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Quantitation and application of bacteriocins in food

by

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Abstract

Several obstacles to widespread use of bacteriocins in food have been identified, including lack of specific, rapid quantitation methods, and little data on their efficacy in food systems. The first objective of this study was to develop a specific, rapid quantitation method for bacteriocins that did not rely on bioassays and their associated limitations. Phage display was chosen to reduce reliance on continued use of animals and produce antibodies to the bacteriocins leucocin A, piscicolin 126, and brochocin-A. Although the antibody libraries generated by phage display were not successful for antibody production, a strong immune response to leucocin A and piscicolin 126 was observed in mice. The second objective of the study was to determine the efficacy of brochocin-C against *Clostridium botulinum* in a model system and in a vacuum-packaged, chopped and formed pork product stored at refrigeration temperature. Group I *Cl. botulinum* was not controlled by brochocin-C, and was found to inactivate brochocin-C and several class IIa bacteriocins by proteolysis. Cell counts revealed that Group II *Cl. botulinum* was controlled by brochocin-C in a model meat system, but was not controlled in the chopped and formed pork product. Powdered smoke and NaCl in the pork product had a synergistic interaction against Group II *Cl. botulinum*, as shown by minimum inhibitory concentration testing. The choice of media for isolation of *Cl. botulinum* from the chopped and formed pork product was important, as the presence of background microflora isolated from the meat was found to impact growth of Group II *Cl. botulinum* on plating media. In the presence of the background microflora, which were

identified by 16S rDNA sequencing as carnobacteria and staphylococci, inclusion of phosphate in the plating medium was found to allow growth of *Cl. botulinum*. Other nutrients such as magnesium, sulphur, or increased protein sources added to the medium had no effect on growth of *Cl. botulinum*. Two of the background microflora strains, *Carnobacterium maltaromaticum* MH3 and *Staphylococcus pasteuri* EIV-21, inhibited *Cl. botulinum*, while one strain, *C. maltaromaticum* MH2, stimulated growth of *Cl. botulinum*.

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Table of Contents

List of Tables

List of Figures

List of Symbols and Abbreviations

A	absorbance
ABC	ATP-binding cassette
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AU	activity units
B.	*Bacillus*
bp	base pairs
Br.	*Brochothrix*
BSA	bovine serum albumin
C.	*Carnobacterium*
CFIA	Canadian Food Inspection Agency
CFU	colony-forming units
Cl.	*Clostridium*
CT	cholera toxin
D	decimal
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
x *g*	times gravity
GRAS	Generally Recognized as Safe
h	hour
HPLC	high performance liquid chromatography
IU	international units
kDa	kilodalton
kHz	kilohertz
KLH	keyhole limpet haemocyanin
L	litre
L.	*Listeria*
Lb.	*Lactobacillus*
Lc.	*Lactococcus*

LD	lethal dose
M	moles per litre
MIC	minimal inhibitory concentration
min	minute
mL	millilitre
mM	millimoles per litre
mol	mole
nM	nanomoles per litre
OD	optical density
p	plasmid or phagemid
PCR	polymerase chain reaction
PFU	plaque-forming units
rev	revolutions
RNA	ribonucleic acid
S.	*Streptococcus*
SEM	standard error of the mean
UV	ultraviolet
v	volume
V	volt
w	weight
µF	microfarad
µL	microlitre
Ω	ohm

Chapter One: **Introduction and Literature Review**

1.1 Food safety

It is estimated that 11 to 13 million cases of foodborne illness occur in Canada annually, which is equivalent to approximately one-third of the population (CFIA, 2010). A similar situation exists in the United States, with an estimate of 76 million cases of foodborne illness annually; of these, 323000 require hospitalization, while 5000 result in death (Mead et al., 1999). Economically, foodborne illness exacts a heavy toll, costing a total of $152 billion (US) annually as a result of health care costs, productivity losses from illness in the workforce, and loss of earning potential due to lowered life expectancy (Scharff, 2010). When added to the costs of implementing recalls and loss of sales through damage to company reputation, it is clear that foodborne illness is a major concern from public health, social, and economic standpoints. Thus, food safety is an important issue for the food industry, academics, and government regulators.

Food safety has also become an important issue for consumers, especially following highly-publicized food recalls and food-associated outbreaks. In a 2008 survey, 45 % of consumers expressed a moderate-to-strong concern about microbial contamination of food, compared to only 12 % in 1984 (Brewer and Presat, 2002; Brewer and Rojas, 2008). Complicating a heightened scrutiny of food safety issues is an increasing concern with the use of preservatives and nitrates in food, with 40 % and 52 %, respectively, of consumers reporting being highly-concerned with these issues (Brewer and Rojas, 2008). Thus, consumers have increased demand for foods with increased food safety, but without the use of perceived “chemical” additives (Sofos, 2008). An alternative to the use of anthropogenic chemical additives is the use of inhibitory substances produced by microorganisms.

1.2 Enhancing food safety and quality using microbial agents

Historically, food preservation has been enhanced by products of microbial metabolism such as organic acids, alcohol, carbon dioxide, and secondary metabolites (Axelsson, 2004). In particular, lactic acid bacteria (LAB) have been

employed as starter cultures in meat, vegetable, and dairy products, and their metabolic activities serve to extend storage life and inhibit pathogenic microorganisms in fermented foods.

The LAB are a diverse group of genera which convert at least 50 % of the carbon from sugars into two isomers of lactic acid, thereby earning the name "lactic acid bacteria" (de Vuyst and Vandamme, 1994; Carr et al., 2002; Axelsson, 2004). Belonging to the clostridial branch of the gram-positive bacteria, LAB are non-spore-forming catalase-negative cocci, bacilli, or coccobacilli with a G+C content of 55 mol % or less (Stiles and Holzapfel, 1997). Food-associated genera included in the LAB group include *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus, Streptococcus, Tetragenococcus, Weissella* (Stiles and Holzapfel, 1997; Axelsson, 2004), and *Bavariicoccus* (Schmidt et al., 2009).

The metabolism of LAB can be described as homofermentative or heterofermentative based on the composition of their metabolites under standard conditions of excess carbohydrate source and limited oxygen (Axelsson, 2004). Homofermentative LAB produce mainly lactic acid using the Emden-Meyerhof-Parnas pathway, with a theoretical yield of 2 mol lactic acid and 2 ATP per mol of glucose consumed (Axelsson, 2004). Heterofermentative LAB use the 6-phosphogluconate pathway to convert carbon substrates such as glucose to equimolar amounts of lactic acid, carbon dioxide and ethanol (de Vuyst and Vandamme, 1994; Axelsson, 2004). Classification as either homo- or heterofermentative is exclusive, but certain conditions of oxygenation and medium composition can cause changes in fermentation end products (Kandler, 1983; Condon, 1987).

In addition to lactic acid, which inhibits microbial growth by lowering the pH, LAB also produce other inhibitory metabolites. Other antimicrobial chemicals produced include hydrogen peroxide (Finnegan et al., 2010), formic acid, acetoin, diacetyl (2,3-butanedione) (Jay, 1982a; Jay, 1982b; Lanciotti et al., 2003), acetaldehyde, benzoate, reutericyclin (Gänzle and Vogel, 2003; Gänzle et

al., 2000) and free fatty acids (Collins et al., 2003; Desbois and Smith, 2010). Many genera of LAB in foods also produce a range of proteinaceous antimicrobial compounds called bacteriocins, some of which have activity against pathogenic microorganisms, and so these compounds have attracted attention as biopreservatives.

1.3 Bacteriocin definition

Bacteriocins are defined as ribosomally-synthesized peptides produced by one microorganism with antimicrobial activity against closely-related organisms, and sometimes including organisms outside their ecological niches (Jack et al., 1995). Bacteriocins are grouped into the ribosomally-synthesized group of peptide antimicrobials, along with certain other archaeal, plant, mammalian, insect, viral, and amphibian antimicrobials (Hancock and Chapple, 1999; Shand and Leyva, 2008). In contrast, many of the peptide antibiotics known for therapeutic uses, such as gramicidin and polymyxin, are non-ribosomally synthesized. These antibiotics are produced by an ordered multi-enzyme complex, and are usually highly modified following synthesis (Kleinkauf and von Döhren, 1990; Hancock and Chapple, 1999). Another key difference between bacteriocins and other antimicrobials is their minimum inhibitory concentration (MIC). Bacteriocins have been shown to have MICs that are lower than that of therapeutic antibiotics (Svetoch et al., 2008), and can be in the nanomolar range (Wiedemann et al., 2001). Although both bacteriocins and therapeutic antibiotics are antimicrobial in action, they differ in many characteristics, as shown in Table 1-1.

Table 1-1 A comparison of bacteriocins and therapeutic peptide antibiotics[1]

Characteristic	Bacteriocin	Therapeutic Peptide Antibiotic
Application	Food	Therapeutic
Synthesis	Ribosomal	Multi-enzyme complex
Activity	Usually narrow, centred around producing strain	Varying spectrum
Host cell immunity	Yes – dedicated immunity protein associated with bacteriocin structural gene	No – independent expression of resistance
Interaction with target	Docking molecules seem to be required	Specific targets
Mode of action	Usually pore formation in cell membrane	Cell membrane, intracellular targets
Toxicity to humans	One reported (cytolysin of *Enterococcus faecalis*)[2]	Yes
Production	In growth phase	In stationary phase, secondary metabolites

[1] adapted from Cleveland et al. (2001); Holo et al. (2002), [2] Cox et al. (2005)

1.3.1 Classification of LAB bacteriocins

Classification of bacteriocins has evolved over the last 18 years as further characterization of the structures of bacteriocins was conducted. Klaenhammer's four-class scheme (1993) based on structure and target cell has been the most widely-used. Classes I, II, and III are still recognized, while class IV, previously thought to contain combine lipid or carbohydrate moieties with a peptide component, has been largely discredited, based on elucidation of bacteriocin structures (Nes et al., 1996).

Class I bacteriocins are the lantibiotics, which are small (< 5 kDa) peptides distinguished by internal ring structures formed by bridging of the unusual amino acids lanthionine and β-methyllanthionine (Klaenhammer, 1993). These unusual amino acid residues are formed upon dehydration of serine or threonine, forming α,β-unsaturated amino acids didehydroalanine or 2,3-didehydrobutyrine (Twomey et al., 2002). When intra-peptide free cysteines become covalently linked to these residues, either lanthionine or β-methyllanthionine is formed. The presence of lanthionine or β-methyllanthionine has been confirmed in at least eight lantibiotics (Twomey et al., 2002). Lantibiotics have been subgrouped into type A, which are cell membrane-active, elongated cationic peptides up to 34 amino acids in length, while type B lantibiotics are globular and shorter in length (19

amino acids), uncharged, and disrupt enzyme function (Jung, 1991; Twomey et al., 2002; Willey and van der Donk, 2007). Nisin, a type A bacteriocin produced by *Lactococcus lactis*, is the most well-characterized lantibiotic.

Class II bacteriocins are small (<10 kDa) heat-stable, non-modified peptides, and are sub-categorized into four groups. Class IIa bacteriocins are cationic, pediocin-like bacteriocins, ranging in size from 37 to 58 amino acids, and often with activity against *Listeria monocytogenes* (Nes and Holo, 2000; Drider et al., 2006). These bacteriocins also have a conserved N-terminus sequence, consisting of $YGNGV(X)C(X)_4C(X)V(X)_4A$, whose disulfide bridge confers structural integrity necessary for antimicrobial activity (Eijsink et al., 1998; Fimland et al., 2000; Drider et al., 2006). Representative class IIa bacteriocins include leucocin A (Hastings et al., 1991; van Belkum and Stiles, 1995), mundticin L (Feng et al., 2009), pediocin PA-1 (also known as AcH) (Biswas et al., 1991; Lozano et al., 1992), piscicolin 126 (Jack et al., 1996; Gursky, 2004), and sakacin A (Holck et al., 1992).

The class IIb bacteriocins are two-peptide bacteriocins, which have two cationic complementary peptides required for optimal activity, are associated with one immunity gene, and have two structural genes that are in the same operon (Eijsink et al., 2002). Although the individual peptides may have minimal activity individually, a synergistic effect requiring a direct physical interaction between the two peptides is characteristic of this group (Oppegård et al., 2007). Two-component bacteriocins produced by LAB include lactocin 705 (Cuozzo et al., 2000; Castellano et al., 2003; Cuozzo et al., 2003), lactococcin G (Moll et al., 1996; Moll et al., 1998), and thermophilin 13 (Marciset et al., 1997), while class IIb bacteriocins produced by other bacteria include brochocin-C, produced by *Brochothrix campestris* (Siragusa and Cutter, 1993; McCormick et al., 1998; Gao et al., 1999).

Class IIc bacteriocins were originally defined as thiol-activated peptides with a double glycine leader sequence (Klaenhammer, 1993), but several groups have proposed different definitions of this class (Nes et al., 1996; van Belkum and

Stiles, 2000; Eijsink et al., 2002; Franz et al., 2007). Class III bacteriocins are large, heat-labile proteins, and include the bacteriocins helveticin J (Joerger and Klaenhammer, 1986; Joerger and Klaenhammer, 1990) and enterolysin A (Hickey et al., 2003; Nilsen et al., 2003). Other workers have subdivided class III into bacteriolysins (IIIa) and non-lytic bacteriocins (IIIb) (Heng et al., 2007). Cyclic bacteriocins have also been proposed as a classification distinction by several workers (Kemperman et al., 2003; Heng et al., 2007).

Classification schemes have proven to be fluid, changing with the discovery of more bacteriocins and characterization of their structures. Although many different classes of bacteriocins have been discovered, bacteriocins belonging to classes I and II have attracted the most interest for use in food products, and will be the focus of this review.

1.4 Biosynthesis and Mechanism of Bacteriocins

1.4.1 Genetic organization and regulation

The genes required for bacteriocin biosynthesis have been found on plasmids (Ahn and Stiles, 1990; Ryan et al., 1996; Ito et al., 2009), megaplasmids (Claesson et al., 2006), transposable elements (Horn et al., 1991), and occasionally in the chromosome (Aymerich et al., 1996; Quadri et al., 1997a). In general, the biosynthetic blueprint for bacteriocin-producing bacteria includes genes encoding proteins for at least four functions: bacteriocin structure, dedicated producer immunity, a dedicated ATP-binding cassette (ABC) transport system for export, and an accessory protein (Drider et al., 2006).

Bacteriocins produced by LAB are expressed initially as pre-peptides, which are composed of an inactive form of the bacteriocin attached to a leader peptide or an N-terminal extension (Twomey et al., 2002). For lantibiotics, there are additional genes encoding proteins for post-translational amino acid modifications and processing. For example, the nisin A gene cluster contains genes encoding the pre-peptide, two enzymes for dehydration and lanthionine modification, a protease for cleavage of the leader peptide, an ABC protein for

secretion, two proteins involved in regulation of expression, and four proteins involved in self-immunity (McAuliffe et al., 2001).

Genes encoding non-lantibiotic bacteriocin structure and self-immunity are usually located in close proximity, likely to ensure translation and expression of the immunity gene temporally close to the structural gene (Nes et al., 1996; Diep and Nes, 2002; Mathiesen et al., 2005). The class IIa bacteriocin carnobacteriocin A is an exception to this, with "considerable separation" between the structural and immunity genes (Worobo et al., 1994). The structural genes for two-peptide bacteriocins are located adjacent to each other on the same operon (Fremaux et al., 1993; Anderssen et al., 1998; McCormick et al., 1998; Franz et al., 2002), with only one immunity gene required (Franz et al., 2002; Garneau et al., 2002; Hu et al., 2010).

Regulation of many bacteriocins has been shown to occur via a two-component regulatory pathway, involving a pheromone signaling molecule, a histidine kinase signal receptor, and a response regulator which modulates gene expression (McAuliffe et al., 2001; Diep and Nes, 2002; Rohde and Quadri, 2006). Nisin takes the role of pheromone in the regulatory network of the *nis* transcriptional unit (Kuipers et al., 1993; Engelke et al., 1994; Kuipers et al., 1995; Ra et al., 1996), while for most class II bacteriocins, signaling molecules are small, unmodified cationic peptides which do not have antimicrobial activity (Saucier et al., 1995; Quadri et al., 1997b; Nilsen et al., 1998; Diep et al., 2000). It was believed that these inducing molecules are constitutively expressed at low levels; thus, when the cell density reaches a critical level, induction of bacteriocin expression occurs, as in a quorum sensing model (Kleerebezem et al., 1997). However, some workers have hypothesized that there may be other as-yet-unidentified environmental factors affecting induction of bacteriocin production (Kleerebezem and Quadri, 2001).

1.5 Biosynthesis and export

For many lantibiotics and class II bacteriocins, processing of the inactive pre-peptide into a functional bacteriocin takes place during export via the

dedicated ABC translocation machinery (Håvarstein et al., 1995). For the ABC-dependent transport, two membrane-bound proteins are required for translocation, a dedicated ABC-transporter (Håvarstein et al., 1995), and an accessory protein of approximately 470 amino acids (Fremaux et al., 1995; Franke et al., 1996; van Belkum and Stiles, 2000). ABC-transporters of bacteriocins do not seem to possess unique structures with related bacteriocins, but instead share features common to all ABC-transporters; namely, a cytoplasmic 200-amino acid conserved ATP-binding C-terminus and a hydrophobic N-terminus which is integrated into the membrane and responsible for proteolytic processing of the leader sequence (Håvarstein et al., 1995; Venema et al., 1995; Ennahar et al., 2000). In the bacteriocin-specific ABC-transporters, cleavage into a biologically-active bacteriocin takes place concomitantly with externalization of the bacteriocin (Håvarstein et al., 1995).

Following translation, the pre-peptide, which has the leader sequence fused to the N-terminus, is inactive while in the cytoplasm (Nes et al., 1996). Lantibiotics (van der Meer et al., 1993), class IIa, and class IIb bacteriocins possess a leader sequence, which is believed to allow recognition for export as well as protecting the producing cell from the toxicity of the active bacteriocin (Nes et al., 1996). The leader sequences of characterized bacteriocins are between 23 – 30 amino acids in length for lantibiotics (McAuliffe et al., 2001), 18 – 27 for class IIa bacteriocins (Ennahar et al., 2000), and 15 – 30 for two-peptide bacteriocins (Oppegård et al., 2007). In lantibiotics, there are two types of leader sequences recognized, differing in charge and consensus sequences (McAuliffe et al., 2001). The consensus sequence of one lantibiotic subgroup resembles that of the class IIa bacteriocins, which is highly conserved and contains a double-glycine sequence in the C-terminus (Håvarstein et al., 1994; McAuliffe et al., 2001), which is believed to be the sequence recognized by the dedicated ABC transport system (Piard et al., 1993; Håvarstein et al., 1995; van Belkum et al., 1997).

However, some bacteriocins have been found to use the general secretory pathway (*sec*) for transport (Leer et al., 1995; Worobo et al., 1995; McCormick et al., 1996; Tomita et al., 1996; Cintas et al., 1997; Herranz and Driessen, 2005). In the case of divergicin A, the N-terminal extension bears no sequence similarity to those of other class I and II bacteriocins. It contains a unique sequence, which, when cleaved by proteases, produces a hydrophobic signaling molecule (Worobo et al., 1995). This molecule allows for export of divergicin A using the *sec*-dependent pathway, and also means that less genetic information is required for divergicin A production, as the dedicated machinery for export is not present as for other bacteriocins. These factors suggest that divergicin A-like bacteriocins may be classified in a unique bacteriocin category (Worobo et al., 1995).

Finally, two bacteriocins produced by *Lc .lactis* BGMN1-5 were found to be secreted via a multi-drug resistance protein, which had not been observed previously (Gajic et al., 2003).

1.6 Mode of action

Bacteriocins produced by LAB are bactericidal in nature, and have varying spectra of activity, though most are membrane-active peptides with activity restricted to gram-positive bacteria (Ennahar et al., 2000). Receptors on the cell membranes of sensitive targeted cells have been shown to be critical for target recognition for most classes of bacteriocins. The lantibiotics nisin and lacticin 3147 have been shown to bind lipid II (Breukink et al., 1999; Wiedemann et al., 2001; Wiedemann et al., 2006), which has a two-fold effect: it renders lipid II unavailable for peptidoglycan synthesis and subsequent cell wall formation (Wiedemann et al., 2001), and forms pores in the cell membrane (Breukink et al., 1999; Breukink et al., 2003). Such pores cause the collapse of the proton motive force (Mitchell, 1966); which disrupts the phosphorylation of ADP to ATP, ion accumulation, protein phosphorylation, and transport of molecules across the cell membrane (Okereke and Montville, 1992; Montville and Bruno, 1994; White, 2000); and causes efflux of ATP, amino acids, and other small metabolites (Héchard and Sahl, 2002).

A target for some class IIa bacteriocins has also been identified. Two proteins involved in the mannose-phosphotransferase system were found to interact with lactococcin A (Diep et al., 2007), which then permeabilized the cell membrane, allowed efflux of solutes from the cell, and caused cell death (Holo et al., 1991). Other workers have found that sensitivity to mesentericin Y105 (Héchard et al., 2001), leucocin A (Ramnath et al., 2000; Ramnath et al., 2004), pediocin PA-1 (Ramnath et al., 2004), and enterocin A (Ramnath et al., 2004) was linked to the mannose-phosphotransferase system. Ramnath (2004) identified this sensitivity as due to expression of one component of the mannose-phosphotransferase system. Recently, a glycerophosphoryl diester phosphodiesterase encoded by *glpQ* was identified as a target in *Enterococcus faecalis* and *L. monocytogenes* (Calvez et al., 2007; Calvez et al., 2008), although in *L. monocytogenes*, a homologous open reading frame with 60.7 % similarity to *glpQ* was investigated. One class II bacteriocin, lacticin Q, was found to affect membrane permeability and subsequent protein leakage by formation of a huge toroidal pore, which did not require a docking molecule (Yoneyama et al., 2009). With a size of 4.6 – 6.6 nm, this pore is much larger than those formed by other bacteriocins such as nisin, which forms pores of 2 – 2.5 nm (Wiedemann et al., 2004; Yoneyama et al., 2009). Regardless of the target, class II bacteriocins damage the cell membrane, and cause cell death via leakage of intracellular solutes (Cotter et al., 2005).

Some bacteriocins include bacterial endospores in their inhibitory spectrum. Nisin has been found to inhibit outgrowth of spores of *Clostridium botulinum* (Scott and Taylor, 1981), *Bacillus anthracis* (Montville et al., 2006; Gut et al., 2008), *B. cereus* and other *Bacillus* spp. (Montville et al., 2006). Brochocin-C, a two-peptide bacteriocin, can also inhibit outgrowth of spores of *Bacillus* spp., *Clostridium* spp., and *Cl. botulinum* (McCormick et al., 1998). Co-inoculation of a class II bacteriocin-producing culture with spores of *Cl. botulinum* may inhibit spore outgrowth, but this appears to be strongly influenced by incubation temperature, producing cell concentration, and *Cl. botulinum* strain (Okereke and Montville, 1991; Rodgers et al., 2003; Rodgers et al., 2004). Outgrowth of *Cl.*

difficile spores was inhibited by lacticin 3147 (Rea et al., 2007). Early research suggested that the dehydroalanine residues of nisin react with sulfhydryl groups on the surface of endospores of *B. cereus*, thereby arresting outgrowth at a low (10 nM) concentration of nisin (Morris et al., 1984). However, little else has been reported about the mechanism of other bacteriocins against spores produced by other organisms.

1.7 Detection and Quantitation

1.7.1 Bioassays

Bioassays using sensitive indicator bacterial strains have been the method of choice for bacteriocin detection and quantitation for many years. Such methods have included direct plating of bacteriocin-treated survivors (Hirsch, 1950), agar diffusion assays (Tramer and Fowler, 1964; Tagg and McGiven, 1971), turbidometric methods (Berridge and Barrett, 1952; Skyttä and Mattila-Sandholm, 1991; Flores et al., 2003; Turcotte et al., 2004; Wu and Li, 2007) , and bioluminescence (Waites and Ogden, 1987; Wahlström and Saris, 1999; Valat et al., 2003a; Reunanen and Saris, 2004; Hakovirta et al., 2006; Immonen and Karp, 2007). In general, quantitative methods, which report the actual concentration of bacteriocin in g per mL, have focused on the lantibiotic nisin, because of the commercial availability of semi-purified preparations, such as Nisaplin®, a 2.5 % (w/w) nisin product manufactured by Danisco (Copenhagen, DK). Semi-quantitative methods have also been reported for nisin and other bacteriocins. Such methods involve a critical dilution assay to determine the most dilute concentration that effects a certain measure of inhibition of a sensitive indicator under defined conditions (Daeschel, 1992), and usually report the value as arbitrary units (AU) mL^{-1}. However, absolute quantitative methods are preferred because of the continuous nature of their measurement, as well as their higher precision (Nuñez et al., 1996; Delgado et al., 2005).

1.7.1.1 Agar diffusion quantitation

A nisin dose-bactericidal effect response curve method constructed by treating cultures of *Streptococcus* spp. with dilutions of purified nisin, and

determining the number of survivors was first reported in 1950 (Hirsch, 1950). Since then, variations on this assay have abounded. Later bioassay methods using solid medium involved boring holes into agar plates seeded with an indicator strain, adding dilutions of nisin to the holes, allowing for an optional pre-diffusion step, and measuring the zones of inhibition following incubation (Tramer and Fowler, 1964; Fowler et al., 1975; Pongtharangkul and Demirci, 2004). Quantitation using this agar diffusion assay was achieved by comparing the diameter of the inhibition zone of unknown samples to the linear portion of a standard curve.

Limitations to these methods have been identified. Variability is an issue, with sources such as the age of the test organism and the diluents used (Hirsch, 1950). In agar diffusion assays, consistency has been compromised by bacteriocin size, which may hinder diffusion through agar and confound results (Tramer and Fowler, 1964). The addition of the surfactant Tween to the agar reduced this problem somewhat (Mocquot and Lefebvre, 1956; Tramer and Fowler, 1964). Later workers improved the sensitivity, accuracy, and precision of this nisin quantitation method by optimizing the test organism, agar concentration and depth, buffer used, and the concentration of Tween 80 used (Wolf and Gibbons, 1996). However, some issues still remain, such as inability to distinguish between different inhibitors or bacteriocins, as products such as acids can also produce inhibition zones. As well, each assay must be optimized for each different bacteriocin because of differences in size and hydrophobicity among bacteriocins, which affects their ability to diffuse through agar. As well, the sensitive indicator organisms used for bacteriocins must be determined individually for each bacteriocin (Papagianni et al., 2006). Inhibition zones may not be uniformly round or may be hazy, leading to difficulties in accurately measuring the zone diameter (Papagianni et al., 2006). Media used may also cause problems in interpretation of results. Insufficient buffering capacity of one medium caused a lowering of pH, leading to inhibition zones in all samples (Lewus and Montville, 1991). Inhibitors present in food samples can also interfere with results (Tramer and Fowler, 1964; Papagianni et al., 2006), thereby

making this assay unsuitable for quantitative analysis in foods, though a modified method for detection of bacteriocins in meat has been described (Saucier and Greer, 2001).

A related method based on the effect of bacteriocins on sensitive indicator organisms involves separating the bacteriocins from other peptides by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then overlayering the gel with a culture of an indicator strain in semi-solid agar (Bhunia et al., 1987; Bhunia and Johnson, 1992). Following incubation, zones of clearing indicate the peptide with antimicrobial activity. In this way, bacteriocins can be detected and approximate sizes of bacteriocins can be deduced, although quantitation cannot be done.

1.7.1.2 Turbidometric quantitation

Turbidometric bioassays using growth inhibition of liquid cultures by bacteriocins have also been used for quantitation (Berridge and Barrett, 1952; Skyttä and Mattila-Sandholm, 1991; Flores et al., 2003; Turcotte et al., 2004; Wu and Li, 2007). In these methods, sensitive cultures in the same growth phase are treated with dilutions of bacteriocin, and after incubating for a defined length of time and halting growth, the turbidity of the culture is determined spectrophotometrically. A standard curve is constructed from the growth response to doses of purified standards, and the quantity of bacteriocin in unknown samples can be determined. Efficiency has been improved by automating the turbidometry readings (Skyttä and Mattila-Sandholm, 1991; Turcotte et al., 2004), and by miniaturizing the method in a microplate assay (Parente et al., 1995; Turcotte et al., 2004). However, these methods still suffer from the limitations of all bioassays; namely, the dependence on a sensitive indicator organism, and the length of time for results, although the assay length is much shorter for these assays than for solid-medium assay. Agar-based methods required 24 to 48 h for results, while turbidometric methods can be completed in 3 to 6 h (Flores et al., 2003; Turcotte et al., 2004). One major concern for both liquid- and solid-phase bioassays is the unavailability of bacteriocins with the

exception of nisin, as such purified or semi-purified preparations are required for standard curves. Other issues with turbidometric methods have been identified, including variability at low bacteriocin doses, influence of lysed and partially-lysed cellular debris on turbidity readings, and influence of the initial concentration of the indicator strain; however, data-processing improvements have minimized the effects of these and other factors (Cabo et al., 1999; Wu and Li, 2007).

When turbidometric and agar diffusion methods were directly compared, turbidometric methods were found to be more sensitive and reproducible (Parente et al., 1995; Papagianni et al., 2006), but the effective range of response was narrower than that of solid medium (Parente et al., 1995). The subjectivity involved with determining the size of inhibition zones on solid agar can be partially counteracted by using an automated image analysis system (Nuñez et al., 1996; Papagianni et al., 2006), but the precision of the agar diffusion assay is still quite low, with a reported coefficient of variation exceeding 20 % in one study (Parente et al., 1995). Turbidometric methods were determined to be more suitable for food applications, because no interferences as seen in agar diffusion assays were observed (Papagianni et al., 2006).

Both bioassay-based methods rely on identification and use of a suitable indicator organism for each bacteriocin to be tested, which was found to be critical to accurate and reproducible quantitation (Papagianni et al., 2006). However, even with the same bacteriocin, there are conflicting reports on the most suitable indicator, based on sensitivity. For instance, *Micrococcus luteus*, first proposed as an appropriate nisin-sensitive indicator by Hirsch (1950), was confirmed as the most sensitive indicator in agar diffusion assays by Wolf and Gibbons (1996). However, other workers determined *Lactobacillus sakei* (Rogers and Montville, 1991; Pongtharangkul and Demirci, 2004) or *Lb. curvatus* (Papagianni et al., 2006) to be more sensitive to nisin than *Micrococcus* spp. *Lb. sakei* was also found to be more sensitive, precise, and reproducible that *M. luteus* or *Br. thermosphacta* (Pongtharangkul and Demirci, 2004). Thus, standardization

of bioassays appears to be difficult for different bacteriocins, as well as the same bacteriocin (nisin).

A related semi-quantitative growth-based bioassay has been developed based on the change in conductance of a culture of a sensitive indicator organism when treated with a bacteriocin-containing preparation (Giraffa et al., 1990). In this method, the supernatant of a bacteriocin-producing strain of *Lb. delbreuckii* subsp. *lactis* was added to a defined number of cells of a *Lb. delbrueckii* subsp. *bulgaricus* indicator strain, and the change in conductance caused by production of low-molecular-weight charged metabolites was monitored using an automated system. The results showed that there was a linear relationship between conductance-based parameters and bacteriocin concentration. Although the authors suggested that this relationship could be used to determine the level of bacteriocin, no verification of this suggestion was performed (Giraffa et al., 1990).

Another form of bioassay has been developed which measures the decrease in metabolic end products produced by sensitive indicator organisms treated with an antimicrobial. Specifically, the production of $^{14}CO_2$ is measured in cultures provided with radiolabeled carbon sources and treated with different concentrations of antimicrobials (Cutler et al., 1989). These researchers observed a reproducible dose-dependent relationship between antimicrobial concentration and $^{14}CO_2$ produced, although this relationship is not linear. However, these workers tested only two therapeutic antibiotics, and so these results may not be applicable to bacteriocins. Also, specialized equipment is required for this assay, which may limit its widespread application.

1.7.1.3 Luminescence bioassay quantitation

A more recent type of bioassay relies on bioluminescence to detect and quantify bacteriocins. One type of bioluminescent method involves measuring the level of intracellular, extracellular, or pooled ATP in cultures of a sensitive indicator strain treated with nisin (Waites and Ogden, 1987; Valat et al., 2003a; Valat et al., 2003b). Methods measuring ATP level have reported linear quantitation in the range of 0.4 – 4 $\mu g\ mL^{-1}$ for nisin in medium using *Lc. lactis*

subsp. *cremoris* as the indicator strain (Valat et al., 2003a), and 2 µg mL^{-1} in beer, water, and wort using *Lb. brevis* as a test organism (Waites and Ogden, 1987). However, these methods require up to 24 h for results, and because all ATP is measured by this method, it is not suitable for use in cultures containing both nisin-producing cells and a sensitive target strain, because the producing strain may also contribute to the ATP pool (Valat et al., 2003a).

A second method using bioluminescence for bacteriocin detection involves using the NisRK/$P_{nisF/nisA}$ signal transduction pathway in a biosensor system. This method uses the features of the NIsin-Controlled gene Expression (NICE) system, which was designed to use the auto-induction feature of the nisin regulatory operon to express other genes (Kuipers et al., 1995; Mierau and Kleerebezem, 2005). For nisin quantitation, the genes encoding a luminescent reporter protein are cloned into a plasmid under the control of either the *nisA* (Reunanen and Saris, 2004; Hakovirta et al., 2006; Immonen and Karp, 2007) or *nisF* (Wahlström and Saris, 1999; Reunanen and Saris, 2003) promoter. In the presence of extracellular nisin, the transmembrane sensor histidine kinase NisK becomes phosphorylated, which results in activation of NisR. NisR then binds to *nisA* and *nisF*, causing transcription of the luminescent genes downstream of the promoters. The level of luminescence produced has a linear relationship with the amount of nisin present, and so a standard curve can be constructed for quantitation of nisin (Immonen and Karp, 2007). One feature of the system is that the genes for nisin production have been disrupted, so that only extracellular nisin is detected. Two different reporter proteins have been used in this type of assay: green fluorescent protein (Reunanen and Saris, 2003; Reunanen and Saris, 2004; Hakovirta et al., 2006) or bacterial luciferase (Wahlström and Saris, 1999; Simon et al., 2001; Immonen and Karp, 2007).

Sensitivities for these methods are among the lowest reported for any nisin quantitation method, both in experimental medium and in foods. In broth, detection limits have been reported to be 2.5 ng mL^{-1} (Reunanen and Saris, 2003), 12.5 pg mL^{-1} (Wahlström and Saris, 1999), 10.5 pg mL^{-1}(Hakovirta et al., 2006), and 0.1-0.3 pg mL^{-1} (Immonen and Karp, 2007). In foods, the detection limits are

slightly higher. The detection limits of liquid products tested are: milk: 45 ng mL^{-1} (Reunanen and Saris, 2003), 75 pg mL^{-1} (Wahlström and Saris, 1999), 200 pg mL^{-1} (Hakovirta et al., 2006), and 0.3 – 3 pg mL^{-1} (Immonen and Karp, 2007); processed cheese: 900 ng g^{-1} (Reunanen and Saris, 2003) and 3.6 ng g^{-1} (Hakovirta et al., 2006); salad dressings: 1 µg g^{-1} (Reunanen and Saris, 2003) and 1 ng g^{-1} (Hakovirta et al., 2006); and 9 ng g^{-1} (Hakovirta et al., 2006) in liquid egg. The only solid food product tested to date is sausage, with a detection limit of 0.9 µg g^{-1} (Reunanen and Saris, 2004). This method has a total reaction time of 3 h (Wahlström and Saris, 1999), which is comparable to turbidometric methods. Such methods have also proven to be highly-specific for nisin and its variants, with no cross-reaction reported to subtilin, carnocin, or sakacin A (Wahlström and Saris, 1999). However, this specificity is also a drawback, as it is not suitable for bioassay of other bacteriocins.

1.7.2 Non-bioassay quantitation

Quantitation of nisin in milk using capillary zonal electrophoresis was achieved, and a sensitivity of 10 µg mL^{-1} was reported in an assay time of 45 min (Rossano et al., 1998). This method separates nisin from other milk proteins based on the difference in protein migration rates on the basis of charge when an electrical field is applied (Skoog and Leary, 1992). Quantitation is done by comparison with a standard curve constructed using purified nisin. One drawback to this method is that nisin must first be extracted from milk using acid and organic solvent, which is time-consuming for multiple samples.

Recently, micellar electrokinetic chromatography was used to quantify nisin in a variety of foods (Stanley et al., 2009; Soliman and Donkor, 2010). This method is similar to capillary zonal electrophoresis, but is revised to allow separation of uncharged molecules. In this method, surfactants such as sodium dodecyl sulphate are added to the solution at a concentration in excess of that required for uncharged molecules to form micelles. The uncharged molecules form the interior of the micelle, while sodium dodecyl sulphate interacts at the interface of the micelle and the surrounding solution. The negative charge of the sulphate allows for movement of micelles in an electrical field. Peptides are

separated by differences in polarity, which causes different partitioning behaviour between the micelle interior and the polar solution (Skoog and Leary, 1992). For nisin, detection limits were higher than those of other methods, with reported limits stated as 0.4 – 0.8 µg mL^{-1} in milk and yogurt products, 0.3 µg mL^{-1} in salad dressings, 0.3 µg mL^{-1} in beer and wine, 0.4 µg mL^{-1} in canned tomatoes, and 0.8 µg mL^{-1} in processed cheese (Soliman and Donkor, 2010). Although the detection limits were higher, the assay time was only 6 min for all products, and a small sample size of 300 µL is required, whereas capillary zone electrophoresis requires extraction of a much larger sample (Rossano et al., 1998; Soliman and Donkor, 2010).

Traditional liquid chromatography/mass spectrometry has also been used to separate and identify bacteriocins such as lacticin 481, mundticin KS, and nisins A, Q, and Z in culture medium (Zendo et al., 2008), but this method was not designed for quantitation, though quantitation may be possible. Similarly, matrix-assisted laser desorption ionization-time of flight coupled to mass spectrometry has also been used for only detection of bacteriocins in culture supernatant (Rose et al., 1999) and bacteriocin-producing cells (Hindré et al., 2003).

1.7.3 Immunochemical quantitation

Immunochemical techniques have been attempted to develop detection methods with increased standardization, immediacy, and improved specificity and sensitivity. Immunochemical methods use the highly-specific interaction between an antibody, either polyclonal or monoclonal, and its corresponding antigen as the basis for detection. Antigens can be classified as either immunogens or haptens. Immunogens are substances that are able to raise an immune response, while haptens are antigens that are unable to do so because of small size or close relationship to self-antigens (Goldsby et al., 2000; Hannigan et al., 2009).

Although proteins are strong immunogens (Lewis, 2001), bacteriocins are considered to be poor immunogens, likely due to their small size. Immunogens usually have one epitope per 5 to 10 kDa of mass (Lewis, 2001), and as most

bacteriocins are between 20 – 60 amino acids in length (Diep and Nes, 2002), it is likely that their size is not sufficient to elicit an immune response. Neither mice (Bhunia et al., 1990; Daoudi et al., 2001; Ingham et al., 2003) nor rabbits (Bhunia et al., 1990) produced antibodies to bacteriocins alone. Antibodies to haptens can be raised by conjugating to a large carrier immunogenic protein such as bovine serum albumin (BSA), cholera toxin (CT), or keyhole limpet haemocyanin (KLH). Antibodies can then be raised to epitopes on the carrier protein, the hapten, and new epitopes formed by combination of the two. Using bacteriocin antigens coupled to larger carrier proteins, antibodies to divercin V41; enterocins A, B, and P; lacticin RM; nisins A and Z; pediocins PA-1/AcH and RS2; and propionicin PLG-1 have been raised. The type of antibody, sensitivity, and significant cross reactions of various immunoassays are detailed in Table 1-2.

Most immunochemical quantitation methods incorporate antibodies in the form of enzyme-linked immunosorbent assays (ELISA). Most ELISAs reported the use of polyclonal antibodies, with only three reports of monoclonal antibodies produced against nisin A, nisin Z, and pediocin RS-2 (Bhunia, 1994; Suárez et al., 1996b; Daoudi et al., 2001). All monoclonal antibodies reported minimal cross-reaction with related bacteriocins, whereas several of the polyclonal antibody-based assays reported significant cross-reaction with multiple bacteriocins (Martínez et al., 2000; Leversee and Glatz, 2001; Richard et al., 2004). Sensitivity in aqueous diluents ranged from less than 10 ng mL^{-1} (Suárez et al., 1996a; Bouksaim et al., 1998; Bouksaim et al., 1999) to 10 µg mL^{-1} (Bhunia, 1994), but most methods detected between 500 and 1000 ng mL^{-1} (Table 1-2).

Usin bacteriocin-specific antibodies, several methods allowed successful quantitation in food products, including processed cheese (Suárez et al., 1996a), milk, and whey (Bouksaim et al., 1999; Daoudi et al., 2001). Suárez et al. (1996a) used nisin-specific monoclonal antibodies in an ELISA to quantify nisin A in processed cheese spread spiked with known amounts of purified nisin A. However, samples required acid extraction prior to quantitation, and although recovery of added nisin was greater than 86 % for all samples in the range of 250

to 5000 ng g^{-1}, there was significant variation (up to 17 %) between assays. Nisin quantitation in spiked samples of milk and whey using nisin-specific antibodies in ELISA also required acid extraction to remove nisin from other constituents before assay (Bouksaim et al., 1999; Daoudi et al., 2001). More than 90 % of added nisin was recovered from milk and whey samples in one assay (Bouksaim et al., 1999).

Instead of the traditional batch ELISA, a variation using a flow-injection system with immobilized monoclonal antibodies on chromatographic beads in a column has been developed for the quantitation of nisin (Nandakumar et al., 2000). In this procedure, there is a continuous flow of reagents over the

Table 1-2 Antibody-based methods for detection and/or quantitation of bacteriocins

Bacteriocin	Carrier	Antibody (type)	Detection limit (ng mL^{-1})	Significant cross-reactivity		Reference
				+	-	
Divercin V41 (synthetic C-terminus, residues18-43)	KLH	Polyclonal (rabbit)	ns	Piscicolin V1b, enterocin P	Bavaricin A, curvacin A, enterocins A and B, mesentericin Y105, nisin Z, sakacin A	Richard et al, (2004)
Enterocin A (synthetic N terminus PH4 (residues 1-14), synthetic C terminus PH5 (residues 37-47)	KLH	Polyclonal (rabbit)	PH4 and PH5: 90	Both: enterocins B and P PH4: sakacin A, pediocin PA-1	Enterocins L50A, L50B, AS-48; lactocin S, nisin A, pediocin A, sakacin P	Martínez et al. (2000)
Enterocin B	BSA	Polyclonal (rabbit)	400-1600 (NCI-ELISA)	nt	Enterocin A	Rose et al. (2001)
Enterocin P (synthetic N-terminal fragments P1 (residues 1-7) and P2 (residues 1-11), synthetic C-terminus P3 (residues 34-42)	KLH	Polyclonal (rabbit)	P1: unable to detect P2: 50 (buffer); 250 (MRS) P3: 10 (buffer); 100 (MRS)	P2: sakacin A	Enterocin A, pediocin PA-1, sakacins A and P	Gutierrez et al. (2004)
Lacticin RM (synthetic internal fragment, residues 75-92)	KLH	Polyclonal (rabbit)	160 (immune-blot) 180 (NCI-ELISA)	None	Nisin A, pediocin A	Keren et al. (2004)
Nisin A	Egg albumen	Polyclonal (sheep)	1.9×10^{-1} iu mL^{-1} (buffer) 231[1] (9250 iu mL^{-1})	Subtilin	Gallidermin, lacticins B and F, pediocin A, Pep5, sakacin A	Falahee et al. (1990), Falahee and Adams (1992)

[1]quantity calculated and reported in Bouksaim et al. (1998), [2] quantity converted using molecular weight reported by Anonymous (2009). Abbreviations: AU-arbitrary units; BSA-bovine serum albumin; CD-competitive direct; CI-competitive indirect; C-competitive; CT-cholera toxin; ELISA-enzyme linked immunosorbent assay; iu-international units; KLH-keyhole limpet haemocyanin; NCI-non-competitive indirect; ns-not stated; nt-not tested; PBS-phosphate-buffered saline; PAG-polyacrylamide

Table 1-2 –continued- Antibody-based methods for detection and/or quantitation of bacteriocins

Bacteriocin	Carrier	Antibody (type)	Detection limit (ng mL^{-1})	Significant cross-reactivity		Reference
				+	-	
Nisin A	KLH	Polyclonal (mouse)	1000 (serum, CD-ELISA) 5-10 (MRS, CD-ELISA)	Nisin Z	Lactocin S, pediocins L50 and PA-1	Suárez et al. (1996a)
Nisin A	CT	Polyclonal (mouse)	1000-5000 (serum, CI-ELISA)	nt	nt	Suárez et al. (1996a)
Nisin A	KLH	Monoclonal (mouse)	10 (CD-ELISA) 250-5000 (per g in processed cheese)	Nisin Z	Lactocin S, pediocins L50 and PA-1	Suárez et al. (1996b)
Nisin A	ns	Polyclonal (rabbit)	65 (NCI-ELISA)	nt	nt	Leung et al. (2002)
Nisin A	None	Polyclonal (rabbit)	Unable to detect	nt	nt	Stringer et al, (1995)
Nisin A	ns	Monoclonal (ns)	10 000-302 000 (flow immunoassay)[2]	nt	nt	Nandakumar et al. (2000)
Nisin Z	KLH BSA	Monoclonal (mouse)	78 (PBS, C-ELISA) 87±5.4 (supernatant, C-ELISA) 106±8.5 (milk, D-ELISA) 90.5±3 (whey, C-ELISA)	nt	nt	Daoudi et al. (2001)
Nisin Z	KLH	Polyclonal (rabbit)	3 (buffer) 155 (milk) (both immunoblot) 0.75 (buffer, ELISA) 1.7 (whey and milk, ELISA) 3.5 (MRS, ELISA)	nt	nt	Bouksaim et al. (1998), Bouksaim et al. (1999)

[1]quantity calculated and reported in Bouksaim et al. (1998), [2] quantity converted using molecular weight reported by Anonymous (2009). Abbreviations: AU-arbitrary units; BSA-bovine serum albumin; CD-competitive direct; CI-competitive indirect; C-competitive; CT-cholera toxin; ELISA-enzyme linked immunosorbent assay; iu-international units; KLH-keyhole limpet haemocyanin; NCI-non-competitive indirect; ns-not stated; nt-not tested; PBS- phosphate-buffered saline; PAG-polyacrylamide

Table 1-2 –continued- Antibody-based methods for detection and/or quantitation of bacteriocins

Bacteriocin	Carrier	Antibody (type)	Detection limit (ng mL^{-1})	Significant cross-reactivity		Reference
				+	-	
Pediocin AcH	BSA	Polyclonal (mouse and rabbit)	Unable to detect	nt	nt	Bhunia et al. (1990)
Pediocin PA-1 (synthetic N-terminus PH1 (residues 1-9)	KLH	Polyclonal (mouse)	Unable to detect whole molecule	nt	nt	Martínez et al. (1997)
Pediocin PA-1 (synthetic N-terminus PH1 (residues 1-9)	KLH	Polyclonal (rabbit)	50 (buffer) 500 (MRS) 10-250 (PBS, CI-ELISA) 10 (MRS, CI-ELISA)	None	Enterocins A, B, AS-48, L50A, L50B; lactocin S, nisin A, pediocin A, sakacin A	Martínez et al. (1999)
Pediocin PA-1 (synthetic C-terminus PH2 (residues 34-44)	KLH	Polyclonal (rabbit)	2500 (immunoblot) 1000 (MRS), 25 (buffer) (NCI-ELISA) 25 (MRS, CD-ELISA)	None	Enterocins A, B, L50A, L50B, AS-48; lactocin S, nisin A; pediocin A	Martínez et al. (1998)
Pediocin RS2	PAG	Monoclonal (mouse)	10 000[1] (32 000 AU mL^{-1})	None	Leuconocin LCM1, nisin A, pediocin A, sakacin A	Bhunia (1994)
Propionicin PLG-1	None	Polyclonal (rabbit)	ns	52 *Propionibacterium* strains	104 *Propionibacterium* strains	Leversee and Glatz (2001)
Pediocin PA-1 (synthetic N-terminus PH1 (residues 1-9)	KLH	Polyclonal (mouse)	Unable to detect whole molecule	nt	nt	Martínez et al. (1997)
Pediocin PA-1 (synthetic N-terminus PH1 (residues 1-9)	KLH	Polyclonal (rabbit)	50 (buffer) 500 (MRS) 10-250 (PBS, CI-ELISA) 10 (MRS, CI-ELISA)	None	Enterocins A, B, AS-48, L50A, L50B; lactocin S, nisin A, pediocin A, sakacin A	Martínez et al. (1999)

[1]quantity calculated and reported in Bouksaim et al. (1998), [2] quantity converted using molecular weight reported by Anonymous (2009). Abbreviations: AU-arbitrary units; BSA-bovine serum albumin; CD-competitive direct; CI-competitive indirect; C-competitive; CT-cholera toxin; ELISA-enzyme linked immunosorbent assay; iu-international units; KLH-keyhole limpet haemocyanin; NCI-non-competitive indirect; ns-not stated; nt-not tested; PBS- phosphate-buffered saline; PAG-polyacrylamide

antibodies, instead of a batch assay. This method reported a much lower sensitivity than conventional ELISAs, but had a shorter assay time, ranging from 15 – 22 min (Nandakumar et al., 2000). Commercially-produced cheese samples containing nisin were analyzed using this method. This method suffers from the same restriction as conventional ELISAs, because an acid extraction pre-treatment is required.

Although the potential of bacteriocins in controlling foodborne spoilage and pathogenic microorganisms has been recognized since the mid-20th century, their full power has not been harnessed. More preparations have received regulatory approval as food additives, and as this practice becomes more commonplace, methods of quantifying bacteriocins will become more important to substantiate concentrations of semi-purified or purified preparations added. For example, according to the FAO/WHO Food Additive Compendium, nisin preparations must have a concentration of at least 900 units per mg of preparation when assayed using a validated method (Joint FAO/WHO Expert Committee on Food Additives, 2007). Limitations of traditional bioassays have been identified, and many researchers have addressed such limitations by using alternative methods.

1.8 Bacteriocins as agents of food protection

Lactic acid bacteria are indigenous to food, and products of their growth, including bacteriocins, have long been recognized as being important for food preservation and protection. Although many types of foods inherently contain LAB capable of bacteriocin production, other foods may require addition of bacteriocins. Bacteriocins can be added to foods either indirectly, by adding a food-grade bacteriocin-producing LAB strain, or directly, by adding a purified or semi-purified bacteriocin preparation. There are only a few bacteriocin preparations approved for use, the most well-known of these is nisin.

Nisin preparations are approved for use in foods in more than 40 countries, usually in products such as processed cheese, pasteurized spreads, pasteurized milk, canned vegetables, and other dairy products (Vandenbergh, 1993; Twomey

et al., 2002). In the United States, it is classified as GRAS, or Generally Recognized as Safe, and is allowed at a concentration of 250 parts per million in pasteurized cheese spreads to inhibit the outgrowth of *Cl. botulinum* spores (FDA, 2006). Nisaplin®, a preparation of 2.5 % (w/w) nisin A in NaCl and dried milk, is the most well-known nisin product. It was first produced by Aplin & Barret in 1962, and is currently manufactured by Danisco (Copenhagen, DK). Nisin preparations are also produced by other companies in China, Ireland, India, and the Netherlands (Jones et al., 2005). Other bacteriocin preparations that have received regulatory approval are ALTA™ 2341 (Quest International, Sarasota, FL; now Kerry Bio-Sciences, Kerry, IL), which contains pediocin PA-1, and Micocin® (Griffith Laboratories, Mississauga, ON), which contains carnobacteriocins (Health Canada, 2010).

The deliberate application of bacteriocins for inhibition of spoilage and pathogenic bacteria in food began as early as 1951, with the use of nisin to control gas production by *Cl. tyrobutyricum* in cheese (Hirsch et al., 1951). Since then, bacteriocins have shown effectiveness in many foods against a variety of bacteria. Bacteriocins have showed promise when applied in dairy products (Gálvez et al., 2008; Sobrino-López and Martín-Belloso, 2008), and fresh meats (Castellano et al., 2008; Zhang et al., 2010).

Emphasis in the use of bacteriocins to control foodborne pathogens in ready-to-eat and minimally-processed foods is evident from the literature. Nisin and class IIa bacteriocins have been tested in cooked and ready-to-eat meat products, and have shown potent activity against *L. monocytogenes* (Budde et al., 2003; Azuma et al., 2007; Jofré et al., 2008). *Lb. sakei* 706, which produces sakacin A, was shown to inhibit *L. monocytogenes* in a vacuum-packaged beef bologna product (Kaban et al., 2010). *B. cereus* was inhibited by the cyclic bacteriocin enterocin AS-48 in ready-to-eat vegetable soups and purees (Grande et al., 2007) and on fresh produce (Cobo Molinos et al., 2008), while *L. monocytogenes* was also controlled on fresh produce (Cobo Molinos et al., 2005). Partially-purified bacteriocins produced by *Lb. casei* and *Lb. acidophilus* were

shown to inhibit both *L. monocytogenes* and *Staphylococcus aureus* in a vegetable dish (pulav) (Jamuna et al., 2005).

The spectrum of activity of bacteriocins, combined with the variety of foods in which they retain activity, makes employing bacteriocins in minimally-processed foods an attractive option for food processors. Consumers' increasing awareness of food safety, as well as concern over food additives, provide opportunities for naturally-produced biopreservatives such as bacteriocins. To date, only nisin has been extensively used in food products; thus, there is ample room for new bacteriocins to be used for maximal food protection.

1.9 Research objectives

Bacteriocins have the potential to be attractive alternatives to "chemical" food additives, which are perceived to be less natural and therefore undesirable to many consumers. The rise in popularity of minimally-processed and organic foods, which minimize or prohibit using additives such as sodium nitrite, has led to interest in bacteriocins to replace these additives.

However, there are several obstacles that must be addressed prior to their widespread use. First, in order for widespread acceptance of bacteriocins in foods, methods of detection and quantitation will be important. If bacteriocins are to be treated as food additives, regulatory agencies may require validated quantitation methods for future food additive petitions, while the food industry requires quantitation methods for economic reasons; namely, by avoiding excessive application of expensive purified or semi-purified bacteriocins to food products. Methods currently being used have many drawbacks, and so specific, rapid methods of quantitation are required.

Second, bacteriocins must be proven to be effective replacements for currently used food additives. One such additive is sodium nitrite, which is used to prevent spore outgrowth and subsequent toxin production by *Cl. botulinum* in products such as vacuum-packaged meat products. Removal of sodium nitrite

leaves such products vulnerable, and so bacteriocins must be able to effectively prevent spore outgrowth of organisms such as *Cl. botulinum*.

The objectives of this study are as follows:

1. Develop a novel method to detect and quantify bacteriocins using antibodies produced by phage display.
2. Determine the efficacy of a class IIb bacteriocin, brochocin-C, to inhibit *Cl. botulinum* spore germination in a model meat system and in a meat system.
3. Determine if brochocin-C is as effective as sodium nitrite or a natural cure at inhibition of *Cl. botulinum* in a meat product.

1.10 References

Ahn, C. and Stiles, M.E. (1990) Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl Environ Microbiol 56, 2503-2510.

Anderssen, E.L., Diep, D.B., Nes, I.F., Eijsink, V.G.H. and Nissen-Meyer, J. (1998) Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. Appl Environ Microbiol 64, 2269-2272.

Anonymous. (2009) Nisin. In: Combined Compendium of Food Additive Specifications, Vol. 4. Joint FAO/WHO Expert Committee on Food Additives, Rome, IT.

Axelsson, L. (2004) Lactic acid bacteria: Classification and physiology. In: S. Salminen, A. von Wright and A. Ouwehand (Eds), Lactic Acid Bacteria: Microbiological and Functional Aspects. Marcel Dekker, Inc., New York, p. 1-66.

Aymerich, T., Holo, H., Håvarstein, L., Hugas, M., Garriga, M. and Nes, I. (1996) Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl Environ Microbiol 62, 1676-1682.

Azuma, T., Bagenda, D.K., Yamamoto, T., Kawai, Y. and Yamazaki, K. (2007) Inhibition of *Listeria monocytogenes* by freeze-dried piscicocin CS526 fermentate in food. Lett Appl Microbiol 44, 138-144.

Berridge, N.J. and Barrett, J. (1952) A rapid method for the turbidimetric assay of antibiotics. J Gen Microbiol 6, 14-20.

Bhunia, A.K. (1994) Monoclonal antibody-based enzyme immunoassay for pediocins of *Pediococcus acidilactici*. Appl Environ Microbiol 60, 2692-2696.

Bhunia, A.K., Johnson, M.C., Bibek, R. and Belden, E.L. (1990) Antigenic property of pediocin AcH produced by *Pediococcus acidilactici* H. J Appl Bacteriol 69, 211-215.

Bhunia, A.K., Johnson, M.C. and Ray, B. (1987) Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J Ind Microbiol Biot 2, 319-322.

Bhunia, A.K. and Johnson, M.G. (1992) A modified method to directly detect in SDS-PAGE the bacteriocin of *Pediococcus acidilactici*. Lett Appl Microbiol 15, 5-7.

Biswas, S.R., Ray, P., Johnson, M.C. and Ray, B. (1991) Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. Appl Environ Microbiol 57, 1265-1267.

Bouksaim, M., Fliss, I., Meghrous, J., Simard, R. and Lacroix, C. (1998) Immunodot detection of nisin Z in milk and whey using enhanced chemiluminescence. J Appl Microbiol 84, 176-184.

Bouksaim, M., Lacroix, C., Bazin, R. and Simard, R.E. (1999) Production and utilization of polyclonal antibodies against nisin in an ELISA and for immuno-location of nisin in producing and sensitive strains. J Appl Microbiol 87, 500-510.

Breukink, E., van Heusden, H.E., Vollmerhaus, P.J., Swiezewska, E., Brunner, L., Walker, S., Heck, A.J.R. and de Kruijff, B. (2003) Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. J Biol Chem 278, 19898-19903.

Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O.P., Sahl, H.G. and de Kruijff, B. (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science 286, 2361-2364.

Brewer, M.S. and Presat, C.J. (2002) Consumer attitudes toward food safety issues. J Food Safety 22, 67-83.

Brewer, M.S. and Rojas, M. (2008) Consumer attitudes towards issues in food safety. J Food Safety 28, 1-22.

Budde, B.B., Hornbæk, T., Jacobsen, T., Barkholt, V. and Koch, A.G. (2003) *Leuconostoc carnosum* 4010 has the potential for use as a protective culture for vacuum-packed meats: culture isolation, bacteriocin identification, and meat application experiments. Int J Food Microbiol 83, 171-184.

Cabo, M.L., Murado, M.A., Gonzalez, M.P. and Pastoriza, L. (1999) A method for bacteriocin quantification. J Appl Microbiol 87, 907-914.

Calvez, S., Prévost, H. and Drider, D. (2008) Identification of a new molecular target of class IIa bacteriocins in *Listeria monocytogenes* EGDe. Folia Microbiol 53, 417-422.

Calvez, S., Rincé, A., Auffray, Y., Prévost, H. and Drider, D. (2007) Identification of new genes associated with intermediate resistance of *Enterococcus faecalis* to divercin V41, a pediocin-like bacteriocin. Microbiology 153, 1609-1618.

Carr, F.J., Chill, D. and Maida, N. (2002) The lactic acid bacteria: a literature survey. Crit Rev Microbiol 28, 281-370.

Castellano, P., Belfiore, C., Fadda, S. and Vignolo, G. (2008) A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. Meat Sci 79, 483-499.

Castellano, P., Raya, R. and Vignolo, G. (2003) Mode of action of lactocin 705, a two-component bacteriocin from *Lactobacillus casei* CRL705. Int J Food Microbiol 85, 35-43.

CFIA. (2010) Causes of Foodborne Illness. http://www.inspection.gc.ca/english/fssa/concen/causee.shtml. Canadian Food Inspection Agency. Last updated 8 June 2010.

Cintas, L., Casaus, P., Håvarstein, L., Hernández, P. and Nes, I. (1997) Biochemical and genetic characterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl Environ Microbiol 63, 4321-4330.

Claesson, M.J., Li, Y., Leahy, S., Canchaya, C., van Pijkeren, J.P., Cerdeño-Tárraga, A.M., Parkhill, J., Flynn, S., O'Sullivan, G.C., Collins, J.K., Higgins, D., Shanahan, F., Fitzgerald, G.F., van Sinderen, D. and O'Toole, P.W. (2006) Multireplicon genome architecture of *Lactobacillussalivarius*. P Natl Acad Sci USA 103, 6718-6723.

Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 71, 1-20.

Cobo Molinos, A., Abriouel, H., Ben Omar, N., Valdivia, E., Lucas Lopez, R., Maqueda, M., Canamero, M.M. and Galvez, A. (2005) Effect of immersion solutions containing enterocin AS-48 on *Listeria monocytogenes* in vegetable foods. Appl Environ Microbiol 71, 7781-7787.

Cobo Molinos, A., Abriouel, H., Lucas López, R., Ben Omar, N., Valdivia, E. and Gálvez, A. (2008) Inhibition of *Bacillus cereus* and *Bacillus weihenstephanensis* in raw vegetables by application of washing solutions containing enterocin AS-48 alone and in combination with other antimicrobials. Food Microbiol 25, 762-770.

Collins, Y.F., McSweeney, P.L.H. and Wilkinson, M.G. (2003) Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int Dairy J 13, 841-866.

Condon, S. (1987) Responses of lactic acid bacteria to oxygen. FEMS Microbiol Lett 46, 269-280.

Cotter, P.D., Hill, C. and Ross, R.P. (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3, 777-788.

Cox, C.R., Coburn, P.S. and Gilmore, M.S. (2005) Enterococcal cytolysin: a novel two component peptide system that serves as a bacteria defense against eukaryotic and prokaryotic cells. Curr Protein Pept Sci 6, 77-84.

Cuozzo, S.A., Castellano, P., Sesma, F.J.M., Vignolo, G.M. and Raya, R.R. (2003) Differential roles of the two-component peptides of lactocin 705 in antimicrobial activity. Curr Microbiol 46, 0180-0183.

Cuozzo, S.A., Sesma, F., Palacios, J.M., de Ruiz Holgado, A.P. and Raya, R.R. (2000) Identification and nucleotide sequence of genes involved in the synthesis of lactocin 705, a two-peptide bacteriocin from *Lactobacillus casei* CRL 705. FEMS Microbiol Lett 185, 157-161.

Cutler, R.R., Wilson, P. and Clarke, F.V. (1989) Evaluation of a radiometric method for studying bacterial activity in the presence of antimicrobial agents. J Appl Microbiol 66, 515-521.

Daeschel, M.A. (1992) Procedures to detect antimicrobial activities of microorganisms. In: B. Ray and M. Daeschel (Eds), Food Biopreservatives of Microbial Origin. CRC Press, Ann Arbor, p. 57-80.

Daoudi, L., Turcotte, C., Lacroix, C. and Fliss, I. (2001) Production and characterization of anti-nisin Z monoclonal antibodies: suitability for distinguishing active from inactive forms through a competitive enzyme immunoassay. Appl Microbiol Biotechnol 56, 114-119.

de Vuyst, L. and Vandamme, E.J. (1994) Antimicrobial potential of lactic acid bacteria. In: L. de Vuyst and E.J. Vandamme (Eds), Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics, and Applications. Chapman & Hall, New York, p. 91-142.

Delgado, A., Brito, D., Fevereiro, P., Tenreiro, R. and Peres, C. (2005) Bioactivity quantification of crude bacteriocin solutions. J Microbiol Meth 62, 121-124.

Desbois, A. and Smith, V. (2010) Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biot 85, 1629-1642.

Diep, D.B., Axelsson, L., Grefsli, C. and Nes, I.F. (2000) The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three-component regulatory system. Microbiology 146, 2155-2160.

Diep, D.B. and Nes, I.F. (2002) Ribosomally synthesized antibacterial peptides in gram positive bacteria. Curr Drug Targets 3, 107-122.

Diep, D.B., Skaugen, M., Salehian, Z., Holo, H. and Nes, I.F. (2007) Common mechanisms of target cell recognition and immunity for class II bacteriocins. P Natl Acad Sci USA 104, 2384-2389.

Drider, D., Fimland, G., Hechard, Y., McMullen, L. and Prevost, H. (2006) The continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 70, 564 - 582.

Eijsink, V., Sheie, M., Middelhoven, P., Brurberg, M. and Nes, I. (1998) Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl Environ Microbiol 64, 3275 - 3281.

Eijsink, V.G.H., Axelsson, L., Diep, D.B., HÃ¥varstein, L.S., Holo, H. and Nes, I.F. (2002) Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Anton van Leeuw Int J G 81, 639-654.

Engelke, G., Gutowski-Eckel, Z., Kiesau, P., Siegers, K., Hammelmann, M. and Entian, K.D. (1994) Regulation of nisin biosynthesis and immunity in *Lactococcus lactis* 6F3. Appl Environ Microbiol 60, 814-825.

Ennahar, S., Sashihara, T., Sonomoto, K. and Ishizaki, A. (2000) Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol Rev 24, 85-106.

Falahee, M.B. and Adams, M.R. (1992) Cross-reactivity of bacteriocins from lactic-acid bacteria and lantibiotics in a nisin bioassay and ELISA. Lett Appl Microbiol 15, 214-216.

Falahee, M.B., Adams, M.R., Dale, J.W. and Morris, B.A. (1990) An enzyme immunoassay for nisin. Int J Food Sci Tech 25, 590-595.

FDA. (2006) Listing of specific substances affirmed as GRAS 21CFR184.1538. http://www.grokfood.com/regulations/184.1538.htm. Last updated 1 April 2006.

Feng, G., Guron, G.K.P., Churey, J.J. and Worobo, R.W. (2009) Characterization of mundticin L, a class IIa anti-*Listeria* bacteriocin from *Enterococcus mundtii* CUGF08. Appl Environ Microbiol 75, 5708-5713.

Fimland, G., Johnsen, L., Axelsson, L., Brurberg, M., Nes, I., Eijsink, V. and Nissen-Meyer, J. (2000) A C-terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. J Bacteriol 182, 2643 - 2648.

Finnegan, M., Linley, E., Denyer, S.P., McDonnell, G., Simons, C. and Maillard, J.-Y. (2010) Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms. J Antimicrob Chemoth 65, 2108-2115.

Flores, S.H., de Mattos Braga, A.L. and Alegre, R.M. (2003) A modified method for the turbidimetric assay of nisin. Braz Arch Biol Techn 46, 479-481.

Fowler, G.G., Jarvis, B. and Tramer, J. (1975) The assay of nisin in foods. In: R.G. Board and D.W. Lovelock (Eds), Some Methods for Microbiological Assay, Vol. 8, Society for Applied Bacteriology Technical Series. Academic Press, New York, p. 91-105.

Franke, C.M., Leenhouts, K.J., Haandrikman, A.J., Kok, J., Venema, G. and Venema, K. (1996) Topology of LcnD, a protein implicated in the transport of bacteriocins from *Lactococcus lactis*. J Bacteriol 178, 1766-1769.

Franz, C.M.A.P., van Belkum, M.J., Holzapfel, W.H., Abriouel, H. and Gálvez, A. (2007) Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol Rev 31, 293-310.

Franz, C.M.A.P., Grube, A., Herrmann, A., Abriouel, H., Starke, J., Lombardi, A., Tauscher, B. and Holzapfel, W.H. (2002) Biochemical and genetic characterization of the two-peptide bacteriocin enterocin 1071 produced by *Enterococcus faecalis* FAIR-E 309. Appl Environ Microbiol 68, 2550-2554.

Fremaux, C., Ahn, C. and Klaenhammer, T.R. (1993) Molecular analysis of the lactacin F operon. Appl Environ Microbiol 59, 3906-3915.

Fremaux, C., Yann, H. and Cenatiempo, Y. (1995) Mesentericin Y105 gene clusters in *Leuconostoc mesenteroides* Y105. Microbiology 141, 1637-1645.

Gajic, O., Buist, G., Kojic, M., Topisirovic, L., Kuipers, O.P. and Kok, J. (2003) Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal multidrug resistance proteins. J Biol Chem 278, 34291-34298.

Gálvez, A., López, R.L., Abriouel, H., Valdivia, E. and Ben Omar, N. (2008) Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Crit Rev Biotechnol 28, 125-152.

Gänzle, M.G. and Vogel, R.F. (2003) Studies on the mode of action of reutericyclin. Appl Environ Microbiol 69, 1305-1307.

Gänzle, M.G., Höltzel, A., Walter, J., Jung, G. and Hammes, W.P. (2000) Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl Environ Microbiol 66, 4325-4333.

Gao, Y., van Belkum, M.J. and Stiles, M.E. (1999) The outer membrane of gram-negative bacteria inhibits antibacterial activity of brochocin-C. Appl Environ Microbiol 65, 4329-4333.

Garneau, S., Martin, N.I. and Vederas, J.C. (2002) Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie 84, 577-592.

Giraffa, G., Neviani, E. and Veneroni, A. (1990) Use of conductance to detect bacteriocin activity. J Food Prot 53, 772-776.

Goldsby, R.A., Kindt, T.J. and Osborne, B.A. (2000) Kuby Immunology. W.H. Freeman and Company, New York.

Grande, M.J., Abriouel, H., López, R.L., Valdivia, E., Omar, N.B., Martínez-Cañamero, M. and Gálvez, A. (2007) Efficacy of enterocin AS-48 against bacilli in ready-to-eat vegetable soups and purees. J Food Prot 70, 2339-2345.

Gursky, L.J. (2004) Bacteriocin production by *Carnobacterium maltaromaticum* UAL26. PhD thesis, Department of Agricultural, Food, and Nutritional Science. University of Alberta, Edmonton, AB, p. 205.

Gut, I.M., Prouty, A.M., Ballard, J.D., van der Donk, W.A. and Blanke, S.R. (2008) Inhibition of *Bacillus anthracis* spore outgrowth by nisin. Antimicrob Agents Chemother 52, 4281-4288.

Gutierrez, J., Criado, R., Citti, R., Martin, M., Herranz, C., Fernandez, M.F., Cintas, L.M. and Hernandez, P.E. (2004) Performance and applications of polyclonal antipeptide antibodies specific for the enterococcal bacteriocin enterocin P. J Agric Food Chem 52, 2247-2255.

Hakovirta, J., Reunanen, J. and Saris, P.E.J. (2006) Bioassay for nisin in milk, processed cheese, salad dressings, canned tomatoes, and liquid egg products. Appl Environ Microbiol 72, 1001-1005.

Hancock, R.E.W. and Chapple, D.S. (1999) Peptide antibiotics. Antimicrob Agents Chemother 43, 1317-1323.

Hannigan, B.M., Moore, C.B.T. and Quinn, D.G. (2009) Immunology. Scion Publishing, Banbury, UK.

Hastings, J.W., Sailer, M., Johnson, K., Roy, K.L., Vederas, J.C. and Stiles, M.E. (1991) Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J Bacteriol 173, 7491-7500.

Håvarstein, L.S., Diep, D.B. and Nes, I.F. (1995) A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol Microbiol 16, 229-240.

Håvarstein, L.S., Holo, H. and Nes, I.F. (1994) The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by gram-positive bacteria. Microbiology 140, 2383-2389.

Health Canada (2010) Canada Gazette Part II. Food and Drug Regulations 144, 2304-2307.

Héchard, Y., Pelletier, C., Cenatiempo, Y. and Frère, J. (2001) Analysis of 54-sigma-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EIIMan) is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiology 147, 1575-1580.

Héchard, Y. and Sahl, H.-G. (2002) Mode of action of modified and unmodified bacteriocins from gram-positive bacteria. Biochimie 84, 545-557.

Heng, N.C.K., Wescombe, P.A., Burton, J.P., Jack, R.W. and Tagg, J.R. (2007) The diversity of bacteriocins in gram-positive bacteria. In: M.A. Riley and M.A. Chavan (Eds), Bacteriocins: Ecology and Evolution. Springer-Verlag, New York, p. 45-92.

Herranz, C. and Driessen, A.J.M. (2005) *Sec*-mediated secretion of bacteriocin enterocin P by *Lactococcus lactis*. Appl Environ Microbiol 71, 1959-1963.

Hickey, R.M., Twomey, D.P., Ross, R.P. and Hill, C. (2003) Production of enterolysin A by a raw milk enterococcal isolate exhibiting multiple virulence factors. Microbiology 149, 655-664.

Hindré, T., Didelot, S., Le Pennec, J.-P., Haras, D., Dufour, A. and Vallée-Réhel, K. (2003) Bacteriocin detection from whole bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 69, 1051-1058.

Hirsch, A. (1950) The assay of the antibiotic nisin. J Gen Microbiol 4, 70-83.

Hirsch, A., Grinsted, E., Chapman, H.R. and Mattick, A.T.R. (1951) A note on the inhibition of an anerobic spore former in Swiss-type cheese by a nisin-producing *Streptococcus*. J Dairy Res 18, 205-206.

Holck, A., Axelsson, L., Birkeland, S.-E., Aukrust, T. and Blom, H. (1992) Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. J Gen Microbiol 138, 2715-2720.

Holo, H., Nes, I., Hildeng, H.H., Fimland, G. and Nissen-Meyer, J. (2002) Unmodified peptide-bacteriocins (Class II) produced by lactic acid bacteria. In: C.J. Dutton, M.A. Haxell, H.A. McArthur, and R.G. Wax (Eds), Peptide Antibiotics: Discovery Modes of Action and Applications. Marcel Dekker, New York, p. 61-87.

Holo, H., Nilssen, O. and Nes, I.F. (1991) Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J Bacteriol 173, 3879-3887.

Horn, N., Swindell, S., Dodd, H. and Gasson, M. (1991) Nisin biosynthesis genes are encoded by a novel conjugative transposon. Mol Gen Genet 228, 129-135.

Hu, C.-B., Malaphan, W., Zendo, T., Nakayama, J. and Sonomoto, K. (2010) Enterocin X, a novel two-peptide bacteriocin from *Enterococcus faecium* KU-B5, has an antibacterial spectrum entirely different from those of its component peptides. Appl Environ Microbiol 76, 4542-4545.

Immonen, N. and Karp, M. (2007) Bioluminescence-based bioassays for rapid detection of nisin in food. Biosens Bioelectron 22, 1982-1987.

Ingham, A., Ford, M., Moore, R.J. and Tizard, M. (2003) The bacteriocin piscicolin 126 retains antilisterial activity *in vivo*. J Antimicrob Chemother 51, 1365-1371.

Ito, Y., Kawai, Y., Arakawa, K., Honme, Y., Sasaki, T. and Saito, T. (2009) Conjugative plasmid from *Lactobacillus gasseri* LA39 that carries genes for production of and immunity to the circular bacteriocin gassericin A. Appl Environ Microbiol 75, 6340-6351.

Jack, R., Tagg, J.R. and Ray, B. (1995) Bacteriocins of gram-positive bacteria. Microbiol Rev 59, 171-200.

Jack, R., Wan, J., Gordon, J., Harmark, K., Davidson, B., Hillier, A., Wettenhall, R., Hickey, M. and Coventry, M. (1996) Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl Environ Microbiol 62, 2897-2903.

Jamuna, M., Babusha, S.T. and Jeevaratnam, K. (2005) Inhibitory efficacy of nisin and bacteriocins from *Lactobacillus* isolates against food spoilage and pathogenic organisms in model and food systems. Food Microbiol 22, 449-454.

Jay, J.M. (1982a) Antimicrobial properties of diacetyl. Appl Environ Microbiol 44, 525-532.

Jay, J.M. (1982b) Effect of diacetyl on foodborne microorganisms. J Food Sci 47, 1829-1831.

Joerger, M.C. and Klaenhammer, T.R. (1986) Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J Bacteriol 167, 439-446.

Joerger, M.C. and Klaenhammer, T.R. (1990) Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. J Bacteriol 172, 6339-6347.

Jofré, A., Aymerich, T., Monfort, J. and Garriga, M. (2008) Application of enterocins A and B, sakacin K and nisin to extend the safe shelf-life of pressurized ready-to-eat meat products. Eur Food Res Technol 228, 159-162.

Joint FAO/WHO Expert Committee on Food Additives. (2007) Compendium of food additive specifications/Joint FAO/WHO Expert Committee on Food Additives, 68th Meeting, Vol. 4. Food and Agriculture Organization of the United Nations, Rome, IT.

Jones, E., Salin, V. and Williams, G.W. (2005) Nisin and the market for commercial bacteriocins. Texas Agribusiness Market Research Center, Texas A&M University, College Station, TX, p. 20.

Jung, G. (1991) Nisin and Novel Lantibiotics. In: G. Jung and H.-G. Sahl (Eds), Nisin and Novel Lantibiotics: Proceedings of the First International Workshop on Lantibiotics. Escom Science Publishers, Leiden, p. 1-34.

Kaban, G., Kaya, M. and Lücke, F.-K. (2010) The effect of *Lactobacillus sakei* on the behavior of *Listeria monocytogenes* in sliced bologna-type sausages. J Food Safety 30, 889-901.

Kandler, O. (1983) Carbohydrate metabolism in lactic acid bacteria. Anton van Leeuw Int J G 49, 209-224.

Kemperman, R., Kuipers, A., Karsens, H., Nauta, A., Kuipers, O. and Kok, J. (2003) Identification and characterization of two novel clostridial bacteriocins, circularin A and closticin 574. Appl Environ Microbiol 69, 1589-1597.

Keren, T., Yarmus, M., Halevy, G. and Shapira, R. (2004) Immunodetection of the bacteriocin lacticin RM: analysis of the influence of temperature and Tween 80 on its expression and activity. Appl Environ Microbiol 70, 2098-2104.

Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12, 39-86.

Kleerebezem, M. and Quadri, L. (2001) Peptide pheremone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of multicellular behaviour. Peptides 22, 1579-1596.

Kleerebezem, M., Quadri, L.E.N., Kuipers, O.P. and de Vos, W.M. (1997) Quorum sensing by peptide pheromones and two-component signal-transduction systems in gram-positive bacteria. Mol Microbiol 24, 895-904.

Kleinkauf, H. and von Döhren, H. (1990) Nonribosomal biosynthesis of peptide antibiotics. Eur J Biochem 192, 1-15.

Kuipers, O.P., Beerthuyzen, M.M., de Ruyter, P.G.G.A., Leusink, E.J. and de Vos, W.M. (1995) Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J Biol Chem 270, 27299-27304.

Kuipers, O.P., Beerthuyzen, M.M., Siezen, R.J. and de Vos, W.M. (1993) Characterization of the nisin gene cluster *nis*ABTCIPR of *Lactococcus lactis*: requirement of expression of the *nis*A and *nis*I genes for development of immunity. Eur J Biochem 216, 281-291.

Lanciotti, R., Patrignani, F., Bagnolini, F., Guerzoni, M.E. and Gardini, F. (2003) Evaluation of diacetyl antimicrobial activity against *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*. Food Microbiol 20, 537-543.

Leer, R.J., van der Vossen, J.B.M., van Giezen, M., van Noort, J.M. and Pouwels, P.H. (1995) Genetic analysis of acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus*. Microbiology 141, 1629-1635.

Leung, P.P., Khadre, M., Shellhammer, T.H. and Yousef, A.E. (2002) Immunoassay method for quantitative determination of nisin in solution and on polymeric films. Lett Appl Microbiol 34, 199-204.

Leversee, J.A. and Glatz, B.A. (2001) Detection of the bacteriocin propionicin PLG-1 with polyvalent anti-PLG-1 antiserum. Appl Environ Microbiol 67, 2235-2239.

Lewis, D.H. (2001) Antigens. In: G.C. Howard and D.R. Bethell (Eds), Basic Methods in Antibody Production and Characterization. CRC Press, New York, p. 11-18.

Lewus, C.B. and Montville, T.J. (1991) Detection of bacteriocins produced by lactic acid bacteria. J Microbiol Meth 13, 145-150.

Lozano, J.C.N., Meyer, J.N., Sletten, K., Pelaz, C. and Nes, I.F. (1992) Purification and amino acid sequence of a bacteriocin produced by *Pediococcus acidilactici*. J Gen Microbiol 138, 1985-1990.

Marciset, O., Jeronimus-Stratingh, M.C., Mollet, B. and Poolman, B. (1997) Thermophilin 13, a nontypical antilisterial poration complex bacteriocin, that functions without a receptor. J Biol Chem 272, 14277-14284.

Martínez, J.M., Kok, J., Sanders, J.W. and Hernandez, P.E. (2000) Heterologous coproduction of enterocin A and pediocin PA-1 by *Lactococcus lactis*: detection by specific peptide-directed antibodies. Appl Environ Microbiol 66, 3543-3549.

Martínez, J.M., Martínez, M.I., Herranz, C., Suárez, A., Fernández, M.F., Cintas, L.M., Rodríguez, J.M. and Hernández, P.E. (1999) Antibodies to a synthetic 1-9-N-terminal amino acid fragment of mature pediocin PA-1: sensitivity and specificity for pediocin PA-1 and cross-reactivity against class IIa bacteriocins. Microbiology 145, 2777-2787.

Martínez, J.M., Martínez, M.I., Suárez, A.M., Herranz, C., Casaus, P., Cintas, L.M., Rodríguez, J.M. and Hernández, P.E. (1998) Generation of polyclonal antibodies of predetermined specificity aginst pediocin PA-1. Appl Environ Microbiol 64, 4536-4545.

Martínez, M.I., Rodríguez, J.M., Suárez, A., Azcona, J.I. and Hernández, P.E. (1997) Generation of polyclonal antibodies against a chemically synthesized N-terminal fragment of the bacteriocin pediocin PA-1. Lett Appl Microbiol 24, 488-492.

Mathiesen, G., Huehne, K., Kroeckel, L., Axelsson, L. and Eijsink, V.G.H. (2005) Characterization of a new bacteriocin operon in sakacin P-producing *Lactobacillus sakei*, showing strong translational coupling between the bacteriocin and immunity genes. Appl Environ Microbiol 71, 3565-3574.

McAuliffe, O., Ross, R.P. and Hill, C. (2001) Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol Rev 25, 285-308.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E. and van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. Appl Environ Microbiol 64, 4757-4766.

McCormick, J.K., Worobo, R. and Stiles, M. (1996) Expression of the antimicrobial peptide carnobacteriocin B2 by a signal peptide-dependent general secretory pathway. Appl Environ Microbiol 62, 4095-4099.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. (1999) Food-related illness and death in the United States. Emerg Infect Dis 5, 607-625.

Mierau, I. and Kleerebezem, M. (2005) 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl Microbiol Biot 68, 705-717.

Mitchell, P. (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol Rev Cambridge Philos Soc 41, 445-502.

Mocquot, G. and Lefebvre, E. (1956) A simple procedure to detect nisin in cheese. J Appl Microbiol 19, 322-323.

Moll, G., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I.F., Konings, W.N. and Driessen, A.J.M. (1998) Mechanistic properties of the two-component bacteriocin lactococcin G. J Bacteriol 180, 96-99.

Moll, G., Ubbink-Kok, T., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I., Konings, W. and Driessen, A. (1996) Lactococcin G is a potassium ion-conducting, two-component bacteriocin. J Bacteriol 178, 600-605.

Montville, T.J. and Bruno, M.E.C. (1994) Evidence that dissipation of proton motive force is a common mechanism of action for bacteriocins and other antimicrobial proteins. Int J Food Microbiol 24, 53-74.

Montville, T.J., de Siano, T., Nock, A., Padhi, S. and Wade, D. (2006) Inhibition of *Bacillus anthracis* and potential surrogate bacilli growth from spore inocula by nisin and other antimicrobial peptides. J Food Prot 69, 2529-2533.

Morris, S.S., Walsh, R.C. and Hansen, J.N. (1984) Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. J Biol Chem 259, 13590-13594.

Nandakumar, R., Nandakumar, M.P. and Mattiasson, B. (2000) Quantification of nisin in flow-injection immunoassay systems. Biosens Bioelectron 15, 241-247.

Nes, I.F., Dzung, B.D., Håvarstein, L.S., Brurberg, M.B., Eijsink, V.G.H. and Holo, H. (1996) Biosythesis of bacteriocins in lactic acid bacteria. Anton van Leeuw Int J G 70, 113-128.

Nes, I.F. and Holo, H. (2000) Class II antimicrobial peptides from lactic acid bacteria. Biopolymers 55, 50-61.

Nilsen, T., Nes, I.F. and Holo, H. (1998) An exported inducer peptide regulates bacteriocin production in *Enterococcus faecium* CTC492. J Bacteriol 180, 1848-1854.

Nilsen, T., Nes, I.F. and Holo, H. (2003) Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. Appl Environ Microbiol 69, 2975-2984.

Nuñez, M., Tomillo, J., Gaya, P. and Medina, M. (1996) Bacteriocin quantification by the critical dilution method: a comparison of arbitrary units with diameter and area of the zone of growth inhibition. Milchwissenschaft 51, 7-10.

Okereke, A. and Montville, T.J. (1992) Nisin dissipates the proton motive force of the obligate anaerobe *Clostridium sporogenes* PA 3679. Appl Environ Microbiol 58, 2463-2467.

Okereke, A. and Montville, T.J. (1991) Bacteriocin-mediated inhibition of *Clostridium botulinum* spores by lactic acid bacteria at refrigeration and abuse temperatures. Appl Environ Microbiol 57, 3423-3428.

Oppegård, C., Rogne, P., Emanuelson, L., Kristiansen, P.E., Fimland, G. and Nissen-Meyer, J. (2007) The two-peptide class II bacteriocins: structure, production, and mode of action. J Mol Microbiol Biotechnol 13, 210-219.

Papagianni, M., Avramidis, N., Filioussis, G., Dasiou, D. and Ambrosiadis, I. (2006) Determination of bacteriocin activity with bioassays carried out on solid and liquid substrates: assessing the factor "indicator microorganism". Microb Cell Fact 5, 30-43.

Parente, E., Brienza, C., Moles, M. and Ricciardi, A. (1995) A comparison of methods for the measurement of bacteriocin activity. J Microbiol Meth 22, 95-108.

Piard, J.-C., Kuipers, O.P., Rollema, H.S., Desmazeaud, M.J. and de Vos, W.M. (1993) Structure, organization, and expression of the *lct* gene for lacticin 481, a novel lantibiotic produced by *Lactococcus lactis*. J Biol Chem 268, 16361-16368.

Pongtharangkul, T. and Demirci, A. (2004) Evaluation of agar diffusion assay for nisin quantification. Appl Microbiol Biot 65, 268-272.

Quadri, L., Kleerebezem, M., Kuipers, O., de Vos, W., Roy, K., Vederas, J. and Stiles, M. (1997a) Characterization of a locus from *Carnobacterium piscicola* LV17B involved in bacteriocin production and immunity: evidence for global inducer-mediated transcriptional regulation. J Bacteriol 179, 6163-6171.

Quadri, L.E.N., Yan, L.Z., Stiles, M.E. and Vederas, J.C. (1997b) Effect of amino acid substitutions on the activity of carnobacteriocin B2. Overproduction of the antimicrobial peptide, its engineered variants, and its precursor in *Escherichia coli*. J Biol Chem 272, 3384-3388.

Ra, S.R., Qiao, M., Immonen, N., Pujana, I. and Saris, P.E.J. (1996) Genes responsible for nisin synthesis, regulation and immunity form a regulon of two operons and are induced by nisin in *Lactococcus lactis* N8. Microbiology 142, 1281-1288.

Ramnath, M., Arous, S., Gravesen, A., Hastings, J.W. and Héchard, Y. (2004) Expression of mptC of *Listeria monocytogenes* induces sensitivity to class IIa bacteriocins in *Lactococcus lactis*. Microbiology 150, 2663-2668.

Ramnath, M., Beukes, M., Tamura, K. and Hastings, J.W. (2000) Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Appl Environ Microbiol 66, 3098-3101.

Rea, M.C., Clayton, E., O'Connor, P.M., Shanahan, F., Kiely, B., Ross, R.P. and Hill, C. (2007) Antimicrobial activity of lacticin 3147 against clinical *Clostridium difficile* strains. J Med Microbiol 56, 940-946.

Reunanen, J. and Saris, P.E.J. (2004) Bioassay for nisin in sausage; a shelf-life study of nisin in cooked sausage. Meat Sci 66, 515-518.

Reunanen, J. and Saris, P.E.J. (2003) Microplate bioassay for nisin in foods, based on nisin-induced green fluorescent protein fluorescence. Appl Environ Microbiol 69, 4214-4218.

Richard, C., Drider, D., Fliss, I., Denery, S. and Prevost, H. (2004) Generation and utilization of polyclonal antibodies to a synthetic C-terminal amino acid fragment of divercin V41, a class IIa bacteriocin. Appl Environ Microbiol 70, 248-254.

Rodgers, S., Kailasapathy, K., Cox, J. and Peiris, P. (2004) Co-incubation of *Clostridium botulinum* with protective cultures. Food Res Int 37, 659-666.

Rodgers, S., Peiris, P. and Casadei, G. (2003) Inhibition of nonproteolytic *Clostridium botulinum* with lactic acid bacteria and their bacteriocins at refrigeration tempeartures. J Food Prot 66, 674-678.

Rogers, A.M. and Montville, T.J. (1991) Improved agar diffusion assay for nisin quantification. Food Biotechnol 5, 161 - 168.

Rohde, B. and Quadri, L. (2006) Functional characterization of a three-component regulatory system involved in quorum sensing-based regulation of peptide antibiotic production in *Carnobacterium maltaromaticum*. BMC Microbiol 6, 93.

Rose, N.L., Sporns, P. and McMullen, L.M. (2001) Production of antibodies against enterocin B for immunological detection and purification purposes. Food Agr Immunol 13, 225-239.

Rose, N.L., Sporns, P. and McMullen, L.M. (1999) Detection of bacteriocins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Appl Environ Microbiol 65, 2238-2242.

Rossano, R., Del Fiore, A., D'Elia, A., Pesole, G., Parente, E. and Riccio, P. (1998) New procedure for the determination of nisin in milk. Biotechnol Tech 12, 783-786.

Ryan, M., Rea, M.C., Hill, C. and Ross, R.P. (1996) An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-sprectrum bacteriocin, lacticin 3147. Appl Environ Microbiol 62, 612-619.

Saucier, L. and Greer, G.G. (2001) Description of a simple detection assay for *in situ* production of bacteriocin in meat. J Food Prot 64, 264-267.

Saucier, L., Poon, A. and Stiles, M.E. (1995) Induction of bacteriocin in *Carnobacterium piscicola* LV17. J Appl Bacteriol 78, 684-690.

Scharff, R.L. (2010) Health-related costs from foodborne illness in the United States. The Produce Safety Project at Georgetown University, Washington, DC, p. 28.

Schmidt, V.S.J., Mayr, R., Wenning, M., Glockner, J., Busse, H.-J. and Scherer, S. (2009) *Bavariicoccus seileri* gen. nov., sp. nov., isolated from the surface and smear water of German red smear soft cheese. Int J Syst Evol Microbiol 59, 2437-2443.

Scott, V.N. and Taylor, S.L. (1981) Effect of nisin on the outgrowth of *Clostridium botulinum* spores. J Food Sci 46, 117-126.

Shand, R.F. and Leyva, K.J. (2008) Archaeal antimicrobials: An undiscovered country. In: P. Blum (Ed) Archaea: New Models for Prokaryotic Biology. Caister Academic Press, Norfolk, UK, p. 233-242.

Simon, L., Fremaux, C., Cenatiempo, Y. and Berjeaud, J.-M. (2001) Luminescent method for the detection of antibacterial activities. Appl Microbiol Biot 57, 757-763.

Siragusa, G.R. and Cutter, C.N. (1993) Brochocin-C, a new bacteriocin produced by *Brochothrix campestris*. Appl Environ Microbiol 59, 2326-2328.

Skoog, D.A. and Leary, J.L. (1992) Principles of Instrumental Analysis. Saunders College, Toronto.

Skyttä, E. and Mattila-Sandholm, T. (1991) A quantitative method for assessing bacteriocins and other food antimicrobials by automated turbidometry. J Microbiol Meth 14, 77-88.

Sobrino-López, A. and Martín-Belloso, O. (2008) Use of nisin and other bacteriocins for preservation of dairy products. Int Dairy J 18, 329-343.

Sofos, J.N. (2008) Challenges to meat safety in the 21st century. Meat Sci 78, 3-13.

Soliman, L.C. and Donkor, K.K. (2010) Method development for sensitive determination of nisin in food products by micellar electrokinetic chromatography. Food Chem 119, 801-805.

Stanley, B., Mehr, K.A., Kellock, T., van Hamme, J.D. and Donkor, K.K. (2009) Separation and determination of closely related lantibiotics by micellar electrokinetic chromatrography. J Sep Sci 32, 2993-3000.

Stiles, M.E. and Holzapfel, W.H. (1997) Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol 36, 1-29.

Stringer, S.C., Dodd, C.E.R., Morgan, M.R.A. and Waites, W.M. (1995) Locating nisin-producing *Lactococcus lactis* in a fermented meat system. J Appl Bacteriol 78, 341-348.

Suárez, A., Rodriguez, J., Hernandez, P. and Azcona-Olivera, J. (1996a) Generation of polyclonal antibodies against nisin: immunization strategies and immunoassay development. Appl Environ Microbiol 62, 2117-2121.

Suárez, A.M., Rodríguez, J.M., Morales, P., Hernández, P.E. and Azcona-Olivera, J.I. (1996b) Development of monoclonal antibodies to the lantibiotic nisin A. J Agr Food Chem 44, 2936-2940.

Svetoch, E.A., Levchuk, V.P., Pokhilenko, V.D., Eruslanov, B.V., Mitsevich, E.V., Mitsevich, I.P., Perelygin, V.V., Stepanshin, Y.G. and Stern, N.J. (2008) Inactivating methicillin-resistant *Staphylococcus aureus* and other pathogens by use of bacteriocins OR-7 and E 50-52. J Clin Microbiol 46, 3863-3865.

Tagg, J.R. and McGiven, A.R. (1971) Assay system for bacteriocins. Appl Microbiol 21, 943.

Tomita, H., Fujimoto, S., Tanimoto, K. and Ike, Y. (1996) Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. J Bacteriol 178, 3585-3593.

Tramer, J. and Fowler, G.G. (1964) Estimation of nisin in foods. J Sci Food Agr 15, 522-528.

Turcotte, C., Lacroix, C., Kheadr, E., Grignon, L. and Fliss, I. (2004) A rapid turbidometric microplate bioassay for accurate quantification of lactic acid bacteria bacteriocins. Int J Food Microbiol 90, 283-293.

Twomey, D., Ross, R.P., Ryan, M., Meaney, B. and Hill, C. (2002) Lantibiotics produced by lactic acid bacteria: structure, function and applications. Anton van Leeuw Int J G 82, 165-185.

Valat, C., Champiat, D., N'Guyen, T.T.T., Loiseau, G., Raimbault, M. and Montet, D. (2003a) Use of ATP bioluminescence to determine the bacterial sensitivity threshold to a bacteriocin. Luminescence 18, 254-258.

van Belkum, M.J. and Stiles, M.E. (2000) Nonlantibiotic antimicrobial peptides from lactic acid bacteria. Nat Prod Res 17, 323-335.

van Belkum, M.J., Worobo, R.W. and Stiles, M.E. (1997) Double-glycine-type leader peptides direct secretion of bacteriocins by ABC transporters: colicin V secretion in *Lactococcus lactis*. Mol Microbiol 23, 1293-1301.

van Belkum, M.J. and Stiles, M.E. (1995) Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. Appl Environ Microbiol 61, 3573-3579.

van der Meer, J.R., Polman, J., Beerthuyzen, M.M., Siezen, R.J., Kuipers, O.P. and de Vos, W.M. (1993) Characterization of the *Lactococcus lactis* nisin A operon genes *nis*P, encoding a subtilisin-like serine protease involved in precursor processing, and *nis*R, encoding a regulatory protein involved in nisin biosynthesis. J Bacteriol 175, 2578-2588.

Vandenbergh, P.A. (1993) Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiol Rev 12, 221-237.

Venema, K., Kok, J., Marugg, J.D., Toonen, M.Y., Ledeboer, A.M., Venema, G. and Chikindas, M.L. (1995) Functional analysis of the pediocin operon of *Pediococcus acidilactici* PAC1.0: PedB is the immunity protein and PedD is the precursor processing enzyme. Mol Microbiol 17, 515-522.

Wahlström, G. and Saris, P.E.J. (1999) A nisin bioassay based on bioluminescence. Appl Environ Microbiol 65, 3742-3745.

Waites, M.J. and Ogden, K. (1987) The estimation of nisin using ATP-bioluminometry. J Inst Brew 93, 30-32.

White, D. (2000) The Physiology and Biochemistry of Prokaryotes. Oxford University Press, New York.

Wiedemann, I., Böttiger, T., Bonelli, R.R., Wiese, A., Hagge, S.O., Gutsmann, T., Seydel, U., Deegan, L., Hill, C., Ross, P. and Sahl, H.-G. (2006) The mode of action of the lantibiotic lacticin 3147 - a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. Mol Microbiol 61, 285-296.

Wiedemann, I., Benz, R. and Sahl, H.-G. (2004) Lipid II-mediated pore formation by the peptide antibiotic nisin: a black lipid membrane study. J Bacteriol 186, 3259-3261.

Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O.P., Bierbaum, G., de Kruijff, B. and Sahl, H.-G. (2001) Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem 276, 1772-1779.

Willey, J.M. and van der Donk, W.A. (2007) Lantibiotics: peptides of diverse structure and function. Annu Rev Microbiol 61, 477-501.

Wolf, C.E. and Gibbons, W.R. (1996) Improved method for quantification of the bacteriocin nisin. J Appl Bacteriol 80, 453-457.

Worobo, R.W., van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. (1995) A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. J Bacteriol 177, 3143-3149.

Worobo, R.W., Henkel, T., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. (1994) Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. Microbiology 140, 517-526.

Wu, Z. and Li, X. (2007) Modification of the data-processing method for the turbidimetric bioassay of nisin. Appl Microbiol Biot 74, 511-516.

Yoneyama, F., Imura, Y., Ohno, K., Zendo, T., Nakayama, J., Matsuzaki, K. and Sonomoto, K. (2009) Peptide-lipid huge toroidal pore, a new antimicrobial mechanism mediated by a lactococcal bacteriocin, lacticin Q. Antimicrob Agents Chemother 53, 3211-3217.

Zendo, T., Nakayama, J., Fujita, K. and Sonomoto, K. (2008) Bacteriocin detection by liquid chromatography/mass spectrometry for rapid identification. J Appl Microbiol 104, 499-507.

Zhang, J.L., Liu, G.R., Li, P.L. and Qu, Y. (2010) Pentocin 31-1, a novel meat-borne bacteriocin and its application as biopreservative in chill-stored tray-packaged pork meat. Food Control 21, 198-202.

Chapter Two: **Phage display for production of bacteriocin-specific antibody fragments**

2.1 Introduction

Although many methods of bacteriocin detection have been described, antibody-based methods are specific and sensitive (see Table 1-2). Although there are five different mammalian antibody classes, most antibodies have similar Y-shaped general structures, consisting of two light (L) chains weighing ~ 25 kDa each and two heavy (H) chains of 50 kDa each, as shown in shown in Figure 2-1A. Heavy chains are composed of three or four constant (C) domains linked to a variable (V) domain, while light chains contain one C domain linked to a V domain (Hannigan et al., 2009). Antigen binding takes place in the upper arm region of the antibody, and antigen specificity is determined by the V_H and V_L domains of the antibody. The five classes of antibodies are grouped into five immunoglobulin (Ig) classes based on the heavy chain identity: IgA, IgD, IgE, IgG, or IgM, with IgG being the major circulating antibody class, at a concentration of 13.5 μg μL^{-1} serum, which is approximately 75 % of the total serum immunoglobulin (Madigan et al., 2003; Hannigan et al., 2009). Traditional antibody-based techniques have employed two types of IgG antibodies: polyclonal or monoclonal.

Polyclonal antibodies are produced as a heterogeneous mixture by different B-lymphocyte cells, each specific for a different epitope, whereas monoclonal antibodies are produced by one B-lymphocyte cell clone grown in cell culture, and therefore are specific for one epitope (Harlow and Lane, 1988). Production of both types of antibodies involves the immunization of laboratory animals with the antigen of interest, periodic monitoring of serum antibody titre via ELISA, and booster immunizations over a period of several months.

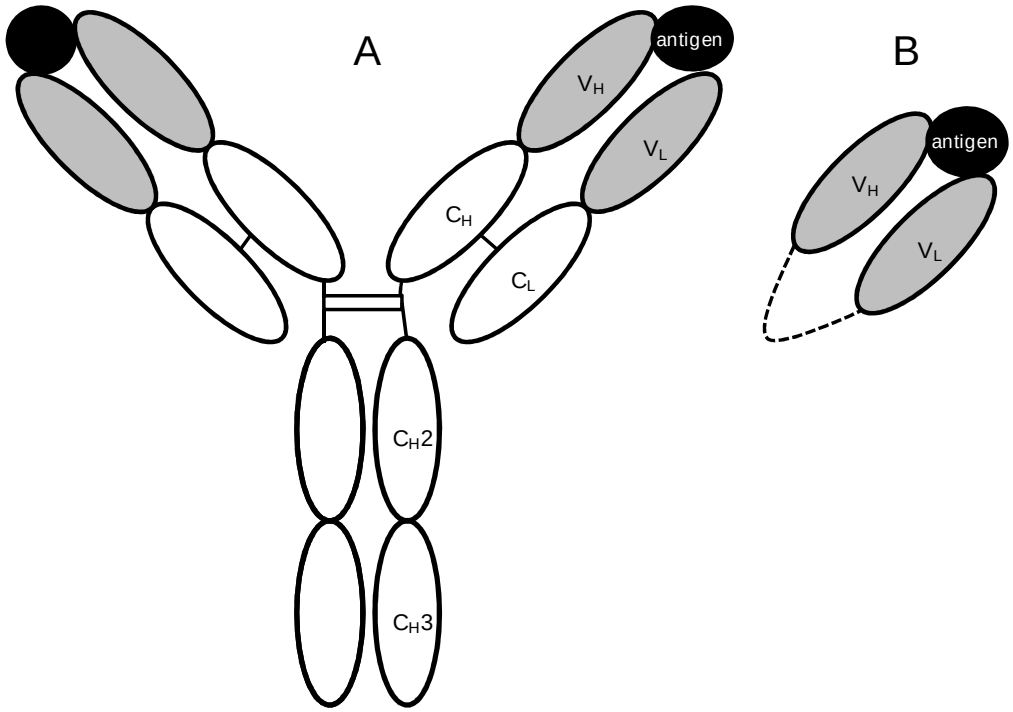

Figure 2-1 Structure of an antibody (A) and single-chain variable fragment antibody (scFv; B). Constant and variable regions are abbreviated as C and V, respectively, while light and heavy chains are shown as L and H. In B, the glycine-rich flexible linking chain is shown by a dotted line.

However, once the antibody titre reaches an acceptable level, methodology of polyclonal and monoclonal production differs. Polyclonal antibodies are purified from blood by methods commonly used for protein purification, including ammonium sulphate precipitation followed by chromatographic methods such as ion-exchange or gel filtration, or application to hydroxyapatite resin to separate antibodies from other serum proteins (Harlow and Lane, 1988). For purification of antigen-specific antibodies from the pool of polyclonal antibodies, antigen affinity column purification is subsequently performed. Rabbits are commonly used for polyclonal antibody production, with an average yield of 10 μg total antibody μL^{-1} serum, of which only 10 % is specific for a given antigen (Harlow and Lane, 1988). Thus, although the purification methodology is well-established, the yield of specific antibodies is low. As well,

polyclonal antibody production relies on a consistent, ongoing supply of immunized animals, which has become an animal welfare issue in recent years.

Monoclonal antibody production is more technically challenging than polyclonal methods. Once an immune response is raised in an animal (usually mice), single antibody-producing cells are fused to myeloma cells, creating hybridoma cells which secrete antibodies with a high specificity (Köhler and Milstein, 1976). If the hybridoma cells are maintained under suitable environmental and nutritional conditions, they will continue to produce monoclonal antibodies indefinitely. However, despite the advantage of producing highly-specific antibodies, a high degree of technical expertise is required for myeloma fusion and hybridoma maintenance.

An alternative method of antibody production is phage display (Smith, 1985). Because of its' ability to readily incorporate foreign DNA, bacteriophage (phage) has been used as a cloning vector in recombinant biology, though its use is restricted to fragments of less than 6 kbp (Model and Russel, 1988). Specifically, filamentous phage or modified forms of phage known as phagemids have been used for phage display. Filamentous phages are composed of a circular single-stranded genome of approximately 6400 nucleotides contained within an elongated protein cylindrical coat, or capsid (Model and Russel, 1988). Eleven proteins are encoded by the genome, five of which are capsid proteins; of these, gene VIII protein (pVIII) is a major capsid protein with 2700 copies, and the remaining four gene product proteins (pIII, pVI, pVII, pIX) are minor coat proteins, with five copies each (Webster, 2001). Proteins VII and IX are located at one end of the capsid, while pIII and pVI are located at the opposite end (Webster, 2001).

Phagemids are smaller, modified phages whose genetic material contains a plasmid origin of replication and an antibiotic resistance marker gene, to simplify selection, in addition to all capsid protein genes (Webster, 2001). These factors, combined with their ease of transformation, make phagemids the preferred choice for phage display.

Phage display involves cloning genes encoding proteins into the phage genome, at the amino terminus of host coat protein genes. When the phage replicates, the fusion protein is expressed on the surface of the phage virion, resulting in a coat protein-foreign protein fusion on the surface of the phage virion (Smith, 1985). Fusions to pIII or pVIII are most common, though fusions to the other minor coat proteins have been constructed (Webster, 2001). Once the foreign protein is displayed, phages containing the desired protein can be selected by an affinity-capture system, in which an antibody to the foreign protein is used to coat a surface, a mixture of phage particles are allowed to bind, and phages lacking the protein are removed through subsequent washing. Phages expressing the desired protein can then be released using an acidic buffer (Smith, 1985). If multiple rounds of this assay are performed (known as panning), purified phages can be harvested, transformed into a suitable bacterial expression host, and used to produce soluble protein. Often, many different genes have been cloned into phages concurrently, creating a library of phages with the ability to express a wide diversity of proteins. In this way, many types of proteins have been produced, including antibodies (McCafferty et al., 1990).

When antibodies are produced via phage display, only the fragments of the antibodies that bind antigen are required; namely, the V_L and V_H domains. Two forms of antibody fragments have been produced, the first being the single-chain variable fragment (scFv), consisting of the two variable domains connected by a flexible glycine-rich linking sequence, as shown in Figure 2-1B (Bird et al., 1988; Huston et al., 1988; McCafferty et al., 1990). The second form is the Fab fragment, consisting of two chains connected by the conserved cysteine residues: the first C_H connected to V_H, and the C_L connected to V_L. Although Fab fragments are more stable than scFv because of the stability conferred by a disulfide bond (Kabat and Wu, 1991), their assembly is more complex, requiring the assembly of two chains, as opposed to the single chain of scFV. In addition, scFv is better-tolerated by bacteria, has a higher affinity for antigens, and is less likely to be degraded, making it a more attractive choice for many applications (Bradbury and Marks, 2004).

To construct a phage display library, antibody genes (typically IgG) are amplified by PCR from RNA extracted from B cells of naive (unimmunized) or immune (immunized) animals (Ørum et al., 1993; Ames et al., 1995), and are cloned into an appropriate phage or phagemid vector. Antibodies originating from immune libraries are important because they have both an increased specificity to and affinity for the antigen used in the immunization (Williamson et al., 1993), but naive libraries can be useful when an antigen is toxic to the animal. Different oligonucleotide primer sets have been developed for amplification of antibody genes, with the sequences optimized for the species of animal from which the library is derived. To preserve the greatest amount of diversity in the library, and thereby increase the chance of selecting the antibody fragment of interest, degenerate primer mixes are often used (Krebber et al., 1997), with primer sequences based on known antibody sequences, such as those found in the Kabat database (Johnson and Wu, 2000; Johnson, 2008).

Antibody fragments to various haptens have been produced, including pesticides and herbicides (Yau et al., 1998; Tout et al., 2001; Kramer, 2002a; Brichta et al., 2003; Olea-Popelka et al., 2005; Li et al., 2006; Sheedy et al., 2006), antibiotics (Burmester et al., 2001; Korpimäki et al., 2002; Yang et al., 2007; Makvandi-Nejad et al., 2010; Zhang et al., 2010), antiparasitics (Zhang et al., 2010), other drugs (Moghaddam et al., 2003; Pan et al., 2006; Alvarez-Rueda et al., 2007; Kobayashi et al., 2008), phytoplanktonic toxins (Nagumo et al., 2004; Finlay et al., 2006; Shaw et al., 2008; Garet et al., 2010), and mycotoxins (Moghaddam et al., 2001; Doyle et al., 2008). Nevertheless, there have been no reports of phage display being used to produce antibody fragments to haptenic bacterial metabolites. Although traditional polyclonal or monoclonal antibodies have been produced to bacteriocins (see Table 1-2), immune response can be variable, and so a consistent source of soluble antibody fragments, such as those produced by phage display, is preferred. In this study, attempts were made to produce an immune phage display library to three bacteriocins, including two class IIa bacteriocins, leucocin A and piscicolin 126, and a class IIb bacteriocin, brochocin-C.

2.2 Materials and methods

2.2.1 Culture maintenance

The strains and vectors, as well as their relevant characteristics, are given in Table 2-1. All bacterial strains were stored at -80°C in 20 % (v/v) glycerol in appropriate medium. *Escherichia coli* strains were stored in Luria-Burtani (LB) broth containing appropriate antibiotics (Difco, Becton Dickinson; Sparks, MD), while *Leuconostoc gelidum* and both carnobacteria were stored in All-Purpose Tween broth (APT, Difco).

Table 2-1 Organisms, vectors, and their relevant characteristics

Bacteria, phage, or phagemid	Characteristics[1]	Source or reference
Carnobacterium divergens NCIMB 702855	Leucocin A-sensitive indicator strain	Worobo et al. (1995), National Collection of Industrial and Marine Bacteria; Aberdeen, SF
Carnobacterium maltaromaticum NCIMB 702852	Leucocin A-sensitive indicator strain	Ahn and Stiles (1990) National Collection of Industrial and Marine Bacteria; Aberdeen, SF
E. coli JM83	F$^-$ *ara* Δ*(lac-proAB) rpsL* (Str^r) *thi* [Φ80dΔ*(lacZ)M15*]; Amber codon non-suppressor strain	ATCC 53607
E. coli TG1	Δ*(lac-proAB)* Δ*(mcrB-hsdSM)5* (rK- mK-) *thi-1 supE* [F´ *traD36 proAB lacI*^{q}*ZΔM15;* Amber codon suppressor strain	Stratagene/Agilent Technologies Canada; Mississauga, ON
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F' *proAB lacI*^{q} ZDM15 Tn*10* [Tet^r]); amber codon suppressor strain	Stratagene
Leuconostoc gelidum UAL187	Leucocin A producer	Hastings et al. (1991)
pAK100	6.4 kbp, cam^r , tet^r	Krebber et al. (1997)
pJB12	7.4 kbp, cam^r , tet^r	Krebber et al. (1997) Burmester and Plückthun (2001)
pUC18	2.7 kbp, Ap^r	Stratagene
VCSM13 helper phage	Interference-resistant	Stratagene

[1] ap^r, ampicillin-resistant; cam^r, chloramphenicol-resistant, Str^r, streptomycin-resistant; tet^r, tetracycline-resistant

2.2.2 Leucocin-A purification

A modified method of Hastings et al. (1991) was used for production and purification of leucocin A. Briefly, 3.2 L of Casamino Acids Medium (Hastings et al., 1991) supplemented with 2 % (w/v) filter-sterilized glucose (Fisher

Scientific Canada; Ottawa, ON) was inoculated with 2 % (v/v) of an overnight culture of *Leuconostoc gelidum* UAL187. Cultures were incubated at 26°C with stirring at 100 rev min^{-1}, with a constant pH of 6.0 under a sterile N_2 headspace in a Minifors 5 L fermentor (Infors HT; Bottmingen, CH). After 24 h fermentation, the bacteriocin-containing supernatant was recovered by centrifugation at 6000 x *g* for 15 min. The supernatant was transferred to sterile flasks, heated at 60°C for 30 min, and the bacteriocin was precipitated using ammonium sulphate [Fisher; 70 % (w/v) saturation, calculated at 4°C] for 12 h with gentle stirring at 4°C. The precipitates were recovered by centrifugation at 6000 x *g* for 15 min at 4°C, followed by resuspension in 100 mL of a solution containing 6 M urea (Fisher) and 10 mM glycine-HCl (final pH 2.5) per L culture supernatant. The fractions were applied (8 mL min^{-1}) to Amberlite XAD-2 (Sigma-Aldrich Canada Ltd; Oakville, ON) columns (6 x 25 cm column) equilibrated with 0.1 % (v/v) trifluoroactetic acid in MilliQ® water (Millipore; Billerica, MA). The columns were washed with 1.5 L of 0.1 % TFA, followed by 1 L each of 25 % and 45 % (v/v) ethanol, and finally 75 % (v/v) ethanol, all at a flow rate of 12 mL min^{-1}. All fractions were tested for antimicrobial activity, and active fractions were concentrated by rotary evaporation (Büchi; Flawil, CH) at 35°C to a final volume of 80 mL L^{-1} culture supernatant, lyophilized to dryness, and resuspended in 1.5 mL MilliQ water per L culture supernatant. Volumes of 0.5 mL were applied onto a Superdex Peptide 10/300 GL column (GE Healthcare; Uppsala, SE) connected to an HPLC system (Varian Prostar, Agilent Technologies Canada; Missisauga, ON), with 50 mM sodium phosphate buffer (7.09 g Na_2HPO_4 and 6.90 g NaH_2PO_4 L^{-1}), 0.15 M NaCl (pH 7) at a flow rate of 1 mL min^{-1} as a mobile phase. Purification was monitored using a UV detector set to 220 nm, and 1-mL fractions were collected from 12 to 45 min. Fractions with antimicrobial activity were pooled, re-purified by gel filtration, and stored at 4°C until use.

2.2.3 Antigen preparation

For immunization, bacteriocins were conjugated to keyhole limpet haemocyanin (KLH) to increase immunogenicity, while for antibody titre ELISA, bovine serum albumin (BSA)-conjugated bacteriocin was used. For leucocin A, a

sulfhydryl group was required on the N-terminus to allow conjugation. This was created by incubating 4.48 µmoles leucocin A (dissolved in 50 mM sodium phosphate buffer, 0.15 M NaCl, 3 mM EDTA) with 44.8 µmoles 2-iminothiolane-HCl (Traut's reagent, Pierce/Thermo Fisher; Rockford, IL) at 23°C for 1 h. Excess Traut's reagent was removed by passing the solution through a D-salt polyacrylamide desalting column (Pierce) equilibrated with phosphate-buffered saline (PBS), pH 7.2, containing 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, and 3mM EDTA (Harlow and Lane, 1988). The thiolated peptide was eluted with 500-µL volumes of PBS containing 0.1 M EDTA (pH 7.4) and peptide-containing fractions were identified by protein quantitation and lyophilized to dryness. BSA or KLH were conjugated to leucocin A using the Imject Maleimide Activated Immunogen kit (Pierce), and unconjugated KLH or BSA were removed by passing the solution through a D-salt dextrose column (Pierce). The conjugated peptide was eluted using the supplied buffer, protein-containing fractions were identified using protein quantitation, and stored at 4°C until use.

Piscicolin 126 and brochocin-A antigens were chemically synthesized by Alberta Peptide Institute (Edmonton, AB) and Sigma-Aldrich Canada (Oakville, ON), respectively. KLH conjugates were prepared for immunization, while BSA-conjugated peptides were prepared for use in antibody titre assays. Conjugation was done at the N-terminus for both carrier proteins. For piscicolin 126, the first 15 amino acids of the C-terminus of piscicolin 126 was synthesized (H_2N-AANLTTGGAAGWNKG-COOH). Both component peptides of brochocin-C were analyzed for antigenic potential and hydropathicity using an antigen prediction tool (Reche, 2007) using a method based on Kolaskar and Tongaonkar, (1990), and a hydropathicity tool (Bowen, 1998) using the method of Kyte and Doolittle (1982). Based on the results, a 21-amino acid fragment at the N-terminus of brochocin-A was chosen (H_2N-LAAGLGAIPGAFVGAHFGVIGGSAA-COOH) as an antigen.

2.2.4 Immunization of mice

For unconjugated leucocin A, a concentration of 85.6 µg mL^{-1} was used, while all peptides conjugated to KLH were used at 100 µg mL^{-1} in sterile PBS, pH 7.4. Initial injection antigens were prepared in a 1:1 ratio of peptide conjugate: Freund's Complete Adjuvant (Pierce), while later booster injections used Freund's Incomplete Adjuvant (Pierce) in place of Complete Adjuvant. For unconjugated leucocin and KLH-leucocin A, five female BALB/c mice (Charles River Laboratories; Wilmington, MA) at five weeks of age were used per immunogen, while for KLH-piscicolin 126(30-44) and KLH-brochocin-A(25-71), six mice each were used. On days 0 and 14, mice were given 2-100 µL injections, one subcutaneous and one intraperitoneal, and on day 21, a 0.1 mL volume of blood was tested for antibody titre. If the titre was not sufficiently high, booster injections were administered two weeks later, with subsequent blood samples taken two weeks later, to a maximum of five booster injections. Once an adequate antibody titre of 1:500 had been reached, a final intravenous tail injection was given using no adjuvant, and three days later, the mice were euthanized, and spleens and blood were collected. Spleens were placed in 5 mL RNAlater solution (Ambion/Applied Biosystems; Streetsville, ON), and were stored at 4°C overnight, then at -20°C until use. All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies (Canada Council on Animal Care, 2002) with approval from the Biosciences Animal Policy and Welfare Committee at the University of Alberta.

2.2.5 Antibody titre quantitation

An ELISA method was used to determine antibody titre. Upon receipt, blood samples were prepared according to Harlow and Lane (1988), with exclusion of sodium azide addition. Samples were incubated at 37°C for 1 h, then at 4°C overnight. Red blood cells were removed by centrifugation at 10 600 x *g* for 10 min at 4°C, removal of the serum supernatant, and another round of centrifugation to remove any remaining cells. Serum was stored at -20°C until use. For ELISA, 96 – well flat-bottom Maxisorp Immunoplates (Nalge Nunc

International; Rochester, NY) were coated with 50 µL of a 5 µg mL^{-1} BSA-peptide conjugate solution, plates were sealed, and incubated overnight at 4°C. Wells were washed twice with sterile PBS, and 100 µL of 1 % (w/v) BSA (Fraction V, Sigma) in PBS were added per well to reduce unspecific binding. Plates were sealed and incubated with gentle rotary shaking at room temperature for 1 h. Blocking solution was removed by washing each well three times with PBS containing 0.05 % (v/v) Tween 20 (PBST) (Batteiger et al., 1982), 200 µL of serially-diluted serum (10^{-2} – 10^{-6}) was added, and plates were incubated at room temperature for 4 h with gentle rotary shaking. Wells were washed twice with PBST, and then 200 µL of goat anti-mouse IgG-peroxidase conjugate (Sigma) diluted 1:10000 in PBS was added. Plates were then incubated with gentle rotary shaking for 2 h at room temperature. After incubation, wells were washed three times with PBST, and then 100 µL of 2,2'-azino-bis (3-ethylbenzythiazoline-6-sulfonic acid) treated with enhancer solution (Sigma) was added. Plates were then incubated with gentle shaking until colour development at 15 min. Absorbance was read on a microtitre plate reader (Varioskan Flash, Thermo Electron Corp; Gormley, ON) at 405 nm. Antibody titre was calculated by plotting the reciprocal of the serum dilution against the A_{405}, and, using the method of Martínez et al. (1998), determining the maximum dilution that produced an absorbance at least twice that of pre-immune control serum.

2.2.6 Protein quantitation

Protein concentration was determined by the bicinchoninic acid microtitre plate method in the BCA protein assay kit (Pierce), using a standard curve prepared with BSA from 25 – 2000 µg mL^{-1} and analyzed using a microtitre plate reader (Varioskan Flash, Thermo Electron Corp.) set at 562 nm.

2.2.7 Antibacterial activity of bacteriocins

A spot-on-lawn bioassay was used to determine antibacterial activity of leucocin-A. Leucocin-A was diluted in a two-fold series with 0.1 % (v/v) trifluoroacetic acid (Sigma), and 10 µL of each dilution was spotted onto APT agar containing 1.5 % (w/v) agar. Plates were coated with a semi-solid APT agar

[0.75 % (w/v) agar] overlay seeded with 1 % (v/v) of an overnight culture of the sensitive indicator organism *C. maltaromaticum* NCIMB 702852 and *C. divergens* NCIMB702855. Plates were incubated anaerobically at 30°C, and were examined at 24 h for zones of clearing. Arbitrary activity units per mL (AU mL^{-1}) were calculated from the reciprocal of the last dilution showing inhibition (McCormick et al., 1998).

2.2.8 Total RNA and mRNA isolation

Approximately 1/3 of each spleen was disrupted using a rotor-stator homogenizer for three pulses of 20 s each. RNA was isolated from mouse splenocytes with the RNEasy mini kit (Qiagen; Mississauga, ON), and was quantified using a Nanodrop 2000c (Thermo Scientific). Purification of mRNA was done using the illustra kit (GE Healthcare; Uppsala, SE), and the resulting mRNA was concentrated by adding 100 µL of a solution containing 10 mM tris-HCl, 1 mM EDTA, and 3 M NaCl, at pH 7.4, to which subsequently 10 mg glycogen mL^{-1} and 2.5 mL ice-cold 98 % ethanol was added. The solution was gently mixed, incubated at -20°C overnight, and centrifuged at 11 950 x *g* for 10 min at 4°C to pellet the mRNA. Following precipitation, mRNA was resuspended in nuclease-free water [diethylpyrocarbonate (DEPC)-treated, Fisher], and immediately used for cDNA synthesis using the first-strand cDNA Synthesis kit (GE Healthcare) and the random primer mix. Samples of cDNA were stored at -20°C until PCR amplification.

2.2.9 PCR amplification of heavy- and light-chain fragment DNA

Antibody fragment DNA (scFv) was amplified using the method of Krebber et al. (1997), as modified by Burmester and Plückthun (2001). Primers were obtained from Integrated DNA Technologies (Coralville, IA), and primer mixes for amplification of light- and heavy-chain scFv were prepared with the same degree of degeneracy and added in the same volumes as in Burmester and Plückthun (2001). For amplification of scFv, 2 µL cDNA template was added to a reaction mixture containing 5 µL 10X buffer, 1.5 mM $MgCl_2$, 2 µM each of primer mixes LB and LF (light-chain) or HB and HF (heavy-chain), 200 µM

dNTP mix, and 2 U Platinum Taq polymerase (Invitrogen Canada, Burlington, ON) in a final reaction volume of 50 µL. Both the VH and VL primers contained sequence encoding the flexible polypeptide linking region $(G_4S)_3$. Products were amplified using the following program: 94°C for 5 min; 5 cycles of 92°C, 55°C, and 72°C for 1 min each; 20 cycles of 92°C, 63°C, and 72°C for 1 min each; with a final extension step of 7 min at 72°C. Annealing temperature was optimized prior to experimentation. PCR products were visualized on a 1.5 % (w/v) agarose gel in 1X Tris-acetate-EDTA buffer, bands corresponding to the expected size were excised, and the DNA extracted using the QiaQuick gel extraction kit (Qiagen). DNA was eluted in 50 µL tris-HCl buffer, pH 8, and subsequently used in single-overlap extension PCR for assembly of the complete scFv gene. When necessary, PCR products were concentrated by ethanol precipitation at -20°C overnight (Sambrook and Russell, 2001).

To assemble the complete scFv gene, 10 ng each of the variable-light and variable-heavy antibody genes was used in a 50 µL reaction mixture containing 5 µL 10X buffer, 1.5 mM $MgCl_2$, 2 µM each of primers scfor and scback, 200 µM dNTP mix, and 2 U Platinum Taq polymerase (Invitrogen Canada). Primers scfor and scback encoded the *Sfi*I restriction site; while cback encodes the FLAG™ sequence which allowed for detection of recombinant antibodies (Knappik and Plückthun, 1994). The product was amplified using the following program: 94°C for 5 min; 7 cycles of 92°C for 1 min, 63°C for 30 s, 58°C for 50 s, and 72°C for 1 min; 23 cycles of 92°C for 1 min, 63°C for 30 s, and 72°C for 1 min; with a final extension step of 7 min at 72°C. The product was visualized on a 1 % (w/v) agarose gel in 1X tris-acetate-EDTA buffer, and DNA of the expected size was extracted as described previously.

2.2.10 Cloning and transformation

The phagemid vectors used for display were pAK100 and pJB12, both obtained from Andreas Plückthun (Krebber et al., 1997; Burmester and Plückthun, 2001). Both the vector and extracted scFv fragments (~ 1 ng each) were digested with 10 U *Sfi*I (New England Biolabs; Pickering, ON) for 4 h at 50°C, analyzed on a 1 % (w/v) agarose gel, and extracted as described previously. Aliquots of

purified vector and scFv fragments were analyzed on a 1 % (w/v) agarose gel to determine concentration, using 10 µL of E-Gel Quantitative DNA Ladder (Invitrogen) as a standard. Digested scFv and vector were ligated using 1.5:1, 5:1, and 10:1 molar ratios of vector to insert using the Rapid DNA Ligation kit (Roche Diagnostics GmbH; Mannheim, DE) and transformed into either electrocompetent or chemically-competent *E. coli* TG1 cells. A transformation efficiency control was also included, which consisted of pUC18 diluted 1:10 in sterile MilliQ water. Electroporation was performed using 1 µL of ligation mixture or pUC18 added to 40 µL cells in a 0.1 cm gap cuvette (Biorad Laboratories; Mississauga, ON), using an electroporation device (Biorad). A single 10-millisecond pulse (25 µF) of 1700 V was delivered, with a resistance of 200 Ω. Following electroporation, 1 mL prewarmed (37°C) Super-Optimal broth with catabolite repression (SOC) (Hanahan, 1983; Sambrook and Russell, 2001) was added, and the cells were incubated at 37°C for 1 h. Controls that utilized ligation mixtures with native pAK100 or pJB12 and non-transformed *E. coli* TG1 cells were also included. Chemically-competent cells were transformed by adding 10 µL ligation mixture to 100 µL cells, and incubating on ice for 20 min, followed by 2 min each at 42°C and on ice. Subsequently, 500 µL SOC broth was added, and cells were incubated at 37°C for 3 h.

Transformants were selected by spreading 150 µL aliquots on the surface of 2xYT agar (Sambrook and Russell, 2001) containing 1 % (w/v) glucose and 30 µg mL^{-1} chloramphenicol. Contaminating unrestricted vector was identified by spreading an aliquot on the surface of 2xYT medium containing 30 µg mL^{-1} chloramphenicol and 12.5 µg mL^{-1} tetracycline. Transformation efficiency controls were spread on the surface of LB medium containing 100 µg mL^{-1} ampicillin. Plates were incubated at 37°C for 24 h, and transformants were pooled for panning.

2.2.11 Library amplification and panning

Transformants were scraped from the medium surface using sterile rubber policemen (Fisher) and 1 mL 2xYT containing 1 % (w/v) glucose and 30 µg mL^{-1} chloramphenicol per plate was used to wash the plate surface. One hundred

microlitres of the pooled suspension was used to inoculate 5 mL of 2xYT medium containing 1 % (w/v) glucose and 30 µg mL^{-1} chloramphenicol, and the culture was incubated at 37°C with shaking at 250 rev min^{-1}. When an OD_{600} of 0.7 was reached, VCSM13 helper phage and isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to give final concentrations of 5 x 10^9 PFU mL^{-1} and 1 mM, respectively. Cultures were incubated at 26°C overnight with shaking, and cell debris was removed by centrifugation at 1000 x *g* for 20 min at 4°C. Phage-containing supernatant was transferred to sterile re-useable 50 mL centrifuge tubes, 2 mL of sterile polyethylene glycol (200 g L^{-1}; Fisher) and NaCl (146.1 g L^{-1}) were added to precipitate the phage. Tubes were then inverted 12 times to mix, and then incubated on ice for 1.5 h. Phagemid was collected by centrifugation at 10 000 x *g* for 20 min at 4°C, and the resulting pellet was resuspended in 500 µL of PBS.

For panning, 100 µL of antigen conjugated to BSA (10 ng $µL^{-1}$) was used to coat eight-well flat-bottomed Maxisorp Immunoplates microtitre plates (Nalge Nunc) overnight at 4°C. Wells were washed three times with sterile PBS to remove unbound antigen, and 200 µL of 3 % (w/v) skim milk powder in PBS were added per well to reduce unspecific binding. Plates were sealed and incubated with gentle rotary shaking at room temperature for 1 h. Blocking solution was removed by washing each well three times with PBS, and 100 µL of the phagemid suspension was added (~10^{11} particles). Plates were then incubated at room temperature for 2 h with gentle rotary shaking. Subsequently, wells were washed 20 times with PBST, and bound phagemid was eluted with 100 µL sodium acetate buffer (0.1 M acetic acid, 0.15 M NaCl, pH 2.8) for 8 min followed by neutralization with 12 µL 2 M Tris buffer, pH 9.5. Neutralized phagemid was used immediately to infect 5 mL log phase *E. coli* TG1 cells (OD_{600} ~ 0.5) for 30 min at 37°C followed by a 30 min incubation at room temperature. Aliquots were then spread onto the surface of 2xYT medium containing 1 % (w/v) glucose and 30 µg mL^{-1} chloramphenicol, and incubated at 30°C overnight. The panning procedure was repeated at least three times, and the

phagemid which remained after three rounds of panning were subjected to ELISA to determine the relative strength of their binding.

2.2.12 ELISA for identification of strong-binding clones

Individual colonies remaining after three rounds of panning were inoculated into 5 mL 2xYT containing 1 % (w/v) glucose and 30 µg mL^{-1} chloramphenicol broth, and incubated at 37°C with shaking to an OD_{600} of 0.5. The protocol for phage library amplification, phagemid precipitation, and preparation of microtitre plates was followed as stated in Section 2.2.11, with two changes: the precipitated phagemid pellet was resuspended in 1 % (w/v) skim milk powder in PBS, and uncoated control wells containing 100 µL PBS were included in the ELISA. Following the incubation step allowing for phagemid binding to antigen, wells were washed three times with PBS and then, three times with PBST. Finally, 100 µL of mouse anti-M13 monoclonal-peroxidase conjugate (GE Healthcare) diluted 1:2500 in 2 % (w/v) skim milk powder in PBS was added. Plates were incubated with gentle rotary shaking for 1 h at room temperature, and then, wells were washed three times each with PBS followed by PBST. Finally, 100 µL of 2,2'-azino-bis (3-ethylbenzythiazoline-6-sulfonic acid) treated with enhancer solution (Sigma) were added. Plates were incubated with gentle shaking until colour development at 30 min, and absorbance was read on a microtitre plate reader (Varioskan Flash, Thermo Electron Corp.) at 405 nm. Strong-binding clones were defined as those producing an absorbance of at least 0.2, with an absorbance at least twice that of the background controls.

2.2.13 Verification of scFv fragment production

Once strong-binding clones were identified, phagemids were isolated using the Qiagen Maxi Prep kit (Qiagen), and were used to transform electrocompetent *E. coli* JM83 cells, using 1 µL phagemid in 40 µL competent cells under the same conditions as previously described. Aliquots of 100 µL were spread onto the surface of 2xYT medium containing 1 % (w/v) glucose and 30 µg mL^{-1} chloramphenicol, and then incubated at 37°C overnight. Transformants containing each phagemid were transferred to 2xYT broth containing 1 % (w/v)

glucose and 30 µg mL^{-1} chloramphenicol, and incubated at 37°C with shaking at 250 rev min^{-1} overnight. Five milliliters of fresh medium was inoculated with 50 µL of overnight culture, and once an OD_{600} of 0.4 was reached, IPTG to final concentrations ranging from 0 to 1 mM was added. Cultures were incubated at 37°C with shaking at 250 rev min^{-1} overnight. Cells (1.5 mL) were harvested by centrifugation at 20 000 x *g* for 2 min, and were lysed using the CelLytic B Plus kit (Sigma). Tris-glycine SDS-PAGE followed by Western blot analysis was performed on the resulting cell lysate.

For tris-glycine SDS-PAGE, samples were prepared by adding 5 µL loading dye (Sigma) to 10 µL of sample, while 10 µL of loading dye was added to 5 µL of a 20 ng $µL^{-1}$ FLAG™-containing control peptide (FLAG™-BAP, Sigma). The control peptide was included to ensure functionality of the primary anti-FLAG™ antibody. Samples and a low molecular weight pre-stained standard (5 µL, Biorad) were heated at 95°C for 4 min and were loaded onto a Mini Protean system (Biorad) with a 5 % (v/v) stacking gel and 12 % (v/v) resolving gel (Sambrook and Russell, 2001). The gel was run at 50 V for 30 min, 90 V for 45 min, and finally 110 V until the dye front was 5 mm from the plate bottom. Proteins were transferred from the gel to a nitrocellulose membrane using the Biorad Protein transfer unit run at 30 V overnight at 4°C. The membrane was rinsed in MilliQ water for 5 min, and blocked with 1% (w/v) polyvinylpyrrolidone 40 [Sigma; Ge et al. (1995)] in PBST for 1 h with gentle rotary shaking at room temperature. The membrane was then incubated in a solution containing a 1:1000 dilution of the primary antibody (Monoclonal anti-FLAG™ M2 antibody, Sigma) in blocking solution [1% (w/v) polyvinylpyrrolidone 40 in PBST] for 2 h at room temperature. The membrane was then washed three times in PBST for 5 min, and incubated in a 1:30000 dilution of goat anti-mouse IgG-peroxidase conjugate (Sigma) made in blocking solution for 1 h at room temperature. The membrane was washed three times for 5 min in PBST. Development was done for 5 min using a chemiluminescent peroxidase conjugate solution (Sigma) added as per supplier directions. Peptide bands were visualized using an imaging system

(Typhoon Trio^{+} Variable Model, GE Healthcare) set to chemiluminescent mode at 600 V with normal sensitivity.

2.2.14 Helper phage production and titring

Helper phage was propagated by inoculating 10 mL of LB broth with a single colony of *E. coli* XL1-Blue grown on supplemented M9 medium containing 5 mM $MgSO_4$ and 0.01 % (w/v) thiamine (Sambrook and Russell, 2001). The culture was incubated at 37°C with shaking at 250 rev min^{-1} until an optical density (OD_{600}) of 0.3 at 600 nm was reached. VCSM13 was added at a multiplicity of infection of 20:1 (phage:cells), and the culture was incubated at 37°C with shaking at 250 rev min^{-1}. Thirty minutes post-infection, kanamycin was added to a final concentration of 70 µg mL^{-1}, and incubation under the same conditions was continued for 8 h. The culture was heated at 65°C for 15 min, and the cell debris was removed by centrifugation at 5311 x *g* for 5 min. Dimethylsulfoxide was added to the supernatant to a final concentration of 7 % (v/v) as a cryoprotectant, the titre of the phage suspension was determined, and aliquots were frozen at -80°C until use.

Helper phage was titred by diluting phage in 50 mM tris-EDTA buffer to achieve dilutions of 10^{-4} to 10^{-7}, 100 µL of each dilution was added to 200 µL of ice-cold *E. coli* XL1-Blue cells at an OD_{600} of 1.0, and dilutions were incubated at 37°C for 15 min to allow phage attachment. Three millilitres of molten (~48°C) sterile 2xYT solidified with 0.7 % (w/v) agarose and supplemented with 5 mM $MgCl_2$ was added, and the dilution was immediately poured onto pre-warmed (37°C) 2xYT medium solidified with 1.5 % (w/v) agar and supplemented with 5 mM $MgCl_2$. Plates were allowed to solidify for 5 min, and were incubated at 37°C overnight. Plaques were counted, and phage titre [plaque-forming units (PFU) mL^{-1}] was calculated.

2.2.15 Antibody specificity

Cross-reactivity of the phages which were identified as strongly-binding leucocin-A as an antigen was determined by ELISA. The peptides tested were brochocin-C, nisin, sakacin A, enterocin 7A, pediocin PA1, brochocin-A-BSA,

brochocin-A-KLH, piscicolin 126-BSA, piscicolin 126-KLH, leucocin A, leucocin A-BSA, and BSA. Production or procurement of brochocin-C, nisin, sakacin A, enterocin 7A, and pediocin PA1 are described in Chapter Four:. One hundred microlitres of each antigen dissolved in PBS (10 ng μL^{-1}) was added in triplicate wells of a Maxisorp microtitre plate (Nalge Nunc), including an ELISA antibody control containing 6.7 x 10^{11} PFU mL^{-1} of VCSM13 helper phage. The same coating, washing, and ELISA protocols described in the previous section were used.

2.3 Results

2.3.1 Purification of leucocin A

The modified method of purifying leucocin A produced a single peak at 7.6 min following re-injection of the pooled fractions containing antibacterial activity (Figure 2-2). Fractions of this highly-purified peptide were collected and used in subsequent experiments. Following conjugation to KLH and desalting to remove excess KLH, fractions were collected and tested for protein content to determine the location of the KLH-leucocin A conjugate. Two broad protein-containing peaks were observed (Figure 2-3). Based on product literature, the first peak was determined to be the conjugate, and the second peak is excess KLH. Analysis by matrix-assisted laser desorption/ionization-time of flight was considered, but due to the wide range of large sizes of KLH (450 – 1300 kDa) relative to the leucocin A (3930 Da), the addition of leucocin A would be difficult to identify (R. Whittal, University of Alberta, personal communication). SDS-PAGE was also attempted, but the maximum mass resolvable by SDS-PAGE [5 % (w/v) acrylamide] is 57 – 212 kDa (Sambrook and Russell, 2001), which is much lower than KLH or the conjugate. Thus, the fraction in the first peak (fraction six) was used for subsequent immunization of mice.

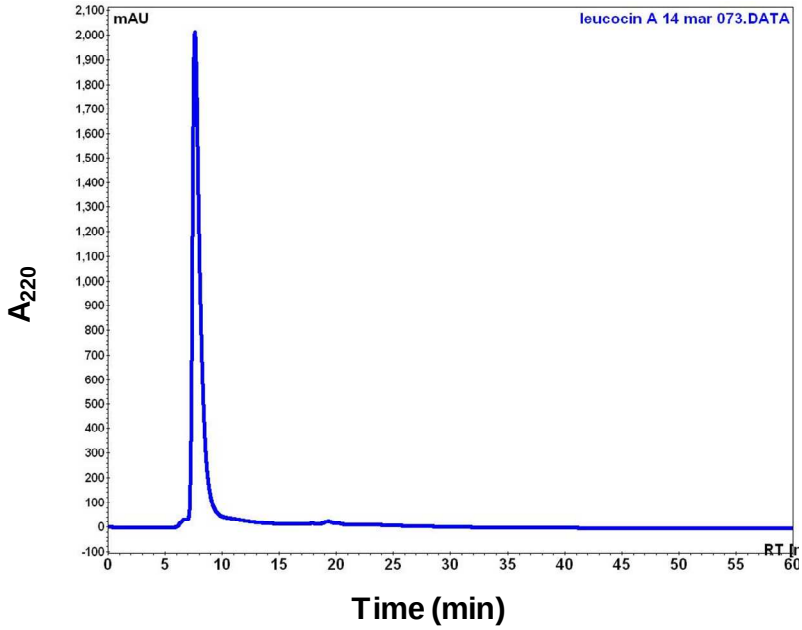

Figure 2-2 HPLC trace showing purified leucocin A following two gel filtration purification steps

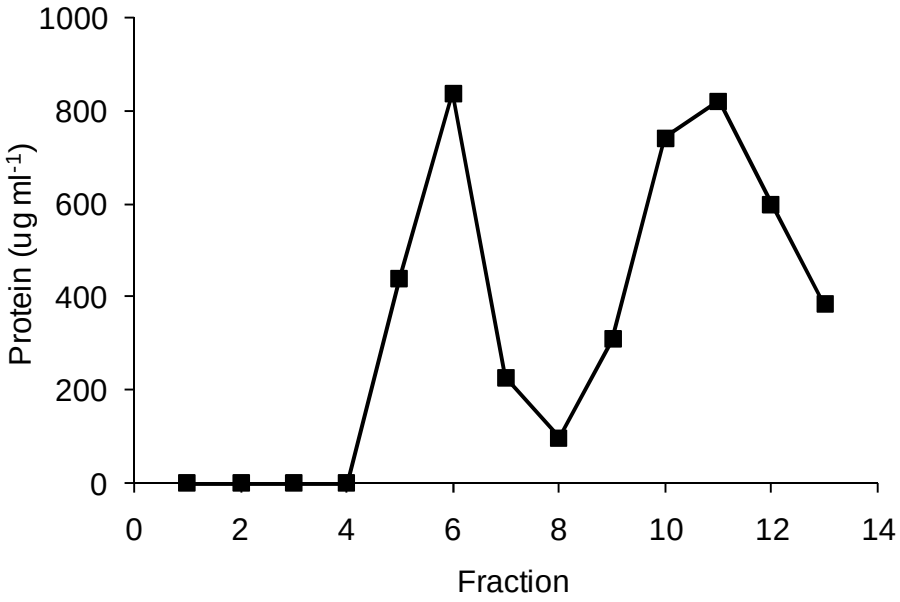

Figure 2-3 Results of purification of KLH-leucocin A conjugate for use as an antigen in mice

2.3.2 Immune response to bacteriocins in mice

Antibodies were raised in mice to unconjugated leucocin A, and KLH conjugates of leucocin A, piscicolin 126, and brochocin-A. Maximum antibody titres were highest for leucocin A conjugated to KLH, and were lowest for the brochocin-A(27-71)-KLH fragment (Table 2-2). Unconjugated leucocin A was able to induce an immune response, though not as high as that produced by its conjugated form. Leucocin A required three booster injections to reach a minimum antibody titre of 1:500, piscicolin 126 required four booster injections, while brochocin-A required five booster injections.

Table 2-2 Maximum serum antibody titres in BALB/c mice immunized with bacteriocin or bacteriocin fragments

Bacteriocin	Portion used as antigen	Antibody titre (mice/total mice)
Leucocin A	Leucocin A	1:5000 (3/5): 1:1000 (2/5)
Leucocin A	KLH-leucocin A	1:10000 (3/5); 1:5000 (2/5)
Piscicolin 126[1]	KLH-(30-44 C-terminus)	1:5000 (2/5); 1:1000 (3/5)
Brochocin-A	KLH-(27-51 C-terminus)	1:500 (1/6)

[1] 1 mouse was euthanized prematurely due to anaphylaxis during final injection

2.3.3 Amplification of V_L and V_H genes, and single overlap extension PCR

Amplification of V_L genes was successful for mice inoculated with leucocin A preparations. A strong band in the expected size range of 375 and 402 bp (Burmester and Plückthun, 2001) was obtained for both leucocin A alone and the leucocin A conjugate (Figure 2-4). An annealing temperature of 55°C was determined to be optimal for both samples. Amplification of V_H genes was also successful, as shown by the bands shown in Figure 2-5 in the expected size range of 386 bp to 440 bp (Burmester and Plückthun, 2001). Annealing temperatures of either 50°C or 55°C were suitable for amplification of V_H genes. One observation is that amplification of V_H genes required freshly-synthesized primer mix, as primer mixes more than several months in age resulted in low PCR product yield (data not shown). Amplification of V_L was more successful than amplification of

V_H, with V_H PCR products often requiring concentration prior to subsequent single overlap extension PCR. A band at the 700 bp position was also observed when V_H was amplified (Figure 2-5).

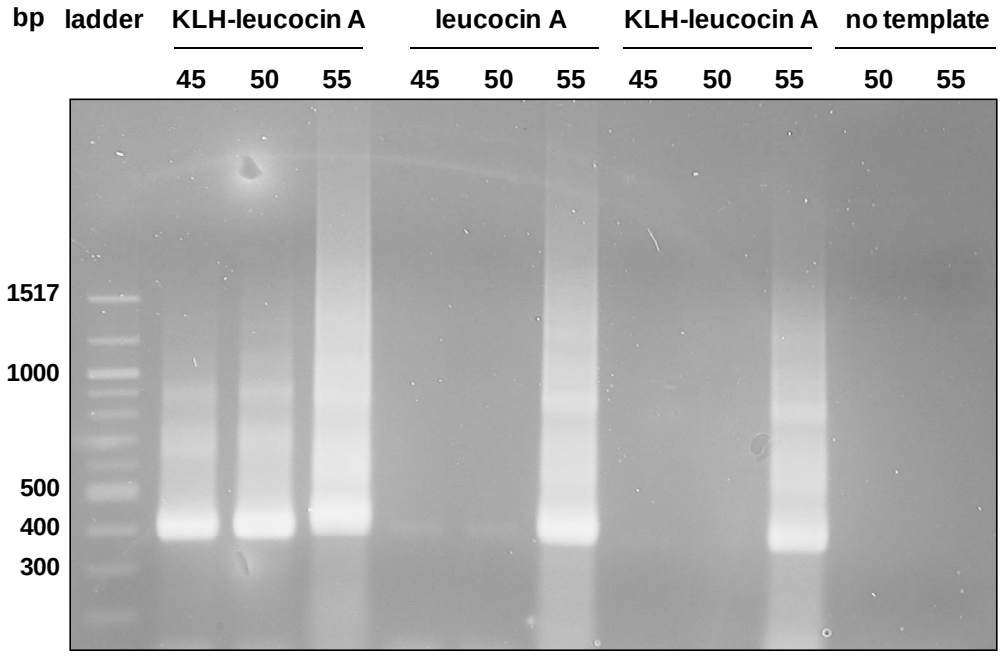

Figure 2-4 Results of PCR amplification of V_L genes from cDNA of mice immunized with leucocin A preparations using different annealing temperatures. Two different preparations of mRNA from the same KLH-leucocin A-immunized mouse are shown in lanes 2-4 and 8-10.

Single overlap extension PCR resulted in a product of the expected size of approximately 800 bp (Figure 2-6). Products of approximately 10 PCR reactions were pooled and concentrated to have sufficient yield to proceed with restriction digestion, ligation, and cloning. For ligation, vector:insert ratios of 1.5:1, 5:1, and 10:1 were tested, and a ratio of 10:1 was found to be optimal for leucocin A and piscicolin 126, as it produced the most transformants.

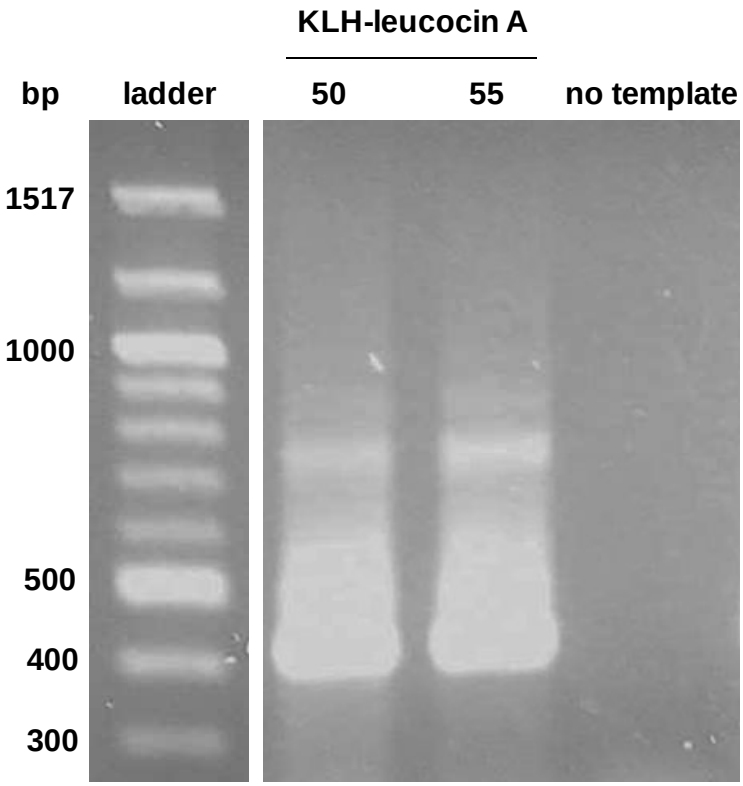

Figure 2-5 Results of PCR amplification of V_H genes from cDNA of mice immunized with KLH-leucocin A using different annealing temperatures.

Two related phagemid vectors, pAK100 and pJB12, were tested to determine which would yield the highest number of transformants in the library. It was observed that restriction digestion of pAK100 resulted in a much clearer product than digestion of pJB12. Running the gel for a longer time did not result in a more defined band (data not shown). Although pAK100 demonstrated a much clearer band following restriction digestion, when transformation of *E. coli* TG1 was performed in a preliminary experiment with KLH-leucocin A samples, pJB12 produced a higher number of transformants (533) as compared to 30 colonies by pAK100, and so pJB12 was selected for all subsequent experiments.

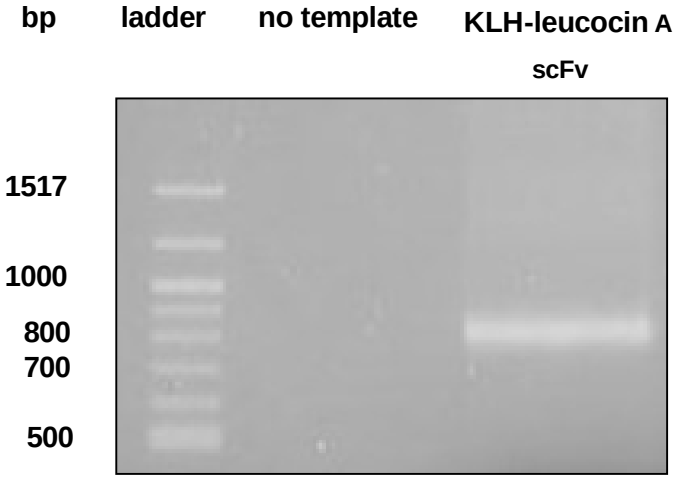

Figure 2-6 Results of single-overlap extension PCR of V_H and V_L genes from cDNA of mice immunized with KLH-leucocin A.

Approximately 20 transformations were performed for the KLH-leucocin A sample, with a total library size of approximately 10^5 clones. Of these, eight clones were determined to strongly bind BSA-leucocin A. The results of the ELISA assay are shown in Figure 2-7.

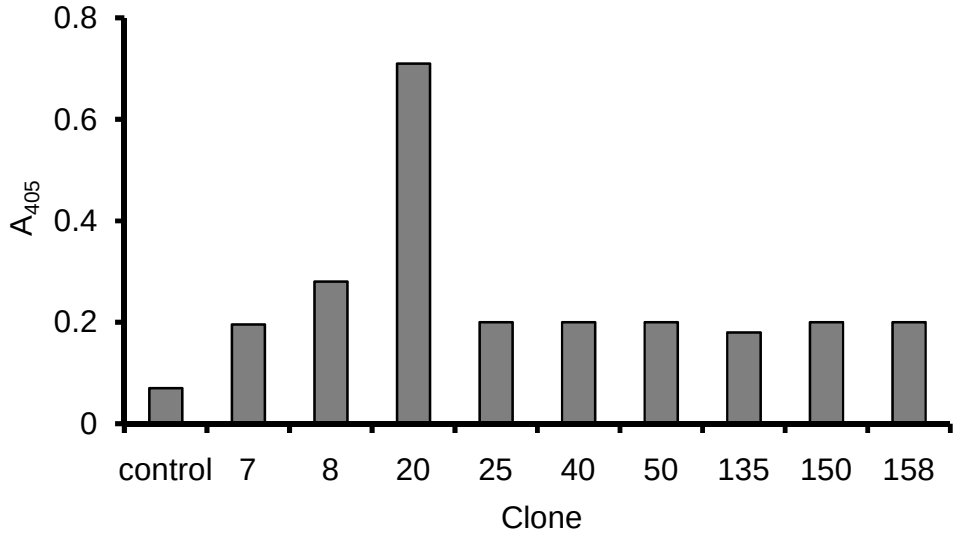

Figure 2-7 Identification of clones of *E. coli* TG1pJB12 with strong ability to bind BSA-leucocin A by ELISA. Strong-binding clones were defined as those producing an absorbance of at least 0.2, with an absorbance at least twice that of the background control.

The KLH-piscicolin 126(30-44) library was approximately the same size as the KLH-leucocin A library. Following three rounds of panning, 126 clones remained, and were tested by ELISA for their ability to bind BSA-piscicolin 126. However, no strongly-binding clones were identified, and so no further work was conducted.

2.3.4 Soluble antibody production

Following transformation into a strain suitable for soluble antibody production, the Western blot analysis revealed that none of the clones had produced a peptide containing the FLAG™ sequence, as shown by a representative Western blot of one clone in Figure 2-8. Although the background is high, no strong band was observed.

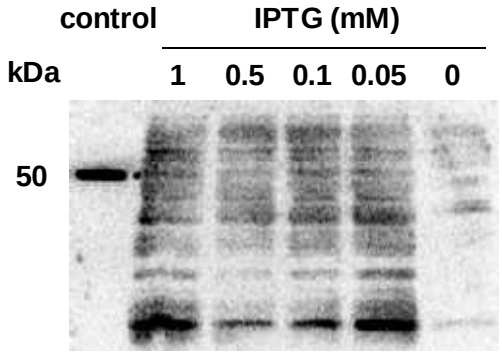

Figure 2-8 Western blot of BSA-leucocin A-binding clone 7 induced by varying concentrations of IPTG. Presence of the FLAG™ peptide is shown by the control peptide in lane one.

As none of the clones were found to produce a FLAG™-containing peptide, the sequence of the scFv insert and the pJB12 vector was determined. Based on these results, it was found that all eight of the clones had retained the original tet^r cassette found in the original phagemid vector, and did not contain a scFv sequence.

To determine the nature of this unexpected binding to BSA-leucocin A-coated plates, further ELISA studies were done using other bacteriocins and bacteriocin fragments of different classes. None of the clones demonstrated

binding of leucocin A when tested (Table 2-3), but three of the eight clones did bind the KLH conjugate of piscicolin 126(30-44). Brochocin-C was bound by five of the eight clones, as well as one clone which had previously been identified as weakly binding leucocin A, but no binding was observed with the brochocin-A C-terminus conjugated to either BSA or KLH. Neither BSA- nor KLH-specific antibodies were responsible for the initial binding, because BSA conjugated to piscicolin 126(30-44) or brochocin A(27-51) and KLH-piscicolin 126(30-44) were not bound. No other bacteriocins were bound by any clones.

2.4 Discussion

Immunochemical methods of detection are suitable for detection of bacteriocins because of their specificity and sensitivity. However, bacteriocins have been found to induce a weak immune response, likely due to their small size (Diep and Nes, 2002). Attempts to raise antibodies to bacteriocins alone have been unsuccessful with the bacteriocins pediocin PA-1(Bhunia et al., 1990), piscicolin 126 (Ingham et al., 2003), and nisin (Daoudi et al., 2001). However, in this study, relatively high antibody titres (1:5000) were observed in mice immunized with the class IIa bacteriocin leucocin A, which is the first report of antibodies to this bacteriocin. The higher antibody titres observed when leucocin A or a 15-amino acid fragment of piscicolin 126 was conjugated to the carrier protein KLH is expected, as carrier proteins are believed to improve immunogenicity by increasing the number of epitopes available for recognition (Lewis, 2001).

This is in agreement with published reports of antibodies to divercin V41(Richard et al., 2004), enterocin A (Martínez et al., 2000), enterocin B (Rose et al., 2001), enterocin P (Gutierrez et al., 2004), lacticin RM (Keren et al., 2004), nisin A (Falahee et al., 1990; Falahee and Adams, 1992; Suárez et al., 1996a; Suárez et al., 1996b; Leung et al., 2002), nisin Z, (Bouksaim et al., 1998; Bouksaim et al., 1999; Daoudi et al., 2001), pediocin PA-1 (Bhunia et al., 1990; Martínez et al., 1997; Martínez et al., 1998; Martínez et al., 1999), and pediocin RS2 (Bhunia, 1994), all of which were produced with conjugation to larger carrier proteins. Thus, the murine immune system can recognize the complete class IIa

Table 2-3 Cross-reactivity of leucocin A-binding clones to different bacteriocins and proteins as indicated by A_{405} in an ELISA

Clone	A_{405}										
	Nisin	Leucocin A	Sakacin A	Enterocin 7A	Pediocin PA-1	BSA-Piscicolin 126(30-44)	KLH-Piscicolin 126(30-44)	Brochocin-C	BSA-Brochocin-A(27-51)	KLH-Brochocin-A(27-51)	BSA
7	0	0	0	0	0	0	0.16	0.25	0	0	0
8	0	0	0	0	0	0	0.13	0.32	0	0	0
20	0	0	0	0	0	0	0.14	0.19	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0
125	0	0	0	0	0	0	0	0.12	0	0	0
150	0	0	0	0	0	0	0	0	0	0	0
158	0	0	0	0	0	0	0	0.42	0	0	0
19*	0	0	0	0	0	0	0	0	0	0	0
143*	0	0	0	0	0	0	0	0.14	0	0	0

* randomly-selected weakly-binding clones included as controls

bacteriocin leucocin A, complete leucocin A conjugated to KLH, or a fragment of another class IIa bacteriocin, piscicolin 126.

Despite an immune response to KLH-leucocin A and KLH-piscicolin 126(30-44) *in vivo*, phagemids bearing strong bacteriocin-specific scFv antibody fragments were not isolated from the murine immune libraries. Several possible explanations for this exist. First, because of the small size of the bacteriocin relative to the carrier KLH, a low proportion of bacteriocin-specific B cells may have been activated. Hapten size limits the number of hapten-specific epitopes available for recognition by the immune system; most epitopes are at least six amino acids in length, so the smaller size of bacteriocins relative to their carriers means less chance of recognition because of a lower number of epitopes (Harlow and Lane, 1988). Second, PCR amplification and linking of V_L and V_H genes may have led to assembly of carrier-specific and bacteriocin-specific antibody chains in the same molecule, resulting in an antibody fragment with low affinity or non-functional capabilities (Kramer, 2002b).

To avoid these problems, Kramer (2002b) enriched for B cells bearing the relevant anti-hapten V genes by immunomagnetic screening prior to cloning and phage display. These workers isolated 0.16 % of the total B cell population by linking the herbicide triazine to paramagnetic particles, and separated B cells capable of binding triazine from all others using a magnet. In this way, the B cells containing the relevant anti-triazine V genes were selected, irrelevant V genes were discarded, and specific soluble scFv fragments were produced (Kramer, 2002b). Compared to an unenriched control library, the enriched library contained a significantly higher number of hapten-specific scFv. Based on the unsuccessful results of the current study, this strategy may be useful for phage display of bacteriocin-specific antibody fragments.

Another possibility for the lack of bacteriocin-specific scFv in this study is the small size of the immune library. Immune libraries can be smaller than naive libraries because their B cells are biased to recognize the antigen of interest; most immune libraries have $10^6 – 10^7$ clones (Brichta et al., 2005), as compared to the larger naive libraries of $10^8 – 10^{11}$ clones (Sblattero and Bradbury, 2000; Brichta

et al., 2005). The libraries in this study were small (10^5), but because strong-binding clones were identified, no further work was completed to expand the size. Future work could expand the library size to improve diversity and locate bacteriocin-specific scFv; however, there is evidence that library size may not be the only factor in the success of phage display for anti-hapten antibody fragments (Yau et al., 2003). Yau et al. (2003) reported the failure of a large (5.4×10^8 clones) naive llama library to yield phage specific for the pesticide picloram, and instead successfully constructed a ribosome-display library specific for this hapten. Thus, forms of antibody display other than phage or phagemid may be necessary for some haptenic antigens.

In previous reports of the production of antibodies to divercin V41, enterocins A and P, lacticin RM, and pediocin PA-1, synthetic fragments of each bacteriocin were capable of inducing immune responses. In this experiment, synthetic fragments consisting of the first 15 amino acids of the C-terminus of piscicolin 126 and 21 amino acids at the N-terminus of brochocin-A were conjugated to KLH, and used as antigens. A relatively high titre of antibodies (1:5000) was raised to the synthetic fragment of piscicolin 126, but antibodies to brochocin-A were present at a 10-fold lower titre (1:500). Although antibodies have been raised to several class IIa bacteriocins, such as enterocin and pediocin, there have been no reports of strong antibodies raised to a hydrophobic, two-peptide bacteriocin such as brochocin-C, though there is one report of weakly-binding antibodies raised in rabbits to a fragment of brochocin-A (Kwok, 1997). Although attempts were made to choose the most immunogenic portion of both brochocins-A and -B using predictive modeling software, the complex nature of immunogenicity precludes prediction of definite immunogenic synthetic peptides (Van Regenmortel, 2001). Animal species may play a role, as shown by the example of a synthetic nine-amino acid N-terminus of pediocin PA-1 conjugated to KLH. Functional polyclonal antibodies could be produced in rabbits (Martínez et al., 1999), but antibodies raised in mice were unable to detect the bacteriocin (Martínez et al., 1997). Absence of a class II protein required for a specific immune response is more common in animals such as laboratory strains of mice

than in outbred animals such as rabbits (Harlow and Lane, 1988); thus, choice of animal for optimal immune response may be important in the case of some bacteriocins.

In the case of brochocin-A, one key point is the hydrophobicity of the peptide. Brochocin-A has a calculated grand average of hydropathicity (GRAVY) (Kyte and Doolittle, 1982; Gasteiger et al., 2003) of 0.880, which means that it is highly hydrophobic. This property has led to difficulties with purification and sequencing (Garneau et al., 2003), and may also cause problems in immune response. Hydrophilic amino acids are favourable in immunogens for several reasons; first, they are more exposed and thus available for recognition, and second, hydrophilic peptides are more readily soluble for coupling reactions (Harlow and Lane, 1988). Some solubility issues were encountered during peptide resuspension in the current study, which also illustrates the hydrophobicity of the conjugate. Thus, the hydrophobic nature of brochocin-A may be one reason for its low immunogenicity.

One unexpected result from this study was the discovery that intact pJB12 phagemid bound bacteriocin in ELISA, although this binding did not appear to be reproducible. In the initial ELISA screening for strong leucocin-A binding clones, eight clones were identified; in a later assay, leucocin A was not bound. However, the C-terminus of piscicolin 126 and intact brochocin-C were bound by several clones. Although non-specific binding may account for this observation, another possibility is that the tet^r cassette recognizes bacteriocins, either complete or a portion thereof, in addition to tetracycline. In pJB12, the tet^r cassette is composed of tetA and tetR (Krebber et al., 1997), which encode a membrane-bound efflux protein (Chopra and Roberts, 2001). However, tetracycline is composed of four fused rings (Chopra and Roberts, 2001), and bears little resemblance to the linear structures of leucocin A, piscicolin A, or brochocin-C. Thus, the nature of this proposed interaction is unclear, and would benefit from further study.

2.5 References

Ahn, C. and Stiles, M.E. (1990) Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl Environ Microbiol 56, 2503-2510.

Alvarez-Rueda, N., Behar, G., Ferré, V., Pugnière, M., Roquet, F., Gastinel, L., Jacquot, C., Aubry, J., Baty, D., Barbet, J. and Birklé, S. (2007) Generation of llama single-domain antibodies against methotrexate, a prototypical hapten. Mol Immunol 44, 1680-1690.

Ames, R.S., Tornetta, M.A., Deen, K., Jones, C.S., Swift, A.M. and Ganguly, S. (1995) Conversion of murine Fabs isolated from a combinatorial phage display library to full length immunoglobulins. J Immunol Methods 184, 177-186.

Batteiger, B., Newhall, W. and Jones, R. (1982) The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J Immunol Methods 55, 297-307.

Bhunia, A.K. (1994) Monoclonal antibody-based enzyme immunoassay for pediocins of *Pediococcus acidilactici*. Appl Environ Microbiol 60, 2692-2696.

Bhunia, A.K., Johnson, M.C., Bibek, R. and Belden, E.L. (1990) Antigenic property of pediocin AcH produced by *Pediococcus acidilactici* H. J Appl Bacteriol 69, 211-215.

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.-M., Lee, T., Pope, S.H., Riordan, G.S. and Whitlow, M. (1988) Single-chain antigen-binding proteins. Science 241, 423-426.

Bouksaim, M., Lacroix, C., Bazin, R. and Simard, R.E. (1999) Production and utilization of polyclonal antibodies against nisin in an ELISA and for immuno-location of nisin in producing and sensitive strains. J Appl Microbiol 87, 500-510.

Bouksaim, M., Fliss, I., Meghrous, J., Simard, R. and Lacroix, C. (1998) Immunodot detection of nisin Z in milk and whey using enhanced chemiluminescence. J Appl Microbiol 84, 176-184.

Bowen, R.A. (1998) Molecular Toolkit: Protein Hydrophobicity Plots. http://www.vivo.colostate.edu/molkit/hydropathy/index.htm. Colorado State University, last updated 20 June 2008.

Bradbury, A.R.M. and Marks, J.D. (2004) Antibodies from phage antibody libraries. J Immunol Methods 290, 29-49.

Brichta, J., Hnilova, M. and Viskovic, T. (2005) Generation of hapten-specific recombinant antibodies: antibody phage display technology: a review. Vet Med-Czech 50, 231-252.

Brichta, J., Vesela, H. and Franek, M. (2003) Production of scFv recombinant fragments against 2,4-dichlorophenoxyacetic acid hapten using naive phage library. Vet Med-Czech 48, 237-247.

Burmester, J. and Plückthun, A. (2001) Construction of scFv fragments from hybridoma or spleen cells by PCR assembly. In: R. Kontermann and S. Dübel (Eds), Antibody Engineering. Springer-Verlag, New York, p. 19-40.

Burmester, J., Spinelli, S., Pugliese, L., Krebber, A., Honegger, A., Jung, S., Schimmele, B., Cambillau, C. and Plückthun, A. (2001) Selection, characterization and X-ray structure of anti-ampicillin single-chain Fv fragments from phage-displayed murine antibody libraries. J Mol Biol 309, 671-685.

Canada Council on Animal Care (2002) Guidelines on Antibody Production. Ottawa, ON.

Chopra, I. and Roberts, M. (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 65, 232-260.

Daoudi, L., Turcotte, C., Lacroix, C. and Fliss, I. (2001) Production and characterization of anti-nisin Z monoclonal antibodies: suitability for distinguishing active from inactive forms through a competitive enzyme immunoassay. Appl Microbiol Biotechnol 56, 114-119.

Diep, D.B. and Nes, I.F. (2002) Ribosomally synthesized antibacterial peptides in gram positive bacteria. Curr Drug Targets 3, 107-122.

Doyle, P.J., Arbabi-Ghahroudi, M., Gaudette, N., Furzer, G., Savard, M.E., Gleddie, S., McLean, M.D., Mackenzie, C.R. and Hall, J.C. (2008) Cloning, expression, and characterization of a single-domain antibody fragment with affinity for 15-acetyl-deoxynivalenol. Mol Immunol 45, 3703-3713.

Falahee, M.B. and Adams, M.R. (1992) Cross-reactivity of bacteriocins from lactic-acid bacteria and lantibiotics in a nisin bioassay and ELISA. Lett Appl Microbiol 15, 214-216.

Falahee, M.B., Adams, M.R., Dale, J.W. and Morris, B.A. (1990) An enzyme immunoassay for nisin. Int J Food Sci Tech 25, 590-595.

Finlay, W.J.J., Shaw, I., Reilly, J.P. and Kane, M. (2006) Generation of high-affinity chicken single-chain Fv antibody fragments for measurement of the *Pseudonitzschia pungens* toxin domoic acid. Appl Environ Microbiol 72, 3343-3349.

Garet, E., Cabado, A.G., Vieites, J.M. and González-Fernández, Á. (2010) Rapid isolation of single-chain antibodies by phage display technology directed against one of the most potent marine toxins: palytoxin. Toxicon 55, 1519-1526.

Garneau, S., Ference, C.A., van Belkum, M.J., Stiles, M.E. and Vederas, J.C. (2003) Purification and characterization of brochocin A and brochocin B(10-43), a functional fragment generated by heterologous expression in *Carnobacterium piscicola*. Appl Environ Microbiol 69, 1352-1358.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D. and Bairoch, A. (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 31, 3784-3788.

Ge, L., Knappik, A., Pack, P., Freund, C. and Plückthun, A. (1995) Expressing antibodies in *Escherichia coli*. In: C.A.K. Borrebaeck (Ed) Antibody Engineering. Oxford University Press, New York, NY, p. 229-266.

Gutierrez, J., Criado, R., Citti, R., Martin, M., Herranz, C., Fernandez, M.F., Cintas, L.M. and Hernandez, P.E. (2004) Performance and applications of polyclonal antipeptide antibodies specific for the enterococcal bacteriocin enterocin P. J Agric Food Chem 52, 2247-2255.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166, 557-580.

Hannigan, B.M., Moore, C.B.T. and Quinn, D.G. (2009) Immunology. Scion Publishing, Banbury, UK.

Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Hastings, J.W., Sailer, M., Johnson, K., Roy, K.L., Vederas, J.C. and Stiles, M.E. (1991) Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J Bacteriol 173, 7491-7500.

Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. and Oppermann, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. P Natl Acad Sci USA 85, 5879-5883.

Ingham, A., Ford, M., Moore, R.J. and Tizard, M. (2003) The bacteriocin piscicolin 126 retains antilisterial activity *in vivo*. J Antimicrob Chemother 51, 1365-1371.

Johnson, G. (2008) The Kabat Database of Sequences of Proteins of Immunological Interest. http://www.kabatdatabase.com/index.html. Last updated 1 July 2008.

Johnson, G. and Wu, T.T. (2000) Kabat database and its applications: 30 years after the first variability plot. Nucleic Acids Res 28, 214-218.

Kabat, E.A. and Wu, T.T. (1991) Identical V region amino acid sequences and segments of sequences in antibodies of different specificities: relative contributions of V(H) and V(L) genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites. J Immunol 147, 1709-1719.

Keren, T., Yarmus, M., Halevy, G. and Shapira, R. (2004) Immunodetection of the bacteriocin lacticin RM: analysis of the influence of temperature and Tween 80 on its expression and activity. Appl Environ Microbiol 70, 2098-2104.

Knappik, A. and Plückthun, A. (1994) An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments. Biotechniques 17, 754-761.

Kobayashi, N., Kato, Y., Oyama, H., Taga, S., Niwa, T., Sun, P., Ohtoyo, M. and Goto, J. (2008) Anti-estradiol-17 beta single-chain Fv fragments: generation, characterization, gene randomization, and optimized phage display. Steroids 73, 1485-1499.

Köhler, G. and Milstein, C. (1976) Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur J Immunol 6, 511-519.

Kolaskar, A.S. and Tongaonkar, P.C. (1990) A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett 276, 172-174.

Korpimäki, T., Rosenberg, J., Virtanen, P., Karskela, T., Lamminmäki, U., Tuomola, M., Vehniäinen, M. and Saviranta, P. (2002) Improving broad specificity hapten recognition with protein engineering. J Agr Food Chem 50, 4194-4201.

Kramer, K. (2002a) Evolutionary affinity and selectivity optimization of a pesticide-selective antibody utilizing a hapten-selective immunoglobulin repertoire. Environ Sci Technol 36, 4892-4898.

Kramer, K. (2002b) Synthesis of a group-selective antibody library against haptens. J Immunol Methods 266, 209-220.

Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H.R. and Pluckthun, A. (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J Immunol Methods 201, 35-55.

Kwok, S.K.A. (1997) Enzyme-linked immunosorbent assay development for advanced glycation end products and brochocin-C. MSc thesis, Department of Agricultural, Food and Nutritional Science. University of Alberta, Edmonton, AB, p. 89.

Kyte, J. and Doolittle, R. (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157, 105-132.

Leung, P.P., Khadre, M., Shellhammer, T.H. and Yousef, A.E. (2002) Immunoassay method for quantitative determination of nisin in solution and on polymeric films. Lett Appl Microbiol 34, 199-204.

Lewis, D.H. (2001) Antigens. In: G.C. Howard and D.R. Bethell (Eds), Basic Methods in Antibody Production and Characterization. CRC Press, New York, p. 11-18.

Li, T., Zhang, Q., Liu, Y., Chen, D., Hu, B., Blake, D.A. and Liu, F. (2006) Production of recombinant ScFv antibodies against methamidophos from a phage-display library of a hyperimmunized mouse. J Agr Food Chem 54, 9085-9091.

Madigan, M.T., Martinko, J.M. and Parker, J. (2003) Brock Biology of Microorganisms. Pearson Education, Toronto, ON.

Makvandi-Nejad, S., Sheedy, C., Veldhuis, L., Richard, G. and Hall, J.C. (2010) Selection of single chain variable fragment (scFv) antibodies from a hyperimmunized phage display library for the detection of the antibiotic monensin. J Immunol Methods 360, 103-118.

Martínez, J.M., Kok, J., Sanders, J.W. and Hernandez, P.E. (2000) Heterologous coproduction of enterocin A and pediocin PA-1 by *Lactococcus lactis*: detection by specific peptide-directed antibodies. Appl Environ Microbiol 66, 3543-3549.

Martínez, J.M., Martínez, M.I., Herranz, C., Suárez, A., Fernández, M.F., Cintas, L.M., Rodríguez, J.M. and Hernández, P.E. (1999) Antibodies to a synthetic 1-9-N-terminal amino acid fragment of mature pediocin PA-1: sensitivity and specificity for pediocin PA-1 and cross-reactivity against class IIa bacteriocins. Microbiology 145, 2777-2787.

Martínez, J.M., Martínez, M.I., Suárez, A.M., Herranz, C., Casaus, P., Cintas, L.M., Rodríguez, J.M. and Hernández, P.E. (1998) Generation of polyclonal antibodies of predetermined specificity aginst pediocin PA-1. Appl Environ Microbiol 64, 4536-4545.

Martínez, M.I., Rodríguez, J.M., Suárez, A., Azcona, J.I. and Hernández, P.E. (1997) Generation of polyclonal antibodies against a chemically synthesized N-terminal fragment of the bacteriocin pediocin PA-1. Lett Appl Microbiol 24, 488-492.

McCafferty, J., Griffiths, A.D., Winter, G. and Chiswell, D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348, 552-554.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E. and van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. Appl Environ Microbiol 64, 4757-4766.

Model, P. and Russel, M. (1988) Filamentous bacteriophage. In: R. Calendar (Ed) The Bacteriophages, Vol. 2, The Viruses. Plenum Press, New York, p. 375-456.

Moghaddam, A., Borgen, T., Stacy, J., Kausmally, L., Simonsen, B., Marvik, O.J., Brekke, O.H. and Braunagel, M. (2003) Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monoacetylmorphine but not morphine. J Immunol Methods 280, 139-155.

Moghaddam, A., Løbersli, I., Gebhardt, K., Braunagel, M. and Marvik, O.J. (2001) Selection and characterisation of recombinant single-chain antibodies to the hapten aflatoxin-B1 from naive recombinant antibody libraries. J Immunol Methods 254, 169-181.

Nagumo, Y., Oguri, H., Tsumoto, K., Shindo, Y., Hirama, M., Tsumuraya, T., Fujii, I., Tomioka, Y., Mizugaki, M. and Kumagai, I. (2004) Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. J Immunol Methods 289, 137-146.

Olea-Popelka, F., McLean, M.D., Horsman, J., Almquist, K., Brandle, J.E. and Hall, J.C. (2005) Increasing expression of an anti-picloram single-chain variable fragment (ScFv) antibody and resistance to picloram in transgenic tobacco (*Nicotiana tabacum*). J Agr Food Chem 53, 6683-6690.

Ørum, H., Andersen, P.S., Øster, A., Johansen, L.K., Riise, E., Bjørnvad, M., Svendsen, I. and Engberg, J. (1993) Efficient method for construction of comprehensive murine Fab antibody libraries displayed on phage. Nucleic Acids Res 21, 4491-4498.

Pan, K., Wang, H., Zhang, H.-b., Liu, H.-w., Lei, H.-t., Huang, L. and Sun, Y.-m. (2006) Production and characterization of single chain Fv directed against B_2-agonist clenbuterol. J Agr Food Chem 54, 6654-6659.

Reche, P. (2007) Tools: Predicting antigenic peptides. http://bio.dfci.harvard.edu/Tools/antigenic.html. Cancer Vaccine Center Bioinformatics, Harvard University. Last updated 28 May 2007.

Richard, C., Drider, D., Fliss, I., Denery, S. and Prevost, H. (2004) Generation and utilization of polyclonal antibodies to a synthetic C-terminal amino acid fragment of divercin V41, a class IIa bacteriocin. Appl Environ Microbiol 70, 248-254.

Rose, N.L., Sporns, P. and McMullen, L.M. (2001) Production of antibodies against enterocin B for immunological detection and purification purposes. Food Agr Immunol 13, 225-239.

Sambrook, J. and Russell, R.W. (2001) Molecular Cloning: A Laboratory Manual, Vol. 3. Cold Spring Harbor Press, Cold Spring Harbour, NY.

Sblattero, D. and Bradbury, A. (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. Nat Biotechnol 18, 75.

Shaw, I., O'Reilly, A., Charleton, M. and Kane, M. (2008) Development of a high-affinity anti-domoic acid sheep scFv and its use in detection of the toxin in shellfish. Anal Chem 80, 3205-3212.

Sheedy, C., Yau, K.Y.F., Hirama, T., MacKenzie, C.R. and Hall, J.C. (2006) Selection, characterization, and CDR shuffling of naive llama single-domain antibodies selected against auxin and their cross-reactivity with auxinic herbicides from four chemical families. J Agr Food Chem 54, 3668-3678.

Smith, G. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228, 1315-1317.

Suárez, A., Rodriguez, J., Hernandez, P. and Azcona-Olivera, J. (1996a) Generation of polyclonal antibodies against nisin: immunization strategies and immunoassay development. Appl Environ Microbiol 62, 2117-2121.

Suárez, A.M., Rodríguez, J.M., Morales, P., Hernández, P.E. and Azcona-Olivera, J.I. (1996b) Development of monoclonal antibodies to the lantibiotic nisin A. J Agr Food Chem 44, 2936-2940.

Tout, N.L., Yau, K.Y.F., Trevors, J.T., Lee, H. and Hall, J.C. (2001) Synthesis of ligand-specific phage-display ScFv against the herbicide picloram by direct cloning from hyperimmunized mouse. J Agr Food Chem 49, 3628-3637.

Van Regenmortel, M.H.V. (2001) Antigenicity and immunogenicity of synthetic peptides. Biologicals 29, 209-213.

Webster, R. (2001) Filamentous phage biology. In: C.F. Barbas III, D.R. Burton, J.K. Scott and G.J. Silverman (Eds), Phage Display: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, p. 1.1-1.37.

Williamson, R.A., Burioni, R., Sanna, P.P., Partridge, L.J., Barbas, C.F. and Burton, D.R. (1993) Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries. Proc Natl Acad Sci USA 90, 4141-4145.

Worobo, R.W., van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. (1995) A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. J Bacteriol 177, 3143-3149.

Yang, Z.Y., Shim, W.B., Kim, M.G., Lee, K.H., Kim, K.S., Kim, K.Y., Kim, C.H., Ha, H.D. and Chung, D.H. (2007) Production and characterization of monoclonal and recombinant antibodies against antimicrobial sulfamethazine. J Microbiol Biotechnol 17, 571-578.

Yau, K.Y.F., Groves, M.A.T., Li, S.H., Sheedy, C., Lee, H., Tanha, J., MacKenzie, C.R., Jermutus, L. and Hall, J.C. (2003) Selection of hapten-specific single-domain antibodies from a non-immunized llama ribosome display library. J Immunol Methods 281, 161-175.

Yau, K.Y.F., Tout, N.L., Trevors, J.T., Lee, H. and Hall, J.C. (1998) Bacterial expression and characterization of a picloram-specific recombinant Fab for residue analysis. J Agr Food Chem 46, 4457-4463.

Zhang, X., Zhang, C., Liu, Y., Yu, X. and Liu, X. (2010) Construction of scFv phage display library with hapten-specific repertories and characterization of anti-ivermectin fragment isolated from the library. Eur Food Res Technol 231, 423-430.

Chapter Three: **Effect of brochocin-C on *Clostridium botulinum* growth in model and meat systems**

3.1 Introduction

Clostridium botulinum is a gram-positive, anaerobic, spore-forming bacillus indigenous to soil and aquatic habitats. It is also a potent pathogen which exerts its pathogenicity via a neurotoxin with an estimated LD_{50} of one ng kg^{-1} in mice (Gill, 1982), making it one of the most toxic substances known. *Cl. botulinum* is subclassified by immunogenticity of toxin produced, with eight toxin serotypes observed (A, B, C_1, C_2, D, E, F, and G). Some strains possess the genes for multiple toxin types (Santos-Buelga et al., 1998; Barash and Arnon, 2004), though expression of a second toxin may not occur (Franciosa et al., 1994; Cordoba et al., 1995; Hutson et al., 1996). Classification is also done on the basis of proteolysis of casein, serum proteins, meat, or coagulated egg (Bell and Kyriakides, 2000), and classification into Groups I through IV based on genotypic and phenotypic traits has been accepted (Hutson et al., 1993; Collins and East, 1998; Lindström and Korkeala, 2006). A summary of the characteristics of the four *Cl. botulinum* groups is presented in Table 3-1.

Although strains of *Cl. botulinum* are classified as one species based on neurotoxin production, 16S rRNA analysis of different *Cl. botulinum* strains has revealed that toxin serotypes A, B and F are closely related, whereas types C, D, E, and G are more closely related to other *Clostridium* spp. (Hill et al., 2007). Most human botulism is caused by ingestion of foodborne toxin; specifically, the toxins produced by *Cl. botulinum* serotypes A, B, and E (Sobel et al., 2004). The spores themselves are not a concern, but the toxin produced during vegetative cell growth is a hazard. Thus, inhibition of spore germination and subsequent outgrowth is key to preventing botulism.

Table 3-1 Properties of the four groups of *Clostridium botulinum*

Properties	Group				Reference
	I	II	III	IV	
Neurotoxin serotype	A, B, F	B, E, F	C_1, C_2, D	G	Bell and Kyriakides (2000)
Proteolytic	Yes	No	No	Weakly	Lynt et al. (1982); Bell and Kyriakides (2000)
Lipolytic	Yes	Yes	Yes	No	Bell and Kyriakides (2000)
Source	Soil, marine and lake sediments	Soil, marine and lake sediments, fish, mammals, birds	Rotting vegetation, soil, lake sediments	Soil, human autopsy specimens	Huss (1980)
Geographical predominance	A – Argentina, China, US B- Europe	B – Europe E – Japan, Canada, Alaska, Denmark, Iran	Bangladesh, subtropical areas	Argentina	Smith (1978); Huss (1980); Bell and Kyriakides (2000); Austin (2003b)
Organism affected	Humans	Human	Waterfowl (C), chickens (C), mink, cattle (A, B, C, D), horses (B, C_1), mules	Humans	Ricketts et al. (1984); Divers et al. (1986); Kinde et al. (1991); Whitlock and Buckley (1997); Whitlock and Williams (1999); Bell and Kyriakides (2000); Kelch et al. (2000); Songer (2005)
Minimum/optimum growth temperature (°C)	10/35-37	3/26-30	nr	nr	Austin (2003b)
Minimum pH	4.3-4.6	5.0-5.1	nr	nr	Austin (2003b)
Maximum NaCl in aqueous phase (%)	10	5	nr	nr	Austin (2003b)
Heat resistance of spores	High ($D_{121\text{-}110°C}$, 0.15-0.18 min)	Moderate ($D_{85°C}$, 1-98 min)	nr	nr	Bell and Kyriakides (2000)

Adapted from Rhodehamel et al. (1992); Lindström and Korkeala (2006) and Peck (2006); nr – not reported

To control *Cl. botulinum* in food, chemical and physical interventions have been developed. Factors such as oxygen level, water activity, pH, temperature, and chemical additives such as sodium nitrite have been manipulated to prevent spore outgrowth (Bell and Kyriakides, 2000). In particular, low-acid canned foods undergo a heat treatment known as the "botulinum cook" or 12-D process, which is a 3 min treatment at 121°C meant to reduce the number of organisms by 12 log units (Pflug and Odlaug, 1978; Berk, 2009). This process was developed by the canning industry to inactivate Group I (proteolytic) *Cl. botulinum* spores, which are more heat-resistant than Group II (non-proteolytic) *Cl. botulinum* (Stumbo et al., 1975). Acidification of products to a pH less than 4.6 (Ito and Chen, 1978), or adding sodium nitrite to cured meats (Johnson and Loynes, 1971; Roberts and Smart, 1974) have also been proven effective. When combined, these treatments have adequately controlled *Cl. botulinum* in commercially-processed foods, as proven by the low number of cases associated with such products. From 1950 to 2005, 405 cases or outbreaks were reported in the United States; of these, 7 % were traced to commercially-processed foods, including restaurants, with failures in commercial processing responsible for only 1 % (CDC, 2007). The most recent figures available indicate that home-processed foods were responsible for all 17 of the botulism cases in the United States in 2008 (CDC, 2010).

However, the advent of minimally-processed, refrigerated foods in the last 20 years led to concerns that *Cl. botulinum* spores may germinate and grow in these foods (Conner et al., 1989), and these concerns have not abated (Lund and Peck, 1994; Peck et al., 1995; Peck and Stringer, 2005; Lindström et al., 2006; Peck, 2006; Peck et al., 2008). Many consumers believe that processed foods containing additives are not healthy (Van Wezemael et al., 2010), and so natural, minimally-processed foods are becoming more popular (Food Safety Inspection Service, 2010). Organic food regulations prohibit the use of sodium nitrite as a food additive in foods labelled as organic in Canada (Canadian General Standards Board, 2008) and the United States (USDA, 2010), whereas in Europe, sodium

nitrite is currently allowed in organic meat products (European Commission, 2008). However, a review of this policy is underway by the European Commission, and there is a proposal under review to eliminate the use of sodium nitrate in organic cured meats by 31 December 2010 (European Commission, 2008; Stones, 2010). Thus, other treatments must be used to control harmful pathogens such as *Cl. botulinum*.

To retain freshness and preserve desirable sensory and nutritional qualities of minimally-processed foods, these products undergo minimal heat treatment, often do not contain chemical preservatives, and may be vacuum-packaged (Peck, 2006). By heating at sub-sporocidal temperatures, competitive native microflora may be killed, potentially leaving *Cl. botulinum* spores as the dominant species in the product. Vacuum-packaging excludes oxygen, which allows for selection of anaerobic microorganisms such as *Cl. botulinum*. "Sous vide", or "cooked under vacuum", treatments do not destroy spores of *Cl. botulinum*, and germination and growth of Group II *Cl. botulinum* can occur during refrigerated storage (Hyytiä-Trees et al., 2000). Removing barriers in minimally processed foods can result in germination, outgrowth, and toxin production by *Cl. botulinum*; particularly, Group II type B *Cl. botulinum* is of concern, as it has been shown to grow and produce toxin at refrigeration temperatures as low as 3.3°C (Eklund et al., 1967).

Novel methods such as high-pressure processing have also been proposed for inactivation of spores of *Cl. botulinum.* Combined with elevated temperatures, high-pressure processing has been tested to determine inactivation of spores of either *Cl. botulinum* or its closely-related surrogate *Cl. sporogenes* in buffer (Reddy et al., 2006; Ahn et al., 2007), mashed carrots (Margosch et al., 2004), crabmeat (Reddy et al., 2003; Reddy et al., 2006), salmon slurry (Ramaswamy and Shao, 2010), and milk (Ramaswamy et al., 2010). However, inactivation of *Cl. botulinum* by high pressure depends on the food product, processing temperature, and the strain of *Cl. botulinum* tested (Bull et al., 2009); thus, more research must be performed under product-specific conditions before

commercial application of high-pressure processing to inactivate spores of *Cl. botulinum* in minimally-processed foods (Margosch et al., 2006).

Reliance on refrigeration to control microbial growth in minimally processed foods is critical to the safety of these products, as there are few other barriers present to control the growth of *Cl. botulinum*. Air temperatures around refrigerated vacuum-packaged meats in a commercial supermarket have been reported to be at or below 5°C for 68 % of samples, but only below 8°C for 23 % of samples (Sagoo et al., 2007). In a recent survey, only 37 % of consumers reported ensuring their home refrigerators maintained a temperature less than 4°C (Lagendijk et al., 2008), and average temperatures in home refrigerators have been reported to be higher than 5°C (Gilbert et al., 2002; Laguerre et al., 2002). Other factors, such as temperature variance by location within the same commercial refrigeration unit (Gilbert et al., 2002) and differences between air and product temperature, mean that there is opportunity for temperature abuse which could allow the growth of Group II *Cl. botulinum.*

Although contamination of meat products with *Cl. botulinum* has not been high in the past (Abrahamsson and Riemann, 1971), with reported average most probable numbers of 0.1 spores kg^{-1} in North America and 2.5 spores kg^{-1} in Europe (Austin, 2003b), this movement towards minimal processing may increase the risk of *Cl. botulinum* intoxication. Types A and B *Cl. botulinum* are most commonly associated with meats (Austin, 2003b), and in places such as central Europe, most foodborne botulism is caused by contaminated meat (Austin, 2003a). Despite its low reported prevalence in prepared foods, type B *Cl. botulinum* has been identified in 62 % and 75 % of fecal samples from pigs and cattle, respectively (Dahlenborg et al., 2001; Dahlenborg et al., 2003), and the opportunity exists for meat to become contaminated due to unhygienic handling practices during slaughter.

Bacteriocins have been proposed as an alternative to other preservatives in pasteurized meats, because of the perception that they are more natural. Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by

bacteria with activity against closely-related bacteria (Jack et al., 1995). One well-studied bacteriocin is nisin, which has been used in foods for over 40 years, and has shown activity against *Cl. botulinum* (Rayman et al., 1981; Scott and Taylor, 1981). Brochocin-C, produced by *Brochothrix campestris* (Siragusa and Cutter, 1993), has also been identified as a potentially useful bacteriocin effective against *Cl. botulinum* spores (McCormick et al., 1998).

However, inactivation of bacteriocins caused by binding to meat constituents has been observed with nisin (Rose et al., 1999; Aasen et al., 2003) and sakacin P (Aasen et al., 2003). Fat had a conflicting impact on efficacy of bacteriocins in foods; in one study, fat significantly reduced the efficacy of nisin (Glass and Johnson, 2004). Aasen et al. (2003) reported that fat had no impact on efficacy of nisin and sakacin P in foods, but that the activity of the same bacteriocins was reduced in the presence of oils. To date there have been no reports of the use of purified brochocin-C being tested in either liquid or solid food products, and no data is available on any interactions with food constituents. The objective of this study was to determine the efficacy of brochocin-C as an anti-botulinal agent in a model meat system, and in a chopped and formed pork product.

3.2 Materials and Methods

3.2.1 Culture maintenance and spore production

Group II strains of *Cl. botulinum* used in this study were 17B, DB2, and 2B (University of Alberta). *Br. campestris* ATCC 43754 (American Type Culture Collection; Manassas, VA) was used to produce brochocin-C, and *Carnobacterium maltaromaticum* NCIMB 702852 (National Collection of Industrial and Marine Bacteria; Aberdeen, SF) was used as the brochocin-C-sensitive indicator strain. *Br. campestris* and *C. maltaromaticum* were stored at -80°C in All-Purpose Tween broth (APT) (Difco, Becton Dickinson; Sparks, MD) containing 20 % (v/v) glycerol, while *Cl. botulinum* strains were stored as spore stocks in sterile 0.9 % (w/v) NaCl at 4°C. Enumeration of both spores and vegetative cells of *Cl. botulinum* was done using Reinforced Clostridial Medium

(RCM; Difco) containing 2.5 % (w/v) agar. Spores of strains 17B and 2B were produced in Trypticase-Peptone-Glucose-Yeast Extract Broth (TPGY) (USDA, 1998) containing, per litre: 50 g tryptone, 5 g peptone, 20 g yeast extract (Difco), 4 g glucose (Fisher Scientific Canada; Ottawa, ON), and 1 g sodium thioglycollate (Sigma-Aldrich Canada Ltd; Oakville, ON). Sporulation medium containing 50 g L^{-1} tryptone and 10 g L^{-1} peptone was used to produce spores of strain DB2. Before use, all media was incubated in an anaerobic chamber for at least 24 h (Coy Laboratory Products Inc; Grass Lake, MI) under an atmosphere of 5 % CO_2, 10 % H_2, and balance N_2 (Praxair Canada; Edmonton, AB) to ensure anaerobic conditions.

For spore production, 100 µL of spore suspensions were inoculated into 5 mL of appropriate medium, heat-shocked in a water bath for 15 min at 55°C, and incubated at 37°C under anaerobic conditions for 48 h. The 5-mL culture was then used to inoculate 250 mL of the appropriate medium, and the culture was incubated at 37°C under anaerobic conditions for 14 d with periodic monitoring by phase contrast microscopy. Spores were harvested by centrifuging at 16270 x *g* for 20 min, followed by ten washes of 100 mL each and a final resuspension (10 mL) in sterile 0.9 % (w/v) NaCl, and heating at 65°C for 1 h (Gao, 2001) to destroy residual toxin and vegetative cells.

3.2.2 Brochocin-C production

An abbreviated method of McCormick et al. (1998) was used for production and partial purification of brochocin-C. Briefly, 90 L of Casamino Acids Medium (Hastings et al., 1991) supplemented with 2.5 % (w/v) glucose was inoculated with 2 % (v/v) of an overnight culture of *Br. campestris*. Cultures were incubated at 26°C at a controlled pH of 6.7 with stirring at 250 rev min^{-1} for 18 h in a Biostat® C Plus fermentor (Sartorius Mechatronics Canada; Mississauga, ON), and the bacteriocin-containing supernatant was recovered by on-line centrifugation. To obtain sufficient brochocin-C for the study, several fermentations of 30 L each were performed. The supernatant was heated at 60°C for 30 min to inactivate any remaining cells, and then extracted twice with 1-

butanol (Fisher Scientific Canada) with a butanol:supernatant ratio of 1:2. The organic phase was mixed in a 1:1 ratio with MilliQ® water (Millipore; Billerica, MA), and concentrated by rotary evaporation (Büchi; Flawil, CH) at 36°C until no butanol remained. Antibacterial activity of brochocin-C was assayed by diluting the semi-purified brochocin-C in a two-fold series with 0.1 % (v/v) trifluoroacetic acid (Sigma), spotting 10 µL of each dilution onto APT agar containing 1.5 % (w/v) agar, and overlaying with semi-solid APT agar [0.75 % (w/v) agar] seeded with 1 % (v/v) of an overnight culture of the sensitive indicator organism *C. maltaromaticum* NCIMB 702852. Plates were incubated anaerobically at 30°C, and were examined at 24 h for zones of clearing. Arbitrary activity units per mL (AU mL^{-1}) were calculated from the reciprocal of the last dilution showing inhibition (McCormick et al., 1998). Following quantitation, the semi-purified brochocin-C was lyophilized.

3.2.3 Model meat system preparation, inoculation, and sampling

A cocktail of Group II *Cl. botulinum* was diluted in 0.9 % (w/v) NaCl to contain 3 x 10^4 CFU mL^{-1} of each strain. Three hundred millilitres of sterile, anaerobic Cooked Meat Medium (CMM; Difco) was supplemented with brochocin-C at concentrations of 0, 800, or 1600 AU mL^{-1} in triplicate. One millilitre of heat-shocked spore cocktail was inoculated into CMM to give a final spore concentration of 100 CFU mL^{-1}, as recommended by Doyle (1991). Solids-free controls were prepared in triplicate by autoclaving CMM, filtering through several layers of cheesecloth to remove large particles, then filter-sterilizing through a 0.22 µm filter before supplementation with 1600 AU mL^{-1} brochocin-C and inoculation. Cultures were incubated anaerobically at 4°C for the duration of the study. On selected days, samples of the liquid phase were removed in an anaerobic chamber for enumeration. The pH of the Group II *Cl. botulinum* cultures was measured at the beginning and end of the experiment using ColorPhast pH indicator strips (EMD Chemicals; Gibbstown, NJ). Total crude fat [% (w/w)] was determined using a Goldfisch fat extractor (Labconco; Kansas

City, MO) by extracting 2 g of ground commercial CMM pellets with petroleum ether for 4 h.

3.2.4 Meat preparation, inoculation, and sampling

The meat product was a chopped and formed pork sausage prepared by Griffith Laboratories (Toronto, ON). Briefly, ground pork was prepared by grinding through a kidney plate and a 1/8" plate respectively, and added to a Hobart mixer (North York, ON). For both trials 1 and 2, a proprietary industry product formulation was used containing equal amounts of sodium erythorbate and sodium chloride (2.28 % w/w) in the finished product. Trial 1 used a formulation incorporating smoke powder [0.12 % (w/w)] and sodium tripolyphosphate (STPP) in all treatments, whereas trial 2 excluded smoke and included STPP-free controls. Treatments with or without STPP, natural cure, sodium nitrite, and brochocin-C (Table 3-2) were prepared and mixed for 1 min before addition of water. The natural cure used was a commercially-available formulation containing extracts of celery, lemon, and green tea, as well as sea salt (Bindmax Proteins; New Berlin, WI). The emulsion was blended for 2 min, stuffed into moisture-proof casings, and cooked in a smokehouse on the steam-cook setting to an internal temperature of 72°C. The product was shipped to the University of Alberta on cold packs, and was stored at -1°C until inoculation.

Table 3-2 Formulation of ingredients in treatments of chopped and formed pork products

Treatment	Ingredient			
	Sodium tripolyphosphate [% (w/w)]	Natural cure [% (w/w)]	Brochocin-C (AU g^{-1})	Nitrite [% (w/w)]
Natural cure + brochocin-C + STPP	0.33	3.0	1600	0
Natural cure + Brochocin-C*	0	3.0	1600	0
Natural cure + STPP	0.33	3.0	0	0
Brochocin-C + STPP	0.33	0	1600	0
STPP only	0.33	0	0	0
Nitrite + STPP	0.33	0	0	6.4
Control – no added antimicrobials*	0	0	0	0

* included only in trial 2

Meat samples were prepared by aseptically opening each chub using a knife dipped in 70 % (v/v) ethanol and passed through a flame. The meat was

aseptically cut into three equal sections, and each section was inoculated on separate days to give three replicates. Each section was sliced into 0.5 cm slices using a sanitized commercial meat slicer (Model X-13, Berkel Equipment; Troy, OH). Each slice was cut in half, giving a surface area of 14.2 cm^2 per sample, and aseptically placed into a vacuum-package bag (oxygen transmission rate of 52 cm^3 m^{-2}; Allied Pak; Toronto, ON). A cocktail containing equal numbers of spores of each strain was heat-shocked (55°C, 15 min) and inoculated onto the surface of each slice to give a final concentration of 100 (trial 1) or 500 (trial 2) spores cm^{-2}. The inoculum was spread evenly over the meat surface by manual manipulation of the package, allowed to soak into the meat surface for 10 min, and the package was sealed using a commercial vacuum packaging machine (Model C200, Multivac Canada; Woodbridge, ON). Samples were incubated at 10°C for the study. On selected days, two duplicate sample packages per treatment were aseptically opened in an anaerobic chamber using a sterile scalpel, and 25 mL of sterile 0.1 % (w/v) peptone was added to each. Samples were manually homogenized for 5 min, and 15 mL was removed from each replicate for cell enumeration.

3.2.5 Proximate analysis of meat

Uninoculated samples of each treatment were weighed, and water activity (A_w) was determined using an Aqualab Series 3 metre (Aqualab Devices; Pullman, WA). Total moisture [% (w/w)] was determined by weighing meat samples before and after freeze-drying for 48 h. Total crude fat [% (w/w)] was determined using a Goldfisch fat extractor (Labconco) by extracting 2 g of freeze-dried ground samples (48 h) with petroleum ether for 4 h. Sodium chloride in the aqueous phase of the product was calculated by dividing the amount of NaCl added per kg final product (2.28 g) by the total weight minus the weight of the total crude fat.

3.2.6 Enumeration of **Cl. botulinum** *and background microflora*

For the model meat broth cultures, samples were serially-diluted in 0.1 % (w/v) peptone and enumerated by spreading onto the surface of RCM medium

containing 2.5 % (w/v) agar. For the pork product, samples were plated in a similar fashion to the model meat system, but three additional types of media were used. *Clostridium botulinum* Isolation Agar (CBI) (Dezfulian et al., 1981) and de Man, Rogosa, Sharpe agar (MRS, Difco) (De Man et al., 1960) were included for the enumeration of *Cl. botulinum* and lactic acid bacteria, respectively. Plate Count Agar (PCA; Difco) was used in later sampling times in trial 1 to determine the total bacterial count. CBI medium was prepared by combining 40 g tryptone (Difco), 7.5 g $Na_2HPO_4 \cdot H_2O$ (Fisher), 2 g NaCl (Fisher), 5 g yeast extract (Fisher), and 20 g agar (Difco) in 950 mL distilled water, sterilizing by autoclaving, and adding 50 mL sterile egg yolk suspension (Difco), and sterile volumes of the following ingredients to give final concentrations (per L) of 0.01 g $MgSO_4$ (Sigma), 2 g D-glucose (Fisher), 0.25 g cycloserine, 0.076 g sulphamethoxazole, and 0.004 g trimethoprim (all antibiotics Sigma). Both diluent and solid media were incubated in the anaerobic chamber overnight prior to use to ensure anaerobic conditions. After inoculation, RCM and CBI plates were incubated anaerobically at 37°C for 48 h prior to counting, while MRS and PCA plates were incubated in a jar containing an anaerobic sachet (GasPak EZ Anaerobic sachet, Becton-Dickinson) at 30°C for 24 h. Colonies displaying lipolytic activity on CBI agar, as evidenced by a translucent halo surrounding the colony, and spreading colonies with irregular, feathered margins on RCM agar were presumptively identified as *Cl. botulinum* based on preliminary work with these strains. Other colonies with different morphologies on CBI and RCM media were counted as background microflora.

3.2.7 Effect of fat on brochocin-C activity

To determine if the presence of fat had an effect on antimicrobial activity of brochocin-C, 10 mL of CMM or solids-free CMM was prepared (described in Section 3.2.3) in triplicate. Brochocin-C was added to a final concentration of 1600 AU mL^{-1}, followed by the addition of 1 mL of autoclaved home-rendered pork fat. Samples were mixed using a vortex for 10 s before and after brochocin-C addition, and were incubated at 25°C for 5 min with shaking at 100 rev min^{-1},

followed by incubation at 25°C overnight with no shaking. The amount of activity of brochocin-C remaining in the aqueous phase was determined by the bioassay method described in Section 3.2.2.

3.2.8 Anti-botulinal activity of ingredients

To determine the antimicrobial potential of several ingredients included in all treatments of the first trial, a minimum inhibitory concentration (MIC) assay was done for vegetative cells and spores of the Group II *Cl. botulinum* strains 17B, DB2, and 2B. An MIC assay was done in a 96-well microtitre plate using serial two-fold dilutions of smoke in sterile TPGY medium, with highest and lowest levels of 0.5 % and 0.002 % (w/v), respectively, to include the concentration used in the product. A range of NaCl concentrations was also tested in conjunction with the smoke assay to determine the MIC at different NaCl concentrations. The range of NaCl concentrations tested was 0, 1, 2, 2.5, 3, and 5 % (w/v), which spanned the concentration in the product. For the assay, each well containing 250 µL of sample was inoculated with 8 µL of either an 18 h culture of vegetative cells (corresponding to ~10^8 CFU mL^{-1}) or a 3 x 10^4 spores mL^{-1} stock of heat-shocked spores (55°C, 15 min) in 0.9 % (w/v) NaCl. Plates were incubated for 18 h at 37°C in an anaerobic atmosphere, and were examined visually for growth. Samples were taken from wells in which no growth was observed, and were spread on the surface of TPGY medium containing 1.5 % (w/v) agar to determine if the effect was either bacterio- or sporostatic, or bacterio- or sporocicidal, and incubated at 37°C for 24 h under anaerobic conditions. All assays were performed in duplicate.

3.2.9 Confirmation of **Cl. botulinum** *and identification of background microflora from pork product*

Representative colonies were chosen from either RCM or CBI, examined using phase contrast microscopy, and inoculated into 5 mL of RCM broth. All manipulations were performed in an anaerobic chamber (Coy Laboratory Products Inc; Grass Lake, MI) containing an atmosphere of 5 % CO_2, 10 % H_2, and balance N_2 (Praxair Canada; Edmonton, AB), and cultures were incubated in an anaerobic

chamber at 37°C for 24 h. The Qiagen DNeasy Blood and Tissue kit (Qiagen; Mississauga, ON) was used for genomic DNA extraction, with a modified pre-treatment for gram-positive bacteria. Briefly, cells were harvested and prepared for DNA extraction by centrifuging 2 mL aliquots at 6000 x *g* for 10 min, washing the cell pellet in 0.5 mL of enzymatic lysis buffer [20 mM Tris-Cl, pH 9.6; 2 mM EDTA; 1.2 % (v/v) Triton X-100], and repeating the previous centrifugation step. Pellets were re-suspended in 180 µL enzymatic lysis buffer containing 50 mg L^{-1} lysozyme, and incubated at 37°C for 1 h, with brief mixing by vortex every 10 min. The remainder of the protocol was performed as per the manufacturer's instructions. DNA was eluted in 100 µL 10 mM Tris-HCl, 0.5 mM EDTA (pH 9.0), and was quantified using the Nanodrop 2000c (Thermo Scientific; Wilmington, DE).

Polymerase chain reaction for 16S rDNA fragments was performed using the general bacterial primers 616V and 630R (Brosius et al., 1981). Briefly, each 50 µL reaction mixture contained 5 µL 10X buffer, 1.5 mM $MgCl_2$, 0.5 µM each of primers 616V and 630R, 200 µM dNTP mix, 1.5 U Platinum Taq polymerase (Invitrogen Canada; Burlington, ON), and 2 µL template DNA. Products were amplified using the following program: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1.5 min, and 72°C for 1.5 min, with a final extension step of 7 min at 72°C. PCR products were purified using the Qiaquick PCR Purification kit (Qiagen), and were eluted in 50 µL nuclease-free water. Products were visualized on a 2 % (w/v) agarose gel in 0.5X Tris-borate EDTA buffer. Sequencing was performed by Macrogen USA (Rockville, MD) using the PCR primers 616V and 630R. Template concentrations were adjusted to 40 ng μL^{-1} in nuclease-free water prior to sequencing. Sequencing results were analyzed using Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information; Bethesda, MD) to identify the isolates.

3.2.10 Statistical analysis

Data from three replicates were subjected to a one-way analysis of variance using a general linear model with Duncan's multiple range test (α=0.05) run on SAS software, version 9.2 (Cary, North Carolina).

3.3 Results

3.3.1 Model meat system

In the model meat system, brochocin-C at both low (800 AU mL^{-1}) and high (1600 AU mL^{-1}) concentrations inhibited the growth of Group II *Cl. botulinum* (Figure 3-1). Counts for all samples except the untreated control remained below the detection limit of log 1 CFU mL^{-1} for the duration of the study, although counts in the 800 AU mL^{-1} sample and 1600 AU mL^{-1} solids-free control briefly reached a maximum of log 1.4 CFU mL^{-1} at 13 d before decreasing to below detection again (Figure 3-1). In cultures lacking brochocin-C, growth began after a brief lag period of 1 d, with a near-maximum cell count of log 7.3 reached by 5 d post-inoculation. Meat solids did not have an effect on growth. In all cultures, the pH dropped slightly to a final pH of 6.5 in all treatments except samples containing 800 AU BrC-C ml^{-1}, which had a final pH of 6.7 ± 0.3. There was no crude fat present in the CMM used in the experiment.

3.3.2 **Cl. botulinum** *growth in chopped and formed pork product*

To overcome any potential interaction of brochocin-C with food constituents, the brochocin-C concentration of 1600 AU g^{-1} was chosen for the chopped and formed pork product. The water activity of all samples was a minimum of 0.96, the fat content ranged from a low of 7.1 % (w/w) in the samples with brochocin-C + STPP to a high of 12.6 % (w/w) in the samples with natural cure + STPP, and the NaCl content ranged from 3.20 to 3.48 % (in aqueous phase) (Table 3-3). In trial 1, *Cl. botulinum* remained at or below the detection limit in all samples enumerated on CBI and RCM media over the 79-day course of the experiment in all samples. Beginning on day 58, enumeration on

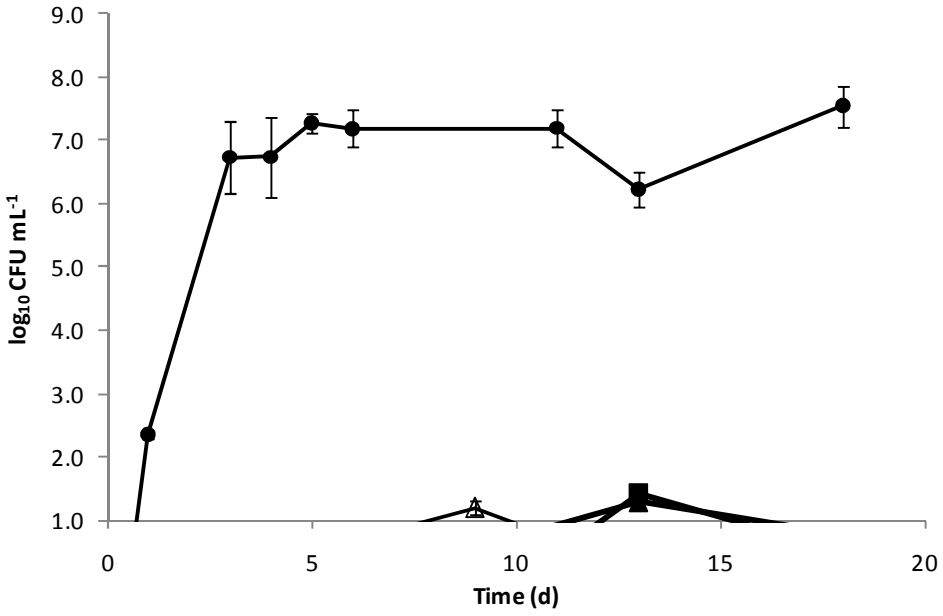

Figure 3-1 Growth of a cocktail of Group II *Cl. botulinum* (strains 17B, 2B, and DB2) in the presence of different concentrations of brochocin-C in a model meat system. Brochocin-C concentration: (●) 0 AU mL^{-1}, (■) 800 AU mL^{-1}, (△) 1600 AU mL^{-1}, (▲) solids-free control + 1600 AU mL^{-1}. Data points represent the mean of n=3, and error bars represent ± SEM. Limit of detection is minimum y-axis value.

PCA was performed to determine the total bacterial count, and counts were found to be below detection for all samples in two replicates from day 58 to day 79. In replicate 2, the sample containing only brochocin-C and the untreated control had total bacterial counts of 2.8 and 2.2 log CFU cm^{-2}, respectively, on day 65. At the end of the experiment (79 days), counts for these samples had increased to 4.0 and 2.7 log CFU cm^{-2}, respectively. The pH of all samples remained relatively constant throughout the experiment, with a final pH of 6.5 to 7.0.

Table 3-3 Proximate analysis of chopped and formed pork product

Treatment	A_w	Moisture [% (w/w)]	Crude fat [% (w/w)]	NaCl [% (w/w) in aqueous phase]
Natural cure + brochocin-C + STPP	0.96	67.0	8.6	3.40
Natural cure + brochocin-C	0.96	65.9	9.1	3.46
Natural cure + STPP	0.97	65.6	12.6	3.48
Brochocin-C + STPP	0.97	71.1	7.1	3.20
STPP only	0.98	67.8	9.3	3.36
Nitrite + STPP	0.97	67.8	9.6	3.36
Control – no added antimicrobials	0.98	nd	nd	nd

nd – not done because of insufficient sample quantity

The lack of growth of both *Cl. botulinum* and background microflora led to identification of ingredients in the formulation common to all treatments, and investigation of these ingredients for antimicrobial activity, as described in Sections 3.2.8 and 3.3.4. Based on these results, the meat product formulation was revised in trial 2 to exclude powdered smoke in all treatments, and include samples that omitted STPP so that any antimicrobial effect of brochocin-C was not masked by inhibition from other ingredients. In addition, the initial inoculum of *Cl. botulinum* spore cocktail was increased from 100 CFU cm^{-2} to 500 CFU cm^{-2} to ensure that microbial growth could be readily detected.

The results of trial 2 are shown in Figure 3-2. When enumeration was performed using CBI medium, the numbers of *Cl. botulinum* (lipolytic colonies) remained relatively constant throughout the experiment for all treatments, beginning with a value of 2.5 ± 0.08 log CFU cm^{-2} in all the samples at the beginning of the experiment, and finishing with a value of 2.2 ± 0.1 log CFU cm^{-2} on day 50 (Figure 3-2A). No significant differences in counts obtained from CBI among samples were observed on any sampling day, with the exception of the untreated control which was higher than samples containing natural cure and brochocin-C after 7 days of storage and was higher than samples containing

natural cure and STPP after 15 days of storage. However, by the end of the experiment, all counts were comparable.

However, when RCM agar was used as the enumeration medium, the results were different from those obtained on CBI medium for several treatments (Figure 3-2B). Counts for all treatments were not significantly different from 0 to 7 days of storage, but following day 7, the counts in the untreated control, sample containing only STPP, and the sample containing brochocin-C + STPP began to decline, reaching a level below detection after 28 days of storage. The counts obtained on RCM for these samples were significantly lower then the counts from samples containing sodium nitrite and natural cure after 28 days of storage. After 50 days of storage, the counts in the samples with STPP and brochocin-C were significantly lower than those treated with sodium nitrite or natural cure.

The results of the enumeration of the background microflora on MRS medium are shown in Figure 3-2C. After a lag phase of 7 days, the counts in the untreated control, STPP only, and brochocin-C + STPP increased rapidly to a maximum concentration of ~7 log CFU cm^{-2} after 21 days of storage. Samples that contained either nitrite or natural cure in various combinations with brochocin-C and STPP reached a final concentration of background microflora near or below 2 log CFU cm^{-2} by the conclusion of the experiment.

Small, white, colonies with regular margins grew on both CBI and RCM medium, and these were later identified as either *Carnobacterium* spp. or *Staphylococcus* spp. by 16S rDNA sequencing (Table 3-4). On CBI agar, these colonies were non-lipolytic. The growth trends for the counts of these small colonies on CBI and RCM were similar to that observed on MRS medium (Figure 3-2C), with high vegetative cell counts (8 log CFU cm^{-2}) observed in samples lacking either sodium nitrite or natural cure after 28 days of storage and continuing to the end of the experiment. Growth of these small colonies in samples containing sodium nitrite or natural cure reached a high of ~4 log CFU cm^{-2} at 14 days of storage, but subsequently decreased throughout the experiment, whereas the cell count remained high in all other samples on both medium types.

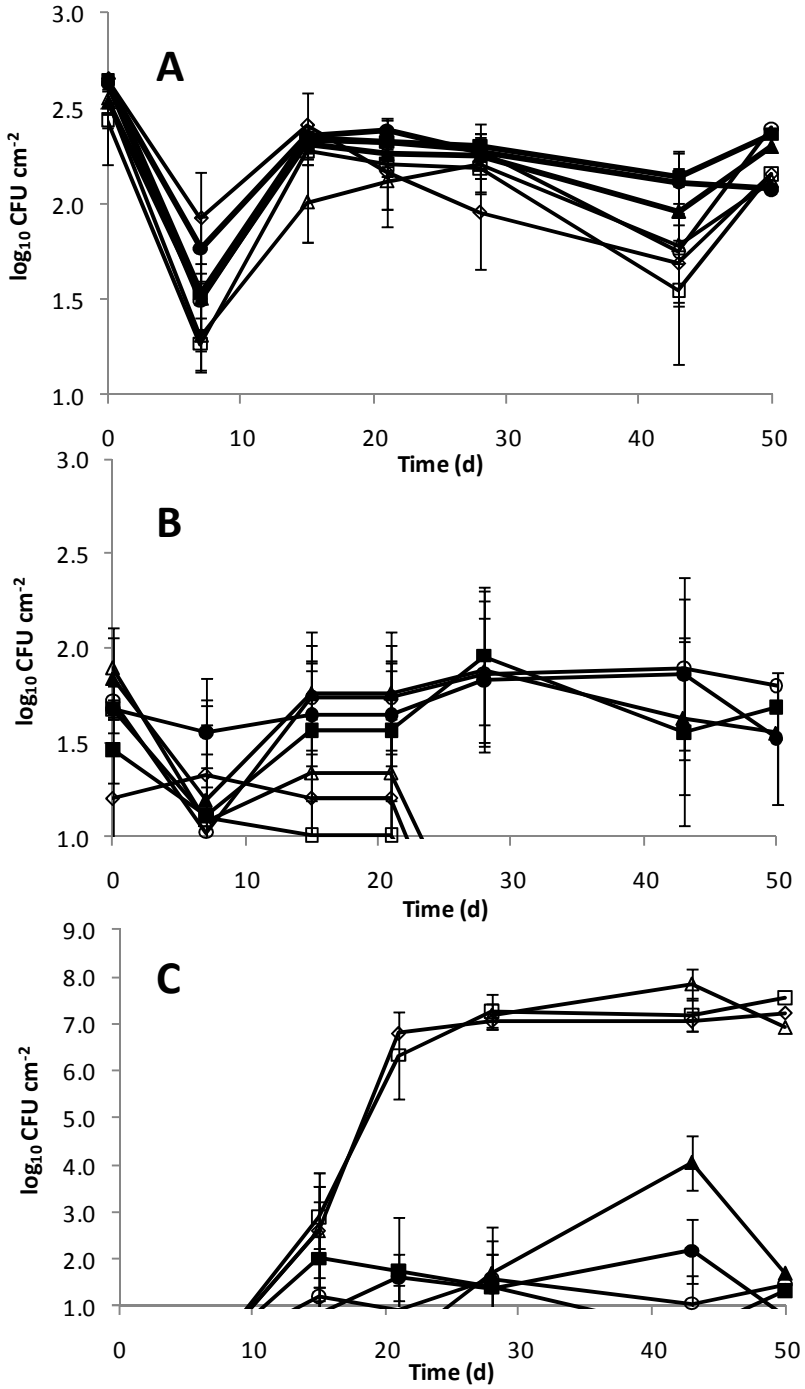

Figure 3-2 Growth of cocktail of Group II *Cl. botulinum* on CBI medium (A) and RCM medium (B), and growth of background microflora on MRS medium (C) originating from chopped and formed pork product with different ingredient formulations. Treatments: (●) natural cure + brochocin-C + STPP, (■) natural cure + STPP, (□) brochocin-C + STPP, (▲) sodium nitrite + STPP, (△) STPP, (○) natural cure + brochocin-C, (◊) control with no added antimicrobials. Lipolytic colonies on CBI were counted as *Cl. botulinum*, data points represent the mean of n=3, and error bars represent ± SEM. Minimum y-axis value is limit of detection.

Table 3-4 Identification by 16S rDNA sequencing and comparison with BLAST database of background microflora isolates originating from a vacuum-packaged pork product

Identification	Maximum identity (%)	Strain designation
Carnobacterium maltaromaticum[1]	99	MH1[2]
C. maltaromaticum	99	MH2[2]
C. maltaromaticum	99	MH3[2]
C. maltaromaticum	99	G117[3]
C. maltaromaticum	97	MF[3]
Staphylococcus pasteuri	97	EIV-21[3]
S. warneri	97	S46[3]

[1] identified as *Lactobacillus maltaromaticum*, now *C. maltaromaticum* (Mora et al., 2003), [2] designated in this study, [3] designated from BLAST search

3.3.3 Effect of fat on brochocin-C activity

Pork fat eliminated the activity of brochocin-C in a model meat system. The results were the same in the presence and absence of meat solids, with growth of the indicator strain in the undiluted samples of the aqueous phase of the samples with added pork fat. In samples lacking pork fat, brochocin-C remained active, with 1600 AU mL^{-1} observed in the aqueous phase of samples containing and lacking solids.

3.3.4 MIC of powdered smoke against Group II Cl. botulinum

Several potential antimicrobial candidates were identified following trial 1 in the pork product: powdered smoke, NaCl, and STPP. The effect of smoke powder and NaCl, at concentrations found in the product, on both spores and vegetative cells of Group II strains of *Cl. botulinum* was investigated. It was observed that at a NaCl concentration of 2.5 % (w/w), the MIC of powdered smoke was at or below the concentration of 0.12 % (w/w) found in the product for two of the three *Cl. botulinum* strains used in the cocktail (Table 3-5). Spores of strain 2B, and both spores and vegetative cells of strain 17B were inhibited by a powdered smoke concentration of 0.125 % (w/w) at 2.5 % (w/w) NaCl, while vegetative cells of 2B were inhibited by 0.063 % (w/w) smoke. Strain DB2 was more resistant to powdered smoke at the same NaCl concentration, requiring 0.25

% and 0.5 % (w/w) powdered smoke to inhibit vegetative cells and spores, respectively. When 3 % (w/w) NaCl was used, which is a level slightly less than that found in the aqueous phase of the meat, the MIC values of smoke powder were further reduced, to less than 0.002 % (w/w) for all spores of strain DB2, and both spores and vegetative cells of strain 17B. Strain 2B was slightly more resistant to smoke powder, with an MIC of 0.03 % (w/w) for spores and vegetative cells.

To determine if the nature of the antimicrobial activity of smoke powder and NaCl was either bacterio- or sporostatic, samples from each combination were enumerated on TPGY medium in the absence of smoke powder. The antimicrobial effect determined to be was bacterio- and sporostatic for vegetative cells and spores, respectively, of all strains of *Cl. botulinum* tested.

Table 3-5 Minimum inhibitory concentrations [in % (w/w)] of commercial powdered smoke against cells and spores of Group II *Cl. botulinum* strains in the presence of different NaCl concentrations.

NaCl (% w/w)	*Cl. botulinum* strain					
	DB2		17B		2B	
	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores
0	>0.5	>0.5	>0.5	>0.5	0.5	0.5
1	0.25	>0.5	>0.5	0.5	0.125	0.5
2	0.125	0.5	0.25	0.25	0.063	0.25
2.5	0.25	0.5	0.125	0.125	0.063	0.125
3	<0.002	0.063	<0.002	<0.002	0.03	0.03
5	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002

3.4 Discussion

Initial characterization studies of brochocin-C found that 800 AU mL^{-1} was sufficient to inhibit *Cl. botulinum* in a spot-on-lawn assay (McCormick et al., 1998), which was the basis of this study. When a concentration of 800 AU mL^{-1} brochocin-C was tested in the model meat product consisting of broth containing 12.5 % (w/v) cooked meat solids, as in CMM (BD, 2009), brochocin-C was effective at inhibiting the growth of *Cl. botulinum*. However, in a solid pork

product containing near 30 % (w/w) solids and double the concentration of brochocin-C, samples containing bacteriocin and STPP did not significantly inhibit the growth of *Cl. botulinum*. Comparison of the formulations of each product indicates that several key differences existed, including product form (solid or liquid) and fat content.

Purified bacteriocins have been incorporated into both homogenized and whole-muscle meat products. Previous work with enterocins A and B (Aymerich et al., 2000) found that bacteriocin efficacy was increased in comminuted products over whole-muscle meats. However, Aasen et al (2003) concluded that bacteriocins, particularly sakacin P and nisin, are most effective when used on meat surfaces, rather than incorporated into ground products. The results of the current study are in agreement with Aasen et al (2003), with no detectable activity of brochocin-C observed in a chopped and formed pork product.

Fat content of the model and meat systems is another key difference. Although beef heart, the largest constituent by weight in CMM (BD, 2009), is known to contain 6.0 % (w/w edible portion) fat (Belitz et al., 2004), commercial CMM was determined to be defatted, as crude fat analysis did not detect any fat. In contrast, all treatments of the chopped and formed pork product contained at least 7 % (w/w) crude fat. However, there are conflicting reports of the influence of fat on bacteriocin activity. In liquid media to which melted milkfat or soybean oil was added, a decrease in anti-botulinal activity of nisin was observed at a concentration of 20 % fat, but a less-consistent effect was observed at 10 % fat (Glass and Johnson, 2004). Similarly, the activity of carnocin H was reduced in the presence of soy oil (Blom et al., 2001), and the anti-listerial activity of *Lactobacillus curvatus* is reduced in the presence of oil (Ghalfi et al., 2006). However, loss of efficacy may be transient, because aqueous solutions of sakacin P and nisin, which had lost activity when mixed with salmon or cod liver oil, regained activity in the aqueous phase after the phases separated (Aasen et al., 2003). It is well-known that bacteriocins have amphipathic properties that effect their mode of action in the cell membrane (Drider et al., 2006), and in the case of

nisin, have also demonstrated potent emulsifying activity (Bani-Jaber et al., 2000).

In solid products such as meats, the influence of fat on bacteriocin activity is not clear. One report indicated equal recovery of sakacin P from salmon and chicken containing 12 % and 1.6 % fat, respectively (Aasen et al., 2003). However, a mixture of semi-purified enterocins A and B demonstrated better inhibition of *Listeria innocua* in higher-fat products such as paté (20 %) and minced pork (13.09 %) than in cooked ham (3.48 %) and chicken breast (1.36 %), respectively (Aymerich et al., 2000). Nisin was most effective in sausages containing 15 % fat, which is comparable to that found by Davies et al. (1999). However, in sausages with higher fat contents (26 – 41 %), the activity was lower (Davies et al., 1999), which fits well with the results of the current study, as the addition of rendered pork fat to aqueous medium was shown to eliminate brochocin-C activity in the model meat system.

In the presence of fat, differences in efficacy among bacteriocins may be caused by differences in physicochemical properties such as hydrophobic profiles. Sakacin P has a calculated grand average of hydropathicity (GRAVY) (Kyte and Doolittle, 1982; Gasteiger et al., 2003) value of -0.574, which indicates that it is much more hydrophilic than nisin (GRAVY=0.415), or either brochocin-A or brochocin-B peptides, with values of 0.880 and 0.595, respectively. The activity of more hydrophobic bacteriocins such as brochocin-C may be impacted to a greater degree in fat-containing foods than more hydrophilic bacteriocins. Lipid-bound bacteriocin appears to lose activity (Aasen et al., 2003), and so this may account for the lack of brochocin-C activity in the chopped and formed pork product. Thus, choice of bacteriocin to be used in a product will depend on the physicochemical properties of the product, such as fat content, as well as the properties of the bacteriocin, such as hydropathicity. Such factors must be considered when designing a biopreservation system for a particular food.

Sodium tripolyphosphate, NaCl, and smoke powder were identified as potential antimicrobial agents in the meat formulation, so product with only STPP

was included as a treatment. Some workers have reported inhibitory effects of phosphate on growth and toxin formation by *Cl. botulinum* (Tompkin, 1983; Wagner and Busta, 1984; Wagner and Busta, 1985; Barbut et al., 1986), but other studies have found that phosphate either stimulated (Roberts et al., 1981a; Barbut et al., 1986) or had no effect on growth and toxin production (Ivey et al., 1978; Barbut et al., 1986). The chemical form of the phosphate is important, as sodium acid pyrophosphate was found to be inhibitory to *Cl. botulinum* in turkey frankfurters, whereas STPP stimulated toxin production (Barbut et al., 1986). Another factor to be considered is pH of the product, as polyphosphate was found to increase toxin production in pasteurized pork slurries with a pH of 5.5 – 6.3 (Roberts et al., 1981a), whereas when the pH was higher, the effect was reversed (Roberts et al., 1981b). Although the enumeration results on RCM medium showed that *Cl. botulinum* decreased when meat samples contained only STPP, the same observation was found for the untreated control. When enumerated on CBI medium, there was no difference in numbers of *Cl. botulinum* recovered from the products; thus, the true effect of STPP remains unclear. As there were no differences in pH between samples, inhibition of *Cl. botulinum* because of pH change can be excluded as the cause of the inhibition.

NaCl is also inhibitory to Group II *Cl. botulinum*, but the concentration required for complete inhibition in the absence of other inhibitory ingredients is relatively high (5 %), and likely would not be palatable for consumers. However, NaCl, in combination with ingredients such as sodium nitrite, sodium isoascorbate, sodium acid pyrophosphate, and potassium sorbate, is inhibitory at lower concentrations (Roberts et al., 1981c; Wagner and Busta, 1984). In this study, the presence of NaCl at a concentration of around 3.2 % in a pork product, without any other potential inhibitors present, was effective in controlling *Cl. botulinum*, which disagreed with the MIC data, which found that 3 % NaCl alone was not effective at controlling *Cl. botulinum*. However, NaCl alone may not be responsible for inhibition of *Cl. botulinum*, because it is possible that the presence of a background microflora may have impacted the growth of *Cl. botulinum*.

When smoke powder is added as an additional barrier, the concentration of NaCl required for inhibition decreases from 5 % to around 3 %, which was the concentration used in the first meat trial. This is in agreement with other studies with *Cl. botulinum* types A and E in fish products (Eklund et al., 1982). Although little has been reported on the efficacy of smoke on inhibition of *Cl. botulinum* in meat, the results in this study indicate that at a concentration of 0.12 %, and in the presence of other inhibitory factors such as NaCl and refrigeration, powdered smoke inhibited Group II *Cl. botulinum*, based on counts that were below detection when smoke was present and the low MIC value observed.

The natural cure used in this experiment contained celery extract, though the exact level in the commercial product is not known. Celery is known to contain both nitrate and nitrite, with average concentrations of 3151 and 0.8 mg kg^{-1} (fresh weight) reported (Walker, 1990), which is concentrated to 2.5 x 10^4 mg kg^{-1} in commercial celery juice powder (Sindelar and Houser, 2009). According to the product literature for the natural cure, it is intended to replace the curing agent sodium nitrite, and gives a final concentration of 200 mg kg^{-1} nitrite in the finished product when used at the recommended concentration (W. Yien, Griffith Laboratories, personal communication). Thus, both treatments containing nitrite should give comparable results, which was the case when samples were enumerated on either RCM or CBI media.

In this study, *C. maltaromaticum*, *S. pasteuri*, and *S. warneri* were identified as part of the microflora of the vacuum-packaged pork product. *C. maltaromaticum* has been isolated from numerous vacuum-packaged muscle products, including chicken (Barakat et al., 2000), beef (Brightwell et al., 2009), smoked salmon (Leroi et al., 1998; Nilsson et al., 1999), and pork (McMullen and Stiles, 1993). *Staphylococcus pasteuri* is a ubiquitous organism originating from human, animal, and foods (Chesneau et al., 1993), while *S. warneri* is found on human skin and some domestic animals (Kloos and Schleifer, 1975; Götz et al., 2006). Both *S. pasteuri* and *S. warneri* have been isolated from sausages (Blaiotta et al., 2004), and so it is not surprising that these organisms were isolated from a

pork product. One result that was surprising was that the background microflora grew on CBI, as this medium is designed to be selective for *Cl. botulinum*.

In this study, treatments containing nitrite from either the natural cure or from sodium nitrite directly demonstrated significant inhibition of *C. maltaromaticum* and *Staphylococcus* spp. Lactic acid bacteria have been shown to be resistant to nitrite (González and Díez, 2002), and in fact a selective medium for lactic acid bacteria was designed based on this presumed resistance (Davidson and Cronin, 1973). However, nitrite has been found to have a variable effect on lactic acid bacteria, homofermentative strains being more resistant to its effects than heterofermentative strains (Dodds and Collins-Thompson, 1984). While relatively high nitrite concentrations (200 – 400 mg L^{-1}) are required for inhibition of some strains (Dodds and Collins-Thompson, 1984; Korkeala et al., 1992), other strains are inhibited by as little as 50 mg L^{-1} (Dodds and Collins-Thompson, 1984). Several studies have reported inhibition of lactobacilli, including *Lactobacillus curvatus* (Verluyten et al., 2003; Ghalfi et al., 2006; Kouakou et al., 2009) and *Lb. sakei* (Leroy and de Vuyst, 1999; Lyhs et al., 1999; Leroy and De Vuyst, 2005). Although the carnobacteria are closely related to the lactobacilli (Collins et al., 1987; Champomier et al., 1989; Mora et al., 2003), and share many phenotypic properties, this is the first report of inhibition of *Carnobacterium* spp. by nitrite. Because of its origins from meat and ability to grow at low temperatures, *Carnobacterium* has been identified as a likely candidate for biopreservation of meats (Ahn and Stiles, 1990). However, the results of this study indicate that preliminary work must be done to ensure that any potential strain is not inhibited by ingredients in the formulation such as nitrite.

One interesting observation from this study was the difference in numbers of *Cl. botulinum* on CBI and RCM media for the untreated control, the samples containing brochocin-C and STPP, and the samples containing only STPP after 21 days of storage. Several possibilities for this disparity have been identified, including bacteriocin production by the non-botulinal isolates *C. maltaromaticum* and *Staphylococcus* spp. found in increased levels in these three treatments, or an

enhanced ability for recovery of *Cl. botulinum* by CBI. Both of these possibilities require further investigation.

In summary, brochocin-C was shown to effectively inhibit outgrowth of spores of *Cl. botulinum* in liquid culture at a concentration of 800 AU mL^{-1}, but was not effective as an antimicrobial in a meat product at twice the concentration. Interactions with food constituents such as fat likely played a role in the loss of activity.

3.5 References

Aasen, I.M., Markussen, S., Møretrø, T., Katla, T., Axelsson, L. and Naterstad, K. (2003) Interactions of the bacteriocins sakacin P and nisin with food constituents. Int J Food Microbiol 87, 35-43.

Abrahamsson, K. and Riemann, H. (1971) Prevalence of *Clostridium botulinum* in semipreserved meat products. Appl Microbiol 21, 543-544.

Ahn, C. and Stiles, M.E. (1990) Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl Environ Microbiol 56, 2503-2510.

Ahn, J., Balasubramaniam, V.M. and Yousef, A.E. (2007) Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. Int J Food Microbiol 113, 321-329.

Austin, J.W. (2003a) Botulism. In: L. Trugo and P.M. Finglas (Eds), Encyclopedia of Food Sciences and Nutition. Elsevier Science Ltd., p. 1413-1418.

Austin, J.W. (2003b) Occurrence of *Clostridium botulinum*. In: L. Trugo and P.M. Finglas (Eds), Encyclopedia of Food Sciences and Nutrition. Elsevier Science Ltd., p. 1407-1413.

Aymerich, T., Garriga, M., Ylla, J., Vallier, J., Monfort, J. and Hugas, M. (2000) Application of enterocins as biopreservatives against *Listeria innocua* in meat products. J Food Prot 63, 721-726.

Bani-Jaber, A., McGuire, J., Ayres, J.W. and Daeschel, M.A. (2000) Efficacy of the antimicrobial peptide nisin in emulsifying oil in water. J Food Sci 65, 502-506.

Barakat, R.K., Griffiths, M.W. and Harris, L.J. (2000) Isolation and characterization of *Carnobacterium*, *Lactococcus*, and *Enterococcus* spp. from cooked, modified atmosphere packaged, refrigerated poultry meat. Int J Food Microbiol 62, 83-94.

Barash, J.R. and Arnon, S.S. (2004) Dual toxin-producing strain of *Clostridium botulinum* type Bf isolated from a California patient with infant botulism. J Clin Microbiol 42, 1713-1715.

Barbut, S., Tanaka, N., Cassens, R.G. and Maurer, A.J. (1986) Effects of sodium chloride reduction and polyphosphate addition on *Clostridium botulinum* toxin production in turkey frankfurters. J Food Sci 51, 1136-1138.

BD. (2009) Difco™ and BBL™ Manual: Manual of Microbiological Culture Media, Vol. 2010. Becton, Dickinson and Company, Sparks, MD.

Belitz, H.-D., Grosch, W. and Schieberle, P. (2004) Food Chemistry. Springer-Verlag, New York.

Bell, C. and Kyriakides, A. (2000) *Clostridium botulinum*: A Practical Approach to the Organism and its Control in Foods. Blackwell Science Inc., Oxford.

Berk, Z. (2009) Thermal processing. In: Z. Berk (Ed) Food Process Engineering and Technology. Academic Press, San Diego, p. 355-373.

Blaiotta, G., Pennacchia, C., Villani, F., Ricciardi, A., Tofalo, R. and Parente, E. (2004) Diversity and dynamics of communities of coagulase-negative staphylococci in traditional fermented sausages. J Appl Microbiol 97, 271-284.

Blom, H., Katla, T., Nissen, H. and Holo, H. (2001) Characterization, production, and purification of carnocin H, a bacteriocin produced by *Carnobacterium* 377. Curr Microbiol 43, 227-231.

Brightwell, G., Clemens, R., Adam, K., Urlich, S. and Boerema, J. (2009) Comparison of culture-dependent and independent techniques for characterisation of the microflora of peroxyacetic acid treated, vacuum-packaged beef. Food Microbiol 26, 283-288.

Brosius, J., Dull, T.J., Sleeter, D.D. and Noller, H.F. (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J Mol Biol 148, 107-127.

Bull, M.K., Olivier, S.A., van Diepenbeek, R.J., Kormelink, F. and Chapman, B. (2009) Synergistic inactivation of spores of proteolytic *Clostridium botulinum* strains by high pressure and heat is strain and product dependent. Appl Environ Microbiol 75, 434-445.

Canadian General Standards Board. (2008) Organic Production Systems General Principles and Management Standards. In: CAN/CGSB-32.310-2006. Government of Canada, Gatineau, ON, p. 1-36.

CDC. (2010) Summary of Notifiable Diseases-United States, 2008. Morbidity and Mortality Weekly Report 57, 1-100.

CDC. (2007) Botulism associated with commercially canned chili sauce --- Texas and Indiana, July 2007. Morbidity and Mortality Weekly Report 56, 1-3.

Champomier, M.-C., Montel, M.-C. and Talon, R. (1989) Nucleic acid relatedness studies on the genus *Carnobacterium* and related taxa. J Gen Microbiol 135, 1391-1394.

Chesneau, O., Morvan, A., Grimont, F., Labischinski, H. and El Solh, N. (1993) *Staphylococcus pasteuri* sp. nov., isolated from human, animal, and food specimens. Int J Syst Bacteriol 43, 237-244.

Collins, M.D. and East, A.K. (1998) Phylogeny and taxonomy of the foodborne pathogen *Clostridium botulinum* and its neurotoxins. J Appl Microbiol 84, 5-17.

Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S. and Jones, D. (1987) Classification of *Lactobacillus divergens, Lactobacillus piscicola,* and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. Int J Syst Bacteriol 37, 310-316.

Conner, D.E., Scott, V.N., Bernard, D.T. and Kautter, D.A. (1989) Potential *Clostridium botulinum* hazards associated with extended shelf-life refrigerated foods: a review. J Food Safety 10, 131-153.

Cordoba, J.J., Collins, M.D. and East, A.K. (1995) Studies on the genes encoding botulinum neurotoxin type A of *Clostridium botulinum* from a variety of sources. Syst Appl Microbiol 18, 13-22.

Dahlenborg, M., Borch, E. and Rådström, P. (2003) Prevalence of *Clostridium botulinum* types B, E and F in faecal samples from Swedish cattle. Int J Food Microbiol 82, 105-110.

Dahlenborg, M., Borch, E. and Rådström, P. (2001) Development of a combined selection and enrichment PCR procedure for *Clostridium botulinum* types B, E, and F and its use to determine prevalence in fecal samples from slaughtered pigs. Appl Environ Microbiol 67, 4781-4788.

Davidson, C.M. and Cronin, F. (1973) Medium for the selective enumeration of lactic acid bacteria from foods. Appl Microbiol 26, 439-440.

Davies, E.A., Milne, C.F., Bevis, H.E., Potter, R.W., Harris, J.M., Williams, G.C., Thomas, L.V. and Delves-Broughton, J. (1999) Effective use of nisin to control lactic acid bacterial spoilage in vacuum-packed bologna-type sausage. J Food Prot 62, 1004-1010.

De Man, J.D., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 23, 130-135.

Dezfulian, M., McCroskey, L.M., Hatheway, C.L. and Dowell, V.R., Jr. (1981) Selective medium for isolation of *Clostridium botulinum* from human feces. J Clin Microbiol 13, 526-531.

Divers, T.J., Bartholomew, R.C., Messick, J.B., Whitlock, R.H. and Sweeney, R.W. (1986) *Clostridium botulinum* type B toxicosis in a herd of cattle and a group of mules. J Am Vet Med Assoc 188, 382-286.

Dodds, K.L. and Collins-Thompson, D.L. (1984) Nitrite tolerance and nitrite reduction in lactic acid bacteria associated with cured meat products. Int J Food Microbiol 1, 163-170.

Doyle, M.P. (1991) Evaluating the potential risk from extended-shelf-life refrigerated foods by *Clostridium botulinum* inoculation studies. Food Technol-Chicago 45, 154-156.

Drider, D., Fimland, G., Héchard, Y., McMullen, L.M. and Prévost, H. (2006) The continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 70, 564-582.

Eklund, M.W., Pelroy, G.A., Paranjype, R., Peterson, M.E. and Teeny, F.M. (1982) Inhibition of *Clostridium botulinum* types A and E toxin production by liquid smoke and NaCl in hot-process smoke-flavoured fish. J Food Prot 45, 935-941.

Eklund, M.W., Wieler, D.I. and Poysky, F.T. (1967) Outgrowth and toxin production of nonproteolytic type B *Clostridium botulinum* at 3.3 to 5.6°C. J Bacteriol 93, 1461-1462.

European Commission. (2008) Commission Regulation laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control. (EC) No 889/2008, p. L250/1-L250/84.

Food Safety Inspection Service, USDA. (2010) Meat and Poultry Labeling Terms. http://www.fsis.usda.gov/factsheets/Meat_&_Poultry_Labeling_Terms/index.asp. Last updated 27 October 2010.

Franciosa, G., Ferreira, J.L. and Hatheway, C.L. (1994) Detection of type A, B, and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. J Clin Microbiol 32, 1911-1917.

Gao, Y. (2001) Brochocin-C resistance in *Listeria monocytogenes*. PhD thesis, Department of Agricultural, Food, and Nutritional Science. University of Alberta, Edmonton, AB, p. 178.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D. and Bairoch, A. (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 31, 3784-3788.

Ghalfi, H., Kouakou, P., Duroy, M., Daoudi, A., Benkerroum, N. and Thonart, P. (2006) Antilisterial bacteriocin-producing strain of *Lactobacillus curvatus* CWBI-B28 as a preservative culture in bacon meat and influence of fat and nitrites on bacteriocins production and activity. Food Sci Technol Int 12, 325-333.

Gilbert, S.E., Whyte, R., Bayne, G., Lake, R.J. and van der Logt, P. (2002) Survey of internal temperature of New Zealand domestic refrigerators. Brit Food J 109, 323-329.

Gill, D.M. (1982) Bacterial toxins: a table of lethal amounts. Microbiol Rev 46, 86-94.

Glass, K.A. and Johnson, E.A. (2004) Antagonistic effect of fat on the antibotulinal activity of food preservatives and fatty acids. Food Microbiol 21, 675-682.

González, B. and Díez, V. (2002) The effect of nitrite and starter culture on microbiological quality of "chorizo"--a Spanish dry cured sausage. Meat Sci 60, 295-298.

Götz, F., Bannerman, T. and Schleifer, K.-H. (2006) The Genera *Staphylococcus* and *Micrococcus*. In: M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt (Eds), The Prokaryotes, Vol. 4. Springer New York, New York, p. 5-75.

Hastings, J.W., Sailer, M., Johnson, K., Roy, K.L., Vederas, J.C. and Stiles, M.E. (1991) Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J Bacteriol 173, 7491-7500.

Hill, K.K., Smith, T.J., Helma, C.H., Ticknor, L.O., Foley, B.T., Svensson, R.T., Brown, J.L., Johnson, E.A., Smith, L.A., Okinaka, R.T., Jackson, P.J. and Marks, J.D. (2007) Genetic diversity among botulinum neurotoxin-producing clostridial strains. J Bacteriol 189, 818-832.

Huss, H.H. (1980) Distribution of *Clostridium botulinum*. Appl Environ Microbiol 39, 764-769.

Hutson, R.A., Zhou, Y., Collins, M.D., Johnson, E.A., Hatheway, C.L. and Sugiyama, H. (1996) Genetic characterization of *Clostridium botulinum* type A containing silent type B neurotoxin gene sequences. J Biol Chem 271, 10786-10792.

Hutson, R.A., Thompson, D.E., Lawson, P.A., Schocken-Itturino, R.P., Böttger, E.C. and Collins, M.D. (1993) Genetic interrelationships of proteolytic *Clostridium botulinum* types A, B, and F and other members of the *Clostridium botulinum* complex as revealed by small-subunit rRNA gene sequences. Anton van Leeuw Int J G 64, 273-283.

Hyytiä-Trees, E., Skytta, E., Mokkila, M., Kinnunen, A., Lindström, M., Lahteenmaki, L., Ahvenainen, R. and Korkeala, H. (2000) Safety evaluation of sous vide-processed products with respect to nonproteolytic *Clostridium botulinum* by use of challenge studies and predictive microbiological models. Appl Environ Microbiol 66, 223-229.

Ito, K.A. and Chen, J.K. (1978) Effect of pH on growth of *Clostridium botulinum* in foods. Food Technol-Chicago 32, 71-72, 76.

Ivey, F.J., Shaver, K.J., Christiansen, L.N. and Tompkin, R.B. (1978) Effect of potassium sorbate on toxinogenesis by *Clostridium botulinum* in bacon. J Food Prot 41, 621-625.

Jack, R., Tagg, J.R. and Ray, B. (1995) Bacteriocins of gram-positive bacteria. Microbiol Rev 59, 171-200.

Johnson, M.A. and Loynes, R. (1971) Inhibition of *Clostridium botulinum* by sodium nitrite as affected by bacteriological media and meat suspensions. Can I Food Sc Tech J 4, 179-184.

Kelch, W.J., Kerr, L.A., Pringle, J.K., Rohrbach, B.W. and Whitlock, R.H. (2000) Fatal *Clostridium botulinum* toxicosis in eleven Holstein cattle fed round bale barley haylage. J Vet Diagn Invest 12, 453-455.

Kinde, H., Bettey, R.L., Ardans, A., Galey, F., D., Daft, B.M., Walker, R.L., Eklund, M.W. and Byrd, J.W. (1991) *Clostridium botulinum* type-C intoxication associated with consumption of processed alfalfa hay cubes in horses. J Am Vet Med Assoc 199, 742-746.

Kloos, W.E. and Schleifer, K.-H. (1975) Isolation and characterization of Staphylococci from human skin II. Descriptions of four new species: *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus horninis*, and *Staphylococcus simulans*. Int J Syst Bacteriol 25, 62-79.

Korkeala, H., Alanko, T. and Tiusanen, T. (1992) Effect of sodium nitrite and sodium chloride on growth of lactic acid bacteria. Acta Vet Scand 33, 27-32.

Kouakou, P., Ghalfi, H., Destain, J., Dubois-Dauphin, R., Evrard, P. and Thonart, P. (2009) Effects of curing sodium nitrite additive and natural meat fat on growth control of *Listeria monocytogenes* by the bacteriocin-producing *Lactobacillus curvatus* strain CWBI-B28. Food Microbiol 26, 623-628.

Kyte, J. and Doolittle, R. (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157, 105-132.

Lagendijk, E., Asséré, A., Derens, E. and Carpentier, B. (2008) Domestic refrigeration practices with emphasis on hygiene: analysis of a survey and consumer recommendations. J Food Prot 71, 1898-1904.

Laguerre, O., Derens, E. and Palagos, B. (2002) Study of domestic refrigerator temperature and analysis of factors affecting temperature: a French survey. Int J Refrig 25, 653-659.

Leroi, F., Joffraud, J.J., Chevalier, F. and Cardinal, M. (1998) A study of the microbial ecology of cold smoked salmon during storage at 8°C. Int J Food Microbiol 39, 111-121.

Leroy, F. and De Vuyst, L. (2005) Simulation of the effect of sausage ingredients and technology on the functionality of the bacteriocin-producing *Lactobacillus sakei* CTC 494 strain. Int J Food Microbiol 100, 141-152.

Leroy, F. and de Vuyst, L. (1999) The presence of salt and a curing agent reduces bacteriocin production by *Lactobacillus sakei* CTC 494, a potential starter culture for sausage fermentation. Appl Environ Microbiol 65, 5350-5356.

Lindström, M., Kiviniemi, K. and Korkeala, H. (2006) Hazard and control of group II (non-proteolytic) *Clostridium botulinum* in modern food processing. Int J Food Microbiol 108, 92-104.

Lindström, M. and Korkeala, H. (2006) Laboratory diagnostics of botulism. Clin Microbiol Rev 19, 298-314.

Lund, B.M. and Peck, M.W. (1994) Heat resistance and recovery of spores of non-proteolytic *Clostridium botulinum* in relation to refrigerated, processed foods with an extended shelf-life. J Appl Bacteriol 76, 115S-128S.

Lyhs, U., Björkroth, J. and Korkeala, H. (1999) Characterisation of lactic acid bacteria from spoiled, vacuum-packaged, cold-smoked rainbow trout using ribotyping. Int J Food Microbiol 52, 77-84.

Lynt, R.K., Kautter, D.A. and Solomon, H.M. (1982) Differences and similarities among proteolytic and nonproteolytic strains of *Clostridium botulinum* types A, B, E, and F: a review. J Food Prot 45, 466-474.

Margosch, D., Ehrmann, M.A., Buckow, R., Heinz, V., Vogel, R.F. and Gänzle, M.G. (2006) High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. Appl Environ Microbiol 72, 3476-3481.

Margosch, D., Ehrmann, M.A., Buckow, R., Gänzle, M.G. and Vogel, R.F. (2004) Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. J Food Prot 67, 2530-2537.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E. and van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. Appl Environ Microbiol 64, 4757-4766.

McMullen, L.M. and Stiles, M.E. (1993) Microbial ecology of fresh pork stored under modified atmosphere at -1, 4.4 and 10°C. Int J Food Microbiol 18, 1-14.

Mora, D., Scarpellini, M., Franzetti, L., Colombo, S. and Galli, A. (2003) Reclassification of *Lactobacillus maltaromicus* (Miller et al. 1974) DSM 20342T and DSM 20344 and *Carnobacterium piscicola* (Collins et al. 1987) DSM 20730T and DSM 20722 as *Carnobacterium maltaromaticum* comb. nov. Int J Syst Evol Microbiol 53, 675-678.

Nilsson, L., Gram, L. and Huss, H.H. (1999) Growth control of *Listeria monocytogenes* on cold-smoked salmon using a competitive lactic acid bacteria flora. J Food Prot 62, 336-342.

Peck, M., Goodburn, K.E., Betts, R.P. and Stringer, S.C. (2008) Assessment of the potential for growth and neurotoxin formation by non-proteolytic *Clostridium botulinum* in short shelf-life commercial foods designed to be stored chilled. Trends Food Sci Technol 19, 207-216.

Peck, M.W. (2006) *Clostridium botulinum* and the safety of minimally heated, chilled foods: an emerging issue? J Appl Microbiol 101, 556-570.

Peck, M.W. and Stringer, S.C. (2005) The safety of pasteurised in-pack chilled meat products with respect to the foodborne botulism hazard. Meat Sci 70, 461-475.

Peck, M.W., Lund, B.M., Fairbairn, D.A., Kaspersson, A.S. and Undeland, P.C. (1995) Effect of heat treatment on survival of, and growth from, spores of nonproteolytic *Clostridium botulinum* at refrigeration temperatures. Appl Environ Microbiol 61, 1780-1785.

Pflug, I.J. and Odlaug, T.E. (1978) A review of z and f-values used to ensure the safety of low-acid canned foods. Food Technol 32, 63-70.

Ramaswamy, H.S. and Shao, Y. (2010) High pressure destruction kinetics of *Clostridium sporogenes s*pores in salmon slurry at elevated temperatures. Int J Food Prop 13, 1074 - 1091.

Ramaswamy, H.S., Shao, Y. and Zhu, S. (2010) High-pressure destruction kinetics of *Clostridium sporogenes* ATCC 11437 spores in milk at elevated quasi-isothermal conditions. J Food Eng 96, 249-257.

Rayman, M.K., Aris, B. and Hurst, A. (1981) Nisin: a possible alternative or adjunct to nitrite in the preservation of meats. Appl Environ Microbiol 41, 375-380.

Reddy, N.R., Tetzloff, R.C., Solomon, H.M. and Larkin, J.W. (2006) Inactivation of *Clostridium botulinum* nonproteolytic type B spores by high pressure processing at moderate to elevated high temperatures. Innov Food Sci Emerg 7, 169-175.

Reddy, N.R., Solomon, H.M., Tetzloff, R.C. and Rhodehamel, E.J. (2003) Inactivation of *Clostridium botulinum* type A spores by high-pressure processing at elevated temperatures. J Food Prot 66, 1402-1407.

Rhodehamel, E.J., Reddy, N.R. and Pierson, M.D. (1992) Botulism: the causative agent and its control in foods. Food Control 3, 125-143.

Ricketts, S.W., Greet, T.R.C., Glyn, P.J., Ginnett, C.D.R., McAllister, E.P., McCaig, J., Skinner, P.H., Webbon, P.M., Frape, D.L., Smith, G.R. and Murray, L.G. (1984) Thirteen cases of botulism in horses fed big bale silage. Equine Vet J 16, 515-518.

Roberts, T.A., Gibson, A.M. and Robinson, A. (1981a) Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized, cured meats: I. Growth in pork slurries prepared from "low" pH meat (pH range 5.5-6.3). J Food Technol 16, 239-266.

Roberts, T.A., Gibson, A.M. and Robinson, A. (1981b) Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized, cured meats: II. Growth in pork slurries prepared from "high" pH meat (pH range 6.3-6.8). J Food Technol 16, 267-281.

Roberts, T.A., Gibson, A.M. and Robinson, A. (1981c) Prediction of toxin production by *Clostridium botulinum* in pasteurized pork slurry. J Food Technol 16, 337-355.

Roberts, T.A. and Smart, J.L. (1974) Inhibition of spores of *Clostridium* spp. by sodium nitrite. J Appl Microbiol 37, 261-264.

Rose, N.L., Sporns, P., Stiles, M.E. and McMullen, L.M. (1999) Inactivation of nisin by glutathione in fresh meat. J Food Sci 64, 759-762.

Sagoo, S.K., Little, C.L., Allen, G., Williamson, K. and Grant, K.A. (2007) Microbiological safety of retail vacuum-packed and modified-atmosphere-packed cooked meats at end of shelf life. J Food Prot 70, 943-951.

Santos-Buelga, J.A., Collins, M.D. and East, A.K. (1998) Characterization of the genes encoding the botulinum neurotoxin complex in a strain of *Clostridium botulinum* producing type B and F neurotoxins. Curr Microbiol 37, 312-318.

Scott, V.N. and Taylor, S.L. (1981) Effect of nisin on the outgrowth of *Clostridium botulinum* spores. J Food Sci 46, 117-126.

Sindelar, J.J. and Houser, T.A. (2009) Alternative Curing Systems. In: R. Tarté (Ed) Ingredients in Meat Products. Springer, New York, p. 379-405.

Siragusa, G.R. and Cutter, C.N. (1993) Brochocin-C, a new bacteriocin produced by *Brochothrix campestris*. Appl Environ Microbiol 59, 2326-2328.

Smith, L.D.S. (1978) The occurrence of *Clostridium botulinum* and *Clostridium tetani* in the soil of the United States. Health Lab Sci 15, 74-80.

Sobel, J., Tucker, N., Sulka, A., McLaughlin, J. and Maslanka, S. (2004) Foodborne botulism in the United States, 1990-2000. Emerg Infect Dis 10, 1606-1611.

Songer, J.G. (2005) Clostridial diseases in domestic animals. In: P. Dürre (Ed) Handbook on Clostridia. Taylor & Francis, Boca Raton, FLA, p. 903.

Stones, M. (2010) DEFRA non committal on EC plans to ban nitrates in organic ham. http://www.foodnavigator.com/Legislation/DEFRA-non-committal-on-EC-plans-to-ban-nitrates-in-organic-ham. Food Navigator. Last updated 6 October 2010.

Stumbo, C.R., Purohit, K.S. and Ramakrishna, T.V. (1975) Thermal lethality guide for low-acid foods in metal containers. J Food Sci 40, 1316-1323.

Tompkin, R.B. (1983) Indirect antimicrobial effects in foods: phosphates. J Food Safety 6, 13-27.

USDA. (2010) National Organic Program 7CFR205.606. http://www.ams.usda.gov/AMSv1.0/nop. Accessed 7 December 2010.

USDA. (1998) Bacteriological Analytical Manual, 8th ed. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm063710.htm. Accessed 28 September 2009.

Van Wezemael, L., Verbeke, W., de Barcellos, M., Scholderer, J. and Perez-Cueto, F. (2010) Consumer perceptions of beef healthiness: results from a qualitative study in four European countries. Brit Med J- Public Health 10, 342.

Verluyten, J., Messens, W. and De Vuyst, L. (2003) The curing agent sodium nitrite, used in the production of fermented sausages, is less inhibiting to the bacteriocin-producing meat starter culture *Lactobacillus curvatus* LTH 1174 under anaerobic conditions. Appl Environ Microbiol 69, 3833-3839.

Wagner, M.K. and Busta, F.F. (1985) Inhibition of *Clostridium botulinum* 52A toxicity and protease activity by sodium acid pyrophosphate in media systems. Appl Environ Microbiol 50, 16-20.

Wagner, M.K. and Busta, F.F. (1984) Inhibition of *Clostridium botulinum* growth from spore inocula in media containing sodium acid pyrophosphate and potassium sorbate with or without added sodium chloride. J Food Sci 49, 1588-1594.

Walker, R. (1990) Nitrates, nitrites and *N*-nitrosocompounds: a review of the occurrence in food and diet and the toxicological implications. Food Addit Contam 7, 717 - 768.

Whitlock, R.H. and Buckley, C. (1997) Botulism. Vet Clin N Am-Equine 13, 107-128.

Whitlock, R.H. and Williams, J.M. (1999) Botulism toxicosis of cattle. In: R.A. Smith (Ed) 32nd Annual Convention of American Association of Bovine Practitioners, Nashville, TN, p. 45-53.

Chapter Four: **Inactivation of bacteriocins by Group II (proteolytic) *Clostridium botulinum***

4.1 Introduction

Bacteriocins have been proposed as alternative treatments for inhibiting outgrowth of *Cl. botulinum* spores in foods. Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by bacteria with activity against closely-related bacteria (Jack et al., 1995). One well-studied bacteriocin is nisin, which has been used in foods for over 40 years, and has demonstrated activity against *Cl. botulinum* (Rayman et al., 1981; Scott and Taylor 1981). More recently, brochocin-C, produced by *Brochothrix campestris* (Siragusa and Cutter, 1993), has also been identified as a potentially useful bacteriocin effective against *Cl. botulinum* spores (McCormick et al., 1998). Brochocin-C is a two-peptide bacteriocin consisting of the two peptides brochocin-A and brochocin-B, which have reported masses of 5244 Da and 3944 Da, respectively (McCormick et al., 1998; Garneau et al., 2003).

However, dependence on one bacteriocin may present a food safety risk, as nisin resistance has been identified in *Cl. botulinum* (Mazzotta et al., 1997; Mazzotta and Montville, 1999). Dehydropeptide-specific enzymes have accounted for nisin resistance in organisms such as *Bacillus cereus* (Jarvis, 1967; Jarvis and Farr, 1971), and although nisin resistance has been demonstrated in *Cl. botulinum* (Rayman et al., 1983), no mechanism has been proposed for any bacteriocin resistance in this organism. Extracellular proteases are produced by *Cl. botulinum*, and are involved in botulinal toxin activation (DasGupta, 1971; DasGupta, 1972; DasGupta and Sugiyama, 1972; Nakane, 1978). In this study, we propose that the proteolytic enzymes of Group I *Cl. botulinum* are responsible for proteolysis of brochocin-C, and demonstrate that the proteases involved are not bacteriocin-specific.

4.2 Materials and Methods

4.2.1 Culture maintenance and spore production

Group I *Cl. botulinum* strains used in this study were *Cl. botulinum* 368B, IIB, 6A, and 62A, while the Group II strain was *Cl. botulinum* 2B (University of Alberta). *Br. campestris* ATCC 43754 (American Type Culture Collection; Manassas, VA) was used to produce brochocin-C, and *Carnobacterium maltaromaticum* NCIMB 702852 (National Collection of Industrial and Marine Bacteria; Aberdeen, SF) was used as the brochocin-C-sensitive indicator strain. *Br. campestris* and *C. maltaromaticum* were stored in APT broth (All-Purpose Tween) (Difco, Becton Dickinson; Sparks, MD) containing 20 % (v/v) glycerol at -80°C, while *Cl. botulinum* strains were stored as spore stocks in sterile 0.9 % (w/v) NaCl at 4°C. Enumeration of both spores and vegetative cells was done using Reinforced Clostridial Medium (RCM; Difco) containing 2.5 % (w/v) agar. Medium for spore production for strain 6A was RCM, while spores of all other strains were produced in Trypticase-Peptone-Glucose-Yeast Extract broth (TPGY) (USDA, 1998) containing, per litre: 50 g tryptone, 5 g peptone, 20 g yeast extract (Difco), 4 g glucose (Fisher Scientific Canada; Ottawa, ON), and 1 g sodium thioglycollate (Sigma-Aldrich Canada Ltd; Oakville, ON). Before use, all media was incubated in an anaerobic chamber for at least 24 h (Coy Laboratory Products Inc; Grass Lake, MI) under an atmosphere of 5 % CO_2, 10 % H_2, and balance N_2 (Praxair Canada; Edmonton, AB) to ensure anaerobic conditions.

For spore production, 100 µL of spore suspensions were inoculated into 5 mL of appropriate medium, heat-shocked in a water bath for 15 min at 75°C (Group I) or 55°C (Group II), and incubated at 37°C under anaerobic conditions for 48 h. The 5-mL culture was then used to inoculate 250 mL of the appropriate medium, and the culture was incubated at 37°C under anaerobic conditions for 14 days with periodic monitoring by phase contrast microscopy. Spores were harvested by centrifuging at 16270 x *g* for 20 min, followed by ten washes (100

mL) and a final resuspension (10 mL) in sterile 0.9 % (w/v) NaCl, and heated at 65°C for 1 h (Gao, 2001) to destroy residual toxin and vegetative cells.

4.2.2 Brochocin-C production

An abbreviated method of McCormick et al. (1998) was used to produce brochocin-C. Briefly, Casamino Acids Medium (CAA) (Hastings et al., 1991) supplemented with 2.5 % (w/v) glucose was inoculated with 2 % (v/v) of an overnight culture of *Br. campestris*. Cultures were incubated at 26°C with stirring at 250 rev min^{-1} for 24 h, and the bacteriocin-containing supernatant was recovered by centrifuging at 8000 x *g* for 20 min. The supernatant was heated at 60°C for 30 min to inactivate any remaining cells, and then extracted twice with 1-butanol (Fisher Scientific Canada) with a butanol: supernatant ratio of 1:2. The organic phase was mixed in a 1:1 ratio with MilliQ® water (Millipore; Billerica, MA), and concentrated by rotary evaporation (Büchi; Flawil, CH) at 36°C until no butanol remained. Antibacterial activity of brochocin-C was assayed by diluting the semi-purified brochocin-C in a two-fold series with 0.1 % (v/v) trifluoroacetic acid (Sigma), spotting 10 µL of each dilution onto APT agar containing 1.5 % (w/v) agar, and overlaying with semi-solid APT agar [0.75 % (w/v) agar] seeded with 1 % (v/v) of an overnight culture of the sensitive indicator organism *C. maltaromaticum* NCIMB 702852. Plates were incubated anaerobically at 30°C, and were examined at 24 h for zones of clearing. Arbitrary activity units per mL (AU mL^{-1}) were calculated from the reciprocal of the last dilution showing inhibition (McCormick et al., 1998).

4.2.3 Model meat system preparation

A cocktail of Group I *Cl. botulinum* was diluted in 0.9 % (w/v) NaCl to contain 3 x 10^4 spores mL^{-1} of each strain. Three hundred millilitres of sterile, anaerobic Cooked Meat Medium (CMM; Difco) was supplemented with brochocin-C at concentrations of 0, 800, or 1600 AU mL^{-1} in triplicate. One millilitre of heat-shocked spore cocktail was inoculated into CMM to give a final spore concentration of 100 spores mL^{-1}, as recommended by Doyle (1991). Solids-free controls were set up in triplicate by autoclaving CMM, filtering

through several layers of cheesecloth to remove large particles, then filter-sterilizing through a 0.22 µm filter before supplementation with 1600 AU mL^{-1} brochocin-C and inoculation. Plate counts of vegetative cells were done on selected days. The pH of the cultures was measured at the beginning and end of the experiment using ColorPhast pH indicator strips (EMD Chemicals; Gibbstown, NJ).

4.2.4 Confirmation of proteolysis

Samples were drawn on day 40 from one replicate of the Group I cultures containing 1600 AU mL^{-1} of brochocin-C, and from one replicate of the solids-free control. Aliquots of each were centrifuged at 20800 x *g* for 5 min, and 10 µL of each of the supernatant and culture was spotted beside 5 µL of brochocin-C on APT agar, and was allowed to dry. Controls consisting of only brochocin-C and only culture were also spotted. Plates were overlayered with 5 mL of semi-solid APT agar inoculated with 1 % (v/v) of an overnight culture of *C. maltaromaticum* NCIMB 702852, incubated at 30°C under anaerobic conditions, and were examined at 24 h for evidence of proteolysis.

Proteolysis of other classes of bacteriocins shown in Table 4-1 was tested by similar methods. Briefly, viable cultures of *Cl. botulinum* strains IIB, 368B, 6A, 62A, and 2B were grown by inoculating 5 mL of RCM with 200 µL of a 3 x 10^4 spores mL^{-1} stock, heat-shocking at 75°C (55°C for 2B) for 15 min, and incubating at 37°C under anaerobic conditions. After 72 h incubation, supernatant was collected by centrifuging 1 mL at 10 000 x *g* for 2 min, and 5 µL of each strain was spotted onto the center of an air-dried 5-µL spot of bacteriocin on APT agar plates.

Additional confirmation of proteolysis of brochocin-C was done by matrix-assisted laser desorption/ionization-time of flight analysis (MALDI-TOF) (Mass Spectrometry Facility, University of Alberta). Brochocin-C used in this analysis underwent additional purification steps beyond the extraction with 1-butanol described in Section 4.2.2. A chloroform extraction followed by gel filtration was performed as described by Garneau et al. (2003). Briefly, following concentration of the 1-butanol extract, the brochocin-C preparation was

Table 4-1 Bacteriocins and indicator organisms used

Bacteriocin (source)	Concentration	Indicator organism used
Brochocin-C (partially purified from *Brochothrix campestris* ATCC 43754)	1600 AU mL^{-1}	*Carnobacterium maltaromaticum* NCIMB 702852
Nisin [as Chrisin (2.5 % nisin), Chr. Hansen; Hørsholm, DK]	25 µg mL^{-1}	*Lactobacillus sakei* Lb 706B[1]
Sakacin A (partially purified from *Lactobacillus sakei* Lb 706)[1]	na	*Listeria monocytogenes* CDC 7762
Leucocin A (partially purified from *Leuconostoc gelidum* UAL187)[2]	na	*C. maltaromaticum* NCIMB 702852
Enterocin 7A (purified)[4]	1.8 mg mL^{-1}	*L. monocytogenes* CDC 7762
Pediocin PA-1 (crude preparation from *Pediococcus acidilactici* PAC1.0)[3]	na	*C. divergens* NCIMB 702855
Subtilosin A (purified)[5]	12.5 µg mL^{-1}	*L. monocytogenes* FS-15[6]
Carnocyclin A (purified)[5]	73.2 µg mL^{-1}	*L. monocytogenes* FS-15

[1] Schillinger and Lücke (1989), renamed as *Lb. sakei* (Trüper and de'Clari, 1997), [2] Hastings et al. (1991); Hastings and Stiles (1991), [3] Gonzalez and Kunka (1987),[4] courtesy of X. Liu, [5]courtesy of Dr. J. Vederas, [6] University of Alberta; na-not available, ATCC-American Type Culture Collection, CDC-Centers for Disease Control, NCIMB-National Collection of Industrial and Marine Bacteria

resuspended in 10 mL MilliQ water per L of original culture supernatant volume. The resuspended preparation was then extracted with 150 mL chloroform (Fisher Scientific Canada) per L original culture supernatant volume, and centrifuged in Teflon® centrifuge bottles (Fisher Scientific Canada) at 1993 x *g* for 4 min at 4°C to break any emulsion formed. The preparation was concentrated by rotary evaporation (Büchi; Flawil, CH) at 30°C to a final volume of ~ 1mL. This concentrate was resuspended in a 1:1 ratio of chloroform:methanol, and was stored under nitrogen at 4°C. The concentrated sample was loaded onto an SR-25 solvent-resistant column (GE Healthcare; Uppsala, SE) containing Sephadex LH-20 resin (GE Healthcare) equilibrated with chloroform:methanol (1:1) at a flow rate of 2 mL min^{-1}. Fractions were collected, and were tested for antimicrobial activity as described in Section 4.2.2.

To prepare samples for MALDI analysis, viable cultures of *Cl. botulinum* strains IIB, 368B, 6A, and 62A were grown by inoculating 5 mL of TPGY broth with 200 µL of a 3 x 10^4 spores mL^{-1} stock, heat-shocking at 75°C for 15 min, and incubating at 37°C under anaerobic conditions. After 24 h incubation, cultures were subcultured [1 % (v/v)] into 5 mL fresh TPGY broth. After 24 h of incubation at 37°C under anaerobic conditions, the supernatant was collected by centrifuging 1 mL at 10 000 x *g* for 5 min. For a heat-denatured control, half of the clarified supernatant was autoclaved at 121°C for 40 min to inactivate proteases produced by *Cl. botulinum*. Following autoclaving, 50 µL of the supernatant was added to 50 µL of semi-purified brochocin-C, and the sample was incubated overnight at 30°C. For treatments in which the proteases retained activity, 50 µL of clarified supernatant was used to treat 50 µL of semi-purified brochocin-C, and the samples were incubated overnight at 30°C. Following incubation, samples were autoclaved (121°C, 40 min) to destroy the botulinum toxin. Samples of brochocin-C were also autoclaved under the same conditions to ensure no loss of brochocin-C from autoclaving.

For MALDI-TOF analysis, 1 µL of sample was spotted on a ground steel MALDI target, air-dried, and 1 µL of a saturated solution of sinapinic acid in 50:50 mixture of acetonitrile:trifluoroacetic acid in water [(0.1 % (v/v)] was added. The spots were washed three times with 5 µL of 0.1 % (v/v) trifluoroacetic acid in water. Mass spectra were obtained on a Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics; Bremen, DE). Ionization was performed with a 1 kHz smartbeam II laser, and the mass spectrometer was used in a reflectron mode of operation.

4.2.5 Identification of protease class

To characterize the protease(s) responsible for inactivation of brochocin-C, protease inhibitors specific for different protease classes were used. The protease inhibitors, protease class inhibited, and the final concentrations are listed in Table 4-3. All protease inhibitors were purchased from Sigma, with the exception of AEBSF and EDTA, which were purchased from Fisher Scientific

Canada. Briefly, 1.2 x 10^3 spores mL^{-1} of Group I *Cl. botulinum* strains IIB, 368B, 6A, and 62A were inoculated into anaerobic RCM broth, heat-shocked at 75°C for 15 min, and incubated at 37°C under anaerobic conditions for 72 h. Supernatant was collected from each strain by centrifuging at 5000 x *g* for 10 min, and volumes of the clarified supernatant were treated with protease inhibitors at the aforementioned concentrations for 1 h at 30°C. An untreated control of each strain was also heat-treated and used. Following inhibitor treatment, 5 µL of the supernatant was spotted directly onto brochocin-C spots on APT agar plates (5-µL spots containing 2.9 x 10^3 AU mL^{-1} brochocin-C). Plates were overlayered with semi-solid APT seeded with 1 % (v/v) of an overnight culture of *C. maltaromaticum* NCIMB 702852, and were incubated at 30°C under anaerobic conditions. Plates were examined at 24 h for clear zones, indicating that proteases had been inhibited.

4.3 Results

4.3.1 Model meat system

In the presence of brochocin-C and cooked meat solids, the cocktail of Group I *Cl. botulinum* strains grew rapidly following a lag phase of 2 days (Figure 4-1). The solids-free control had a much longer lag phase nearing 13 days, at which point growth began, reaching a maximum concentration of 9 log CFU mL^{-1}. All other treatments reached a maximum cell concentration at 28 days, with a value of 8 log CFU mL^{-1}. Brochocin-C up to a concentration of 1600 AU mL^{-1} did not inhibit outgrowth of Group I *Cl. botulinum* spores and subsequent vegetative cell growth. Cultures did not demonstrate a pH decrease in any treatment except the treatment lacking meat solids, which showed a pH of 6.5 ± 0.5. Treatments containing 0, 800, and 1600 AU brochocin-C mL^{-1} had final pHs of 7.7 ± 0.3, 7.5, and 7.3 ± 0.3, respectively.

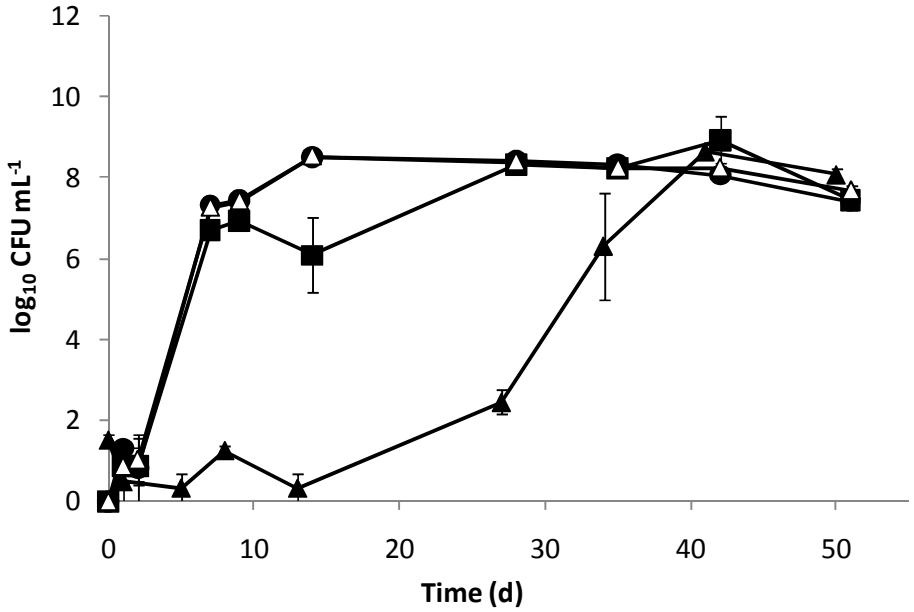

Figure 4-1 Growth of a cocktail of Group I *Cl. botulinum* in the presence of different concentrations of brochocin-C in a model meat system. Brochocin-C concentration: (●) 0 AU mL^{-1}, (■) 800 AU mL^{-1}, (△) 1600 AU mL^{-1}, (▲) solids-free control + 1600 AU mL^{-1}. Data points represent the mean of n=3, and error bars represent ± SEM.

4.3.2 Confirmation of proteolysis

To confirm that proteolysis was the mechanism of brochocin-C inactivation, samples of supernatant taken from cultures were spotted adjacent to brochocin-C, and loss of bacteriocin activity was observed (Figure 4-2). Both clarified culture supernatant and culture containing cells exhibited inactivation of brochocin-C, as demonstrated by the characteristic "flattening" of the zone of clearing (Figure 4-2). When other bacteriocins were tested, all four Group I strains inactivated the class IIa bacteriocins sakacin A and pediocin PA-1, and three strains inactivated leucocin A (Table 4-2). Nisin, enterocin 7A, subtilosin A, and carnocyclin A retained their activity, indicating no inactivation by *Cl. botulinum* supernatants. The supernatant from the Group II strain 2B did not inactivate any of the bacteriocins tested.

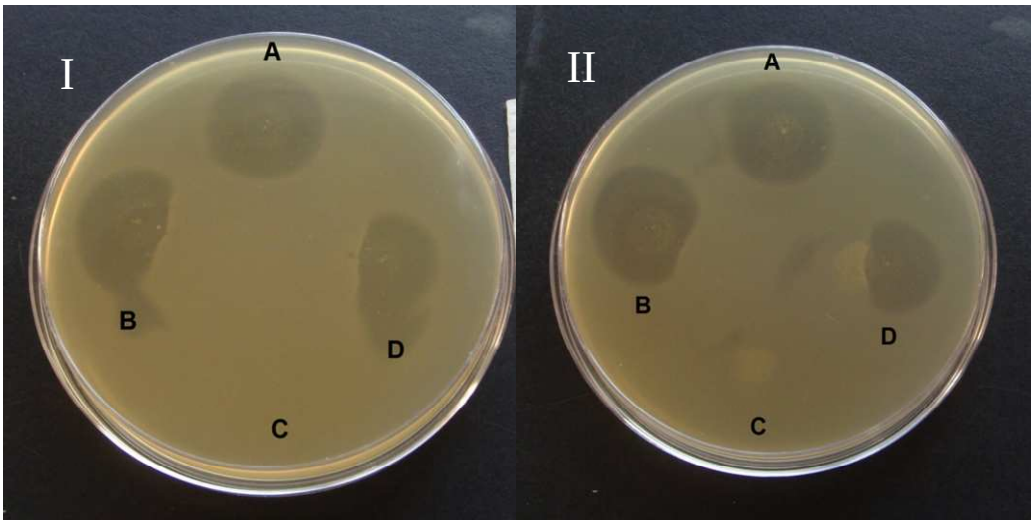

Figure 4-2 Inactivation of brochocin-C by Group I *Cl. botulinum*. I. In solids-free medium, II. In medium containing solids. A, brochocin-C only; B, brochocin-C + Group I *Cl. botulinum* cocktail culture supernatant; C, Group I *Cl. botulinum* cocktail culture only; D, brochocin-C + Group I *Cl. botulinum* cocktail culture. Petri dishes are 15 mm diameter**.**

Table 4-2 Growth of indicator strains exposed to bacteriocins treated with supernatants of *Cl. botulinum*

Bacteriocin	Class of bacteriocin	Indicator strain	*Cl. botulinum* strain					
			IIB	368B	6A	62A	2B	none
Nisin	I	*Lb. sakei* Lb 706B	-	-	-	-	-	-
Pediocin PA-1	IIa	*C. divergens* NCIMB 702855	+	+	+	+	-	-
Sakacin A	IIa	*L. monocytogenes* CDC 7762	+	+	+	+	-	-
Leucocin A	IIa	*C. maltaromaticum* NCIMB 702852	+	+	+	-	-	-
Brochocin-C	IIb	*C. maltaromaticum* NCIMB 702852	+	+	+	+	-	-
Enterocin 7A	IIc	*L. monocytogenes* CDC 7762	-	-	-	-	-	-
Subtilosin A	IV	*L. monocytogenes* FS-15	-	-	-	-	-	-
Carnocyclin A	IV	*L. monocytogenes* FS-15	-	-	-	-	-	-

+ indicates growth of indicator organism, - indicates no growth of indicator organism. Growth of indicator organism signifies inactivation of bacteriocin, while lack of growth signifies active bacteriocin.

To determine if the enzymes present in the supernatant of the strains of *Cl. botulinum* were responsible for inactivation of brochocin-C, MALDI-MS analysis was performed. This analysis revealed that only one of the component peptides, brochocin-A (5242 Da, monoisotopic molecular ion), was visualized in all untreated samples, but brochocin-B was not observed. All strains of *Cl. botulinum* showed the same pattern, so only the results of *Cl. botulinum* 62A are shown (Figure 4-3). Mass spectra of samples that contained supernatant that had been heat-treated prior to addition to brochocin-C revealed the presence of a peak at 5242 Da (Figure 4-3). After treatment with *Cl. botulinum* supernatant that had not been heat-treated prior to addition to brochocin-C, the peak at 5242 Da disappeared. Autoclaving brochocin-C with no added *Cl. botulinum* supernatant had no effect on the presence of brochocin-A (data not shown).

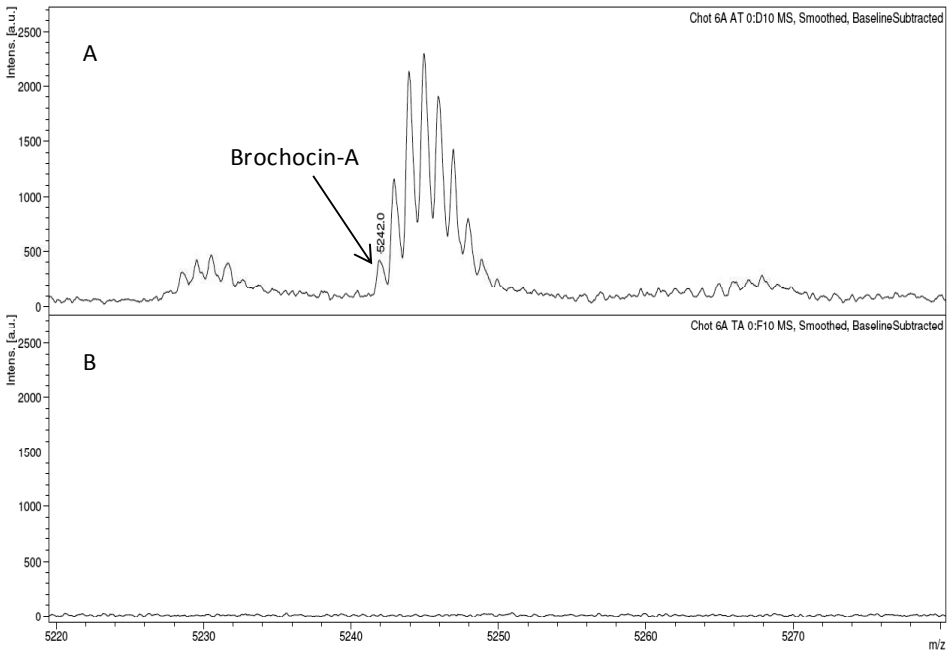

Figure 4-3 Destruction of brochocin-A in samples treated with supernatant of *Cl. botulinum* 62A as shown by MALDI-MS analysis. The sample autoclaved pre-treatment is shown in A, and the sample autoclaved post-treatment is shown in B. Position of brochocin-A is indicated (5242 Da, monoisotopic molecular ion). Brochocin-B was not observed by this method.

4.3.3 Mode of proteolysis

To determine the class(es) of protease(s) responsible for brochocin-C inactivation, inhibitors of different classes of proteases were used to treat culture supernatants of Group I *Cl. botulinum* strains. If a specific class of protease is present in the strain of *Cl. botulinum* strain tested, it is inactivated by a protease inhibitor of that class, leaving the bacteriocin brochocin-C with full activity against growth of the indicator organism.

Differences in proteases produced among the strains tested were evident, as shown in Table 4-3. At least one protease type in each *Cl. botulinum* strain was inactivated by the protease inhibitors tested in this assay, with the exception of strain 6A. Inhibition of the indicator strain was observed when the supernatant of *Cl. botulinum* strain IIB was treated with cysteine protease inhibitors, and this was the only class of proteases inhibited in strain IIB. Strains 62A and 368B displayed an inhibition spectrum similar to each other, with both producing all classes of proteases with the exception of serine proteases, which were not produced by 368B. One interesting result was that the proteolytic activity of the supernatant of *Cl. botulinum* 62A was eliminated by the serine protease inhibitor PMSF, but not by the serine protease inhibitor AEBSF.

Table 4-3 Growth of indicator strain *C. maltaromaticum* NCIMB 702852 exposed to brochocin-C and the culture supernatant of Group I *Cl. botulinum* treated with protease inhibitors. Absence of growth of the indicator strain demonstrates that a specific class of protease was inactivated by the protease inhibitor.

Protease inhibitor	Final concentration (mM)*	Target	*Cl. botulinum* strain			
			IIB	368B	6A	62A
4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)	1	Serine proteases	+	+	+	+
Bestatin	0.001	Leucine and alanyl Aminopeptidases	+	-	+	-
N-(*trans*-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64)	0.01	Cysteine proteases	-	-	+	-
EDTA	1	Metalloproteases	+	-	+	-
Pepstatin	0.001	Aspartic proteases	+	-	+	-
1,10-phenanthroline	10	Zinc metalloproteases	+	-	+	-
phenylmethanesulfonyl fluoride (PMSF)	1	Serine and cysteine proteases	+	+	+	-
Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK)	0.1	Trypsin-like serine proteases, clostripain	+	-	+	-
No inhibitor control			+	+	+	+

* concentration recommended either by manufacturer or by Salveson and Nagase (2001). + indicates growth of indicator organism, - indicates no growth of indicator organism. Growth of indicator signifies protease inhibitor inactive against protease, while no growth indicates protease inhibitor active against protease.

4.4 Discussion

Resistance to bacteriocins is common in gram-negative organisms due to protection conferred by the lipopolysaccharide-containing outer cell membrane (Jack et al., 1995), but it has also been observed in gram-positive organisms. Nisin resistance in gram-positive bacteria has been the most-studied, and has been reported in *Listeria monocytogenes* (Crandall and Montville, 1998), *B. cereus* (Jarvis, 1967; Jarvis and Farr, 1971), *Streptococcus thermophilus* (Alifax and Chevalier, 1962; Garde et al., 2004), *S. bovis* (Mantovani and Russell, 2001), *Lactobacillus casei* (Breuer and Radler, 1996), and *Cl. botulinum* (Rayman et al., 1983).

Nisin resistance resulting from enzymatic action has been observed. In *B. cereus*, a dehydropeptide reductase was identified which acted upon the C-terminal dehydroalanyllysine, reducing it to alanyllysine, and reducing its' efficacy by 76 % (Jarvis and Farr, 1971). More recently, proteolysis been identified as a mechanism of bacteriocin resistance in *Lactococcus lactis*. Sun et al. (2009) found that the 35-kDa nisin resistance protein in a non-nisin-producing strain of *Lc. lactis* was responsible for inactivating nisin via a specific proteolytic cleavage of the peptide bond between residues 28 and 29. Interestingly, the nisin resistance protein is specific for these two residues, one of which is a β-methyllanthionine ring. In both cases, enzymatic inactivation of nisin was due to specificity towards the dehydropeptide amino acid residues unique to the class I lantibiotics. Because of this specificity, it is likely that the protease(s) believed responsible for brochocin-C inactivation in Group I strains of *Cl. botulinum* are different than those described earlier in either *B. cereus* (Jarvis and Farr, 1971) or *Lc. lactis* (Sun et al., 2009).

Cl. botulinum has been reported to produce proteases that are able to hydrolyse casein (Elberg and Meyer, 1939; Bonventre and Kempe, 1960) and liquefy gelatin (Elberg and Meyer, 1939; Iida, 1964), and in fact proteolysis of such substrates is one of the main properties differentiating botulinal groups (Bell

and Kyriakides, 2000). Aminopolypeptidases have been observed by several workers, including those which demonstrated target sequence specificity (Elberg and Meyer, 1939; Millonig, 1956) and one that only cleaved proteins at the linkage proximal to the amino group (Millonig, 1956). More specific studies revealed that *Cl. botulinum* Type B strain Lamanna produces a protease specific for the carboxyl group of arginine and lysine residues (DasGupta, 1971; DasGupta, 1972), and in fact was identified as the protease responsible for cleavage and subsequent activation of the less-active botulinum progenitor toxin. Thus, extracellular proteases are known to be produced by *Cl. botulinum*, but no studies of their action on peptides such as bacteriocins have been reported.

Until the results reported in this paper, proteolysis as a mechanism of bacteriocin inactivation has not been observed in *Cl. botulinum*. In fact, Mazzotta and Montville (1999) determined that proteolysis was not responsible for nisin inactivation in a proteolytic strain of *Cl. botulinum*. The current study agreed with their conclusion in regards to nisin, because nisin was not inactivated by cell-free supernatants of *Cl. botulinum*, but proteolysis of the class II bacteriocins brochocin-C, sakacin A, leucocin A, and pediocin PA-1 was observed in this study. Previous observations (see Chapter 3) revealed that Group II *Cl. botulinum* strains were inhibited by brochocin-C, which lends further support to the conclusion that proteolysis is responsible for bacteriocin inactivation.

Although all the bacteriocins inactivated by *Cl. botulinum* were class II bacteriocins, enterocin 7A, a class IIc bacteriocin (Liu, 2010), was not inactivated, suggesting that the protease(s) responsible possess some degree of specificity. According to the results of the protease inhibitor assay, *Cl. botulinum* strain 6A did not produce proteases of any class; however, data from the spot-on-lawn assay shows that brochocin-C is inactivated, while MALDI-MS analysis indicates that one of the component peptides of brochocin-C (brochocin-A) is destroyed. Further research is required to determine the reason for the conflicting results in this strain, and to elucidate the proteases responsible for bacteriocin inactivation

of the *Cl. botulinum* strains 368B and 62A, both of which produce multiple classes of proteases.

The circular structures of subtilosin A and carnocyclin A offer an explanation for their resistance to proteolysis. In characterization studies of the circular bacteriocins carnocyclin A (Martin-Visscher et al., 2008), gassericin A (Kawai et al., 2003), circularin A (Kawai et al., 2003; Kawai et al., 2004), enterocin A (Joosten et al., 1996), and microcin J25 (Blond et al., 1999), all bacteriocins were resistant to one or more proteases tested. The rigid conformation resulting from circularizing the bacteriocins renders specific proteolytic target sites inaccessible, thereby conferring resistance to proteolysis (Maqueda et al., 2008).

One interesting observation is that in strain 62A, the sulfonyl fluoride serine protease inhibitor AEBSF, but not PMSF, inhibited proteolysis. These two protease inhibitors share many common targets (Salvesen and Nagase, 2001), and therefore similar action against the serine protease(s) produced by *Cl. botulinum* is expected. Similar observations were noted in previous studies of a protease from *Candida boidinii* (Stewart et al., 2002) and in protein storage studies (Sharma and Luthra-Guptasarma, 2009). Although AEBSF and PMSF are serine protease inhibitors, only PMSF inhibited proteases. One possible explanation given is that the phenyl group present in PMSF but not in AEBSF is necessary for enzyme binding and subsequent inhibition (Stewart et al., 2002), which may be the case with the serine protease produced by *Cl. botulinum* 62A.

This study is the first to demonstrate that proteolysis by *Cl. botulinum* is a mechanism of resistance to class II bacteriocins. Although bacteriocins have been touted as more “natural” alternatives to chemically-synthesized preservatives, the results of this study clearly indicate that careful research must be undertaken to identify routes of resistance, as well as pathogens possessing such mechanisms. One potential avenue of circumventing proteolysis by *Cl. botulinum* is to replace known proteolytic target sites with sequences that are not targeted by the proteases, which may allow previously-unusable bacteriocins to be used to

inhibit Group I *Cl. botulinum*.

4.5 References

Alifax, P.R. and Chevalier, R. (1962) Etude de la nisinase produite par *Streptococcus thermophilus*. J Dairy Res 29, 233-240.

Bell, C. and Kyriakides, A. (2000) *Clostridium botulinum*: A Practical Approach to the Organism and its Control in Foods. Blackwell Science Inc., Oxford.

Blond, A., Peduzzi, J., Goulard, C., Chiuchiolo, M.J., Barthelemy, M., Prigent, Y., Salomon, R.A., Farias, R.N., Moreno, F. and Rebuffat, S. (1999) The cyclic structure of microcin J25, a 21-residue peptide antibiotic from *Escherichia coli*. Eur J Biochem 259, 747.

Bonventre, P.F. and Kempe, L.L. (1960) Physiology of toxin production by *Clostridium botulinum* types A and B IV.: activation of the toxin. J Bacteriol 79, 24-32.

Breuer, B. and Radler, F. (1996) Inducible resistance against nisin in *Lactobacillus casei*. Arch Microbiol 165, 114-118.

Crandall, A.D. and Montville, T.J. (1998) Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl Environ Microbiol 64, 231-237.

DasGupta, B.R. (1972) Role of a protease in natural activation of *Clostridium botulinum* neurotoxin. Infect Immun 6, 587-590.

DasGupta, B.R. and Sugiyama, H. (1972) Isolation and characterization of a protease from *Clostridium botulinum* type B. Biochim Biophys Acta 268, 719-729.

DasGupta, B.R. (1971) Activation of *Clostridium botulinum* type B toxin by an endogenous enzyme. J Bacteriol 108, 1051-1057.

Doyle, M.P. (1991) Evaluating the potential risk from extended-shelf-life refrigerated foods by *Clostridium botulinum* inoculation studies. Food Technol-Chicago 45, 154-156.

Elberg, S.S. and Meyer, K.F. (1939) The extracellular proteolytic system of *Clostridium parabotulinum*. J Bacteriol 37, 541-565.

Gao, Y. (2001) Brochocin-C resistance in *Listeria monocytogenes*. PhD thesis, Department of Agricultural, Food, and Nutritional Science. University of Alberta, Edmonton, AB, p. 178.

Garde, S., Ávila, M., Medina, M. and Nuñez, M. (2004) Fast induction of nisin resistance in *Streptococcus thermophilus* INIA 463 during growth in milk. Int J Food Microbiol 96, 165-172.

Garneau, S., Ference, C.A., van Belkum, M.J., Stiles, M.E. and Vederas, J.C. (2003) Purification and characterization of brochocin A and brochocin B(10-43), a functional fragment generated by heterologous expression in *Carnobacterium piscicola*. Appl Environ Microbiol 69, 1352-1358.

Hastings, J.W., Sailer, M., Johnson, K., Roy, K.L., Vederas, J.C. and Stiles, M.E. (1991) Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J Bacteriol 173, 7491-7500.

Hastings, J.W. and Stiles, M.E. (1991) Antibiosis of *Leuconostoc gelidum* isolated from meat. J Appl Bacteriol 70, 127-134.

Iida, H. (1964) Experimental studies on toxin production in *C. botulinum*. Japan J Bacteriol 19, 463-468.

Jack, R., Tagg, J.R. and Ray, B. (1995) Bacteriocins of gram-positive bacteria. Microbiol Rev 59, 171-200.

Jarvis, B. and Farr, J. (1971) Partial purification, specificity and mechanism of action of the nisin-inactivating enzyme from *Bacillus cereus*. BBA-Enzymology 227, 232-240.

Jarvis, B. (1967) Resistance to nisin and production of nisin-inactivating enzymes by several *Bacillus* species. J Gen Microbiol 47, 33-48.

Joosten, H., Nunez, M., Devreese, B., Van Beeumen, J. and Marugg, J. (1996) Purification and characterization of enterocin 4, a bacteriocin produced by *Enterococcus faecalis* INIA 4. Appl Environ Microbiol 62, 4220-4223.

Kawai, Y., Arakawa, K., Itoh, A., Saito, B., Ishii, Y., Nishimura, J., Kitazawa, H., Itoh, T. and Saito, T. (2003) Heterologous expression of gassericin A, a bacteriocin produced by *Lactobacillus gasseri* LA39. Anim Sci J 74, 45-51.

Kawai, Y., Kemperman, R., Kok, J. and Saito, T. (2004) The circular bacteriocins gassericin A and circularin A. Curr Protein Pept Sc 5, 393-398.

Liu, X. (2010) Biochemical identification of bacteriocins from *Enterococcus faecalis* 710C. MsC thesis, Department of Agricultural, Food and Nutritional Science. University of Alberta, Edmonton, AB, p. 111.

Mantovani, H.C. and Russell, J.B. (2001) Nisin resistance of *Streptococcus bovis*. Appl Environ Microbiol 67, 808-813.

Maqueda, M., Sánchez-Hidalgo, M., Fernández, M., Montalbán-López, M., Valdivia, E. and Martínez-Bueno, M. (2008) Genetic features of circular bacteriocins produced by gram-positive bacteria. FEMS Microbiol Rev 32, 2-22.

Martin-Visscher, L.A., van Belkum, M.J., Garneau-Tsodikova, S., Whittal, R.M., Zheng, J., McMullen, L.M. and Vederas, J.C. (2008) Isolation and characterization of carnocyclin A, a novel circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL307. Appl Environ Microbiol 74, 4756-4763.

Mazzotta, A.S. and Montville, T.J. (1999) Characterization of fatty acid composition, spore germination, and thermal resistance in a nisin-resistant mutant of *Clostridium botulinum* 169B and in the wild-type strain. Appl Environ Microbiol 65, 659-664.

Mazzotta, A., Crandall, A. and Montville, T. (1997) Nisin resistance in *Clostridium botulinum* spores and vegetative cells. Appl Environ Microbiol 63, 2654-2659.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E. and van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. Appl Environ Microbiol 64, 4757-4766.

Millonig, R.C. (1956) Isolation of an aminopeptidase from type B *Clostridium botulinum*. J Bacteriol 72, 301-307.

Nakane, A. (1978) Proteases produced by a proteolytic mutant of *Clostridium botulinum* type E. J Gen Microbiol 107, 85-91.

Rayman, K., Malik, N. and Hurst, A. (1983) Failure of nisin to inhibit outgrowth of *Clostridium botulinum* in a model cured meat system. Appl Environ Microbiol 46, 1450-1452.

Rayman, M.K., Aris, B. and Hurst, A. (1981) Nisin: a possible alternative or adjunct to nitrite in the preservation of meats. Appl Environ Microbiol 41, 375-380.

Salvesen, G.S. and Nagase, H. (2001) Inhibition of proteolytic enzymes. In: R. Beynon and J.S. Bond (Eds), Proteolytic Enzymes: A Practical Approach. Oxford University Press, New York, p. 105-130.

Schillinger, U. and Lücke, F.-K. (1989) Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl Environ Microbiol 55, 1901-1906.

Scott, V.N. and Taylor, S.L. (1981) Effect of nisin on the outgrowth of *Clostridium botulinum* spores. J Food Sci 46, 117-126.

Sharma, M. and Luthra-Guptasarma, M. (2009) Degradation of proteins upon storage at near-neutral pH: indications of a proteolytic/gelatinolytic activity associated with aggregates. BBA-Gen Subjects 1790, 1282-1294.

Siragusa, G.R. and Cutter, C.N. (1993) Brochocin-C, a new bacteriocin produced by *Brochothrix campestris*. Appl Environ Microbiol 59, 2326-2328.

Stewart, M.Q., van Dijk, R., Veenhuis, M. and Goodman, J.M. (2002) Monomeric alcohol oxidase is preferentially digested by a novel protease from *Candida boidinii*. BBA-Mol Cell Res 1542, 160-172.

Sun, Z., Zhong, J., Liang, X., Liu, J., Chen, X. and Huan, L. (2009) Novel mechanism for nisin resistance via proteolytic degradation of nisin by the nisin resistance protein NSR. Antimicrob Agents Chemother 53, 1964-1973.

Trüper, H.G. and de'Clari, L. (1997) Taxonomic note: necessary correction of specific epithets formed as substantives (nouns) "in apposition". Int J Syst Bacteriol 47, 908-909.

USDA. (1998) Bacteriological Analytical Manual, 8th ed. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm063710.htm. Accessed 28 September 2009.

Chapter Five: **Effect of media formulation on growth of *Clostridium botulinum* in the presence of competitive microflora**

5.1 Introduction

Many chemical and physical interventions have been used to inhibit spore germination and subsequent outgrowth of *Clostridium botulinum* in food. Strategies have included use of aerobic conditions, as *Cl. botulinum* is an anaerobe. Other strategies employed include lowering water activity by addition of salt or other solutes, reducing pH to less than 4.6, adding sodium nitrite, or maintaining a low storage temperature to control spore outgrowth and subsequent vegetative cell growth (Bell and Kyriakides, 2000). Often, multiple interventions are used in order to ensure that microorganisms which may not be inhibited by one individual barrier will be inhibited by a combination of barriers, which is known as the hurdle concept (Leistner, 1992). By employing multiple hurdles, these treatments have proven effective in controlling *Cl. botulinum* in commercially-prepared foods. However, changing consumer preferences has led to modification of product formulations, which may improve the ability of *Cl. botulinum* to grow and produce toxin. Removal of, or reduction of the concentration of, sodium nitrite and reduced salt levels are two examples of this trend in the food industry. Thus, *Cl. botulinum* is considered an important foodborne pathogen.

Testing for *Cl. botulinum* in food products can be done by assaying for the presence of the viable organism, the neurotoxin resulting from its growth, or both. The gold standard for determining if food has allowed growth of *Cl. botulinum* is the mouse bioassay (Health Protection Branch, 1989; Kautter et al., 1992). However, there are several issues with this assay. The presence of other toxic substances in the sample or infection stemming from the injection can also cause death, so results must be interpreted correctly. In addition, animal welfare is also a concern. Typically, testing for growth of *Cl. botulinum* in samples is done by culture methods. Growth medium that approximates the food products in which *Cl. botulinum* was a major problem (meat) was developed, first using actual meat

extract preparations (Robertson, 1915; Henry, 1918; Quagliaro, 1977), which was later replaced by a commercially-prepared dehydrated cooked meat medium (Difco, Becton Dickinson; Sparks, MD). Complex medium for *Cl. botulinum* was first described in the literature in 1928, and incorporated a high protein content in the form of peptone and dried egg white (Dack et al., 1928). Since that time, media has been formulated with a high protein content from extracts of beef and yeast, combined with protein or casein hydrosylates such as soya peptone, tryptone, peptone, or casitone. Several manufacturers have produced clostridial media, including Reinforced Clostridial Medium (RCM; Difco) (Hirsch and Grinsted, 1954; Barnes et al., 1963), McClung Toabe Agar (Difco) (McClung and Toabe, 1947), and Clostridial Count Agar (Nissau Pharmaceutical; Tokyo, JP), but these media are not selective for *Cl. botulinum*. Differential Reinforced Clostridial Medium (Gibbs and Freame, 1965) is RCM that allows for partial selectivity for clostridia because of the presence of sodium acetate, while differentiation of clostridia is achieved by blackening of the medium from the formation of iron sulphide (BD, 2009).

Attempts have been made to improve the selectivity of media for *Cl. botulinum* by addition of antibiotics such as cycloserine (Dezfulian et al., 1981; Mills et al., 1985; Gibson, 1986; Carminati et al., 2001; Bagenda et al., 2008), kanamycin (Gibson, 1986; Bagenda et al., 2008), oleoandomycin (Gibson, 1986), polymyxin (Rahmati and Labbe, 2008), sulphadiazine (Gibson, 1986), trimethoprim (Dezfulian et al., 1981; Mills et al., 1985), or sulphamethoxazole (Dezfulian et al., 1981; Mills et al., 1985; Carminati et al., 2001; Bagenda et al., 2008). Selection for Group I *Cl. botulinum* was achieved by addition of gentamicin and nalidixic acid to RCM (Gibson, 1986). These antibiotics inhibit the growth of Group II *Cl. botulinum* (Swenson et al., 1980; Gibson, 1986). Neomycin was found to inhibit a type E strain of Group II *Cl. botulinum*, but not Group I (types A and B) strains (Spencer, 1969).

The addition of egg yolk to media allows for differentiation of *Cl. botulinum*, based on the opaque zones surrounding presumed *Cl. botulinum* colonies. The opaque zones are a result of lipase activity of *Cl. botulinum*, and

several media formulations are based on this reaction (McClung and Toabe, 1947; Dezfulian et al., 1981; Mills et al., 1985; Sagua et al., 2009). The most common of these selective media is *Clostridium botulinum* isolation agar (CBI), developed for the enumeration of *Cl. botulinum* in fecal samples (Dezfulian et al., 1981). CBI contains egg yolk and antibiotics in a basal medium of McClung Toabe agar, which contains proteose peptone as its protein source and phosphates for buffering. A revised version of this medium based on heart infusion to replace proteose peptone with a lower concentration of egg yolk has been developed (Mills et al., 1985), and is known as Botulinum Selection Medium. *Cl. botulinum* Selection medium or Botulinum Selection Medium have been used for *Cl. botulinum* enumeration in many studies (Rodriguez and Dezfulian, 1997; Dufresne et al., 2000; Pavic et al., 2001; Daifas et al., 2003; Bianco et al., 2008; Sagua et al., 2009).

Most researchers do not use a standard medium for isolation or enumeration of *Cl. botulinum* (or its closely-related surrogate organism *Cl. sporogenes*), despite the recommendation by authorities such as Health Canada to use either McClung Toabe agar or CBI agar (Health Protection Branch, 1989; J. Austin, Health Canada, personal communication). Review of the literature has identified many different medium formulations used for *Cl. botulinum* or *Cl. sporogenes* isolation or enumeration, including Clostridial Count Agar (Kimura et al., 2008), trypticase-peptone-glucose-yeast extract medium (TPGY) (USDA, 1998; Glass and Johnson, 2004; Mah et al., 2009), TPGY + 0.1 M phosphate (Peck et al., 2010), tryptic soy medium (Ágoston et al., 2009), tryptic soy medium amended with salicin (Rodgers, 2004; Rodgers et al., 2004), peptone-yeast extract-glucose-starch medium (Stringer et al., 1999; Stringer et al., 2009), RCM (Hibbert and Spencer, 1970; Barnes, 1985; Jineja et al., 1995; Joannou et al., 1998; Carminati et al., 2001; Margosch et al., 2004), RCM with hemin (Matos et al., 2008), brain heart infusion (Okereke and Montville, 1991), blood agar (Johnston et al., 2005), yeast extract medium (Odlaug and Pflug, 1977), Wynne-egg yolk medium (Hauschild and Hilsheimer, 1977), and nutrient-salt medium (Lekogo et al., 2010). More detailed investigation into the minimal nutrient

requirements of representative strains in groups I (proteolytic) and II (non-proteolytic) *Cl. botulinum* has also been done (Whitmer and Johnson, 1988), but such formulations may not be practical for enumeration of multiple strains of *Cl. botulinum.* These formulations were optimized for the nutrient requirements of a limited number of strains within each group, and may not be suitable for recovery of organisms or spores of *Cl. botulinum* from a complex ecological niche such as food. As a result, the complex media have been the choice of most investigators. However, in most cases, no justification for the use of the medium chosen is given, and differences in formulations among media raises questions about the accuracy of the results and makes it difficult to compare results among different experiments.

In a previous challenge study in which group II *Cl. botulinum* was isolated from a chopped and formed pork product (Chapter 3, this thesis), recovery of *Cl. botulinum* was significantly higher on CBI than RCM in samples that contained high numbers (~10^8 CFU mL^{-1}) of a background microflora. To determine the underlying reason for this large discrepancy in counts, the growth of Group II *Cl. botulinum* on CBI and RCM were compared, and factors influencing the recovery of *Cl. botulinum* on these media were investigated.

5.2 Materials and Methods

5.2.1 Culture maintenance

Group II strains of *Cl. botulinum* used in this study were 17B, DB2, and 2B (University of Alberta). Spores were prepared and enumerated as described in Section 3.2.1, and were stored in 0.9 % (w/v) NaCl at 4°C until use. Strains of *Carnobacterium maltaromaticum* (MH1, MH2, MH3, MF, and G117), *Staphylococcus warneri*, and *S. pasteuri* were stored at -80°C in All-Purpose Tween (Becton Dickinson; Sparks, MD) broth containing 20 % (v/v) glycerol.

5.2.2 Media analysis and amendments

5.2.2.1 Media composition for recovery of *Cl. botulinum* from chopped and formed pork product

To determine the impact of media composition on recovery of *Cl. botulinum* from a chopped and formed pork product, commercial RCM was amended individually with egg yolk; the antibiotics cycloserine, sulphamethoxazole, and trimethoprim; $MgSO_4$, or total protein source to the concentration of each found in CBI (Dezfulian et al., 1981), as shown in Table 5-1. The total protein source of commercial RCM includes yeast extract, beef extract, and peptone (23 g L^{-1}), while the total protein source of CBI (45 g L^{-1}) originates from tryptone and yeast extract. To formulate commercial RCM with increased protein source, the amounts of beef extract and peptone were doubled to 20 g L^{-1} each, and 5 g L^{-1} of yeast extract, giving a total protein source of 45 g L^{-1}. The pH of all media was determined and adjusted to 7.0 ± 0.5 if necessary prior to use.

5.2.2.2 Media composition for growth of vegetative cells and spores of *Cl. botulinum* in presence of background microflora

To investigate the effect of media composition on the growth of vegetative cells and spores of *Cl. botulinum* in the presence of background microflora isolates, five media were used in deferred inhibition assays: CBI (Dezfulian et al., 1981), commercial RCM, commercial RCM amended with $MgSO_4$ at the concentration found in CBI (Table 5-1), and commercial RCM amended with either $MgCl_2$ or K_2SO_4 at the same concentration as $MgSO_4$. Replacing $MgSO_4$ with equimolar amounts of $MgCl_2$ or K_2SO_4 tested the effect of magnesium and sulphur separately on growth of strains of *Cl. botulinum*. The pH of all media was determined and adjusted to 7.0 ± 0.5 if necessary prior to use.

5.2.2.3 Media composition for growth of heat-shocked and non-heat-shocked spores of *Cl. botulinum* in presence of background microflora

Further investigation into the effect of media composition on growth of heat-shocked and non-heat-shocked spores of *Cl. botulinum* in the presence of background microflora was conducted in deferred inhibition assays. Six different

media based on commercial RCM were tested, including unamended commercial RCM, commercial RCM containing the following ingredients at the same concentrations as found in CBI: $MgSO_4$ (80 μM), $MgCl_2$ (80 μM; same Mg^{2+} concentration as $MgSO_4$), $Na_2HPO_4 \cdot 2H_2O$ (84 mM), and NaCl (11 mM) (Table 5-1). To determine if ion strength of salts such as $Na_2HPO_4 \cdot 2H_2O$ was responsible for observed differences in growth between RCM and CBI, $Na_2HPO_4 \cdot 2H_2O$ was replaced by NaCl at the same concentration (84 mM).

To assess the effect of removing acetate as a buffering agent in commercial RCM, five media with formulations similar to that of RCM were assembled in the laboratory using individual ingredients rather than commercial RCM. The individual ingredients on which the laboratory-prepared RCM is based are shown in Table 5-1, with the removal of sodium acetate and NaCl. All laboratory-prepared RCM was formulated with 84 mM $Na_2HPO_4 \cdot 2H_2O$, which is the concentration found in CBI, to replace sodium acetate for buffering. Amendments were added to this base formulation to test the effect of varying the amount of total protein source, NaCl, and Na^+. Total protein source was increased to 45 g L^{-1} (the concentration in CBI), using the method described previously in Section 5.2.2.1. NaCl was added to achieve the same NaCl concentration as found in commercial RCM (86 mM) and CBI (11 mM). To assess the effect of Na^+ concentration, NaCl (2 mM) was added to the base formulation to increase the Na^+ concentration in the laboratory-prepared medium containing $Na_2HPO_4 \cdot 2H_2O$ (84 mM) to the concentration of Na^+ contributed by NaCl in commercial RCM (86 mM). The effect of total protein source and Na^+ was also tested by increasing the protein source content to 45 g L^{-1}, and adding NaCl to achieve a total Na^+ concentration equal to that of commercial RCM. The pH of all media was determined and adjusted to 7.0 ± 0.5 if necessary prior to use.

5.2.3 Analysis of medium ingredients

Elemental analysis was performed by the Analytical and Instrumentation Laboratory (University of Alberta) to determine the sulphur content of commercial RCM (Difco). Magnesium content of commercial RCM and sodium

content of commercial RCM, tryptone, yeast extract, beef extract, and peptone were determined by inductively coupled plasma mass spectroscopy using an Elan 6000 analyzer (Perkin-Elmer; Waltham, MA) at the Radiogenic Isotope Facility (University of Alberta).

Table 5-1 Composition of RCM and CBI media used in this study

Ingredient	Source	RCM			CBI		
		g L^{-1}	mM		g L^{-1}	mM	
			total	Na^+		total	Na^+
Yeast extract	Difco	3		0.73	5		1.3
Tryptone	Difco	0			40		40.5
Beef extract	Difco	10		8.0	0		
Peptone	Difco	10		7.1	0		
Glucose	Fisher	5	28		2	11	
NaCl	Fisher	5	86	86	2	34	34
Soluble starch	Nichols Chemical Co., Montreal, QC	1			0		
Sodium acetate	Sigma	3	37		0		
$Na_2HPO_4{\cdot}2H_2O$	Fisher	0			7.5	42	84
$MgSO_4$	Sigma	0			0.2	0.08	
Cysteine hydrochloride	Sigma	0.5	2.9		0		
Egg yolk (50 %)	Difco	0			50 mL		
Cycloserine	Sigma	0			0.25	2.4	
Sulfamethoxazole	Sigma	0			0.076	0.3	
Trimethoprim	Sigma	0			0.004	0.01	

5.2.4 Recovery of Cl. botulinum *from a chopped and formed pork product*

Samples used for plating experiments originated from the chopped and formed pork product as described in Chapter 3. Briefly, meat samples were manually homogenized for 5 min in 25 mL 0.1 % (w/v) peptone, serially-diluted in the same diluent, and enumerated on CBI, and commercial RCM with the amendments detailed in Section 5.2.2. All plating was done in an anaerobic chamber (Coy Laboratory Products Inc; Grass Lake, MI) under an atmosphere of 5 % CO_2, 10 % H_2, and balance N_2 (Praxair Canada; Edmonton, AB).

5.2.5 Deferred inhibition assays against Cl. botulinum

The role of different media formulations in growth or inhibition of *Cl. botulinum* in the presence of background microflora was investigated using a deferred inhibition assay (Ahn and Stiles, 1990) with seven strains of

carnobacteria and staphylococci previously isolated as the background microflora of a chopped and formed pork product (Table 3-4). Background microflora isolates were inoculated into 5 mL APT broth, incubated for 18 h at 30°C, and subcultured [1% (v/v)] into 5 mL fresh APT broth. Cultures were subcultured three times prior to the deferred inhibition assay.

5.2.5.1 Deferred inhibition of spores and vegetative cells of *Cl. botulinum* in presence of background microflora on amended media

In deferred inhibition assays using spores and vegetative cells of *Cl. botulinum*, the background microflora isolates were spotted onto the surface of CBI or commercial RCM with amendments described in Section 5.2.2.2. A Cathra replicator equipped with 1-mm pins (Cathra International Inc; St. Paul, MN), which delivers a volume of 0.1 μL (Rousseau and Harbec, 1987), was used, and spots were allowed to dry for 10 min. Plates were incubated anaerobically for 24 h at 30°C in an anaerobic jar containing three anaerobic sachets (GasPak EZ Anaerobe System, Becton Dickinson) until growth was apparent. The plates were then overlayered with 5 mL of either sterile semi-solid agar [0.75 % (w/v) in 0.85 % (w/v) NaCl] containing ~10^4 CFU mL^{-1} of *Cl. botulinum* or sterile semi-solid agar [0.75 % (w/v)] agar containing 3 x 10^8 heat-shocked (55°C, 15 min) spores mL^{-1}. Plates were incubated anaerobically at 37°C for 48 h prior to observation for inhibition or growth of *Cl. botulinum*. Assays were performed in triplicate.

5.2.5.2 Deferred inhibition of heat-shocked and non-heat-shocked spores of *Cl. botulinum* in the presence of background microflora on amended media

In deferred inhibition assays investigated the response of heat-shocked and non-heat-shocked spores of *Cl. botulinum* to the growth of background microflora, the background microflora isolates were spotted onto the surface of amended commercial RCM or laboratory-prepared RCM described in Section 5.2.2.3 using the method described in the preceding paragraph. Plates were overlayered with 5 mL sterile semi-solid agar [0.75 % (w/v)] agar containing either heat-shocked (55°C, 15 min) or non-heat-shocked spores at a concentration of 3 x 10^8 spores mL^{-1}. Plates were incubated anaerobically at 37°C for 48 h prior

to observation for inhibition or growth of *Cl. botulinum*. Assays were performed in triplicate.

5.3 Results

5.3.1 Analysis of medium ingredients

The results of the chemical analysis of select nutrients in ingredients in commercial RCM and ingredients of CBI are shown in Table 5-2. The protein concentration of RCM is nearly half that of CBI, and the concentration of Na^+ and PO_4^{2-} in RCM is lower than that found in CBI. Chemical analysis of commercial RCM revealed that Mg^{2+} and S^{2-} are higher in RCM than in CBI.

Table 5-2 Chemical analysis of selected constituents of Reinforced Clostridial Medium and *Clostridium botulinum* isolation agar

Ingredient or ion	RCM		CBI	
	g L^{-1}	mM	g L^{-1}	mM
Protein source[1]	23		45	
Na^+		102[2]		146[2]
Mg^{2+}		0.25[2]		0.08[1]
S^{2-}		2.1[2]		0.08
PO_4^{2-}		0		42[1]

[1] calculated concentration, [2] analyzed concentration

5.3.2 Recovery of **Cl. botulinum** *from pork product*

Addition of egg yolk, cycloserine, sulfamethoxazole, trimethoprim, magnesium, sulphate to commercial RCM, or increasing the protein concentration to the concentration found in CBI did not improve recovery of *Cl. botulinum* from the pork product. No growth was observed on any amended commercial RCM, compared to a count of 2 log CFU mL^{-1} on CBI medium. Because of limited pork samples and very low levels of *Cl. botulinum* recovered following these initial experiments, further work was done using *Cl. botulinum* spores grown in the presence of the seven background microflora isolates.

5.3.3 *Deferred inhibition against* Cl. botulinum

5.3.3.1 Deferred inhibition of spores and vegetative cells of *Cl. botulinum* in presence of background microflora on amended media

When vegetative cells and spores of *Cl. botulinum* were exposed to background microflora isolates in deferred inhibition assays on amended commercial RCM, the background microflora affected the growth of *Cl. botulinum*, as shown in Table 5-3. Two background microflora isolates, C. *maltaromaticum* MH3 and *S. pasteuri* EIV-21, did not grow anaerobically on CBI agar, so their effect on *Cl. botulinum* on this medium type could not be assessed. However, these two strains inhibited the growth of spores and viable cells of *Cl. botulinum* 2B on all amended commercial RCM formulations, as well as the spores of *Cl. botulinum* 17B and DB2 on unamended commercial RCM. *S. pasteuri* EIV-21 also inhibited growth of spores of *Cl. botulinum* 17B and DB2 on all commercial RCM formulations, growth of vegetative cells of strain 17B on all commercial RCM formulations, and growth of vegetative cells of *Cl. botulinum* DB2 on commercial RCM containing either $MgSO_4$ or K_2SO_4. The other strains of background microflora tested did not inhibit *Cl. botulinum* as effectively, as growth of either spores or vegetative cells of all three *Cl. botulinum* strains was observed on at least one RCM formulation.

Media amendments changed the inhibitory effect of the background microflora on growth of *Cl. botulinum*. The vegetative cells and spores of all three *Cl. botulinum* strains grew on CBI in the presence of all *C. maltaromaticum* and staphylococci, but none of the medium formulations based on commercial RCM allowed growth of both vegetative cells and spores of all strains. Some amendments allowed growth on commercial RCM; in the presence of *C. maltaromaticum* MH3, addition of $MgCl_2$ or K_2SO_4 to commercial RCM allowed growth of spores or vegetative cells of *Cl. botulinum* 17B, respectively, while addition of K_2SO_4 to commercial RCM allowed growth of vegetative cells of strain DB2 (Table 5-3). Although unamended commercial RCM did not allow growth of vegetative cells of *Cl. botulinum* 2B in the presence of any of the carnobacteria, addition of K_2SO_4 allowed growth of some of the vegetative cells.

Table 5-3 Effect of amended media on the growth of vegetative cells or heat-shocked spores of *Cl. botulinum* in the presence of background microflora isolates of *Carnobacterium maltaromaticum* and *Staphylococcus* spp. using deferred inhibition assays.

***Cl. botulinum* 17B**

Background microflora isolate	Medium									
	CBI		RCM		RCM + $MgSO_4$		RCM + $MgCl_2$		RCM + K_2SO_4	
	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores
C. maltaromaticum MH1	+	+	+	+	+	-	+	+	+	+
C. maltaromaticum MH2	+	+	+	+	+	+	+	+	+ (NG)	+
C. maltaromaticum MH3	+ (NG)	+ (NG)	-	-	-	-	-	+	+	-
C. maltaromaticum G117	+	+	+	+	+	-	+	+	-	+
C. maltaromaticum MF	+	+	+	+	+	-	+	+	-	+
S. pasteuri EIV-21	+ (NG)	+ (NG)	-	-	-	-	-	-	-	-
S. warneri S46	+	+	+	+	+	-	+	+	+	+

***Cl. botulinum* 2B**

Background microflora isolate	Medium									
	CBI		RCM		RCM + $MgSO_4$		RCM + $MgCl_2$		RCM + K_2SO_4	
	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores
C. maltaromaticum MH1	+	+	-	+	-	+	NS	+	+	+
C. maltaromaticum MH2	+	+	-	+	+	+	NS	+	+	+
C. maltaromaticum MH3	+ (NG)	+ (NG)	-	-	-	-	NS	-	-	-
C. maltaromaticum G117	+	+	-	+	-	+	NS	+	+	+
C. maltaromaticum MF	+	+	-	+	-	+	NS	+	+	+
S. pasteuri EIV-21	+ (NG)	+ (NG)	-	-	-	-	NS	-	-	-
S. warneri S46	+	+	+	+	+	+	NS	+	+	+

***Cl. botulinum* DB2**

Background microflora isolate	Medium									
	CBI		RCM		RCM + $MgSO_4$		RCM + $MgCl_2$		RCM + K_2SO_4	
	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores
C. maltaromaticum MH1	+	+	NS	+	-	+	NS	+	+	-
C. maltaromaticum MH2	+	+	NS	+	-	+	NS	+	+ (NG)	+
C. maltaromaticum MH3	+ (NG)	+ (NG)	NS	-	-	-	NS	-	+	-
C. maltaromaticum G117	+	+	NS	+	-	+	NS	+	-	+
C. maltaromaticum MF	+	+	NS	+	-	+	NS	+	-	+
S. pasteuri EIV-21	+ (NG)	+ (NG)	NS	-	-	-	NS	-	-	-
S. warneri S46	+	+	NS	-	-	-	NS	+	+	-

NG indicates no growth of background microflora isolate on medium, NS indicates insufficient growth of *Cl. botulinum* on medium, + indicates growth of *Cl. botulinum*, - indicates inhibition of *Cl. botulinum*

There were differences in growth among strains of *Cl. botulinum* on the same medium, particularly in the case of vegetative cells (Table 5-3). Vegetative cells of strain 17B grew well on commercial RCM with added $MgCl_2$ in the presence of *S. warneri* and all carnobacteria except *C. maltaromaticum* MH3, but vegetative cells of *Cl. botulinum* strains 2B and DB2 grew only isolated colonies that did not form a confluent lawn. This prevented observation of inhibition zones near the background microflora isolates on this medium. Vegetative cells of *Cl. botulinum* 2B were inhibited by all strains of carnobacteria and *S. pasteuri* on commercial RCM, but vegetative cells of strain 17B grew in the presence of four of the five carnobacteria.

There was also a difference between growth of spores and vegetative cells of the same strain of *Cl. botulinum* on the same medium Most spores of *Cl. botulinum* strains 2B and DB2 grew on commercial RCM, and commercial RCM amended with either $MgSO_4$ or $MgCl_2$, whereas most vegetative cells did not grow (Table 5-3). In contrast, the growth pattern of vegetative cells and spores of *Cl. botulinum* 17B was not very different on the same media (Table 5-3) with the same background microflora. On commercial RCM and commercial RCM containing $MgCl_2$, vegetative cells of *Cl. botulinum* DB2 did not form a confluent lawn, which was also the case for *Cl. botulinum* strain 2B in commercial RCM containing $MgCl_2$. However, spores of both strains grew readily on the same media.

5.3.3.2 Deferred inhibition of heat-shocked and non-heat-shocked spores of *Cl. botulinum* in the presence of background microflora on amended media

Because spores are more likely than vegetative cells to contaminate food products, further work was done using only *Cl. botulinum* spores. Heat-shocked spores were used to replicate the conditions of the spores directly after inoculation into the chopped and formed meat product. Spores that were not subjected to a heat-shock treatment were also tested to simulate spores that did not undergo a recent stimulation of germination. Both commercial RCM containing amendments and laboratory-prepared RCM containing amendments were tested, to determine the impact of substituting $Na_2HPO_4 \cdot 2H_2O$ for sodium acetate for

buffering. The effect of adding different concentrations of NaCl and Na^+ was also assessed in both media. The results for *Cl. botulinum* 17B, 2B, and DB2 are shown in Table 5-4, Table 5-5, and Table 5-6, respectively.

For *Cl. botulinum* 17B, spores which had not been heat-shocked did not grow on any medium based on commercial RCM, with the exception of the medium with added $Na_2HPO_4 \cdot 2H_2O$ (Table 5-4). All heat-shocked spores were inhibited on commercial RCM containing added NaCl, and most heat-shocked spores were inhibited on unamended commercial RCM or commercial RCM containing added $MgSO_4$ or $MgCl_2$. Media amendments had a slight effect on growth of heat-shocked spores, as addition of $MgCl_2$ to commercial RCM resulted in growth of spores of strain 17B spores in the presence of *C. maltaromaticum* MH2 and *S. warneri* S46. When laboratory-prepared media containing $Na_2HPO_4 \cdot 2H_2O$ in place of sodium acetate was used, spores grew in the presence of all background microflora (Table 5-4).

The results of the deferred inhibition assay using *Cl. botulinum* strain 2B in the presence of background microflora are shown in Table 5-5. Spores of this strain which had not been heat-shocked were inhibited on all media based on commercial RCM, with the exception of the medium containing added $Na_2HPO_4 \cdot 2H_2O$ (in the presence of all background microflora) or 11 mM NaCl (in the presence of *C. maltaromaticum* MH2). Addition of $MgCl_2$ allowed growth of heat-shocked spores in the presence of *C. maltaromaticum* strains MH1 and MF, and *S. pasteuri*. Growth was observed all heat-shocked spores in the presence of one background microflora isolate, *C. maltaromaticum* MH2, on all media based on commercial RCM. Several interesting observations were seen with *Cl. botulinum* strain 2B when plated on laboratory-prepared RCM (Table 5-5). All background microflora strains except *C. maltaromaticum* G117 and *S. pasteuri* inhibited growth of non-heat-shocked spores of *Cl. botulinum* 2B on laboratory-prepared medium containing 11 mM NaCl; however, addition of 86 mM NaCl allowed growth in the presence of *C. maltaromaticum* MH1, MH2, or MH3.
In the case of *C. maltaromaticum* G117, *Cl. botulinum* 2B was inhibited by 2 mM NaCl, but grew in the presence of 86 mM NaCl, and 123 mM NaCl.

Table 5-4 Effect of RCM containing amendments on the growth of heat-shocked and non-heat-shocked spores of *Cl. botulinum* 17B in the presence of strains of *Carnobacterium maltaromaticum* and *Staphylococcus* spp.

Background microflora isolate	Commercial RCM +												Laboratory-prepared RCM (– 37 mM CH_3COONa + 84 mM $Na_2HPO_4 \cdot 2H_2O$) +									
	Unamended		80 µM $MgSO_4$[1]		80 µM $MgCl_2$[2]		84 mM $Na_2HPO_4 \cdot 2H_2O$[1]		11 mM NaCl[1]		84 mM NaCl[3]		86 mM NaCl[4]		2 mM NaCl[5]		45 g L^{-1} protein sources[1]		45 g L^{-1} protein sources[1] + 123 mM NaCl[6]		11mM NaCl[1]	
	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS
C. maltaromaticum MH1	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
C. maltaromaticum MH2	+	-	-	-	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
C. maltaromaticum MH3	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
C. maltaromaticum G117	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
C. maltaromaticum MF	-	-	+	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
S. pasteuri EIV-21	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
S. warneri S46	-	-	-	-	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+

[1] concentration same as in CBI medium, [2] Mg^{2+} concentration same as CBI, [3] same NaCl concentration as $Na_2HPO_4 \cdot 2H_2O$, [4] concentration in commercial RCM, [5] Na^+ balanced from removing sodium acetate and adding $Na_2HPO_4 \cdot 2H_2O$, [6] Na^+ concentration same as commercial RCM; HS indicates heat-shocked spores; NHS indicates non-heat-shocked spores; + indicates growth of *Cl. botulinum*, - indicates inhibition of *Cl. botulinum*; NS indicates insufficient growth of *Cl. botulinum* on medium

Table 5-5 Effect of RCM containing amendments on the growth of heat-shocked and non-heat-shocked spores of *Cl. botulinum* 2B in the presence of strains of *Carnobacterium maltaromaticum* and *Staphylococcus* spp.

Background microflora isolate	Commercial RCM +												Laboratory-prepared RCM (– 37 mM CH_3COONa + 84 mM $Na_2HPO_4 \cdot 2H_2O$) +									
	Unamended		80 µM $MgSO_4$[1]		80 µM $MgCl_2$[2]		84 mM $Na_2HPO_4 \cdot 2H_2O$[1]		11 mM NaCl[1]		84 mM NaCl[3]		86 mM NaCl[4]		2 mM NaCl[5]		45 g L^{-1} protein sources[1]		45 g L^{-1} protein sources[1] + 123 mM NaCl[6]		11mM NaCl[1]	
	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS
C. maltaromaticum MH1	+	-	-	-	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-
C. maltaromaticum MH2	+	-	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-
C. maltaromaticum MH3	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-
C. maltaromaticum G117	-	-	-	-	-	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	+	+
C. maltaromaticum MF	-	-	-	-	+	-	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+	-
S. pasteuri EIV-21	-	-	-	-	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
S. warneri S46	-	-	-	-	-	-	+	+	-	-	-	-	+	-	+	+	+	-	+	+	+	-

[1] concentration same as in CBI medium, [2] Mg^{2+} concentration same as CBI, [3] same NaCl concentration as $Na_2HPO_4 \cdot 2H_2O$, [4] concentration in commercial RCM, [5] Na^+ balanced from removing sodium acetate and adding $Na_2HPO_4 \cdot 2H_2O$, [6] Na^+ concentration same as commercial RCM; HS indicates heat-shocked spores; NHS indicates non-heat-shocked spores; + indicates growth of *Cl. botulinum*, - indicates inhibition of *Cl. botulinum*; NS indicates insufficient growth of *Cl. botulinum* on medium

Table 5-6 Effect of RCM containing amendments on the growth of heat-shocked and non-heat-shocked spores of *Cl. botulinum* DB2 in the presence of strains of *Carnobacterium maltaromaticum* and *Staphylococcus* spp.

Background microflora isolate	Commercial RCM +												Laboratory-prepared RCM (– 37 mM CH_3COONa + 84 mM $Na_2HPO_4 \cdot 2H_2O$) +									
	Unamended		80 µM $MgSO_4$[1]		80 µM $MgCl_2$[2]		84 mM $Na_2HPO_4 \cdot 2H_2O$[1]		11 mM NaCl[1]		84 mM NaCl[3]		86 mM NaCl[4]		2 mM NaCl[5]		45 g L^{-1} protein sources[1]		45 g L^{-1} protein sources[1] + 123 mM NaCl[6]		11mM NaCl[1]	
	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS	HS	NHS
C. maltaromaticum MH1	-	-	-	-	-	-	+	+	-	-	-	-	+	NS	+	NS	+	NS	+	NS	+	NS
C. maltaromaticum MH2	+	-	+	-	+	-	+	+	+	-	+	-	+	NS	+	NS	+	NS	+	NS	+	NS
C. maltaromaticum MH3	-	-	-	-	-	-	+	+	-	-	-	-	+	NS	+	NS	+	NS	+	NS	+	NS
C. maltaromaticum G117	-	-	-	-	-	-	+	+	+	-	-	-	+	NS	+	NS	+	NS	+	NS	+	NS
C. maltaromaticum MF	-	-	+	-	-	-	+	+	-	-	-	-	+	NS	+	NS	+	NS	+	NS	+	NS
S. pasteuri EIV-21	-	-	+	-	-	-	+	+	+	-	-	-	+	NS	+	NS	+	NS	+	NS	+	NS
S. warneri S46	-	-	+	-	+	-	+	+	-	-	-	-	+	NS	+	NS	+	NS	+	NS	+	NS

[1] concentration same as in CBI medium, [2] Mg^{2+} concentration same as CBI, [3] same NaCl concentration as $Na_2HPO_4 \cdot 2H_2O$, [4] concentration in commercial RCM, [5] Na^+ balanced from removing sodium acetate and adding $Na_2HPO_4 \cdot 2H_2O$, [6] Na^+ concentration same as commercial RCM; HS indicates heat-shocked spores; NHS indicates non-heat-shocked spores; + indicates growth of *Cl. botulinum*, - indicates inhibition of *Cl. botulinum*; NS indicates insufficient growth of *Cl. botulinum* on medium

Table 5-6 shows the results of the deferred inhibition assay using *Cl. botulinum* DB2 on different media in the presence of background microflora. Using media based on commercial RCM, non-heat-shocked spores only grew on media containing $Na_2HPO_4{\cdot}2H_2O$. As was the case with *Cl. botulinum* 2B, the background isolate *C. maltaromaticum* MH2 stimulated growth of heat-shocked spores of strain DB2 on all media based on commercial RCM. Addition of $MgSO_4$ or $MgCl_2$ allowed growth of heat-shocked spores of strain DB2 in the presence of *C. maltaromaticum* MF, *S. pasteuri*, and *S. warneri*, while addition of 11 mM NaCl allowed growth of the heat-shocked spores in the presence of *C. maltaromaticum* G117 and *S. pasteuri*. As was the case with *Cl. botulinum* strains 17B and 2B, all heat-shocked spores of *Cl. botulinum* DB2 grew on all formulations of laboratory-prepared RCM in the presence of all background microflora (Table 5-6). However, spores of strain DB2 that had not been heat-shocked did not form a confluent lawn on laboratory-prepared media, so no determination of inhibition could be made.

5.4 Discussion

RCM has been used for enumeration of many clostridia, including *Cl. botulinum*, since its formulation was first reported (Hirsch and Grinsted, 1954). Originally, the formulation was designed for enumeration of *Cl. butyricum* based on a general yeast-glucose medium with the addition of 1 g L^{-1} soluble starch to adsorb growth inhibitors such as free fatty acids (Olsen and Scott, 1950), 0.5 g L^{-1} cysteine as a reducing agent, and 5 g L^{-1} sodium acetate. Sodium acetate was added based on a previous study that showed that acetate is required for growth of the lactate-fermenting butyric acid bacterium *Cl. lacto-acetophilum* [now known as *Cl. beijerinckii* (Cummins and Johnson, 1971)] when grown on lactate-containing medium (Bhat and Barker, 1947). However, other researchers have shown that addition of acetate to medium can inhibit growth of *Cl. beijerinckii*, *Cl. butyricum*, and *Cl. perfringens* (Weenk et al., 1995). Group II *Cl. botulinum* is closely related to *Cl. beijerinckii* and *Cl. butyricum* (Hill et al., 2007), and so

acetate may also be inhibitory to Group II *Cl. botulinum*. Although inhibition of *Cl. botulinum* by acetate has not been reported in a direct assay, Montville (1981) did observe lower counts of Group I *Cl. botulinum* on RCM than on a botulinum assay medium (Huhtanen, 1975), which is in agreement with the results of the present study in which lower numbers of Group II *Cl. botulinum* were recovered from a chopped and formed pork product on RCM than on CBI.

One difference between CBI and RCM is the presence of egg yolk in CBI. The purpose of egg yolk in CBI is to differentiate between lipase-producing *Cl. botulinum* and other organisms that may grow on this medium (Dezfulian et al., 1981). Addition of egg yolk to recovery medium has also been shown to increase the number of *Cl. botulinum* recovered after heating, which was attributed to the presence of lysozyme (Hauschild and Hilsheimer, 1977; Peck et al., 1992). However, lysozyme has not been found in chicken egg yolk (Burley and Vadehra, 1979; Guilmineau et al., 2005), although it is present as a major protein in the outer vitelline membrane surrounding the yolk (Boeck and Stockx, 1986; Mann, 2008). During the processing of egg yolk, lysozyme originating from the vitelline membrane may contaminate the yolk and provide a source of lysozyme for spores; however, there is no direct evidence of this in the literature, and is unlikely (J. Zhang, Neova Technologies, personal communication). Regardless, in the current study, addition of egg yolk to commercial RCM had no effect on recovery of *Cl. botulinum* from the chopped and formed pork product. This agrees with the results of several studies that found that addition of lysozyme did not enhance recovery of spores of Group II *Cl. botulinum* after treatment with high-pressure and heat (Reddy et al., 2010) or heat alone (Scott and Bernard, 1985). Lysozyme has been shown to improve recovery of heated spores of Group I (Alderton et al., 1974) and Group II *Cl. botulinum* (Alderton et al., 1974; Peck et al., 1992). In the current study, the spores were not exposed to heat sufficient to induce injury, but were in the presence of competing microflora. Thus, the type of prior sample treatment (heating, competitive microflora, high-pressure, etc.) may be important choosing media formulations for growth or enumeration of *Cl.*

botulinum. Even with the same *Cl. botulinum* strains, recoveries may differ based on sample treatment, as spores of strains 17B and 2B which had been heated at 85°C were recovered at higher numbers on media containing egg yolk (Peck et al., 1992), while in the current study, recovery of the same two strains from a chopped and formed pork product in which there were competitive microflora was not improved by addition of egg yolk.

The amount of nitrogen, in the form of protein hydrosylates, is also not the cause of decreased growth of Group II *Cl. botulinum* on RCM, because addition of nitrogen sources to commercial RCM to the concentration found in CBI did not allow growth of the Group II *Cl. botulinum* in this study. The results of Montville (1981) are in agreement with the results of the current study, as addition of tryptone to RCM allowed no additional recovery of Group I *Cl. botulinum* spores over unamended RCM. Montville (1981) concluded that Botulinum Assay Medium (Huhtanen, 1975) was a better choice than RCM for recovery of unstressed *Cl. botulinum* spores. Although the authors did not suggest a reason why Botulinum Assay Medium is more efficient at spore recovery, this medium does contain 14.4 mM K_2HPO_4, so this may account for increased recovery observed with its use.

Numerous ion and nutrient requirements for spore germination and/or outgrowth have been identified for several spore-forming organisms. One such required nutrient is sulphur, either in the forms of sulphate or cysteine, which was required at a minimum concentration of 0.05 mM sulphur for post-germinative development of *Bacillus megaterium* spores (Hyatt and Levinson, 1957). *Clostridium botulinum* isolation agar contains 0.08 mM $MgSO_4$, which is above the minimum requirements; however, RCM does not contain added sulphates, but does contain 2.1 mM S^{2-}, originating mainly from cysteine hydrochloride. The availability of this form of sulphur to Group II *Cl. botulinum* is unknown, but sulphur does not appear to limit growth of Group II *Cl. botulinum*, as addition of neither $MgSO_4$ nor K_2SO_4 increased growth.

Magnesium has also been identified as a nutrient required for spore germination or outgrowth. When added to a turkey meat system, magnesium improved the growth of Group I *Cl. botulinum* (Barbut et al., 1986), though no studies were done with magnesium in the enumeration medium. Cazemier et al. (2001) found that counts of heat-injured spores of *B. subtilis* were increased by 0.5 log CFU mL^{-1} when magnesium was added to a final concentration of 203 μM in the recovery medium. A defined minimal medium for Group II *Cl. botulinum* contained 300 μM magnesium; however, it was not indicated if less magnesium was sufficient for growth (Whitmer and Johnson, 1988). The results of the current study did not show increased recovery of Group II *Cl. botulinum* when magnesium was added to RCM. This result, combined with the results of the elemental analysis that show that unamended RCM contains 250 μM magnesium, leads to the conclusion that magnesium limitation in RCM does not explain the reduced ability for Group II *Cl. botulinum* to grow on this medium.

One key difference between RCM-based media and both Botulinum Assay Medium (Huhtanen, 1975) and CBI (Dezfulian et al., 1981) is the presence of phosphate in CBI instead of acetate as a buffering agent. Some of the early formulations of RCM, such as that devised by Bhat and Barker (1947), included phosphate as a buffering agent, but the later version of RCM reported by Hirsch and Grimstead (1954) omitted phosphate, leaving acetate as the sole buffering agent. No reason for this change was identified. The role of acetate in RCM and its derivative Differential RCM is unclear in product literature; in RCM, acetate is stated to be a buffering agent, while in Differential RCM acetate is reported to be a selective agent (BD, 2009). Acetate may be ineffective as a buffering agent in RCM, as the useful buffering range of sodium acetate/acetic acid is only pH 3.7 - 5.6 (Physical and Theoretical Chemistry Laboratory, 2003) whereas the reported pH of RCM is 6.8 ± 0.2 (BD, 2009); however, no other buffering agents are present.

Based on the results of the current study, it is likely that absence of phosphate in RCM limits recovery of Group II *Cl. botulinum* from systems with

competitive microflora. Addition of phosphate to commercial RCM allowed growth of all three strains of *Cl. botulinum* tested in this study. However, when purified spores of the same three strains that had not been inoculated into meat were enumerated on RCM and CBI agar, there was no difference in counts between the two media (data not shown). The results of the current work and the work of Montville (1981) point to phosphate-containing media as more suitable for recovery of both groups I and II *Cl. botulinum*, particularly for cultures originating from food.

Phosphate has previously been shown to improve recovery of heated spores of *B. cereus*, but recovery of unheated spores was unaffected (Blocher and Busta, 1982). Spores of several strains of Group I *Cl. botulinum* were recovered more effectively using Botulinum Assay Medium (Huhtanen, 1975) than RCM with or without heat shocking the spores at 80°C (Huhtanen, 1975; Montville, 1981). Phosphate has not been reported to be a germinant for *Cl. botulinum* spores, but has been identified as a co-germinant (along with Na^+) for food poisoning isolates of *Cl. perfringens* (Paredes-Sabja et al., 2009). Hyatt and Levinson (1957) observed that spores of *B. megaterium* germinated in phosphate-free medium, but no post-germinative development occurred. They suggested that phosphate may play a role in spore outgrowth. Similarly, spores of *B. cereus* have been shown to germinate when supplied with other germinants in a buffered solution of 3.3 mM phosphate, but they did not germinate with deionized water as the solvent (Rode and Foster, 1962). The same study also concluded that sodium is required for germination of this strain. Sodium does not appear to be required for growth of the Group II *Cl. botulinum* in this study, as there was over 100 mM Na^+ in unamended RCM, and addition of Na^+ to the concentration found in CBI did not increase growth.

The background microflora isolated from the chopped and formed pork product appeared to have an impact on growth of *Cl. botulinum*. In particular, *C. maltaromaticum* MH3 and *S. pasteuri* inhibited both spores and vegetative cells of Group II *Cl. botulinum*, and this inhibition was not countered by the addition of

ions or nutrients to commercial RCM, with the exception of phosphate. *C. maltaromaticum* produces at least seven different bacteriocins (Leisner et al., 2007), including piscicolin 126 (Jack et al., 1996; Gursky et al., 2006), carnocyclin A (Martin-Visscher et al., 2008), carnobacteriocin BM1 (Quadri et al., 1994), carnobacteriocin B2 (Quadri et al., 1994), and piscicocin V1b (Bhugaloo-Vial et al., 1996). There are no reports of bacteriocins produced by *S. pasteuri*; however, production of bacteriocins by *S. warneri* has been reported (Sashihara et al., 2000; Minamikawa et al., 2005; Prema et al., 2006). *S. pasteuri* and *S. warneri* are closely-related, and belong to the same cluster group based on 16S rRNA sequencing (Takahashi et al., 1999); thus, the production of bacteriocins by *S. warneri* is possible. Bacteriocins have been shown to inhibit spores of *Cl. botulinum* (Okereke and Montville, 1991; Rodgers et al., 2003; Rodgers et al., 2004), and so bacteriocin inhibition is a possibility.

Bacteriocin production may account for the observed inhibition of *Cl. botulinum*, but no characterization of any such products was completed in the current study. If bacteriocin production does account for the inhibition, the reason for phosphate reversing the inhibition of *Cl. botulinum* is unknown. In fact, the addition of inorganic phosphate to cultures of *Lactococcus lactis* subsp. *lactis* significantly stimulated nisin production in several studies (Kozlova et al., 1972; Kozlova et al., 1979; De Vuyst and Vandamme, 1993). This effect was attributed to the effect of pH buffering (Kozlova et al., 1972; Kozlova et al., 1979). The pH of all media used in the current study was 7.0 ± 0.5 prior to use (Section 5.2.2), but sodium phosphate has a higher pH buffering range of 5.8 – 8.0 , compared to sodium acetate (3.7 – 5.6) (Physical and Theoretical Chemistry Laboratory, 2003). This difference may have impacted bacteriocin production by the isolates of background microflora. Future research should be conducted to investigate the presence of bacteriocins from these strains, and also evaluate the effect of pH buffering on bacteriocin production by carnobacteria and staphylococci.

Although several of the background microflora isolates inhibited *Cl. botulinum*, one strain, *C. maltaromaticum* MH2, appeared to stimulate the growth

of the *Cl. botulinum* strains tested. This type of stimulation of growth of *Cl. botulinum* by any LAB has not been reported in the literature. There is one report of increased growth of a Group I *Cl. botulinum* strain in the presence of the aerobic microorganisms *Acinetobacter lwoffi* and a *Pseudomonas* sp. (Giménez et al., 1988). As well, the presence of *Pseudomonas mendocina* in cultures of *Cl. argentinense*, a toxigenic clostridial species, caused a four-fold higher biomass level than that found when *Cl. argentinense* was grown alone (Centorbi and Silva, 2000). This increase was attributed to the production of a complex alginate-like carbon source by *P. mendocina* that was used by *Cl. argentinense* (Centorbi and Silva, 2008). However, based on the results of the current study, there is no clear explanation for the increased growth of *Cl. botulinum* in the presence of *C. maltaromaticum* MH2.

From the results of the current study, it is clear that choice of medium ingredients is an important consideration when working with spore-forming organisms, particularly those of significant import in food. In this study, the presence of phosphate in CBI medium was shown to allow growth of spores of Group II *Cl. botulinum* in the presence of competing microflora. Another commonly-used medium, RCM, was shown to be ineffective in allowing for growth of these spores, unless phosphate was added. The mechanism by which phosphate allows recovery is unknown, and requires further research. Also, the effect of phosphate on recovery of other *Cl. botulinum* groups should be assessed, because Groups I and II *Cl. botulinum* strains are genetically quite divergent (Collins and East, 1998; Hill et al., 2007). Interestingly, one manufacturer of RCM, Becton Dickinson, reports good recovery of *Cl. botulinum* ATCC 3502, a group I organism, on RCM but not on Differential RCM (BD, 2009). Other manufacturers do not test *Cl. botulinum* at all, though Oxoid (Nepean, ON), PML Biologicals (Wilsonville, OR), and Merck Chemicals (Darmstadt, DE) all report good or very good growth of the closely-related organism *Cl. sporogenes* on RCM. None of the manufacturers report testing any group II *Cl. botulinum*, and so its use may not suitable.

This research has shown that RCM is not an appropriate medium for the isolation of *Cl. botulinum* from food. Results observed using RCM as an isolation medium could underestimate the growth of Group II *Cl. botulinum* in refrigerated foods.

5.5 References

Ágoston, R., Soni, K.A., McElhany, K., Cepeda, M.L., Zuckerman, U., Tzipori, S., Mohácsi-Farkas, C. and Pillai, S.D. (2009) Rapid concentration of *Bacillus* and *Clostridium* spores from large volumes of milk, using continuous flow centrifugation. J Food Prot 72, 666-668.

Ahn, C. and Stiles, M.E. (1990) Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl Environ Microbiol 56, 2503-2510.

Alderton, G., Chen, J.K. and Ito, K.A. (1974) Effect of lysozyme on the recovery of heated *Clostridium botulinum* spores. Appl Microbiol 27, 613-615.

Bagenda, D.K., Hayashi, K., Yamazaki, K. and Kawai, Y. (2008) Characterization of an antibacterial substance produced by *Pediococcus pentosaceus* Iz3.13 isolated from Japanese fermented marine food. Fisheries Sci 74, 439-448.

Barbut, S., Tanaka, N. and Maurer, A.J. (1986) Effects of varying levels of chloride salts on *Clostridium botulinum* toxin production in turkey frankfurters. J Food Sci 51, 1129-1131.

Barnes, E.M. (1985) Isolation methods for anaerobes in foods. Int J Food Microbiol 2, 81-87.

Barnes, E.M., Despaul, J.E. and Ingram, M. (1963) The behaviour of a food poisoning strain of *Clostridium welchii* in beef. J Appl Bacteriol 26, 415-427.

BD. (2009) Difco™ and BBL™ Manual: Manual of Microbiological Culture Media, Vol. 2010. Becton, Dickinson and Company, Sparks, MD.

Bell, C. and Kyriakides, A. (2000) *Clostridium botulinum*: A Practical Approach to the Organism and Its Control in Foods. Blackwell Science Inc., Oxford.

Bhat, J.V. and Barker, H.A. (1947) *Clostridium lacto-acetophilum* nov. spec. and the role of acetic acid in the butyric acid fermentation of lactate. J Bacteriol 54, 381-b-391.

Bhugaloo-Vial, P., Dousset, X., Metivier, A., Sorokine, O., Anglade, P., Boyaval, P. and Marion, D. (1996) Purification and amino acid sequences of piscicocins V1a and V1b, two class IIa bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. Appl Environ Microbiol 62, 4410-4416.

Bianco, M.I., Lúquez, C., de Jong, L.I.T. and Fernández, R.A. (2008) Presence of *Clostridium botulinum* spores in *Matricaria chamomilla* (chamomile) and its relationship with infant botulism. Int J Food Microbiol 121, 357-360.

Blocher, J.C. and Busta, F.F. (1982) Constituents of media for recovery of sporeformers from foods. Arch Lebensmittelhyg 33, 138-142.

Boeck, S.d. and Stockx, J. (1986) Egg white lysozyme is the major protein of the hen's egg vitelline membrane. Int J Biochem 18, 617-622.

Burley, V.W. and Vadehra, D.V. (1979) Chromatographic separation of the soluble proteins of hen's egg yolk: an analytical and preparative study. Anal Biochem 94, 53-59.

Carminati, D., Perrone, A. and Neviani, E. (2001) Inhibition of *Clostridium sporogenes* growth in mascarpone cheese by co-inoculation with *Streptococcus thermophilus* under conditions of temperature abuse. Food Microbiol 18, 571-579.

Cazemier, A.E., Wagenaars, S.F.M. and Steeg, P.F.t. (2001) Effect of sporulation and recovery medium on the heat resistance and amount of injury of spores from spoilage bacilli. J Appl Microbiol 90, 761-770.

Centorbi, H.J. and Silva, H.J. (2000) Enhancement of growth and toxin production of *Clostridium argentinense* by co-culture with *Pseudomonas mendocina*. Microbios 398, 15-22.

Centorbi, H.J. and Silva, H.J. (2008) Slowly utilized carbon sources enhance botulinic specific toxicity by co-culture of *Clostridium argentinense* with the non-pathogen *Pseudomonas mendocina*. World J Microbiol Biotechnol 24, 1823-1828.

Collins, M.D. and East, A.K. (1998) Phylogeny and taxonomy of the foodborne pathogen *Clostridium botulinum* and its neurotoxins. J Appl Microbiol 84, 5-17.

Cummins, C.S. and Johnson, J.L. (1971) Taxonomy of the clostridia: wall composition and DNA homologies in *Clostridium butyricum* and other butyric acid-producing clostridia. J Gen Microbiol 67, 33-46.

Dack, G.M., Wood, W.L. and Dehler, S.A. (1928) Toxin production and proteolytic activity of *Clostridium botulinum* in peptone and peptone egg white medium. J Infect Dis 42, 176-178.

Daifas, D.P., Smith, J.P., Blanchfield, B., Cadieux, B., Sanders, G. and Austin, J.W. (2003) Challenge studies with proteolytic *Clostridium botulinum* in yeast and chemically leavened crumpets packaged under modified atmosphere atmospheres. J Food Safety 23, 107-125.

De Vuyst, L. and Vandamme, E.J. (1993) Influence of the phosphorus and nitrogen source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations using a complex medium. Appl Microbiol Biot 40, 17-22.

Dezfulian, M., McCroskey, L.M., Hatheway, C.L. and Dowell, V.R., Jr. (1981) Selective medium for isolation of *Clostridium botulinum* from human feces. J Clin Microbiol 13, 526-531.

Dufresne, I., Smith, J.P., Liu, J.N., Tarte, I., Blanchfield, B. and Austin, J.W. (2000) Effect of films of different oxygen transmission rate on toxin production by *Clostridium botulinum* type E in vacuum packaged cold and hot smoked trout fillets. J Food Safety 20, 251-268.

Gibbs, B.M. and Freame, B. (1965) Methods for the recovery of clostridia from foods. J Appl Bacteriol 28, 95-111.

Gibson, A.M. (1986) Selective recovery of proteolytic strains of *Clostridium botulinum* types A and B from model cured meat systems. Food Microbiol 3, 213-222.

Gill, D.M. (1982) Bacterial toxins: a table of lethal amounts. Microbiol Rev 46, 86-94.

Giménez, M.A., Solanes, R.E. and Gimenez, D.F. (1988) Efectos metabiótico y sinérico de algunas bacterias aerobias estrictas sobre el crecimiento y toxinogénesis dr *Clostridium botulinum* tipo A. Rev Fac Cs Agrarias 24, 59-68.

Glass, K.A. and Johnson, E.A. (2004) Antagonistic effect of fat on the antibotulinal activity of food preservatives and fatty acids. Food Microbiol 21, 675-682.

Guilmineau, F., Krause, I. and Kulozik, U. (2005) Efficient analysis of egg yolk proteins and their thermal sensitivity using sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing and nonreducing conditions. J Agr Food Chem 53, 9329-9336.

Gursky, L.J., Martin, N.I., Derksen, D.J., van Belkum, M.J., Kaur, K., Vederas, J.C., Stiles, M.E. and McMullen, L.M. (2006) Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. Arch Microbiol 186, 317-325.

Hauschild, A.H.W. and Hilsheimer, R. (1977) Enumeration of *Clostridium botulinum* spores in meat by a pour-plate method. Can J Microbiol 23, 829-832.

Health Protection Branch, Health Canada. (1989) Compendium of analytical methods. Official methods of microbiological analysis of foods. Polyscience Publications Inc., Ottawa, ON.

Henry, H. (1918) An investigation of the cultural reactions of certain anaerobes found in wounds. J Pathol Bacteriol 21, 344-385.

Hibbert, H.R. and Spencer, R. (1970) An investigation of the inhibitory properties of sodium thioglycollate in media for the recovery of clostridial spores. J Hyg-Camb 68, 131-135.

Hill, K.K., Smith, T.J., Helma, C.H., Ticknor, L.O., Foley, B.T., Svensson, R.T., Brown, J.L., Johnson, E.A., Smith, L.A., Okinaka, R.T., Jackson, P.J. and Marks, J.D. (2007) Genetic diversity among botulinum neurotoxin-producing clostridial strains. J Bacteriol 189, 818-832.

Hirsch, A. and Grinsted, E. (1954) Methods for the growth and enumeration of anaerobic spore-formers from cheese, with observations on the effect of nisin. J Dairy Res 21, 101-110.

Huhtanen, C.N. (1975) Some observations on a Perigo-type inhibition of *Clostridium botulinum* in a simplified medium. J Milk Food Technol 38, 762-763.

Hyatt, M. and Levinson, H. (1957) Sulfur requirements for postgerminative development of *Bacillus megaterium* spores. J Bacteriol 74, 87-93.

Jack, R., Wan, J., Gordon, J., Harmark, K., Davidson, B., Hillier, A., Wettenhall, R., Hickey, M. and Coventry, M. (1996) Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl Environ Microbiol 62, 2897-2903.

Jineja, V.K., Marmer, B.S., Phillips, J.G. and Miller, A.J. (1995) Influence of the intrinsic properties of food on thermal inactivation of spores of nonproteolytic *Clostridium botulinum*: Development of a predictive model. J Food Safety 15, 349-364.

Joannou, C.L., Cui, X.-Y., Rogers, N., Vielotte, N., Torres Martinez, C.L., Vugman, N.V., Hughes, M.N. and Cammack, R. (1998) Characterization of the bactericidal effects of sodium nitroprusside and other pentacyanonitrosyl complexes on the food spoilage bacterium *Clostridium sporogenes*. Appl Environ Microbiol 64, 3195-3201.

Johnston, M.D., Lawson, S. and Otter, J.A. (2005) Evaluation of hydrogen peroxide vapour as a method for the decontamination of surfaces contaminated with *Clostridium botulinum* spores. J Microbiol Meth 60, 403-411.

Kautter, D.A., Solomon, H.M., Lake, D.E., Bernard, D.T. and Mills, D.C. (1992) *Clostridium botulinum* and its toxins. In: C. Vanderzant and D.F. Splittstoesser (Eds), Compendium of methods for the microbiological analysis of foods. American Public Health Association, Washington, p. 605-621.

Kimura, B., Kimura, R., Fukaya, T., Sakuma, K., Miya, S. and Fujii, T. (2008) Growth and toxin production of proteolytic *Clostridium botulinum* in aseptically steamed rice products at pH 4.6 to 6.8, packed under modified atmosphere, using a deoxidant pack. J Food Prot 71, 468-472.

Kozlova, Y.I., Golikova, T.I., Baranova, I.P. and Egorov, N.S. (1979) Investigation of the influence of KH_2PO_4 on the growth of *Streptococcus lactis* and nisin synthesis at constant pH values of the medium. Mikrobiologiya 48, 443-446.

Kozlova, Y.I., Egorov, N.S., Baranova, I.P. and Maksimov, V.N. (1972) Metabolic kinetics of *Streptococcus lactis* on initial and optimal media. Mikrobiologiya 41, 1007-1012.

Leisner, J.L., Laursen, B.G., Prévost, H., Drider, D. and Dalgaard, P. (2007) *Carnobacterium*: positive and negative effects in the environment and in foods. FEMS Microbiol Rev 31, 592-613.

Leistner, L. (1992) Food preservation by combined methods. Food Res Int 25, 151-158.

Lekogo, B.M., Coroller, L., Mathot, A.G., Mafart, P. and Leguerinel, I. (2010) Modelling the influence of palmitic, palmitoleic, stearic and oleic acids on apparent heat resistance of spores of *Bacillus cereus* NTCC 11145 and *Clostridium sporogenes* Pasteur 79.3. Int J Food Microbiol 141, 242-247.

Mah, J.-H., Kang, D.-H. and Tang, J. (2009) Comparison of viability and heat resistance of *Clostridium sporogenes* stored at different temperatures. J Food Sci 74, M23-M27.

Mann, K. (2008) Proteomic analysis of the chicken egg vitelline membrane. Proteomics 8, 2322-2332.

Margosch, D., Ehrmann, M.A., Buckow, R., Gänzle, M.G. and Vogel, R.F. (2004) Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. J Food Prot 67, 2530-2537.

Martin-Visscher, L.A., van Belkum, M.J., Garneau-Tsodikova, S., Whittal, R.M., Zheng, J., McMullen, L.M. and Vederas, J.C. (2008) Isolation and characterization of carnocyclin A, a novel circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL307. Appl Environ Microbiol 74, 4756-4763.

Matos, T.J.S., Bruno-Soares, A., Jensen, B.B., Barreto, A.S. and Hojberg, O. (2008) Growth inhibition of bacterial isolates recovered from two types of Portuguese dry smoked sausages (chouriço). Meat Sci 80, 1352-1358.

McClung, L.S. and Toabe, R. (1947) The egg yolk plate reaction for the presumptive diagnosis of *Clostridium sporogenes* and certain species of the gangrene and botulinum groups. J Bacteriol 53, 139-147.

Mills, D.C., Midura, T.F. and Arnon, S.S. (1985) Improved selective medium for the isolation of lipase-positive *Clostridium botulinum* from feces of human infants. J Clin Microbiol 21, 947-950.

Minamikawa, M., Kawai, Y., Inoue, N. and Yamazaki, K. (2005) Purification and characterization of warnericin RB4, anti-*Alicyclobacillus* bacteriocin, produced by *Staphylococcus warneri* RB4. Curr Microbiol 51, 22-26.

Montville, T.J. (1981) Effect of plating medium on heat activation requirement of *Clostridium botulinum* spores. Appl Environ Microbiol 42, 734-736.

Odlaug, T.E. and Pflug, I.J. (1977) Recovery of spores of *Clostridium botulinum* in yeast extract agar and pork infusion agar after heat treatment. Appl Environ Microbiol 34, 377-381.

Okereke, A. and Montville, T.J. (1991) Bacteriocin-mediated inhibition of *Clostridium botulinum* spores by lactic acid bacteria at refrigeration and abuse temperatures. Appl Environ Microbiol 57, 3423-3428.

Olsen, A.M. and Scott, W.J. (1950) The enumeration of heated bacterial spores I. Experiments with *Clostridium botulinum* and other species of *Clostridium*. Aust J Sci Res Ser B 3, 219-233.

Paredes-Sabja, D., Udompijitkul, P. and Sarker, M.R. (2009) Inorganic phosphate and sodium ions are cogerminants for spores of *Clostridium perfringens* type A food poisoning-related isolates. Appl Environ Microbiol 75, 6299-6305.

Pavic, S., Lastre, D., Bukovski, S., Hadziosmanovic, M., Miokovic, B. and Kozacinski, L. (2001) Convenience of *Clostridium botulinum* agar for selective isolation of B type *Clostridium botulinum* from dry cured Dalmatian ham during family outbreak. Arch Lebensmittelhyg 52, 43-45.

Peck, M.W., Plowman, J., Aldus, C.F., Wyatt, G.M., Penaloza Izurieta, W., Stringer, S.C. and Barker, G.C. (2010) Development and application of a new method for specific and sensitive enumeration of spores of nonproteolytic *Clostridium botulinum* types B, E, and F in foods and food materials. Appl Environ Microbiol 76, 6607-6614.

Peck, M.W., Fairbairn, D.A. and Lund, B.M. (1992) The effect of recovery medium on the estimated heat-inactivation of spores of non-proteolytic *Clostridium botulinum*. Lett Appl Microbiol 15, 146-151.

Physical and Theoretical Chemistry Laboratory. (2003) Common buffers. http://msds.chem.ox.ac.uk/buffers.html. Oxford University. Last updated 16 December 2010.

Prema, P., Bharathy, S., Palavesam, A., Sivasubramanian, M. and Immanuel, G. (2006) Detection, purification and efficacy of warnerin produced by *Staphylococcus warneri*. World J Microbiol Biotechnol 22, 865-872.

Quadri, L., Sailer, M., Roy, K., Vederas, J. and Stiles, M. (1994) Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. J Biol Chem 269, 12204-12211.

Quagliaro, D.A. (1977) An improved cooked meat medium for the detection of *Clostridium botulinum*. J AOAC Int 60, 563-569.

Rahmati, T. and Labbe, R. (2008) Levels and toxigenicity of *Bacillus cereus* and *Clostridium perfringens* from retail seafood. J Food Prot 71, 1178-1185.

Reddy, N.R., Tetzloff, R.C. and Skinner, G.E. (2010) Effect of media, additives, and incubation conditions on the recovery of high pressure and heat-injured *Clostridium botulinum* spores. Food Microbiol 27, 613-617.

Robertson, M. (1915) Notes upon certain anaerobes isolated from wounds. J Pathol Bacteriol 20, 327-349.

Rode, L.J. and Foster, J.W. (1962) Ions and the germination of spores of *Bacillus cereus* T. Nature 194, 1300-1301.

Rodgers, S. (2004) Novel approaches in controlling safety of cook-chill meals. Trends Food Sci Tech 15, 366-372.

Rodgers, S., Kailasapathy, K., Cox, J. and Peiris, P. (2004) Co-incubation of *Clostridium botulinum* with protective cultures. Food Res Int 37, 659-666.

Rodgers, S., Peiris, P. and Casadei, G. (2003) Inhibition of nonproteolytic *Clostridium botulinum* with lactic acid bacteria and their bacteriocins at refrigeration tempeartures. J Food Prot 66, 674-678.

Rodriguez, A. and Dezfulian, M. (1997) Rapid identification of *Clostridium botulinum* and botulinal toxin in food. Folia Microbiol 42, 149-151.

Rousseau, D. and Harbec, P.S. (1987) Delivery volumes of the 1- and 3-mm pins of a Cathra replicator. J Clin Microbiol 25, 1311.

Sagua, M.D., Lúquez, C., Barzola, C.P., Bianco, M.I. and Fernández, R.A. (2009) Phenotypic characterization of *Clostridium botulinum* strains isolated from infant botulinum cases in Argentina. Rev Argent Microbiol 41, 141-147.

Sashihara, T., Kimura, H., Higuchi, T., Adachi, A., Matsusaki, H., Sonomoto, K. and Ishizaki, A. (2000) A novel lantibiotic, nukacin ISK-1, of *Staphylococcus warneri* ISK-1: Cloning of the structural gene and identification of the structure. Biosci Biotech Bioch 64, 2420-2428.

Scott, V.N. and Bernard, D.T. (1985) The effect of lysozyme on the apparent heat resistance of nonproteolytic type B *Clostridium botulinum*. J Food Safety 7, 145-154.

Spencer, R. (1969) Neomycin-containing media in the isolation of *Clostridium botulinum* and food poisoning strains of *Clostridium welchii*. J Appl Bacteriol 32, 170-174.

Stringer, S.C., Webb, M.D. and Peck, M.W. (2009) Contrasting effects of heat treatment and incubation temperature on germination and outgrowth of individual spores of nonproteolytic *Clostridium botulinum* bacteria. Appl Environ Microbiol 75, 2712-2719.

Stringer, S.C., Haque, N. and Peck, M.W. (1999) Growth from spores of nonproteolytic *Clostridium botulinum* in heat-treated vegetable juice. Appl Environ Microbiol 65, 2136-2142.

Swenson, J.M., Thornsberry, C., McCroskey, L.M., Hatheway, C.L. and Dowell, V.R., Jr. (1980) Susceptibility of *Clostridium botulinum* to thirteen antimicrobial agents. Antimicrob Agents Chemother 18, 13-19.

Takahashi, T., Satoh, I. and Kikuchi, N. (1999) NOTE: Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. Int J Syst Bacteriol 49, 725-728.

USDA. (1998) Bacteriological Analytical Manual, 8th ed. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm063710.htm. Accessed 28 September 2009.

Weenk, G.H., van den Brink, J.A., Struijk, C.B. and Mossel, D.A.A. (1995) Modified methods for the enumeration of spores of mesophilic *Clostridium* species in dried foods. Int J Food Microbiol 27, 185-200.

Whitmer, M.E. and Johnson, E.A. (1988) Development of improved defined media for *Clostridium botulinum* serotypes A, B, and E. Appl Environ Microbiol 54, 753-759.

Chapter Six: **General Discussion and Conclusions**

Lactic acid bacteria have been used for many years to delay food spoilage and enhance food safety, both by competitive inhibition of spoilage and pathogenic microorganisms, as well as through production of antimicrobials such as bacteriocins. Key product categories such as organic, 'natural', and minimally-processed foods may be at increased risk of foodborne illness because of the absence of chemical hurdles to microbial growth in these products. To maintain a safe food supply without such additives, alternatives must be found. Increased emphasis on more "natural" food preservatives has created a niche for bacteriocins in food preservation and safety.

To date, deliberate bacteriocin application in foods has been limited, with only several semi-purified or purified preparations being marketed for this purpose. Until recently, nisin has captured the major market share of these preparations, based on a long history of use and commercial availability (Jones et al., 2005). Nisin has been approved for use since 1969 by the FAO/WHO (Joint FAO/WHO Expert Committee on Food Additives, 2007; Anonymous, 2009), and since 1988 in the United States by the FDA (FDA, 2006). However, resistance to nisin has been reported in pathogenic bacteria such as *L. monocytogenes* (Crandall and Montville, 1998) and *Cl. botulinum* (Mazzotta et al., 1997; Mazzotta and Montville, 1999). Thus, there has been more emphasis on diversification of the bacteriocin arsenal available for food protection, as evidenced by the recent approval of Micocin®, a product containing class IIa bacteriocins (Health Canada, 2010).

For bacteriocins other than nisin to be harnessed effectively in food products, several issues must be addressed. One such issue is lack of specific, validated methods for detection and quantitation of bacteriocins. Although manuals such as the FAO/WHO Food Additive Compendium recognize a validated method for nisin quantitation, this method is bioassay-based, and therefore is subject to the limitations of such methods; namely, the requirement for a suitable sensitive indicator organism, lack of specificity, and the time

required for results. Antibody-based methods such as ELISA eliminate many of the limitations of bioassays, but have their own difficulties. ELISA requires a reliable, consistent source of antibodies, which can be difficult when multiple immunizations of different animals are performed. Phage display of antibody fragments specific for bacteriocins would provide a consistent, renewable supply of antibodies independent of animal immunizations. In addition, the technical ease of polyclonal antibody production and the specificity of monoclonal antibodies are combined in phage display.

In the current study, attempts were made to produce antibodies to the bacteriocins leucocin A, piscicolin 126, and brochocin-A by phage display. The first step toward this goal was to produce an immune response to these bacteriocins in mice, which was successful for all three bacteriocins. Antibodies for the class IIa bacteriocin leucocin A conjugated to KLH were raised in mice to a maximum titre of 1:10000, which is a relatively high titre (Harlow and Lane, 1988; Burmester and Plückthun, 2001). Antibodies were also raised to KLH conjugates of a C-terminal fragment of piscicolin 126 and an N-terminal fragment of brochocin-A, though the titres were lower than those obtained with antibodies for leucocin A. This was the first report of antibodies to either leucocin A or piscicolin 126 in any animal model, which demonstrates that these two bacteriocins are sufficiently immunogenic to produce antibodies by traditional polycolonal or monoclonal methods if desired.

Although amplification of scFv antibody fragments from mouse splenocytes and cloning of these fragments into phagemid was successful, the resulting phage library bearing antibody fragments did not produce phage capable of specifically and strongly binding leucocin A or piscicolin 126. The antibody titre of brochocin-A was quite low (1:500), and so phage display was not attempted with this bacteriocin. Although these attempts at producing antibodies with phage display were unsuccessful, strategies to overcome potential obstacles have been described, including enriching for B cells bearing desired anti-bacteriocin genes prior to phage display (Kramer, 2002) or using other forms of antibody display,

such as ribosomal display (Schaffitzel et al., 1999; Yau et al., 2003; Groves et al., 2006). Future work could incorporate these strategies for successful development of antibody production techniques that reduce reliance on animals.

Despite the failure of phage display to produce antibody fragments to the class IIa bacteriocins tested in this study, the successful production of antibodies to unconjugated leucocin A in mice following immunization is of interest. Previous work by other researchers failed to raise antibodies to other unconjugated class IIa bactericions (Bhunia et al., 1990; Ingham et al., 2003). Comparison of the differences in sequence and structure between leucocin A and the non-immunogenic class IIa bacteriocins may allow for determination of the features acting as epitopes. This information may subsequently be used to design bacteriocins that are capable of maximum immunogenicity. Some attempts have been reported in the literature to increase the spectrum, stability, or activity of bacteriocins by engineering amino acid changes into bacteriocins (Johnsen et al., 2000). Depending on the changes incorporated, an additional benefit from such engineering could be to increase immunogenicity of bacteriocins; for instance, by replacing hydrophobic residues with more hydrophilic amino acids (Harlow and Lane, 1988). Such altered bacteriocins could then be used in the production of antibody display libraries from immunized animals. Another potential outcome of engineering changes in amino acids of bacteriocins is increasing resistance to proteases (O'Shea et al., 2010; Rink et al., 2010) such as those produced by Group II *Cl. botulinum*. This would increase the repertoire of useful bacteriocins available for application in foods to control protease-producing pathogens such as *Cl. botulinum*.

Comparable efficacy to chemical additives is another issue that must be addressed for bacteriocins to replace chemical additives in foods. This is particularly relevant to products that possess physical or chemical characteristics that render them vulnerable to pathogens, as is the case with *Cl. botulinum* growth in minimally-processed, vacuum-packed meat products. Because of increasing health concerns over nitrite usage in processed meats, food manufacturers are

searching for alternatives to the addition of sodium nitrite to inhibit the outgrowth of *Cl. botulinum* spores. To avoid labelling cured meat products with nitrite or nitrate as chemical additives, some meat processors are formulating their products with celery powder or sea salt as flavouring ingredients. These ingredients act as a "natural" source of nitrite and nitrate; thus, processors avoid adding sodium nitrite or nitrate to the product ingredients declaration. However, the efficacy of "natural cure" to control the outgrowth of *Cl. botulinum* spores in meat products has not been reported in the research literature. Another approach to removing sodium nitrite or nitrate is to use bacteriocins. Brochocin-C is a two-peptide bacteriocin that inhibited the outgrowth of *Cl. botulinum* spores in a bioassay under laboratory conditions (McCormick et al., 1998), as well as in a model meat system (Chapter 3, this thesis), and it was proposed as an anti-botulinal agent in a meat product.

In this study, brochocin-C was tested in a model meat system composed of commercial cooked meat medium, and effectively inhibited Group II *Cl. botulinum* in this system. However, the same concentration did not have any effect on Group I *Cl. botulinum*. Further investigation led to the discovery that Group I *Cl. botulinum* inactivated brochocin-C, as well as several class IIa bacteriocins, and that this inactivation was the result of proteolysis. The proteolysis observed was relatively specific, as not all bacteriocins tested were inactivated, likely due to lack of specific amino acid targets or protective circular structures possessed by unaffected bacteriocins. Several different classes of proteases may be responsible for inactivation of bacteriocins, and although the production of proteases by *Cl. botulinum* has been reported (DasGupta, 1971; DasGupta, 1972; DasGupta and Sugiyama, 1972; Nakane, 1978), the current study established that such proteases inactivate antimicrobial peptides such as bacteriocins. More detailed study is required to determine which protease classes are responsible for inactivation of bacteriocins.

Brochocin-C was purified in sufficient quantities for use as an additive in a chopped and formed pork product, and the same product was formulated without sodium nitrite but with "natural" cure to assess the anti-botulinal effects

of a commercially-available “natural” cure. However, brochocin-C did not provide the expected anti-botulinal effects in the pork product. When spores of Group II *Cl. botulinum* were added to the product, there was no significant difference in counts among treatments containing sodium nitrite, natural cure, brochocin-C, or a control containing no antimicrobials, as all counts remained at the same concentration throughout the 50-day experiment when enumerated on CBI medium. However, in combination, some ingredients in the chopped and formed pork product were identified as being inhibitory to Group II *Cl. botulinum*; namely, powdered smoke and NaCl. In an MIC assay with both ingredients, the concentration of smoke and NaCl found in the pork product inhibited several strains of Group II *Cl. botulinum*. Although commercial smoke products have been shown to inhibit *Cl. botulinum* (Eklund et al., 1982), this is the first report examining the synergistic interaction between these two ingredients in a meat product.

Although brochocin-C was not effective as an anti-botulinal ingredient in the chopped and formed pork product, its proven interaction with pork fat in this study (Chapter 3, this thesis) has offered insight into interactions of bacteriocins with food constituents. In the presence of pork fat, the activity of brochocin-C was eliminated in the aqueous phase. Further research is required to determine if other interactions occur in food products, as interaction of bacteriocins with food components is one of the barriers to their application in food systems. The interaction of brochocin-C with fat makes it unavailable for antimicrobial activity; thus, its application in food systems that contain fat is not recommended for control of outgrowth of *Cl. botulinum* spores. These results draw attention to the importance of considering the potential chemical properties of the bacteriocin being considered (such as hydrophobicity), and chemical and physical characteristics of the intended product into which it will be incorporated (fat content), in order to achieve maximum food safety.

During the course of the research on the anti-botulinal effects of brochocin-C in a meat product, differences were observed in the recovery of Group II *Cl. botulinum* on media that are commonly used for isolation of *Cl.*

botulinum from foods. RCM, a commonly-used medium for enumeration of *Cl. botulinum*, did not efficiently recover Group II *Cl. botulinum* from a pork product, whereas another medium, CBI, was efficient at recovery of *Cl. botulinum*. Previous studies attributed higher recovery of *Cl. botulinum* to the presence of lysozyme in egg yolk added to the medium (Hauschild and Hilsheimer, 1977; Peck et al., 1992), but addition of egg yolk did not increase recovery of Group II *Cl. botulinum* from this product. When other ingredients found in CBI were added to RCM, no increase in recovery was observed.

The presence of a background microflora had an impact on the recovery of Group II *Cl. botulinum* from the pork product. The differences in medium composition and influence of background microflora were investigated to understand their impact on the recovery and growth of *Cl. botulinum*. The ability of RCM to support growth of *Cl. botulinum* in the presence of different strains of background microflora was greatly enhanced by addition of phosphate to the medium. Addition of other nutrients such as magnesium, NaCl, sulphur, or additional protein sources had little impact on the growth of *Cl. botulinum* when exposed to the background microflora. Thus, the presence of phosphate in CBI was identified as the factor which allowed for increased recovery of *Cl. botulinum* from the pork product, and also allowed growth of *Cl. botulinum* in the presence of background microflora isolated from the same product. However, further research is required to determine the mechanism by which phosphate acts. In addition, the novel observation that growth of background microflora combined with medium composition may impact growth of *Cl. botulinum* requires further investigation to determine the nature of this relationship. The discovery that RCM is not optimal for recovery or growth of *Cl. botulinum* has significance for both the food industry and academics, because this medium or its derivatives have been used for isolation and enumeration of *Cl. botulinum* from food. Careful consideration must be given to the choice of medium for growth of *Cl. botulinum* in the presence of background microflora to avoid underestimating the growth of *Cl. botulinum*.

Several strains of carnobacteria and staphylococci that effectively inhibited Group II *Cl. botulinum* were isolated from the pork product. Although the carnobacteria are known to produce bacteriocins (Ahn and Stiles, 1990; Gursky, 2004), it is unknown whether these particular strains are capable of bacteriocin production. Investigation of the nature of the inhibition may identify additional bacteriocins with potent activity against *Cl. botulinum*. Surprisingly, one strain of *C. maltaromaticum* was found to stimulate growth of Group II *Cl. botulinum.* To date, there is only one report of two organisms that had this effect on *Cl. botulinum* (Giménez et al., 1988), and so this discovery requires further investigation to determine the reason for this stimulation of growth.

Overall, of the three objectives identified for this project, two have been fulfilled. A phage display method for quantitation of bacteriocins was not achieved, though some insights into immunogenicity of bacteriocins were gained. Brochocin-C was found to be effective at inhibiting Group II *Cl. botulinum* strains in a model meat system, but was inactive against Group I *Cl. botulinum* because of proteolysis by these strains. Brochocin-C application in a chopped and formed pork product did not reduce Group II *Cl. botulinum*, though other factors such as powdered smoke in the presence of salt were found to be inhibitory. However, other insights were gained into the limitations of media for accurately enumerating *Cl. botulinum* that originate from food systems, and the importance of considering this factor when performing challenge studies in food.

6.1 References

Ahn, C. and Stiles, M.E. (1990) Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl Environ Microbiol 56, 2503-2510.

Anonymous. (2009) Nisin. In: Combined Compendium of Food Additive Specifications, Vol. 4. Joint FAO/WHO Expert Committee on Food Additives, Rome, IT.

Bhunia, A.K., Johnson, M.C., Bibek, R. and Belden, E.L. (1990) Antigenic property of pediocin AcH produced by *Pediococcus acidilactici* H. J Appl Bacteriol 69, 211-215.

Burmester, J. and Plückthun, A. (2001) Construction of scFv fragments from hybridoma or spleen cells by PCR assembly. In: R. Kontermann and S. Dübel (Eds), Antibody Engineering. Springer-Verlag, New York, p. 19-40.

Health Canada. (2010) Canada Gazette Part II. Food and Drug Regulations 144, 2304-2307.

Crandall, A.D. and Montville, T.J. (1998) Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl Environ Microbiol 64, 231-237.

DasGupta, B.R. (1972) Role of a protease in natural activation of *Clostridium botulinum* neurotoxin. Infect Immun 6, 587-590.

DasGupta, B.R. and Sugiyama, H. (1972) Isolation and characterization of a protease from *Clostridium botulinum* type B. Biochim Biophys Acta 268, 719-729.

DasGupta, B.R. (1971) Activation of *Clostridium botulinum* type B toxin by an endogenous enzyme. J Bacteriol 108, 1051-1057.

Eklund, M.W., Pelroy, G.A., Paranjype, R., Peterson, M.E. and Teeny, F.M. (1982) Inhibition of *Clostridium botulinum* types A and E toxin production by liquid smoke and NaCl in hot-process smoke-flavoured fish. J Food Prot 45, 935-941.

FDA. (2006) Listing of specific substances affirmed as GRAS 21CFR184.1538. http://www.grokfood.com/regulations/184.1538.htm. Last updated 1 April 2006.

Giménez, M.A., Solanes, R.E. and Gimenez, D.F. (1988) Efectos metabiótico y sinérico de algunas bacterias aerobias estrictas sobre el crecimiento y toxinogénesis dr *Clostridium botulinum* tipo A. Rev Fac Cs Agrarias 24, 59-68.

Groves, M., Lane, S., Douthwaite, J., Lowne, D., Gareth Rees, D., Edwards, B. and Jackson, R.H. (2006) Affinity maturation of phage display antibody populations using ribosome display. J Immunol Methods 313, 129-139.

Gursky, L.J. (2004) Bacteriocin production by *Carnobacterium maltaromaticum* UAL26. PhD thesis, Department of Agricultural, Food, and Nutritional Science. University of Alberta, Edmonton, AB, p. 205.

Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Hauschild, A.H.W. and Hilsheimer, R. (1977) Enumeration of *Clostridium botulinum* spores in meat by a pour-plate method. Can J Microbiol 23, 829-832.

Ingham, A., Ford, M., Moore, R.J. and Tizard, M. (2003) The bacteriocin piscicolin 126 retains antilisterial activity *in vivo*. J Antimicrob Chemother 51, 1365-1371.

Johnsen, L., Fimland, G., Eijsink, V. and Nissen-Meyer, J. (2000) Engineering increased stability in the antimicrobial peptide pediocin PA-1. Appl Environ Microbiol 66, 4798-4802.

Joint FAO/WHO Expert Committee on Food Additives. (2007) Compendium of food additive specifications/Joint FAO/WHO Expert Committee on Food Additives, 68th Meeting, Vol. 4. Food and Agriculture Organization of the United Nations, Rome, IT.

Jones, E., Salin, V. and Williams, G.W. (2005) Nisin and the market for commercial bacteriocins. Texas Agribusiness Market Research Center, Texas A&M University, College Station, TX, p. 20.

Kramer, K. (2002) Synthesis of a group-selective antibody library against haptens. J Immunol Methods 266, 209-220.

Mazzotta, A.S. and Montville, T.J. (1999) Characterization of fatty acid composition, spore germination, and thermal resistance in a nisin-resistant mutant of *Clostridium botulinum* 169B and in the wild-type strain. Appl Environ Microbiol 65, 659-664.

Mazzotta, A., Crandall, A. and Montville, T. (1997) Nisin resistance in *Clostridium botulinum* spores and vegetative cells. Appl Environ Microbiol 63, 2654-2659.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E. and van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. Appl Environ Microbiol 64, 4757-4766.

Nakane, A. (1978) Proteases produced by a proteolytic mutant of *Clostridium botulinum* type E. J Gen Microbiol 107, 85-91.

O'Shea, E.F., O'Connor, P.M., Cotter, P.D., Ross, R.P. and Hill, C. (2010) Synthesis of trypsin-resistant variants of the *Listeria*-active bacteriocin salivaricin P. Appl Environ Microbiol 76, 5356-5362.

Peck, M.W., Fairbairn, D.A. and Lund, B.M. (1992) The effect of recovery medium on the estimated heat-inactivation of spores of non-proteolytic *Clostridium botulinum*. Lett Appl Microbiol 15, 146-151.

Rink, R., Arkema-Meter, A., Baudoin, I., Post, E., Kuipers, A. and Nelemans, S.A. (2010) To protect peptide pharmaceuticals against peptidases. J Pharmacol Toxicol 61, 210-218.

Schaffitzel, C., Hanes, J., Jermutus, L. and Plückthun, A. (1999) Ribosome display: an in vitro method for selection and evolution of antibodies from libraries. J Immunol Methods 231, 119-135.

Yau, K.Y.F., Groves, M.A.T., Li, S.H., Sheedy, C., Lee, H., Tanha, J., MacKenzie, C.R., Jermutus, L. and Hall, J.C. (2003) Selection of hapten-specific single-domain antibodies from a non-immunized llama ribosome display library. J Immunol Methods 281, 161-175.