ABSTRACT

CHENG, CHRISTOPHER JASON. Controlling *Listeria monocytogenes* in Cold Smoked Salmon with the Antimicrobial Peptide Salmine. (Under the direction of Clinton Stevenson).

Cold smoked salmon contains no Listericidal steps during processing. This ready to eat seafood product is processed by brining fillets in a 3-5% salt solution and then smoking at 37ºC for 6 to 24 hr to achieve a shelf life of typically 2-4 weeks. *Listeria monocytogenes* (LM) is a psychrotrophic and halotolerant pathogen that can survive this process and is a major safety concern for smoked salmon producers. It is endemic in the environment and can easily contaminate the fish during and after processing and continue to grow during warehousing, distribution and storage.

Salmine is a cationic antimicrobial peptide derived from the milt of salmon that has been shown to inhibit the growth of LM *in vitro*; however its antimicrobial efficacy has not been tested in smoked salmon. This peptide could slow or prevent the growth of LM after contamination thus extending the shelf life past 4 weeks. It would also add value to a waste product produced when raising salmon. This ingredient has the potential to be listed as a natural ingredient on packaging labels of smoked salmon products since it is derived from salmon tissues.

Cold smoked salmon was treated with 0.5, 2.5, and 5 mg/g salmine/g salmon and then inoculated with approximately $1x10^3$ cells of LM. The viable counts were then enumerated after 0, 1, 4, 7, 13, 22, and 32 days storage at 4°C. Salmine concentrations ranging 2.5-5 mg/g delayed the growth of LM on smoked salmon samples. No growth of LM was observed on these samples for the first 7 days. On days 13 and 22, LM counts on these

samples were significantly *($p < 0.05$)* lower than the counts on the untreated samples by >1 log. By day 32, however, the antimicrobial effect of salmine was not apparent.

Antimicrobial films composed of k-carrageenan and agar were used in an effort to improve the antimicrobial efficacy of salmine. Cold smoked salmon samples were dipped into film forming solutions composed of 1% biopolymer and 10mg/mL salmine. They were then inoculated with approximately $1x10^3$ cells of LM. The viable counts were then enumerated after 0, 1, 4, 7, 13, 22, and 32 days at 4°C storage. The edible films did not enhance the antimicrobial efficacy of salmine. This is possibly due to too much binding between the peptide and the biopolymer components of the film. These results suggest there is potential for salmine to be used as a natural hurdle to inhibit growth of LM due to post process contamination; however, future research is warranted to extend its effect throughout the shelf life of smoked salmon products.

Controlling *Listeria monocytogenes* in Cold Smoked Salmon with the Antimicrobial Peptide Salmine

by
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BIOGRAPHY

Chris was born on November 21, 1989 in Munster, Indiana. He eventually graduated from Munster High School in 2008, and attended Purdue University for his undergraduate studies. In 2012, he obtained his Bachelor's in Science in Food Science. In the fall of 2012, Chris attended North Carolina State University for his Master's degree in Food Science under Dr. Clint Stevenson.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 1

1. Smoked Salmon

Smoked salmon is a delicacy that is part of the growing seafood market. It is currently estimated to be worth $16.2 billion in the United States, and it is projected to grow to $19.5 billion by 2018 (Frank 2013). Smoked salmon is first cured by wet brining, dry brining, or direct injection. In wet brining, the salmon is dipped into a brining solution with salt, sugar and other spices. Dry brining involves applying salt, sugar, and other seasonings directly to the meat without water. The third way is a direct injection of the brine into the meat. In each method, a final water phase salt concentration of 3-5% is desirable which lowers the water activity to 0.95 (Huss, Embarek, Jeppesen 1995). After brining, the salmon is subjected to cold smoking or hot smoking (Figure 1). Cold smoking maintains the fresh raw fish texture of the salmon and is more common than hot smoking. It entails heating salmon to 37°C for 6-24 hours. Hot smoking salmon involves cooking until internal temperatures reach 60-70°C. The meat is then cooled, vacuum packed, and stored at refrigeration temperatures for distribution.

Smoked salmon is a ready to eat product. It falls under the FDA's definition of ready to eat foods, as it is "in a form that is edible without additional preparation to achieve food safety" (FDA 2013). Consequently, *Listeria monocytogenes* contamination is a major food safety concern, as it can contaminate this product and cause serious illness to consumers.

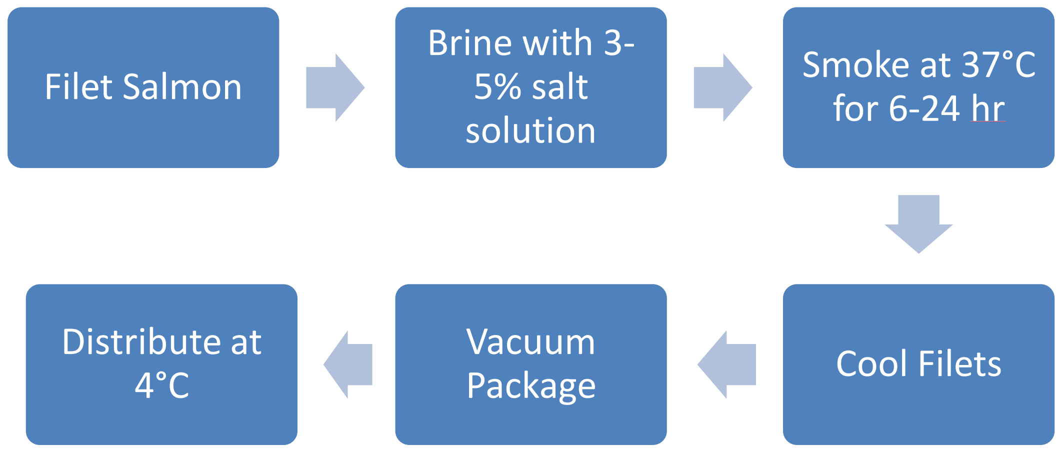


Figure 1: Smoked Salmon Processing Flow Diagram

2. *Listeria monocytogenes* (LM)

Listeria monocytogenes (LM) is a pathogenic gram positive organism facultative anaerobic bacterium that is a major concern in ready to eat food products. LM is psychrotrophic and can grow at temperatures that range from 1°C to 45°C (Kramarenko et al. 2013). Refrigerated ready to eat products contaminated with LM could have a high enough population to cause illness which is estimated to be about 1000 CFU/g (Farber and Peterkin 1991). Ingestion of LM can cause listeriosis, a serious infection for vulnerable populations like the young, old, pregnant, and immunocompromised. This disease is characterized by gastrointestinal distress, fever, and muscle aches among healthy individuals. In vulnerable populations, there are more severe symptoms. Infections among pregnant women can lead to serious threats to the fetus and may cause stillbirth or miscarriages. Immunocompromised populations may suffer from meningitis and septicemia (CDC 2013).

This bacterium is a major concern for food processors, especially those that process ready to eat foods. This bacterium is readily isolated from plants, soil, sewage, environmental water, and processing plants (Farber and Peterkin 1991). It is difficult to remove all environmental LM contamination from food plants as some strains can form biofilms which can survive many sanitation chemicals like QUAT, chlorine, and peroxides (Farber and Peterkin 1991; Pan, Breidt, Kathariou 2006). Because this pathogen is so ubiquitous in the environment, it can contaminate smoked salmon before and during processing (Embarek 1994).

LM can be isolated from smoked salmon after processing. There was a high variation in the percentage of smoked salmon samples testing positive for LM (Eklund et al. 1995). In the United States, it was found that 12-16% of tested samples of smoked salmon were positive with LM (Cartwright et al. 2013; FAO 2004; Rorvik, Caugant, Yndestad 1995) with contamination levels approximately at 10 CFU/g or less of LM (FAO 2004; Huss, Jorgensen, Vogel 2000). It is estimated that there are 0.053 cases of listeriosis per 1 million servings of cold smoked fish (FAO 2004). In comparison, there are one tenth as many cases for the same amount of servings of milk, and it is estimated that there are 0.0000021 cases per 1 million servings for fermented meat products, which is 5 orders of magnitude lower than smoked fish (FAO 2004).

3. Control of *Listeria monocytogenes* in Smoked Salmon

Traditional processing methods cannot guarantee the inactivation of LM that may have contaminated smoked salmon. Brining both enhances flavor and prevents most pathogen growth. However, it is ineffective at inhibiting the halotolerant LM. The water phase salt content of smoked salmon usually ranges from 3-5%, which has no effect in inhibiting growth of LM (Peterson et al. 1993). Because this bacterium can survive in the brine solution, the brining step itself can be a source of contamination of the fish (Autio et al. 1999; Eklund et al. 1995). Dry curing is not a better alternative, as there is no difference in pathogen inhibition between dry curing and wet curing (Peterson et al. 1993).

Cold smoking salmon can reduce the LM load in the short term (< 24hr), but it is unreliable at completely eliminating all LM on the meat (Rorvik, Caugant, Yndestad 1995). At 37°C, the heat of the cold smoking process is not lethal to LM (Hitchins and Jinneman 2011). The smoke itself can be bactericidal to pathogens on the surface of the meat only if the filets are wet. Air drying the filets before smoking allowed LM cells time to attach under the pellicle and survive the smoking process (Eklund et al. 1995).

In addition to the fact that LM can survive both the brining and cold smoking process, this pathogen is psychotropic. At refrigeration temperatures, LM can grow one log cycle a week in vacuum packed cold smoked salmon, which may reach lethal levels during storage and distribution (Huss, Embarek, Jeppesen 1995). This is a major issue for manufacturers of smoked salmon, because in the United States, there is a zero tolerance policy of finding LM in a 25g sample of Ready-to-Eat foods (Pan, Breidt, Kathariou 2006).

Currently there are two methods to minimize the risk of listeriosis from consuming smoked salmon (Huss, Embarek, Jeppesen 1995). The first method is to minimize LM contamination by strictly adhering to a detailed Hazard Analysis Critical Control Point (HACCP) system and Good Manufacturing Practices (GMP). However, this is not a good option by itself in controlling LM as no critical control point can be identified to control this pathogen (Huss, Embarek, Jeppesen 1995). The second method is to store the fish at freezing temperatures to prevent LM outgrowth and lower the shelf life to a maximum of 3 weeks to ensure the product is consumed before LM can grow to dangerous levels (Huss, Embarek, Jeppesen 1995).

4. Recently Studied Methods in Controlling *Listeria monocytogenes* in Cold Smoked Salmon

4.1 Alternative Processing

Alternative non thermal processing such as irradiation, modified atmosphere packaging, and high pressure processing can be used to reduce microbial load. High pressure processing (HPP) is a technique to inactivate enzymes and microbes by applying pressures ranging from 100-1000 MPa onto food products (Cheftel 1995). This is done by submerging the product into a liquid that has low compressibility, such as water, inside a sealed container. The pressure is raised and held for about 10 minutes for smoked salmon (Medina et al. 2009). One advantage of this process is that it is more energy efficient than thermal processing without as much degradation of the organoleptic properties of food. For example, raising the pressure of a system to 400 MPa takes the equivalent amount of energy to raise the temperature to about 30ºC (Cheftel 1995). Processing cold-smoked salmon at 250 and 450 MPa for 10 minutes combined with a treatment of lactoperoxidase kept the viable count of LM below detectable limits for 35 days at 5°C. However, these treatments did change some the color and the firmness of the salmon (Montiel et al. 2012). One major disadvantage of utilizing this process is its high capital cost. It is estimated that industrial scale equipment can cost anywhere from 0.6-5 million dollars (Hogan, Kelly, Sun 2005).

Irradiation is another non-thermal process that results in less degradation in quality of the product when compared to thermal processing. It has been found that 2 kGy of X-Ray irradiation was able to completely inhibit any outgrowth of LM in smoked salmon stored at 5°C (Mahmoud 2012). Similar results were obtained when electron beams were utilized instead of X-rays (Su, Duan, Morrissey 2004). Despite the effectiveness of this process at ensuring the safety of smoked salmon, there is lack of consumer acceptance of irradiated foods (Bruhn 1995).

Both high pressure processing and electron beam irradiation were evaluated to see how well they could meet the International Commission on Microbiological Specifications for Food's Food Safety Objective of ensuring that only 100 CFU/g of LM is present in smoked salmon at the time of consumption. Application of electron beam irradiation of 1.5 kGy at 5°C obtained the food safety objective of 0 LM cells per 25 g sample (Medina et al. 2009). For high pressure processing, holding the product at 450 MPa for 10 min achieved the objective as well (Medina et al. 2009).

4.2 Organic Acids

Other antimicrobial treatments such as essential oils, bacteriocins, and organic acids can also be used to slow or prevent growth of pathogens. Organic acids such as lactic, acetic, propionic, citric, and benzoic acid are the most common classical antimicrobial agents in food. For these ingredients to be effective, they must be in their undissociated forms, so they can permeate across the bacterial membrane (Brul and Coote 1999). This usually requires a relatively low pH. Once inside the cell, the higher pH of the cytoplasm deprotonates the organic acids. The charged molecules cannot permeate out of the cell. The buildup of these charged molecules in the cell are thought to disrupt internal cellular homeostasis and other metabolic processes. Organic acids have been found to be able to control LM growth in smoked salmon. Brines with potassium lactate, sodium acetate, or sodium diacetate when injected into salmon meat at 2-3% completely prevented LM growth when vacuum packed and stored at 10°C (Vogel et al. 2006).

Organic acids have been shown to synergize well with irradiation at controlling growth of LM in cold smoked salmon (Table 1). E-Beam irradiation and the combination of nisin with CO_2 packaging seemed to be the two most effective methods as it had a bacteriostatic effect on LM (Medina et al. 2009; Nilsson, Gram, Huss 1999; Su, Duan, Morrissey 2004). The combination of sodium diacetate and potassium lactate was also effective at reducing the bacterial count (Vogel et al. 2006). However, there is a negative stigma with consumers on the usage of irradiation and perceived artificial chemical preservatives. The use of natural antimicrobial compounds would be preferable.

Table 1: Evaluation of Organic Acids and Alternative Processing on Log Reduction of LM in Smoked Salmon

Reference	Method	Concentration	Log Reduction vs. control at ~15 days	Storage Temp (C)	Time	Notes
(Vogel et al. 2006)	Sodium acetate	0.14%	0.5-1	10	25	Bacteriostatic
	Sodium acetate Sodium Diacetate	0.21%	2	10	25	Bacteriostatic
		0.14%	1	10	25	Bacteriostatic
	Sodium Diacetate K Lactate	0.21%	1	10	25	Bacteriostatic
		2.00%	2			
	K Lactate K lactate + Na Diacetate	3.00%	5			
		2% PL, 0.4% SDA	5	10	25	Bacteriostatic for 30 days
	K lactate + Na Diacetate	2.5% PL, 0.18% SDA	5	10	25	Bacteriostatic for 30 days
		3% PL, 0.21% SDA	5	10	25	Bactericidal
		3% PL, 0.21 SA	5	10	25	Bactericidal
(Medina et al. 2009)	E-Beam Irradiation	1 kGy	2	5		Bactericidal
		2 kGy	4.5	5		Bactericidal
		3 kGy	5.5	5		Bactericidal
		4 kGy	6	5		Bactericidal
	HPP at 450 Mpa	5 min	3.5	5		Bacteriostatic
		15 min	3.5	5		bacteriostatic
		25 min	4	5		Bactericidal

4.3 Natural Antimicrobials

Artificial preservatives have a negative stigma with consumers who instead, prefer more familiar and natural ingredients (Dickson-Spillmann, Siegrist, Keller 2011). Consumer acceptance and rejection of preservatives and other additives are often fickle and can stem from something as simple as familiarity as well as the pronunciation of the compound (Grunert 2005). It has been found that additives with hard to pronounce names are perceived as risky (Song and Schwarz 2009). Thus, potentially novel food preservatives that are naturally occurring compounds with antimicrobial properties have been identified. These include essential oils, bacteriocins, and eukaryotic antimicrobial peptides.

4.3.1 Essential Oils

Essential oils have been found to exhibit antioxidant and antimicrobial properties. Many of these compounds are already Generally Recognized as Safe (GRAS) according to CFR 21 170.3, and can be a natural alternative to artificial preservatives such as sodium nitrate (Burt 2004). Essential oils are hydrophobic liquids that contain volatile aromatic compounds produced by flowering plants to defend against predators and microbes (Burt 2004). Unlike a triglyceride, there is no single defined chemical structure that describes essential oils. An essential oil extracted from any given plant will be composed of a wide variety of compounds of which can contain an equally diverse set of functional groups including aldehydes, phenols, ketones, esters, oxides, epoxides, methyl esters, or terpene hydrocarbons (Burt 2004).

The antimicrobial activity of essential oils has been extensively tested *in vitro*. It is theorized that the hydrophobic essential oil compounds are able to embed themselves between the lipid bilayer of a bacterium, thus causing critical cellular fluids to leak out of the cell. However, their efficacy in food systems has been shown to be less effective, and a greater concentration of essential oils is required to reach the same effect (Burt 2004). The factor that most limits the use of essential oils is that these compounds cause a significant sensory change at the required levels of usage to inhibit microbes (Bagamboula, Uyttendaele, Debevere 2004; Brul and Coote 1999).

There have been limited studies investigating the effects of essential oils in smoked seafood. It was found that when thyme oil was added to a brine solution with liquid smoke flavoring, both the total viable and the psychrotrophic counts were decreased (Alcicek 2011). In another study, oregano essential oil was infused to a potato peel film to regulate the release of the antimicrobial into the meat. It was found that the oregano oil infused film was able to significantly reduce the outgrowth of LM during storage under refrigerated conditions (Tammineni, Ünlü, Min 2013).

4.3.2 Bacteriocins

Bacteriocins are antimicrobial peptides produced by bacteria to inhibit other closely related strains. These chemicals are distinguished from antibiotics in various ways. Bacteriocins are synthesized in the ribosome with a narrow spectrum of activity while antibiotics are secondary metabolites and their spectrums vary. Bacteriocins do not harm the host cell and, unlike antibiotics, have no known toxicity to humans. Unlike essential oils, one bacteriocin has already been proven effective enough to be commercialized as a food preservative. Nisin is the only FDA approved bacteriocin currently used in food (Burrowes et al. 2004). It is often used as an antimicrobial agent in processed cheese to prevent the growth of various *Clostridium* bacteria that were present in the initial ingredients and survived the heat treatment of the cheese. In addition to processed cheese, Nisin is used in many other food products such as salad dressing, alcoholic beverages, canned vegetables, and meat (Delves-Broughton 2005). This peptide inhibits bacteria by creating pores in the cytoplasmic membrane, which disrupts the proton motive force. While nisin is effective against many gram positive bacteria, it is ineffective at inhibiting gram negative strains (Delves-Broughton 2005).

One study tested the efficacy of nisin with modified atmosphere packaging. It was found that the bacteriocin used at 1000 IU/g was able to delay the growth of LM in vacuum packed cold smoked salmon. Using a modified atmosphere with 30% N_2 and 70% CO_2 was able to slow the growth of LM (Table 2). The addition of nisin decreases the growth of LM in a concentration dependent manner (Nilsson, Henrik Huss, Gram 1997).

Other bacteriocins have been also been tested in smoked salmon. The bacteriocin divercin was found to cause a 2 log reduction of LM at both 4°C and at 8°C in cold smoked salmon (Duffes et al. 1999). The same researchers also tried inoculating salmon meat with *C. piscicola* which is a bacteriocin producing bacterium. They found that at 8°C there was a strong inhibition of LM, and using the bacteria was more effective than adding the pure bacteriocin. However, at 4°C the bacterial treatment was less effective than the pure bacteriocin (Duffes et al. 1999).

Table 2: Evaluation of Bacteriocins on Log Reduction of LM in Smoked Salmon

Reference	Method	Concentration	Log Reduction vs. control at ~15 days	Time	Notes
(Duffes et al. 1999)	Nisin	$10x10^5$ AU/g	2	21 Days	Bacteriostatic
	Diversin	10 $x10^5$ AU/g	Maintained <1 log	21	Bactericidal
		10 $x10^4$ Au/g	Maintained <1 log	21	Bactericidal
	Piscicocin	2% w/v	Maintained <1 log	21	Bactericidal
	Carnobacterium	$10x10^{4-5}$ CFU/g		21	Bacteriostatic
(Nilsson, Gram, Huss 1999)	CO_2		2	10	Slowed growth for 10-20 days
	Nisin	500iu	0.5	10	only slowed growth for 10 days
		1000iu	5	15	Slowed growth for 15 days
	CO_2 + Nisin	500iu	6	45	Slowed Growth
		1000iu	7	45	Slowed Growth

4.3.3 Protamine

Protamine is an antimicrobial peptide (AMP) that compresses the DNA of sperm cells of fish. It has great potential to be used in food systems as an antimicrobial. It is commercially extracted from the milt of fish. The peptide has many similar properties to the currently used antimicrobial nisin both are low molecular weight cationic peptides that inhibit bacteria by disrupting the cytoplasmic membrane. Protamine from different fish species has different names. For example, protamine from herring is called clupeine, while protamine from salmon is called salmine. The peptide has about 30 amino acid residues of which nearly two thirds are arginine. It is currently used in the medical field to counteract the blood thinning effects of heparin (Despotis et al. 1999). Its antimicrobial properties were shown to be relatively heat stable and most inhibitory at low salt concentrations and neutral to alkaline pH (Islam, Itakura, Motohiro 1984; Uyttendaele and Debevere 1994).

Protamine may be the best alternative antimicrobial to be used in smoked salmon. It has been found to inhibit both gram positive and gram negative bacteria (Table 3), whereas nisin is ineffective on gram negative bacteria. Nevertheless protamine seems to be especially effective against gram positive bacteria (Uyttendaele and Debevere 1994; Vaara and Vaara 1983). Another advantage of using this peptide over other antimicrobials is its potential to not affect the label. Salmine is extracted from the milt tissues of salmon and the application of this peptide could keep the ingredient label unchanged and can be perceived by consumers as more natural than bacteriocins. Another benefit of using this peptide is that would add value to a waste product from the aquaculture industry. It has been found that on average, 137mL of milt/kg body weight can be obtained from Atlantic salmon (Gjerde 1984). Commercialization of this peptide would convert this waste material into a valuable resource.

Table 3: Minimum Inhibitory Concentration of Protamine on Various Bacteria

Reference	Bacterial Species	MIC ug/mL	Gram Stain
(Hansen and Gill 2000)	*Escheria coli*	750	-
	Listeria monocytogenes	500	+
(Islam, Itakura, Motohiro 1984)	*Bacillus coagulans*	75	+
	Bacillus licheniformis	200	+
	Bacillus megaterium	75	+
	Bacillus subtilis mesentericus	150	+
	Bacillus subtilis niger	125	+
	Bacillus subtilis ruber	200	+
	Enterobacter aerogenes	650	-
	Lactobacillus casei	150	+
	Lactobacillus plantarum	100	+
	Streptococcus faecalis	400	+
(Johansen, Gill, Gram 1995)	*Aeromonas sobria*	>40000	-
	Brochothrix thermosphacta	20	+
	Pseudomonas fluorescens	3000	-
	Staphylococcus Aureus	500	+
	Vibrio anguillarum	1000	-
	Vibrio parahaemolyticus	500	-
	Aeromonas salmonicidae	4000	-
(Johansen, Gill, Gram 1996)	*Listeria moncytogenes O32*	1000	+
	Listeria moncytogenes O57	1000	+
	Shewanella putrefaciens A11	1000	-
	Shewanella putrefaciens A2	1000	-
	Shewanella putrefaciens A22	1000	-
	Shewanella putrefaciens A6	500	-
(Uyttendaele and Debevere 1994)	*Bacillus cereus*	1000	+
	Listeria monocytogenes	700	+
(Vaara 1992)	*Salmonella typhimurium*	100	-

5. General Mode of Action of Antimicrobial Peptides

AMP's are relatively short strands of less than 100 amino acids produced by all eukaryotic organisms for microbial defense. Various AMP's have been found to inhibit a wide range of organisms (Table 3) including gram positive bacteria, gram negative bacteria, fungi, protozoans, parasites, and even some viruses (Jenssen, Hamill, Hancock July 2006). There have been AMP's isolated from various fish which have a wider range of activity and are effective at inhibiting a greater number of microorganisms. These compounds can potentially be a better alternative to both essential oils and bacteriocins. They have the potential to not affect sensory properties as much as essential oils, and some AMP's are effective at inhibiting both gram negative and gram positive bacteria (Table 3). However, there are some limitations to using these peptides in food. Like essential oils, these compounds are not as effective in food systems as they are *in vitro*.

Bacteria are protected by a cytoplasmic membrane composed of a lipid bilayer and membrane proteins. All bacteria except *Mycobacterium* have a cell wall composed of peptidoglycan. Gram-positive bacteria have a thicker peptidoglycan cell wall with lipoteichoic acids. Gram-negative bacteria have a thinner cell wall and have an outer membrane with lipopolysaccharides (LPS) (Pasupuleti, Schmidtchen, Malmsten 2012). The outer membrane of gram-negative bacteria prevents large macromolecules from passing through it, as it only allows limited diffusion of hydrophobic compounds (Vaara and Vaara 1983). Gram-negative bacteria are naturally more resistant to cytoplasmic membrane targeting antimicrobials because of its complex outer membrane. AMP's can pass through the outer membrane through the self-promoted uptake pathway (Hancock 1997), in which cationic AMP's interact with the LPS of the outer membrane. AMPs interact with divalent cationic binding sites on the LPS and competitively displace Mg^{2+} and Ca^{2+} ions (Hancock 1997). This process disorganizes the structure of the LPS on the outer membrane and then causes transient cracks in the bacterial membrane at which point more AMP's can pass through. Gram positive bacteria like LM which lack an outer membrane are much more susceptible to cationic antimicrobial peptides (Hancock 1997).

Most AMP's target the cytoplasmic bacterial membrane (Pasupuleti, Schmidtchen, Malmsten 2012). There are three proposed models of inhibition: barrel-stave, torroidal, and carpet (Figure 2) (Bechinger and Lohner 2006). For the barrel-stave and torroidal models, it is thought that the positively charged peptide is electrostatically attracted to the negatively charged cytoplasmic membrane. Once the peptide comes into contact with the membrane, the peptide folds itself into an alpha helical structure, and when a high enough concentration of peptides adsorbs onto the membrane surface, a pore forms in the following manner. According to the barrel-stave model, the peptides form a very ordered barrel like structure through the membrane creating a pore that intercellular fluids can leak out of (Bechinger and Lohner 2006). The torroidal pore model suggests that pore formation occurs either through the AMP imbedding itself into the membrane or through positive curvature strain (Bechinger and Lohner 2006). The pores formed through either of these models can cause leaking of intercellular fluid and the loss of membrane potential. According to the carpet model, AMP's aggregate onto the bacterial membrane much like the barrel stave and the torroidal model (Figure 2); but instead of imbedding themselves into the membrane; the peptides form a carpet like structure on the outer surface of the membrane by electrostatically binding to the phospholipid head group and directly dissolving the membrane (Oren and Shai 1998). The structure causes the membrane to break into pieces through micellization (Bechinger and Lohner 2006). Non-membrane targeting AMP's pass through the bacterial membrane without damaging it and have been observed inhibiting protein synthesis, nucleic acid synthesis, enzymatic activity, and cell wall synthesis (Zhang, Rozek, Hancock 2001).

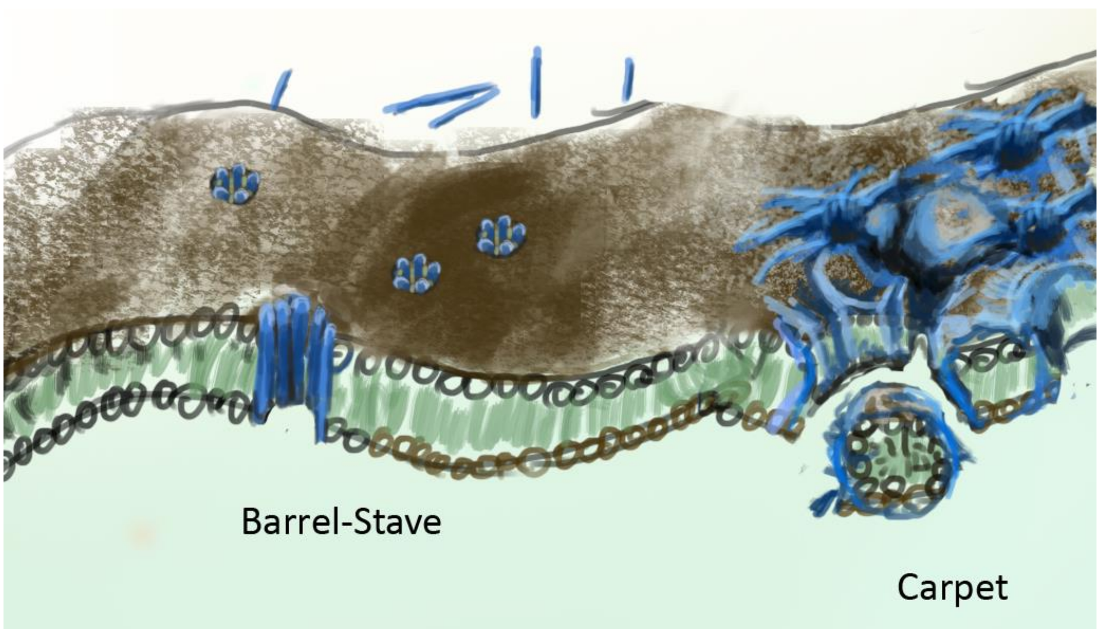


Figure 2: Protamine's Mode of Action
Illustration of the Barrel-Stave and Carpet model

6. The Effect of Electrostatic Interactions on Protamine

The positive charge of protamine allows it to inhibit bacteria, but it will have nonspecific electrostatic interactions with many negatively charged molecules in neutral to acidic environments. When protamine was added to tryptic soy broth, it precipitated in a pH dependent manner. At a pH below 6, there was no inhibitory effect on the bacterial growth (Potter, Hansen, Gill 2005). Up to 90% of protamine was precipitated at pH levels 6.5-8, and lowering the pH decreased the incidence of precipitation. Only 15% of the protamine was precipitated at a pH as low as 5.0. At a neutral pH, protamine had the highest anti-bacterial effect despite the fact that the majority of the molecules were precipitated out of solution (Hansen and Gill 2000). This was attributed to the fact that at neutral pH the AMP had the highest affinity with the bacterial membrane despite the precipitation effect.

In food systems, positively charged peptides would have unwanted interactions with negatively charged food components, especially those with high amounts of proteins. Such interactions would prevent AMP activity on the bacterial membrane. Protamine was modified in one experiment to reduce its charge density by reacting the peptide with 1,2-cyclohexanedione (Potter, Hansen, Gill 2005). The compound bound the charged guanidino groups of the arginine residues. It was found that when testing inhibitory effects of AMP's in milk and beef, protamine that was modified to reduce its charge density had a significantly better inhibitory effect against LM and total coliforms than unmodified protamine (Potter, Hansen, Gill 2005). In the same study, it was found that decreasing the charge on protamine did not decrease its precipitation with TSB. Protamine that was modified to have a reduced charge also had a high degree of binding to the membrane of LM than unmodified protamine. This suggests that there are more interactions with other components besides electrostatic ones (Potter, Hansen, Gill 2005).

The importance of electrostatic interactions on the efficacy of protamine was further demonstrated when protamine has been tested in food model media composed of TSB gelled by the protein gelatin. Two types of gelatin were used with isoelectric points ranging from 4.7–5.2. It was found that when the media was gelled with gelatin as opposed to low charged agarose, the protamine was less effective in inhibiting bacterial growth. The gelatin gel with a lower isoelectric point had a greater negative impact on antimicrobial efficacy, which suggests that the protamine preferred to bind to the gel rather than the bacterial cell (Hansen and Gill 2000). This suggests that food which is mostly negatively charged will interfere with the ability of protamine to interact with bacteria.

It is expected that smoked salmon will also have unwanted electrostatic interactions with protamine. The major muscle proteins actin and myosin both have isoelectric points below the typical pH of raw salmon which is typically around 5.5-6. These proteins would be in the unprotonated form and have negative charges. Such negative charges would electrostatically interact with the positively charged protamine.

7. Edible Films

Edible films are thin matrices formed from an edible polymer such as carbohydrates, proteins, or lipids and can be a potential method to improve the antimicrobial efficacy of protamine. Films are used to form a thin layer on the surface of a food product to improve either safety or quality. They are typically composed from 1 or more types of polymers to provide the basic structure, and often other chemicals like glycerol, polyethylene glycol, and sugar are used as plasticizers (Cagri, Ustunol, Ryser 2004). Films incorporated with antimicrobial agents can help improve the shelf life of many products.

Edible antimicrobial films can potentially improve the efficacy of salmine in smoked salmon. Previous research has shown that films can improve the efficacy of various antimicrobials such as lysozyme, organic acids, and bacteriocins like nisin in foods like smoked salmon in comparison to a simple surface treatment (Cagri, Ustunol, Ryser 2004; Goswami, Han, Holley 2009; Jiang, Neetoo, Chen 2011; Min et al. 2005). In smoked salmon, films can potentially control the diffusion rate of salmine into fish and maintain a high localized concentration on the surface of the food where contamination typically occurs (Coma 2008). By doing so, the films minimize unwanted electrostatic interactions between protamine and the food matrix.

7.1 Polysaccharide Films

Agar and carrageenan are both hydrocolloids extracted from red seaweed. Agar is composed of agarose and agaropectin. Most types of agar contain no charged functional groups. Carrageenan is a common functional ingredient in the food industry for its ability to thicken, form gels, and stabilize food systems. It is composed of sulfated galactose units. There are many types of carrageenans that vary in sulfonation and charge density. Kappa-carrageenan has one sulfate group per disaccharide unit. Alginate and k-carrageenan films in combination with sodium diacetate and sodium lactate have both been shown to slow the growth of aerobic bacteria in smoked salmon (Neetoo, Ye, Chen 2010). However these hydrocolloids themselves do not have inherent antimicrobial activity (Neetoo, Ye, Chen 2010). This was attributed to the interactions between the antimicrobial and the alginate. It was hypothesized that the interactions promoted an optimum release rate of the antimicrobial compound into the salmon to inhibit the growth of LM (Neetoo, Ye, Chen 2010).

7.2 Diffusion of Antimicrobial Compounds

When an antimicrobial like protamine is applied to a piece of food, it will diffuse from the surface towards the center until an equilibrium concentration is reached according to Fick's first law of diffusion as seen below.

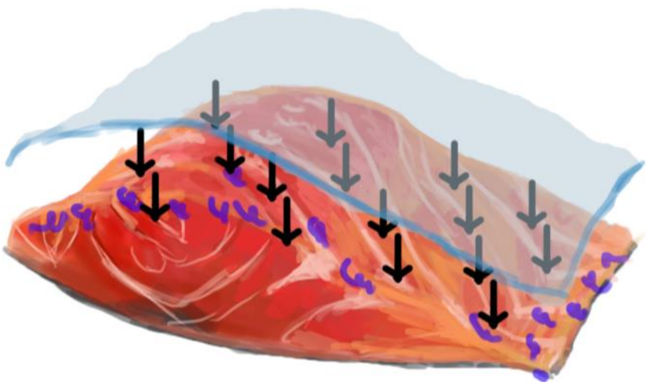
$$J = -D\frac{d\Phi}{dx}$$

Where J is flux which measures the amount diffused over time, D is the diffusion coefficient, Φ is the concentration, and x is the length or position. The first law states that the total amount of solute that diffuses through a given unit area in a given time is proportional to the concentration gradient. In other words, the greater the concentration difference, the faster something will diffuse to equilibrium.

The diffusion of antimicrobial compounds from an edible film into a food matrix can also be characterized by Fick's second law of diffusion. However, interactions between the antimicrobial and the films could affect the diffusion coefficient and modify the final flux. The release rate of antimicrobial compounds can be affected by many extrinsic factors such as temperature and pH. Higher temperatures have increased the release rate of lysozyme from whey protein films into smoked salmon (Min, Rumsey, Krochta 2008). Intrinsic factors of the edible film could also have an effect. Nonspecific interactions such as electrostatic, dipole, and hydrophobic interactions could all influence the rate that this peptide diffuses from the film into the salmon meat.

Because protamine is highly positively charged, it is likely that electrostatic interactions are the biggest factor in the diffusion rate. A film composed of negatively charged polymers like k-carrageenan would theoretically lower the diffusion rate due to strong interactions between protamine and the polymer (Figure 3). A neutrally charged film like agar would have a much lower effect on the diffusion rate.

a)



b)

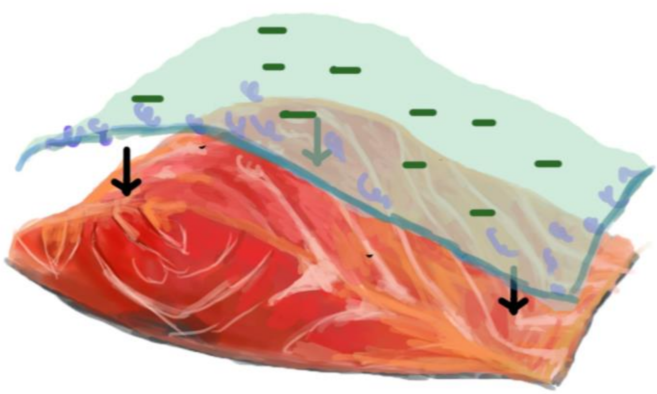


Figure 3: Diffusion Rate of Antimicrobials as Affected by Edible Film Composition
a) Neutrally charged films has no effect on diffusion rate of antimicrobial depicted in blue, b) Negatively charged films slows antimicrobial diffusion rate

8. Summary

Listeria monocytogenes (LM) is a major safety concern for smoked salmon producers, as it can survive both the brining and smoking process in cold smoked salmon production. Salmine is a cationic antimicrobial peptide derived from the milt of salmon that has been shown to inhibit the growth of LM *in vitro*. Commercialization of this peptide would add value to a waste product produced when raising salmon while improving the safety of smoked salmon. Edible films may improve the antimicrobial efficacy of salmine by maintaining a high concentration at the surface of the fillet where contamination typically occurs.

The objective of this study was to quantify the anti-listeria activity of salmine in smoked salmon and determine the effect of charged films on its antimicrobial activity. The antimicrobial activity of salmine was hypothesized to be influenced by both the concentration of salmine used and the charge of the edible film. Higher concentrations of salmine would have a greater antimicrobial effect than lower concentrations. Negatively charged films would bind the positively charged salmine and slow its release rate into the salmon fillet thus improves its efficacy over time.

Chapter 2

Abstract

Cold smoked salmon is a ready to eat seafood product processed by brining fish in a 3-5% salt solution and then smoking at 37°C for 6 to 24 hr. *Listeria monocytogenes* (LM), a psychrotrophic and halotolerant pathogen, can survive all of these processes and is a major safety concern for smoked salmon producers. Salmine is a cationic antimicrobial peptide derived from the milt of salmon, and it has been shown to inhibit the growth of LM *in vitro*. Commercialization of this peptide would also add value to a waste product produced when raising salmon. The purpose of this study was to determine the anti-listeria activity of salmine in smoked salmon by measuring the viable counts of LM over time.

Cold smoked salmon was treated with a 0, 0.5, 2.5, and 5 mg/g salmine solution and then inoculated with approximately $1x10^3$ cells of LM. The viable counts were then enumerated after 0, 1, 4, 7, 13, 22, and 32 days at 4°C storage. It was found that 5 mg/g salmine delayed the growth of LM on smoked salmon samples. No growth of LM was observed on these samples for the first 7 days. On days 13 and 22, LM counts on these samples were significantly $(p < 0.05)$ lower than the counts on the untreated samples by >1 log. These results suggest there is potential for salmine to be used as a natural hurdle to inhibit growth of LM due to post process contamination; however, future investigations for extending this effect throughout the shelf life of smoked salmon products are warranted.

Introduction

Cold smoked salmon is a ready to eat seafood product that has no thermal kill step. It is produced by brining in a salt solution then smoking at 37°C for times ranging between 6-24 hours and its shelf life is typically has a range of 2-4 weeks. *Listeria monocytogenes* (LM), a psychrotrophic and halotolerant pathogen, can survive all of these processes, and is a major safety hazard for smoked salmon producers. Ingestion of LM can cause listeriosis, a serious infection for vulnerable populations (the young, old, pregnant, and immunocompromised). This disease is characterized by gastrointestinal distress, fever, and muscle aches. In vulnerable populations, there are more severe symptoms. Infections among pregnant women can lead to serious threats to the fetus and may cause stillbirth or miscarriages. Immunocompromised populations may suffer from meningitis and septicemia. It is estimated that there is approximately 1600 illnesses and 260 deaths due to listeriosis every year in the United States (CDC 2013).

The water phase salt content of smoked salmon usually ranges from 3-5% which has no effect in inhibiting growth of LM (Peterson et al. 1993). The cold smoking process cannot guarantee the removal of this pathogen either because the low temperature doesn't inactivate the pathogen. The smoke can kill the LM on the surface of the fish, but the bacterium is still able to grow during the smoking process if it is embedded within the surface of the meat (Eklund et al. 1995). There is a zero tolerance policy for LM in ready to eat foods in the United States (Huss, Embarek, Jeppesen 1995), thus there is a need for a technology that minimizes the growth of this pathogen caused by post process contamination.

Antimicrobial peptides (AMP) are short (20-30 residues) cationic peptides with antimicrobial properties that may be used to prevent growth of LM due to post process contamination. Protamine is a cationic AMP derived from fish milt tissues (Uyttendaele and Debevere 1994). This peptide has many similar properties to the currently used antimicrobial nisin, as both are low molecular weight cationic peptides that inhibit bacteria by disrupting the cytoplasmic membrane (Bechinger and Lohner 2006). There are potential benefits in using this peptide over other AMP's. Salmine is the protamine extracted from the sperm cells of salmon and could be used as a natural antimicrobial agent. Another potential benefit of using this peptide is the opportunity for it to add value to a traditional waste product of aquaculture.

While salmine has been found to inhibit *LM in vitro*, few studies tested the efficacy of this peptide in true food systems (Conte et al. 2007; Islam, Itakura, Motohiro 1984; Islam, Motohiro, Itakura 1985; Uyttendaele and Debevere 1994). Protamine derived from herring (clupeine) caused a 2-3 log reduction in LM in milk over 16 days (Potter, Hansen, Gill 2005). Those findings show that this peptide can be effective at inhibiting LM in a real food system like cold smoked salmon.

The objective of this study was to quantify the anti-listeria and anti-bacterial activity of salmine in smoked salmon by measuring the log reduction of *LM* and total viable count over time. It was hypothesized that smoked salmon samples inoculated with *LM* and treated with salmine would have a lower Listeria and total viable count than samples without salmine treatment. Higher concentrations of salmine should have a greater antimicrobial effect than lower concentrations.

Materials and Methods

Preparation of Bacterial Inocula

Listeria monocytogenes strains of Scott A., J0161, LW-A46 were used. Scott A and J0161 have been associated with major LM outbreaks. LW-A46 is a strain that had been isolated from salmon. The three strains were grown by incubating individually aerobically in brain heart infusion (BHI) at 37 °C for 18 h. The culture was then centrifuged for 4 minutes. The resulting pellet was then suspended in sterile 0.85% NaCl solution and the OD_{600} of each strain solution was adjusted to 0.5. The three strains suspended in saline solution were then combined to yield a cocktail of approximately $1x10^8$ CFU/mL. The cocktail was then diluted to $1x10^4$ CFU/mL with 0.85% NaCl solution.

Treatment and Inoculation of Smoked Salmon

Cold smoked salmon purchased from a wholesale grocery outlet was stored at 4°C on the day of LM inoculation and salmine treatment. Salmon filets were aseptically cut into 2 g samples with an approximate surface area of 2 square inches. Salmine solutions were prepared at a concentration of 0, 1, 5, and 10 mg/mL and filtered with 22 nm membrane filters. Salmon samples were then treated with salmine by dropping 1 mL of a solution onto the fish to yield final concentrations of 0, 0.5, 2.5, and 5 mg salmine/g salmon sample. They were then allowed to air dry in a biosafety cabinet for 10 minutes. Each of these treatments was repeated in triplicate. After salmine treatment, each salmon sample was then inoculated with $1x10^3$ cells of LM to simulate typical LM contamination by randomly dropping 0.1 mL of culture onto the meat and allowing it to air dry in the biosafety cabinet. Samples were then vacuum packaged and stored at 4°C to simulate typical smoked salmon storage during distribution, wholesale and retail storage. Because the typical shelf life of smoked salmon is about 2-4 weeks, the *LM* was then enumerated at 1, 4, 7, 13, 22, and 32 days using Listeria selective media (oxford formulation). Colonies of LM appeared dark green with black halos.

Microbial Analysis

LM was enumerated by adding 18 mL sterile buffered peptone water into the vacuum package containing the 2g salmon sample. It was then homogenized with a stomacher at 200RPM for 30 seconds. Appropriate serial dilutions were prepared in sterile buffered peptone water and 0.1mL of the final solution was spread on Listeria selective media (oxford formulation) in duplicate. Plates were then incubated for 48h at 37°C.

The total viable counts of smoked salmon were enumerated using the same method except plate count agar was used instead of Listeria selective media. The samples were also not inoculated with the LM cocktail.

Statistical Analysis

One way analysis of variance (ANOVA) with Tukey-HSD means comparison was performed to determine significant difference of salmine concentrations *($p<0.05$)* using JMP statistical software.

Results

The LM in all salmon samples grew in a sigmoidal fashion (Figure 4). There was an initial lag phase of about 3 days, a log growth phase of over 20 days, and a stationary phase once LM grew to about 8 log CFU/g after 32 days.

The antimicrobial efficacy of salmine was determined by the concentration of the salmine treatment. Treating salmon with salmine at 5 mg/g delayed the log phase of LM. The lag phase of these samples was extended by about 6 days. Significant growth was observed after 22 days on samples treated with 5 mg/g salmine, whereas similar growth happened earlier on day 13 on samples treated with 0 and 0.5 mg/g salmine (Table 4). LM counts on these samples on days 13 and 22 were significantly lower than the counts on the untreated samples by >1 log *($p < 0.05$)*. On day 32, there was no significant difference between any salmine treatment and the control.

Table 4: *Listeria monocytogenes* Counts (log CFU/g) in Smoked Salmon as Affected by Salmine

Mean of triplicate plate counts ± standard deviation of LM in smoked salmon at 4°C. Capital letters denote significance groupings between days. Lower case letters denote significance groupings between salmine treatment levels.

Salmine Treatment (mg/g salmon)	Time (days)						
	0	1	4	7	13	22	32
0	$3.0±0.1^{Aa}$	$3.0±0.1^{Aa}$	$3.0±0.2^{Aa}$	$3.4±0.4^{Aa}$	$5.8±1.0^{Ab}$	$8.2±0.3^{Ac}$	$8.6±0.4^{Ac}$
0.5	$3.2±0.2^{Aa}$	$3.0±0.1^{Aa}$	$3.0±0.1^{Aab}$	$3.4±0.04^{Ab}$	$5.2±0.2^{ABc}$	$8.0±0.2^{ABd}$	$9.0±0.1^{Ae}$
2.5	$3.0±0.1^{Aa}$	$3.0±0.1^{Aa}$	$3.0±0.2^{Aa}$	$3.1±0.3^{Aa}$	$4.4±1.3^{ABb}$	$8.7±0.03^{Ac}$	$8.5±0.3^{Ac}$
5	$3.1±0.1^{Aa}$	$3.0±0.2^{Aa}$	$3.0±0.5^{Aa}$	$3.2±0.2^{Aa}$	$3.4±0.7^{Ba}$	$6.5±1.5^{Bb}$	$8.6±0.2^{Ac}$

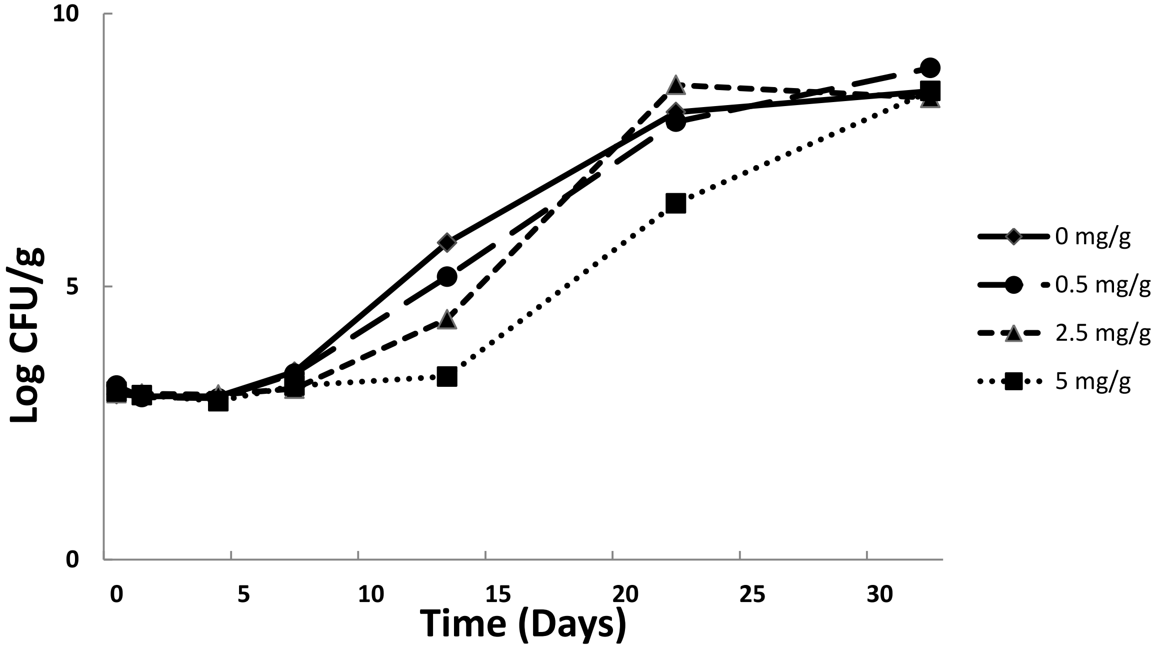


Figure 4: Effect of Salmine Concentrations Ranging From 0-5 mg/g Salmon on the Growth of LM over 32 Days

Table 5: Total Viable Counts (log CFU/g) in Smoked Salmon as Affected by Salmine
Mean of triplicate plate counts of bacteria in smoked salmon at 4°C. Capital letters denote significance groupings between salmine treatment levels.

Salmine Treatment (mg/g salmon)	Time (days)						
	0	1	4	7	13	22	32
0	<2.0	<2.0	<2.0	5.7±1.1^{A}	8.1±0.5^{A}	8.3±0.2^{A}	8.6±0.1^{A}
2.5	<2.0	<2.0	<2.0	3.3±0.2^{B}	7.3±1.1^{AB}	8.2±0.2^{A}	8.2±0.5^{A}
5	<2.0	<2.0	<2.0	3.6±0.4^{B}	5.7±0.9^{B}	7.8±0.6^{A}	8.1±0.5^{A}

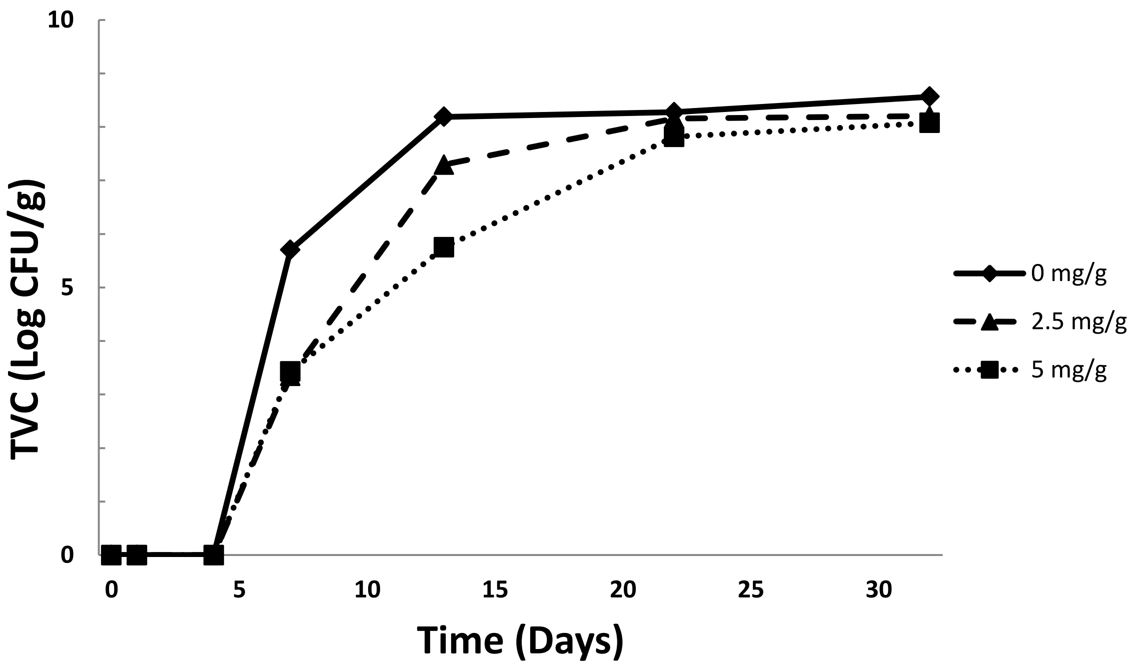


Figure 5: Effect of Salmine Concentrations Ranging from 0-5 mg/g Salmon on Total Viable Count

Salmine had a lesser effect on the growth of natural flora of smoked salmon as compared to its effect on LM growth. Like LM, there was an initial lag phase of about 4 days before exponential growth occurred. The natural flora grew quicker than LM, reaching the stationary phase after about two weeks. Salmine was able to slow the growth of the natural flora. Both the 2.5 and 5 mg/g treatments had significantly *($p < 0.05$)* lower bacterial counts than the control on day 7. However, on day 14, only the 5 mg/g treatment had a significantly *($p < 0.05$)* lower bacterial count. By day 22, all treatments reached a stationary phase of about 8 log CFU/g.

Discussion

Smoked salmon samples treated with sufficient levels of salmine prolonged the lag phase of LM and slowed the log phase growth of both LM and natural flora. However, by day 32, LM counts in all salmon samples reached 8-9 log CFU/g, which is the stationary phase of LM growth (Duffes et al. 1999; Kang et al. 2012; Nilsson, Henrik Huss, Gram 1997; Vogel et al. 2006). There was a similar effect observed for the total viable counts, as salmine had no effect on bacterial growth by day 22. The antimicrobial effects of salmine did not last through the typical shelf life of cold smoked salmon, which is about 3-4 weeks.

LM can grow rapidly in untreated smoked salmon, e.g. this bacterium can grow up to 5 log CFU/g within the first week of incubation when vacuum packaged at 4°C (Nilsson, Gram, Huss 1999; Nilsson, Henrik Huss, Gram 1997). However, this did not occur in this study, as there was a lag period of nearly 3 days for all samples including the control. The growth rate of LM was also much slower as counts only grew 5 log CFU/g after nearly three weeks. This is most likely due to variation in the natural microflora of the smoked salmon slowing the growth of LM (Datta et al. 2008).

Salmine had a smaller inhibitory effect on LM when compared to other antimicrobial treatments which were able to prevent the growth of LM in smoked salmon for up to 4 weeks (Nilsson, Henrik Huss, Gram 1997). Bacteriocins at high enough concentrations have been shown to completely inhibit the outgrowth of LM. For example, application of 500-1000 IU of nisin to cold smoked salmon initially reduced the bacterial load with subsequent slowed growth (Nilsson, Henrik Huss, Gram 1997). Piscicocins at $10x10^4$ have also been found to be bactericidal to LM (Duffes et al. 1999). Organic acids also had a much more potent antimicrobial effect, eg potassium lactate and sodium diacetate had a bacteriostatic effect on LM for up to 30 days (Vogel et al. 2006).

The cationic nature of salmine and the salt of the smoked salmon could negatively affect the antimicrobial efficacy of salmine. The positive charge of salmine is responsible for its antimicrobial properties, as it has electrostatic interactions with bacterial membrane (Bechinger and Lohner 2006; Epand and Vogel 1999; Oren and Shai 1998; Potter, Hansen, Gill 2005). That same property would cause the peptide to have unwanted interactions with negatively charged food components like the various proteins found in the muscle tissue, which would interfere with its antibacterial activity. Higher concentrations of protamine are required to overcome the interactions with the food matrix. *In vitro* the minimum inhibitory concentration (MIC) of salmine acting on LM ranged from 0.5-1 mg/mL. In smoked salmon, at least 2.5 mg/g sample salmine was needed to affect the growth of LM smoked salmon. Higher concentrations of cations from salt such as sodium and magnesium have also negatively impacted the antimicrobial efficacy of protamine *in vitro* (Pink et al. 2003; Uyttendaele and Debevere 1994). The minimum inhibitory concentration of protamine ranges from 2.5 mg/mL at 3% salt to over 5mg/mL at 5% salt *in vitro* (Uyttendaele and Debevere 1994). Smoked salmon typically has between 3-5% salt content, and the salt content of the smoked salmon used in this experiment was 3%. It is also possible that by day 32 of this study, the salmine that was applied on the surface of the smoked salmon diffused towards the center of the meat thus lowering the effective concentration of the antimicrobial.

At that point, not enough antimicrobial was present to overcome the electrostatic interactions with the food matrix and inhibit bacterial growth. To utilize this peptide as an antimicrobial in smoked salmon, some method should be developed to maintain high concentrations at the surface of the fish.

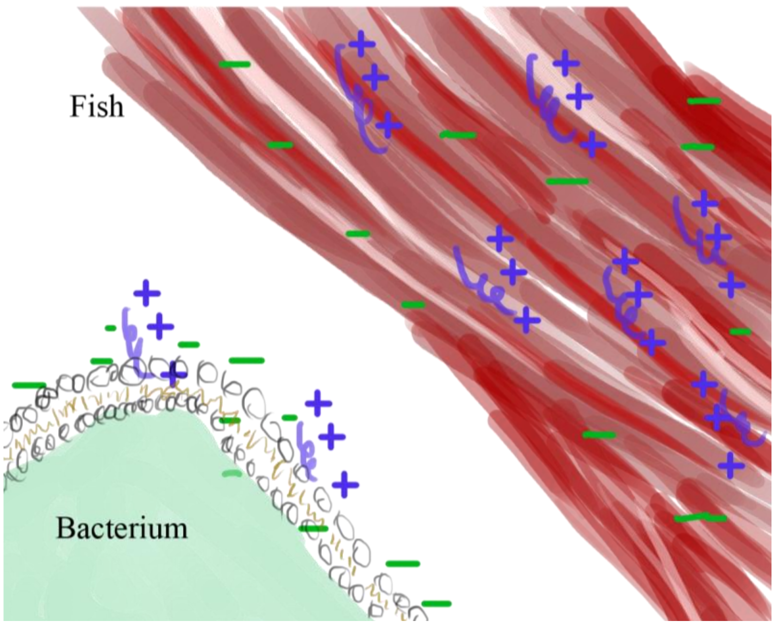


Figure 6: Salmine in Vivo Conditions
Salmine in smoked salmon has lower antimicrobial efficacy when compared to *in vivo* conditions which is likely due to unwanted binding with the fish.

An edible film or a colloidal delivery system could be used to improve antimicrobial efficacy. Films can immobilize salmine and prolong its effects at the surface of the meat. These technologies can also minimize interactions with the food matrix. If those technical challenges could be overcome, salmine could potentially be used as a natural hurdle to inhibit growth of LM due to post process contamination.

Conclusion

Salmine extended the lag phase and delay the outgrowth of LM in cold smoked salmon and it inhibited the growth of the natural bacterial flora. However, for both LM and total viable counts, salmine alone failed to have an effect throughout the entire 4 week period, so it failed to last through the typical shelf life of smoked salmon. Therefore by itself, salmine cannot be used to prevent the growth of LM in cold smoked salmon.

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Chapter 3

Abstract

Antimicrobial films composed of carrageenan and agar were used in an effort to improve the antimicrobial efficacy of salmine. Cold smoked salmon samples were dipped into film forming solutions composed of 1% biopolymer and 10mg/mL salmine. They were then inoculated with approximately 1×10^3 cells of LM. The viable counts were then enumerated after 0, 1, 4, 7, 13, 22, and 32 days at 4°C storage. Salmon samples coated in edible films composed of agar and k-carrageenan did not have significantly *($p > 0.05$)* lower LM counts when compared to samples dipped in a salmine solution. Salmine combined with biopolymers also had lower antimicrobial activity *in vitro* when compared to salmine alone. This is possibly due to electrostatic binding between the peptide and the biopolymer components of the film. Based on these results, some other method should be used to improve the antimicrobial efficacy of salmine in cold smoked salmon.

Introduction

Protamine is a low molecular weight cationic antimicrobial peptide (AMP) that inhibits bacteria by disrupting the cytoplasmic membrane. Salmine is protamine extracted from the milt tissues of salmon. There is potential that application of this peptide as an antimicrobial agent may control growth of *Listeria monocytogenes while also maintaining a clean product label*. Another potential benefit of using this peptide is the value-added potential of using a traditional waste product of aquaculture as a food ingredient.

Salmine was able to slow the growth of LM in smoked salmon by up to 3 log CFU/g over 21 days, but failed to have an effect on LM growth over 32 days which is the typical shelf life of smoked salmon (Chapter 2). The discrepancy in antimicrobial efficacy is usually attributed to the cationic salmine electrostatically binding to the negatively charged food components (Conte et al. 2007).

Films may also minimize unwanted electrostatic interactions between salmine and the food matrix by providing a slow release mechanism of the antimicrobial from the film into the food. It will diffuse from the surface towards the center until an equilibrium concentration is reached according to Fick's first law of diffusion. The release rate of antimicrobial compounds can be affected by many extrinsic factors such as temperature and pH (Choi et al. 2005; Min, Rumsey, Krochta 2008; Teerakarn et al. 2002). Intrinsic factors of the edible film could also affect the release rate of salmine. The charge of the polymers used to form the film would have different electrostatic interactions with the antimicrobial peptide. Negatively charged films would bind the positively charged salmine and slow its release rate into the salmon fillet. The objective of this study is to determine the effect of charged biopolymer films on the antilisterial activity of salmine in cold smoked salmon.

Materials and Methods

Preparation of Bacterial Inoculant

LM strains of Scott A., J0161, LW-A46 were used in this experiment. Scott A and J0161 were strains associated with major LM outbreaks. LW-A46 is a strain isolated from salmon. The three strains were grown by incubating individually in BHI at 37 °C for 18 h. The culture was then centrifuged for 4 minutes. The resulting pellet was then suspended in sterile saline solution and the OD600 of each strain solution was set to 0.5. The three strains suspended in saline solution were then combined to yield a cocktail of approximately $1x10^8$ CFU/mL. The cocktail was then diluted to $1x10^4$ CFU/mL.

Film Preparation

Four types of films were prepared: negatively charged k-carrageenan, neutrally charged agar, a 1:2 blend, and a 2:1 blend. Film forming solutions were prepared by dissolving 0.5g of each polymer into 25mL of deionized water and then autoclaved. Salmine solutions were separately prepared by dissolving 0.5g of salmine into 25mL water, filter sterilized, then incorporated into the polymer solution.   The final solution had a 1% (w/v) biopolymer concentration and 10mg/mL of salmine.

Treatment and Inoculation of Smoked Salmon

Cold smoked salmon fillets purchased from a wholesale grocery outlet were stored at 4° C on the day of LM inoculation and salmine treatment. Salmon filets were aseptically cut into 2 g samples. Salmon samples were treated by dipping into film forming solutions. They were then allowed to air dry in a biosafety cabinet for 10 minutes. Each of these treatments was repeated in triplicate. After salmine treatment, each salmon sample was then inoculated with $1x10^3$ cells of LM to simulate typical LM contamination by randomly dropping 0.1 mL of culture onto the meat and allowing it to air dry in the biosafety cabinet. Samples were then vacuum packaged and stored at 4°C to simulate typical smoked salmon storage during shipping and retail storage. Because the typical shelf life of smoked salmon is about 3-4 weeks, the *LM* was then enumerated at 1, 4, 7, 13, 22, and 32 days using Listeria selective media (oxford formulation).

Microbial Analysis

LM was enumerated by placing the 2g salmon sample in 18mL buffered peptone water and stomaching it for 2 minutes. Appropriate serial dilutions were performed and 0.1mL of the final solution was spread on Listeria selective media (oxford formulation). Plates were then incubated for 48h at 37°C.

In Vitro Determination of Antimicrobial Efficacy

The antimicrobial efficacy of the edible films was determined using the broth dilution method outlined by Uyttendaele (Uyttendaele and Debevere 1994). Tubes of tryptic soy broth were combined with film forming solution. The final 5mL mixture had 1% (w/v) biopolymer and a salmine concentration of 10mg/mL. Each tube was then inoculated with 10uL of LM cocktail with an approximate concentration of $1x10^5$ CFU/mL. After incubating at 37°C for 24hr, LM counts were enumerated using Listeria selective media (oxford formulation).

Preparation of Smoked Salmon

Cold smoked salmon purchased from a wholesale grocery outlet was stored at 4° C on the day of salmine treatment. Salmon fillets were cut into cylindrical shapes with a diameter of 9cm and a thickness of 2.5cm. Edible films infused with salmine were then placed onto the salmon pieces, vacuum packaged, and then stored in 4°C for 10, 25, 48, 120, and 168 hours.

Statistical Analysis

One way analysis of variance (ANOVA) with Tukey-HSD means comparison was performed to determine significant difference of salmine concentrations using JMP statistical software.

Results

Table 6: *Listeria monocytogenes* Counts in Smoked Salmon as Affected by Agar and k-Carrageenan (kCGN)
Mean of triplicate plate counts ± standard deviation of LM in smoked salmon at 4°C. Letters denote significance groupings between salmine treatment levels.

Treatment	Days						
	0	1	4	7	13	22	32
Agar	2.9±0.2[a]	2.8±0.2[a]	3.0±0.1[a]	4.1±0.2[a]	5.0±0.6[a]	7.5±2.0[a]	8.1±1.1[a]
1:2 kCGN: Agar	2.9±0.1[a]	2.6±0.2[a]	3.0±0.3[a]	3.8±0.2[a]	5.8±0.9[a]	7.0±0.4[a]	8.5±0.7[a]
2:1 kCGN: Agar	2.8±0.2[a]	2.9±0.1[a]	2.9±0.3[a]	3.9±0.1[a]	6.3±0.1[a]	8.3±0.4[a]	8.2±0.4[a]
kCGN	3.0±0.1[a]	2.7±0.1[a]	2.8±0.1[a]	3.3±0.8[a]	5.0±1.0[a]	7.6±1.0[a]	9.0±0.2[a]
Water	2.8±0.1[a]	2.9±0.1[a]	2.8±0.1[a]	4.0±0.3[a]	6.3±0.3[a]	6.9±0.8[a]	8.5±0.3[a]

The LM in all salmon samples grew in a typical sigmoidal fashion (Figure 4). Significant growth was observed by the first week, and the LM grew to 7-8 log CFU/g by day 32. As expected, the addition of kCGN and agar alone to smoked salmon did not have a significant *($p > 0.05$)* effect on the growth of LM at any time point (Figure 4). However, salmine alone also did not have a statistically significant inhibitory effect (Figure 4). Edible films incorporated with salmine also did not have significantly lower LM counts when compared to their respective controls.

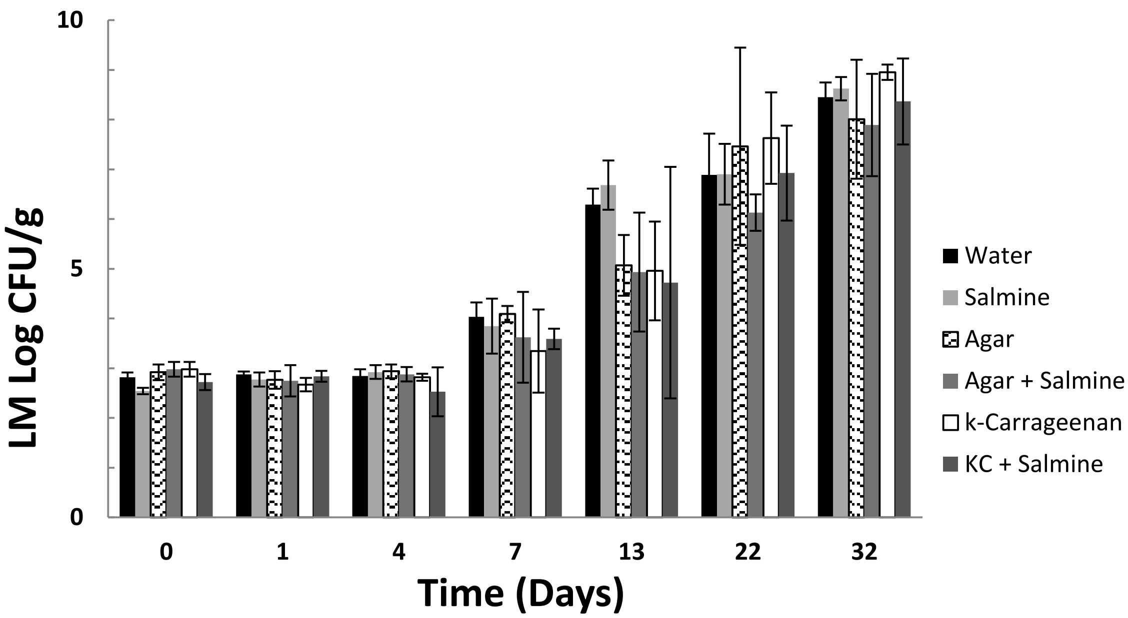

Figure 7: Effect of Agar and k-Carrageenan Films with Salmine on LM Growth
Error bars depict standard deviation

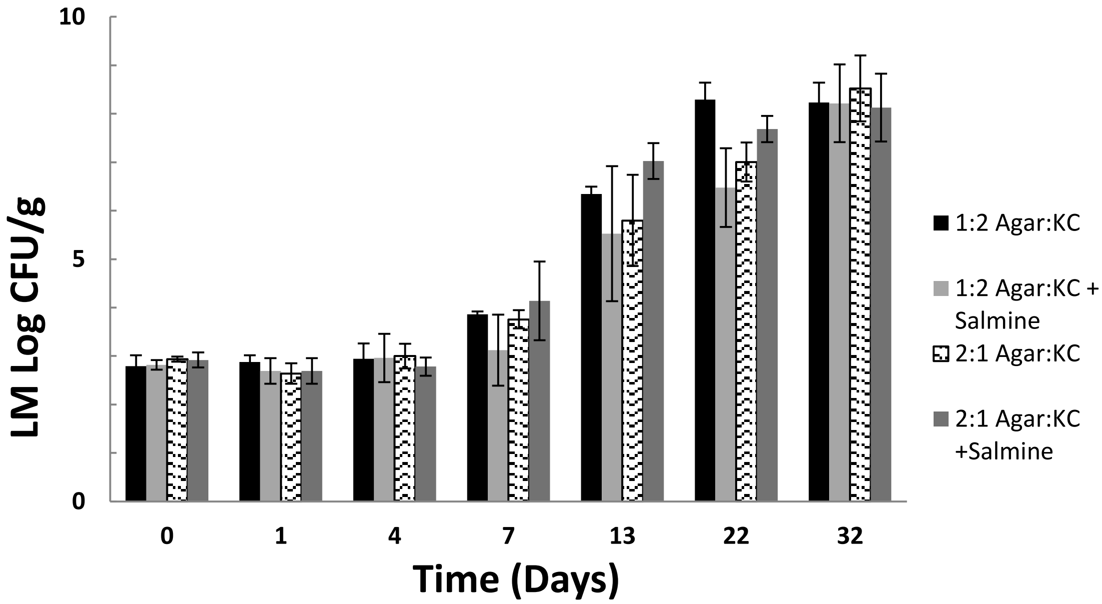

Figure 8: Effect of Multi Component Edible Films with Salmine on LM Growth
Error bars depict standard deviation

Table 7: *Listeria monocytogenes* Counts in Smoked Salmon as Affected by Agar and k-Carrageenan (kCGN) Films Incorporating Salmine

Mean of triplicate plate counts ± standard deviation of LM in smoked salmon at 4°C. Letters denote significance groupings between salmine treatment levels.

Treatment	Days						
	0	1	4	7	13	22	32
Agar + Salmine	3.0±0.1^{a}	2.8±0.3^{a}	2.9±0.1^{a}	3.6±1.0^{a}	4.9±1.1^{a}	6.1±0.4^{a}	7.9±1.0^{a}
1:2 kCGN: Agar + Salmine	2.9±0.2^{a}	2.7±0.3^{a}	2.8±0.2^{a}	4.1±0.8^{a}	7.0±0.4^{a}	7.7±0.3^{a}	8.1±0.7^{a}
2:1 kCGN: Agar + Salmine	2.8±0.1 ^{a}	2.7±0.2^{a}	2.9±0.5^{a}	3.1±0.73^{a}	5.5±1.1 ^{a}	6.5±0.36^{a}	8.2±1.0^{a}
kCGN + Salmine	2.7±0.2^{a}	2.8±0.1^{a}	2.5±0.5^{a}	3.6±0.2^{a}	4.7±2.3^{a}	6.9±1.0^{a}	8.4±0.9^{a}
Salmine	2.5±0.1^{a}	2.8±0.1^{a}	1.2±1.1^{a}	3.9±0.6^{a}	6.7±0.5^{a}	6.9±O.6 ^{a}	8.6±0.2^{a}

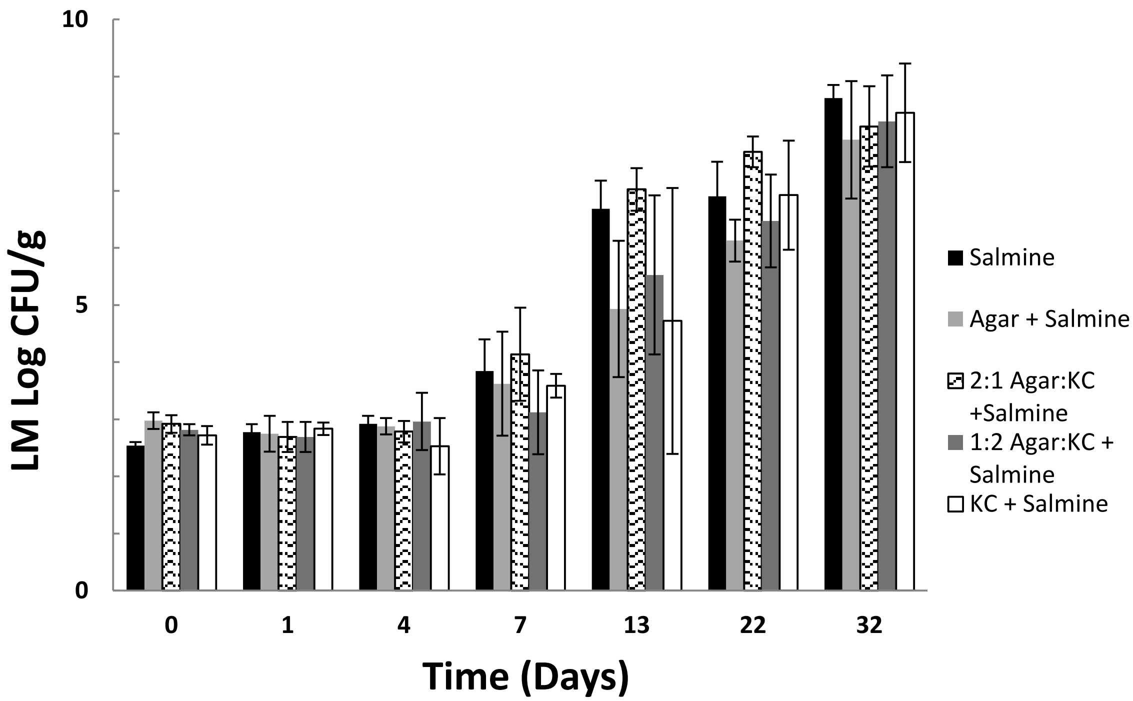

Figure 9: Effect of Agar and k-Carrageenan (kCGN) Films on Antilisterial Activity of Salmine in Cold Smoked Salmon
Error bars depict standard deviation

Films incorporating salmine did not have statistically significantly *($p > 0.05$)* lower LM counts when compared to salmon samples treated with salmine alone (Figure 9). The LM in these samples also grew in a typical sigmoidal fashion with a lag phase lasting about a week and growing up to 8 log CFU/g by 4 weeks (Table 7).

Phase separation occurred when the salmine solution was combined with the biopolymer solution. Large insoluble complexes immediately formed when salmine was mixed with kCGN and completely separated from the supernatant (Figure 10). When salmine was added to an agar solution, it changed from a clear solution to a cloudy solution (Figure 10). Salmine still precipitated with kCGN in the 2:1 agar:kCGN film forming solution, but the agar kept the solid particles mixed with the supernatant (data not shown). The higher concentrations of kCGN in the 1:2 agar:kCGN and kCGN created even larger complexes (Figure 10 c,d).

a). Agar b). 2:1 Agar:kCGN c). 1:2Agar:kCGN d). kCGN

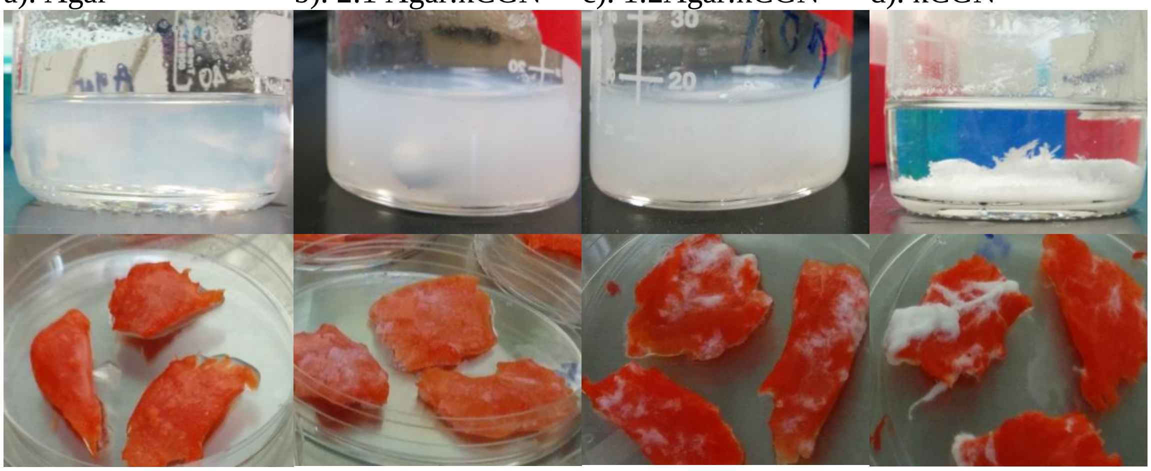

Figure 10: Smoked Salmon Samples Treated With Edible Films with Salmine

Salmine combined with agar or kCGN had an inhibitory effect on LM *in vitro*. Salmine alone was bactericidal and reduced LM counts below detectable limits (Table 8).

Table 8: In vitro Inhibitory Effect of Salmine Combined with Agar and kCGN

Treatment	Turbidity Observed	Estimated LM Counts (log cfu/mL)
Agar + Salmine	No	>4
kCGN + Salmine	No	>4
kCGN + Salmine (Precipitate Only)	No	>4
Salmine	No	<2
Water	Yes	8
kCGN	Yes	8
Agar	Yes	8

Discussion

Samples treated with salmine alone did not have significantly lower LM counts compared to samples treated with water. This result differed from previous studies which showed that a salmine solution at this concentration was able to extend the lag phase of LM in this system (Chapter 2). This may be due to the different method of application of the antimicrobial. Salmon samples were dipped in a salmine solution for this study; however, the samples in the other study were treated with the antimicrobial through dropwise addition. This may have allowed for more time for the peptide to attach to the surface of the fish.

Agar and k-carrageenan (kCGN) films failed to improve the antimicrobial efficacy of salmine (Figure 10). K-Carrageenan has a negatively charged sulfate group per disaccharide unit and it gels in the presence of divalent cations. It is expected that the positively charged salmine would precipitate with the hydrocolloid. Addition of salmine to kCGN solutions prevented any film formation as little to no kCGN was left in the supernatant to form a film.

Films composed of agar alone also did not significantly improve salmine efficacy. Previous research has suggested that forces other than electrostatic interactions can cause binding between salmine and other compounds. Potter found that reducing the charge of clupeine (a protamine derived from herring) did not affect its binding with the components of tryptic soy broth. They suggested that the increased hydrophobicity of the modified protamine could explain those interactions (Potter, Hansen, Gill 2005). This suggests that there are more interactions with other components besides electrostatic ones.

The antimicrobial activity of salmine was reduced when it was combined with agar or kCGN *in vitro* (Table 8). Salmine alone had a bactericidal effect on LM. However, when it was combined with kCGN or agar, it only slowed the growth of LM. Higher concentrations of antimicrobial are needed to observe an inhibitory effect in foods when compared to *in vitro* conditions (Chapter 2). It is likely that the lack of significant differences in LM counts in salmon is due to salmine not being released from these complexes at a high enough concentration.

Salmon samples coated with agar, 1:2 agar:kCGN, and kCGN antimicrobial films had large standard deviations in LM counts, particularly on day 13. This may be due to an inhomogeneous distribution of salmine through the film forming solution. Increasing the kCGN concentration tended to cause a greater variance in LM counts as larger complexes were formed. At that point, it was difficult to ensure that the complexes were uniformly distributed throughout every salmon sample. Some salmon samples may have had higher concentrations of salmine. The kCGN/salmine complexes more evenly distributed in the 2:1

agar:kCGN film forming solution which may explain its lower variation in LM counts (Figure 10).

Previous research has shown that films can improve the efficacy of various antimicrobials such as lysozyme and organic acids by maintaining a high localized concentration of the antimicrobial on the surface of the fish where contamination typically occurs (Cagri, Ustunol, Ryser 2004; Goswami, Han, Holley 2009; Jiang, Neetoo, Chen 2011; Min et al. 2005). However, lysozyme and organic acids have different modes of action when compared to salmine, as they do not electrostatically bind to the bacterial membrane (Epand and Vogel 1999). Nisin is thought to have a similar mode of action as salmine, and it has been found that calcium alginate films failed to improve its antimicrobial efficacy as well (Datta et al. 2008).

Conclusion

Edible films composed of agar and kCGN failed to improve the antimicrobial efficacy of salmine in cold smoked salmon. Phase separation occurred when salmine was combined with kCGN which is probably due to electrostatic interactions. Such binding between the biopolymers and salmine is likely lowering its antimicrobial activity on LM both *in vitro* and in smoked salmon. Some other method should be investigated to improve the antimicrobial efficacy of this peptide.

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