antioxidants (50 or 250 ppm EGCG, 1% or 20% GTP). The film covered the well in such a way that its inner surface was in contact with the oil with no air bubbles entrapped underneath (Figure 1). High vacuum grease was applied on the edges of the well to provide a tight seal between the film and well. Another ¼ inch thick acrylic plate was placed on top of the films and lower

plate then both plates were screwed together to hold the film pieces in place to ensure that gases only diffuse in and out of oil through the film surface.

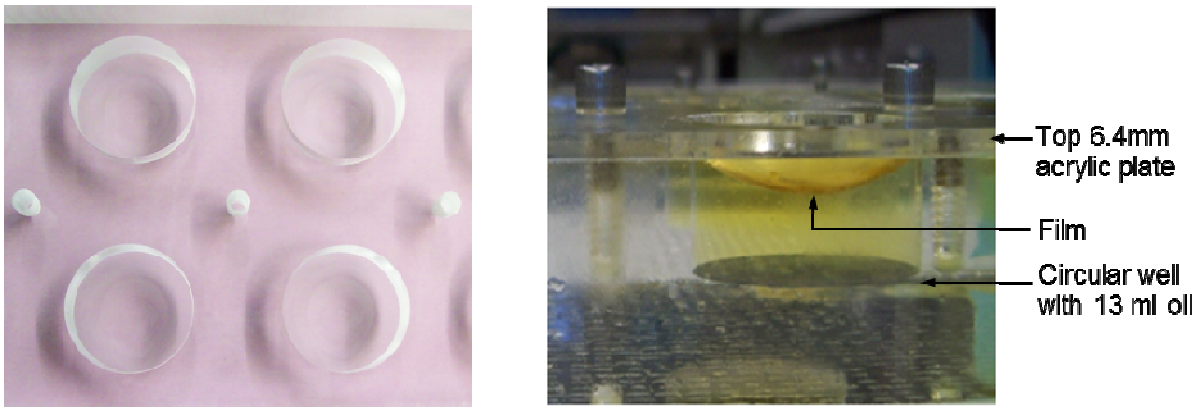

Figure 1: Experimental setup showing fabricated well for oxidation of cod liver oil covered with film

Monitoring primary and secondary oxidation products

Oxidation of cod liver oil was monitored by measuring primary and secondary oxidation products. Peroxide value (PV) was measured following AOAC method 965.33 (AOAC 2005). The TBARS (thiobarbituric acid reactive substances) assay was performed on the oil to monitor secondary oxidation products (Bower and others 2009). To 400 mg oil, 3.5 ml cyclohexane (EMD Chemicals, Gibbstown, NJ) and 9 ml of 7.5% trichloroacetic acid (Sigma, St. Louis, MO) with 0.17% thiobarbituric acid (Sigma, St. Louis, MO) was added. The remaining steps were the same as in the protocol.

DSC analysis on stored cod liver oil

Cod liver oil stored at 40^{o}C for 20 days while covered with films containing antioxidants were sampled every fifth day and analyzed by modulated DSC (Q200, TA Instruments, New Castle, DE) under isothermal conditions at four different temperatures (110, 120, 130 and 140^{o}C). The oxidation induction time (OIT) was determined following protocol from Tan and others (2001). Activation energy (Ea, kJ/mole), based on Arrhenius equation, was calculated from plots of natural log of (1/OIT) vs. inverse of absolute temperature (K).

Correlation coefficients

Correlation coefficients were calculated to see if there is any relationship between oxidation parameters (PV and TBARS values) and the concentration of antioxidants. Graph is plotted between oxidation parameter values and the concentration of antioxidants for all the sampling days and the R^2 values calculated.

Statistical analysis

For cod liver oil oxidation studies, pooled ANOVA was conducted assuming randomized complete block design. Statistical Analysis Software version 9.2 (SAS Institute, Cary, NC) was used to analyze data. For each treatment, there were triplicate samples at every time point (n=1 with 3 replicates). Each sample was analyzed in duplicates and quadruplicates for PV and TBARS assay, respectively. The DSC analysis of oil was performed on a single sample in duplicates. For studying the physical properties of the films, pair wise comparisons (equivalent to Fishers LSD) were conducted. Each mean ± standard error was the average of 6 replicates (2 samples each from 3 separate films) for WVTR and 8

replicates (4 samples each from 2 separate films) for tensile properties. The OTR experiments were conducted in duplicate (one sample each from 2 films). The total phenolics and DPPH assay on the films was conducted with five analytical replicates per film sample. The thermal analysis on films was performed on 4 replicates (2 samples each form 2 separate films).

Results and discussion

Antioxidant activity of films

The films prepared with GTP and EGCG possessed antioxidant activity as shown in Figure 2. Radical scavenging activity of the films changed as a function of time and type of antioxidant. Control films did not show any radical scavenging activity and hence data are not presented. Films with 50 and 250 ppm EGCG showed 28.6±6.4 and 67.1±4.7 % radical scavenging activity on day 0; however their activity was significantly reduced ($P < 0.05$) to 0 and 22.8±4.5 % by day 20, respectively. On day 0 films with 20% GTP showed 77.6±0.9 % activity while the films with 1% GTP showed 18.9±1.3 % activity. By day 20, there was no significant drop ($P > 0.05$) in the radical scavenging activity of these films. Significant drop ($P < 0.05$) in activity of films with EGCG is because EGCG is a cis-configured catechin and oxidizes following first order kinetics (Li and others 2011). Films with GTP retained activity most likely due to multiple antioxidants present in green tea including a mixture of cis and trans-configured catechins (Sundararajan and others 2011). Trans-configured catechins have a complex degradation mechanism often involving epimerization and degradation (Li and others 2011). Similar mechanisms might have been responsible for the prolonged activity of films containing green tea.

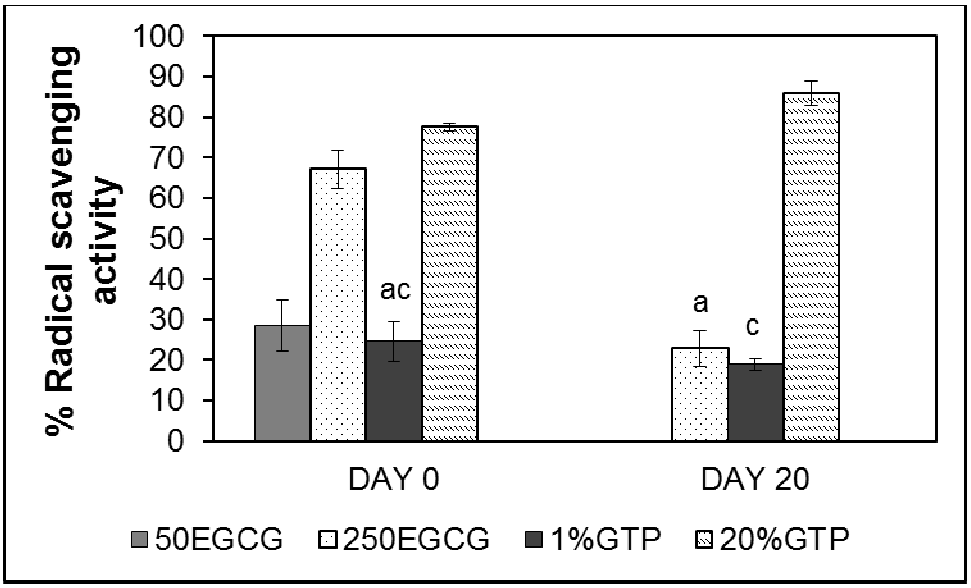

Figure 2: Radical scavenging activity of films with green tea powder (GTP) and EGCG during 20 day storage (50% RH, 23±2^{o}C). Means with different letters and no letters are significantly different ($P < 0.05$). Means were calculated from five analytical replicates taken from a film.

Total phenolic content of films

The total phenolic content (TPC) of the films was determined by Folin–Ciocalteu (FC) phenol reagent (Figure 3). As the amount of green tea in the films increased, the amount of phenolic compounds increased. This resulted in higher radical scavenging activity (Figure 2) of films with higher phenolic compounds. The same trend was observed with the films containing EGCG. Increase in the antioxidant activity due to phenolic compounds was also observed in another study (Siripatrawan and Harte 2010).

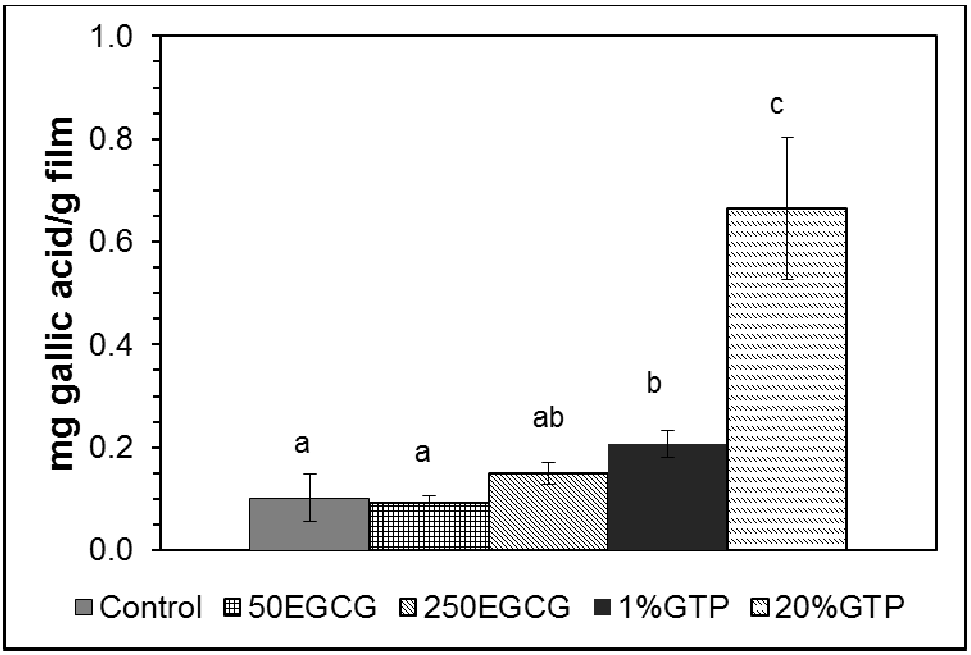

Figure 3 – Total phenolic content of films with different concentrations of EGCG and green tea powder (GTP). Means followed by different letters are significantly different ($P < 0.05$). Means were calculated from five analytical replicates taken from a film.

Tensile properties of films

Tensile properties can be used to relate the mechanical properties of the films to their chemical structure (McHugh and Krochta 1994). Tensile properties of the films with different concentrations of antioxidants are shown in Table 1. The TS and EM of film increased while the %E decreased with increasing EGCG concentrations. Incorporation of 1% GTP increased the TS and EM of the films compared to control. GTP at 20% resulted in decreased TS and EM. There was no significant difference in the %E of the films containing 1% GTP, however 20% GTP decreased ($P < 0.05$) the %E value. Extent of crystallinity in the film structure has an effect on the TS of the films (Park and Zhao 2004). Incorporation

of EGCG and green tea might have altered the film's crystallinity and hence the TS. It is evident that the antioxidants incorporated had no plasticizing effect on the film matrix as %E did not increase, although higher concentrations reduced it. Filling of voids in the film matrix and the resulting reduction in stretch ability can cause lower %E of the films (Park and Zhao 2004). As expected, the film EM (stiffness) followed the same trend as the TS since the stiffness varies with the strength of the film.

Table 1: Tensile properties of films with EGCG and GTP

Film sample	Tensile strength (MPa)	Elastic modulus (MPa)	% Elongation	Thickness (mm)
Control	29.0±3.7[a,1,3]	700±74[a,1,3]	5.7±0.6[a,1]	0.107±0.009
50ppm EGCG	36.5±3.0[a,b]	799±60[a]	6.0±0.5[a]	0.110±0.007
250ppm EGCG	48.6±5.3[b]	1386±104[c]	2.9±0.7[b]	0.106±0.011
1% GTP	45.8±3.82	972±742	5.7±0.61	0.111±0.008
20% GTP	23.0±3.33	760±663	3.1±0.52	0.102±0.007

Means followed by different letters and numbers are significantly different ($P < 0.05$). Letters represent significant differences ($P < 0.05$) in the means of EGCG films while numbers represent the significant differences in means of GTP films compared to the control films. Means were calculated from 8 replicates (four each taken from two separate films).

Gas barrier properties of films

The WVTR of films with different antioxidants is shown in Table 2. There was no significant difference in the WVTR of the films due to antioxidant incorporation. Phenolic compounds may interact with the film matrix (Siripatrawan and Harte 2010) and alter the free volume which possibly caused the small change observed ($P > 0.05$) in the WVTR of

the films. The WVTR depends on the diffusivity and solubility of water molecules in the film matrix (Sablani and others 2009). In general, the WVTR of cold water fish gelatin films were lower than those of warm water fish and mammalian counterparts. The higher hydrophobicity (lower WVTR) of gelatins derived from cold water fish species has been attributed to lower contents of proline and hydroxyproline (Avena-Bustillos and others 2006).

Table 2: Water vapor and oxygen transmission rates of films with EGCG and GTP

Sample	Water vapor transmission rate (g/m^2 h)	Oxygen transmission rate (cc/m^2 day)
Control	41.88 ±10.86	2.47±0.48
50ppm EGCG	47.41 ±2.09	4.68±2.49[a]
250ppm EGCG	48.90 ±2.20	4.31±0.11
1% GTP	46.97 ±1.68	3.69±0.74
20% GTP	45.12 ±2.59	1.26±0.58[b]

Data represents means ± standard error of means. Means without letters are not significantly different ($P > 0.05$). Means are calculated from six and two replicates for water vapor and oxygen transmission rate, respectively.

Only films with 50 ppm EGCG and 20% GTP were significantly different in OTR (Table 2). The incorporated antioxidants might not have been at a sufficiently high concentration to affect the molecular mobility within the film structure. However, Bae and others (2009) reported reductions in the oxygen permeability of fish gelatin films due to nano-clay incorporation. They explained that the film structure might be better oriented and intercalated due to the incorporation of antioxidants and hence forcing the oxygen molecules

to pass through a more tortuous pathway that reduces permeability. Cold water fish gelatin films provide better barrier to oxygen than warm water fish gelatin and mammalian gelatin films due to differences in triple helix structure (Aveena-Bustillos and others 2011). Films with triple helix structure have more water sorption at the surface, leading to film plasticization and increased mobility which in turn increases the permeability.

DSC analysis on films

Incorporation of GTP increased the T_g and energy required to induce glass transition (Table 3). Materials with flexible bond structure have lower T_g compared to materials with rigid structure (Gabbott 2008). Incorporation of green tea into the films might have induced certain degree of cross linking (due to presence of polyphenols) in the film matrix leading to an increase in T_g. Hence more energy was needed for this transition to occur. Decrease in the T_g was observed in films with EGCG and GTP compared to control. The GTP (water activity of about 0.24) and EGCG used in this study did not show glass transition possibly due to low plasticizer (water) content. However, Li and others (2011) reported glass transition for GTP stored at higher relative humidity conditions, which most likely contributed to plasticization. The reason for not being able to detect T_g in our GTP samples might be due to less plasticizer.

Table 3: Glass transition temperature (T_g) and enthalpy to induce the change for films containing antioxidants

Sample	Glass transition temperature (^oC)	Enthalpy change during glass transition (J/g film)	Water activity (aw)
Control	24.8±0.8[ac]	4.86±0.29[a]	0.42±0.01
50ppm EGCG	21.3±1.7[b]	5.94±1.58[ac]	0.41±0.07
250ppm EGCG	20.5±0.8[b]	5.53±2.03[c]	0.41±0.01
1% GTP	27.1±0.3[c]	9.72±1.84	0.38±0.01
20% GTP	26.0±1.6[c]	14.23±2.22[d]	0.38±0.01

Values represent means ± standard error of means calculated from two replicates (two samples taken from two films). Means with different letters are significantly different ($P < 0.05$). Water activity was measured at 23±1^{o}C.

Oxidation of cod liver oil during storage

Cod liver oil contains polyunsaturated fatty acids (approx 22.5%, USDA 2012) which are susceptible to oxidation. Peroxidation of fatty acids leads to the formation of lipid peroxides (primary oxidation products) (Hornero-Mendez and others 2001). The PV of uncovered oil increased to 231.6±2.6 millieq/kg oil by day 15 and tended to decrease thereafter (Figure 4) due to decomposition of peroxides. However, the PV of oil covered with the film remained below 7.0 millieq/kg oil throughout the storage period.

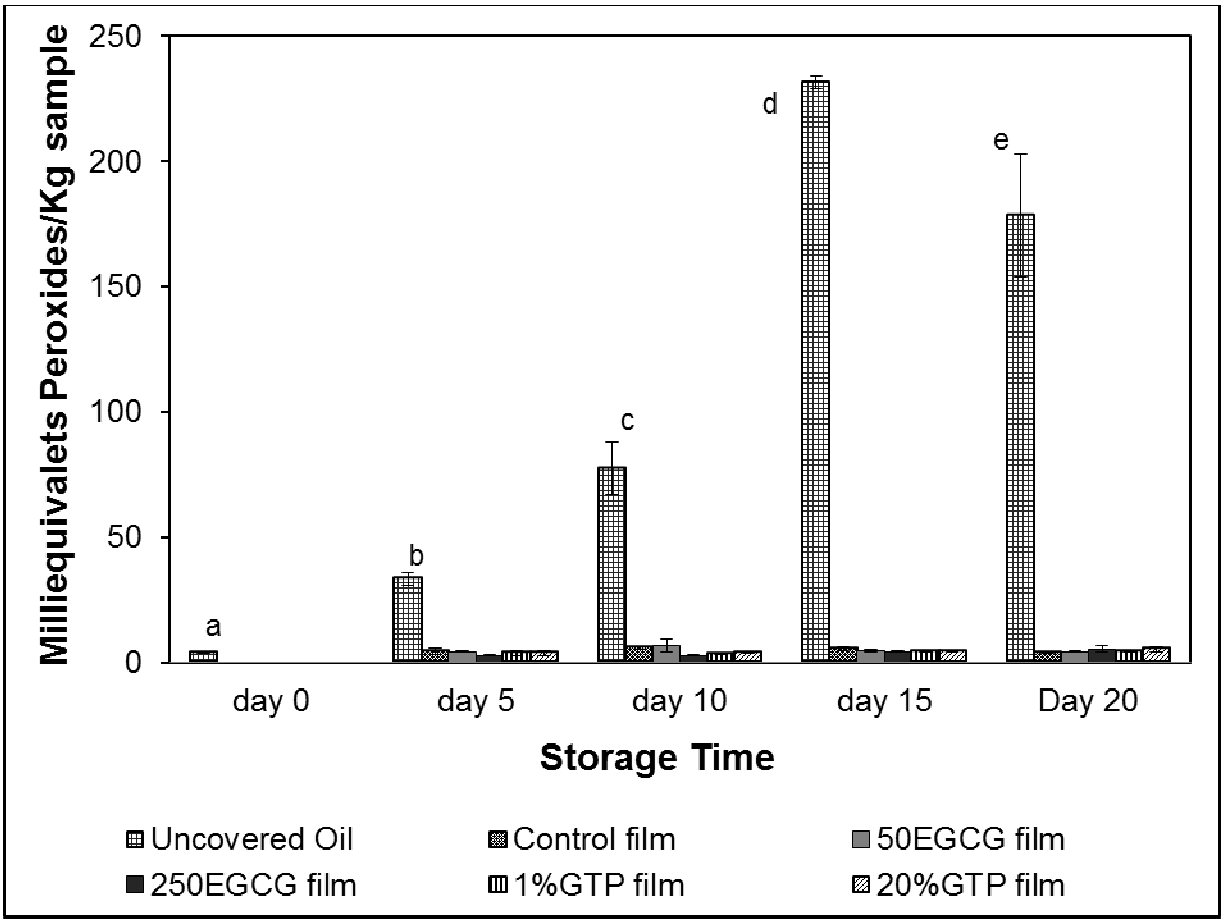

Figure 4 - Peroxide values of uncovered and cod liver oil covered with films (containing antioxidants) stored at 40°C for up to 20 days. Values represent means ± standard error of means. Treatments means followed by different letters are significantly different ($P < 0.05$). Means were calculated from 12 replicates (four replicates each from three samples).

The major substrate in TBARS test is malonaldehyde, but other oxidation products also react with thiobarbituric acid to produce pink color (Sahidi and Zhong 2005). TBARS value for uncovered oil increased considerably with storage time and reached 4.7 g/kg oil by day 20 due to availability of oxygen (Figure 5). For oil covered with films, there was no significant difference ($P > 0.05$) in TBARS values with storage time and among the

samples. The TBARS values remained below 0.37 g/kg oil throughout the storage period. For the concentrations of antioxidants used, no noticeable difference in the extent of oxidation was observed. Correlation coefficients were computed for oxidation parameters (TBARS and PV) and concentration of antioxidants. The correlation coefficients for concentration of EGCG in the films and the oxidation parameters were in the range of 0.003 to 0.577. The parameters have a negative correlation (value of oxidation parameters decreased as the concentration of EGCG in the film increased). The correlation coefficients for concentration of GTP in the films and the oxidation parameters were in the range of 0.298 to 0.99. We were surprised to see that the parameters have a positive correlation (value of oxidation parameters increased as the concentration of GTP increased). However, there was no significant difference in the values of oxidation parameters of oil due to change in the antioxidant concentration of the films as seen is Figures 4 and 5. It is likely that the protective effect of the film in retarding the oxidation of cod liver oil is predominantly from the oxygen barrier property rather than presence of antioxidants in the amounts used.

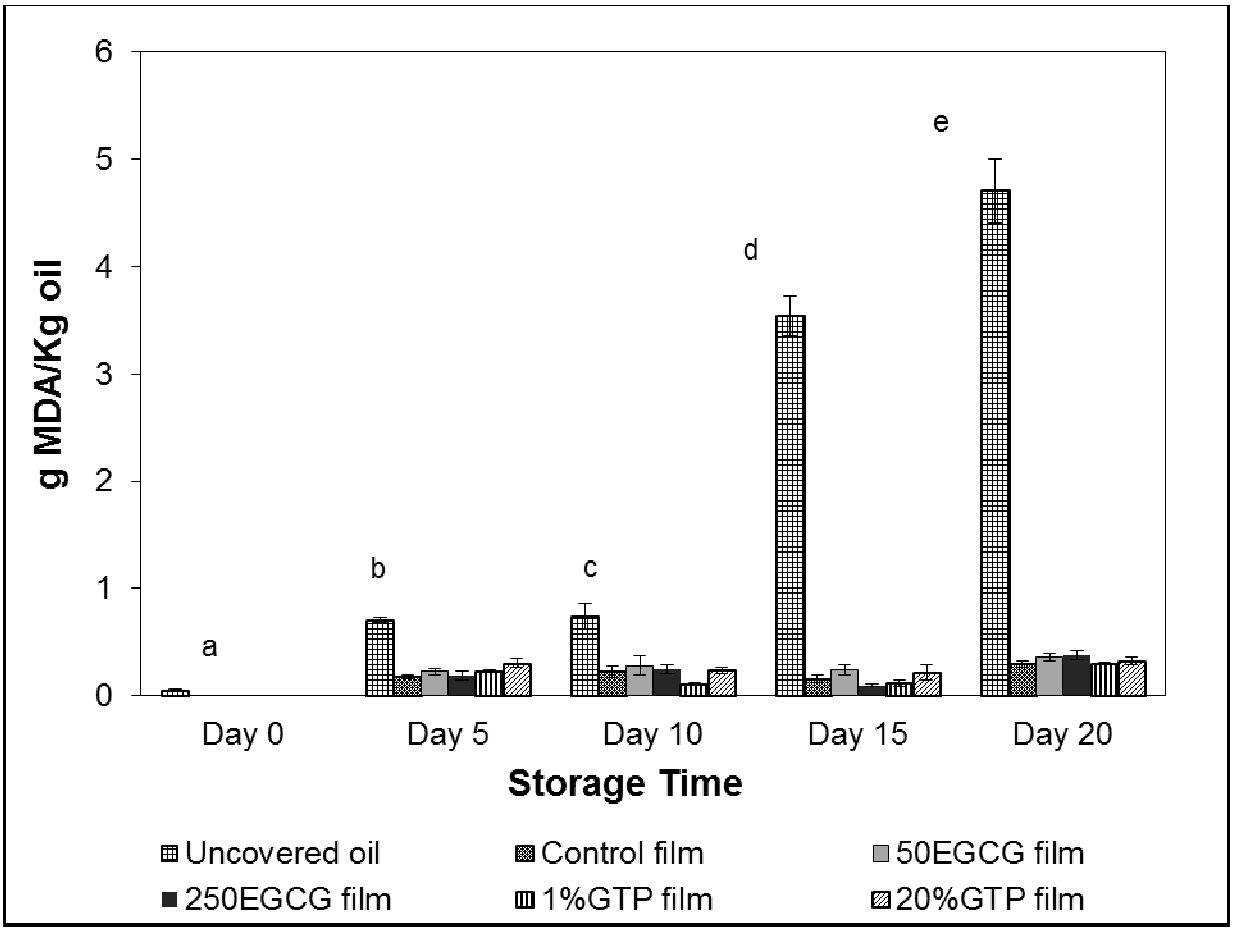

Figure 5 - TBARS values (g MDA/kg oil) of uncovered and cod liver oil covered with films (containing antioxidants) stored at 40^{o}C for up to 20 days. Values represent means ± standard error of means. Means followed by different letters are significantly different ($P <$ 0.05). Means were calculated from 6 replicates (two replicates each from three samples).

In dry food systems, the film structure is more tightly packed, so oxygen barrier effect is better compared to a wet system. Addition of antioxidants further enhances the oxygen barrier effect due to reduced mobility in the films (Bonilla and others 2012). Ayaranci and Tunc (2004) observed a reduction in the oxygen permeability of cellulose based films with addition of ascorbic, stearic and citric acids. As seen in this study and as supported by

Bonilla and others (2012), chemical effects of antioxidants on the oxidation process can be masked by the oxygen barrier property of the films. Even though the films were in contact with cod liver oil, not enough plasticization might have occurred to cause a change in the oxygen permeability of the films. Hence the antioxidant effect of green tea and EGCG was not evident. Ataris and others (2010) showed that the addition of essential oils to sodium caseinate films had no added advantage in protecting the sunflower oil from oxidation although their experimental design had a headspace between the film and the oil.

DSC analysis of oxidation of cod liver oil

Table 4 shows the Ea of oxidation of oil covered with films containing EGCG and GTP. Higher Ea implies more susceptibility to oxidation since a given change in temperature can cause a large shift in reaction rates (Tan and others 2001). Even though the Ea of cod liver oil treated with control film and films containing antioxidants were significantly different ($P < 0.05$) from day 5 and day 20, the change in magnitude was not that high considering the variables (storage time and temperature, and sampling) in the experiment. The differences in Ea of different treatments on each sampling day were significant ($P < 0.05$) for the majority of samples. Except for the Ea of fish oil covered with films containing 50 ppm and 250 ppm EGCG on day 10 and 20% GTP on day 20, all the other samples had Ea values in the ±8 kJ/mole range. There is no clear trend (as observed in PV and TBARS values) in activation energy data to show the effect of treatments. However, Ea on day 5 were not markedly different from day 20 for all the treatments (except for the 20% GTP on day 20). Also, the Ea on day 20 is within ±5.6 kJ/mole range (except for the 20% GTP) for oil covered with

control films and films containing antioxidants. There was no significant difference in the extent of oil oxidation whether it was covered with control film or film with antioxidants.

Table 4: Activation energy (kJ/mole) of uncovered oil and oil covered with films containing different concentrations of antioxidants.

Storage time (days)	Control	EGCG*		GTP*	
		50ppm	250ppm	1%	20%
5	25.19±0.21^{a,1,4}	25.00±0.38^{a,1}	23.66±0.01^{a,2}	26.26±0.02^{a,3}	24.98±0.00^{a,4}
10	17.88±0.20^{b,1}	38.44±0.41^{b,2}	32.99±0.00^{b,3}	26.05±0.03^{b,4}	25.61±0.02^{b,4}
15	22.50±0.01^{c,1}	25.35±0.01^{a,c,2}	26.15±0.11^{c,3}	25.58±0.05^{c,2}	26.05±0.00^{c,3}
20	26.04±0.01^{d,1}	26.33±0.03^{c,2}	20.72±0.05^{d,3}	25.42±0.04^{c,4}	12.51±0.06^{d,5}

Values represent means ± standard error of means. Means were calculated from duplicates (duplicate analysis of a single sample). Letters and numbers represent significant differences ($P < 0.05$) in the means of columns and rows respectively. *EGCG and GTP refer to epigallocatechin gallate and green tea powder, respectively.

Conclusion

Antioxidant trout skin gelatin films developed in this study was shown to delay the oxidation in cod liver oil model system. Similar approaches can be extended to increase shelf life of fish products. Gelatin films, both control and those containing EGCG (50 and 250 ppm) or GTP (1 and 20% w/w) reduced the oxidation of cod liver oil. The PV and TBARS values of fish oil covered with film were significantly ($P < 0.05$) different from uncovered oil. Incorporation of 50 ppm EGCG and 20% GTP varied ($P < 0.05$) the oxygen barrier property of the films but did not influence the oxidation of the covered oil. The protective effect of the films was more due to the oxygen barrier property than the presence

of antioxidants. The antioxidants incorporated into the film matrix were most likely not available to show a chemical effect in the oil due to low film-to-oil diffusion rates compared to oxygen transmission through the film. Incorporation of antioxidants did not enhance or alter the physical and barrier properties of the films to an extent to affect the application of films to food systems.

Acknowledgements

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Chapter 3

Bovine and fish gelatin coatings incorporating tannins: Effect on physical properties and oxidative stability of salmon fillets

Abstract

Fish gelatin provides an alternative source of gelatin for Halal and Kosher applications and is prion and zoonotic agent free. However, applications of fish gelatin have been limited due to inferior mechanical and barrier properties. The physical properties of fish gelatins can be improved by crosslinking using natural polyphenolic compounds such as tannic acid. The objectives of this study were to develop fish gelatin films incorporated with tannic acid and evaluate their antioxidant, thermal, tensile, water vapor permeability and water solubility properties. Also, the effect of tannin incorporated gelatins on the oxidative stability of salmon fillets is examined at 4 and 10^{o}C. Comparative data with bovine gelatins were generated. Fish gelatin (6.75% wt/wt) films were prepared at a gelatin:tannic acid ratio (wt/wt) of 1:0.05, 1:0.10 and 1:0.15. Tensile strength of bovine gelatin control was approximately 49 MPa and that of fish gelatin was 21 MPa. Tensile strength of bovine gelatins increased with tannic acid incorporation ($P > 0.05$) and did not vary significantly for fish gelatin films. Percent elongation of films increased and elastic modulus decreased with tannic acid incorporation. Water solubility of bovine gelatin films was reduced significantly ($P < 0.05$) and there is no effect of tannin on the solubility of fish gelatin films. The water vapor permeability was not significantly different for both the gelatins ($P > 0.05$) and the values ranged between 1.62 and 2.01 g mm/kPa h m^2. Bovine and fish gelatin films with highest level of tannic acid showed an increase in glass transition temperature of

approximately 12 and 6^{o}C, respectively. Films with tannic acid possess antioxidant activity and are able to reduce oxidation (TBARS values) in gelatin coated refrigerated salmon held for 12 days.

Introduction

Shelf life of meat and seafood can be extended if microbial growth and oxidative reactions are limited. As consumer rejection of synthetic additives is becoming more common, natural preservative systems are sought. Due to high water activity, neutral pH, presence of autolytic enzymes and relatively high concentration of free amino acids, fish such as salmon can be spoiled easily (Duna et al., 2010). Storage under controlled atmosphere can be effective for fish. For instance, in muscle foods stored under modified atmosphere, the microbial counts were well within the limit for 3 weeks, but deterioration in muscle color was observed after 12 days (Antionewski et al., 2007). Lipid oxidation can lead to off odors due to rancid volatiles, loss of muscle color due to oxidation of oxymyoglobin and drip accumulation (liquid oozing out of stored muscle) leading to deterioration in appearance (Antionewski et al., 2007). Fish muscle contains polyunsaturated fatty acids (He et al., 1997) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Several health benefits are associated with these fatty acids; however, these foods are easily susceptible to oxidation and strategies to control oxidation would be beneficial for extending the supply chain for these perishable foods.

Use of antioxidant systems such as plant phenolics and plant extracts on fish fillets to reduce oxidation was investigated. Direct treatment of fish fillets with antioxidants have been reported in several studies. Vacuum packed sardines (rosemary extract) (Ozogul et al.,

2010), bonito fillets (grape seed and green tea extracts) (Yerlikaya and Gokoglu 2009), blue sprat (tea polyphenol) (Seto et al., 2005) and salmon fillets (isoeugenol solution) (Tuckey et al., 2009) were treated with antioxidants to retard oxidation. Instant green tea powder was added to mackerel fillets (Alghazeer et al., 2008), and green tea extracts were added to minced mackerel muscle (He et al., 1997; Tang et al., 2001) and minced carp (Dembele et al., 2010) to extend fish shelf life. Solutions of chitosan with vitamin E were able to reduce oxidation in lingcod fillets (Duna et al., 2010).

Use of edible coatings and films incorporated with active components (like antioxidants or antimicrobials) will result in prolonged activity of the active components on foods than direct application by dipping or spraying (Min et al., 2005). Edible films can act as barriers to oxygen, water and limit lipid oxidation. Muscle foods can be wrapped in a preformed gelatin film, dipped or sprayed with gelatin film forming solution. On industrial scale, dipping muscle foods in gelatin solutions seems to be more practical. Significant improvements in oxidative stability of fish fillets dipped in chitosan-megrim skin gelatin solution (Caballero et al., 2005) and gelatin-benzoic acid solution (Ou et al., 2001) have been observed. Treated plastic films are also effective. For example, low density polyethylene films with barley husk extracts were able to reduce the TBARS values of salmon fillets stored for 12 month at -20^{o}C (Abreu et al., 2010). Chitosan microcapsules with horseradish extract coated onto ethylene vinyl acetate film (Jung et al., 2009) resulted in an extended shelf life of Spanish mackerel stored at 5^{o}C for 9 days due to reduced oxidation.

Bovine and porcine gelatins are well studied for use as coatings to extend quality of meat and seafood products. Recently, fish gelatin gained importance as an alternative source to mammalian gelatin due to socio-cultural and safety/sanitary reasons (Gomes Estaca et al., 2009). Fish processing waste can account to about 75% of total catch and 30% of this consists of fish skin and bones with gelatin (Gomez-Guillen et al., 2002). However, use of fish gelatins is limited as they have inferior rheological properties due to low number of proline and hydroxyproline residues and fewer inter and intra chain cross links compared to mammalian gelatins (Gomez Estaca et al., 2009). These films are naturally hydrophilic and absorb large quantities of water, resulting in more plasticization and inferior properties, which affect the applications of these films for extending shelf life of muscle foods.

Gelatins are crosslinked with a variety of cross linking agents. Most of these studies were intended to reduce solubility of gelatins and enhance strength properties. Crosslinking will result in the formation of new covalent bonds between reactive groups resulting in enhanced properties. Often, plant phenolics are a good choice as crosslinkers as they are natural compounds and also possess antioxidant activity. Tannic acid is a plant phenolic with multiple phenolic groups and can react with proteins resulting in improved film forming ability. Crosslinking bovine gelatin films with tannic acid (Zhang et al., 2010b) resulted in reduction of film solubility by about 80%. Equilibrium moisture uptake of crosslinked films was lower than that of the control films. However, cross linking did not limit the water uptake of films. Other crosslinkers including enzymes like transglutaminase (Piotrowska et al., 2008), white grape juice and coffee (Strauss et al., 2004), phenolic extract from Acacia bark (Haroun and Toumy, 2010), genipin (Bigi et al., 2002) and caffeic acid (Zhang et al., 2010a) were used to cross link food grade gelatins. These systems reduced the water

solubility and altered properties of films to different extents. From the scope of the literature, use of fish gelatin coatings and films to extend shelf life of fish products has been limited. The objectives of this work is to develop gelatin coatings incorporated with tannins and investigate the effect on fish gelatin properties and suitability of these as antioxidative coatings on salmon fillets.

Materials and methods

Film preparation with tannic acid

Commercial cold water fish gelatin (Norland Fish Products, Cranbury, NJ) and bovine skin gelatin (Sigma, St. Louis, MO, USA) were used in film preparation. Film forming solution (FFS) was formulated to contain 6.75% gelatin, and tannic acid (Sigma, St. Louis, MO, USA) at concentrations of 0, 5, 10 and 15% (wt/wt of gelatin). Initially, gelatin and tannic acid solutions were prepared separately by dissolving in warm deionized water. Tannic acid solution was added in 8-10 increments to the gelatin solution while being stirred at approximately 70^{o}C for 30 min. The FFS was denatured in 90^{o}C water bath for 30 min and cooled to room temperature. Glycerol (Sigma, St. Louis, MO, USA) was added to the FFS at a concentration of 25% (wt/wt of gelatin) followed by degassing. Amount of dry solids in each film preparation was kept constant (3 g) to maintain uniform film thickness. The FFS was poured into Teflon plates and left to dry for 24 h at 25^{o}C, 15% RH. Dried films were stored at 50% RH until use.

Tensile properties of films

Tensile properties including tensile strength (TS), elastic modulus (EM) and percent elongation at break (%E) were measured using standard method D 882-01 (ASTM, 2001). To facilitate mounting on the clamps of a texture analyzer (TA-XT2, Stable Micro System Ltd., Surrey, UK), the prepared films were cut into 50 mm (long) x 8 mm (wide) and conditioned in a 50% RH chamber for 2 days at 22±2^{o}C before testing. A 5 kg load cell and crosshead speed of 50 mm/min was used for determining the tensile properties.

Water vapor permeability of films

Modified Gravimetric Cup method based on ASTM standard E 96-92 (McHugh et al., 1993) was used to determine the water vapor transmission rate (WVTR) of films. Desiccation chambers fitted with fans to attain air velocity of 152m/min were maintained at 0% RH and 22±2^{o}C. Polymethylmethacrylate circular test cups with lid and screws were filled with deionized water (6 ml). Circular discs were cut from the films and placed in between the circular test cup lids. They were screwed tight to form a seal. Reduction in weight due to loss of water was recorded every 2 hours for 8 hours and the last reading was at 24th hour. Calculated water loss with time was divided by cup area (m^2) to give water vapor transfer rate (g/h-m^2). Permeance (g/kPa-h-m^2) was obtained by dividing water vapor transfer rate with partial pressure at the inner surface of the film. Permeance was multiplied by average film thickness to yield water vapor permeability (WVP) (g-mm/kPa-h-m^2).

Water solubility of films

Film soluble matter was determined by the modified method of Zhang et al (2010b). Film (1 g) was taken in a centrifuge tube and 50 ml water at 90^{o}C was added to it. The centrifuge tubes were immersed in water bath at 90^{o}C for 30 min. The tubes were then cooled and centrifuged (Sorvall Legend Mach 1.6, Thermo Scientific, Waltham, MA) for 15 min at 2000*g*. The supernatant was discarded and the pellet was dried for 6 h at 106^{o}C. The % soluble matter was the difference in the weight of the film taken and the dried pellet times one hundred.

Glass transition temperature (T_g) of films

Glass transition temperatures of films were measured using a Modulated Differential Scanning Calorimeter (MDSC) (DSC Q200, TA Instruments, New Castle, DE). Sample (10±1 mg) was taken in standard DSC pan, placed in the furnace with nitrogen flow rate of 20 ml/min. Sample was subjected to heating rate of 10^{o}C/min between temperature range of -50 to 150 °C. Heat flow data was collected and analyzed using instrument software (Universal Analysis 2000, v. 4.3, TA Instruments, New Castle, DE) to calculate the T_g.

Antioxidant activity of films

Film antioxidant activity was determined following DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Bao et al., 2009) using 0.072 mM/L DPPH reagent in ethanol. Films were stored in a 50% RH chamber at 23±1^{o}C and sampled on day 0, 10 and 20. Percent DPPH quenched was calculated by [1-(AS-AO)/AC)]*100); where AS, AC, AO are the absorbance values of sample, control, and solution of 5.5 ml ethanol and 500 µl sample, respectively.

Effect of tannin incorporated gelatins on the oxidative stability of salmon fillets

Atlantic salmon fillets (*Salmo salar*) were obtained from a local retailer (Pullman, WA). Fillets were sliced using a sterile knife into pieces of approximately 15±0.5 g and stored at 3^{o}C for one day until use. Pieces of same shape and size were sliced so that all pieces would have similar surface area (approximately 25 cm^{2}). Fillet pieces were dipped in fish and bovine gelatin solution with tannic acid. Samples were dried under a fan for 15 min. After drying, samples were placed in foam meat trays (Genpack, Gens Falls, NY) and covered with a Saran® wrap to mimic packaging in retail display. Samples were stored at 4 and 10^{o}C for 12 days and analyzed for TBARS every third day. Treatments included uncoated fillets (positive control), fillets dipped in control FFS (negative control) and fillets dipped in FFS solutions with 5, 10 and 15% tannic acid.

Modified protocol for TBARS assay was adopted from Kim et al. (2012). Briefly, 15±0.3 g fish fillet was blended (Waring Blender, Model HGBTAC30, Warring Commercial, Torrington, CT) with 40 ml of 1.5% trichloroacetic acid (Sigma, St. Louis, MO) for 15 seconds. Blended mixture (20 ml) was taken and centrifuged (Sorvall Legend Mach 1.6, Thermo Scientific, Waltham, MA) for 15 min at 3300 *g*. Supernatant (2ml) was added to 2 ml of 20 mM thiobarbituric acid (Sigma, St. Louis, MO) solution. The mixture was heated in a 95^{o}C water bath for 30 min and cooled to room temperature. Solution was filtered through 0.45 µm Whatman (Maidstone, Kent, UK) GD/X syringe filters. The absorbance of samples was taken at 532 nm and TBARS values (mg MDA/Kg fish) were calculated using the extinction coefficient.

Statistical analysis

Pooled ANOVA assuming randomized complete block design was used for salmon oxidation studies. Data analysis was done using statistical analysis software version 9.2 (SAS Institute, Cary, NC). For determining TBARS, each treatment consisted of triplicate samples at every time point. Pair wise comparisons equivalent to Fishers LSD were used for physical properties of the films. Each mean ± standard error was the average of 2, 5, 6, 8 and 10 replicates for glass transition temperature, DPPH radical scavenging activity, water vapor permeability, tensile properties and water solubility of films, respectively.

Results and discussion

Tensile properties

Incorporation of tannic acid to gelatin films altered tensile properties. Tensile strength increased ($P > 0.05$) and % elongation decreased ($P > 0.05$) with increasing concentration of tannic acid in bovine gelatin films (Fig 1, 2). In case of fish gelatin films, there was a slight decrease ($P < 0.05$) in tensile strength compared to control. Percent elongation of fish gelatin samples with intermediate concentrations of tannic acid (5% and 10%) increased ($P > 0.05$) compared to control and decreased ($P < 0.05$) for films with 15% tannic acid. Increase in the TS and decrease in the % elongation of crosslinker-incorporated gelatin films has been reported by other authors (Cao et al., 2006; Kim et al., 2005; Rivero et al., 2010). Increase in tensile strength can be due to the crosslinkers stabilizing the film matrix, which in turn decreases film elongation. Incorporation of tannic acid increased ($P > 0.05$) film

stiffness (Fig 3), which is evident by an increase in the elastic modulus of bovine and fish gelatin samples.

Tensile strength increased from 84 to 88 MPa as the concentration of tannic acid increased from 0 to 40 mg/g gelatin in bovine gelatin films (Cao et al., 2006). The same study showed a reduction of approximately 2 MPa in the elastic modulus of the samples with increase in tannin concentration. Kim et al. (2005) reported an increase of tensile strength from 44 to 68 MPa and no significant change in the elastic modulus of the samples with the incorporation of condensed tannins in gelatin chitosan matrix. Rivero et al. (2010) reported an increase in tensile strength of chitosan films incorporated with tannic acid. Crosslinkers change physical properties to varying degrees. However, direct comparison of data among different gelatin samples is not reliable due to different formulations, test conditions and gelatin composition.

Tensile strength and elastic modulus of bovine gelatin samples are significantly higher compared to fish gelatin samples (Fig 1, Fig 3). This can be explained by the difference in the amino acid composition. Proline and hydroxylproline content is higher in bovine gelatin compared to fish gelatin (Avena-Bustillos et al., 2006, 2011; Gomez-Gullien et al., 2007; Gomez-Estaca et al., 2009). These amino acids stabilize the triple helix structure resulting in increased tensile strength and elastic modulus. Hydroxyproline has major role in stabilizing the triple helix structure due to H-bonding to its hydroxyl group. High content of proline and hydroxyproline is believed to be the reason for highly viscous properties for mammalian gelatins (Aveena-Bustillos et al., 2006).

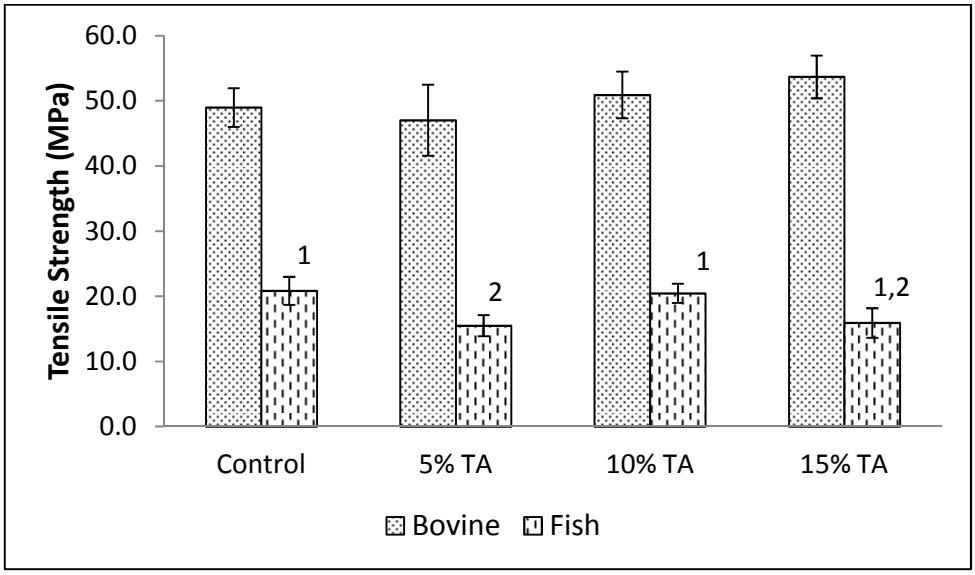

Fig 1. Tensile strength (MPa) of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=8). Means with same numbers and without numbers are not significantly different ($P > 0.05$).

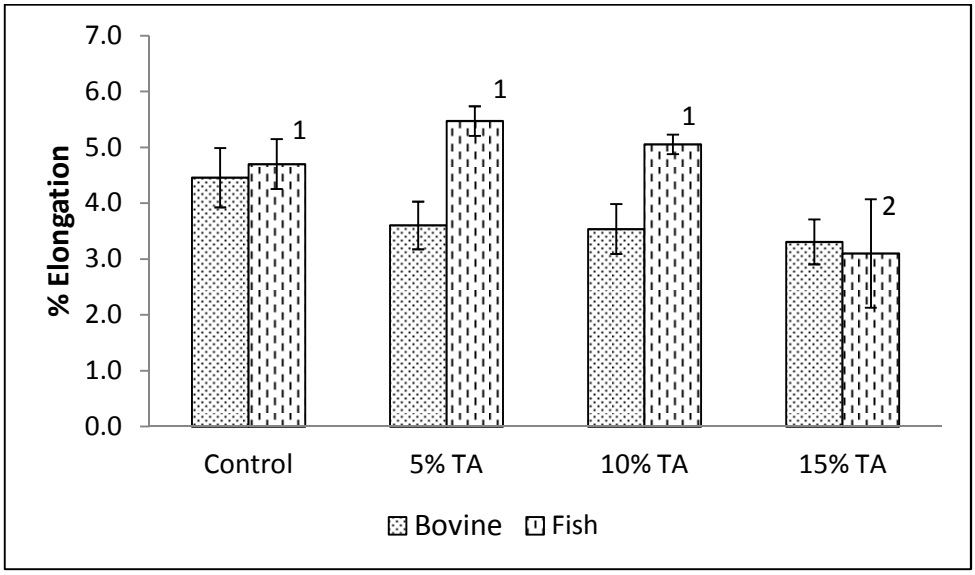

Fig 2. Percent elongation of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=8). Means with same numbers and without numbers are not significantly different ($P > 0.05$).

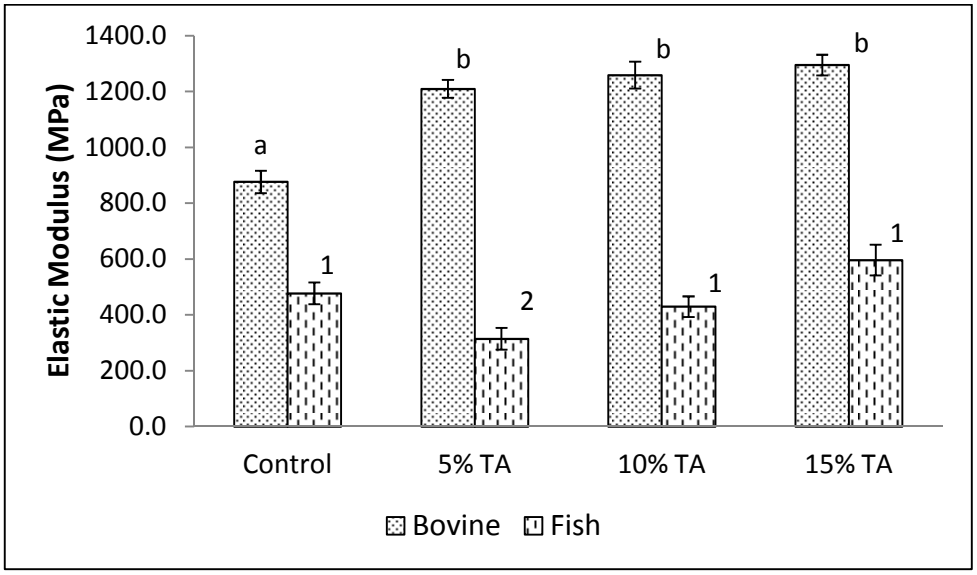

Fig 3. Elastic modulus (MPa) of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=8). Letters and numbers represent significant difference in the means of fish and bovine gelatin samples, respectively. Means with same letters or numbers and without letters or numbers are not significantly different ($P > 0.05$).

Water vapor permeability

Incorporation of tannic acid did not make a significant ($P > 0.05$) difference in the WVP of the bovine and fish gelatin films. However, samples with 15% tannic acid have lower WVP compared to controls. Difference was significant in case of bovine gelatin films. Tannic acid has numerous hydroxyl groups which can interact with water (Cao et al., 2006). As a result, the WVP of samples did not change significantly. No significant change in the WVP of tannic acid crosslinked bovine gelatins (Cao et al., 2006) and transglutaminase crosslinked fish gelatins (Piotrowska et al., 2008) was reported.

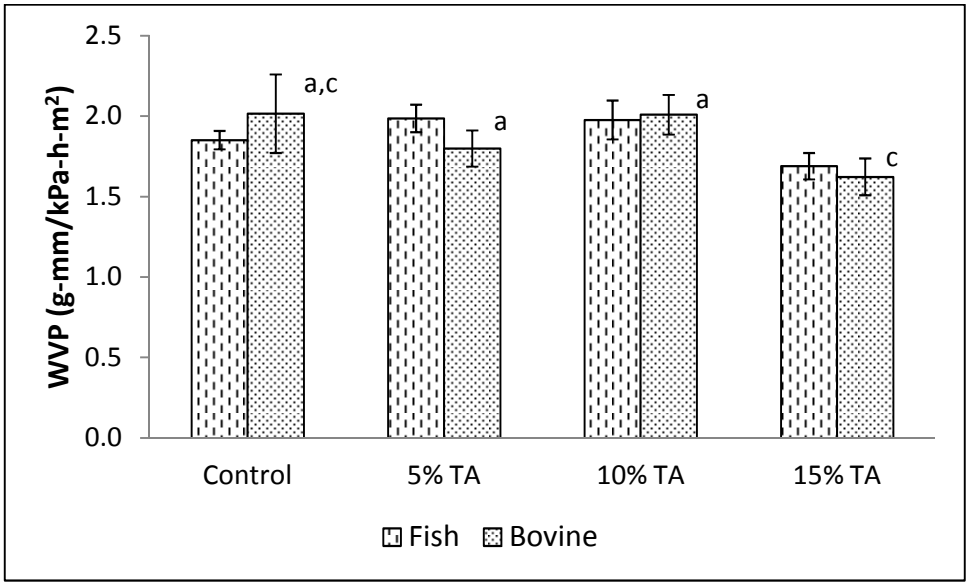

Fig 4. Water vapor permeability of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=6). Means with same letters and without letters are not significantly different ($P > 0.05$).

Water solubility

Solubility of bovine gelatin films reduced significantly ($P < 0.05$) with incorporation of tannic acid. The percent soluble matter was reduced from 55.4±3.9 to 12.9±1.7 with incorporation of 15% tannic acid in bovine gelatin films. In case of fish gelatin, solubility did not change significantly ($P > 0.05$) with incorporation of tannic acid. Film solubility increased for intermediate concentrations of tannic acids and reached value close to the control for fish gelatin films with 15% tannic acid. Increased degree of crosslinking can result in decreased combination of gelatin with water (Cao et al., 2006).

Approximately 8% decrease in solubility of tannin crosslinked chitosan was reported by Rivera et al. (2010). Solubility of bovine gelatin films crosslinked with tannic acid (3 wt %)

decreased significantly (Zhang et al. 2010b). Formation of covalent crosslinks in the film matrix was suggested as possible reason for reduction in film solubility. Reduction in solubility from 99 to 27% was reported for cod fish gelatin films crosslinked with 0.3 mg/ml transglutaminase (Piotrowska et al., 2008). Kolodziejska et al. (2006) reported reduction in solubility of chitosan-fish gelatin films crosslinked with transglutaminase. It is likely that the film solubility depends on the extent and type of crosslinking and the method used to determine solubility (boiling time and temperature).

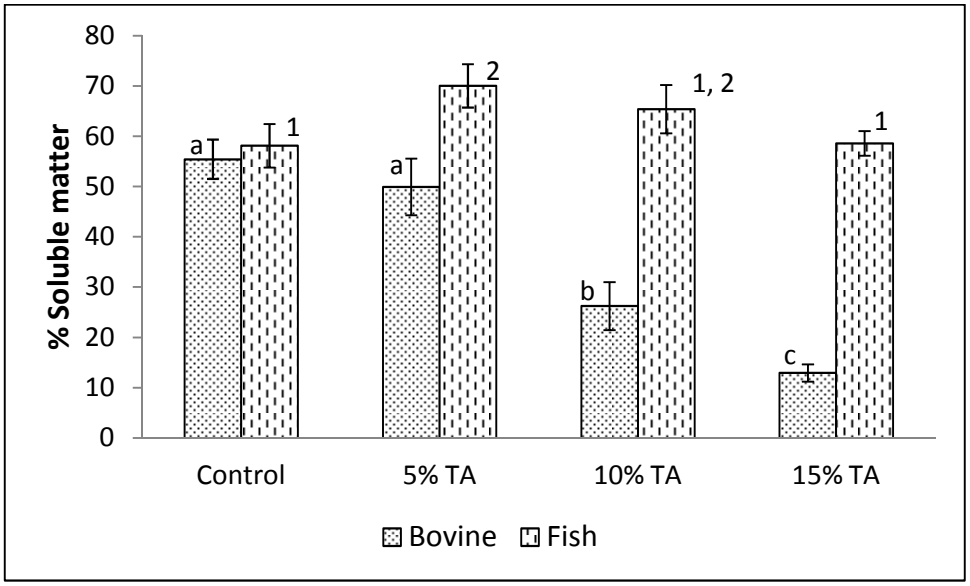

Fig 5. Water solubility of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=10). Letters and numbers represent significant difference in the means of bovine and fish gelatin samples, respectively. Means with same letters or numbers and without letters or numbers are not significantly different ($P > 0.05$).

Glass transition temperature

Material transition from a glassy or brittle state into a rubbery state over a temperature range is described by glass transition. Incorporation of tannic acid increased the glass transition temperature by 12 and 6^{o}C for bovine and fish gelatin films, respectively. When tannins are incorporated into the films, the film matrix becomes more rigid, which in turn increases the energy needed for transition into a rubbery state.

Table 1. Glass transition temperature (T_g) of bovine and fish gelatin films with tannic acid.

Treatments	Bovine gelatin films	Fish gelatin films
	Glass transition temperature (°C)	
Control	26.5±0.39	31.6±1.4
5% TA	33.68±1.05	28.7±1.0
10% TA	33.42±1.09	34.4±2.7
15% TA	38.75±0.77	37.8±0.7

Values represent means ± standard error of means (n=2).

Antioxidant activity of films

Bovine and fish gelatin controls showed 10% DPPH radical scavenging activity. Bovine gelatin controls retained this level of activity throughout the 20 day storage while the fish gelatin controls did not. Samples with tannic acid possess 70 to 90% radical scavenging activity. Level of tannic acid in the films had no significant effect ($P > 0.05$) on the radical scavenging activity of the films. Cao et al. (2006) reported an improvement in the properties of tannic acid crosslinked bovine gelatin films during 90 day storage. It was proposed that tannic acid interacts with gelatin in a step-by-step manner, resulting in improved properties (Cao et al., 2006; Frazier et al., 2003). Similar phenomenon might be responsible for changes observed in this study to retain radical scavenging activity during storage. However, it should be noted that effect of storage on other film properties was not investigated in this study.

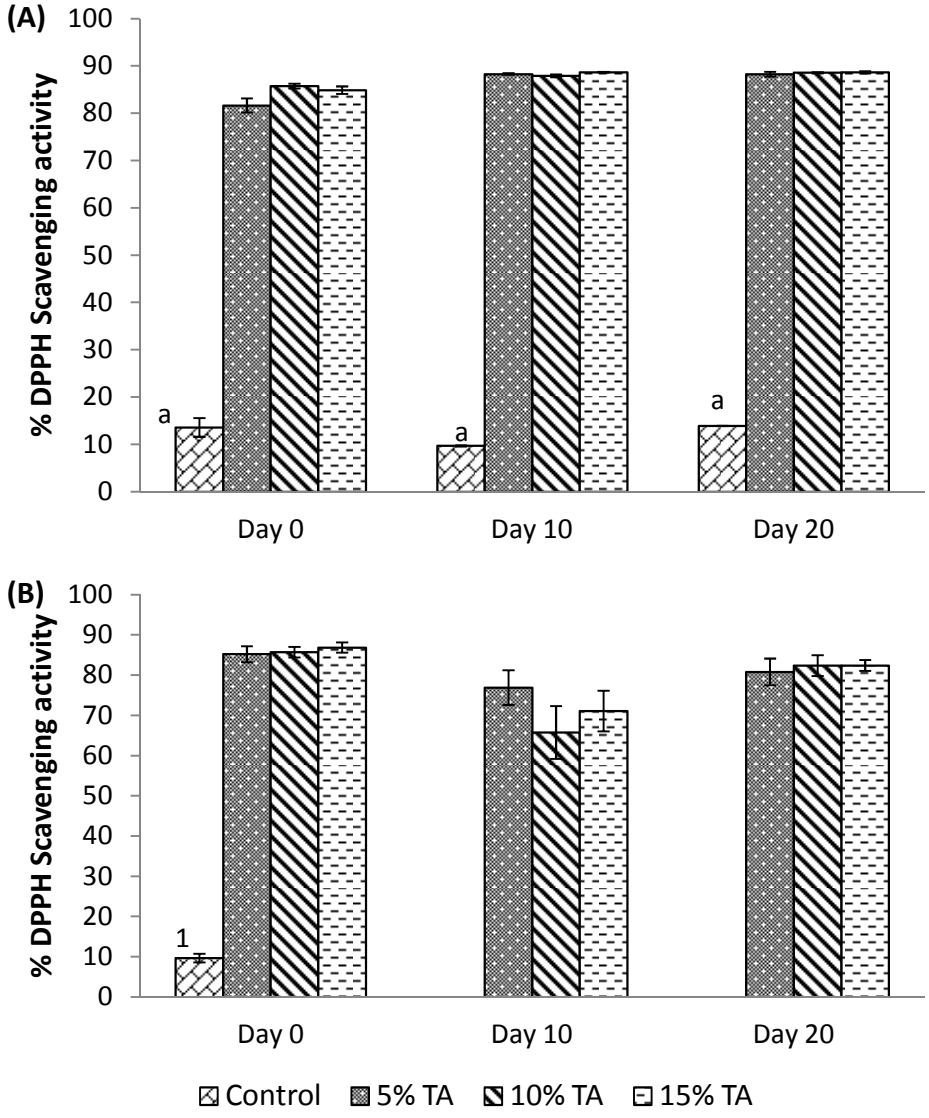

Fig 6. DPPH radical scavenging activity of tannic acid (0, 5, 10, and 15%) incorporated bovine (A) and fish (B) gelatin films. Letters and numbers represent significant difference in the means of bovine and fish gelatin samples, respectively. Means (n=5) with same letters or numbers and without letters or numbers are not significantly different ($P > 0.05$).

Effect of tannin incorporated gelatins on the oxidative stability of salmon fillets

There was a tendency for salmon fillets treated with bovine and fish gelatin solutions to show lower TBARS values compared to untreated controls (Table 2), but the trend was not strong and the results were similar after day 9. At 4^{o}C storage, bovine and fish gelatin treatments resulted in lower TBARS values than untreated samples and controls at day 3, 6 and 9. However, the differences were not significant ($P > 0.05$). Similar results were reported for samples stored at 10^{o}C.

It is likely that the treatment effect is being masked by the variability in the assay. Variability in TBARS can be due to several reasons. Degradation of fatty acids results in the formation of malondialdehyde (MDA). It is a minor component of fatty acids with double bonds. The MDA is used as indicator of oxidative rancidity in biological samples. Monoenolic form of MDA reacts with methylene groups of TBA and forms a complex (Mendens et al., 2009), which has absorption maxima at 532nm. However, TBA also reacts with other compounds like alkenals, alkadienes, pyridines, pyrimidines, sucrose and urea (Shahidi and Zhong, 2005). The generic term TBARS is used to describe these substances. Also, TBA reacts with other food compounds including carbohydrates, amino acids and pigments (Mendens et al., 2009). The TBARS assay can lack accuracy in certain food systems due to these factors. Underestimation of oxidation products in the sample can occur when MDA reacts with amino acids, proteins and glycogen in the food (Jo and Ahn, 1998). However, this method is widely used to assess oxidative spoilage in foods due to its simplicity.

Table 2. TBARS (mg MDA/Kg fish) values of salmon fillets stored at 4 and 10°C treated with bovine and fish gelatin solutions incorporated with 5, 10 and 15% (wt/wt of gelatin) tannic acid.

Storage time	Treatment	4°C		10°C	
		Bovine gelatin	Fish gelatin	Bovine gelatin	Fish gelatin
		mg MDA/Kg fish			
Day 0		1.16±0.15		1.29±0.09	
	Untreated	1.33±0.55	1.24±0.29	0.77±0.34	0.95±0.43
	Control	1.07±0.18	1.38±0.18	0.75±0.22	1.03±0.49
Day 3	5% TA	0.41±0.16	0.39±0.19	0.42±0.23	0.30±0.12
	10% TA	0.51±0.20	0.27±0.12	0.34±0.07	0.22±0.10
	15% TA	0.2±0.06	0.24±0.13	0.36±0.13	0.18±0.05
	Untreated	0.55±0.09	0.66±0.36	0.39±0.12	0.73±0.27
	Control	1.02±0.24	0.61±0.11	0.59±0.01	0.23±0.06
Day 6	5% TA	0.30±0.03	0.31±0.15	0.05±0.00	0.18±0.11
	10% TA	0.13±0.04	0.09±0.03	0.15±0.05	0.04±0.00
	15% TA	0.08±0.03	0.06±0.01	0.12±0.05	0.04±0.01
	Untreated	1.01±0.66	0.82±0.31	0.64±0.29	0.19±0.12
	Control	0.86±0.20	0.80±0.28	0.56±0.30	0.20±0.14
Day 9	5% TA	0.26±0.03	0.19±0.02	0.38±0.13	0.30±0.13
	10% TA	0.46±0.16	0.40±0.31	0.04±0.00	0.07±0.03
	15% TA	0.78±0.69	0.53±0.45	0.61±0.13	0.12±0.06
	Untreated	0.21±0.02	0.56±0.16	0.19±0.06	0.10±0.03
	Control	0.86±0.19	1.17±0.53	0.32±0.13	0.16±0.09
Day 12	5% TA	0.29±0.15	0.84±0.64	0.17±0.09	0.09±0.02
	10% TA	0.58±0.26	0.21±0.07	0.22±0.16	0.12±0.05
	15% TA	0.57±0.25	0.14±0.04	0.07±0.05	0.15±0.02

Values represent means ± standard error of means (n=3).

Conclusion

Incorporation of tannic acid resulted in reduction in solubility of bovine gelatin films and other minor changes to fish and bovine gelatin film properties. Treatments reduced the TBARS values of salmon fillets at best during the first 9 days of storage. However, general trend in data was not evident due to variability in the assay. Perhaps a more sophisticated assay such as HPLC would be more suitable to study treatment effects of the added antioxidant.

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Chapter 4

Inhibition of *Listeria monocytogenes* on rainbow trout (*Oncorhynchus mykiss*) using trout skin gelatin edible films containing nisin

Abstract

Contaminated food can pose high risk of listeriosis for susceptible populations. *Listeria monocytogenes* has been isolated from fresh, frozen and processed seafood products. Our goal in this study was to develop nisin-containing edible films using trout skin gelatin and test their effectiveness against *L. monocytogenes* on raw trout fillets (stored at 4^{o}C and 10^{o}C) as a strategy for reducing the risk of contamination. Films with 18 μg nisin/cm^{2} showed consistent inhibition and were chosen for storage studies conducted at 4 and 10^{o}C for 30 days. Trout fillets were challenged with 2 log CFU *L. monocytogenes*/g before or after coating with nisin-containing films. Films with nisin reduced *L. monocytogenes* counts below the detection limit (1 log CFU/g) at 4^{o}C. At 10^{o}C, a 0.3 to 1.1 log reduction was observed compared to controls by the end of storage. The effectiveness of treatments depended upon the concentration of nisin and storage temperature. The developed edible films have the potential to reduce pathogens on seafood and can be incorporated with a variety of antimicrobials.

Introduction

Listeriosis causes over 1600 cases annually in the USA with a projected average cost per case of $1 million for patient care and the associated costs of product recalls and market withdrawals (CDC 2011, Cagri et al. 2002). An average of 132 cases of listeriosis is

reported to occur annually in Canada (Public Health Agency of Canada, 2012). In 1999, 26 listeriosis cases with 7 deaths were reported in France due to contaminated pork tongue in jelly (WHO, 2013). Recent recalls have included seafood, salads, produce and dairy products. During a two month period between July and September 2012, *L. monocytogenes* was the cause of 30 food related recalls (FDA 2012). High risk population including pregnant women, infants, and people with compromised immune system are at greater risk of contracting listeriosis (Rocourt and Cossart, 1997). There is zero tolerance policy for this microorganism in ready-to-eat foods in the United States (FDA 2011).

An FDA/USDA risk assessment included seafood in the list of foods at high risk of causing human listeriosis on a per annum basis (Datta et al. 2008, Brett et al. 1998, Ericsson et al. 1997, Farber et al. 2000). Up to 25% of frozen seafood, smoked salmon, and raw fish may be contaminated with *L. monocytogenes* (Norton et al. 2000, Hoffman et al. 2003) including rainbow trout (Rocourt and Cossart, 1997). In one study, 17% of cold-smoked and 50% of salt-cured rainbow trout tested were contaminated with *L. monocytogenes* (Johansson et al. 1999). In a more recent study with fish roe products, 11.4% of cod roe (tarako) and 10% of salmon roe (ikura) were found to be contaminated with *L. monocytogenes* (Handa et al. 2005). Presence of *L. monocytogenes* in seafood is often traced back to contamination in processing environments (Shin et al. 2008, Vogel et al. 2001, Rorvik et al. 1995). Numerous factors contribute to the incidence of *L. monocytogenes* in seafood, including methods of processing, cleaning and sanitation in processing facilities and worker food handling practices. This indicates that improved sanitation controls resulting from the implementation of HACCP programs are critical and that greater efforts are needed to reduce risk.

There has been a great deal of interest in methods to control foodborne pathogens using naturally occurring antimicrobial agents and minimizing the use of synthetic chemical preservatives. Approaches to control *L. monocytogenes* on seafood include the addition of lactic acid bacteria (LAB) cultures which are capable of producing bacteriocins with antilisterial activity (Nilsson et al. 2004, Tome et al. 2008), addition of organic acid salts (Neetoo et al. 2008a) and bacteriocins (Al-Holy et al. 2005, Szabo and Cahill 1999). Selected LAB produce several compounds including organic acids, hydrogen peroxide, diacetyl, bacteriocins, antibiotics, acetaldehydes, oxygen metabolites and carbon dioxide which result in the inhibition of *L. monocytogenes* (Montville and Winkowski, 1997). However, LAB can reduce the shelf life of products by resulting in higher volatile nitrogen concentrations (Tome et al. 2006). Use of powdered bacteriocins is limited by their low solubility (Nilsson et al. 2004). Organic acids impart undesirable sensory attributes to the product (Shin et al. 2008). Since the approaches listed here have limitations, alternative control methods against *L. monocytogenes* are sought.

Control of several foodborne pathogens, including *L. monocytogenes,* with edible films containing antimicrobial compounds has been investigated. Different natural antimicrobials, either singly or in combination with others, can be incorporated into edible films to enhance their antimicrobial properties. Edible films can improve food safety by reducing the risk of microbiological contamination by prolonging antimicrobial activity on food surfaces. Edible films can modulate the diffusion of antimicrobial agents to the surface of food. Spraying of antimicrobial agents onto the surface of a food is usually less effective because of the rapid diffusion of the antimicrobial agent into the bulk of the food and the limited contact time with the food surface (Pranoto et al. 2005). Growth of microorganisms can be prevented or

minimized by antimicrobial edible films during storage of perishable seafood products. Min et al. (2005) showed that whey protein films incorporating a lactoperoxidase system could inhibit the growth of *L. monocytogenes* on the surface of smoked salmon and could preserve smoked salmon for 35 days at 4^{o}C and for 14 days at 10^{o}C. Similar antilisterial challenge studies were conducted on cold-smoked salmon by using alginate films and nisin-chitosan-coated plastic films (Neetoo et al. 2008a, Neetoo et al. 2008b, Ye et al. 2008). Antilisterial activity of whey protein isolate films with nisin and glucose oxidase was demonstrated by Murillo-Martinez et al. (2013). Chitosan coatings with cinnamon oil were used to enhance the microbiological shelf life of rainbow trout with a one log reduction of psychrotrophs and a two log reduction in total viable counts (Ojagh et al. 2010). Nisin and sodium lactate were shown to control *L. monocytogenes* on rainbow trout stored at 8^{o}C for 17 days achieving a 1.5 log reduction (Nykanen et al. 2000). Growth of *L. monocytogenes* was reduced by 2 logs on cold-smoked salmon stored at 4^{o}C using potato peel waste-based films containing oregano oil (Tammineni et al. 2013).

Nisin is the most popular bacteriocin and is produced by some strains of *Lactococcus lactis*. Since the 1950's, nisin has been used commercially in the UK and other countries, including the United States, where it was approved as a food additive in 1988. Nisin belongs to the Group I bacteriocins which are small peptides with sulfhydryl rings (Montville and Winowski, 1997). Nisin is used as a food preservative for processed cheeses, dairy desserts and canned foods. It has anti-bacterial activity against Gram-positive bacteria such as *Bacillus* spp., *Staphylococcus* spp. (Ray and Daeschel, 1994) and *Listeria* spp. (Brewer et al. 2002, Schillinger et al. 2001). Nisin can bind to negatively charged cell membranes creating pores and leakage of cell contents leading to cell death (Bruno et al. 1992).

There are numerous studies reported on the use of edible films to increase shelf life of meat products. However, from the scope of literature review, such studies are limited with aquatic food products. The objective of this study was to investigate the effect of trout gelatin films containing natural antimicrobial nisin on the growth of *L. monocytogenes* inoculate onto the surface of trout fillets.

Materials and methods

Gelatin extraction

For extraction of gelatin from trout skins, a modified method of Chiou et al. (2006) was followed. Trout skins (Big Bend Trout Inc., Buhl, ID) were thawed and washed three times with ice-cold water (2 to 5^{o}C) and with ice-cold 0.8 N NaCl. The ratio of weight of skins to volume of wash solution in each step was 1:6. Skins were stirred for 40 min in ice-cold 0.2 N NaOH and washed with tap water. This step was repeated three times. After NaOH wash, skins were washed using ice-cold 0.2 N H_2SO_4 and ice-cold 0.7% citric acid in a similar manner as with the NaOH solution. After completion of all the washings, the skins were totally immersed in distilled water at 45^{o}C for overnight. The skins were removed from the solution and the solution was filtered using Whatman filter #4 and subsequently freeze-dried (Freeze Mobile 600L, Virtis Company, Gardiner, NY) to obtain gelatin.

Preparation of gelatin films containing nisin

Film formation protocol was adopted from Krishna et al. (2011). Gelatin solution (6.75% w/w) was stirred for 30 min at room temperature. The solution was denatured for 30 min in a 90^{o}C water bath and cooled to room temperature. Glycerol (Sigma, St. Louis, MO, USA)

was added as a plasticizer (gelatin:glycerol ratio = 1:0.2). Nisin (MP Biomedicals, Solon, OH, USA), at different concentrations, was dissolved in sterile deionized water and added to the film-forming solution. The solution was degassed under vacuum until no visible air bubbles were present. The degassed solution was poured onto Teflon plates (16 cm internal diameter, 4 mm groove depth and 5 mm thickness) and left to dry for 24 h at 25^{o}C, 30% RH. To attain a uniform film thickness of 0.1 mm in all films, a constant dry solid content of 3 g was maintained. The area of each film was approximately 200 cm^2. The prepared films were stored until further use in a 50% RH chamber kept at room temperature.

Bacterial strains, growth conditions and enumeration

A cocktail of *L. monocytogenes* strains ATCC 19114, ATCC 7644 and ATCC 19113 was prepared. The strain 19114 was originally isolated from animal tissue and the strains 7644 and 19113 are human clinical isolates (ATCC, 2012) selected for their importance in foodborne outbreaks and adaptation to low temperature conditions. Each strain was grown to an A_{600} value of 0.8 to 1 at 37^{o}C in 10 mL Tryptic Soy Broth with 1% yeast extract (Becton Dickinson, Sparks, MD, USA). Equal volumes (10 mL each) of three cultures were combined and centrifuged (AccuSpin400 bench top centrifuge, Fisher Scientific, Pittsburgh, PA, USA) at 2,500 *g* for 30 min. The pellet was suspended in 30 mL of 0.2% buffered peptone water (Becton Dickinson, Sparks, MD, USA), vortexed and centrifuged again under same conditions. A washing step with 0.2% buffered peptone water was repeated three times to remove residual media. After the final wash, *L. monocytogenes* pellet was suspended in 0.2% buffered peptone water to prepare a cell suspension with approximately 5 log CFU/mL for inoculation in subsequent experiments. Tryptic Soy Agar with Yeast Extract (TSAYE;

Becton Dickinson, Sparks, MD, USA) was used for determination of minimum inhibitory concentration (MIC) of nisin and for demonstrating the antilisterial activity of the films. For enumeration of *L. monocytogenes* during microbial challenge studies, Tryptic Soy Agar, PALCAM agar (Becton Dickinson, Sparks, MD, USA) and TSA-PALCAM overlay plates (Kang and Fung 1999) were used. Overlay plates were prepared with PALCAM agar on the bottom and TSA on the top. These plates were incubated for 24 h at 37^{o}C and enumerated.

Antilisterial activity of nisin on microbiological growth media

A stock solution of 1.5 mg nisin/mL (activity of 1.2 x 10^8 IU/g) in deionized water was prepared (Padgett et al. 2000). The stock solution was diluted (1:1 to 1:40) to 0.75 to 0.03 mg nisin/mL. Agar-spot-on-lawn method was used to determine the sensitivity of strains towards nisin (Dimitrieva-Moats and Ünlü, 2011). Briefly, TSAYE spread plates were inoculated with 10^8-10^9 CFU/mL of *L. monocytogenes* to produce a lawn. These plates were spotted with 10 µL of the prepared nisin dilutions, incubated for 24 h at 37^{o}C and observed for zones of inhibition.

Antilisterial activity of gelatin films containing nisin on microbiological growth media

Gelatin films were prepared at 10 different concentrations of nisin (0, 0.125, 0.187, 0.25, 0.50, 0.75, 1.0, 1.25, 3.75, and 6.25 mg nisin/film). This translates to nisin concentrations of 0, 0.6, 0.9, 1.0, 2.0, 3.0, 5.0, 6.0, 18, and 31 µg/cm^2 of film. Disks with a diameter of 0.7 cm were made from the films using a cork borer. These film discs were placed on TSAYE plates with a lawn of *L. monocytogenes* (Zivanovic et al. 2005) generated using both spread

and pour-plating techniques. The plates were incubated for 24 h at 37^{o}C and the diameter of the zone of inhibition was measured.

Microbiological challenge studies with trout

Fresh rainbow trout (*Oncorhynchus mykiss*) was purchased from a local grocery store in Pullman, WA, USA. Fillets were sliced into 25±0.5 g pieces, placed in sterile Petri plates and air dried in biological safety hood (recommended for BSL-2 practices) for 10 min. The sliced fillet samples were challenged with 1.85 to 2.03 log CFU *L. monocytogenes*/g fillet. The level of inoculation was determined based on preliminary experiments with fresh fish. An inoculum of 100 µL was spotted at 20-25 locations on the surface of fish fillet and spread with a sterile hockey stick. Samples were allowed to air dry for 30 min in a biological safety hood. Two modes of inoculations were used. Half of the samples were inoculated first, dried and then covered with films containing 18 µg nisin/cm^2 (designated as inoculate and coat: I+C). The other half were coated with film (18 µg nisin/cm^2) first, inoculated and dried (designated as coat and inoculate: C+I). Petri plates containing inoculated fish were placed in UV-sterilized Ziploc® bags and were stored at 4 and 10^{o}C for up to one month. The 10^{o}C storage was selected to determine the effect of moderate temperature abuse. Preliminary studies (data not shown) indicate that 30 days (at 4^{o}C) was a week past the limit of shelf-life for this food product. Microbiological analysis was performed on the 0, 5, 10, 20 and 30th day. Untreated trout fillets and those covered with films (no nisin) served as controls.

Enumeration of *L. monocytogenes*

At each time interval, trout samples were placed in sterile stomacher bags (Fisher Scientific, Pittsburgh, PA, USA) with 225 mL of 0.2% buffered peptone water. Samples were homogenized using a stomacher (400 Circulator, Seward, London, UK) for 2 min at 230 RPM. After homogenization, 1 mL of aliquot from the sample was serially diluted with 9 mL of sterile buffered peptone water and plated on TSA (non-selective medium), PALCAM (selective medium) and TSA-PALCAM overlay plates. Colonies on plates were enumerated after incubation at 37^{o}C for 24 h.

Statistical analysis

Inhibition zone tests (n=3) were conducted with duplicate analyses per test. Microbial challenge experiments were performed in duplicate (n=2) with duplicate plating at each time point. For statistical analysis of microbial challenge studies, completely randomized design was used. Data were analyzed using one-way analysis of variance (ANOVA) procedure using Minitab (Minitab 14, Minitab Inc., State College, PA, USA). When the effect was significant ($P < 0.05$), the mean separation was determined using Tukey's pair wise comparison. Tukey's pair-wise comparison with a family error of $\alpha = 0.05$ was used to determine significance of the means.

Results and discussion

Nisin activity

When the agar-spot-on-lawn technique was used, the lowest concentration of nisin stock that showed clear zone of inhibition was 0.04 mg/mL. Since higher nisin concentration would be needed to show antimicrobial effect due to diffusion limitations in food systems, films with a range of nisin concentration (0.6 to 31 μg nisin/cm^2) were prepared. The diameter of inhibition zones were not significantly different ($P > 0.05$) from each other over the range of 0.6 to 5 μg nisin/cm^2 (Table 1). However, zones of inhibition were significantly different ($P < 0.05$) for films with 6, 18, and 31 μg nisin/cm^2 each of which showed a clear zone of inhibition with measurements of 0.88±0.1, 1.24±0.06 and 1.41±0.06 cm, respectively. Zones of inhibition were more obvious in the spread-plate technique than the pour-plate technique especially at lower concentrations of nisin (Fig. 1). It is likely that the bacterial cell density was higher in pour plates compared to spread plates. Also, L. monocytogenes grew on and under the surface of agar in pour plates. The intermediate concentration of 18 μg nisin/cm^2 was used for subsequent experiments.

Table 1. Zones of inhibition on a lawn of *L. monocytogenes* in the presence of trout gelatin films with different concentrations of nisin

	Amount of nisin in film (μg/cm^2)									
	0.0	0.6	0.9	1.0	2.0	3.0	5.0	6.0	18.0	31.0
Diameter of zone of inhibition (cm)	No zone	0.62± 0.12	0.63± 0.04	0.69± 0.08	0.69± 0.05	0.68± 0.04	0.71± 0.01	0.88± 0.01[a]	1.245± 0.06[b]	1.41± 0.06[c]

Values represent means ± standard error of means (n=3). Means followed by different letters (a, b, c) are significantly different ($P < 0.05$) and means with no letters are not significantly different ($P > 0.05$). Means were calculated from 6 replicates from 3 independent experiments.

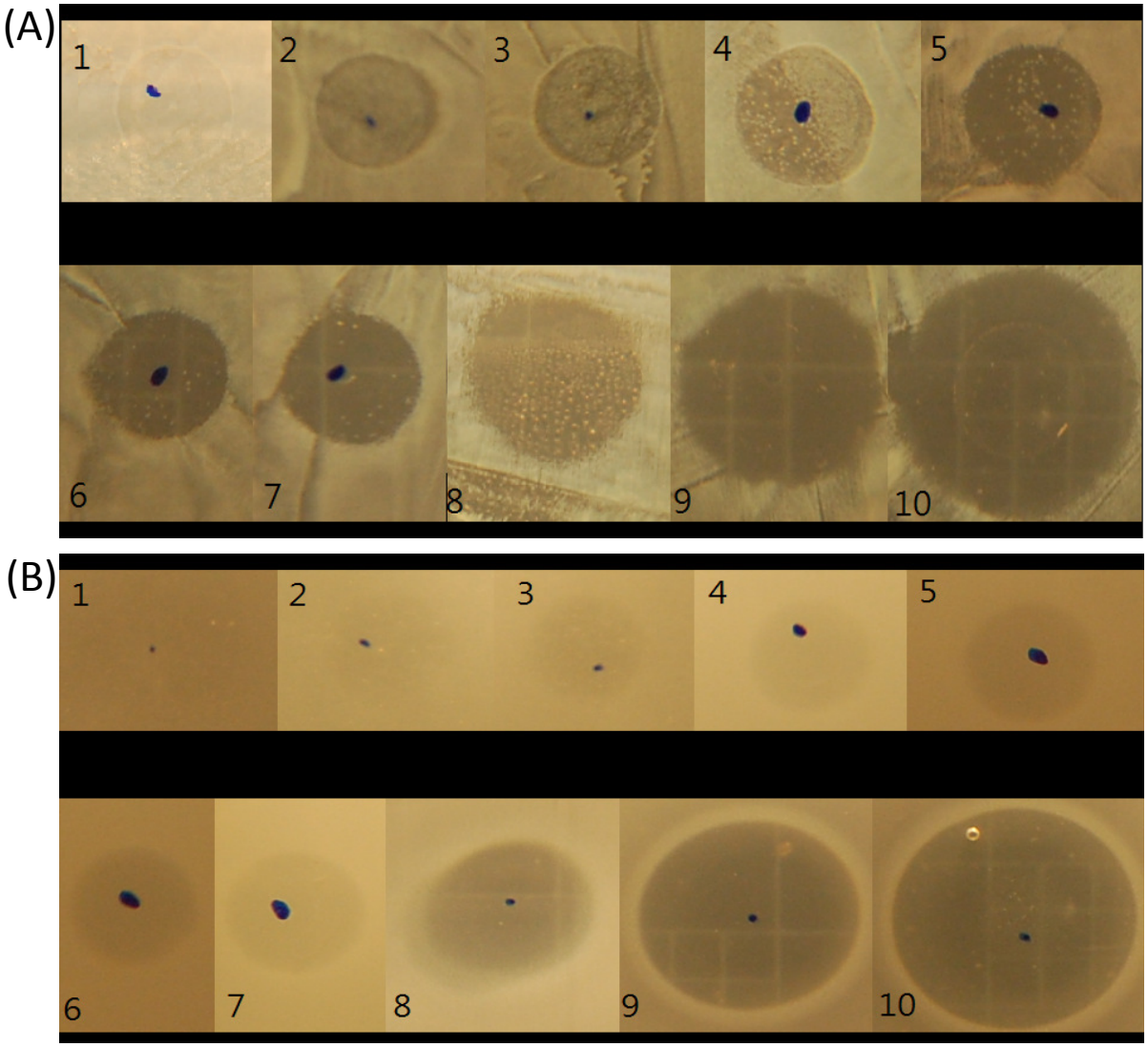

Fig 1: Zones of inhibition around trout gelatin film discs with different concentrations of nisin using spread plating (a) and pour plating technique (b) for plating *L. monocytogenes* cells (n=3). Numbers 1-10 correspond to nisin concentrations ranging from 0 to 31 µg nisin/cm^2.

Microbiological challenge studies on trout

Growth of *L. monocytogenes* on inoculated-uncovered trout

L. monocytogenes was not detected in fresh trout as purchased. For the inoculated-uncovered trout control stored at 4°C, counts of *L. monocytogenes* increased from 2±0.1 to 7.43±0.1 log CFU/g by day 30 (Fig. 2a). At 10°C, counts increased to 7.56±0.27 log CFU/g by day 20 and decreased to 6.05±0.1 CFU/g by day 30 of storage (Fig. 2b) possibly due to the presence of competitive microorganisms.

Antilisterial activity of films with nisin

Growth of *L. monocytogenes* on trout covered with nisin-containing films was below the detection limit (< 1 log CFU/g) at 4°C storage (Fig. 2a). On the I+C and C+I control films (no nisin), *L. monocytogenes* growth increased to 5.37±1.0 and 4.72±0.1 log CFU/g, respectively, by the end of storage time (Fig. 2a). This shows that films with nisin effectively inhibited growth of *L. monocytogenes* at 4°C.

Nisin-containing films inhibited growth of *L. monocytogenes* for five days at 10°C with cell numbers being approx. 3 log CFU/g at day 10, 4 log CFU/g at day 20 and 5 log CFU/g by day 30 (Fig. 2b). Control films provided some reduction compared to uncovered trout by 1 to 2 log at each time point with the cell numbers for I+C and C+I reaching 6.04±0.8 and 5.3±0.25 log CFU/g, respectively by day 30. This might be due to the lack of access of bacterial cells to the surface of trout fillet because of the film. Cell numbers for nisin-containing and non-nisin-containing films were significantly different. These results indicate that temperature control during storage is critical and that antimicrobial treatments can be

much less effective with a relatively small change in storage temperature. A storage temperature of 10^{o}C was chosen in our study because 20-25% home refrigerators in the US are between 7 to 10^{o}C (FDA, 2009). Trout held at 10^{o}C had a short shelf life and spoilage became apparent at around day 7 (data not shown).

In general, listeria counts were higher in I+C samples coated with control film (4 and 10^{o}C) and nisin film (4^{o}C) compared to C+I samples. With I+C mode of inoculation, bacterial cells would be in direct contact with the surface of trout fillet ensuring abundant nutrient availability. In addition, *L. monocytogenes* might have been protected from the antimicrobial effect due to the rough surface of fish fillet (Min et al. 2005) reducing contact of nisin with bacterial cells. Data shown in Figure 2a, b are for recovery of *L. monocytogenes* on TSA-PALCAM overlay plates. There was a trend for *L. monocytogenes* counts to be higher on TSA-PALCAM overlay plates compared to PALCAM plates at all time points in this storage study. High cell recovery on overlay plates was reported compared to selective media (Kang and Fung, 1999). In addition, soluble matter (approx. 85%) of films might have affected the antimicrobial activity of films.

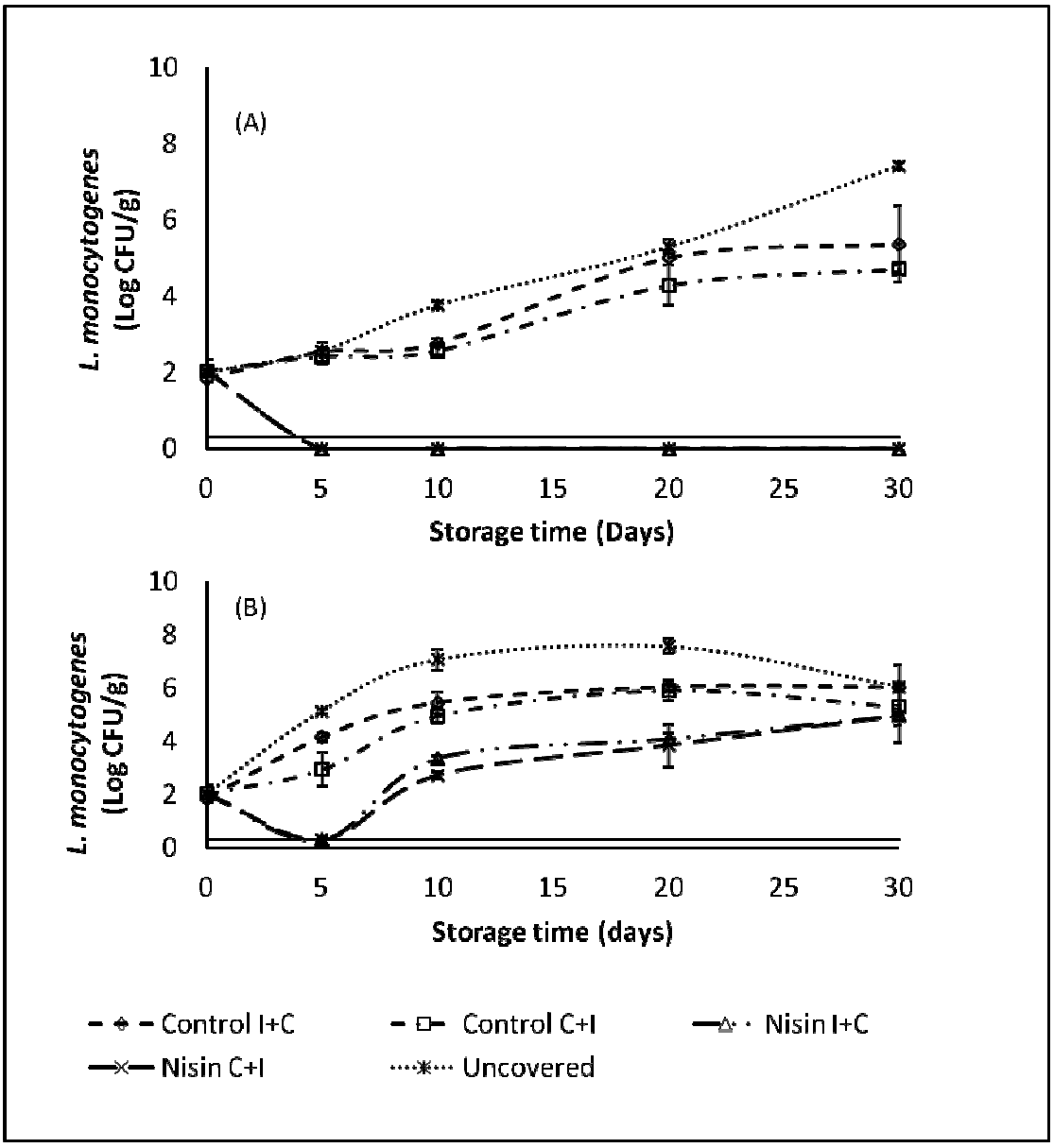

Fig 2. Counts of *L. monocytogenes* on trout fillets during storage at 4°C (a) and 10°C (b). Treatments include: uncovered, control film (no nisin) and nisin film (18 μg nisin/cm^2) covered samples. Values are represented by means ± standard error of means (n=2). Detection limit is 1 log CFU/g. The two modes of inoculation are denoted as C+I (Coat and Inoculate) and I+C (Inoculate and Coat).

Total aerobic plate counts

There was no significant difference ($P > 0.05$) in the number of total aerobic bacteria between treatments (Figure 3a, 3b). Counts were between 8 to 9 log CFU/g by the end of storage period at both temperatures. Fish spoilage under refrigeration conditions is caused by various microbiota, including LAB, *Photobacterium phosphoreum* (Budsburget al. 2003) and the members of the family *Enterobacteriaceae*. LAB can grow rapidly under refrigeration (Civera et al. 1995, Leroiet al. 1998, Hansen and Huss 1998) and certain LAB are tolerant to nisin (Montville and Winkowski, 1997). The LAB are likely among the spoilage microbes observed in this study. Nisin itself is not effective against Gram-negative bacteria. Off odors resulting from spoilage were detected prominently in samples stored at 10^{o}C beginning at day 7 indicative of the presence of organic acid production and proteolytic decomposition.

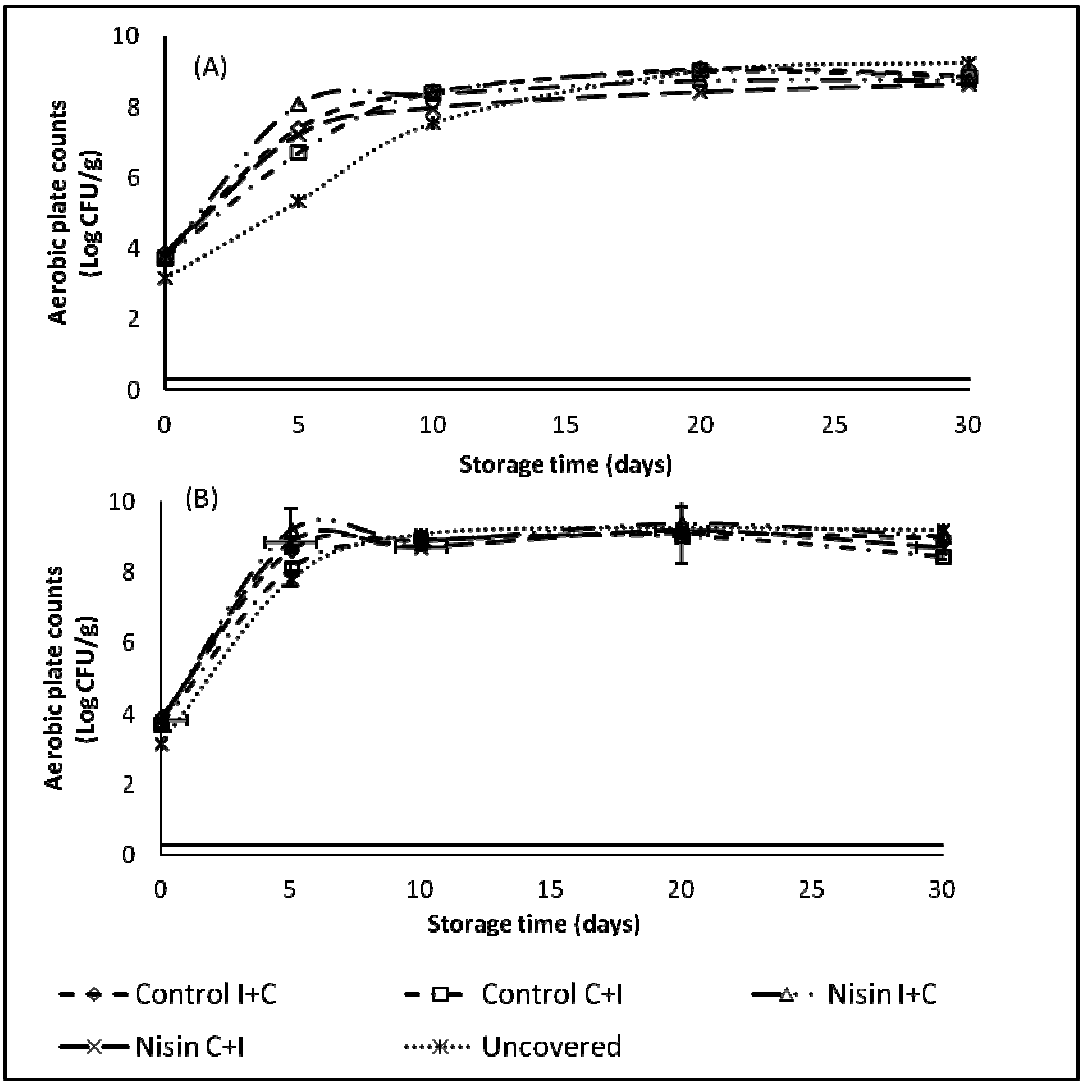

Fig 3. Aerobic plate counts on trout fillets during storage at 4°C (a) and 10°C (b). Treatments include: uncovered, control film (no nisin) and nisin film (18 μg nisin/cm^2) covered samples. Values are represented by means ± standard error of means (n=2). The two modes of inoculation are denoted as C+I (Coat and Inoculate) and I+C (Inoculate and Coat).

Conclusion

L. monocytogenes growth on trout fillets stored at 4^{o}C could be controlled by the application of trout gelatin films containing nisin (18 μg nisin/cm^{2}). The effectiveness of nisin-containing films depended on storage time, temperature and initial microbial load. Aerobic microbiota was not reduced with film treatment. Our future work will focus on improving the physical properties of fish gelatin films incorporated with nisin and other natural antimicrobials. Various microorganisms can be inhibited using edible films incorporated with different antimicrobials. However, attention must be paid to the interactions between antimicrobial agents as they may increase or decrease antimicrobial activity. Since food safety is a combination of several factors like temperature and water activity, use of antimicrobial edible films would be an additional hurdle to ensure food safety.

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Chapter 5

Recommendations for Future Work

Antioxidant fish gelatin films were developed by incorporating green tea powder and EGCG into the films. Films were effective in reducing lipid oxidation of cod liver oil. To enhance the fish gelatin film properties, tannic acid was incorporated into the films. However, incorporating tannic acid did not change film properties significantly. Effect of tannic acid incorporated gelatins (bovine and fish) on oxidative parameters of salmon fillets was determined using TBARS assay. The TBARS values were lower for treatments; however, there was variation in data. Antimicrobial fish gelatin films developed using nisin were able to control growth of *L. monocytogenes* on trout fillets. Based on the research done, the following recommendations for future work are being made.

Studying the microbiology of fish fillets in conjunction with lipid oxidation studies can be beneficial. Specifically, monitoring the microbial growth on fresh salmon treated with gelatin solutions incorporated with various antioxidants can explain the change in oxidative parameters of fish fillets during storage. Correlation between microbiological growth and oxidative parameters, if any, can be explained by conducting a study of this type.

Antioxidant or antimicrobial activity of edible films can vary with the nature of application (dipping in coatings vs. using preformed films). Examining the effect of dipping fish fillets in gelatin solutions vs. covering fillets with preformed films (containing antioxidants and/or antimicrobials) can reveal any differences due to the nature of coating.

There is significant variation in the oxidative parameters of fresh salmon fillets during storage. It is sometimes difficult to study the treatment effect due to such variations, resulting from the type and location of fat in the fish tissue sampled. For example, the fat in the tissue along the lateral line may be more susceptible to oxidation because of the high concentration of heme iron compared to the adipose fat associated with a fish fillet taken from the ventral portion of the fish. Conventional assays for oxidation may not be suitable due to interference from food components and microbial metabolites. Use of a more sophisticated method, such as HPLC, to determine the oxidative parameters of seafood during storage can help study the treatment effects better.

There are several studies on crosslinking bovine and porcine gelatins. Such studies on fish gelatins are limited. Studying the reaction chemistries of natural crosslinkers, such as tannic acid, and fish gelatin can help develop crosslinked fish gelatins with enhanced functional properties. Application of fish gelatins as coating to meat and seafood is greatly limited by their water sensitivity. Crosslinked gelatins tend to be less sensitive to water and can be used in a variety of applications.