Application of Microcin N and Tridecaptin A_1 to Control Bacterial Pathogens

by

Katherine Lynn Satchwell

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Department of Agriculture, Food and Nutritional Sciences

University of Alberta

Abstract

Bacterial pathogens are responsible for a number of infectious diseases affecting humans and animals. Peptide based antimicrobial interventions may be an effective means to control pathogens. Enterohaemorrhagic *Escherichia coli* and *Salmonella enterica* are foodborne pathogens that are commonly implicated in outbreaks stemming from contaminated meat products. *Listonella anguillarum* is the etiological agent responsible for vibriosis in farmed salmon. The antimicrobial peptides, Microcin N (McnN) and Tridecaptin A_1 ($TriA_1$), were investigated for their ability to control these pathogens in foodstuff, designated for human and aquatic consumption, respectively. McnN is a bacteriocin produced by a non-pathogenic strain of *E. coli* that displays activity against *E. coli* and *Salmonella* species. $TriA_1$ is a cationic linear lipopeptide produced by *Paenibacillus terrae* that displays potent antimicrobial activity against gram-negative microorganisms. Synthesis of an analogue of $TriA_1$, Octyl-Tridecaptin A_1, produced via acylation of octanoic acid to the N-terminus of the peptide was performed by collaborators and was found to exhibit antimicrobial activity similar to the native $TriA_1$.

Hydrophobic interaction chromatography was used to partially purify Microcin N from the supernatant of *E. coli* MC4100 pGOB18. A compound similar in molecular mass to of McnN was identified in the partially purified McnN fraction using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. High performance liquid chromatography and ion chromatography failed to yield pure McnN. The partially pure preparation of McnN obtained from hydrophobic interactions chromatography failed to reduce counts of 5-strain cocktails of *E. coli* O157:H7 when applied to raw or cooked ground beef and incubated for 72 h at 8°C, respectively. Additionally, 5-strain cocktails of *S. enterica* were not inhibited by the partially pure McnN preparation under the same storage conditions in raw or cooked ground beef. While the results with McnN were not as expected, future work to isolate

pure bacteriocin could provide an antimicrobial effective against gram-negative foodborne pathogens. The application of antimicrobials to foodstuffs for the control of bacterial pathogens is a viable means to reduce the burden associated with contamination of these organisms.

$TriA_1$ was purified from the supernatant of *P. terrae* B-NRRL 30644 using hydrophobic interactions chromatography and confirmed using high performance liquid chromatography. Both the antimicrobial preparation (AMP) and Oct-$TriA_1$ were active against *L. anguillarum in vitro* in spot-on-lawn assays. The minimum inhibitory concentration of Oct-$TriA_1$ was determined against a collection of foodborne and aquatic organisms, and MIC values ranged from 0.6 to 38 μg/mL. Lyophilization of the AMP provided a water-soluble powdered preparation that could be concentrated for application. Both the concentrated AMP and Oct-$TriA_1$ were active against *L. anguillarum* when assessed directly using spot-on-lawn assays and when treated commercial feed was over-layered with agar containing the indicator strain. Encapsulation of antimicrobials using alginate was successfully performed, though Oct-$TriA_1$ was superior in terms of consistent inhibition of *L. anguillarum.* Oct-$TriA_1$ did not interact with constituents in salmon feed whereas Polymyxin B and Polymyxin E were inactive against *L. anguillarum* after exposure to commercial feed. The concentration of $TriA_1$ in the concentrated AMP is likely the limiting factor in the variability of activity of encapsulated products against indicators. Sufficient quantities are needed to elicit an antimicrobial effect when working with medicated feed. Based on these findings $TriA_1$ is an effective antimicrobial for use against pathogens that afflict Atlantic salmon. Oct-$TriA_1$ has great potential for industrial application as an antimicrobial for medicated feed formulation for use in challenge studies with Atlantic salmon.

Preface

Some of the research conducted for this thesis forms part of a national research collaboration, by Dr. Lynn McMullen (Food Microbiology) at the University of Alberta, Dr. Simon Jones (Fisheries) at the Department of Fisheries and Oceans Canada and with Dr. John Vederas (Chemistry) acting as lead collaborator, at the University of Alberta with funding generously provided by the Natural Sciences and Engineering Research Council of Canada.

Portions of Chapter 2 have been published as an abstract and presented at the International Association of Food Protection Annual Conference: (Satchwell, L., C.T. Lohans, J. C. Vederas and L. M. McMullen. 2014. Isolation and purification of microcin N — a bacteriocin effective against *Salmonella enterica* and *Escherichia coli,* Aug 3-6, 2014, Indianapolis, IN). Two undergraduate students, Aleicia M. Mushins and Emilie J. Dubois, were trained in the food microbiology laboratory during the course of this program and assisted in the execution of experiments and collection of data, specifically the experiments with raw and cooked ground beef using Microcin N as antimicrobial interventions, respectively.

Portions of Chapter 3 have been published as an abstract and presented at International Association of Food Protection Annual Conference: (Satchwell, K.L., S.A. Cochrane, J. C. Vederas and L. M. McMullen. 2015. Tridecaptin A1: a novel antimicrobial to inhibit foodborne pathogens, July 24-27, 2015, Portland, OR). Dr. Stephen Cochrane from the Department of Chemistry provided synthetic analogues of Octyl-Tridecaptin A_1 for antimicrobial testing.

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List of Abbreviations

AMP	Antimicrobial preparation
APT	All purpose tween
ATCC	American Type Culture Collection
CFU	Colony forming units
CFIA	Canadian Food Inspection Agency
DFO	Department of Fisheries and Oceans Canada
DIFR	Danish Institute for Fisheries Research
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphospahte
EHEC	Enterohaemorrhagic *Escherichia coli*
FMCC	Food Microbiology culture collection
LB	Luria Burtani
log	logarithmic
MALDI-TOF MS	Matrix assisted laser desorption time of flight mass spectroscopy
M63	Minimal M63 media
Mcn24	Microcin 24
McnN	Microcin N
Oct-TriA_1	Octyl-Tridecaptin A_1
PCA	Plate Count Agar
PCR	Polymerase chain reaction
PHAC	Public Health Agency of Canada
Sol-AMP	Re-solubilized lyophilized antimicrobial preparation
TriA_1	Tridecaptin A_1
USDA	United States Department of Agriculture
WHO	World Health Organization

Chapter 1. Introduction and Literature Review

Bacterial pathogens are responsible for a number of infectious diseases affecting humans and animals. Foodborne pathogens are responsible for approximately 4.0 million cases of foodborne disease acquired annually in Canada (Thomas et al., 2008; Thomas et al., 2013). Reducing the incidence of foodborne disease has two-fold importance: reduction in health care associated losses (cost of illness, loss of productivity, medical costs and loss of life) and reducing costs associated with recalls and stigma for industry (direct recall and demand reduction costs) (Scharff, 2011).

Modern consumers are demanding foods that have unambiguous labels and are free from synthetic preservatives. The Market research company, Innova Market Insights© classified the top ten global food, beverage and nutrition trends for 2015 and identified the number one market consumer consideration as "From clean to clear label" (Boothroyd, 2014). Antimicrobial compounds derived via fermentation of bacterial species offer a promising solution to combat foodborne pathogens.

The Public Health Agency of Canada identified *Salmonella* spp. and enterohaemorrhagic *Escherichia coli* (EHEC) as the second and fourth most frequently acquired bacterial foodborne pathogens, respectively (Thomas et al., 2013). EHEC and *S. enterica* are gram-negative, non-spore forming, non-fastidious, facultatively anaerobic, mesophilic, enteric organisms. EHEC produce shiga-like toxins that give rise to severe disease, as these toxins are responsible for the development of hemolytic uremic syndrome and hemorrhagic colitis. In 2012, the vast majority of recall notices associated with *E. coli* O157:H7 were linked to beef products (110 of 113 recalls; CFIA, 2014). While recalls from 2012-2014 with *Salmonella* spp. as the causative agent were primarily associated with fresh produce, nuts and spices, poultry processors have committed to following strict processing standards for the control of *Salmonella*, for domestic consumer safety and international export (CFIA, 2014; CFIA-Annex U, 2014).

Bacterial pathogens present problems not only in the food supply, but are also problematic within agriculture and aquaculture industries. Modern agricultural practices increase the risk of infectious disease due to large animal-feeding operations, close proximity of animals, increased animal stress/susceptibility to disease and frequency of transmission of disease within

these environments (Samrah et al., 2006). The use of antibiotics for food-producing animals has greatly improved animal health through the prevention and spread of infectious disease in these settings (Diarra and Malouin, 2014). Antibiotics are frequently administered in low doses as growth promoters and prophylactic agents, in addition to administration for treatment of bacterial disease in agricultural animals (Schwarz et al., 2001). The extensive use of antimicrobials in agricultural industries is not without fault, as the use of low concentrations of antibiotics in agriculture selects for resistant organisms (Martinez, 2009). The increased frequency of antimicrobial resistant bacteria is considered the most daunting public health risk of our time (WHO, 2015). Responsible and prudent use of antimicrobial compounds is necessary to prevent the acquisition of antibiotic resistance in microorganisms. In addition to sensible use of antimicrobials, efforts to use compounds that are structurally unrelated to compounds currently used in clinical settings is necessary to further reduce the severity and prevalence of cross-resistance of antimicrobial compounds.

The Canadian salmon aquaculture industry represents the 4th largest producer of farmed salmon globally and Atlantic salmon (*Salmo salar)* exports are the largest Canadian aqua-cultural export (Fisheries and Oceans Canada, 2014). Of the vast number of infectious diseases that may affect aquaculture species, roughly 34% are bacterial infections (Lafferty et al., 2015). Interestingly, more infectious diseases occur in the Northern hemisphere making control and management of infectious disease an important consideration for Canadian aquaculture industries. This may be due to enhanced surveillance and reporting of diseases in northern hemispheres. Infectious bacteria, including *Aeromonas salmonicida, Renibacterium salmoninarum,* and *Vibrio salmonicida* infect salmon populations in Holarctic environments, making control and prevention strategies of economic and aquatic importance (Lafferty et al., 2015). *Listonella anguillarum* (formerly *Vibrio anguillarum*) is of particular importance as this pathogen is problematic on a global scale to a number of marine fish, including salmon, eels, turbot, plaice, dover-sole, bream, mullet, catfish and tilapia (Lafferty et al., 2015; Roberts, 2012). While these bacterial pathogens are not zoonoses, prevention and treatment of bacterial infections will lead to improved animal health and greater food security through mitigation of losses due to morbidity and mortality.

Application of the following novel antimicrobial peptides, microcins and tridecaptins to food and feedstuffs to aid in the prevention of bacterial disease was explored in the research reported in this thesis. Antimicrobial peptides offer a promising solution to decrease the risk associated with foodborne pathogens in meat products (Patton et al., 2008; Cotter et al., 2005). Bacteriocins, a class of antimicrobial peptides, are ribosomally synthesized by some microorganisms and exhibit a narrow spectrum of activity, generally active against closely related species (Cotter et al., 2005). Tridecaptins are novel antimicrobials not currently used in the food or aquaculture industries, thus exploiting *Paenibacillus* spp. for use in these industries presents an interesting and unique solution to animal and human health outcomes. Research on antimicrobials to provide the food and aquaculture industries with additional hurdles to control the growth and survival of pathogens are necessary. *Paenibacillus terrae* NRRL B- 30644 will receive particular attention for their ability to produce tridecaptin A_1.

1.1. The Canadian meat industry: At a glance

Beef is an economically important industry for Canadians and Albertans; in 2008, Canada produced 3.6 billion pounds of beef, contributing $26 billion to the Canadian economy, with 40% of the beef herd coming from Alberta (Canadian Beef Information Center, 2009). In 2015, Canadian beef exports totaled $2.2 billion, equivalent to 322,000 tonnes (Canadian Meat Council, 2016). The Canadian meat industry is currently the 6th largest producer of beef globally. Alberta is home to large federally inspected slaughter facilities, responsible for the processing of thousands of cattle per day, such as JBS and Cargill Meat Processing Solutions. In 2012, the Alberta economy suffered dramatic losses after the recall of contaminated meat from the XL Foods Inc. meat processing facility in southern Alberta (Lewis et al., 2013). This was the largest food recall in Canadian history, resulting in 1.8 million kg of beef being recalled and a $4 million settlement by XL foods (Graveland, 2015). To quantify this in terms of animal life wasted, this equates to a minimum of 12,000 head of cattle (Lewis et al., 2013). Furthermore, the agent responsible for the recall, *Escherichia coli* O157:H7, sickened 18 people.

According to the United States Center for Disease Control (U.S. C.D.C.) ground beef is the fourth most common cause of *Salmonella* spp. outbreaks (Andrews, 2014), although

Salmonella spp. are not considered adulterants in non-intact beef, this is not the case for EHEC serotypes O157:H7, O26, O45, O103, O111, O145 and O121 (United States Department of Agriculture, 2011). This new regulation means that any raw ground beef or precursors testing positive for these strains is prohibited from entering commerce in the US marketplace. Canadian processors must also comply with these regulations if they are shipping products to the USA, thus control of these pathogens is of paramount importance as 20,045 tonnes of beef were exported to the USA in January 2016, totaling $131,739,000 (Agriculture and Agri-Food Canada, 2016).

To understand the economic and social consequences, of contamination of food with pathogenic bacteria, one must understand the routes of contamination of food and foodborne illness. *E. coli* and *Salmonella* spp. are members of the intestinal microbiota of cattle—they inhabit the lower digestive tract and contribute to the commensal microbiota of cattle (Kim and Wells, 2016). During the slaughter and processing of cattle, feces and ruminal contents may contaminate meat, after evisceration and hide removal—cattle often have feces caked on hides when entering slaughter facilities. Though processing facilities have many carcass interventions to prevent microbial survival (hot water washes, lactic and peroxyacetic acid washes), pathogens may survive and be present in products leaving the processing plant. Contamination from pathogens is a major concern within the Canadian food supply, particularly the beef industry where contamination with these organisms may occur during slaughter and processing. Grinding muscle tissue disperses contamination throughout the product when making ground beef. Ground beef is an ideal growth-medium for bacteria as it has a pH between 5.6-5.7, contains abundant nutrients and minerals necessary for growth, in addition to having high water activity (Watters, 2002). Meat surface temperatures within retail displays can range from 1.7-10°C (Greer et al., 1994).

1.2. Microbiology of ground beef

Beef is a vehicle of transmission of *E. coli* and *Salmonella* spp. and interventions aimed at reducing the prevalence pathogens in products where multiple pathogens may be a risk are of particular importance. In most cases, contamination likely occurs through fecal contamination during animal production and slaughter operations and carcasses may become contaminated

during hide removal, evisceration or through cross-contamination of equipment or workers' hands. Currently, commonly used, approved carcass interventions include trimming, steam vacuuming, steam pasteurization, water washes and organic acid washes (Patton et al., 2008).

The typical microbiota of raw ground beef consists mainly of psychrotrophic organisms including *Achromobacter, Acinetobacter, Aeromonas, Alcaligenes, Athrobacter, Bacillus, Brochothrix, Campylobacter, Carnobacterium, Chromobacterium, Citrobacter, Clostridium, Corynebacterium, Enterobacter, Enterococcus, Escherichia, Flavobacterium, Hafnia, Klebsiella, Kluyvera, Kocuria, Kurthia, Lactobacillus, Lactococcus, Leuconostoc, Listeria, Microbacterium, Moraxella, Paenibacillus, Pantoea, Proteus, Providencia, Pseudomonas, Shewanella, Staphylococcus, Streptococcus, Vibrio, Weissella, and Yersinia* (Nychas et al., 2008). *Pseudomonas, Moraxella, Acinetobacter, Flavobacterium* are common spoilage organisms of fresh ground beef (López-Tomás et al., 2006). Depending on storage conditions of raw ground beef for sale in retail displays, mesophilic organisms, such as *E. coli* or *S. enterica,* may proliferate if products are temperature abused, and survival in low numbers can be problematic. Additional hurdles to prevent microbial growth are necessary to prevent growth of foodborne pathogens in raw meat.

1.2.1. Enterohaemorrhagic *Escherichia coli*

E. coli are gram-negative, catalase-positive, oxidase-negative, non-spore forming, non-fastidious, motile, rod-shaped bacterium that are facultatively anaerobic, demonstrating growth at temperatures as low as 7.5°C and a pH as low as 4.0 (Buchanan and Doyle, 1997; Brenner and Farmer, 2005a). *E. coli* is a natural and essential part of the commensal intestinal microbiota of mammals and most *E. coli* strains are not pathogenic (Brenner and Farmer, 2005a). Different classes of pathogenic *E. coli* organisms that are associated with foodborne disease include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), EHEC, enteroaggregrative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC). The presence or absence of certain virulence factors define the pathogenicity of different strains. *E. coli* O157:H7, the most well-known strain of EHEC, is a pathogen with an infectious dose that is extremely low, with estimates that between 2-1,000 cells may cause an *E. coli* O157:H7 infection (Buchanan and Doyle, 1997).

EHEC is of particular importance with regards to food safety and the food supply, not only because it has such a low infective dose but also because the consequences of infection in susceptible individuals are severe. EHEC are capable of producing shiga-like toxins (Stx1 and Stx2), also known as verotoxins, which are are cytotoxic as their presence inactivates protein synthesis and cells are unable to recover from exposure to these toxins, even after the toxin has cleared (Thorpe, 2004). This organism is capable of producing severe gastroenteritis that presents clinically as bloody diarrhea and 3-15% of cases of bloody diarrhea caused by EHEC result in development of hemolytic uremic syndrome (Razzaq, 2006). Hemolytic uremic syndrome is an acute renal failure caused by damage induced to red blood cells and is fatal in 3-5% of cases (Buchanan and Doyle, 1997). EHEC infection most commonly affects the immuno-compromised, small children and the elderly; however, it can affect all age groups and accounts for 15% of cases of bloody diarrhea (Buchanan and Doyle, 1997).

EHEC have a host of virulence factors that make them especially dangerous including the capacity to produce Shiga-like toxins, Stx1 and Stx2, the Locus of Enterocyte Effacement pathogenicity island, and O157 plasmid and other factors (Lim *et al,* 2010). The cytotoxins that EHECs produce are deemed shiga-like toxins because they exhibit a relatedness to the shiga toxin produced by *Shigella dysenteriae,* as Stx1 has a one amino acid difference from Stx while Stx2 is approximately 55% homologous to Stx on the primary structure level (Gamage et al., 2004). Stx2 is more pathogenic to humans and is associated with development of hemorrhagic colitis and hemolytic uremic syndromes (Lim et al., 2010). EHECS may harbor more than one prophage in their bacterial chromosomes, as these genetic elements are highly mobile and they are referred to as double lysogens in those cases (Fogg *et al,* 2012; Herold et al., 2004). In addition to genes that encode for virulence factors that make these organisms particularly dangerous, they have a suite of other genes that allow them to persist in a variety of environments—such as genes that garner protection for heat shock, osmotic stress, acid resistance, and cellular elongation.

Prior to the 2011 USDA amendment, *E. coli* O157:H7 was the only EHEC considered an adulterant by the USDA and FDA. Raw beef trim destined for retail distribution containing these strains will not be allowed to enter the food supply chain and will be subjected to food

recall (USDA, 2011). These all six EHEC serotypes have all been implicated in causation of human disease, and are gaining attention as the source of enterohemorrhagic colitis not associated with well-known *Escherichia coli* O157:H7. Like *Escherichia coli* O157:H7, these six serotypes are capable of producing shiga-like toxin that is responsible for the risk and severity of infection with these organisms (UDSA, 2011). This action marks the enforcement of the Obama Administrations' public health measures to safeguard the food supply, prevent foodborne illness and to improve consumer's knowledge about the food they eat (USDA, 2011). It should be noted that currently under Canadian regulations, neither the Food and Drugs Act or the Meat Inspection Act require the testing of raw meat products for the presence of *Escherichia coli* O157:H7 or any of the other serotypes newly classified as adulterants by the USDA (Health Canada, 2000), but if the Canadian beef industry seeks to export beef to the U.S., these testing requirements are necessary.

EHEC are commonly implicated in outbreaks stemming from non-intact meat products such as ground beef, but have also been linked to raw milk, unpasteurized apple juice, dry-cured salami, produce from manure-fertilized gardens, potatoes, radish and alfalfa sprouts, yogurt, sandwiches, spinach, cookie dough and water (Razzaq, 2006; CDC, 2016). Recently in Canada, recalls due to the presence of *E. coli* O157:H7 have been linked to ground beef, sirloin beef burgers, lean ground veal, peanuts and walnuts (CFIA, 2014a).

1.2.2. *Salmonella enterica*

Salmonella spp. are a gram-negative, motile, facultatively anaerobic, non-spore forming, rod-shaped, enteric pathogen, which like *E. coli* is a member of the *Enterobacteriaceae* family, specifically the *Salmonellae* genus (Brenner and Farmer, 2005b). *Salmonella* was first isolated from the digestive tract of swine infected with swine fever in 1855 by Dr. Theobald Smith (Eng et al., 2015). The nomenclature of *Salmonella* is contentious and is still up for debate by many. Currently the CDC and WHO Collaborating Center use the following system for *Salmonella* nomenclature: *Salmonella* is divided into two species (based on 16S rRNA analysis): *S. enterica* and *S. bongori,* both species have strains capable of causing disease in humans. *S. enterica* is further divided into the following subspecies: I. *S. enterica* subsp. *enterica,* II. *S. enterica* subsp. *salame,* IIIa. *S. enterica* subsp. *arizonae,* IIIb. *S. enterica* subsp. *diarizonae*, IV. *S.*

enterica subsp. *houtenae* and VI. *S. enterica* subsp. *indica.* This classification can be misleading because *S. enterica* contains over 2587 known serovars and previously each serovar was considered a separate species (Brenner et al., 2000; Guibourdenche et al., 2010). Genomic relatedness and biochemical screening then further stratify *S. enterica* serovars. *S. enterica* subsp. *enterica* is further divided into five sero-groups comprising 59% of *Salmonella* serotypes, based on O-antigen sero-groups: A, B, C1, C2, D and E (Brenner et al., 2000). The most common serotypes are implicated in foodborne disease in the US in 2013 were: *S. enterica* subsp. *enterica* ser. Enteritidis (19%), *S. enterica* subsp. *enterica* ser. Typhirumium (14%), *S. enterica* subsp. *enterica* ser. Newport (11%), *S. enterica* subsp. *enterica* ser. Javiana (10%), *S. enterica* subsp. *enterica* ser. I 4,[5],12,i- (6%), *S. enterica* subsp. *enterica* ser. Heidelberg (3%) and all other accounting for remaining 38% (CDC, 2016). Strains from these sero-groups comprise 99% of clinical isolates from humans and mammals (Brenner et al., 2000). *Salmonella* is a zoonotic organism and has emerged with multi-drug antibiotic resistance, due in part, to the use of antibiotics in the feed of farm and agriculture animals (Chalon et al., 2011). One can appreciate the scope the *S. enterica* serovar and understand why public health concern lies in controlling this species.

Salmonella spp. can cause two types of disease by infection: non-typhoidal salmonellosis or typhoid fever, both caused by *Salmonella* infection. Non-typhoidal *Salmonella* infection can cause severe illness with unpleasant symptoms but it is usually a self-limiting disease in those with healthy immune systems. The onset of non-typhoidal salmonellosis is usually 6-72 hours and the infective dose may be as low as a single cell (Hammack, 2012). Morality with this infection is less than 1% and complications from non-typhoidal salmonellosis in cases where reactive arthritis occurs happen in approximately 2% of culture-proven cases and onset is 3-4 weeks after the onset of acute symptoms (Hammack, 2012). Typhoid fever on the other hand, has a higher infective dose of approximately 10^3 CFU and has a delayed onset of 1-3 weeks and the mortality rate associated with typhoid fever is around 10% (Hammack, 2012). Symptoms of typhoid or enteric fever include headache, abdominal pain, diarrhea or constipation and fever, usually low grade to begin moving to high grade in the second week of illness (Eng et al., 2015). Typhoid-salmonellosis has a usual duration of 2-4 weeks and complications from this infection may include septicaemia, septic arthritis and chronic infection of the gallbladder, which would

cause this individual to become a carrier. Typhoid salmonellosis is not a foodborne disease, though can be transmitted through contaminated food via carriers (Brenner and Farmer, 2005b; Connor and Schawrtz, 2005). Gastroenteritis caused by *S. enterica* is marked during this progression of events by sudden nausea, vomiting, abdominal cramps, diarrhea, headache, chills and fever, sometimes lasting 5-7 days (PHAC, 2016).

Salmonella spp. are widely dispersed in nature and has been isolated from a number of different natural environments. Foods which have been linked to *Salmonella* contamination include beef, poultry, eggs, milk and dairy products, fish, shrimp, spices, yeast, coconut, sauces, unpasteurized salad dressings, cake mixes, cream-filled desserts, dried gelatine, peanut butter, cocoa, fresh produce and any foods contaminated by handlers who are carriers (Hammack, 2012; Koohmaraie et al., 2012; Jackson et al., 2013). Researchers found a prevalence of 4.2% of *Salmonella* in commercial ground beef, with the primary source of *Salmonella* contamination in beef from hide and lymph nodes (Bosilevac et al., 2009; Koohmaraie et al., 2012). *S. enterica* need to be combated with innovative approaches through microbial hurdle treatment in food processing in order to reduce recalls, outbreaks and disease.

1.3. Antimicrobial peptides produced by bacteria: bacteriocins

Bacteriocins, a class of antimicrobial peptides, are ribosomally synthesized peptides, to which the producer has an immunity mechanism and they exhibit a narrow spectrum of activity as they are generally active against closely related species and have low toxicities and high potency (Etayash et al., 2016; Cotter et al., 2013; Cotter et al., 2005). Bacteriocins are a group of structurally diverse peptides, with distinct mechanisms of action and specific microbial targets.

Bacteriocins produced by gram-positive bacteria, are classified into groups based on whether they undergo post-translational modification and subsequent structural rearrangement. Class I bacteriocins, also known as Lantibiotics, are characterized by their unusual ringed structures, consisting of lanthionine and β-methyllanthionine residues (Lohans and Vederas, 2011). Lantibiotics generally exert their antimicrobial activity through interaction with Lipid II on the cell membrane of the susceptible organism. The bacterial cell wall is comprised of alternating units of the disaccharides, N-Acetyl Glucosamine and N-Acetyl Muramic acid (GlcNAc-MurNAc) to which a pentapeptide (L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine) is

attached (Tesser and Nivard, 1964; de Kruijff et al., 2008). Cell wall precursors are assembled within the cytosol and are transported by Lipid II across the cell membrane for cell wall assembly, in a series of enzymatic steps (de Kruijff et al., 2008). Lantibiotic interaction with lipid II has a two-fold inhibitory mechanism in that it interacts with lipid II, preventing the transfer of the pentapeptide subunit preventing further synthesis of the bacterial cell wall; and secondly, as lipid II moves back across the membrane to facilitate transport of the pentapeptide subunit, the lantibiotic-lipid II complex causes the formation of pores in the cell membrane, resulting in a loss of cellular contents and membrane potential (de Kruijff et al., 2008). Examples of lantibiotics include: Nisin, lacticin 3147, paenicidin A and paenicidin B (Hart et al., 2016; García-Ruiz et al., 2013; Lohans et al, 2012, Lohans et al, 2014). It is noteworthy that other classes of bacteriocins exist, namely, class IIa (pediocin-like or *Listeria*-active: leucocin A, pediocin PA1; Derksen et al., 2008), class IIb (two-peptide bacteriocins: Enterocin 7; Lohans et al., 2013a), class IIc (carnocyclin A: Martin-Visscher et al., 2011; Gong et al, 2009), and class IId (non-pediocin single linear peptides: lactococcin Z; Ishibashi et al., 2015).

The bacteriocins of gram-negative bacteria (colicins and microcins) have unique structural motifs that separate them from the bacteriocins from gram-positive bacteria; they have unique target organisms and unique mechanisms of action. Bacteriocins secreted by gram-negative bacteria, called colicins (30-80 kDa) or microcins (1-10 kDa), are produced predominately by *E. coli* and other *Enterobacteriaceae,* and are distinguished on the basis of molecular size (Guarner and Malageleda, 2003; Duquesne et al*.,* 2007; O'Brien, 1996). According to Rebuffat (2011), colicins are bactericidal proteins produced by *E. coli* harboring a colicinogenic plasmid. Colicinogenic plasmids contain a structural gene (*cxa*), immunity protein (*cxi* or *imX)* and a gene encoding lysis proteins. Regulation of colicin production is mediated by the SOS response, which plays a role in the response of host bacterial cells to DNA damage. Colicins have three different modes of action: 1. formation of voltage-dependent channels in the inner membrane of gram-negative bacteria; 2. nuclease action within cytoplasm of sensitive cells; or 3. degradation of peptidoglycan (Gillor et al., 2004; Rebuffat, 2011). Most colicins hijack cell-surface receptors to gain access to susceptible cells. Colicins are considered to have three functional protein domains, each responsible for a distinct step in the mode of action: central domain is involved in binding to the receptor of target cell, the N-terminal domain is

responsible for translocation, enabling entry of bacteriocin to susceptible cells, and lastly the C-terminal domain is the active, killing region of the peptide (Rebuffat, 2011).

Microcins, as a class, have molecular masses less than 10 kDa, are fairly heat stable, resistant to fluctuations in pH, resistant to denaturation by proteases, and are ribosomally synthesized peptides, with non-inducible production with dedicated export systems (Vassiliadis et al., 2011, Duquesne et al*.,* 2007). Microcins demonstrate a narrow spectrum of activity and are active against species that are closely related to the producing organism. Microcins are separated into two separate classes based on molecular weight. Unlike colicins, microcins are generally produced under nutrient depletion or cellular stress (Vassiliadis et al., 2011). Microcins are encoded by genetic machinery located on plasmids and in some cases, on chromosomes. Classification of microcins involves three criteria: 1. the presence, nature and localization of posttranslational modifications; 2. gene cluster organization; and 3. sequences of leader peptides. Class I microcins consist of peptides that have a molecular mass below 5 kDa and undergo extensive posttranslational modification. Examples of class I microcins include: Microcin B, Mirocin C7-C51 and Microcin J25 (Shukundina et al., 2014; Heddle et al., 2001; Metliskaya et al., 1995). Class II microcins have masses between 5-10 kDa and further classified into subgroups: IIa and IIb. Class IIa microcin peptides are plasmid-encoded, contain disulfide linkages but undergo no post-translational modification (Rebuffat, 2011; Duquesne et al*.,* 2007). Examples of class IIb microcins include Microcin L (Pons et al., 2004) Microcin V (Azpiroz and Laviña, 2007), Microcin N (Corsini et al., 2010) and newly discovered Microcin PDI (Zhao et al., 2015). Class IIb microcin peptides are chromosomally encoded peptides that undergo a *C*-terminal post-translation modification (generally siderophore) (Vassiliadis et al., 2011; Duquesne et al*.,* 2007). Recent advances in the class IIb microcins have involved the creation of a recombinant strain, *Lactobacillus plantarum* 8148-ColV, capable of producing McnV and pediocin PA-1 simultaneously through fusion of the Pediocin PA-1 leader peptide with *McnV* (Ma et al., 2016). It is of importance to note that McnN (Mcn24) does not perfectly match the classification criteria; McnN has not been isolated or biochemically characterized to date, and does not contain a disulfide bond or posttranslational modifications (Vassiliadis et al., 2011). McnN was placed into class IIb based on genetic cluster organization. Microcins require target cell receptor interaction in order to illicit mechanism of action: class I microcins inhibit bacterial

enzymes while class II microcins target the inner membrane of susceptible cells, permeabilizing the inner membrane (Zhao et al., 2015; Vassiliadis et al., 2011).

1.3.3. Microcin N

Microcin N (McnN; 7217.76 Da), previously known as Mcn24, is produced by *Escherichia coli* MC4100 pGOB18 and is active against EHEC and *S. enterica* (Corsini et al., 2010; Wooley et al., 1999; Zihler et al., 2009; Frana et al., 2004). Class IIa microcin peptides are encrypted by gene clusters that consist of four genes, all encoded on plasmids. In the case of McnN, the plasmid, p24-2, is a 43.54-kb conjugative plasmid isolated from an uropathogenic strain, *E. coli* 2424 originally found to produce Mcn24 (O'Brien and Mahanty, 1994). McnN production is not SOS inducible but its production is initiated at the beginning of the stationary phase of growth (O'Brien, 1996). Extracts of Mcn24 were able to degrade linear and covalently closed plasmid DNA, highlighting the potential DNAse activity of Mcn24 (O'Brien, 1996). McnN has been hypothesized to consist of 90 amino acids; however, its actual structure has not been elucidated. The Mcn24 gene cluster contains four genes located in a single operon, required for functionality, *mtfS, mtfI, mtfA,* and *mtfB*, and they are required for formation of the precursor peptide, immunity protein, and export proteins, respectively (O'Brien and Mahanty, 1994; Duquesne et al*.,* 2007). However, further research highlighted discrepancies between experimental molecular mass of Mcn24 and theoretical mass of Mcn24 and researchers re-sequenced the pGOB18 plasmid and found that *mcnN* is different from previously reported *mtfS* (Corsini et al*.,* 2010), thus Mcn24 was renamed McnN. The structural gene encoding McnN has three additional guanine nucleotides when compared to the structural gene for Mcn24 (Corsini et al*.,* 2010). The corrected sequence encodes for a peptide that is 75 residues in length. An additional change in the sequence of the microcin genetic locus in pGOB18 is the insertion of an adenine in the *mdbA* gene. This insertion is responsible for a frame shift mutation that generates a new protein with only 60.2% identity to the previously reported sequence (Corsini et al*.,* 2010). In susceptible cells, the uptake of Mcn24 is dependent on the presence of *SemA* and/or *TonB*, both are genes that encode for membrane proteins within *E. coli* and are involved in microcin resistance and sensitivity, respectively (O'Brien, 1996). *E. coli* strains harboring the *semA* outer membrane receptor are resistant to the actions of Mcn24 (O'Brien, 1996). The relatively small

nature of these peptides and the effectiveness against enteric gram-negative pathogens makes them promising candidates for applications to improve food safety (Duquesne et al., 2007). Previous research investigated the use of a variant of McnN, Mcn24, in swine production and found that it inhibited the growth and survival of *Salmonella in vivo,* but it was not able to prevent shedding of *S.* Typhimurium during swine production though it was able to inhibit *S.* Typhimurium in chickens (Frana, 2004; Frana et al., 2004; Wooley et al., 1999). However, studies with McnN in meat have not been done. Given these findings, McnN is an ideal candidate to explore application to control *E. coli* and *S. enterica* in meat systems.

1.4. The Canadian salmon industry

Aquaculture encompasses the farming of aquatic species for commercial benefit in controlled and selected environments (Storey, 2005). The Canadian aquaculture industry is an active industry, totaling to $838.4 million in operating revenues, contributing $300.6 billion to the Canadian economy in 2011 (Statistics Canada, 2012). Finfish sales contribute 89.8% of that total operating revenue and salmon dominates the productive species in Canada, at 63% of production by volume in 2011 (Statistics Canada, 2012). In 2011, Canada produced 102,064 tonnes of salmon, compared with 6,511 tonnes of trout, 25,509 tonnes of mussels and 10,880 tonnes of oysters (Statistics Canada, 2012). The value of this salmon was $606,775,000 with British Columbia, New Brunswick and Nova Scotia as the provinces that contribute most to salmon production in Canada (Statistics Canada, 2012). Given that salmon are such an economically important commodity to the Canadian aquaculture industry, unanticipated losses due to premature mortality pose dire consequences.

The following description of salmon farming is summarized from the Canadian Aquaculture Industry Alliance (2016). To produce salmon, premium females are chosen to serve as the breeding stock. Each female fish produces 10,000 eggs that are manually fertilized and incubated at a fresh-water hatchery. Once the eggs hatch, juvenile salmon remain at the hatchery up to 18 months to grow and eventually undergo the smolting process. Once the salmon smolt, they are transferred from freshwater hatcheries to open net-pens that float in the open ocean. The fish grow in these open net pens for an additional 18 months, or until they reach harvest weights of around 4.5 kg.

The Canadian salmon industry started in the 1980's in British Columbia's Sunshine Coast region, initially with Pacific salmon species including Chinook (*Oncorhynchus tshawytscha)* and Coho *O. kisutch)* were the predominant species produced; however, over time, Atlantic salmon *(S. salar)* took over as the dominant species produced (Morrison and Saksida, 2013). The transition to rearing Atlantic salmon occurred for two primary reasons: industry farms relocated to more northern locations due to warming water temperature and subsequent harmful algal blooms and both species of farmed Pacific salmon are more prone to disease than farmed Atlantic salmon (Morrison and Saksida, 2013). Pacific salmon are prone to higher occurrences and severity of infection by *R. salmoninarum*, the causative agent of bacterial kidney disease. Due to the nature of growth and life cycle of the pathogen, fish are affected later in their lifecycle, greatly affecting stocks just shy of market weight. This is not to imply that farmed Atlantic salmon are free from disease; diseases experienced by this species are usually caused early in the saltwater lifecycle by gram-negative bacterial species rampant in the Pacific Northwest: *A. salmonicidia, L. anguillarum* and *Y. ruckeri* comprise a few of the problematic pathogens (Morrison and Saksida, 2013). Researchers found a prevalence of *L. anguillarum* ranged from 31% and 15% in wild juvenile Chinook and Coho salmon, respectively (Arkoosh et al*.,* 2004). Management of aquaculture stocks is of increasing importance as escaped farmed salmon can have dramatic effects on wild populations. Farmed salmon tend to have a higher incidence of *V. anguillarum* and other diseases that spread to wild populations when fish escape into the environment (COSEWIC, 2006). Of 11 diseases and disease agents investigated in Maritimes (excluding sea lice), 10 were reported from aquaculture sites and 3 later reported in wild stocks (COSEWIC, 2006). Responsible management of aquaculture sites and stocks is necessary to prevent the spread of disease to wild, vulnerable, endangered populations of Atlantic salmon. Wild stocks of Atlantic salmon in the Bay of Fundy are rapidly declining and the species is currently listed as endangered; thus the use and responsible application of antimicrobials to prevent the spread of disease may help mitigate continued losses of wild salmon from these regions.

1.5. Salmon pathogen: *Listonella anguillarum*

Listonella anguillarum (previously *V. anguillarum*) is a gram-negative, non-spore forming, motile, halophilic, facultatively anaerobic, psychrotrophic bacterium that is the

causative agent of vibriosis in salmonid species (Lawlor et al., 2009). *L. anguillarum* occurs frequently in marine and estuarine farming environments though it can also be problematic in freshwater farms (Lawlor et al., 2009). Vibriosis, the bacterial septicemia caused by *L. anguillarum,* occurs predominately in farmed Atlantic salmon, reaching peak levels in the summer, characterized by internal and external hemorrhages and anemia and is said to be one of the most important bacterial infections in fish globally (Bjelland A.M. et al., 2013; Crisafi et al., 2014; Myhr et al., 1991). The disease was first reported in 1893 as *Anguilla anguilla* in eels (Frans et al., 2011). *L. anguillarum* is found various cultured and wild fish stocks and currently 23 distinct O serotypes have been identified, each displaying different host specificity and pathogenicity (Frans et al., 2011). Serotypes O1 and O2 predominately affect salmon with 98.3% of diseased Atlantic salmon isolates belong to these serovars (Myhr et al., 1991). *L. anguillarum* has the most significant impact on cultured fish as these animals undergo more stress through elevated water temperatures and low dissolved oxygen with seasonal variations and confinement in open-net pens (Altinok et al., 2015).

L. anguillarum has two separate circular chromosomes (3.0 and 1.2 Mbp, respectively) that are thought to give rise to its enhanced survival in numerous environments that make it a virulent pathogen (Frans et al., 2011). As an illustration of the adaptability of *L. anguillarum,* this organism is capable of surviving in saltwater, freshwater and mixed estuaries in addition to an ability to survive in a range of conditions. Water temperature and salinity determine host colonization, abundance, distribution, survival and adherence to host mucosa (Crisafi et al., 2014). In addition to two chromosomes, *L. anguillarum* has a virulence plasmid (pJM1 or pEIB1; 65-67 kbp) that encodes siderophore-dependent iron sequestering system (Frans et al., 2011). In conditions of high osmotic stress (5% NaCl), increased temperatures (15°C) and iron deficiency, *L. anguillarum* isolates demonstrated increased expression of virulence genes (*toxR, fur, OMP, angR, fatA, tonB2* and *empA)* (Crisafi et al., 2014). These findings are important because they illustrate that stressed organisms have the potential to be more virulent pathogens. With increasing concern about global warming and warming oceans, it can be hypothesized that we may see increased incidence of *L. anguillarum* outbreaks in aquaculture operations.

Histopathologies of vibriosis from *L. anguillarum* in salmon present most extensively in vasculature and haematopoietic tissues; bacterial cells are consistently located in blood vessels and hemorrhaging tissues in salmon infected, especially kidneys, spleen, liver, cardiac muscle and loose connective tissue (Ransom et al., 1984). Hemorrhages were frequently observed in muscle tissue, gills and throughout the digestive tract of infected salmon. Necrosis presents in many tissues including posterior mucosal regions of the digestive tract, spleen and kidneys and colonies are isolated from blood samples of infected fish. Researchers estimate that based on the amount of lesions present in the posterior digestive tract, that the route of infection is through the gastrointestinal tract (Ransom et al., 1984). It is of note that the pH of the digestive tract of salmon ranges from 2.1-3.8 in the stomach, 6.6-7.0 in the ascending and descending intestine and 7.5-8 in the rectum (Ransom et al., 1984). Clinical signs of vibriosis caused by *L. anguillarum* include weight loss, lethargy, hemorrhaging on ventral and lateral sides of salmon and inflamed and dark abrasions that ulcerate and bleed (Frans et al., 2011). Due to the swift progression of disease, many fish affected die without showing clinical signs of vibriosis (Frans et al, 2011).

1.6. Peptide antibiotics produced by bacteria: Lipopeptides

Lipopeptides are linear or cyclic peptides that contain a lipophilic hydrocarbon tail, located on the N-terminus of the peptide (Cochrane and Vederas, 2016). Most lipopeptides have narrow clinical use due to systemic toxicity concerns and have been limited to topical uses, though a recent systematic review of polymyxins has shown that the incidence of nephrotoxicity an neurotoxicity is less frequent and severe than what has been previously reported (Falagas and Kasiakou, 2005). Lipopeptides are gaining attention and popularity due to diverse applications within food, pharmaceutical, cosmetic and biotechnology industries (Mandal et al., 2013). Lipopeptides produced by *Paenibacillus* spp. are secondary metabolites produced by non-ribosomal peptide synthases (NRPS) (Finking and Marahiel, 2004). Peptides produced via NRPS synthesis versus ribosomal synthesis differ because the NRPS systems lack the proof reading mechanisms present in ribosomal peptide synthesis, and several hundred substrates are available to peptides produced via NRPS systems which yields a group of structurally diverse proteins and peptides (Finking and Marahiel, 2004). The genetics for NRPS systems are not as widely distributed across bacterial species and are present only in *Bacillus* spp., *Paenibacillus* spp. and *Streptomyces* spp. (Lohans et al., 2015). Research efforts of this thesis have primarily focused

on work with tridecaptins, but in an effort to provide detail about a related class of lipopeptides, polymyxins will be described. Polymyxins are cyclic cationic lipopeptides, containing five to six 2,4-diaminobutyric acid residues, which give rise to the positive charge of these molecule (Cochrane and Vederas, 2016). They exert strong activity against gram-negative bacteria through the following mechanism: interaction with lipid A component of lipopolysaccharide (LPS), causing a disturbance in the outer membrane that ultimately permeabilizes and disrupts the inner membrane (Dijkmans et al., 2015). Polymyxins are used as a last resort for multi-drug resistant bacterial infections and Polymyxin E (Colistin; Figure 1-1) is one of the few options antibiotics active against multi-drug resistant bacteria (Falagas and Kasiakou, 2006; Dijkmans et al., 2015).

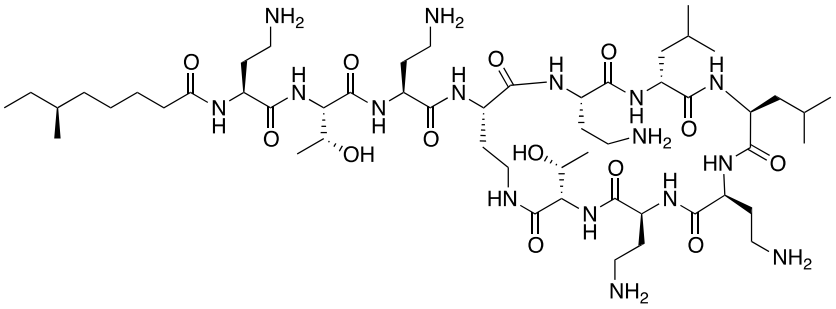

Figure 1-1. Chemical structure of Polymyxin E (Colistin). Figure provided by Dr. Stephen Cochrane.

Tridecaptins are a group of linear cationic lipopeptides structurally related to the polymyxins (Cochrane et al., 2013). These peptides were first discovered the 1970's (Shoji et al; 1978; Kato et al; 1978; Shohi et al; 1979), but little attention was given until recently when researchers at the University of Alberta conducted studies on the mode of action and chemical synthesis of Tridecaptin A_1 (Tri A_1; Figure 1-2) originally isolated from *Paenibacillus terrae* NRRL B-30644 (previously *Bacillus circulans* NRRL B-30644; Lohans et al., 2014).

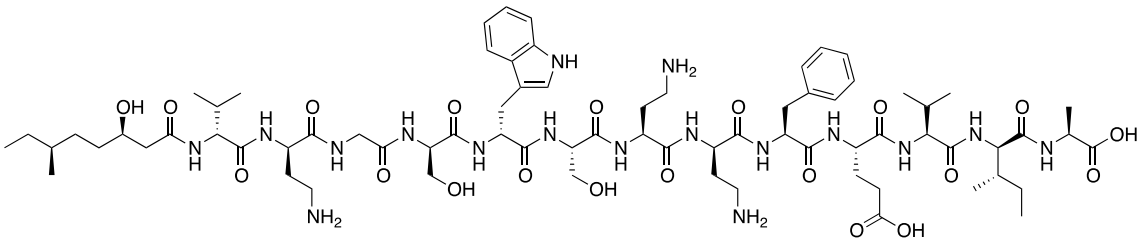

Figure 1-2. Chemical structure of Tridecaptin A_1. Figure provided by Dr. Stephen Cochrane.

In addition to $TriA_1$, *P. terrae* NRRL B-30644 also produces paenicidin B, a class I lantibiotic (Lohans et al., 2014). Octyl-tridecaptin A_1 (Oct-$TriA_1$; Figure 1-3) is a synthetic analogue of $TriA_1$, synthesized by lipid tail modifications of the N-terminus by acylation with octanoic acid (Cochrane et al., 2013).

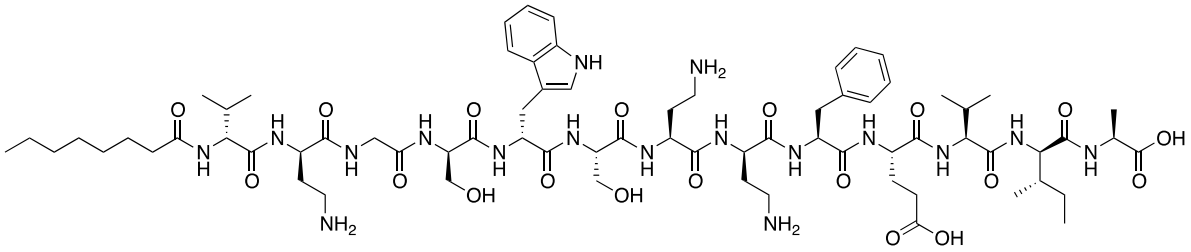

Figure 1-3. Chemical structure of Octyl-Tridecaptin A_1. Figure provided by Dr. Stephen Cochrane.

Lipopeptides are especially effective antimicrobial compounds because they are very effective at disrupting membranes, the mechanism by which they exert bactericidal effects (Cochrane and Vederas, 2016). Microbes have difficulty developing resistance mechanisms to antibacterial agents that affect the membrane as rearrangement of the phospholipid bilayer (Cochrane and Vederas, 2016). Tri A_1 elicits inhibitory effects against both gram-negative and gram-positive microorganisms, though potency is greatest against gram-negative organisms (Lohans et al., 2013b). $TriA_1$ operates through a membrane disruption mechanism by interacting preferentially with lipid II of gram-negative bacteria (Cochrane, 2015). Tridecaptins are stable at temperatures up to 95°C, are resistant to gastrointestinal proteases, show selective and strong activity against gram-negative bacteria, have low hemolytic activity and cytotoxicity (Cochrane, 2015). Native $TriA_1$ can be purified from the supernatant of *P. terrae*, giving an efficacious means for application of these peptides at relatively low cost. Tridecaptins have not been investigated for activity against gram-negative aquatic pathogens to date.

1.7. Administration of antimicrobials to salmon

Antimicrobial use within Canadian salmon farming differs from other agri-food (pork, beef, dairy, poultry, aquaculture) sectors because administration of antimicrobial compounds must be by veterinarian prescription, only for clinical disease, use must be reported to and monitored by Fisheries and Oceans Canada (DFO) and must be delivered to food producing fish

via medicated feed (Morrison and Saksida, 2013). The most noted difference between salmon farming and other agri-food industries is the restriction of antimicrobial use as prophylactic agents. Currently, the only approved antibiotics for use in salmon aquaculture in Canada and the US are: Aquaflor® (Florfenicol; Merck Animal Health, Summit, NJ), Romet-30® (Sulfadimethoxine/ormetoprim; Aquatic Health Resources, Minnetonka MI), Tribrissen-40 powder® (Sulfadiazine/trimethoprim; Merck Animal Health) and Terramycin Aqua® (Oxytetracycline; Phibro Animal Health, Regina, SK) (Morrison and Saksida, 2013; Storey, 2005) . Obtaining approval for a new class of antibiotic may be a challenge, but would be advantageous in the fight against the spread of multi-drug resistant bacteria. Tridecaptins are not structurally related to current classes of approved antibiotics (Table 1-1) and their use and approval would limit the acquisition of cross-resistance in aquatic microbes.

Table 1-1.Comparison of antimicrobials approved for use in Canadian salmon farming and Tridecaptin A_1

Antimicrobial	Antibiotic compound	Mechanisms of action	Microbial target	Resistance?
Aquaflor®	Florfenicol	Protein synthesis	Gram-negative	Yes: White et al., 2000
Romet-30®	Sulfadimethoxine /ormetoprim	Nucleotide biosynthesis	Gram-negative	Yes: Cooper et al., 1993
Tribrissen-40 powder®	Sulfadiazine /trimethoprim	Nucleotide biosynthesis	Gram-negative /gram-positive	Yes: Cooper et al., 1993
Terramycin Aqua®	Oxytetracycline	Protein synthesis	Gram-negative /gram-positive	Yes: Hargrave et al., 2008
Tridecaptin A_1	Tridecaptin A_1	Cell wall synthesis/ cytoplasmic membrane integrity	Gram-negative	Resistance not determined; Lohans et al., 2012

Given that regulation requires antimicrobial compounds be administered to salmon via medicated feed, approaches to incorporate antimicrobial compounds using a medicated feed approach were explored, though it should be noted numerous methods of drug administration to salmon exist. These methods include (a) water medication: bath treatment (suitable for closed-pen and land based facilities where high numbers of animals are affected), immersion or dipping

of individual sick fish and flushing (removing treated water after a given time point); (b) in feed medication: pelleted medicated feed, surface-coated pelleted feed, spray-coated pelleted feed or microencapsulation of antimicrobials; (c) Gavage or forced oral administration of antimicrobials (d) injection: intramuscular, intraperitoneal, dorso-median sinus or; (e) implantation of antimicrobial compounds (Treves-Brown, 2000). The most cost effective routes of antimicrobial application include surface treatment of pelleted feed and encapsulation of antimicrobials.

There are a number of regulatory considerations for the use of antimicrobial compounds in aquaculture; presenting a barrier to the implementation of many pharmaceutical drugs that have been proven to be effective in aquatic species are not approved for use in humans or animals, thus cannot be prescribed for use under the US FDA Animal Medicinal Drug Use Clarification Act (AMDCUA; Storey, 2005). Significant resources are required to obtain approval of a New Animal Drug Application (NADA) and approval costs can be upwards of $40 million USD. These costs include experimental data that the products are safe, efficacious and will not pose environmental concerns (to humans or species of interest), to meet the statutory requirements of each NADA (Storey, 2005). The requirements for a NADA are the same regardless of animal species and given the small size of the aquaculture industry compared with other agri-food industries, justification of the expense for many pharmaceutical ventures is lacking. There are ways to mitigate the expense of drug development, for example off the lable use of antimicrobials prescribed by a veterinarian and demonstration of efficacious use. Continued research on novel antimicrobials is necessary to help streamline pharmaceutical development and approval processes.

When antimicrobials are dispensed to salmon in husbandry practices there are multiple fates of the antimicrobials in question. Administration of medicated feeds to open-net pen aquaculture farms may be eaten by diseased fish and treat the bacterial infection it is intended for. Mediated feed may pass through open net pens and may be available to wild fish stocks through direct ingestion or bioaccumulation (Scott, 2004). Alternatively, antimicrobials can leach into surrounding water from medicated feed or by being excreted un-metabolized (Scott, 2004). It is well established that diseased fish feed poorly and researchers estimate that 40.5% of feed may pass through aquaculture net pens uneaten (Scott, 2004). Researchers also found that

using alginate coatings prevented leaching of antimicrobials compared with oil coatings of medicated feed between 61% and 26.6% for trimethoprim and oxytetracycline, respectively, following 15 min water immersion (Duis et al., 1994).

Encapsulation is the process of capturing and protecting bioactive compounds, lives cells, and pharmaceuticals in a matrix for delivery into foods or other materials (Nedovic et al., 2011). The principle behind encapsulation involves using a solid matrix or core material to separate bioactive components, bacterial cells or other agents of interest from the environment. Microencapsulates are generally spherical semi-permeable networks, ranging in size from micrometers to millimeters (Kaliasapathy, 2002). Alginate hydrocolloids, harvested from brown algae, are an attractive matrix because of the ease of preparation, they are non-toxic, biodegradable and food grade. Safe and efficacious use of alginates for administration of compounds and probiotics to aquatic species has been demonstrated in a variety of settings and species (Ghosh et al., 2016; Pirarat et al., 2015; Ghosh et al., 2015).

Sodium alginate is the sodium containing, water soluble salt of alginic acid, which is the predominant structural component of *Ascophyllum, Durvillaea, Ecklonia, Laminaria, Lessonia, Macrocytsis, Phaeophyceace, Sargassum* and *Trubinaria* brown seaweeds (McHugh, 2003a; Paolucci et al., 2015). Alginates are desirable encapsulation matrices as when dissolved in water, they are able to form gels in the presence of divalent cations, without heating. These gels are also relatively stable, as they do not melt when heated (McHugh, 2003b). Alginate polymers are composed of two monomeric units, β-D-mannuronic acid and α-L-guluronic acid. Alginate polymers are composed of three regions or blocks: G-blocks contain only repeating α-L-guluronic acid monomers, M-blocks are contain only repeating units of β-D-mannuronic acid monomers while MG-blocks consist of alternating units of β-D-mannuronic acid and α-L-guluronic acids (McHugh, 1987). In an aqueous suspension, sodium alginate forms water-insoluble gels by cross-linking divalent cations, such as calcium, between COO^- groups found in G-blocks, which link different polysaccharide chains (McHugh, 1987; Paolucci et al., 2015). The buckled G-block chains (forming a structure analogous to a corrugated egg-box) contains crevices where cations arrange themselves, coordinated between two separate G-block linear polymers, termed cooperative binding (McHugh, 1987).

Alginates may be used to form films or beads of gel. Beads of alginate gels are formed when a sodium alginate solution (1-4%) is extruded into calcium chloride solutions (0.05-0.1M), resulting in the formation of an immediate skin on the exterior of the alginate droplet (McHugh, 1987; Trabelsi et al., 2014). As calcium ions gradually infuse inward, a gel forms. Drying alginate gels prior to use increases gel strength, durability and reduces swelling on reintroduction of moisture. Producing alginate gels via extrusion forms capsules that are spherical beads, with a solid core (Vemmer and Patel, 2013). Encapsulation protects compounds from environmental factors, extends shelf-life, and may help maintain metabolic activity of active components (Vemmer and Patel, 2013). Formation of solid alginate encapsulates is the most widely used polymer matrix for microbial control agents (Vemmer and Patel, 2013). Recently, application of alginates as method of immunization of Atlantic salmon was explored with considerable success. Release of proteins from encapsulates was found to be greatest at pH 9, which is close to the pH of the distal colon of salmon (pH 7-8) (Ghosh et al., 2015; Ransom et al., 1984). The expected site of infection with *L. anguillarum* in the distal colon, release of antimicrobial peptides bound in alginate would be greatest in this part of the digestive tract, thereby offering the greatest chance of inhibition. Given these considerations, encapsulation using alginate as a matrix is deemed a suitable choice for encapsulating sensitive antimicrobials to aid in the delivery to salmon, as it requires no specialized equipment for production, is cost-effective and has demonstrated effectiveness with respects to consumption, gastrointestinal transit and peptide release in Atlantic salmon (Ghosh et al., 2015).

1.8. Research objectives and hypotheses

The **long-term objective** of my research is to further the understanding of the antimicrobial peptides, microcin N with an emphasis on its application as an antimicrobial in meat products to enhance the quality and safety of meat products; and the use of $TriA_1$ in salmon feed for was explored for its ability to retain antimicrobial potency when exposed to feed and to design an delivery method for feeding salmon.

The **short-term objectives** of this research are to:

1. Determine the spectrum of activity and minimum inhibitory concentrations or arbitrary units of McnN and $TriA_1$ against the foodborne pathogens and aquatic salmon pathogens, respectively.

2. Test the ability of McnN and $TriA_1$ to control the growth of pathogens in meat and in salmon feed.

The specific hypotheses of this research are:

1. McnN will inhibit the growth of the foodborne pathogens, *E. coli* and *S. enterica.* $TriA_1$ will inhibit the growth of salmon pathogens such as *L. anguillarum.*
2. McnN and $TriA_1$ when applied *in vivo* will inhibit *E. coli* and *S. enterica* in meat and *L. anguillarum* in salmon feed, respectively.

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Chapter 2. Assessment of the potential of purified Microcin N to inhibit *Escherichia coli* O157:H7 and *Salmonella enterica* in raw and cooked ground beef

2.1. Introduction

Escherichia coli and *Salmonella enterica* are enteric organisms found commonly in the gastrointestinal tracts of mammalian animals. Enterohaemorrhagic *E. coli* (EHEC) and *S. enterica* are human pathogens and are commonly associated with foods of animal origin. In attempts to provide a safer food supply, research into the use of novel antimicrobials has emerged in recent years. Recently, there have been a number of outbreaks related to EHEC in the Canadian meat industry: the largest meat recall in Canadian history occurred in 2012, resulting in 1.8 million kg of beef being recalled and a $4 million settlement by XL foods (Graveland, 2015). The meat industry needs effective methods to control pathogenic bacteria in meats.

Microcins are antimicrobial peptides produced and secreted by a number of strains of *E. coli* and are active against members of the *Enterobacteriaceae* family, including EHECs and *Salmonella* spp. (Zhiler et al., 2009; Jordi et al., 2001; O'Brien et al., 1994) and may be useful to control these pathogens in meats. Microcins have a molecular weight of <10kDa, are generally quite hydrophobic and are stable to heat, pH extremes and proteases (Duquesne et al., 2007). Microcins are produced under conditions of cellular stress, such as nutrient depletion and are produced during stationary phase growth. Microcins are generally resistant to denaturation by heat, extreme pH's and proteases and demonstrate activity in the nanomolar range (Duquesne et al., 2007), making them especially good candidates for use as antimicrobial agents in food.

Microcin N (McnN), a bacteriocin produced by *E. coli* MC4100 pGOB18, has potential to control the growth and survival of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 and *Salmonella enterica* (Zihler et al., 2009; Frana et al., 2004; O'Brien, 1996; Wooley et al., 1999). McnN also referred to in literature as Colicin 24/Microcin 24 (Mcc24), originally isolated from *Escherichia coli* 2424 is classified as a Class IIa microcin and is closely related to Microcin V and Microcin L (Pons et al., 2004; O'Brien and Mahanty, 1994). To harness the antimicrobial potential of these peptides, purification and isolation of this microcin is necessary in meeting the

aims of potential application within food systems. The objective of this study was to purify McnN for use as an antimicrobial in raw and cooked ground beef. Little is known about the stability of McnN in different food systems. It is hypothesized that McnN will effectively inhibit the growth and survival of *E. coli* O157:H7 and *S. enterica* in ground beef. The objectives of this work were to develop a purification protocol for McnN and apply the purified product to raw and cooked ground beef to eliminate *E. coli* O157:H7 and *Salmonella*.

2.2. Materials and Methods

Bacterial strains, growth media and growth conditions. All bacterial strains used in this study are outlined in Table 2-1. Strains were stored at -80°C in LB broth (Difco™; Becton, Dickinson and Company, Mississauga, ON) with 40% glycerol. Prior to use in experiments strains were sub-cultured twice. *E. coli* MC4100 pGOB18 was grown from frozen stocks in 10 mL LB broth at 37°C for 24 h at 250 rpm. The strain was subcultured into 1 L minimal M63 (Appendix A; pH 7) supplemented with 0.2% glucose, 0.05% casamino acids (Bacto™; Becton, Dickinson and Company) and 0.05% Tryptone (Bacto™; Becton, Dickinson and Company) and incubated at 37°C for 24 h. Minimal media was used to grow *E. coli* MC4100 pGOB18 to reduce background interference with spent culture media in downstream purification and chromatographic separation. Following incubation, cultures were centrifuged for 15 m at 16,270 *g* at 4°C. The supernatant was used in downstream McnN purification.

Cocktails of five strains of *S. enterica* and of *E. coli* O157:H7 were prepared after cells of *S. enterica* (ATCC 8324; 8326; 10708; 13076; 13311) and non-shiga toxin producing *E. coli* O157:H7 (00-3581; 02-0304; 02-0627; 02-0628; 02-1840) were grown in LB broth for 24 h at 250 rpm. Individual strains were sub-cultured into fresh LB and incubated for 24 h before centrifugation (Allegra 25R Centrifuge, Model 208V, Beckman and Coulter, Palo Alto, CA) for 5 m at 5,311 *g* at 25°C to obtain cell pellets. Pellets were re-suspended in 1 mL fresh LB after the supernatant was discarded. Each of the resuspended cells of *S. enterica* or *E. coli* were combined into a sterile test tube and serially diluted in 0.85% saline to provide an inoculum level of 10^5 CFU/mL for application to ground meat. Starting inoculum level of respective cocktails was confirmed by plating serial dilutions on LB agar.

Table 2-1. Bacterial strains used in this work.

Strain	Characteristics	Source, if known	Reference
Escherichia coli MC4100 pGOB18	McnN producer		Corsini et al., 2010
Escherichia coli O157:H7 00-3581	Gram-negative, lactose fermenting coliform, Δstx1/stx2	Public Health Agency of Canada (PHAC)	Holley and Luciano, 2011
Escherichia coli O157:H7 02-0304	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Escherichia coli O157:H7 02-0627	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Escherichia coli O157:H7 02-0628	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Escherichia coli O157:H7 02-1840	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Salmonella enterica subsp. *enterica* serovar Cholerasuis ATCC 10708	Gram-negative, non-lactose fermenting enteric human pathogen	American Type Culture Collection (ATCC)	Davison et al., 1996
Salmonella enterica subsp. *enterica* serovar Enteriditis ATCC 13076	Gram-negative, non-lactose fermenting enteric human pathogen	ATCC	Stolle and Beck., 1988
Salmonella enterica subsp. e*nterica* serovar Typhimiruim ATCC 13311	Gram-negative, non-lactose fermenting enteric human pathogen	ATCC	Stolle and Beck., 1988
Salmonella enterica subsp. *enterica* serovar Gaminara ATCC 8324	Gram-negative, non-lactose fermenting enteric human pathogen	ATCC	Tindall et al., 2005
Salmonella enterica subsp. *enterica* serovar Heidelberg ATCC 8326	Gram-negative, non-lactose fermenting enteric human pathogen	ATCC	Tindall et al., 2005

DNA extraction and polymerase chain reaction amplification. Polymerase chain reaction (PCR) was used to isolate amplicon products to confirm the sequence of the McnN structural gene, as discrepancies in the literature regarding the sequence of McnN and Mcn24 are evident (Corsini et al., 2010; Frana, 2004; O'Brien, 1996). Plasmid DNA was isolated from an overnight culture of *E. coli* MC4100 pGOB18 grown in LB broth for 24 h at 37°C, using the DNeasy

Blood and Tissue Kit (Qiagen, Hilden, Germany). The Nanodrop Photospectrometer (Model 2000C, ThermoScientific, Waltham, MA) was used to analyze quality and yield of DNA after extraction. Briefly, primers were designed to target the structural gene of McnN (P985-Forward: 5'-GAT ATG CTT CAT ATA TCC ATG GCT AA-3' and P1529-Reverse: 5'-GTG TCT GTA CAC GAT TAC CAT AG-3'; product size of 529 bp) and were synthesized by Integrated DNA Technologies (Coralville, IO). Individual PCR reactions contained 1 µL 10 mM dNTPs, 5 µL PCR buffer, 2 µL 50 mM $MgCl_2$, 1 µL template DNA, 38.5 µL nuclease-free H_2O and 1 µL 20 pmol/µL of primers P985-F and P1529-R, respectively. To this reaction mixture, 0.5 µL Taq polymerase was added, for a total reaction volume of 50 µL. Applied Biosystems GeneAmp PCR system 9700 (ThermoFisher Scientific, Singapore) thermocycler instrumentation used for PCR reactions under following conditions: hot start at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 45 s, annealing at 40 for 40 s, and elongation at 72°C for 60 s. Reaction was held at 72°C for 7 min following cycle completion. Successful PCR reactions were confirmed, in duplicate, using gel electrophoresis in an 2% agarose gel to visualize products stained with SYBERsafe DNA gel stain (Invitrogen, Carlsbad CA) using AlphaImager HP (Cell Biosciences, Santa Clara CA) using 1kb Plus DNA Ladder (Invitrogen, Carlsbad CA) as reference (data not shown). PCR reaction products were stored at 4°C prior to shipment to Macrogen (Rockville, MD) for sequencing. The amino acid sequence of the microcin isolated in this study, denoted as $McnN^*$ was aligned with two other published peptide sequences obtained from National Center for Biotechnology Information (NCBI) to determine sequence similarities among McnN, Mcn24 and the microcin isolated in this study. A ClustalW multiple alignment of Microcin N (accession number: FJ895580.1), Microcin 24 (accession number: U47048) and the Microcin from *E. coli* MC4100 pGOB18 was done using default parameters in Geneious (Biomatters, Auckland, New Zealand).

Microcin N purification. Attempts to purify McnN following the procedure published by Corsini et al. (2004) were unsuccessful and a new purification protocol was developed. Briefly, 1 L of the culture supernatant of *E. coli* MC4100 pGOB18, that had been grown in LB broth for 24 h, was centrifuged (16,270 *g*), treated with protease inhibitors according to the manufacturer's instructions (cØmplete lysis-B (2x), EDTA free; Roche Applied Science). The supernatant was

loaded onto an activated Amberlite® XAD16N™ resin (Sigma-Aldrich®, St. Louis, MO) column that had been washed with 500 mL 0.1% trifluoroacetic acid (TFA). McnN was eluted using increasing concentrations of isopropyl alcohol (IPA; 0%, 20%, 50% and 80%+0.1% TFA) at a flow rate of 10 mL/m. The fractions were concentrated using a SPD Speedvac® SPD111V (Thermo Savant, Holbrook, NY). Antimicrobial activity was confirmed in the fraction eluted with 80% IPA, referred to as the crude McnN fraction, by spot-on-lawn assay using *S. enterica* ATCC 10708 and *E. coli* DH5α as the indicator organisms. *S. enterica* ATCC 10708 and E coli DH5α were chosen representative indicators as they were the most resistant to McnN, thus providing the minimum baseline of McnN inhibition. The concentrated 80% IPA + 0.1% TFA elutant from hydrophobic interactions chromatography, with observed antimicrobial activity, will be referred to as the crude McnN preparation.

Purification of McnN using high performance liquid chromatography (HPLC) followed hydrophobic interactions chromatography was performed. An aliquot of the semi-purified crude McnN fraction (0.5 mL) was injected into an HPLC (Varian Prostar analytical system, Model 210, Walnut Creek CA) equipped with a Rheodyne 7225i injector fitted with a 1 mL sample loop. The sample was separated on a Vydac C_{18} analytical peptide and protein column (300 **Å** pore diameter, 5 μm particle size, 4.6 x 250 mm) using water with 0.1% TFA and Acetonitrile (ACN) with 0.1% TFA as the mobile phases, with a gradient of 5-95% ACN. The flow rate was 1 mL/min of the mobile phases. Proteins were detected using Varian Prostar UV detector at 220 and 230 nm. In addition to the Vydac C_{18} column, a C_4 Analytical (Beckman Coulter HPLC System Gold, Palo Alto, CA; 1 mL/min, 2-98% gradient, 0.1% TFA and ACN with 0.1% TFA) and C_4 Preparatory columns (Gilson HPLC, 8 mL/min, 2-95% gradient, 0.1% TFA and ACN with 0.1% TFA), were used for purification. HPLC was repeated in a minimum of three times for each column tested.

A cation exchange SP-Sepharose column was also used to purify McnN, modeled after a method from Lohans et al., (2013). Briefly a SP-Sepharose column (Sigma Life Science, Sweden, 20 mL resin, 2.5 cm x 20 cm) was equilibrated with 100 mL Buffer 1 (20 mM sodium phosphate, 500 mL, pH 6.9) at a flow rate of 4 mL/min. Next, 50 mL of the crude McnN preparation was loaded onto the column with a flow rate of 2.5 mL/min. Stepwise gradients of increasing NaCl in Buffer 2 (0.2 M NaCl with 20 mM sodium phosphate) and Buffer 3 (1.0 M

NaCl with 20 mM sodium phosphate) were used to elute adsorbed proteins from the column. Fractions collected from buffer elutions 1, 2 and 3 were concentrated and desalted using C_{18} zip tip pipette tips (EMD Millipore, Darmstadt, Germany) prewashed with 3 x 10 µL of a 60:40 ratio of ACN and 0.1% Formic Acid (FA), followed by 3 x 10 µL 0.1% FA. Each sample was loaded onto tips (30 x 10 µL sample), which were washed with 0.1% FA 30 x 10 µL to desalt. Samples were eluted using 60:40 ACN:0.1% FA (3x 10 µL). Samples were concentrated on a rotary evaporator for 15 min prior to MALDI-TOF analysis. Desalted, concentrated samples from buffers 1, 2 and 3 were also used in spot-on-lawn assays to assess antimicrobial activity. McnN was confirmed by MALDI-TOF MS analysis to elute in Buffer 2 using 0.2 M NaCl. HPLC separation of Buffer 2 was performed with a Vydac C_{18} Peptide and Protein column, with a defined clear peak eluting at 23 min. Buffer 2 was subsequently concentrated and desalted using 5 g of Amberlite XAD 16N resin, equilibrated in IPA. The resin was washed with 60 mL of 0.1% TFA prior to loading Buffer 2 to column at a flow rate of 10 mL/min. The column was washed with 32 mL of miliQ water and 32 mL 80% IPA + 0.1% TFA. The 80% IPA fraction was concentrated *in vacuo* to approximately 6 mL. Concentrated, desalted buffer 2 fraction was subjected to spot-on-lawn analysis and MALDI-TOF MS for confirmation of presence of McnN.

Mass spectrometry analysis. The fractions of potential microcin collected from HPLC were analyzed using MALDI-TOF MS by analyzing the spectra produced by the molecular ions with an AB Sciex Voyager Elite MALDI-TOF MS (Foster City, CA). The ionization matrix was a two-layer method consisting of equal parts 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma Aldrich) and acetone, mixed in equal parts with the sample. The second matrix layer consisted of equal parts 0.1% trifluoroacetic acid, methanol and sinapinic acid. The spectra were recorded in positive ion mode, using linear method. Fractions from HPLC purification that had antimicrobial activity but no peak corresponding with the mass of McnN were subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS). LC-MS/MS was used to determine amino acid sequence of samples using an UPLC (ultra performance liquid chromatography, Waters USA) coupled with a Q-TOF (quadrupole-time of flight) Premier mass spectrometer (Waters, Milford, MA USA), similar to a method by Tulini *et al* (2014). Samples of 5 µL were analyzed using a liner gradient of 5-80% ACN with 0.1% formic acid (FA; v/v) with a

flow rate of 300 nL/min on a nano-analytical column (75 μm x 100 nm, Atlantis dC_{18} nanoAcquity, Waters). Mass spectra and amino acid sequences were analyzed to determine peptides present in samples, according to observable fragments.

Bacteriocin activity assays and determination of arbitrary units of activity. Determination of the volume of crude McnN to be used *in vivo* preparation was performed in a method similar to Wiegand et al., (2008). Cocktails of 5 strains of *E. coli* O157:H7 and of 5 strains of *S. enterica* were serially diluted in cation-adjusted Mueller-Hinton broth (CAMHB; Oxoid, Basingstoke, Hampshire, England) to 10^5 CFU/mL. The CAMHB (50 μL) was added to each well of a 96-well microtitre plate and 50 μL microcin N fraction was added in negative control wells and initial test wells. Two-fold dilutions were done across the plate and all wells were topped up with 50 μL of an indicator organism. Columns containing negative and positive controls, antimicrobial and broth, and culture and broth, respectively, were included on each plate. Plates were incubated at 37°C for 24 h and arbitrary units (AU) of McnN were determined as last column concentration of McnN prior to visible cell growth. Arbitrary units (AU) are defined as the reciprocal of the greatest inhibitory dilution, as calculated per mL (Ahn and Stiles, 1990).

Ground beef preparation. To determine if the semi-purified preparations that had activity *in vitro* were active *in vivo*, ground beef was prepared aseptically. A beef round was trimmed of the outermost 6 mm of muscle, flaming a scalpel and forceps as necessary, to expose intact inner muscle tissue. The meat was cut into 6 mm by 6 mm cubes with a sterile butchers knife and ground using a Kitchen Aid Meat Grinder Attachment (Kitchen Aid, Mississauga ON) that had been sprayed with 70% ethanol prior to grinding. A small amount of meat was passed through grinder to remove any contaminants and residual ethanol and was discarded. Meat was ground and packed vacuum package bags in 20 g portions. Samples were vacuum packaged (Model C200; Multivac Canada Inc., Woodbridge, ON) and stored at -20°C until thawed for use. For experiments using raw ground beef, samples were thawed, inoculated and treated with antimicrobials as appropriate. For experiments using cooked ground beef, thawed beef was cooked in a water bath set to 100°C until an internal temperature of 85°C was recorded using Tinytag Thermocouples, using one sample to monitor temperature by inseting probe through vaccum packs into the center of beef sample (Gemini Data Loggers, Chichesters West Sussex,

England). Samples of vacuum packaged cooked beef were immediately placed into an ice bath to cool the beef to 25°C prior to inoculation. A 0.1 mL aliquot of 5-strain cocktails of *E. coli* O157:H7 or *S. enterica* were applied to ground meat for a target inoculation of 10^4 CFU/g. Samples were massaged by hand for 10 sec to distribute the inoculum. Crude Microcin N preparation (4 mL) was applied to of the meat samples (raw cooked or ground beef). Samples were massaged for 30 sec to incorporate the antimicrobial preparation. Treated samples were enumerated directly after application of the McnN preparation and after 72 h storage at 7±1°C. Serial dilutions were prepared in 0.1% peptone and samples were planed onto Plate Count Agar (PCA; Difco™), Violet Red Bile Agar (VRBA; Difco™), and MacConkey Agar (MAC; Difco™). Extracts from raw ground beef samples that had been treated with crude McnN were subjected to MALDI-TOF MS analysis as outlined above to assess presence of peptide. Experiments with cooked and raw ground beef were completed in triplicate.

2.3. Results and Discussion

Microcin N structural gene sequence confirmation. The discrepancy between the published molecular weights and amino acid sequence of Mcn24 and McnN necessitated verification of McnN of the microcin produced by *Escherichia coli* MC4100 pGOB18, used in this study. The presence of the structural gene for McnN production in *E. coli* MC4100 pGOB18 was confirmed through gel electrophoresis of PCR amplicons (data not shown). Sequencing of amplicons confirmed that the microcin produced by *E. coli* MC4100 pGOB18 is in 97.8% consensus alignment with that of McnN, when comparisons using ClustalW amino acid sequence alignment were performed (Figure 2-1).

The structural gene for McnN isolated in this study lacks the first and second residues, methionine and tyrosine, respectively. It is unknown if the absence of these amino acids in the leader sequence will affect the function of mature McnN as the cleavage site of the leader peptide from the McnN precursor 17th residue (Duquesne et al., 2007), between an alanine and a glycine shifts with the deletion to the 15th residue. Consistent with the findings of Corsini et al. (2004), McnN was different from the previously published sequence of Mcn24. Corsini et al. (2010) sequenced the pGOB18 plasmid and found one deletion and three insertion events in the putative regulator gene and structural gene for McnN (*mcnN)*. They noted three nucleic acid

insertions with respect to the previously published Mcn24 structural gene (*mtfS)* in the N-terminal domain of *MftS* (Corsini et al., 2010). Consistent with the previous findings, McnN had mutations that altered the final precursor and mature peptide sequences, when compared to Mcn24. It should be noted that the amino acid sequence of Mcn24 had the 44th residue deleted and residues 40-50 did not align with the peptide sequence of McnN. Based on these findings, purification of McnN from this strain for downstream application was undertaken. As the gene for structural microcin production isolated in this study is analogous to that for McnN, the peptide isolated in this work will be referred to as McnN.

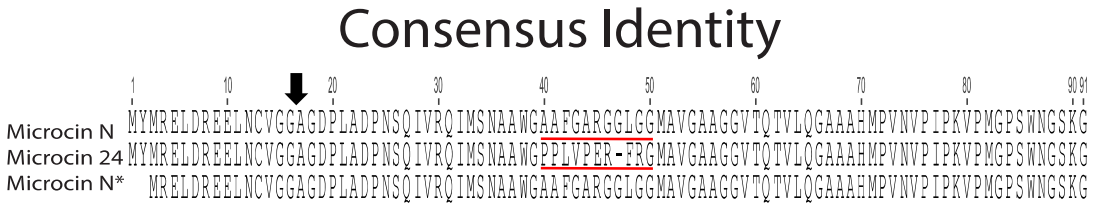

Figure 2-1. Multiple alignments of *N- and C*-terminal Microcin N and Microcin 24 precursor peptide. A ClustalW multiple amino acid alignment and consensus identity of Microcin N (Gene bank accession number: FJ895580.1), Microcin 24 (Gene bank accession number: U47048) and the sequence of the microcin isolated in this study from *E.* coli MC4100 pGOB18 using default parameters in Geneious software, Microcin N* (Biomatters, Auckland, New Zealand).

Bacteriocin purification. Purification of McnN following the work of Corsini et al. (2010) was unsuccessful thus a new method for purification was developed. The initial purification of the supernatant of *E. coli* MC4100 pGOB18 using Amberlite XAD16N provided an crude preparation where antimicrobial activity was observed in the 80% IPA + 0.1% TFA fraction (Table 2-2). MALDI-TOF MS analysis of this crude fraction yielded a compound with a mass of 7217.76 and 7221.1 Da in analyses of different chromatographic separation attempts (Figure 2-2A). Singly and doubly charged species differ from the published mass of McnN by 53.8 Da.

Table 2-2. Antimicrobial activity determined by spot-on-lawn assays of *E. coli* MC4100 pGOB18 and fractions from hydrophobic interaction chromatography. Indicators included *E. coli* DH5α and *S. enterica* ATCC 10708.

Antimicrobial Activity	*E. coli* DH5α	*S. enterica* ATCC 10708
Escherichia coli MC4100 pGOB18	+	+
0% IPA	-	-
20% IPA	-	-
50% IPA	-	-
80% IPA + 0.1% TFA	+	+

– No inhibition zone, + Inhibition zone

The difference between the published mass of McnN and the peptide isolated in this study was 53.8-57.14 Da. The difference between the masses of the microcin isolated from the crude Amberlite column purification differs from the published mass by the approximate mass of a glycine residue (57.1 Da). Analysis of the peptide sequence revealed a terminal glycine residue in position 91 (Figure 2-1). Cleavage of this terminal glycine residue could explain the difference in the masses of McnN reported by Corsini et al. (2010) and those that are reported in this study. Based on these findings, further purification using the crude preparation derived from the Amberlite purification was used to isolate pure McnN.

Results of spot-on-lawn antimicrobial assay of HPLC fractions, collected at 5-minute intervals, are shown in Figure 2-3 and Table 2-3. High performance liquid chromatography of the crude McnN preparation obtained from the hydrophobic interaction chromatography using C_4A, C_4P, C_{18} and C_{18}A columns failed to yield a fraction that had antimicrobial activity that corresponded to a clearly resolved compound on the chromatograms (Figure 2-3). Fractions were collected every 5 min and tested for activity over the duration of the 60 min HPLC run, using a C18A column. Antimicrobial activity was observed in fractions collected corresponding to + symbol listed on Table 2-2.

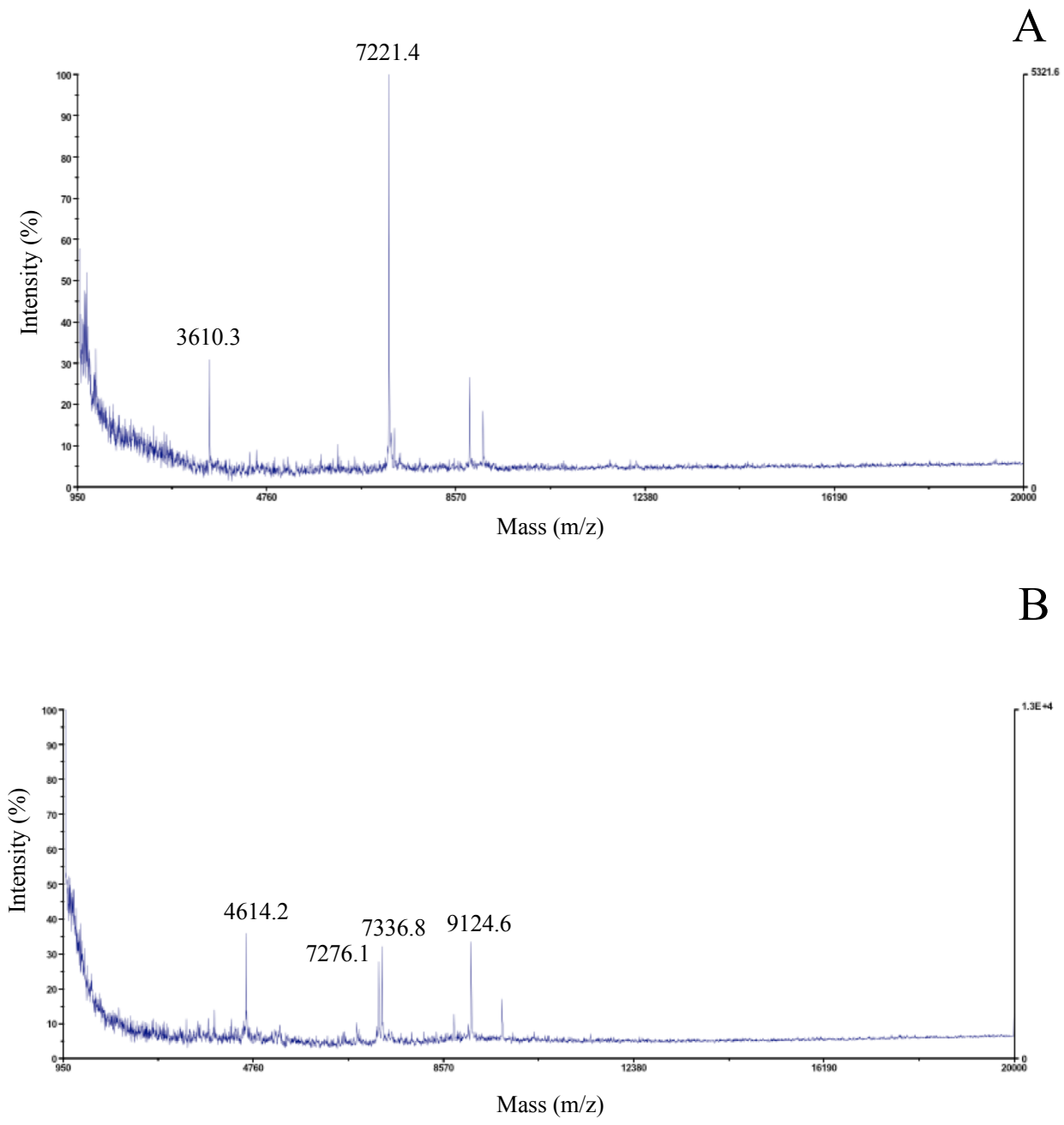

Figure 2-2. MALDI-TOF mass spectra of partially pure Microcin N preparation obtained from Hydrophobic interactions chromatography (A) and eluent of Buffer 2 from Cation-Exchanger Chromatography (B).

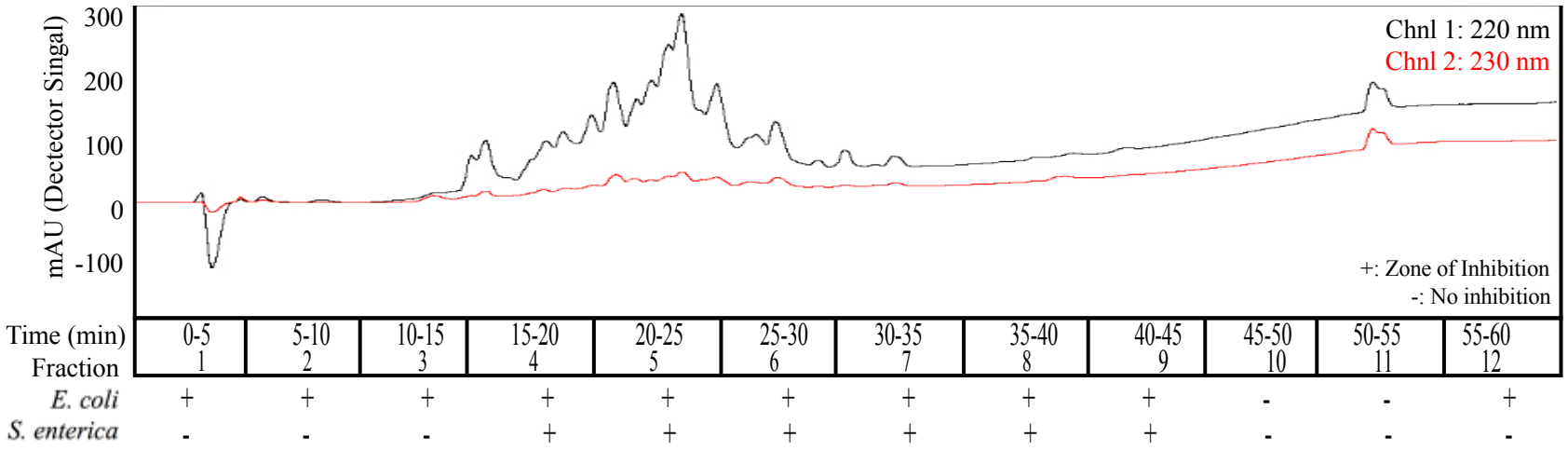

Figure 2-3. Representative chromatogram of HPLC purification of McnN of the fraction obtained from hydrophobic interactions chromatography.

Chromatograms from the different columns over the course of multiple HPLC attempts yielded broad and closely associated peaks with consistently poor resolution. The chromatogram in Figure 2-3 shows multiple peaks situated on what appear to be an oxidative shoulder; however, hydrophobic interaction chromatography provided a crude preparation that was pure enough for resolution of McnN using HPLC. It should be noted that other hydrophobic peptides are often poorly retained on C_{18} RP HPLC columns (Scanlon and Finlayson, 2004). The crude analytical trace of c(RSRNR), a hydrophobic cyclic pentapeptide (Scanlon and Finlayson, 2004) has an analytical trace that somewhat resembles the trace observed for McnN in Figure 2-3. Attempts to optimize chromatographic separation of McnN using C_4 columns were done, as hydrophobic peptides that are retained longer than 25 min are recommended (Scanlon and Finlayson, 2004). Unfortunately, use of C_4 columns failed to improve resolution. Fractions collected from separation of the crude preparation had inconsistent antimicrobial activity when compared to resolved compounds on the chromatogram. Antimicrobial activity was consistently noted in the later fractions (i.e. fraction 8 and 9) where no peaks were observed on the chromatogram. This trend was consistently observed to varying degrees, among the different columns used. Contamination of columns and mobile phases was ruled out as the source of antimicrobial activity, thus an interaction of the crude McnN preparation with the column could be the explanation for the unexpected antimicrobial activity. With the C_4A, C_4P, C_{18} and C_{18}A columns, antimicrobial activity was observed in the fractions collected from the midpoint of the runs and onward. A potential explanation of this phenomenon could be that McnN was smearing in the column and elutes in all subsequent fractions. Scanlon and Finlayson (2004) note that a common phenomenon that occurs is that hydrophobic peptides can smear at high acetonitrile concentrations, which was observed in this study, as activity was evident in fractions where the ACN concentration was 50% and higher.

MALDI-TOF MS analysis of fractions 1-12 from the C_{18}A column did not yield any peaks corresponding to the mass of McnN reported in this study, regardless of observation of antimicrobial activity. MALDI-TOF MS analysis of fractions with antimicrobial activity collected from HPLC failed to yield a peak with a mass corresponding to McnN (Table 2-2; C_4A, C_{18}, C_{18}A; fractions 6-12). Fraction 12 consistently showed the greatest antimicrobial activity in bioassays using HPLC fractions.

Table 2-3. Comparison of antimicrobial activity of fractions collected from HPLC instruments using C_4A, C_4P, C_{18} and C_{18}A columns using spot-on-lawn assays of injection of crude McnN preparation. Fraction 1 refers to sample collected 0-5 minutes after HPLC injection; fraction 2: 5-10 min; fraction 3: 10-15 min; fraction 4: 15-20 min; fraction 5: 20-25 min; fraction 6: 25-30 min; fraction 7: 30-35 min; fraction 8: 35-40 min; fraction 9: 40-45 min; fraction 10: 45-50 min; fraction 11: 50-55 min: fraction 12: 55-60 min.

	Column				
Fraction	**C4A**	**C4P***	**C18**	**C18A**	**SP-Sepharose C18**
1	-	-	+	-	-
2	-	-	+	-	-
3	-	-	+	-	-
4	-	+	+	-	-
5	-	+	+	-	-
6	+		+	+	-
7	+		+	-	-
8	+		+	+	-
9	+		+	+	-
10	+		-	+	+
11	+		+	-	+
12	+		-	+	+

*Instrumentation used with C_4P had automatic fraction collection

Due to the poor resolution of the crude McnN preparation using HPLC purification attempts, a cation-exchange column was used in attempts to obtain pure McnN. Cation-exchange columns can be an effective means for protein purification as the amphoteric nature of peptides allow the charges to interact with a negatively charged column matrix (Rosenberg, 2005). The pI of McnN is 10.6 and at a pH below the pI, the protein is positively charged, thus a cation exchange column was determined to be the ion-exchange column of choice. HPLC of buffer 2 from the SP-Sepharose Cation-Exchange column revealed a cleanly resolved peak eluting at 23 minutes, which could be McnN (Figure 2-4).

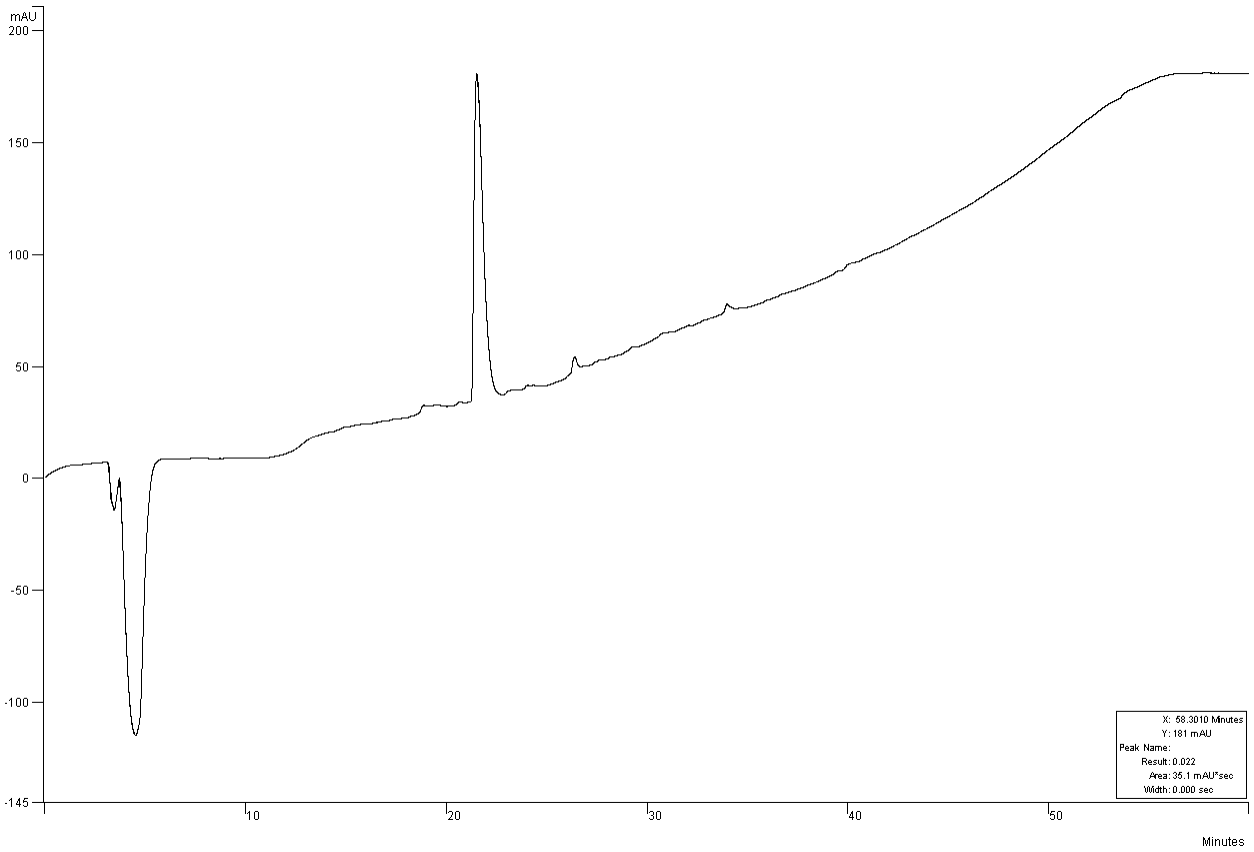

Figure 2-4. Chromatogram of HPLC purification of the eluent of Buffer 2 from an SP-Sepharose cation-exchange column.

MALDI-TOF MS analysis of the eluent of Buffer 2 revealed the presence of a compound with a mass of 7276.1 (Figure 2-2B). The difference in mass of the fraction collected in the crude McnN fraction and the eluent of buffer 2 of cation-exchnage chromatography is 54.7 Da lower than the peak corresponding to McnN in the crude McnN fraction. This could be a result of the crude analyte of McnN picking up a manganese cation resulting in the mass of 7221.4 Da (Figure 2-2A). Unfortunately, fractions collected using the cation-exchange SP-Sepharose column failed to yield antimicrobial activity when tested against *S. enterica* ATCC 10708. Elution of the crude McnN fraction using a cation-exchange column yielded compounds that differed in mass from the McnN isolated from Amberlite column purification by approximately 55.0 and 115.7 Da (Figure 2-2B). In contrast to the attempts to purify McnN with HPLC purification using a variety of columns and instruments, purification of the crude McnN fraction using a cation-exchange column did provide a fraction that contained a compound with a mass close to that of McnN. The desalted eluent of Buffer 2 was subjected to a subsequent HPLC and

12 fractions were collected and tested using a spot-on-lawn assay. The concentrated buffer 2 and subsequent 5 later fractions collected demonstrated activity against *S. enterica* ATCC 10708 (Table 2-3); however MALDI-TOF MS analysis of fractions 8-12 and the concentrated elutant of buffer 2 failed to yield a compound with a mass corresponding to that of McnN. The inconsistent results of antimicrobial activity and isolation of a compound with a mass corresponding to the mass of McnN necessitated further exploration of the source of antimicrobial activity in the samples. Concentrated samples of buffer 2 were sent for LC-MS/MS analysis to determine the source of antimicrobial activity and fragments of casein (fragment 108-119 and 193-206) were detected by sequence analysis (*QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP PFLQPEVMGV SKVKEAMAPK HK*EMPFPKYP VEPF*TESQSL TLTDVENLHL PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL SQSKVLPVPQ KAVPYPQRDM PIQAFLL* YQ PVLGPVRGPF PI*FVS*), with the size of the peptide around 11 kDa (portions of sequence indicated in italics are retrieved from the public database, while those not italicized are the sequenced portions). Casein fragments are likely left in crude preparation after fermentation of M63 media, which contains Casamino acids as a nitrogen source. Casein fragments are also known for their antimicrobial activity against *E. coli, Pseudomonas aeurginosa, Kleibsella pneumonia, Enterococcus faecalis, Bacillus subtilis* and *Staphylococcus aureus* (Arruda et al., 2012). Similarly, researchers at the University of Alberta have isolated fragments of casein that were akin to the peptides isolated in this study as a result of metabolic activity from *Carnobacterium maltaromaticum* UAL26 (previously *C. piscicola*) grown in BHI media (Rosario, 2001).

Similar to purification attempts using HPLC, the cation-exchange did not yield a fraction that contained antimicrobial activity where a compound corresponding to the mass of McnN was observed. HPLC of the elutant from cation-exchange column presented a similar situation, with apparent peptide smearing and resulting activity *in vitro*. In all purification attempts, antimicrobial activity was observed in numerous fractions, but the source of activity could not be confirmed with certainty to McnN in downstream purification analysis. Generally hydrophobic interaction chromatography, cation-exchange chromatography, C_{18} HPLC is successful in purifying a wide variety bacteriocins (Pingitore et al., 2007); however, the characteristics of McnN have proven it to be a challenging peptide to isolate. McnN belongs to class IIa microcins, and is similar to McnV and McnL (Duquesne et al., 2007). Morin et al. (2011), developed a

purification protocol for McnL that resulted in pure McnL for use in mode-of-action assay, through solid-phase extraction using Sep-Pak C_{18} cartridges followed by two successive C_8 Reverse-Phase HPLC assays. Sep-Pak cartridges were also used in the purification by Corsisni et al (2010) for the purification of McnN but attempts to reproduce this protocol were unsuccessful. Purification of McnV (previously Colicin V) was undertaken by Fath *et al* (1994) using precipitation and HPLC and they were able to obtain fractions with antimicrobial activity, but activity was not limited to fractions corresponding to peaks that matched that of ColV, eluting after 40% acetonitrile gradients. Authors noted that McnV (ColV) adsorbs to C_{18} columns, and a large fraction of the protein remains bound to the column after the first elution. Eluting the column again under the same parameters without injecting additional McnV yielded a clean trace with one resolved peak, with antimicrobial activity. Future work with highly adsorbent peptides, such as McnN, could be eluted by a second run on the same column without further sample injection.

Inhibition of pathogens in raw and cooked ground beef. For challenge studies in raw and cooked ground beef, the concentrated 80% IPA + 0.1% TFA fraction (referred to as crude McnN) was used. The crude McnN preparation was active at 320,000 AU/mL against both the 5-strain cocktails of *E. coli* O157:H7 and of *S. enterica*. Sixteen times the AU was used in the beef challenge studies, for a total of 1.28×10^6 AU, equivalent to 4 mL of the crude McnN preparation or 64,000 AU/g. Treatment of raw ground meat with McnN did not result in significant reductions in cell counts after 0 h of storage (Figure 2-5).

After 72 h of storage, no significant reductions in survival of pathogens occurred in samples that were treated with McnN (Figure 2-6). MALDI-TOF analysis of the McnN preparation prior to application on meat showed a peak with a mass corresponding to that of McnN; however, analysis of samples of McnN exposed to raw ground beef failed to show any peaks corresponding to McnN.

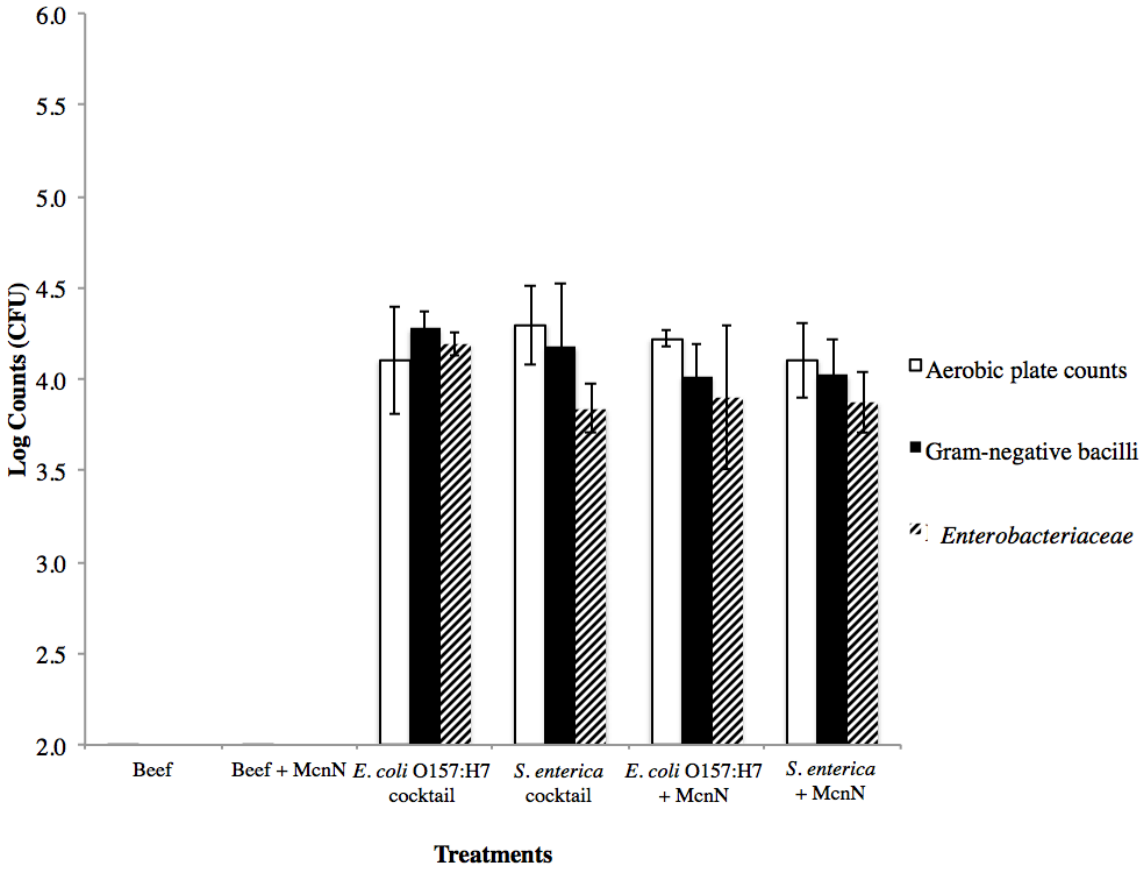

Figure 2-5. Mean log (CFU) counts of ground beef inoculated with 5-strain cocktails of either *E. coli* O157:H7 or *S. enterica* with or without crude microcin N (1.28 x 10^6 AU) and enumerated immediately after treatment. Samples were enumerated on Plate Count (total aerobic bacteria), MacConkey (gram-negative bacilli) and Violet Red Bile (*Enterobacteriaceae*) agars. n=3.

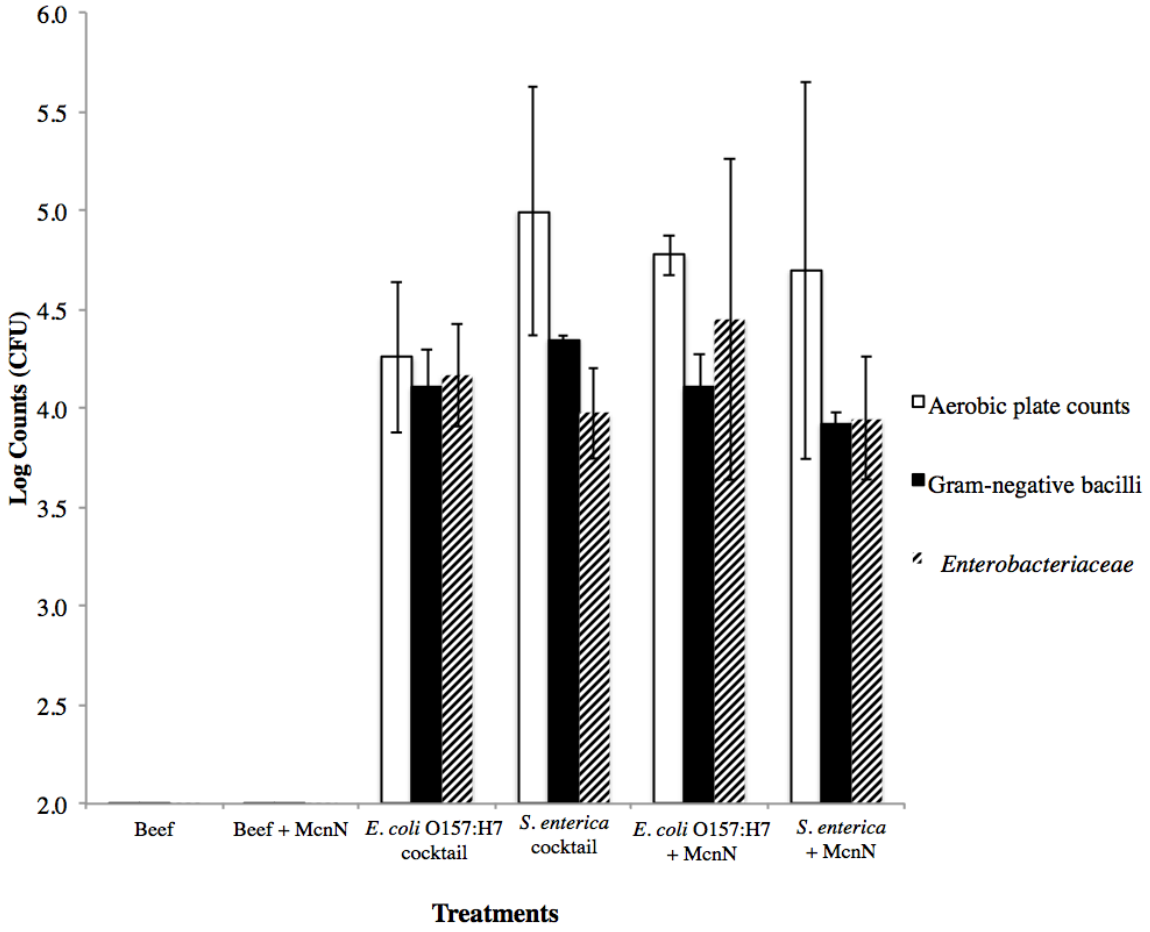

Figure 2-6. Mean log (CFU) counts of ground beef inoculated with 5-strain cocktails of either *E. coli* O157:H7 or *S. enterica* with or without crude microcin N (1.28 x 10^6 AU) after 72 h of storage at 8°C. Samples were enumerated on Plate Count (total counts), MacConkey (Gram-negative bacilli) and Violet Red Bile (*Enterobacteriaceae*) agars. n=3.

Sable et al. (2000) purified Microcin J25 and tested the activity against *E. coli* O157:H7 *in situ* against mincemeat, milk and egg yolk extracts and found that after 24 h that no viable cells were recovered in samples treated with 6.25 µg/mL per product (total of three samples). Researchers inoculated products with 10^3 CFU/mL of *E. coli* O157:H7, treated with McnJ25 and incubated at 37°C. The authors failed to present the data from the inoculation and treatment of the different samples and the manuscript does not mention specifics of the mincemeat preparation. Additionally, 10^3 CFU/mL is nearly the detection limit of bacterial enumeration and data from inoculated, untreated control samples cannot be used to verify whether reductions stated compare to recovery from control samples. Based on the discussion of results, it appears

McnJ25 is effective against *E. coli* in a meat preparation (Sable et al., 2000). A possible explanation for the absence of inhibition of cocktails in raw meat treated with crude McnN could be that some agent in raw meat is responsible for the inactivation of McnN. To assess this experiments utilizing cooked ground beef were performed.

Microcin N inhibition in cooked ground beef. Treatment of cooked ground beef with crude McnN did not result in significant reductions in cell counts after 0 h of storage (Figure 2-7).

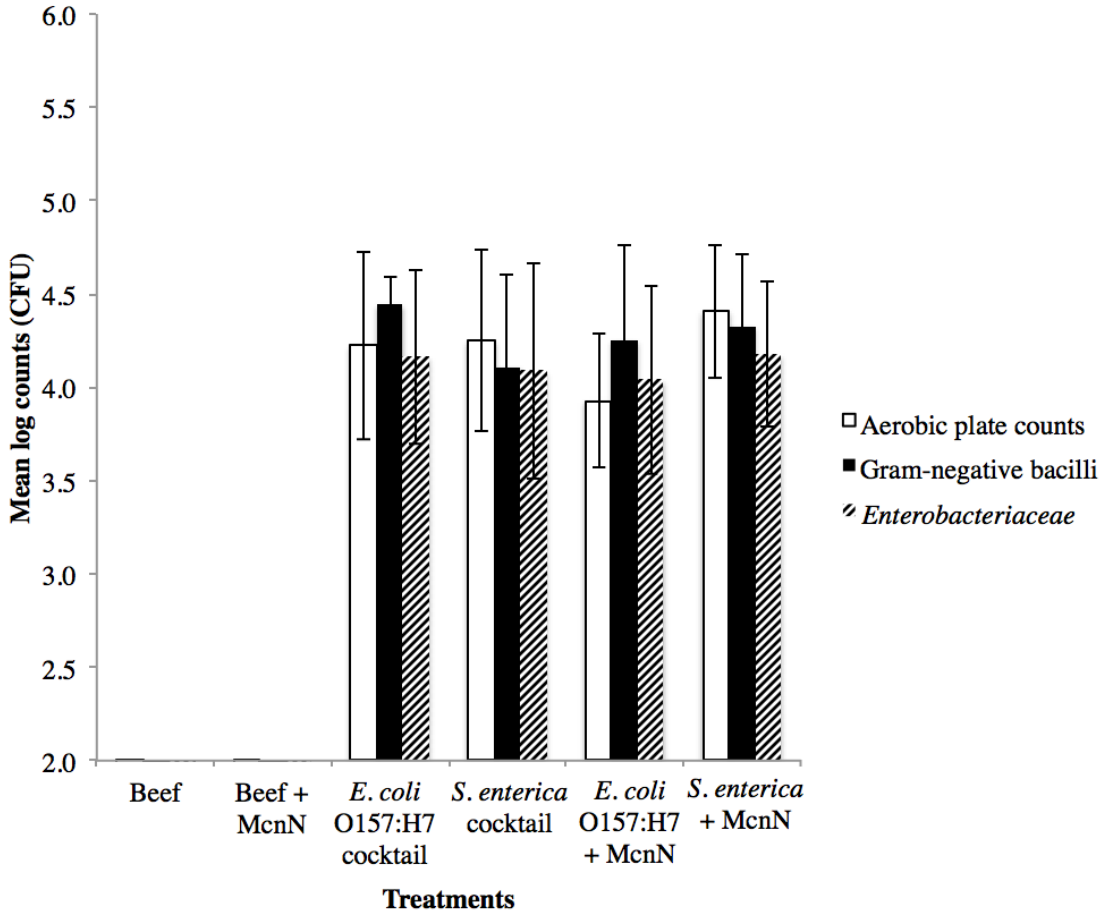

Figure 2-7. Mean log (CFU) counts of cooked ground beef inoculated with 5-strain cocktails of either *E. coli* O157:H7 or *S. enterica* with or without crude microcin N (1.28 x 10^6 AU) and enumerated immediately after treatment. Samples were enumerated on Plate Count (total counts), MacConkey (Gram-negative bacilli) and Violet Red Bile (*Enterobacteriaceae*) agars. n=3.

Similar counts of *E. coli* O157:H7 and *S. enterica* cocktails were detected in both raw and cooked ground beef after enumeration. After 72 h of storage, no significant reductions in survival of pathogens occurred in samples that were treated with crude McnN (Figure 2-8). Enumeration of *E. coli* O157:H7 and *S. enterica* cocktails was similar in cooked and raw ground beef stored for 72 h, indicating that denaturation of meat proteins does not affect pathogen survival. Aliquots of crude McnN were tested for activity against the 5-strain cocktails of *E. coli* O157:H7 and *S. enterica* and preparations were inhibitory against these strains *in vitro*. During cooking of the beef samples, fat deposits accumulated within the vacuum packs, which may be a possible explanation for the lack of antimicrobial activity *in vivo*. It is possible that the crude McnN preparation adsorbed to the protein and fat and thus did not interact with cultures to illicit an inhibitory effect. It is widely documented that the application of bacteriocins is limited to food products with little to no protein and fat present as bacteriocins adsorb to these macronutrients, causing antagonistic effects (Boziaris et al., 1998; Aasen et al., 2003; Chumchalová et al., 1998; Gänzle et al., 1999). Activity of sakacin P and nisin in homogenates of raw chicken and raw salmon could not be detected, though once homogenates were treated with urea, activity was restored after 10 min and 4 h, which indicates adsorbance of bacteriocins to the interface of meat emulsions (Aasen et al., 2003). The authors speculate that bacteriocins are most effective when applied to whole foods to prevent the emulsifying effect of amphiphilic bacteriocins with other charged and hydrophobic amino acids. An explanation for the lack of activity of crude McnN in ground meat could be adsorption of the bacteriocin to protein and lipid. Future work with crude McnN could involve testing whole intact, surface inoculated steaks to see if the antimicrobial preparation is more effective in this manner. Some strains of *S. enterica* have develop spontaneous resistance to Mcn24 through spontaneous mutation of the *mar* operon, which is responsible for efflux pumps for export of antibiotics in *Salmonella* (Carlson et al., 2001). These authors noted resistance to Mcn24 after overnight exposure to the bacteriocin. Strains of *E. coli* O157:H7 are also able to develop resistance to colicins and some strains have the ability to produce colicins (Schamberger and Diez-Gonzales, 2005). An alternative explanation for the lack of activity of McnN could be the ability of these strains to develop resistance through random mutation or through immunity mechanisms encoded on genomic operons for colicin and microcin production. Future work could involve screening of isolates for genetic elements encoding immunity to these bacteriocins.

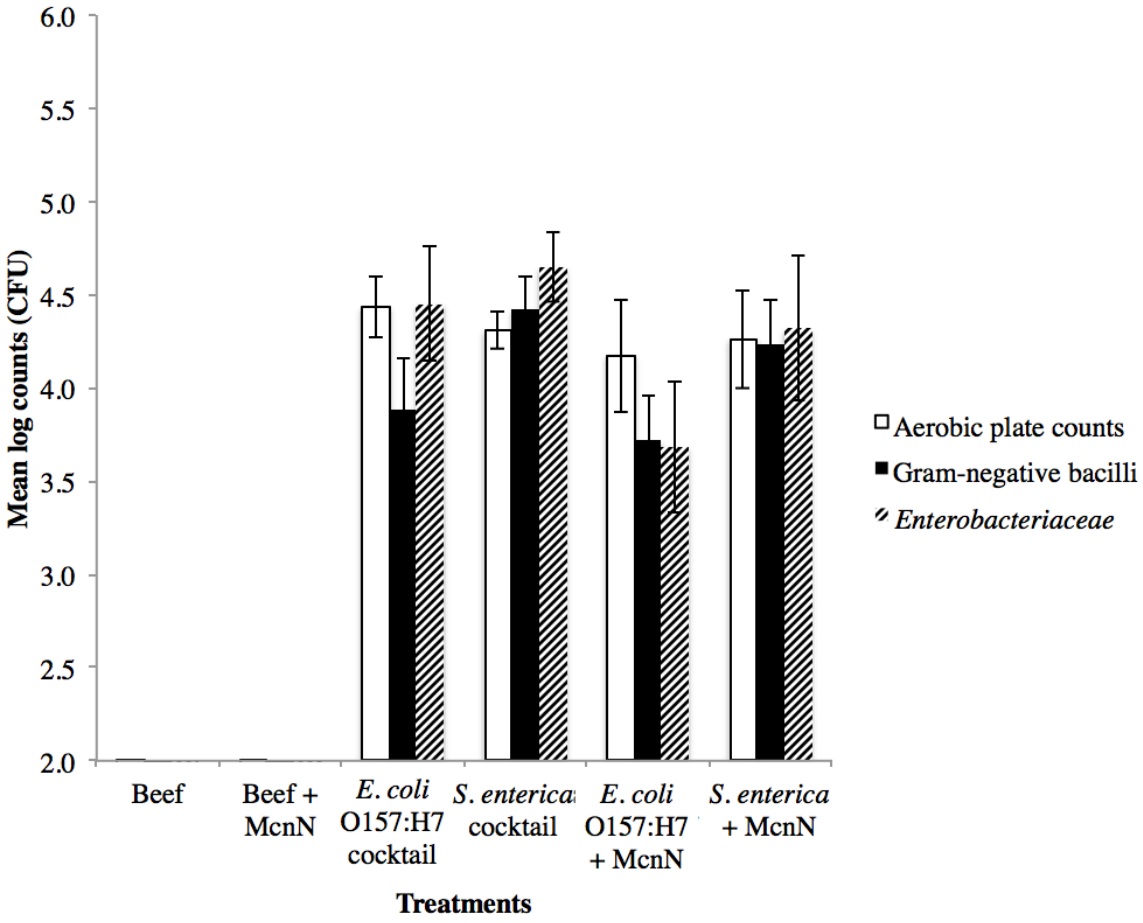

Figure 2-8. Mean log (CFU) counts of cooked ground beef inoculated with 5-strain cocktails of either *E. coli* O157:H7 or *S. enterica* with or without crude microcin N (1.28 x 10^6 AU) after 72 h of storage at 8°C. Samples were enumerated on Plate Count, MacConkey and Violet Red Bile Agars. n=3.

2.4. Conclusions

The main focus of this work was to create a standard protocol for the purification and isolation of the microcin produced by *Escherichia coli* MC4100 pGOB18, which was somewhat successful. A microcin, of similar weight to Microcin N was purified using hydrophobic interaction chromatography, followed by further purification using HPLC.

Hydrophobic interaction chromatography produced a crude, partially purified concentrated fraction with antimicrobial activity. HPLC with C_4A, C_4P, C_{18}, and C_{18}A columns failed to yield a fraction with antimicrobial activity that corresponded to the mass of McnN. In

later fractions of all HPLC eluents, antimicrobial activity was observed. This was likely due to smearing of the hydrophobic McnN on the RP-HPLC column. Cation-exchange chromatography also failed to isolate a compound the mass of McnN that had antimicrobial activity. Due to inconsistencies with HPLC purification, crude Microcin N fraction was used at 16 times the MIC in a challenge study with raw and cooked ground beef. Treatment of raw or cooked ground meat with McnN did not result in significant reductions in cell counts.

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Chapter 3. Purification, encapsulation and application of Tridecaptin A_1, an antimicrobial active against salmon bacterial pathogens

3.1. Introduction.

The Canadian salmon aquaculture industry represents the 4th largest producer of farmed salmon globally with Atlantic salmon (*Salmo salar)* representing the largest Canadian aqua-cultural export (Fisheries and Oceans Canada, 2014). Of the vast number of infectious diseases that may affect aquaculture species, roughly 34% are bacterial infections (Lafferty et al., 2015). Interestingly, more infectious diseases of salmon occur in the Northern hemisphere making control and management of infectious disease an important consideration for Canadian aquaculture industries. *Listonella anguillarum (*formerly *Vibrio anguillarum)* is of particular importance as this pathogen is problematic on a global scale for salmon, eels, turbot, plaice, dover-sole, bream, mullet, catfish and tilapia (Lafferty et al., 2015; Roberts, 2012). *L. anguillarum,* the causative agent of vibrosis*,* is a gram-negative, non-spore forming, mesophilic and halophilic facultatively anaerobic bacterium. The disease caused by *L. anguillarum* is haemorrhagic septicaemia, characterized by weight loss, lethargy, ulcers on ventral and lateral areas of fish, and swollen lesions that can often affect eyes (Frans et al., 2011). In 1997, the World Bank estimated that global disease losses in aquaculture totaled in the $3 billion USD per year range (Subasinghe et al., 2000). While these bacterial pathogens are not zoonoses, prevention and treatment of bacterial infections in salmon will lead to improved animal health and greater food security through mitigation of loses due to morbidity and mortality.

Tridecaptin A_1 ($TriA_1$) is a novel antimicrobial lipopeptide that is naturally produced by *Paenibacillus terrae* NRRL B-30644 (Lohans et al*.,* 2014). In addition to $TriA_1$, *P. terrae* B-NRRL 30644 also produces Paenicidin B, a class I lantibiotic bacteriocin. Lantibiotics are generally active against gram-positive organisms while tridecaptins demonstrate inhibitory activity against both gram-negative and gram-positive microorganisms, though potency is greatest against gram-negative organisms (Cochrane et al*.,* 2014-1; Cochrane et al*.,* 2014-2). Exploiting a strain that produces two antimicrobial compounds is advantageous as organisms of differing cell wall structure can be targeted simultaneously. Broad-spectrum antibiotics often rely on the same mode of action across multiple targets; utilizing an antimicrobial preparation that

contains multiple antimicrobials could be tested across a range of targets with heightened efficacy. While it is known class I bacteriocins are generally not active against gram-negative organisms with intact outer membranes (Martin-Visscher et al., 2011), $TriA_1$ could cause damage to the outer membrane of gram-negative cells allowing Paenicidin B to act for a synergistic bactericidal effect. Cochrane et al., have shown analogues of $TriA_1$ are effective sensitizers of gram-negative bacterial cell membranes, lowering the MIC of rifampicin over 500-fold against *E. coli* and sensitizing cells to nisin and gallidermin, well-studied lantibiotics (2014-3). *P. terrae* B-NRRL 30644 is not currently used in food or aquaculture but application of *P. terrae* and the antimicrobials it produces for use in these industries presents an interesting and unique solution to animal health outcomes and overall food security.

Octyl-Tridecaptin A_1 (Oct-$TriA_1$) is a synthetic analogue of $TriA_1$ and is synthesized with lipid tail modifications of the N-terminus by acylation with octanoic acid (Cochrane et al., 2014-1). Chemical synthesis of Oct-$TriA_1$ allows for production of larger quantities of tridecaptin than are possible by isolation from microbial fermentation. Oct-$TriA_1$ was identified after synthesis and testing of a library of $TriA_1$ analogues as it allowed for the production of structurally simpler analogues of native $TriA_1$ that retain similar antimicrobial potency as the native peptide (Cochrane et al., 2014-1). Oct-$TriA_1$ is a novel lipopeptide and has not been tested against a number of foodborne and aquatic pathogens.

Administering pharmaceutical compounds to fish presents a unique problem as control of dose per animal, environmental contamination and acquisition of antimicrobial resistance are all issues to take into consideration. Methods of drug administration in aquaculture include treatment of water (immersion or dipping, hyperosmotic infiltration, flushing and bath treatment), medicated feed, gavage or oral administration, injection or implantation (Treves-Brown, 2000). Medicated feed is an appealing option as control over release of antimicrobials into the aquatic environment can be closely regulated, compared to water treatment methods, is less stressful than gavage, injection and implants and can be relatively inexpensive to produce. In addition to topically treating feed with antimicrobial compounds, encapsulation of target compounds can aid in the delivery of compounds to aquaculture species. Encapsulation is the process of capturing and protecting bioactive compounds, lives cells, and pharmaceuticals in a matrix for delivery into foods or other materials (Nedovic *et al.,* 2011). The principle behind

encapsulation involves using a solid matrix or core material to separate bioactive components, bacterial cells or other agents of interest from the environment. Microencapsulates are generally spherical semi-permeable networks, ranging in size from micrometers to millimeters (Kailasapathy, 2002). Alginate hydrocolloids, harvested from brown algae, are an attractive matrix because of the ease of preparation, require no specialized equipment, they are non-toxic, biodegradable, food grade and thus are an attractive encapsulate worth exploration in tridecaptin delivery to salmon.

It is hypothesized that purification of $TriA_1$ produced by *P. terrae* NRRL B-30644 will provide an antimicrobial effective against *L. anguillarum* when encapsulated and in medicated feed. The objectives of this work were to develop a purification and delivery strategy for $TriA_1$ and Oct-$TriA_1$ to salmon feed for the prevention and treatment of bacterial disease.

3.2. Materials and Methods

Bacterial strains, growth media and growth conditions. Relevant bacterial strains used in this study are outlined in Table 3-1. Strains were stored at -80°C in LB, APT or MRS broth (Difco™; Becton, Dickinson and Company, Mississauga, ON) with 40% glycerol. Prior to use in experiments strains were sub-cultured twice. *P. terrae* NRRL B-30644 was grown from frozen in 10 mL All Purpose Tween (APT) broth (Difco™) incubated anaerobically at 30°C for 48 h, using two BD GasPak EZ anaerobe container system sachets AnaeroPack® System anaerobic jar (7.0 L, No. 50-70; Mitsubishi Gas Chemical Company, Tokyo, Japan) The strain was sub-cultured into 1 L APT broth and incubated at 30°C for 48 h. Following incubation, cultures were centrifuged for 15 m at 16,720 *g* at 4°C. The supernatant was used in downstream purification of tridecaptin A_1.

Table 3-1. Bacterial strains used in this work.

Strain	Characteristics	Source	Reference
Aeromonas salmonicida subsp. salmonicida ATCC 33658	Causitive agent of furunculosis in salmon	ATCC	Miller et al., 2006
Bacillus cereus ATCC 14579	Facultative anerobe, agent of foodborne illness	ATCC	
Bacillus subtilis ATCC 6051		ATCC	Okamoto et al., 1997
Brochothrix thermosphacta ATCC 11509	Meat spoilage organism	ATCC	
Clostridium botulinum A6	Strict anaerobic spore-forming organism, produces botulinum neutotoxin	Health Canada	
Clostridium botulinum A62	Strict anaerobic spore-forming organism, produces botulinum neutotoxin	Health Canada	
Clostridium botulinum B17	Strict anaerobic spore-forming organism, produces botulinum neutotoxin	Health Canada	
Clostridium botulinum IIB13983	Strict anaerobic spore-forming organism, produces botulinum neutotoxin	Health Canada	
Enterococcus faecalis 710C	Lactic acid bacterium	University of Alberta Food Microbiology Culture Collection (FMCC)	
Enterococcus faecalis BFE 900	Lactic acid bacterium	FMCC	
Escherichia coli AW 1.7	Heat and pressure resistant *E. coli*	FMCC	
Escherichia coli O103 PARC 444	Shiga toxin producing *E. coli*	Public Health Agency of Canada (PHAC)	
Escherichia coli O111 PARC 447	Shiga toxin producing *E. coli*	PHAC	
Escherichia coli O145-6465	Shiga toxin producing *E. coli*	PHAC	
Escherichia coli O157:H7 00-3581	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Escherichia coli O157:H7 02-0304	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Escherichia coli O157:H7 02-0627	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Escherichia coli O157:H7 02-0628	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Escherichia coli O157:H7 02-1840	Gram-negative, lactose fermenting coliform, Δstx1/stx2	PHAC	Holley and Luciano, 2011
Escherichia coli O157:H7 1935 FUA 1303	Shiga toxin producing *E. coli*	PHAC	
Escherichia coli O26 PARC 448	Shiga toxin producing *E. coli*	PHAC	
Escherichia coli O145 03-6430	Shiga toxin producing *E. coli*	PHAC	

Table 3-1 continued.

Flavobacterium psychrophilum 110119-112	Causitive agent of bacterial coldwater disease in salmon	Danish Institute for Fisheries Research (DIFR)	
Flavobacterium psychrophilum 110525-1/1A	Causitive agent of bacterial coldwater disease in salmon	DIFR	
Flavobacterium psychrophilum 27/3B	Causitive agent of bacterial coldwater disease in salmon	DIFR	
Flavobacterium psychrophilum 37/5A	Causitive agent of bacterial coldwater disease in salmon	DIFR	
Flavobacterium psychrophilum ATCC 49418	Causitive agent of bacterial coldwater disease in salmon	ATCC	Soule et al., 2005
Lactobacillus acidophilus ATCC 4356	Lactic acid bacterium	ATCC	
Lactobacillus casei ATCC 11578	Lactic acid bacterium	FMCC	
Lactobacillus curvatus ATCC 25601	Lactic acid bacterium	FMCC	
Lactobacillus sakei UAL 1218	Gram-positive beef spoilage organism	FMCC	Leisner et al., 1996
Listeria monocytogenes CDC 7762	Causitive agent of listeriosis	Health Canada	
Listeria monocytogenes FS 104	Causitive agent of listeriosis	FMCC	
Listeria monocytogenes FS 105	Causitive agent of listeriosis	FMCC	
Listeria monocytogenes FS 110	Causitive agent of listeriosis	FMCC	
Listonella anguillarum ATCC 19264	Causitive agent of vibirosis in salmon	ATCC	Beaulieu et al., 2010
Listonella anguillarum Case no. 99282	Causitive agent of vibirosis in salmon	DFO	
Paenibacillus terrae NRRL B-30644	Produces: Tridecaptin A1, Paenicidin B	U.S. Agricultural Research Service Culture Collection (NRRL)	Lohans et al., 2014
Salmonella enterica subsp. *enterica* serovar Cholerasuis ATCC 10708	Gram-negative, non-lactose fermenting enteric human pathogen	American Type Culture Collection (ATCC)	Davison et al., 1996
Salmonella enterica subsp. *enterica* serovar Enteriditis ATCC 13076	Gram-negative, non-lactose fermenting enteric human pathogen	ATCC	Stolle and Beck., 1988
Salmonella enterica subsp. *enterica* serovar Gaminara ATCC 8324	Gram-negative, non-lactose fermenting enteric human pathogen	ATCC	Tindall et al., 2005
Salmonella enterica subsp. *enterica* serovar Heidelberg ATCC 8326	Gram-negative, non-lactose fermenting enteric human pathogen	ATCC	Tindall et al., 2005

Table 3.1 continued.

Salmonella enterica subsp. e*nterica* serovar Typhimiruim ATCC 13311	Gram-negative, non-lactose fermenting enteric human pathogen	ATCC	Stolle and Beck., 1988
Staphylococcus aureus 6538 AT-1	Causitive agent of foodborne intoxication	FMCC	
Staphylococcus aureus ATCC 13565	Causitive agent of foodborne intoxication	ATCC	
Weissella confusa ATCC 10881	Meat spoilage organism	ATCC	
Yersinia ruckeri ATCC 29473	Causitive agent of enteric redmouth disease in salmon	ATCC	Verner et al., 2009

Aeromonas salmonicida ATCC 33658*, Listonella anguillarum* ATCC 19264, *Listonella anguillarum* 99282 and *Yersinia ruckeri* ATCC 29473 were subcultured from frozen stocks and were grown in 5 mL Cation-Adjusted Mueller Hinton broth (CAHMB) at 25°C for 24 h. *Salmonella enterica* ATCC 8324, ATCC 8326, ATCC 10708, *S. enterica* ATCC 13076, *S. enterica* ATCC 13311, *Escherichia coli* O157:H7 00-3581, *E. coli* O157:H7 02-0304, *E. coli* O157:H7 02-0627, *E. coli* O157:H7 02-0628, *E. coli* O157:H7 02-1840, *E. coli* O157:H7 1935 FUA 1303, *E. coli* O26 PARC 448, *E. coli* O103 PARC 444, *E. coli* O111 PARC 447, *E. coli* O145-6465, *E. coli* O145 03-6430 and *E. coli* AW 1.7 were grown in CAHMB broth from frozen at 37°C for 24 h at 200 rpm. *Bacillus cereus* ATCC 14579, *B. subtilis* ATCC 6051, *Carnobacterium maltaromaticum* UAL 307 and NCIMB 702852, *Listeria monocytogenes* FS 104, *L. monocytogenes* FS 105, *L. monocytogenes* FS 110, *L. monocytogenes* CDC 7762, *P. polymyxa* NRRL B-30509, *Staphylococcus aureus* ATCC 6538 and *S. aureus* ATCC 13565 were grown in 5 mL APT broth and incubated at 37°C for 24 h. *C. maltaromaticum* was grown at 25°C. Spores of *Clostridium botulinum* A6, A62, IIB13983 and B17 were grown anaerobically in 5 mL of APT broth for 48 h at 30°C. Strains were sub-cultured using 0.01% (v/v) inoculum into 5 mL fresh APT. *Brochothrix thermosphacta* ATCC 11509, *Enterococcus faecalis* BFE 900 and 710C, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* ATCC 11578, *Lactobacillus curvatus* ATCC 25601, *Lactobacillus sakei* UAL 1218 and *Weissella confusa* ATCC 10881 were grown in 5 mL Lactobacilli MRS broth and incubated at 37°C for 24 h. All strains were sub-cultured into 5 mL fresh broth and incubated at above specified conditions before spot-on-lawn and minimum inhibitory assays.

Spot-on-lawn assay and determination of the minimum inhibitory concentration (MIC). Spot-on-lawn assays were used as an initial screening to determine which strains were sensitive to the antimicrobials produced by *P. terrae* NRRL B-30644. Briefly, 10 µL of 100 µM Oct-Tri A_1 (Cochrane *et al.,* 2014) and 10 µL of Antimicrobial preparation (AMP) were spotted onto CAHMB agar (1.5%) that had been seeded with 0.01% (v/v) inoculum of an indicator strain and plates were allowed to dry before incubation. APT agar was used for assays for inhibition of gram-positive bacteria while LB agar was used for gram-negative bacteria. After 24 h incubation, plates were assessed for zones of inhibition where antimicrobials were applied. Determination of the MIC of pure Oct-TriA_1 was done by a method similar to Wiegand et al. (2008). Indicator strains were serially diluted in CAMHB to 10^5 CFU/mL. CAMHB (50 µL) was added to each well of a 96-well microtiter plate and 50 µL of 1 mM Oct-TriA_1 was added to wells as a negative control and test wells (on separate plates; Appendix B). Two-fold dilutions were done across the plate and all wells (except negative controls) had 50 µL of an indicator organism added. Wells containing negative and positive controls, antimicrobial and broth, and culture and broth were included on each plate. Plates were incubated for 25°C for 24 h and MIC's were determined as the concentration of antimicrobial in the last column prior to visible cell growth. All MIC assays were repeated in triplicate for each indicator strain.

Antimicrobial purification. TriA_1 was purified based on a method created by Lohans *et al* (2014) with some modifications. Briefly, 1 L of the culture supernatant of *P. terrae* NRRL-B 30644 that had been grown for 48 h at 30°C was loaded onto an Amberlite® XAD16N™ resin (Sigma-Aldrich®, St. Louis, MO) column that had been washed with 500 mL 0.1% trifluoroacetic acid (TFA), at a flow rate of 10 mL/min Antimicrobials were eluted using increasing concentrations of isopropyl alcohol (IPA; 0%, 20% and 80%+0.1% TFA). Fractions were concentrated using a SPD Speedvac® SPD111V (Thermo Savant, Holbrook, NY) and antimicrobial activity was confirmed in the 80% IPA fraction by spot-on-lawn assay. The 80% IPA + 0.1% TFA fraction was concentrated from 250 mL to 50 mL using a rotary evaporator (125 MPa, 40°C; Büchi Vac® V-511, Flawil, Switzerland). This concentrated preparation is hereafter referred to as the "Antimicrobial Preparation" (AMP). Purification using reverse-phased High Performance Liquid Chromatography (HPLC) of the AMP was done to confirm the presence of TriA_1 in AMP. The sample (1 mL) was injected onto a Vydac C_{18} analytical peptide and protein column (300 angstrom pore diameter, 5 um particle size, 4.6 x 250 mm) using water

with 0.1% TFA and acetonitrile with 0.1% TFA as the mobile phases, with a gradient of 20-95% acetonitrile at a flow rate of 1 mL/ min. Proteins were detected with a UV detector at 220 nm.

Fish feed preparation and treatment. EWOS Dynamic salmon feed (4-5 mm pellet diameter, containing 25-30% fish meal, 27% oil, 45% crude protein, 20% crude fat, 2% crude fiber, 3000 IU/kg Vitamin A, 3000 IU/kg Vitamin D_3, 200 IU/kg Vitamin E, 2.1% calcium, 1.3% phosphorous, 0.5% sodium; EWOS Canada, Surrey BC) was autoclaved for 15 minutes. Feed was treated with 100 µL of 1 mM Oct-TriA_1, 1 mM PolyB, 1mM PolyE, AMP or sterile distilled water, followed by incubation at 6°C for 24 h. Treated feed was placed onto petri dishes and over layered with pre-seeded CAMHB agar (1.5% w/v) containing *A. salmonicida* ATCC 33658, *L. anguillarum* 99282, *L. anguillarum* ATCC 19264 or *Y. ruckeri* ATCC 29473. After the agar was allowed to set, plates were incubated overnight at 25°C. Plates were assessed for visible zones of inhibition extending past feed pellets as a measure of antimicrobial stability and efficacy. Treated feed (3 pellets for each antimicrobial) was subjected to mechanical homogenization using 0.5 g of sterilized silica beads in 1 mL of sterile distilled water using a Bead Beater ((Mini-BeadBeater-8; Biospec Products, Bartlesville, OK) at 6°C for 60 s. Following centrifugation at 17,500 *g* for 60 s, the supernatant was tested for antimicrobial activity using spot-on-lawn assays.

Alginate Encapsulation. A sterile 1% (w/v) sodium alginate (Modernist Pantry, Portsmouth, NH) solution was prepared by adding alginate to distilled water followed by autoclaving at 121°C. Antimicrobial encapsulates [20% (v/v)] were prepared by adding AMP, 1 mM Oct-TriA_1, 1 mM PolyB or 1 mM PolyE to sodium alginate and each solution was vortexed to mix, and incubated for 10 min at 25°C. Incubation allowed full dissipation of bubbles entrapped in polymer matrix with incorporation of antimicrobials into alginate matrix. Sodium alginate-antimicrobial mixtures were added drop wise using a sterile syringe to 200 mL, 0.05M calcium chloride (Modernist Pantry, Portsmouth, NH) cross-linking solution to encapsulate. Alginate-antimicrobial encapsulates remained in the crosslinking solution for 30 min prior to collection and testing. Samples of antimicrobials alone, alginate-antimicrobial preparations prior and after crosslinking and before and after exposure to feed were tested for activity using a spot-on-lawn assay against sensitive indicators. Control encapsulates (alginate encapsulates without

antimicrobials) were prepared as negative controls and tested for activity. To assess antimicrobial efficacy of encapsulates against indicator strains, encapsulated products were over-layered with CAMHB agar containing *A. salmonicida* ATCC 33658*, L. anguillarum* ATCC 19264, *L. anguillarum* 99282 or *Y. ruckeri* ATCC 29473 (0.01% inoculum) and incubated overnight at 25° prior to assessment of activity C. A feed mash was prepared by soaking sterilized pellets in 100 µL sterile distilled H_2O per pellet overnight at 6°C. Encapsulated products were added to the feed mash and incubated for 30 min at 25°C. Pellets were removed from the feed mash and were subjected to enzymatic lysis and mechanical homogenization to recover antimicrobials. Alginate Lyase (Sigma-Aldrich, St. Louis MO) was prepared according to manufactures instructions to achieve 1 un/mL of enzymatic activity. Alginate lyase (150 µL) was added to 1 g of each encapsulate preparation in 500 µL sterile H_2O. After incubation at 37°C for 30 min, products were added to 0.5 g sterilized silica beads and homogenized for 60 s at 6°C. Tubes containing products and silica beads were centrifuged at 17,500 *g* for 60 s. Supernatant was tested using spot-on-lawn assays against indicator organisms. Testing of antimicrobial treated feed, encapsulates, extracts from encapsulates and pure antimicrobials was completed in triplicate.

Lyophilization and application of the concentrated, re-solubilized antimicrobial preparation (Sol-AMP). To enhance the efficacy of the AMP, the AMP was concentrated by lyophilization. Aliquots (25 mL) of the AMP were frozen at -80°C overnight and transferred to a FreeZone 18 Liter Console Freeze Dry System (Labconco, Kansas City, MO) for 96 h for lyophilization. For 1 L of culture ferment, 50 mL of AMP produced 1.08 g of AMP powder. Concentrated solutions of AMP were prepared from lyophilized AMP by adding sterile distilled water to the AMP. Solutions of lyophilized AMP (Sol-AMP) preparations ranging from 0-15% (w/v) were tested against *A. salmonicida* ATCC 33658*, L. anguillarum* 99282, *L. anguillarum* ATCC 19264 and *Y. ruckeri* ATCC 29473 using spot-on-lawn assays. Concentrated AMP preparations [0-15% (w/v)] were applied to salmon feed (50 µL/pellet) and allowed to dry for 48 h at 37°C. Treated feed was over layered with CAMHB agar containing a 0.1% inoculum of indicator organisms and incubated overnight at 25°C before assessing for zones of inhibition extending past salmon feed. Lyophilization and treatment of feed assays were repeated in triplicate.

3.3. Results and Discussion

Spot-on-lawn and minimum inhibitory concentration assays. Oct-TriA$_1$ was active against all gram-negative microorganisms tested when tested using spot-on-lawn assays (Table 3-2). The 100 μM Oct-TriA$_1$ preparation did not inhibit *L. monocytogenes* strains or *Lb. sakei* UAL1218 (data not shown). Using a 1 mM concentration Oct-TriA$_1$ improved inactivation rates against *L. monocytogenes* (Table 3-2). When 1 mM Oct-TriA$_1$ was tested against the panel of lactic acid bacteria, no effect was observed against *Lb. casei* ATCC 4356, *Lb. curvatus* ATCC 25601, *Lb. sakei* UAL 1218, *E. faecalis* 710C, *E. faecalis* BFE 900 and *W. confusa* ATCC 10881.

Table 3-2. Spectrum of activity of 1 mMOct-TriA$_1$ against aquatic and foodborne bacteria assessed using spot-on-lawn assays. +: inhibitory activity observed; -: no antimicrobial effect observed. n=2.

Strain	Zone of Inhibition	Strain	Zone of Inhibition
Aeromonas salmonicida ATCC 33658	+	*Enterococcus faecalis* BFE900	-
Bacillus cereus 14579	+	*E. faecalis* 710C NL	-
Bacillus subtilis 6051	+	*Lactobacillus sakei* UAL1218	-
C. maltmaroticum UAL307	+	*Lb. casei* ATCC 4356	-
Clostridium botulinum A6	+	*Lb. curvatus* ATCC 25601	-
C. botulinum A62	+	*L. monocytogenes* FS 104	+
C. botulinum IIB13983	+	*L. monocytogenes* FS 105	+
C. botulinum B17	+	*L. monocytogenes* FS 110	+
Escherichia coli DH5α	+	*L. monocytogenes* CDC 7762	+
E. coli AW1.7	+	*Listonella anguillarum* ATCC 19264	+
E. coli O103 PARC 444	+	*Paenibacillus polymyxa* NRRL 30509	-
E. coli O111 PARC 447	+	*Staphylococcus aureus* 13565 LK200	+
E. coli O145 03-6430	+	*S. aureus* ATCC 6538	+
E. coli O145-6465	+	*Salmonella enterica ssp.* Enterica ATCC 10708	+
E. coli O157:H7 00-3581	+	*S.* Enterica ATCC 8326	+
E. coli O157:H7 02-0304	+	*S.* Enterica ATCC 13076	+
E. coli O157:H7 02-0627	+	*S.* Enterica ATCC 13311	+
E. coli O157:H7 02-0628	+	*S.* Enterica ATCC 8324	+
E. coli O157:H7 02-1840	+	*Weissella confusa* 10881	-
E. coli O157:H7 1935 FUA 1303	+	*Yersinia ruckeri* ATCC 29473	+
E. coli O25 PARC 448	+		

MIC assays were carried out based upon inhibitory spectra identified in spot-on-lawn assay, beginning with the highly sensitive salmon pathogens, *A. salmonicida* ATCC 33658, *L. anguillarum* ATCC 19264, *L. anguillarum* 99282 and *Y. ruckeri* ATCC 29473. MIC's of Oct-TriA$_1$ against salmon pathogens ranged from 6 to 24 μg/mL for *L. anguillarum* ATCC 19264 and *F. psychrophilum* 110525-1/1A (Table 3-3). *L. anguillarum* ATCC 19264 was identified as most sensitive to Oct-TriA$_1$Establishing the MIC of Oct-TriA$_1$ was a critical first step in identifying the target pathogen of interest and in efforts to utilize minimal amounts of peptide in salmon experimentation done by collaborators at the Department of Fisheries and Ocean, Canada. The MIC of Oct- TriA$_1$ against *L. anguillarum* 99282 was 24 μg/mL, which was higher than the MIC against *L. anguillarum* ATCC 19264.

Table 3-3. Minimum inhibitory concentrations of 1 mM octyl-tridecaptin A$_1$ against pathogenic aquatic and foodborne microorganisms. n=3, n=2*.

Foodborne organism	MIC (μg/mL)	Aquatic organism	MIC (μg/mL)
Bacillus cereus ATCC 14579	38	*Aeromonas salmonicida* ATCC 33658	1.2
B. subtilis ATCC 6051	38	*Flavobacterium psychrophilum* ATCC 49418	1.2
Clostridium botulinum A6*	2.4	*F. psychrophilum* 27/3B	1.2
C. botulinum A62*	2.4	*F. psychrophilum* 37/5A	1.2
C. botulinum IIB13983*	2.4	*F. psychrophilum* 110119-112	1.2
C. botulinum B17*	2.4	*F. psychrophilum* 110525-1/1A	2.4
Listeria monocytogenes FS 104	38	*Listonella anguillarum* ATCC 19264	0.6
L. monocytogenes FS 105	38	*L. anguillarum* case no. 99282	2.4
L. monocytogenes FS 110	38	*Yersinia ruckeri* ATCC 29473	1.2
L. monocytogenes CDC 7762	38		
Staphylococcus aureus AT-1	4.8		
S. aureus ATCC 13565	2.4		

MIC values of Oct-TriA$_1$ against gram-positive foodborne pathogens ranged from 2.4 to 38 μg/mL with strains of *C. botulinum* and *S. aureus* ATCC 13565 being most sensitive while strains of *B. cereus* ATCC 14579, *B, subtilis* ATCC 6051 and strains of *L. monocytogenes* were least sensitive. MIC testing using gram-positive foodborne pathogens was carried out because it has been established previously that tridecaptins are effective against gram-negative foodborne pathogens, such as *Campylobacter jejuni*, (Lohans et al., 2014) but little was previously known about spectrum and inhibitory effect of TriA$_1$ on gram-positive foodborne pathogens. TriA$_1$

interacts selectively with lipid II on the inner membrane of gram-negative bacteria and results in pore formation (Cochrane, 2015). This pore formation ultimately results in a disruption of the proton gradient and loss of proton motive force that results in the killing effect of $TriA_1$ (Cochrane, 2015). The substantial increase in activity of Oct-$TriA_1$ against *C. botulinum* and *S. aureus* as compared with *L. monocytogenes* may be attributed to differences in the structure of lipid II in *C. botulinum* and *S. aureus*.

Tridecaptin A_1 purification and preliminary testing. Modification of the pre-established method of $TriA_1$ purification (Lohans et al., 2014) was done to capture both $TriA_1$ and Paenicidin B within the AMP for application in prevention of bacterial infection in salmon aquaculture. The concentrated AMP after hydrophobic interactions chromatography demonstrated antimicrobial activity when tested against the salmon pathogens *A. salmonicida* ATCC 33658*, L. anguillarum* ATCC 19264, *L. anguillarum* 99282 and *Y. ruckeri* ATCC 29473. Tridecaptin A_1 eluted between 30-35 min, in accordance with previously published HPLC methodology for $TriA_1$ (Lohans et al., 2014), confirming presence of the antimicrobial in AMP (data not shown). Due to the relative ease of preparation and economical nature of AMP compared to chemical synthesis of Oct-$TriA_1$, use and application of the AMP for control of salmon pathogens was further investigated.

Antimicrobial activity against salmon pathogens alone and in salmon feed. During preliminary assessment of AMP activity, it was observed that AMP lost antimicrobial activity on exposure to commercial salmon feed and when encapsulated, both when treated feed or encapsulates were over-layered or extracts of treated feed were tested against indicators using spot-on-lawn assay (data not shown). To assess whether the concentration of AMP was insufficient to have an effect on indicator strains after treatment on salmon feed, concentrated lyophilized, re-solubilized AMP (Sol-AMP) were prepared and assessed. Future experimentation utilized Sol-AMP in place of AMP. Oct-$TriA_1$, polymyxin B and polymyxin E and AMP all demonstrate antimicrobial activity when tested directly against salmon pathogens using spot-on-lawn assays (Table 3-4). Zones of inhibition of AMP and Sol-AMP against *A. salmonicida* were smaller than observed zones against other pathogens and consistent inhibition with was variable, indicating *A. salmonicida* is not as susceptible as other strains to these preparations (Table 3-4).

All of the strains tested were inactivated by all of the antimicrobials tested *in vitro:* Oct-TriA$_1$, AMP, 15% Sol-AMP, Polymyxin B and Polymyxin E.

Table 3-4. Activity of 1 mM Oct-TriA1, 1 mM polymyxin B, 1 mM polymyxin E (Colistin) and AMP using spot-on-lawn assay. Tested against salmon pathogens grown in cation-adjusted Mueller Hinton agar. Where variability was observed, +/- reports 2 of 3 positive results; -/+ reports 2 of 3 negative results. n=3.

	Antimicrobial Alone			
Antimicrobial	*A. salmonicida* ATCC 33658	*L. anguillarum* ATCC 19264	*L. anguillarum* 99282	*Y. ruckeri* ATCC 29473
Oct-TriA$_1$ (1mM)	+	+	+	+
Polymyxin B (1mM)	+	+	+	+
Polymyxin E (1mM)	+	+	+	+
AMP	+/-	+	+	+
Sol-AMP (15%)	-/+	+	+	+

Activity of lyophilized antimicrobial preparation. Of the strains tested, the original AMP (prior to lyophilization) did not inhibit *A. salmonicida* ATCC 33658*, L. anguillarum* ATCC 19264 and *Y. ruckeri* ATCC 29463 though it was active against *L. anguillarum* 99282, *E. coli* ATCC 25922 and *P. aeurginosa* ATCC 27853 (Table 3-5). Lyophilization and subsequent concentration of AMP (Sol-AMP) yielded a concentrated aqueous solution that was active against all indicators, suggesting that the lack of activity in AMP was due to concentration. In aquatic strains tested, 5% AMP was the lowest concentration of powdered antimicrobial, observed in *L. anguillarum* ATCC 19264 while *A. salmonicida* ATCC 33658 had required the highest minimum concentration of Sol- AMP, to inhibit growth, observed at 9%. All strains tested were inhibited at concentrations of Sol-AMP from above 9% (w/v). *A. salmonicida* ATCC 33658 appears to be least sensitive to AMP with both strains of *L. anguillarum* ranking as the most sensitive aquatic organisms. The potency of TriA$_1$ in powdered AMP is roughly 1.9 mg/g powder. *E. coli* ATCC 25922 and *P. aeurginosa* ATCC 27853 were included in this assay as gram-negative controls as they are model organisms for antimicrobial susceptibility testing (Weigand et al., 2008). Due to limited substrate availability, testing moving forward utilized only the aquatic pathogens of interest. Given this information, it is likely that concentration of antimicrobials in AMP is the limiting factor with respects to observed inhibitory activity and increasing concentrations of Sol-AMP in feed may resolve the lack of antimicrobial activity from AMP, particularly as the AMP prior to lyophilization did not inhibit all strains (Table 3-5). 15%

(w/v) Sol-AMP was chosen to as experimental concentration in future assays as it demonstrated activity against all microorganisms tested, and was well above inhibition limits.

Table 3-5. Activity of Lyophilized Antimicrobial Preparation (Sol-AMP) against salmon pathogens. Lyophilized AMP of increasing concentrations tested directly against salmon pathogens using spot-on-lawn assay.

	Antimicrobial Alone					
Sol-AMP (w/v)	*A. salmonicida* ATCC 33658	*L. anguillarum* ATCC 19264	*L. anguillarum* 99282	*Y. ruckeri* ATCC 29473	*E. coli* ATCC 25922	*P. aeruginosa ATCC 27853*
0%	-	-	-	-	-	-
1%	-	-	-	-	-	-
2%	-	-	-	-	-	-
3%	-	-	-	-	+	-
4%	-	-	-	-	+	-
5%	-	+	-	-	+	-
6%	-	+	+	-	+	+
7%	-	+	+	+	+	+
8%	-	+	+	+	+	+
9%	+	+	+	+	+	+
10%	+	+	+	+	+	+
11%	+	+	+	+	+	+
12%	+	+	+	+	+	+
13%	+	+	+	+	+	+
14%	+	+	+	+	+	+
15%	+	+	+	+	+	+
AMP	-	-	+	-	+	+

Based on these preliminary results, sterile EWOS commercial salmon feed was treated with increasing concentrations of lyophilized AMP (0-15% w/v) and assessed for zones of inhibition surrounding over-layered pellets. Following incubation, none of the pellets treated with lyophilized AMP prevented the growth of any of the salmon pathogens tested (Table 3-6), even when corresponding concentrations were shown to be inhibitory alone. Solutions of the AMP were tested in against the same indicators and lyophilized AMP solutions alone did exhibit an antimicrobial effect against all salmon pathogens. The chemical nature of the antimicrobial preparation derived from hydrophobic interactions chromatography may cause hydrophobic compounds, including $TriA_1$, to adsorb to lipids and proteins present in the commercial salmon feed. Lipopeptides are known for having surfactant and emulsifying chemical properties (Mandal et al., 2013; Stein, 2005). The feed contains a minimum 28% fat, and the fat may

interact with components of the lyophilized AMP. Preparation of medicated feed with antimicrobials by applying antimicrobials using vegetable or fish oil to coat feed, could be a strategy that may be effective for treating feed with AMP containing $TriA_1$ in future work.

Table 3-6. Antimicrobial activity of a lyophilized antimicrobial preparation (AMP) on feed and alone against salmon pathogens. EWOS commercial salmon feed treated with lyophilized AMP of increasing concentrations was over-layered with cation-adjusted Mueller Hinton agar inoculated with indicator organisms. The same lyophilized AMP (10-15%) without exposure to feed was tested against salmon pathogens using spot-on-lawn assays. n=3.

Lyophilized AMP on feed (w/v)	**Indicator organism**			
	A. salmonicidia ATCC 33658	*L. anguillarum* ATCC 19264	*L. anguillarum* 99282	*Y. ruckeri* ATCC 29473
0%	-	-	-	-
1%	-	-	-	-
2%	-	-	-	-
3%	-	-	-	-
4%	-	-	-	-
5%	-	-	-	-
6%	-	-	-	-
7%	-	-	-	-
8%	-	-	-	-
9%	-	-	-	-
10%	-	-	-	-
11%	-	-	-	-
12%	-	-	-	-
13%	-	-	-	-
14%	-	-	-	-
15%	-	-	-	-
Lyophilized AMP				
10%	+	+	+	+
11%	+	+	+	+
12%	+	+	+	+
13%	+	+	+	+
14%	+	+	+	+
15%	+	+	+	+

When antimicrobials were applied to sterile EWOS salmon feed and over layered with indicators, the pure analogues of $Oct\text{-}TriA_1$, polymyxin B, polymyxin E and Sol-AMP (15%) had antimicrobial effects against the majority of the pathogens tested (Table 3-7), perceived as zones

of inhibition extending past pellets. Activity against *L. anguillarum* 99282 was only observed when Oct-TriA$_1$ and the Sol-AMP (15%) were used as antimicrobials. *Y. ruckeri* demonstrated variable activity to the 15% Sol-AMP preparation, as observed in Table 3-6. In assays using pure analogues (Table 3-7), 100 µL volumes of antimicrobials were applied to feed prior to incubation while 50 µL volumes of increasing concentrations Sol-AMP were applied to feed (Table 3-6). The difference in volume of Sol-AMP may explain the difference observed inhibition by in the 15% Sol-AMP in Table 3-6 and Table 3-7. Pellets did not absorb Sol-AMP in 100 µL volumes, thus the smaller volumes of solutions were used in attempts to use preserve reagent reserves.

Table 3-7. Activity of EWOS commercial salmon feed treated with 100 µL antimicrobials and over layered with cation-adjusted Mueller Hinton agar containing indicator organisms. Where variability was observed, +/- reports 2 of 3 positive results; -/+ reports 2 of 3 negative results. n=3.

Antimicrobial	Indicator organism			
	A. salmonicida ATCC 33658	*L. anguillarum* ATCC 19264	*L. anguillarum* 99282	*Y. ruckeri* ATCC 29473
Oct-TriA$_1$ (1mM)	+	+	+	+
Polymyxin B (1mM)	+	+	-	+
Polymyxin E (1mM)	+	+	-	+
Sol-AMP (15%)	+	+	+	+/-
Blank	-	-	-	-

Given the relative ease of treatment of feed with polymyxins, Oct-TriA$_1$ and the Sol-AMP and observed activity of feed treated with these antimicrobials, the pure analogues or Sol-AMP appear to be the obvious choice of antimicrobials for medicated feed preparation. Neither the polymyxins nor the tridecaptins are currently used in aquaculture to treat bacterial disease. Currently, the US and Canada have only four antibiotics approved for use in aquaculture species designated for human consumption: oxytetracycline, Florfenicol, sulfadimethozine/ormetoprim and Sulfamerazine/trimethoprim (Morrison and Saksida, 2013; Kelly, 2013). Polymyxins and tridecaptins are structurally related to any of the four approved antimicrobials approved for use, which may translate as reduced risk of the spread of antimicrobial drug resistance harbored by bacteria. The limiting factor in using the pure compounds is the expense, as producing 1 g of Oct-TriA$_1$ costs approximately $570 (Cochrane, personal communication), thus capitalizing on purification techniques to yield the AMP is much more economical. Treatment of pellets with AMP alone did not result in inhibition against indicator strains (data not shown). Given these

results, surface treatment of feed with high concentrations Sol-AMP may be a viable option for delivery of native $TriA_1$ and paenicidin B to salmon, though the risk of antimicrobials leaching into water in surface-treated pellets is higher than other methods of medicated feed preparation, reducing likelihood of *in situ* response (Treves-Brown, 2000). Variability in inhibition observed from treated feed over layered with indicators may be explained by differences in concentrations of Sol-AMP present in pellets at time of overlay. To assess the possibility of AMP adsorption to pellets to explain variable inhibitory activity when treated feed was over-layered led to efforts to explore alternate methods of delivery of antimicrobials to salmon via encapsulation were done.

Encapsulation of Antimicrobials. Entrapment of Oct-$TriA_1$ in alginate generated encapsulates that had antimicrobial activity when over-layered with salmon pathogens with zones of inhibition that extended approximately 1 mm around encapsulates (Table 3-8). When encapsulates containing 15% (w/v) Sol-AMP were over-layered with agar containing indicators, activity was highly variable. Zones of inhibition with Sol-AMP containing encapsulates were observed only where 4 or more encapsulates were grouped together. This suggests that the concentration of Sol-AMP is not great enough to illicit an antimicrobial effect in a singular encapsulate. Again, polymyxins B and E do not demonstrate activity against *L. anguillarum* 99282, while Oct-$TriA_1$ does, similar to the treated feed results.

Table 3-8. Antimicrobial activity of encapsulates made with different antimicrobials and over-layered with cation-adjusted Mueller Hinton agar containing a 0.1% inoculum of different salmon pathogens. n=3.

Antimicrobial	Indicator Organism			
	A. salmonicida ATCC 33658	*L. anguillarum* ATCC 19264	*L. anguillarum* 99282	*Y. ruckeri* ATCC 29473
Oct-$TriA_1$(1mM)	+/-	+	+	+
Polymyxin B (1mM)	+	+	-	+
Polymyxin E (1mM)	+	+	-	+
Sol-AMP (15%)	+/-	+/-	+/-	+/-
Blank	-	-	-	-

Assuming an 100% encapsulation efficiency, AMP encapsulates prepared from 15% (w/v) Sol-AMP preparations would contain only 3% of the lyophilized AMP, though literature shows the encapsulation efficiency is much lower, found to be approximately 67% in one study

(Liu et al., 2016). To rule out loss of antimicrobial compounds during the encapsulation process, samples at each step of the encapsulation process were extracted and tested with spot-on-lawn assays (Table 3-9). In almost all cases, homogenized extracts of commercial salmon feed treated with antimicrobials only retained activity when treated with Oct-TriA$_1$, though this activity was variable amongst indicators across experimental replicates. A possible explanation for the lack of activity in homogenized pellet extracts of polymyxin B and polymyxin E treated feed is the emulsification of the polymyxins with fat present in the feed. Surfactant properties of lipopeptide produced by *B. subtilis* to acts an emulsifier of fat have gained attention (Mandal et al., 2013). A lipopeptide recently isolated from *B. licheniformis* NIOT-AMKV06 was shown to have superior emulsification properties of kerosene and crude oil (Lawrance et al., 2014). It has been shown that activity of the lipopeptide, polymyxin B is greatly reduced in the presence of sodium-phospholipids, namely sodium salts of saturated fatty acids, due to micelle formation entrapping the antibiotic (Mamber et al., 1994). The commercial salmon feed is composed of 27% oil coming from fish, vegetable, and poultry (John McNicol, EWOS Canada Ltd. Quality Assurance Manager; Personal communication) and would likely contain saturated fatty acids that could form micelles that the polymyxins may bind to or be trapped within. Alternatively, Polymyxin B sulfate has also been shown to interact with milk solids-not fat in solid and liquid media, rendering the antibiotic inactive against strains of interest, likely by interaction of carboxyl groups with milk solids (Al-Shaikhli et al., 1964). It is well known that many antibiotics under go interactions with food-constituents, limiting bioavailability, as is the case for casein/calcium and ciproflaxin and calcium and tetracycline (Pápai et al, 2010; Jung et al., 1997). Whatever the mechanism, it is highly likely that the polymyxins are interacting with some constituent of the homogenized feed, resulting in the lack of activity observed against indicator strains.

When antimicrobials were tested for activity after addition to alginate prior to cross-linking, all synthetic antibiotics were active against indicators, with the exception of polymyxin B and polymyxin E against *L. anguillarum* 99282 (Sodium alginate + antimicrobial; Table 3-9). This trend was observed whether polymyxin B and polymyxin E were encapsulated or applied to feed alone. *L. anguillarum* 99282 was not sensitive to these lipopeptides, consistent with previous findings. In contrast, Oct-TriA$_1$ was able to inhibit *L. anguillarum* 99282 whether it had been exposed to feed alone, mixed with alginate or encapsulated. Oct-TriA$_1$, polymyxin B, polymyxin E and 15% Sol-AMP were encapsulated in an alginate matrix using calcium chloride

as chelating agent. Encapsulation using alginate was chosen as means of entrapment of antimicrobials because of the ease of preparation, safety, they are biodegradable, exhibit superior gel formation, mechanical stability, and are produced from marine brown algae so are derived from naturally occurring sources (Kaliasapathy, 2002; Lee et al., 2013). Given the widespread application of alginates as a matrix of choice for encapsulation of other composites in food, cosmetic and pharmaceutical industries (Chan et al., 2006; Furtado et al*.,* 2012; Annan et al*.,* 2008) it was deemed an appropriate choice of matrix.

On cross-linking, the extract from encapsulated 15% (w/v) Sol-AMP failed to illicit an antimicrobial effect against any of the indicator organisms; however, extracts of encapsulated 1 mM Oct-TriA_1, 1 mM polymyxin B and 1 mM polymyxin E caused observed antimicrobial effects., Encapsulates were subjected to enzymatic lysis prior to testing. Preliminary testing of encapsulates using Alginate Lyase, mechanical homogenization with a bead beater and extraction showed that mechanical homogenization with enzymatic pre-treatment liberated antimicrobials. When AMP encapsulates were exposed to a sterilized feed mash, no antimicrobial effect was observed, execpt polymyxin B against *A. salmonicida* and *Y. ruckeri* and polymyxin E retained activity only against *Y. ruckeri,* respectively (Encapsulated antimicrobial + feed; Table 3-9). The cross-linking encapsulation step occurred in a minimum volume of 200 mL of 0.5 M $CaCl_2$. The alginate-Sol-AMP solution may have been diluted in the cross-linking stage, explaining the loss of activity from the sodium alginate and antimicrobial and the encapsulated antimicrobial (Table 3-9). During the cross-linking process, calcium exposed to the interface of alginate spheres undergoes reactions with guluronic acid dense regions, forming egg-box junctions (McHugh, 1987). Calcium cations become entrapped between guluronic acid regions of separate polymer chains, resulting in cross-linking of individual polymers and results in gelation. Upon gelation an osmotic gradient is created between the calcium cross-linking solution and the inner sphere, providing a driving force for calcium to continue to migrate through the gelling sphere (Lee *et al.,* 2012).

Table 3-9. Antimicrobial activity of extracts of antimicrobial treated feed, encapsulates, and encapsulates exposed to feed against different salmon pathogens. EWOS commercial salmon feed was treated with 100 µL antimicrobials and subjected to mechanical homogenization prior to collecting supernatant for testing against indicators with spot-on-lawn assays. Encapsulates were subjected to enzymatic lysis and mechanical homogenization with and without exposure to salmon feed prior to collecting supernatant for testing against indicators with spot-on-lawn assays. Where variability was observed, +/- reports 2 of 3 positive results; -/+ reports 2 of 3 negative results. n=3.

	Indicator organisms							
Antimicrobial	**Antimicrobial + Feed**				**Sodium alginate + Antimicrobial (20% v/v)**			
	A. salmonicida ATCC 33658	*L. anguillarum* ATCC 19264	*L. anguillarum* 99282	*Y. ruckeri* ATCC 29473	*A. salmonicida* ATCC 33658	*L. anguillarum* ATCC 19264	*L. anguillarum* 99282	*Y. ruckeri* ATCC 29473
Oct-TriA$_1$ (1mM)	-	+/-	+/-	+/-	+	+	+	+
Polymyxin B (1mM)	+/-	-	-	-	+	+	-/+	+
Polymyxin E (1mM)	-	-	-	-	+	+	-/+	+
Sol-AMP (15%)	-	-	-	-	-	-	-/+	-/+
Blank	-	-	-	-	-	-	-	-
	Encapsulated Antimicrobial				**Encapsulated Antimicrobial + Feed**			
Oct-TriA$_1$ (1mM)	-	+	+	+/-	-	-	-	-
Polymyxin B (1mM)	+/-	+	-	+	-/+	-	-	+/-
Polymyxin E (1mM)	+/-	+	-	+	-	-	-	-/+
Sol-AMP (15%)	-	+/-	-	-	-	-	-	-
Blank	-	-	-	-	-	-	-	-

It could be that during this process, that the calcium cations more favorably interact with the alginate matrix, squeezing out the positively charged $TriA_1$ from the newly formed encapsulates into the cross-linking bath. This effect may not be observed in encapsulates containing 1 mM solutions of other antimicrobials because the concentration sufficient so that a slight dilution effect is countered by higher concentrations of pure compounds. Additionally, the Sol-AMP contains a number of other metabolites and hydrophobic compounds purified during hydrophobic interactions chromatography. This mixture of hydrophobic compounds may displace $TriA_1$ and destabilize the gelled network as studies have shown when drug compounds are larger than calcium cations, a positively charged microenvironment is created within encapsulates as the larger molecules displace calcium to the center of encapsulates (Chan et al., 2006). Future work could asses alginate encapsulation cross-linking and $TriA_1$ permeability as a function of time in encapsulates. Additionally, the use of the powdered lyophilized AMP instead of Sol-AMP may yield a more concentrated antimicrobial preparation that demonstrates activity against all indicators. Only pure Oct-$TriA_1$ after encapsulation retained activity against *L. angjuillarum* 99282, the clinical isolate of interest though this activity was diminished on exposure to feed (Table 3-9) Over layered encapsulates and over layered feed consistently had activity with Oct-$TriA_1$ against *L. anguillarum* 99282, thus extraction of antimicrobial compounds may require further investigation. The lack of activity of Sol-AMP in the encapsulated products and in treated feed compared with synthetic analogues may be the result of concentrations of active antimicrobials that are too low to have an antimicrobial effect. Based on the results of this work, Oct-$TriA_1$ preparations applied directly to feed or encapsulated are most likely to be most effective at controlling *L. anguillarum* 99282 infection in Atlantic salmon challenge studies.

3.4. Conclusions

The main focus of this work was to purify $TriA_1$ from the supernatant of *P. terrae* B-NRRL 30644 to explore avenues of application for this antimicrobial in the prevent the growth of pathogens that infect salmon through addition of $TriA_1$ to the salmon diet. A successful purification method was developed that demonstrated antimicrobial activity against bacterial pathogens. Work undertaken as part of this chapter demonstrated the effectiveness of both the

synthetic analogue, Oct-TriA$_1$, the AMP containing TriA$_1$ and lyophilized AMP containing TriA$_1$ (Sol-AMP) were active against *L. anguillarum*, among other salmon pathogens. Oct-TriA$_1$ was the only form of tridecaptin used in this study that retained antimicrobial activity on exposure to salmon feed. Attempts to encapsulate the AMP using alginate as a means of protection and delivery of antimicrobials proved effective, though extraction and retention of activity was lost. Further work could explore the agent in salmon feed responsible for inactivation or adsorption of polymyxins from feed. Additionally, Topical treatment of feed with fish oil containing the lyophilized AMP may prove to be an effective means of delivering these active antimicrobials to salmon. Application of Oct-TriA$_1$ via medicated feed is currently the best option for application of TriA$_1$ for the treatment of *L. anguillarum* infections in salmon, though application of purified TriA$_1$ would have been much more economical. Future work could explore preparation of medicated feed with lyophilized AMP through extrusion. Further work is necessary to determine to determine shelf life of Oct-TriA$_1$ medicated feed. The potential of Oct-TriA$_1$ as a novel antimicrobial to prevent bacterial disease in aquaculture is promising.

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Chapter 4. Overall discussion and conclusions

Hydrophobic interaction chromatography (HIC) was used to purify both antimicrobials used with this work, McnN and $TriA_1$. Isolation and characterization of McnN was unsuccessful, likely due to adsorption of the peptide to the column in high performance liquid chromatography (HPLC) separation attempts. MALDI-TOF MS analysis revealed a peptide of similar size to McnN in the crude preparation obtained from HIC separation and this fraction demonstrated antimicrobial activity against *S. enterica* and *E. coli* indicators. Application of the crude McnN containing fraction to raw and cooked ground beef failed to result in reductions of *S. enterica* and *E. coli* O157:H7 cocktails when stored for 72 h at 8°C. Further work should be done to obtain pure McnN for use in downstream experiments.

HIC was a technique successfully utilized to purify $TriA_1$ and paenicidin B from the culture ferment of *P. terrae* B-NRRL 30644. The presence of $TriA_1$ in the antimicrobial preparation (AMP) obtained from HIC separation was confirmed both by using HPLC and through assessment of antimicrobial activity against sensitive indicators. The synthetic analogue of Oct-$TriA_1$ was found to be active against a number of organisms of food and aquatic origins. Determining the minimum inhibitory concentrations (MIC) of Oct-$TriA_1$ against individual organisms provided insight about which aquatic pathogens would be best to target *in situ.* Given that *L. anguillarum* was most sensitive to Oct-$TriA_1$, it was decided that this would be the strain that would be involved in challenge studies with Atlantic salmon, work completed by collaborators. Lyophilization of the AMP and re-suspension in aqueous solution at higher concentrations yielded a preparation (Sol-AMP) that demonstrated activity against the aquatic pathogens, including *L. anguillarum.* Concentration of $TriA_1$ appears to be a limiting factor with respects to antimicrobial efficacy. Future work could include addition of the lyophilized AMP to a feed-mash for extrusion for enhanced antimicrobial effectiveness. Encapsulation using sodium alginate yielded a stable matrix suitable for delivery of Oct-$TriA_1$ to salmon. Palatability of encapsulates containing antimicrobials may need to be addressed in future work, to ensure salmon would consume antimicrobials for them to have the desired effect. Homogenization of feed treated with antimicrobials caused a loss in activity in polymyxin B and polymyxin E, which may be the result of a chemical interaction of the polymyxins with lipid or protein present in the commercial feed pellets. Given these findings, Oct-$TriA_1$ appears to be the most robust

antimicrobial tested with respects to stability, antimicrobial retention and recovery after treatment. These results are promising; use of the lyophilized AMP may be an economical means to administer $TriA_1$ to salmon afflicted with *L. anguillarum* infections via extruded medicated feed.

Determining the spectrum of activity and MIC of $TriA_1$ may prove useful for future research with this $TriA_1$ including prospective bacterial candidates and applications. The use and application of $TriA_1$ in foods as an antimicrobial and preservative is not recommended due to the robust and resilient nature of the peptide. Typically, antimicrobial peptides for use in human food are non-toxic and are susceptible to degradation by digestive enzymes. Thus use of these compounds poses minimal risk of affecting the human intestinal microbiota, potentially conferring antimicrobial resistance through frequent consumption and adversely affecting the consumer. Tridecaptins are resistant to gastrointestinal proteases, have selective and strong activity against gram-negative bacteria and have low hemolytic activity and cytotoxicity (Cochrane, 2015). The human gastrointestinal microbiota are dominated by 5 phyla with Bacteriodetes (gram-negative species), Firmicutes (gram-positive), Actinobacteria (gram-positive), Proteobacteria (gram-negative species), and Verrucomicrobia (gram-positive) being the most dominant phyla, respectively (Donaldson et al, 2016). Genera in the small intestine are limited to *Lactobacillaceae* and *Enterobacteriaceae*, due to constraints of the upper digestive tract including high quantities of bile acids, high concentrations of oxygen, lower pH and a higher transit rate than the distal colon. Introducing a stable antibiotic such as $TriA_1$ to food would likely devastate *Enterobacteriaceae* populations in the small intestine, which could lead to microbial dysbiosis and increases in opportunistic pathogen infection. Compounding this affect, members of Bacteriodetes, in the colon, including *Bacteroidaceae*, *Prevotellaceace* and *Rikenellaceae* may also be affected by free $TriA_1$, compounding microbial dysbiosis and subsequent pathologies. Preventing foodborne disease is important; however, utilizing a powerful antimicrobial that may have utility to control bacterial infections (including multi-drug resistant infections) would be a more suitable use for the application of $TriA_1$.

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Appendices

Appendix A- Culture growth media recipes

Minimal M63 media (modified from O'Brien, 1996), 1 L x M63 salts:

13.6 g KH_2PO_4 (Potassium phosphate monobasic)	0.1 M
2.0 g $(NH_4)_2SO_4$ (Ammonium sulfate)	0.015 M
0.5 mg $FeSO_4 \cdot 7H_2O$ (Ferrous sulfate heptahydrate)	1.8×10^{-6} M
15 g Agar (if making M63 minimal agar)	1.5%

Directions:
Dissolve reagents in 1 L distilled water. Adjust pH to 7.0 prior to sterilization.
Autoclave at 121°C for 15 min.

After autoclaving, once media has cooled, aseptically add the following filter sterilized reagents:

1 mL 20% $MgSO_4$ (Magnesium sulfate)	0.02%
0.5 mL 1% Thiamine-hydrochloride	0.0005%
10 mL 20% Glucose	0.2%
2.5 mL 20% Caseamino acids	0.05%
2.5 mL 20% Bacto-tryptone	0.05%

The pH of M63 minimal media after addition of additional components to the working solution is approximately 7.20. Addition of glucose, tryptone and caseamino acids required for *E. coli* MC4100 pGOB18 growth.

Appendix B- Minimum inhibitory concentration sample calculation

1) C= (m/V) • (1/MW)

Where:

C= Molar concentration or Molarity (mol/L or M)

m= Mass (g)

V= Volume (L)

MW= Molecular weight (g/mol)

C= (1.520 mg or 0.00152 g) • 1 .

1 mL 1520 g/mol = 1mM Oct-TriA$_1$

2) $C_1V_1=C_2V_2$

Where:

C_1= Concentration of stock solution (mol/L)

V_1= Volume of stock solution removed (L)

C_2= Final concentration of diluted solution (mol/L)

V_2= Final volume of diluted solution (L)

(1mM Oct-TriA$_1$ •5x10^{-5} L)= C$_2$ (1x10^{-4} L) C$_2$= 0.5 mM

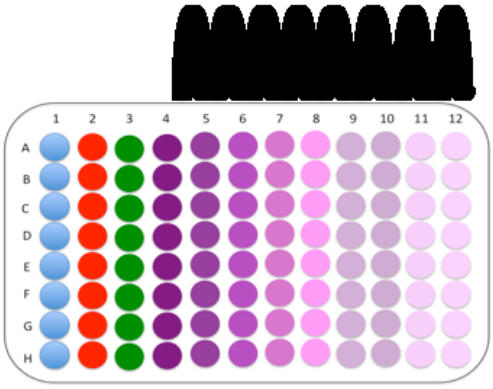
3) C= n/V

0.5 mM= 0.0005 M = n/ 0.00005 L

n= 2x10^{-8}mol; n=m/M 2x10^{-8} mol=m / 1520 g/mol

m=0.000038 g =

After 2-fold dilutions across series plate, concentration in wells is as follows:



Column 1: Blank: CAMHB only (100 µL)
Column 2: Negative Control: 0.5 mM Oct-TriA1
Column 3: Positive Control: CAMHB 50 µL + 50 µL culture
Column 4: 0.25 mM Oct-TriA1 or 38 µg/mL
Column 5: 0.125 mM Oct-TriA1 or 19 µg/mL
Column 6: 0.0625 mM Oct-TriA1 or 9.5 µg/mL
Column 7: 0.03125 mM Oct-TriA1 or 4.75 µg/mL
Column 8: 0.015625 mM Oct-TriA1 or 2.375 µg/mL
Column 9: 0.0078125 mM Oct-TriA1 or 1.1875 µg/mL
Column 10: 0.00390625 mM Oct-TriA1 or 0.59375 µg/mL
Column 11: 0.001953125 mM Oct-TriA1 or 0.29688 µg/mL
Column 12: 0.000976563 mM Oct-TriA1 or 0.14844 µg/mL