Histone H5: Bioinspiration for novel antimicrobial peptides

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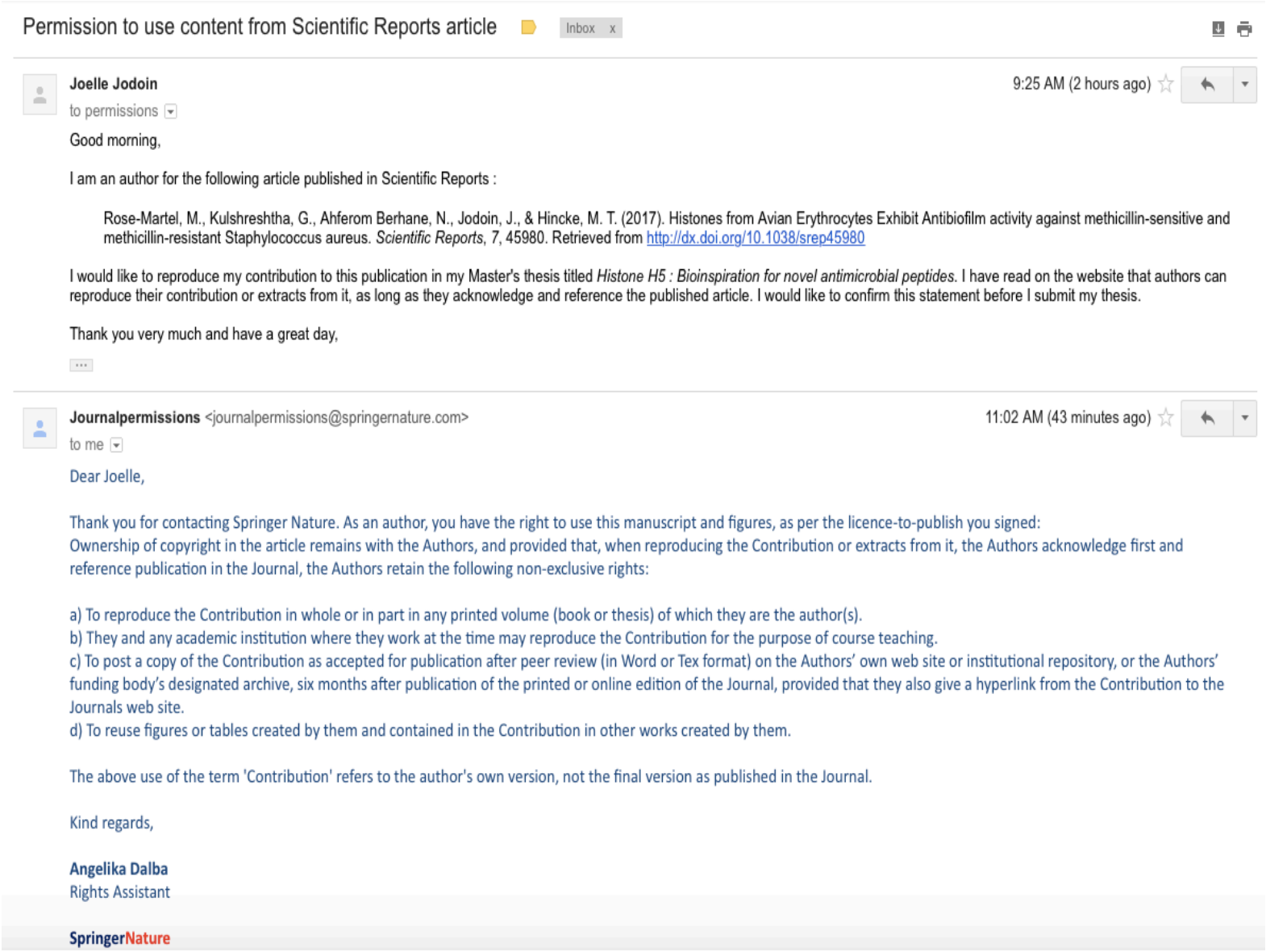

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ABSTRACT

Modern medicine is challenged continuously by the increasing prevalence of multi-drug resistant bacteria. Therefore, the development of alternatives to traditional antibiotics is an urgent necessity. Cationic antimicrobial peptides (CAMPs) are components of the innate immune defense system. Histones, generally known as proteins that package and regulate the transcription of DNA, share all of the essential antimicrobial traits of CAMPs, and could be promising alternatives to antibiotics. In this study, I investigated the antimicrobial properties of nucleated-erythrocyte-specific linker histone H5 and its derived peptides. Histone H5 was extracted and purified from chicken erythrocytes using an acid extraction followed by ion exchange chromatography using a step salt gradient; the purity (>95%) was verified by densitometry and proteomics analysis. Purified histone H5 demonstrated potent antimicrobial activity against various Gram-positive and Gram-negative planktonic bacteria, including resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), as well as anti-biofilm activity against *Listeria monocytogenes* and *Pseudomonas aeruginosa*. Furthermore, scanning electron microscopy (SEM) revealed significant damage to *L. monocytogenes* and *P. aeruginosa* bacterial cell surfaces after histone H5 treatment. The potential for histone toxicity towards mammalian cells was investigated with a hemolytic assay which determined that even at the highest concentration tested (1 mg/mL), histone H5 was non-hemolytic. An in *silico* analysis determined the predicted antimicrobial domain of histone H5 of which six histone H5-derived peptides with potential antimicrobial activity were identified. These six histone H5-derived peptides were synthesized and tested

against bacterial pathogens to determine their antimicrobial properties. Although the H5-derived peptides were identified within the predicted antimicrobial domain of histone H5, they did not possess more potent antimicrobial activity than the full length protein. Overall, this study demonstrates that histone H5 and histone H5-derived peptides could be promising candidates in the development of novel anti-infective therapeutics.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
AMP	Antimicrobial Peptide
APD	Antimicrobial Peptide Database
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid
BHI	Brain-Heart Infused
BSA	Bovine Serum Albumin
CAMP	Cationic Antimicrobial Peptide
CBD	Calgary Biofilm Device
CD	Circular Dichroism
CFIA	Canadian Food Inspection Agency
CFU	Colony Forming Unit
CPRC	Canadian Poultry Research Council
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EPS	Exopolysaccharide
FDR	False Discovery Rate
HCl	Hydrochloric Acid
HMDS	Hexamethyldisilizane
HPLC	High-Performance Liquid Chromatography
HUS	Hemolytic Uremic Syndrome
ICU	Intensive Care Unit
LB	Luria-Bertani
LC/MS/MS	Liquid Chromatography Tandem-Mass Spectrometry
LPS	Lipopolysaccharides
LTA	Lipoteichoic Acids
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration

MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
MS/MS	Tandem-Mass Spectrometry
NET	Neutrophil Extracellular Trap
OD	Optical Density
PBS	Phosphate Buffered Saline
R & D	Research & Development
RBC	Red Blood Cell
RNA	Ribonucleic Acid
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SPPS	Solid-Phase Peptide Synthesis
TCA	Trichloroacetic Acid
TFA	Trifluoroacetic Acid
VRE	Vancomycin-Resistant *Enterococcus*
WHO	World Health Organization

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1.0 INTRODUCTION

1.1 Antimicrobial resistance

1.1.1 The urgent problem of antimicrobial resistance

The discovery of antibiotics has greatly changed global health by effectively treating infections caused by bacteria. However, the increase of antibiotic resistant bacteria has currently become a great worldwide health challenge. Causes of the antibiotic resistance crisis include the overuse and misuse of antibiotics, incorrectly prescribed antibiotics, economic and regulatory obstacles that have prevented the development of new antibiotics by the pharmaceutical industry and the substantial reduction of new antibiotic approvals (Ventola 2015; Cars et al. 2011). Additionally, the extensive use of antibiotics in agriculture has a major impact on the spread of antimicrobial resistance. In fact, an estimated 80% of all antibiotics sold in the USA are used in livestock to control and treat bacterial infections as well as for growth promotion purposes (Phillips et al. 2004; Ventola 2015). Antibiotics used in animals bred for human consumption can lead to the development of antibiotic-resistant foodborne pathogens followed by their transmission to humans as food contaminants (Economou & Gousia 2015). In addition to the increased risk of acquired illness from foodborne pathogens, efficient treatment of infections significantly decreases with the heightened development of antibiotic resistance within bacterial populations. In fact, it is estimated that approximately 700,000 deaths per year worldwide can be attributed to antimicrobial resistance, and that this death toll will increase to 10 million per year by 2050 (O'Neill 2014). Additionally, the augmentation of antibiotic resistant microbes has economic consequences on health care systems worldwide. For example, the World Health

Organization (WHO) estimates that the US health system spends between 21 and 34 billion dollars per year, and patients stay an additional 8 million days in hospitals on account of the antimicrobial resistance problem (World Health Organization 2014).

1.1.2 Bacterial pathogens

To emphasize and prioritize the importance of research and development of new and effective treatments against antibiotic-resistant bacteria, the WHO recently identified the most important resistant bacteria at a global level. Among the twelve listed bacterial pathogens, *Pseudomonas aeruginosa* ranks at the top of the list as a pathogen requiring critical priority for the development of new or alternative antibiotics (World Health Organization 2017). *P. aeruginosa* is a Gram-negative opportunistic bacterium that is persistent in causing infections in community and hospital settings on account of its ability to tolerate an array of different environments while requiring minimal nutrition (Lister et al. 2009). Although community-acquired infections persist, serious infections caused by *P. aeruginosa* are predominately nosocomial. In fact, 10% of all nosocomial infections are caused by *P. aeruginosa* (Aloush et al. 2006; National Nosocomial Infection Surveillance System, 2004). Being an opportunistic bacterial pathogen, *P. aeruginosa* infections cause serious problems for patients in the intensive care unit (ICU), responsible for 10% of urinary tract infections, 3% of bloodstream infections, 21% of pneumonias and 13% of ear, eye, throat and nose infections in ICUs in the USA from 1992 to 1997 (Lister et al. 2009; Richards et al. 1999).

Other pathogens requiring critical priority for new treatments are the *Enterobacteriaceae* bacterial family, including *Escherichia coli* (World Health

Organization 2017). *E. coli* is a Gram-negative rod-shaped bacterium and an important component of the normal intestinal microflora in humans. However, *E. coli* can also be a versatile pathogen causing various intestinal and extra-intestinal illnesses (Kaper et al. 2004). For instance, infection by the enterohemorrhagic strain, *E. coli* O157:H7, can lead to hemorrhagic colitis and hemolytic uremic syndrome (HUS), characterized by hemolytic anemia, a low platelet count and renal failure (Kaper et al. 2004; Rangel et al. 2005). Cattle are a major reservoir of *E. coli* O157:H7 and accordingly, the principle routes for foodborne transmission of the bacteria are through unpasteurized milk and contaminated bovine meat, particularly ground beef (Karch et al. 2005). *E. coli* O157:H7 is responsible for 73,000 illnesses annually in the USA, and from 1982 to 2002 there were 350 outbreaks leading to 40 deaths (Rangel et al. 2005).

Bacterial species of high priority for the development of new anti-infectives, according to the WHO, are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) and *Salmonella* spp. (World Health Organization 2017). *Salmonella* was the leading cause of bacterial foodborne illnesses in the United States from 2000 to 2008, resulting in an estimated 1 million illnesses, 20,000 hospitalizations and 380 deaths (Scallan et al. 2011). Among the most common serotypes, associated with at least 15% of all clinically reported salmonellosis in humans, is *Salmonella typhimurium* (DiMarzio et al. 2013). Vancomycin-resistant *Enterococcus* (VRE) is a major nosocomial pathogen worldwide, however rates of VRE infections are highest in North America. From 2009 to 2010, enterococcal infections were the second most common cause of hospital-acquired infections in the USA, and 35.5% of these enterococcal infections were resistant to vancomycin (Sievert et al.

2013; Driscoll & Crank 2015). Clinical manifestations of VRE can include presence of bacteria in the bloodstream, urinary tract infections, skin and skin structure infections, intra-abdominal and pelvic infections, infective endocarditis and central nervous system infections (Driscoll & Crank 2015). *Staphylococcus aureus* is one of the principle causes of post-surgical wound infections and is associated with a variety of illnesses such as pneumonia, endocarditis, meningitis and sepsis (Kardas-Sloma et al. 2011; Waness 2010). Furthermore, methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with longer hospital stays as well as higher morbidity and mortality rates, compared to patients infected with methicillin-sensitive *Staphylococcus aureus* (MSSA) (Lodise & Mckinnon 2007). Additionally, from 1995 to 2007, there has been a 17-fold increase of MRSA colonization incidence and infections in Canadian hospitals (Simor et al. 2010).

Other pathogens of concern include *Bacillus cereus* and *Listeria monocytogenes*, major Gram-positive foodborne pathogens. *B. cereus* is spore-forming and produces a large number of virulence factors, such as hemolysins and proteases (Schoeni & Wong 2005). *B. cereus* causes two types of food associated illnesses (emetic and diarrheal syndromes) as well as various local and systemic infections (Drobniewski 1993). This pathogen's ability to adapt to heat, cold and chemical environments makes *B. cereus* contamination difficult to control in the food processing industry (Schlegelova et al. 2003). Hence, *B. cereus* contamination occurs commonly among a variety of food products, including pasta, rice, chicken, seafood, vegetables, fruits and especially, pasteurized milk and dairy products (Schoeni & Wong 2005). Infection by *Listeria monocytogenes* causes listeriosis, a disease associated with a range of clinical

manifestations, from febrile gastroenteritis to bacteremia, meningitis and sepsis, depending on the severity of the infection (Hernandez-Milian & Payeras-Cifre 2014). From 1995 to 2004, severe infections by *L. monocytogenes* resulted in 43 deaths in Canada (Clark et al. 2010) and an estimated 260 deaths in the United States from 2000 to 2008 (Scallan et al. 2011). Like *B. cereus*, *L. monocytogenes* is an important concern for the food industry since it has the ability to survive and proliferate in harsh conditions, such as high salt concentrations, a wide pH range and cold temperatures (Hernandez-Milian & Payeras-Cifre 2014). Consequently, food (meat, poultry, dairy and vegetable products) is the major route of transmission for listeriosis, responsible for 99% of acquired listeriosis illnesses in the USA (Scallan et al. 2011). Furthermore, Conter and colleagues demonstrated that 11.7% of 120 *L. monocytogenes* strains isolated from food and food-processing environments were resistant to antibiotics, acquired by antibiotic resistance genes from Gram-positive bacteria (Conter et al. 2009). As a result of the developing antibiotic resistance of *L. monocytogenes*, alternative therapeutics will become necessary to ensure the effective treatment of listeriosis, an already deadly illness.

The importance of research and development of new treatments effective against antibiotic-resistant bacterial pathogens has recently been emphasized by the World Health Organization (World Health Organization 2017). The global threat of antimicrobial resistance is clearly present; therefore, the development of new antibiotics or alternatives to traditional antibiotics is an urgent necessity (Bush et al., 2011; Cars et al., 2011).

1.2 Bacterial biofilms

In addition to the prevalence of antibiotic resistant bacteria, bacterial biofilms are also of great concern. Bacterial biofilms are a collection of unicellular organisms attached to a solid surface and enclosed in an exopolysaccharide (EPS) matrix (Mah & O'Toole 2001; Donlan 2002). Compared to freely suspended planktonic bacteria, biofilms have an increased resistance to antibiotics. In fact, it has been demonstrated that bacterial biofilms are 10 to 1,000 times more resistant to antibiotics than planktonic bacteria (Davies 2003; Mah & O'Toole 2001). Several hypotheses have been suggested in order to explain the reduced susceptibility of biofilms to antibiotics. Firstly, it has been proposed that the EPS matrix may prevent antibiotics from penetrating into the biofilm and consequently limit access to the bacterial cells (Mah & O'Toole 2001; Stewart & Costerton 2001; Davies 2003). Decreased metabolic activity and growth rates, especially in bacteria deep in the biofilm, also contribute to antibiotic resistance (Hoiby et al. 2010). Finally, biofilm-growing bacteria are more tolerant to antimicrobial compounds on account of certain protective factors they express, such as upregulated efflux pumps and stress response regulons (Davies 2003). As a result of this enhanced antibiotic resistance, biofilms are of great concern in human diseases; gingivitis, endocarditis and cystic fibrosis are a short list of common examples (Hoiby et al. 2010). In addition, biofilms have the capacity to colonize and infect items like catheters, shunts, respirators, contact lenses, prosthetic joints, pacemakers and artificial heart valves (Davies 2003).

1.3 Cationic antimicrobial peptides (CAMPs)

1.3.1 General properties and mechanism of action

Antimicrobial peptides (AMPs) are widely distributed among a diverse range of organisms (prokaryotes, vertebrates, invertebrates and plants) and are an important component of the innate immune defense system (Radek & Gallo 2007). These peptides display antimicrobial activities against bacteria, fungi, viruses and other pathogenic microorganisms (Zasloff 2002). Most antimicrobial peptides are short in length (12-50 amino acids), have an overall positive charge (net charge from +2 to +9) and contain a substantial proportion of hydrophobic residues (≥30%); these peptides are termed cationic antimicrobial peptides (CAMPs) (Brogden et al. 2003; Cho et al. 2009; Fjell et al. 2011; Jindal et al. 2015; Stark et al. 2002). CAMPs are often classified according to their secondary structures: amphipathic alpha-helical peptides (most CAMPs), beta-sheet peptides and linear peptides (Pasupuleti et al. 2012). Beta-sheet peptides have a rigid structure and are therefore more ordered in aqueous solution, while alpha-helical peptides are often unordered in aqueous solution and undergo conformational changes into amphipathic helical structures upon interaction with bacterial membranes (Yeaman & Yount 2003; Mahlapuu et al. 2016).

CAMPs display bacteriolytic activity by causing disruption of the microbial membrane and/or by acting on intracellular bacterial targets (Zasloff 2002; Hancock & Sahl 2006). Even when antimicrobial activity is achieved by means of intracellular targets, initial AMP and bacterial membrane interaction is required (Mahlapuu et al. 2016; Bahar & Ren 2013). Bacterial surfaces are negatively charged due to the presence of phospholipids such as phosphatidylglycerol, cardiolipin and

phosphatidylserine, which have negatively charged head groups (Yeaman & Yount 2003). Additionally, lipopolysaccharides (LPS), in the outer membrane of Gram-negative bacteria, and lipoteichoic acids (LTA), anchored to the peptidoglycan layer of Gram-positive bacteria, further enhance the negative charge of the bacterial cell wall (Armstrong et al. 1958; Kamio & Nikaido 1976). Consequently, positively charged AMPs can form electrostatic interactions with these anionic surfaces (Matsuzaki 1999). In contrast to bacteria, mammalian cell membranes have a neutral net charge and contain a high level of cholesterol (Yeaman & Yount 2003; Zasloff 2002; Lai & Gallo 2009). The presence of cholesterol stabilizes the phospholipid bilayer of cells, while the formation of electrostatic interactions is impossible between CAMPs and the neutrally charged mammalian cells (Zasloff 2002; Mahlapuu et al. 2016). As a result of these fundamental differences between bacterial and mammalian cell membranes, CAMPs have selective activity towards bacteria (Yeaman & Yount 2003).

Since Gram-negative bacteria possess outer membranes, CAMPs must first translocate through these membranes via self-promoted uptake in order to reach the bacterial membranes (Hancock & Lehrer 1998). Once in contact with the Gram-positive or Gram-negative bacterial membranes, the CAMPs undergo conformational changes into amphipathic structures, if not already present (Mahlapuu et al. 2016; Yeaman & Yount 2003). Then, electrostatic interactions between the peptides and bacteria are formed (as described above), while the hydrophobic domains of the CAMP interact with the lipid bilayer, allowing CAMP insertion into the bacterial membrane (Mahlapuu et al. 2016; Hancock & Sahl 2006). Different models have been suggested to describe the subsequent mechanism of action in which CAMPs cause membrane permeabilization:

the barrel-stave, carpet or toroidal-pore models (Yeaman & Yount 2003) (**Figure 1**). Each model ultimately leads to pore formation within the bacterial membrane, which leads to leakage of metabolites and ions, membrane dysfunction, followed by membrane rupture and rapid lysis of the cells (Yeaman & Yount 2003; Brogden 2005). Membrane permeabilization also permits access into the cytoplasm where certain CAMPs act on intracellular targets, such as the machinery of protein synthesis and folding, DNA/RNA synthesis, cell wall production and/or enzymatic processes (Brogden 2005; Yeaman & Yount 2003).

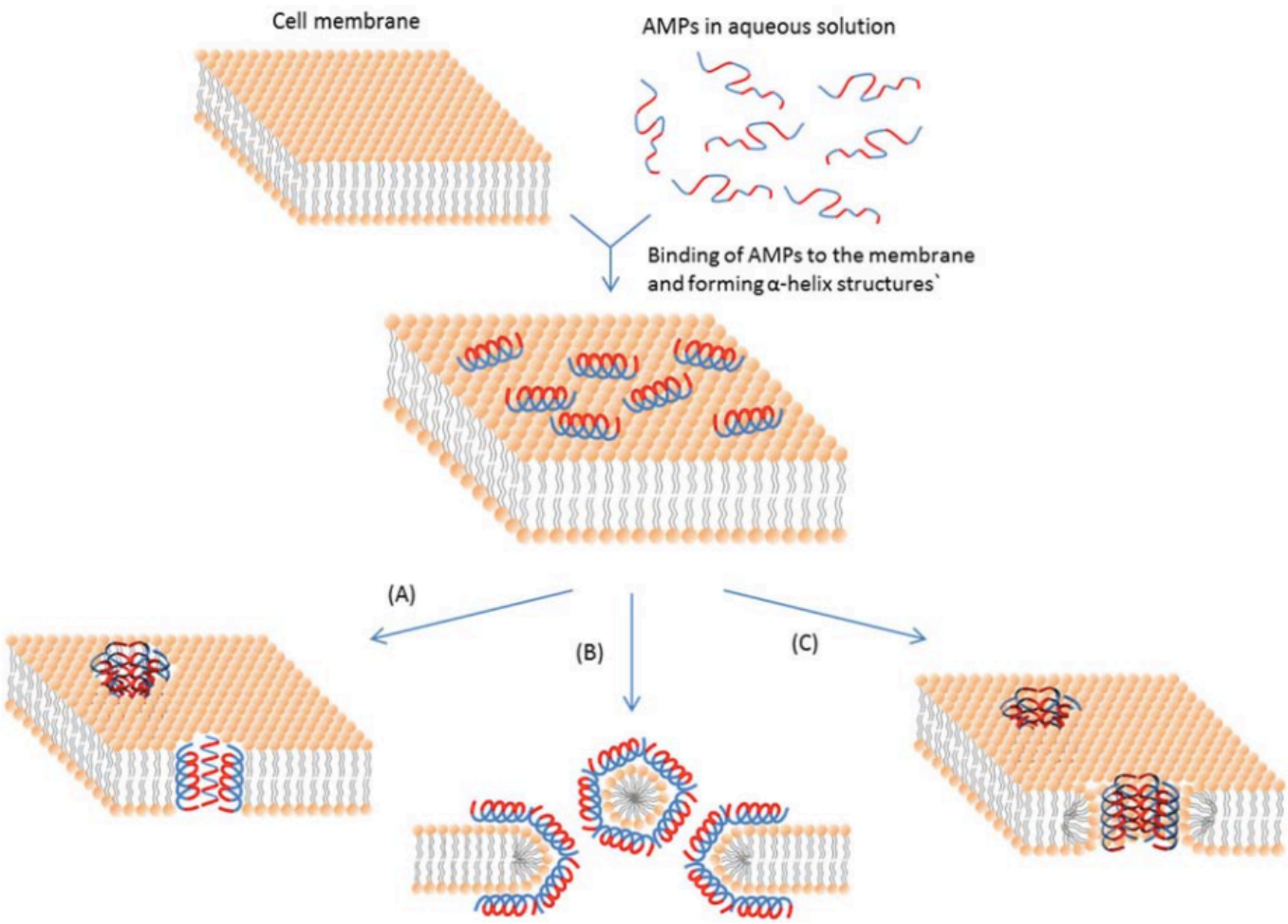


Figure 1. Schematic representation of the different mechanisms of action of CAMPs. Different models leading to bacterial membrane permeabilization by CAMPs: A) Barrel-stave model, B) Carpet model and C) Toroidal-pore model. The hydrophobic (blue) and the hydrophilic (red) portions of CAMPs are shown. Figure adapted from: (Bahar & Ren 2013).

In contrast to antibiotics, antimicrobial activities of CAMPs aim for multiple low-affinity targets, which complicates the development of bacterial resistance mechanisms (Lai & Gallo 2009; Mahlapuu et al. 2016). However, bacterial resistance to CAMPs does occasionally occur via constitutive resistance (biofilm formation and electrostatic shielding) and inducible resistance (efflux pumps and modifications of bacterial membrane molecules and intracellular targets) (Bahar & Ren 2013; Yeaman & Yount 2003). Nonetheless, bacterial membranes are the main targets for CAMP antimicrobial activity, which are components of microorganisms that cannot be easily modified (Kawasaki & Iwamuro 2008). Consequently, development of resistance to CAMPs by bacteria is very limited compared to that observed against antibiotics, although such antimicrobial peptides have been acting upon bacteria for hundreds of millions of years (Bahar & Ren 2013; Zasloff 2002). Accordingly, CAMPs are interesting potential candidates to overcome the global threat of antibiotic resistance.

1.3.2 AMPs as therapeutic agents

Antimicrobial peptides possess multiple appealing characteristics, including rapid bactericidal activity, an unusual broad range of activity and limited bacterial resistance, which makes them prospects as therapeutic anti-infectives (Mahlapuu et al. 2016; Hancock & Sahl 2006). However, only a few AMPs have been approved for clinical use, such as polymyxins. Polymyxins are used as topical agents for the prevention and treatment of local infections and as an intravenous treatment of infections caused by drug-resistant Gram-negative bacterial pathogens (Zavascki et al. 2007). Additionally, multiple AMPs are currently undergoing clinical trials for development as new treatments against various bacterial infections. Omiganan (a derivative of indolicidin),

Pexiganan (an analog of magainin isolated from the African clawed frog) and Lytixar (a synthetic peptide) are all examples of antimicrobial peptides being assessed as topical agents to treat infections (Hancock & Sahl 2006; Mahlapuu et al. 2016). In addition to the peptides mentioned above, many others are proceeding through discovery, development and finally, clinical trials (Mahlapuu et al. 2016).

1.4 Histones

Histones are commonly known as proteins with very important roles in gene transcription regulation in eukaryote cells (Wolffe & Guschin 2000). Nucleosomes are structures found in chromosomes and are composed of DNA, core histones and linker histones. Each nucleosome has 147 base pairs of DNA wrapped around an octomeric complex formed by core histones (H2A, H2B, H3 and H4) (Khorasanizadeh 2004). Linker histones (H1 and H5) secure the DNA strand tightly around the octomeric complex (Khorasanizadeh 2004). Linker histone H5 is a nucleated-erythrocyte-specific linker histone (Perucho et al. 1982) that coexists with linker histone H1 in avian erythrocytes. Histone H1 represents 9.7% ± 1 while histone H5 represents 18% ± 1 of a whole histone mixture extracted from chicken erythrocytes (Rose-Martel et al. 2017). Histone proteins share all of the essential traits of cationic antimicrobial peptides. They are hydrophobic, cationic and can form amphipathic alpha-helical structures (Kawasaki & Iwamuro 2008). Consequently, many reports have demonstrated antimicrobial activities of histones. The first was a study in 1958 which established that histones extracted from calf thymus had bactericidal activity (Hirsch 1958). Since then, histones and histone-derived fragments extracted from many different organisms have been identified as antimicrobial agents. For example, a histone H1 N-terminal fragment found

in the skin mucus of the Atlantic salmon (Lüders et al. 2005), histones H2A, H2B.V and H2B C-terminal fragment from the chicken liver (Li et al. 2007), histones H1 and H2B from the chicken reproductive system (Silphaduang et al. 2006) and buforin I, a 39-amino acid peptide derived from histone H2A, secreted into the stomach of an Asian toad (Park et al. 1996). Additionally, histones are one of the main components of neutrophil extracellular traps (NETs) released by neutrophils in order to elevate the concentration of antimicrobials at sites of infection (Brinkmann et al. 2004). Hence, this evidence demonstrates that histones are not only present within the nucleus, but that they can also act outside of the nucleus as antimicrobial peptides.

Unlike mammals, most nonmammalian vertebrates (birds, reptiles, fish and amphibians) have nucleated red blood cells (Claver & Quaglia 2009) (**Figure 2**). The anucleated erythrocyte is considered more evolutionary «advanced» (Snyder & Sheafor 1999). Mammals have smaller end-blood-vessels than nonmammalian vertebrates, and the presence of a nucleus may prevent erythrocytes from passing through these small capillaries (Snyder & Sheafor 1999). Consequently, nucleated red blood cells, as found in birds, contain DNA that is highly compacted by histones. In the poultry industry, avian blood is a significant waste product. Therefore, due to the abundance and ease of harvest of avian blood, this could be an ideal source of purified histones. Recent findings from our laboratory clearly indicate that a histone mixture (H1, H2A, H2B, H3, H4 and H5), extracted from chicken erythrocytes, possesses antimicrobial activity against a variety of Gram-negative and Gram-positive planktonic bacteria (Rose-Martel & Hincke 2014), as well as eradicating activity against Gram-positive bacterial biofilms (Rose-Martel et al. 2017). However, investigation for a specific histone responsible in

whole or in part for the antimicrobial activity of the histone mixture extracted from chicken erythrocytes was not completed. Determination of which histone, and more specifically which histone-derived peptide, has the most potent antimicrobial activity could lead to the identification of a novel CAMP. Interestingly, histone H5 is a nucleated-erythrocyte-specific linker histone (Perucho et al. 1982) of which, to our knowledge, the antimicrobial properties have never been previously investigated. Histone H5 from chicken erythrocytes is 190 amino acids in length, with a hydrophobic ratio of 28%, a total net charge of +61 and an isoelectric point of +12. Considering these properties, the amino acid sequence of histone H5 and the general cationic and hydrophobic properties of other antimicrobial histones, we hypothesize that histone H5 has antimicrobial activity. Therefore, the goal of this project was to gain insight into the antimicrobial properties of histone H5 and of histone H5-derived peptides. Essentially, this project has the potential to identify a novel CAMP and, therefore, to provide a R & D pathway to a therapeutic alternative to antibiotics, which is crucial for human health.

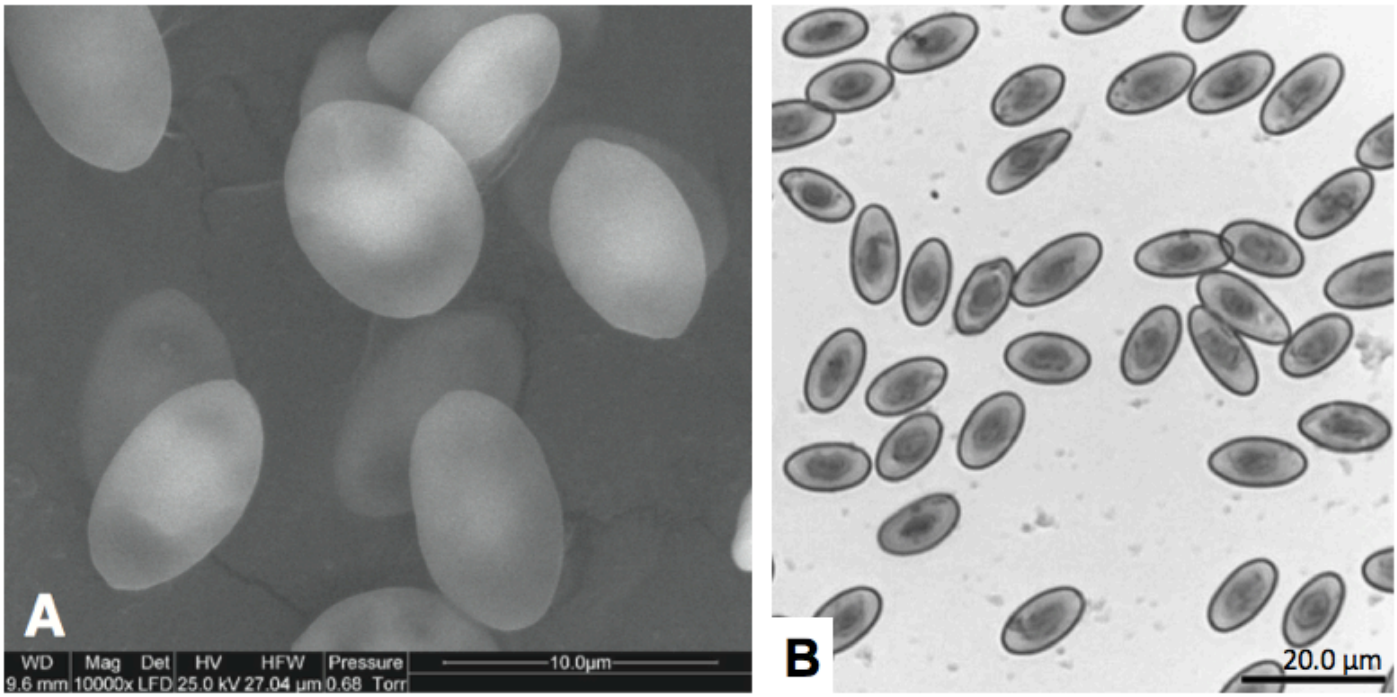


Figure 2. Chicken red blood cell morphology. Visualization of chicken nucleated RBCs by scanning electron microscopy (A) and by light microscopy (stained with May-Grünwald-Giemsa) (B). Figure adapted from: (Kutwin et al. 2014).

1.5 Hypothesis and Experimental aims

1.5.1 Hypothesis

Histone H5 has antimicrobial activity against Gram-positive and Gram-negative bacteria.

1.5.2 Objectives/Experimental aims

1. Extract and purify histone H5 from chicken erythrocytes.
2. Determine the minimal inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of purified histone H5 against a range of Gram-positive and Gram-negative planktonic bacteria.
3. Determine the minimum biofilm eradication concentrations (MBEC) of purified histone H5 against Gram-positive and Gram-negative bacterial biofilms.
4. Identify and verify the specific regions of histone H5 with potential antimicrobial activity.
5. Determine which histone H5-derived peptide has the most potent antimicrobial activity.

2.0 MATERIALS AND METHODS

2.1 Extraction and purification of histone H5 from chicken erythrocytes

2.1.1 Collection and storage of RBCs

Broiler chickens (5-7 weeks of age) were euthanized by decapitation at local slaughterhouses in accordance with Canadian Food Inspection Agency (CFIA) regulations. Blood was collected immediately after decapitation with 1.5 mg EDTA/mL. Next, chicken erythrocytes were separated from the freshly collected chicken whole blood by centrifugation (300 x g for 10 minutes) and washed with 1X PBS (three times). Cells were flash-frozen using liquid nitrogen and stored at -80˚C.

2.1.2 Acid extraction of linker histones (H1 & H5)

The protocol for this step of the project is based on previous work from our laboratory (Rose-Martel & Hincke 2014). Erythrocytes corresponding to 25-100 mL of blood (depending on the extraction) were lysed with 10 X the volume of hypotonic lysis buffer (10 mM Tris-Cl pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$ and 1 mM DTT) for 1 hour at 4°C with light agitation. The intact nuclei were pelleted at 10,000 x g for 10 minutes at 4°C and the supernatant was removed. The nuclei were resuspended with 250 mL of hypotonic lysis buffer and the nuclei were pelleted at 10,000 x g for 10 minutes at 4˚C; this was repeated three times. The contamination by hemoglobin in the supernatant after each wash was detected by a spectral scan (absorbance from 300-500 nm) using the EON microplate spectrophotometer (BioTek, Winooski, VT, USA). When no apparent absorbance peak was detected at 400 nm, the nuclei were treated with 100 mL of 10% perchloric acid ($HClO_4$) for 1 hour at 4°C with light agitation. The nuclear debris was pelleted at 16,000 x g for 10 minutes at 4°C and discarded. Trichloroacetic

acid (TCA) (49.5 mL, 100%) was added to the supernatant in small increments in order to precipitate the linker histones. The suspension was incubated overnight at 4˚C. The next morning, histones were pelleted at 16,000 x g for 10 minutes at 4°C and the supernatant was discarded in acid waste. Next, histone pellets were washed three times with acetone at 16,000 x g for 10 minutes at 4°C. The linker histones were dissolved in 10 mL of sterile ddH_2O and passed through an Amicon Ultra centrifugal filter unit with a 3,000 Da molecular weight cut off (Millipore Corporation, Billerica, MA, USA) at 750 x g for 30 minutes. The retained histones were flash frozen and lyophilized overnight.

2.1.3 Histone H5 purification using ion exchange chromatography

The protocol for this step of the project was developed from a previous article published in *Protein expression and purification* (Garcia-Ramirez et al. 1990) but with certain modifications. The instrument used for this step was the Model EP-1 Econo Pump from Biorad, at a flow rate of 2 mL/min (1.6 mm tubing) and the system was kept at 4˚C. The column was packed with a 1:1 slurry of CM-Sephadex C-25 resin (GE Healthcare) and the initial buffer (0.35 M NaCl, 10 mM Tris-HCl, pH 8.8). The lyophilized linker histones from the previous step were dissolved directly into the initial buffer, and loaded onto the column. Next, the elution buffer A (0.75 M NaCl, 10 mM Tris-HCl, pH 8.8) was run through the system (10 X column volume) to elute histone H1. In order to verify total elution of histone H1 from the column, absorbance at 230 nm and 280 nm of the solution exiting the system was checked. At this point, the elution buffer B (1.4 M NaCl, 10 mM Tris-HCl, pH 8.8) was run through the system (10 X column volume) to elute histone H5. Once again, absorbance at 230 nm and 280 nm was

verified. The histone H5 sample was concentrated using an Amicon Ultra centrifugal filter unit with a 3,000 Da molecular weight cut off (Millipore Corporation, Billerica, MA, USA). SDS-PAGE analysis was then performed in order to visualize if histone H1 was still present in the histone H5 sample. Depending on the initial amount of linker histones, the sample had to be put through the system 2 to 4 times to collect purified histone H5. Once the purified H5 sample was obtained, dialysis of the sample was performed to eliminate the buffer from the sample. Next, the concentration of the sample was determined with the BCA protein assay (Pierce, Thermofisher) in which bovine serum albumin (BSA) (Pierce, Thermofisher) served as the standard. Finally, the sample was aliquoted (100 µg), flash frozen and lyophilized.

2.1.4 Densitometry and proteomics analysis of the H5 samples

For the densitometry analyses, the H5 sample (1.5 µg of protein) was analyzed by SDS-PAGE using a 4-12% NuPAGE® Bis-Tris mini gel from Life Technologies. The sample and buffer preparations as well as the protocol was completed according to the manufacturer's instructions. The gel was stained with Coomassie Brilliant Blue and the bands were analyzed using ImageJ densitometry software (version 1.48, NIH, USA). For the proteomics analyses, the H5 sample (1.5 µg of protein) was loaded on 4-12% NuPAGE® Bis-Tris mini gel as described previously. However, the sample was only run for 5 minutes, not allowing the protein bands to separate, and stained. This unresolved band was excised and sent to the Proteomics Platform of the Eastern Quebec Genomics Centre (Laval, QC) for LC/MS/MS proteomics analysis. This service included protein in-gel digestion, mass spectrometry and Mascot database searching.

All MS/MS samples were analyzed using Mascot software (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search the TAX_GallusGallus_CI_9031_20170116 database (unknown version, 33116 entries) with trypsin digestion. Validation of MS/MS based peptide and protein identification was completed by Scaffold software (version Scaffold_4.7.3, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability by the Protein Prophet algorithm (Nesvizhskii et al. 2003) and contained at least 2 identified peptides.

2.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values

2.2.1 Preparation of bacterial suspension

The bacterial strains tested included Gram-positive bacteria (*Bacillus cereus* (ATCC 11778), methicillin-sensitive *Staphylococcus aureus* (ATCC 6538), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 29247), *Listeria monocytogenes* (ATCC 19112), *Enterococcus faecalis* (clinical isolate) and vancomycin-resistant *Enterococcus* (ATCC 51299)) and Gram-negative bacteria (*Escherichia coli* K12 (ATCC 29425), *Escherichia coli* (O157:H7), *Pseudomonas aeruginosa* (ATCC 15442) and *Salmonella typhimurium* (ATCC 1535)). Bacterial colonies from glycerol stocks were plated on LB agar plates (BHI agar plates for *L. monocytogenes*) and incubated overnight at 37°C. A single colony from these plates was grown in 3 mL of LB broth

(BHI broth for *L. monocytogenes*) overnight at 37°C and shaken at 250 rpm. The overnight culture was diluted 1:50 in fresh LB broth (BHI broth for *L. monocytogenes*), incubated at 37˚C and shaken at 250 rpm until it displayed exponential growth (OD_{600} = 0.2). The bacterial suspension was pelleted at 3,000 x g, 4°C for 10 minutes and washed with PBS (twice) and then adjusted to 10^5 - 10^6 CFUs/mL in PBS.

2.2.2 Broth microdilution assay

The protocol for this step of the project was based on established methods (Rose-Martel & Hincke 2014). The antimicrobial activity of the histone H5 sample or the histone H5-derived peptides against planktonic bacteria was evaluated using a broth microdilution assay. Histone H5 or the peptides were dissolved in sterile water, pH 7.4, serially two-fold diluted and aliquoted in a 1:1 (v/v) ratio with bacteria in Eppendorf tubes. Kanamycin (all bacteria except for VRE) or ampicillin (used for VRE only) served as a positive control for inhibition, and sterile water (pH 7.4) as a negative control for inhibition. The tubes were then incubated for 3 hours at 37°C and shaken at 200 rpm. Next, 100 µL of each tube and 100 µL of LB broth (BHI broth for *L. monocytogenes*) was plated in wells of a 96-well microplate. The microplate was incubated in the EON microplate reader overnight at 206 oscillations/min and at 37°C. During this period, the EON microplate spectrophotometer and Gen5 data analysis software (BioTek, Winooski, VT, USA) were used to monitor the growth of bacteria by measuring the optical density at 600 nm every 30 minutes for 18 hours. The lowest protein concentration without visible bacterial growth was designated as the minimum inhibitory concentration (MIC). For each bacterial species tested, three independent trials, each performed in triplicate was completed (n=3).

2.2.3 Minimum bactericidal concentration

Bacteria (10 µL) from the wells at MIC, MIC x 2 and MIC x 3 concentrations of H5 were plated on LB agar (BHI agar for *L. monocytogenes*) and incubated at 37°C for 18 hours. The lowest concentration of histone H5 without a single colony was designated as the minimum bactericidal concentration (MBC).

2.3 Scanning electron microscopy (SEM)

The same protocols as described in sections 2.2.1 and 2.2.2 (preparation of the bacterial suspension and the broth microdilution assay) were followed to prepare the histone H5-treated bacteria and the untreated control cells for SEM. After the 3-hour incubation of the Eppendorf tubes containing a 1:1 (v/v) ratio of planktonic bacteria and histone H5 (or 1:1 (v/v) ratio of planktonic bacteria and sterile H_2O for untreated control cells), 100 µL of this solution was added directly to an Isopore™ polycarbonate membrane filter (0.2 µm pore size, EMD Millipore Co Cork, IRL) which was placed in a Swinny Stainless Steel 13 mm Filter Holder (EMD Millipore, MA, USA) connected to vacuum suction. The filter was then removed and placed onto a Kimwipe™ (Kimberly-Clark™ Professional Kimtech Science™) in a glass cell culture dish. The filters were fixed with 5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.5 (VWR, Radnor, PA, USA) overnight at 4 °C. The fixative was removed and the samples were dehydrated using sequential ethanol washes of 20, 40, 60, 80, 90, 95, and twice in 100% for 10 min each. Filters were then treated with 1:2 - hexamethyldisilizane (HMDS):100% ethanol, 2:1 - HMDS:100% ethanol and 100% HMDS (twice) for 10 min each (Sigma Aldrich, Oakville, ON, Canada). Filters were air dried overnight in the fume hood, sputter coated

with gold to ~10-15 nm thickness and viewed using a Tescan Vega-II XMU VPSEM instrument at the Carleton University Nano Imaging Facility (Ottawa, Ontario, Canada). For each bacterial species tested, three independent trials, each performed in duplicate was completed (n=3).

2.4 Determination of minimum biofilm eradication concentration (MBEC) of purified histone H5 versus bacterial biofilms

The preparation of the bacterial suspension was the same as described in section 2.2.1 (determination of the MIC and MBC), except that the bacterial suspension was adjusted to 10^5 CFUs/ml in LB broth (BHI broth for *L. monocytogenes*), not PBS. Wells of a MBEC™ Assay's Biofilm Inoculator (Innovotech, Edmonton, AB), which contains pegs on the lid that serve as a surface for the biofilm to form, was inoculated with 150 µL of bacterial inoculum. The plate was incubated for 24 hours at 37°C and shaken at 110 rpm for *Pseudomonas aeruginosa* bacterial biofilms or incubated for 72 hours at 22°C and shaken at 60 rpm for *Listeria monocytogenes* bacterial biofilms. Following incubation, planktonic cells were rinsed away by immersing the 96-peg lid in a sterile 96-well microplate with 200 µL of sterile PBS per well. Biofilms were then challenged with 200 µL of histone H5 (dissolved in sterile water, pH 7.4), and serially two-fold diluted in a sterile 96-well microplate. Gentamicin and kanamycin served as positive controls for *P. aeruginosa* and *L. monocytogenes*, respectively, whereas sterile water (pH 7.4) served as a negative control. The device was incubated for 2 hours at 37°C, 110 rpm for *P. aeruginosa* and at 22°C, 60 rpm for *L. monocytogenes*. The 96-peg lid was then rinsed twice as described above and placed in a sterile 96-well microplate with 200 µL of LB broth per well (BHI broth for *L. monocytogenes*). The

microplate was sonicated for 10 minutes (*P. aeruginosa*) or 30 minutes (*L. monocytogenes*) in order to dislodge the biofilms from the pegs, after which the peg-lid is replaced with a sterile, normal lid. This microplate was incubated in the EON microplate reader overnight at 206 oscillations/min and at 37°C. The EON microplate spectrophotometer and Gen5 data analysis software (BioTek, Winooski, VT, USA) were used to monitor the growth of bacteria by measuring the optical density at 600 nm every 30 minutes for 24 hours (*P. aeruginosa*) or 48 hours (*L. monocytogenes*). The lowest histone H5 concentration without visible bacterial growth was designated as the minimum biofilm eradication concentration (MBEC). For each bacterial species tested, three independent trials, each performed in triplicate was completed (n=3). Additionally, a standard curve for the number of viable bacteria in the inoculum versus bacterial growth lag time was generated, since every microplate contained serially ten-fold diluted bacteria obtained from the uninhibited biofilm control (sterile H_2O). Therefore, bacterial growth inhibition for each histone H5 concentration tested was determined from this standard curve.

2.5 Hemolytic assay

This assay is based on a previously described protocol (Kim et al. 2011). The potential for hemolytic activity was assessed using rat red blood cells (RBCs) which were obtained from the University of Ottawa Animal Care Facility and in accordance with the University of Ottawa Animal Care Committee guidelines. 1 mL of rat blood was centrifuged at 1,000 x g for 10 minutes to pellet the RBCs. The serum was removed and the RBCs were washed with PBS, pH 7.4, and centrifuged at 1,000 x g for 10 minutes at room temperature (this was repeated three times). The RBCs were

resuspended in PBS to a final volume of 1 ml and then diluted 1:10 in PBS. The diluted RBCs were incubated in a 1:1 (v/v) ratio with different concentrations of histone H5 and incubated at 37°C with shaking at 200 rpm for 1 hour. Controls include PBS and PBS, 0.05% Triton-X, representing no hemolysis and 100% hemolysis, respectively. After the 1-hour incubation, the samples were centrifuged at 1,000 x g for 5 minutes and the absorbance of the supernatant was measured at 540 nm to monitor the release of hemoglobin. A total of three independent trials, each performed in triplicate was completed (n=3). The percent hemolysis was calculated according to the following equation:

$$Hemolysis\ (\%) = \left(\frac{OD\ 540\ nm\ sample - OD\ 540\ nm\ no\ hemolysis}{OD\ 540\ nm\ 100\%\ hemolysis - OD\ 540\ nm\ no\ hemolysis}\right) \times 100$$

2.6 Identification of histone H5 sequences with potential antimicrobial activity

2.6.1 Bioinformatics analysis

The next step in the project was to identify the active domain(s) of histone H5 responsible for its antimicrobial activity. In general, antimicrobial peptides are short in length (12-50 amino acids), have an overall positive charge (net charge from +2 to +9), contain a substantial amount of hydrophobic residues (≥ 30%) and can fold into amphipathic alpha-helical structures (Stark et al. 2002; Cho et al. 2009; Fjell et al. 2011; Jindal et al. 2015). Considering these characteristics, a bioinformatics analysis was performed to identify histone H5-derived peptides with potential antimicrobial properties. The full length histone H5 protein sequence (total of 190 amino acid residues) was divided into sections of 20 amino acids (a.a) sequentially (example: section #1 would

correspond to a.a. number 1 to 20 in the original H5 sequence and section #2 corresponds to a.a. number 21 to 40, etc.). Each sequence of 20 amino acids was then entered into the Antimicrobial Peptide Database (APD: http://aps.unmc.edu/AP/main.php) in order to obtain its properties related to antimicrobial activity, such as charge, hydrophobicity, the capacity to form alpha-helices, the number of hydrophobic residues on the same surface and the most similar AMP in the database. This original database is widely accepted and utilized in research and education (Wang et al. 2016). The database includes > 2900 AMPs, and categorizes peptides according to specific criteria: (i) natural peptides with a known sequence, (ii) antimicrobial activity and (iii) less than 200 amino acids in length (Wang et al. 2016). In addition, the database also contains a frequently updated prediction interface which predicts if a sequence has the possibility to be an AMP based on parameters defined by all of the natural peptides entered in the database (Wang et al. 2016). Using these bioinformatics tools, we wanted to predict if a peptide has the potential to be antimicrobial. This analysis was also completed with sequential 20 amino acid sections starting at amino acid number 10 in the histone H5 sequence (example: section #1 would correspond to a.a. number 11 to 30 in the original H5 sequence and section #2 corresponds to a.a. number 31 to 50, etc.) and again with 30 amino acid sequences starting from a.a. number 1 and a.a. number 15 in the original histone H5 sequence. This *in silico* analysis identified six histone H5-derived peptides with predicted antimicrobial activity.

2.6.2 Synthesis of the histone H5-derived peptides

The next step was to synthesize the predicted histone H5-derived peptides. This was carried out by Dr. Ajoy Basak and his student Chunyu Lu, University of Ottawa, using the solid-phase peptide synthesis method (SPPS) (Merrifield 1963) with the MultiPep automated peptide synthesizer (Intavis Bioinformatics Instruments). A total of 5 mg of lyophilized powder for each peptide was produced, which was enough to perform a preliminary peptide screening in order to identify the most potent antimicrobial histone H5-derived peptide. Additionally, peptides with a >95% purity were synthesized by GenScript's service of custom peptide synthesis, to obtain a total of 10-14 mg of lyophilized powder.

2.7 Circular dichroism (CD) analysis

The protocol followed for circular dichroism spectrometry is based on previously described methods (Greenfield 2006). Circular dichroism (CD) measurements were performed using a Jasco-715 Spectropolarimeter (Japan Spectroscopic Co., Tokyo), in which the wavelengths scanned were 185 to 260 nm, using a scan speed of 100 nm/min, a bandwidth and data pitch of 1.0 nm, with a quartz cell (0.1 cm path length). Histone H5 and histone H5-derived peptides were dissolved in sterile ddH_2O, pH 7.4, or 30 mM SDS. The CD spectrum of sterile ddH_2O (buffer of the samples) was used as the blank. Each CD spectrum represents the average of two independent measurements, each in quintuplicate. The CD data was converted to molar ellipticity [θ], which was calculated using the following equation:

$$[\theta] = \frac{CD\ in\ mdeg}{10 \times path\ length\ in\ cm \times Cr}$$

where $Cr = number\ of\ residues \times molar\ concentration\ (mol.l^{-1})$

In order to estimate secondary structure content, averaged CD spectra for each peptide were analysed using the CDSSTR software (Johnson 1999). This program analyzes the molar ellipticity [θ] values at each wavelength and compares them with a library of known protein secondary structures (α-helix, β-sheet, turns, etc.) in order to estimate the percentages of the various secondary structural components of the tested protein samples.

2.8 Statistical analysis

The Student's T-test was used to determine the statistical significance of bacterial growth inhibition between the MIC and MBC values of histone H5 and the histone mixture and to determine significant variations in bacterial biofilm growth and planktonic bacterial growth when calculating dose-dependent logarithmic inhibition. The Student's T-test was also used to determine significant hemolytic activity of histone H5 compared to the PBS (no hemolysis) control and to compare the MIC values of the three versions of histone H5-derived peptide H5(61-90). In every case, a P value ≤ 0.05 was necessary for statistical significance.

3.0 RESULTS

3.1 Assessment of purified histone H5 by densitometry and proteomics analysis

A histone mixture (H1, H2A, H2B, H3, H4 and H5) was extracted from chicken RBC nuclei using a 0.4 N sulfuric acid treatment and TCA precipitation (**Figure 3a**). A proteomics assessment of this histone mixture has previously been completed by our laboratory. SDS-PAGE analysis revealed seven distinct bands in which the proteomics analysis revealed that the 25 kDa band is the avian-erythrocyte-specific histone H5 representing 18% ± 1 of the sample (Rose-Martel et al. 2017). Linker histones (histones H1 and H5) are selectively soluble in perchloric acid (Garcia-Ramirez et al. 1990). Therefore, separation of the linker histones from the core histones was achieved with 0.1 N perchloric acid extraction and TCA precipitation (**Figure 3b**). Histone H5 was separated and purified from the histone H1 proteins by ion exchange chromatography using a step salt gradient (**Figure 3c**). A total of five independent histone H5 preparations were completed; the purity of these histone H5 lots were assessed by densitometry and proteomics analysis (**Table 1**, **Figure S1**). Densitometry and proteomics analysis of all five lots demonstrate an average histone H5 purity of 98.6% ± 0.9 and 96.9% ± 1.8, respectively, with negligible contamination by histone H1 variants (H1.11R, H1.11L, H1.01, H1.03).

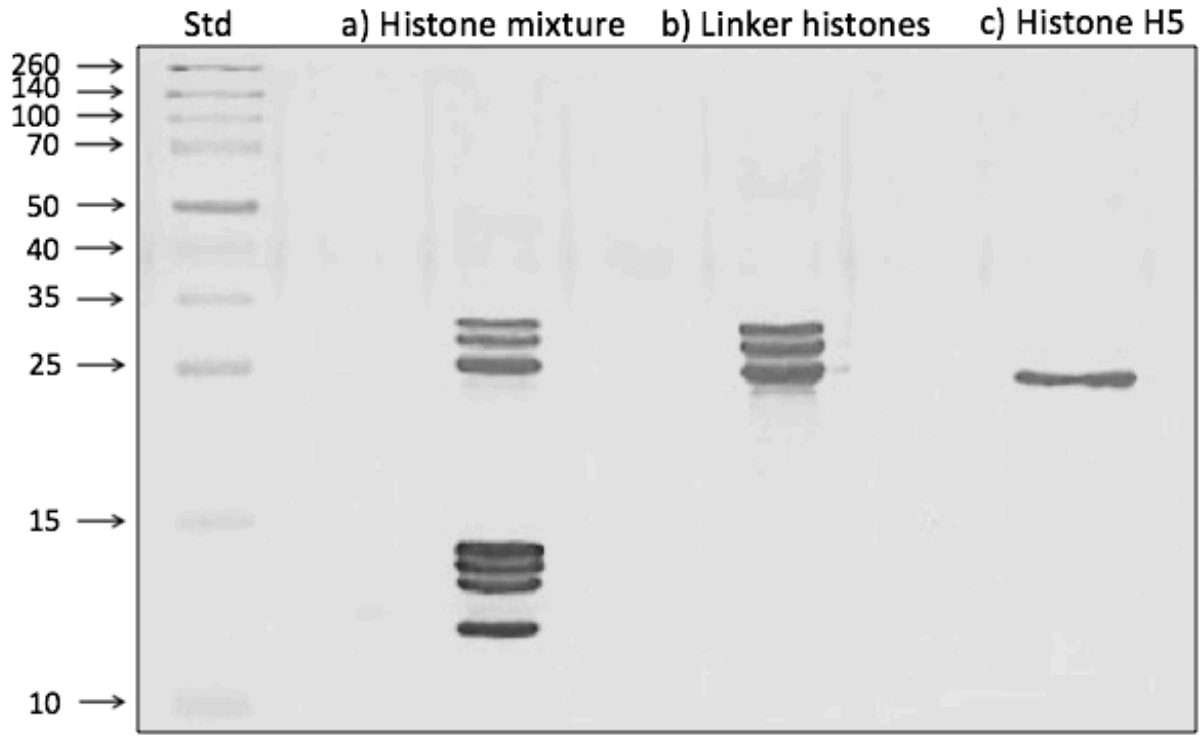


Figure 3. SDS-PAGE of the histone mixture, linker histones and histone H5 extracted from chicken erythrocytes. Protein (1 µg) extracted from chicken erythrocytes separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gel (Laemmli 1970). The first lane (a) represents the histone mixture extracted by 0.4 N sulfuric acid, the second lane (b) represents the linker histones extracted using 10% perchloric acid and the third lane (c) represents the purified histone H5 sample obtained by 10% perchloric acid extraction / ion exchange chromatography. All lanes represent the same gel. Std: molecular weight standards (kDa).

Table 1. Densitometry and proteomics analysis of histone H5. Summary of the densitometry and proteomics analyses showing the purity of the histone H5 sample lots used in this study.

Histone H5	Densitometry	Proteomics (LC/MS/MS)	Yield (µg H5/ g RBCs)
Extraction #1	98.9 %	96.0 %	0.5
Extraction #2	98.4 %	96.8 %	8.5
Extraction #3	97.0 %	95.9 %	41.1
Extraction #4	99.2 %	95.9 %	18.2
Extraction #5	99.3 %	100 %	286.0

3.2 Antimicrobial activity of histone H5 versus planktonic bacteria

Antimicrobial activity of histone H5 against a variety of Gram-positive and Gram-negative planktonic bacteria was evaluated using a broth microdilution assay. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values from each independent experiment (each in triplicate), as well as the average MIC and MBC values (mean ± SD, n=3) for every bacterial species are shown in **Table 2** and **Table 3**. The antimicrobial activity of histone H5 has been tested against six Gram-positive and four Gram-negative bacterial species. As demonstrated by **Figure 4** and **Figure 5**, bacterial growth inhibition by histone H5 was dose-dependent. The MIC values for histone H5 against Gram-positive bacteria *B. cereus*, *E. faecalis*, *L. monocytogenes*, MRSA, *S. aureus* and VRE are 3.8 ± 1.7 µg/mL, >32 µg/mL, 4 ± 0 µg/mL, 2.4 ± 0.8 µg/mL, 3.8 ± 0.4 µg/mL and 4 ± 1.2 µg/mL, respectively. *E. faecalis* was the least susceptible to histone H5 inhibition, requiring over 32 µg/mL which was the highest histone H5 concentration tested. The MIC values for all other Gram-positive pathogens were not significantly different from each other, demonstrating similar susceptibilities to histone H5, including antibiotic-resistant bacteria MRSA and VRE. The MIC values for histone H5 versus Gram-negative bacteria *E. coli* K12, *E. coli* O157:H7, *P. aeruginosa* and *S. typhimurium* are 4.9 ± 1.5 µg/mL, 4.9 ± 1.5 µg/mL, 2.9 ± 1 µg/mL and 1.9 ± 1.8 µg/mL, respectively. All of the Gram-negative bacterial pathogens tested for growth inhibition by histone H5 showed similar susceptibilities to the purified H5 protein.

Previous work from our laboratory has determined the MIC and MBC values of a histone mixture (H1, H2A, H2B, H3, H4 and H5) extracted from chicken erythrocytes

(Rose-Martel & Hincke 2014). These previously published results are summarized and compared to the MIC and MBC values of histone H5 in **Table 4**. The MIC values of the histone mixture versus Gram-positive planktonic bacteria are 6 ± 1 µg/mL, 8 ± 2 µg/mL and 700 ± 100 µg/mL for *S. aureus*, MRSA and *E. faecalis*, respectively. For the Gram-negative planktonic bacteria, the histone mixture had MIC values of 21 ± 3 µg/mL (*E. coli* (K12)), 3.6 ± 0.4 µg/mL (*S. typhimurium*) and 5 ± 1 µg/mL (*P. aeruginosa*). Comparing the MIC and MBC values, there was a trend of increased antimicrobial activity for the purified histone H5 protein compared to the histone mixture. Furthermore, histone H5 has significantly higher antimicrobial activity than the histone mixture against MRSA and *E. coli* (K12) ($P < 0.05$).

Table 2. Minimum inhibitory concentration (MIC) values of histone H5 against planktonic bacteria. MIC values of purified histone H5 extracted from chicken erythrocytes versus Gram-positive and Gram-negative planktonic bacteria determined by broth microdilution assays. Results from every independent trial each performed in triplicate are shown (n=3). The average of every replicate is shown in the last column (mean ± SD, n=3).

	MIC values (µg/mL)			
Planktonic bacteria	**Trial #1**	**Trial #2**	**Trial #3**	**Mean ± SD**
Gram-positive				
B. cereus	5.33	4	2	**3.8 ± 1.7**
E. faecalis	> 32	> 32	> 32	**> 32**
L. monocytogenes	4	4	4	**4 ± 0**
MRSA	2	3.33	2	**2.4 ± 0.8**
S. aureus	4	4	3.33	**3.8 ± 0.4**
VRE	3.33	3.33	5.33	**4 ± 1.2**
Gram-negative				
E. coli (K12)	6.67	4	4	**4.9 ± 1.5**
E. coli (O157:H7)	4	6.67	4	**4.9 ± 1.5**
P. aeruginosa	2.67	4	2	**2.9 ± 1**
S. typhimurium	4	1	0.83	**1.9 ± 1.8**

Table 3. Minimum bactericidal concentration (MBC) values of histone H5 against planktonic bacteria. MBC values of purified histone H5 extracted from chicken erythrocytes versus Gram-positive and Gram-negative planktonic bacteria determined by broth microdilution assays. Results from every independent trial each performed in triplicate are shown (n=3). The average of every replicate is shown in the last column (mean ± SD, n=3).

	MBC values (µg/mL)			
Planktonic bacteria	**Trial #1**	**Trial #2**	**Trial #3**	**Mean ± SD**
Gram-positive				
B. cereus	5.33	4	2	**3.8 ± 1.7**
E. faecalis	> 32	> 32	> 32	**> 32**
L. monocytogenes	4	5.33	4	**4.4 ± 0.8**
MRSA	2	4	2	**2.7 ± 1.2**
S. aureus	4	4	4	**4 ± 0**
VRE	3.33	3.33	6.67	**4.4 ± 1.9**
Gram-negative				
E. coli (K12)	8	8	4	**6.7 ± 2.3**
E. coli (O157:H7)	4	6.67	4	**4.9 ± 1.5**
P. aeruginosa	2.67	4	2	**2.9 ± 1**
S. typhimurium	4	1	0.83	**1.9 ± 1.8**

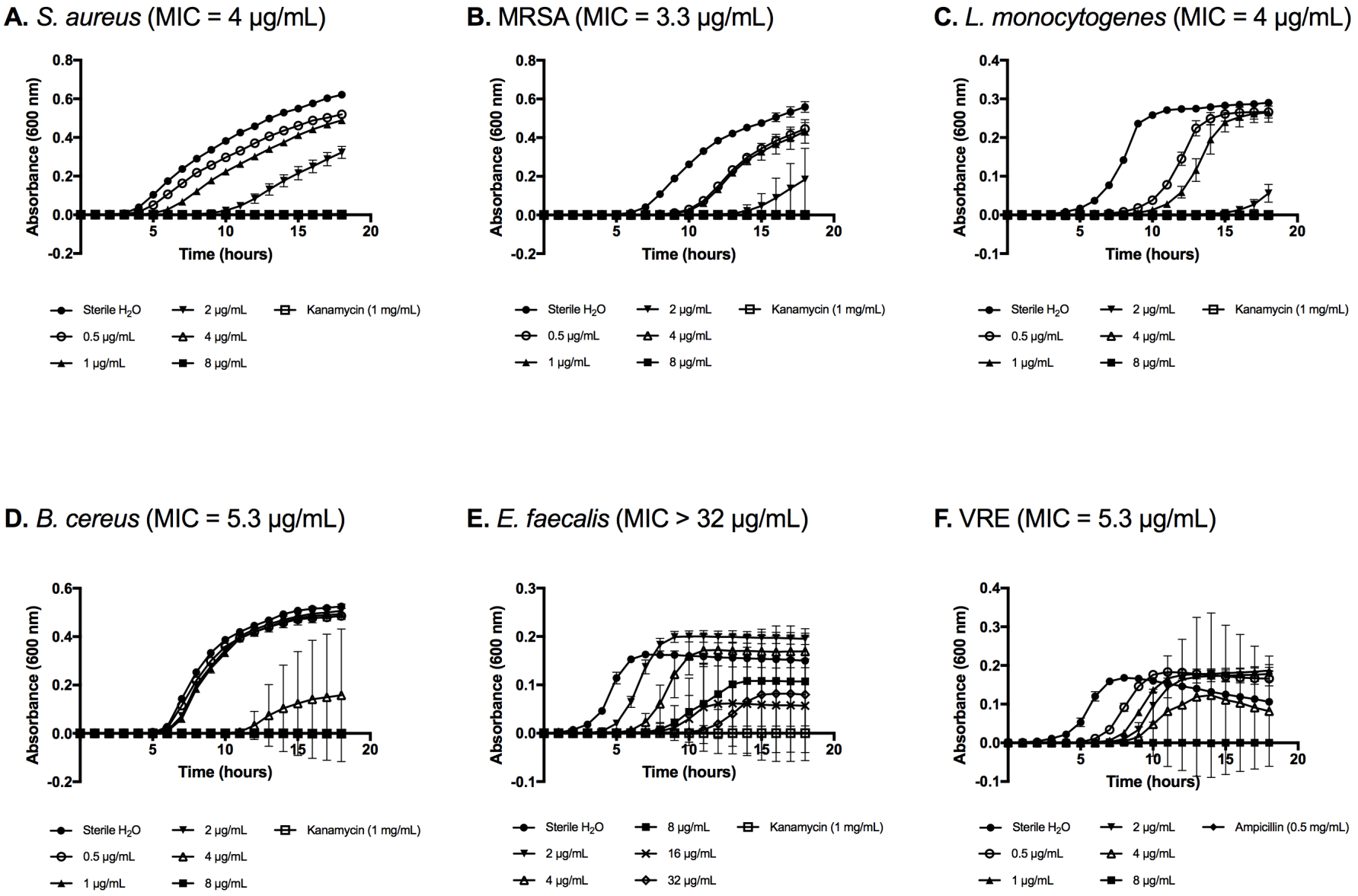


Figure 4. Dose-dependent growth inhibition of Gram-positive bacteria versus histone H5. Minimum inhibitory concentrations (MICs) of histone H5 versus *S. aureus* (A), MRSA (B), *L. monocytogenes* (C), *B. cereus* (D), *E. faecalis* (E) and VRE (F) determined by broth microdilution assays. Sterile H_2O, pH 7.4, was the negative control for inhibition. Kanamycin (1 mg/mL) or ampicillin (0.5 mg/mL) was the positive control for inhibition. Results are representative of three independent trials, each performed in triplicate (n=3).

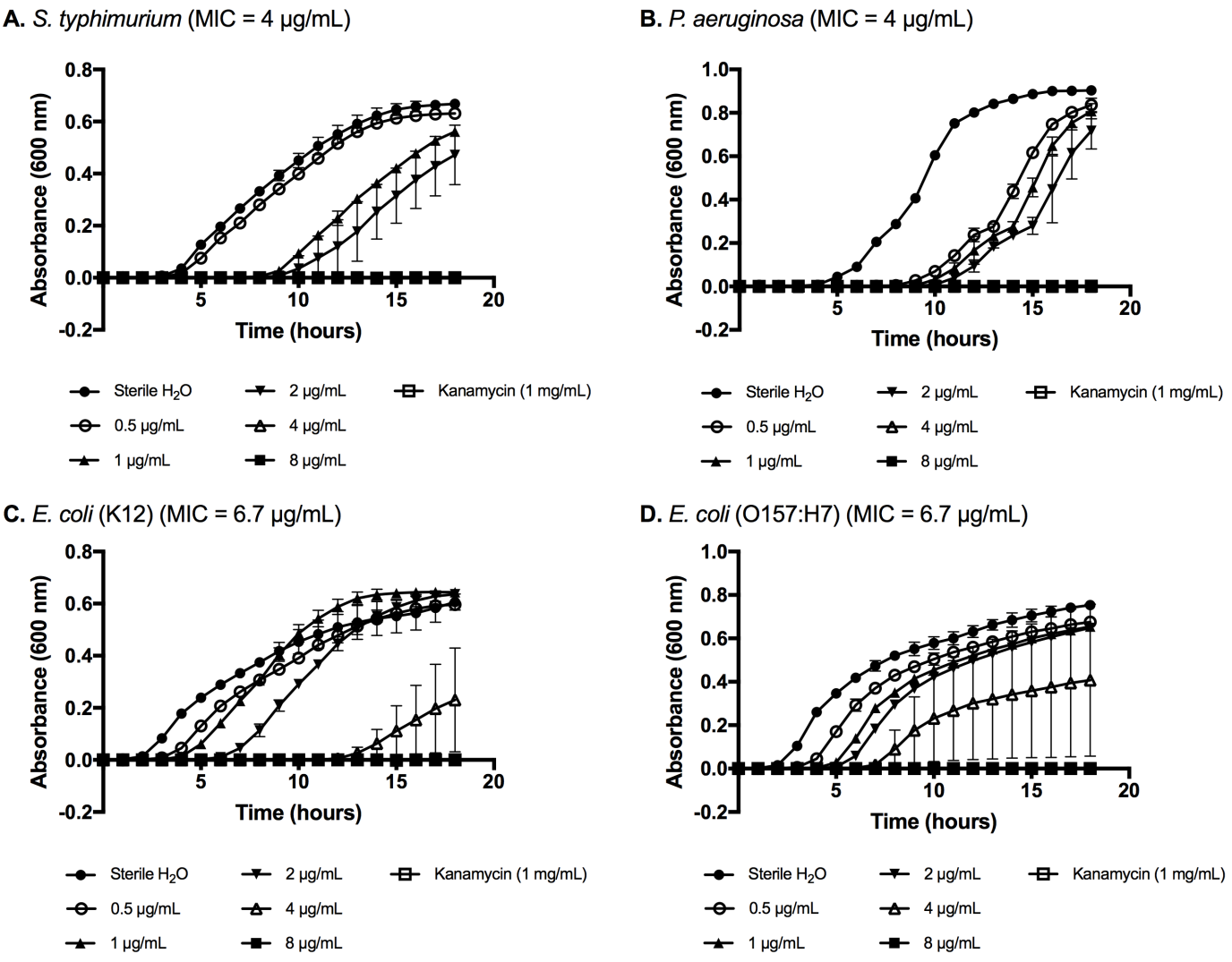


Figure 5. Dose-dependent growth inhibition of Gram-negative bacteria versus histone H5. Minimum inhibitory concentrations (MICs) of histone H5 versus *S. typhimurium* (A), *P. aeruginosa* (B), *E. coli* (K12) (C) and *E. coli* (O157:H7) (D) determined by broth microdilution assays. Sterile H_2O, pH 7.4, was the negative control for inhibition. Kanamycin (1 mg/mL) was the positive control for inhibition. Results are representative of three independent trials, each performed in triplicate (n=3).

Table 4. Summary of MIC and MBC values of histone H5 against planktonic bacteria. Minimum inhibitory concentration (MIC) values (mean ± SD, n=3) and minimum bactericidal concentration (MBC) values (mean ± SD, n=3) of purified histone H5 extracted from chicken erythrocytes versus Gram-positive and Gram-negative planktonic bacteria determined by broth microdilution assays. The MIC and MBC values (mean ± SD, n=3) of the histone mixture were determined in previous work from our laboratory (Rose-Martel, 2015). There is a statistically significant difference between histone H5's MIC values and the histone mixture's MIC values against MRSA and *E. coli* K12 (Student's T-test, P value < 0.05).

	Histone mixture		Histone H5	
Planktonic bacteria	**MIC (µg/mL)**	**MBC (µg/mL)**	**MIC (µg/mL)**	**MBC (µg/mL)**
Gram-positive				
B. cereus	-	-	3.8 ± 1.7	3.8 ± 1.7
E. faecalis	700 ± 100	1100 ± 200	> 32	> 32
L. monocytogenes	-	-	4 ± 0	4.4 ± 0.8
MRSA	8 ± 2	8 ± 2	2.4 ± 0.8 *	2.7 ± 1.2
S. aureus	6 ± 1	6 ± 1	3.8 ± 0.4	4 ± 0
VRE	-	-	4 ± 1.2	4.4 ± 1.9
Gram-negative				
E. coli (K12)	21 ± 3	21 ± 3	4.9 ± 1.5 *	6.7 ± 2.3
E. coli (O157:H7)	-	-	4.9 ± 1.5	4.9 ± 1.5
P. aeruginosa	5 ± 1	5 ± 1	2.9 ± 1	2.9 ± 1
S. typhimurium	3.6 ± 0.4	5 ± 1	1.9 ± 1.8	1.9 ± 1.8

3.3 Scanning electron microscopy (SEM)

In order to study the effect of histone H5 on bacterial membranes, Gram-positive and Gram-negative planktonic bacteria incubated with histone H5 at their corresponding MIC values were visualized by scanning electron microscopy (SEM). Representative SEM images of *Listeria monocytogenes* and *Pseudomonas aeruginosa* untreated (H_2O control) and histone H5-treated bacterial cells are shown in **Figure 6** and **Figure 7**. Untreated *L. monocytogenes* (**Figure 6b**) and *P. aeruginosa* (**Figure 7b**) bacteria showed normal and smooth surface structures, whereas clear morphological differences were observed after the histone H5 treatment in both bacterial species. Multiple indentations in the bacterial membrane, membrane wrinkling and pronounced deformations were visualized in the *L. monocytogenes* cells after being exposed to 4 µg/mL of histone H5 (MIC value = 4 µg/mL) (**Figure 6c – 6d**). Similarly, *P. aeruginosa* bacterial cells subjected to 4 µg/mL of histone H5 treatment (MIC value = 2.9 ± 1 µg/mL) showed indentations in the bacterial membrane, small pore formation (**Figure 7c**), and fibrous material possibly originating from bacterial content leakage (**Figure 7d**). Similar observations were made in each of three independent trials, each performed in duplicate. All of the observations made within the Gram-positive and Gram-negative bacterial species treated with the purified histone H5 protein are indicative of a membrane dependent mechanism of bactericidal activity.

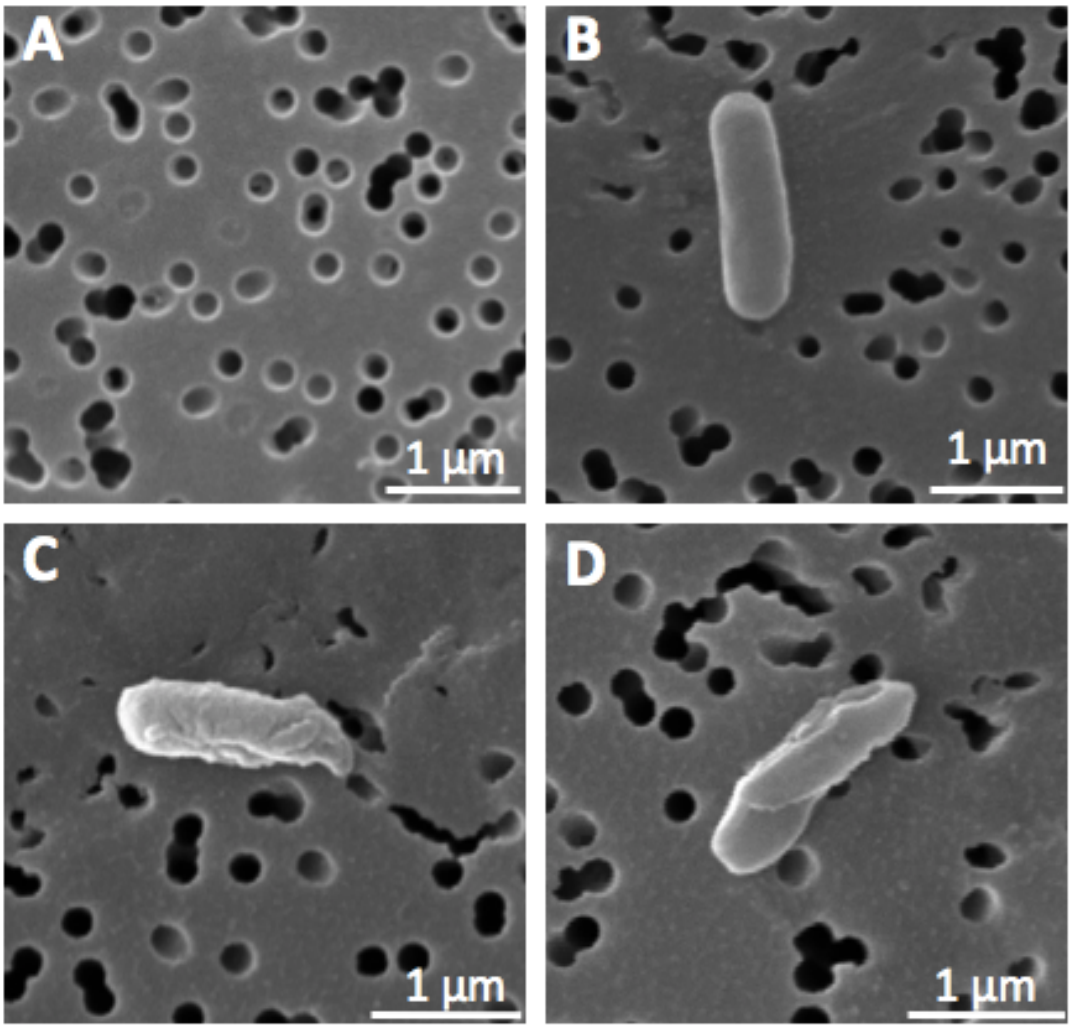


Figure 6. Scanning electron microscopy (SEM) of histone H5-treated *Listeria monocytogenes* planktonic cells. (A) untreated filter; (B) bacteria treated with H_2O (untreated control cells); (C) and (D) treatment with 4 µg/mL of histone H5 (corresponding to the MIC value) showing multiple indentations in the cellular membrane. Results are representative of three independent trials (n=3). All at 30,000 X magnification.

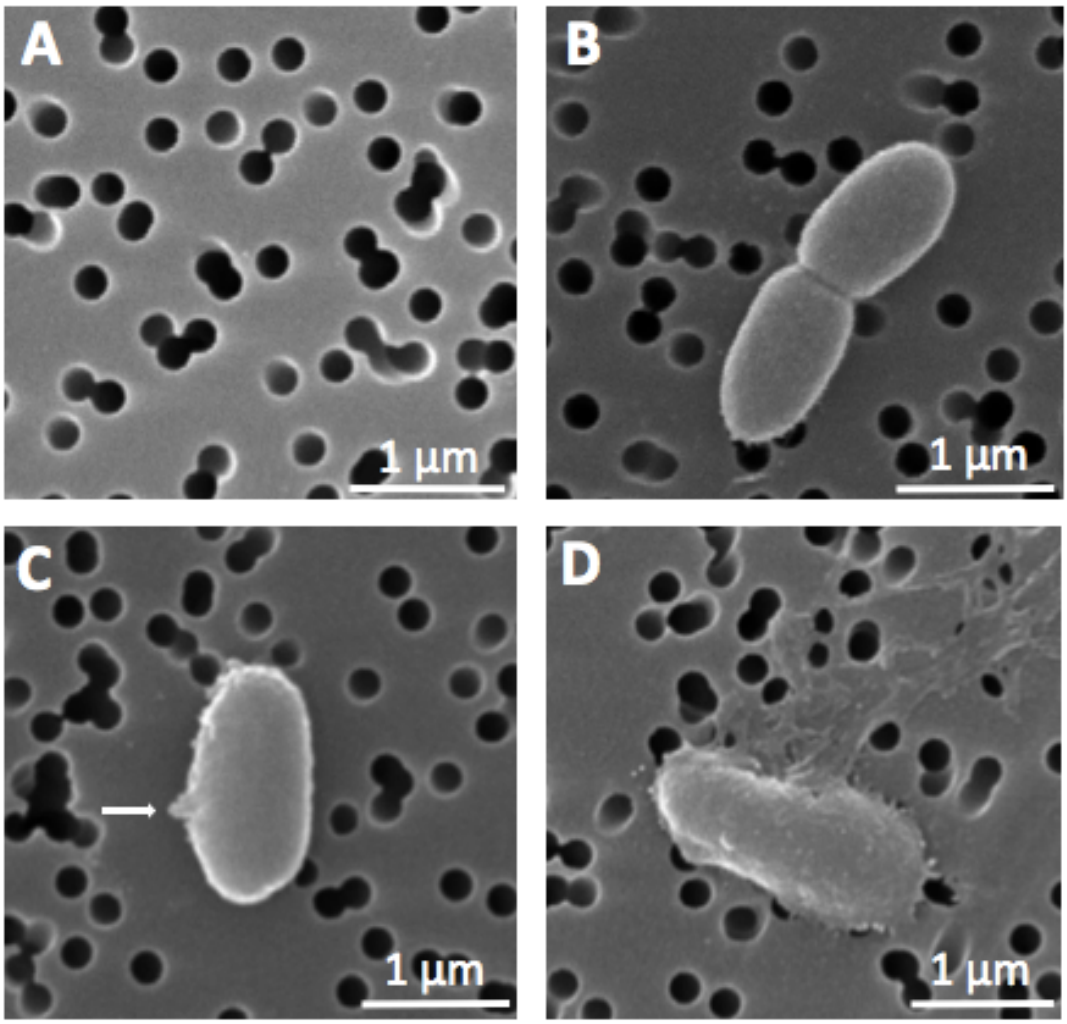


Figure 7. Scanning electron microscopy (SEM) of histone H5-treated *Pseudomonas aeruginosa* planktonic cells. (A) untreated filter; (B) bacteria treated with H_2O (untreated control cells); (C) and (D) treatment with 4 µg/mL of histone H5 (MIC value = 2.9 ± 1 µg/mL) showing multiple indentations in the cellular membrane as well as pore formation (indicated by the white arrow (C)). Results are representative of three independent trials (n=3). All at 30,000 X magnification.

3.4 MBEC assay – Evaluating histone H5 anti-biofilm activity

The anti-biofilm activity of histone H5 against *P. aeruginosa* and *L. monocytogenes* biofilms was assessed using the MBEC Assay's Biofilm Inoculator (formerly the Calgary Biofilm Device (CBD)), a plastic lid with 96 pegs and a corresponding 96-well microplate. Between 10^6 – 10^7 CFUs/peg was obtained for *P. aeruginosa* after a 24-hour incubation in the Biofilm Inoculator, while 10^5 CFUs/peg was obtained for *L. monocytogenes* following a 72-hour incubation. The minimum biofilm eradication concentration (MBEC) values from each independent experiment (each in triplicate), as well as the average MBEC values (mean ± SD, n=3) for the two bacterial species tested, are shown in **Table 5**. The MBEC value for the purified H5 protein against *L. monocytogenes* is 19.1 ± 13.1 µg/mL, while *P. aeruginosa* has an unknown MBEC value >128 µg/mL. Anti-biofilm activity against *P. aeruginosa* was assessed by monitoring the increase in lag time for bacterial growth at each histone H5 concentration tested. The increase in lag time can be correlated with the initial concentration of viable bacteria by using standard bacterial growth curves generated by serially ten-fold diluted bacterial cultures obtained from the uninhibited biofilm control sample. Although histone H5 was unable to completely eradicate *P. aeruginosa* biofilms at a concentration of ≤128 µg/mL, a dose-dependent response to the increasing concentrations of histone H5 can be observed (**Figure 8b** and **Figure 9**). Furthermore, a significant growth inhibition was detected at 32 µg/mL (0.7 ± 0.2 log inhibition), 64 µg/mL (1.6 ± 0.5 log inhibition) and 128 µg/mL (2.3 ± 0.9 log inhibition) of histone H5 treatment compared to the untreated control cells (0 µg/mL of histone H5) (**Figure 9**, P value < 0.04). Previous work from our laboratory evaluated the anti-biofilm activity of a histone mixture (H1,

H2A, H2B, H3, H4 and H5) against *P. aeruginosa* (Rose-Martel 2015). It was determined that >512 µg/mL of the mixture would be required to completely eradicate the bacterial biofilms, and interestingly, none of the histone mixture concentrations tested (8 µg/mL to 512 µg/mL) demonstrated significant growth inhibition (**Figure S2**). In fact, when treated with 16, 64 and 256 µg/mL of the histone mixture, *P. aeruginosa* biofilms showed significant increase in growth compared to the uninhibited control (**Figure S2**).

Table 5. Summary of MBEC values of histone H5 against bacterial biofilms. Minimum biofilm eradication concentration (MBEC) values of purified histone H5 extracted from chicken erythrocytes versus Gram-positive and Gram-negative bacterial biofilms determined by MBEC assays. Results from every independent trial each performed in triplicate are shown (n=3). The average of every replicate is shown in the last column (mean ± SD, n=3).

	MBEC values (µg/mL)			
Bacterial biofilms	**Trial #1**	**Trial #2**	**Trial #3**	**Mean ± SD**
Gram-positive				
L. monocytogenes	26.67	26.67	4	**19.1 ± 13.1**
Gram-negative				
P. aeruginosa	> 128	> 128	> 128	**> 128**

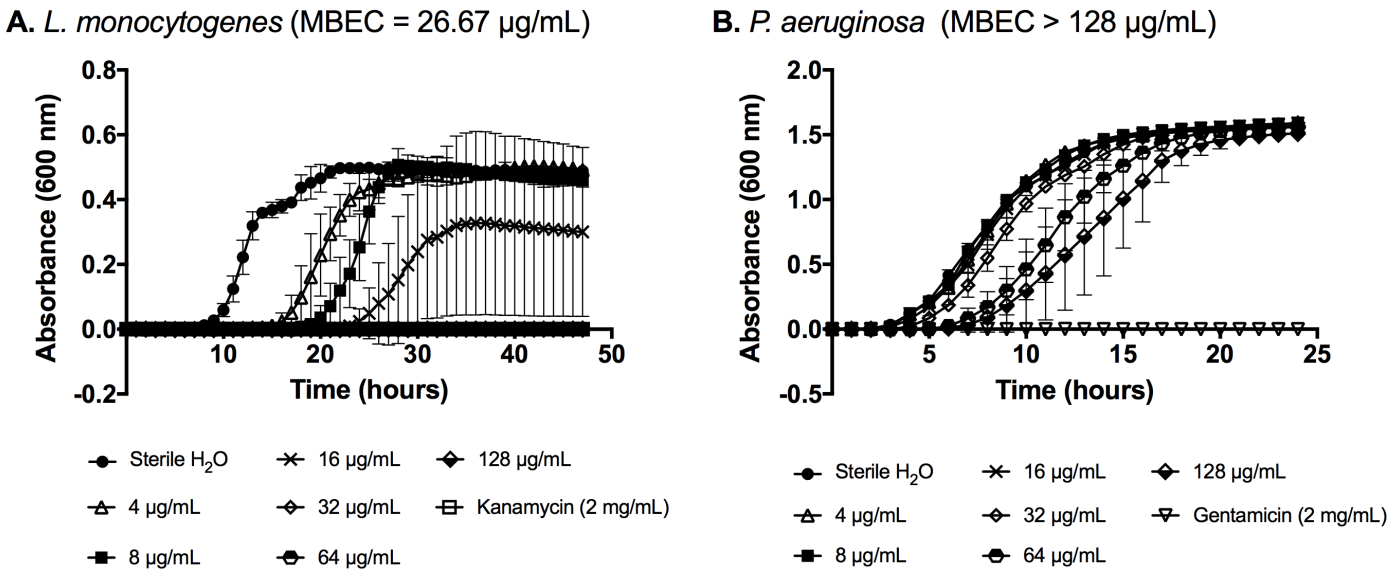


Figure 8. Dose-dependent growth inhibition of Gram-positive and Gram-negative bacterial biofilms versus histone H5. Minimum biofilm eradication concentrations (MBECs) of purified histone H5 extracted from chicken erythrocytes versus *L. monocytogenes* (A) and *P. aeruginosa* (B) determined by MBEC assays. Sterile H_2O, pH 7.4, was the negative control for inhibition. Kanamycin (2 mg/mL) or gentamicin (2 mg/mL) was the positive control for inhibition. Results are representative of three independent trials, each performed in triplicate (n=3).

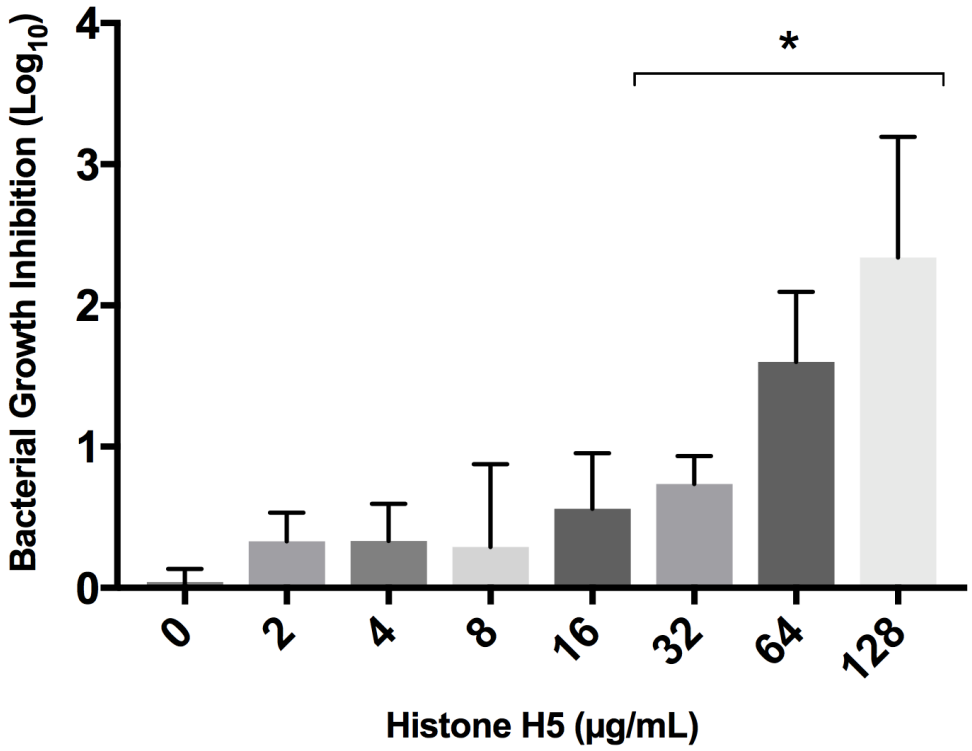


Figure 9. Dose-dependent logarithmic growth inhibition of *Pseudomonas aeruginosa* bacterial biofilms versus histone H5. Growth inhibition of *P. aeruginosa* versus concentrations of purified histone H5 extracted from chicken erythrocytes was determined by the MBEC assay. Results are a summary of three independent trials, each performed in triplicate (n=3). Statistical analysis was done by Student's T-Test, (*) indicates $P \leq 0.04$ compared with the control (0 µg/mL of histone H5).

3.5 Assessing hemolytic activity of histone H5 against mammalian erythrocytes

The potential for histone H5 toxicity towards mammalian cells was assessed using a hemolytic assay. A wide range of histone H5 concentrations were tested (from 0.005 to 1 mg/mL) against rat erythrocytes (**Table 6**). The absorbance (OD 540 nm) values (mean ± SD) were 0.59 ± 0.04 and 5.19 ± 0.38 for the PBS control and the 0.05% Triton-X control, respectively. Statistical analysis revealed that treatments with 1, 0.5, 0.025, 0.0125 and 0.005 mg/mL of histone H5 were not significantly higher than the PBS control. Therefore, even at the highest concentration tested (1 mg/mL), histone H5 is non-hemolytic.

Table 6. Hemolytic activity of histone H5 versus mammalian RBCs. A wide range of histone H5 concentrations were tested (from 0.005 - 1 mg/mL) against rat erythrocytes. Controls include PBS and 0.05% Triton-X, representing no hemolysis and 100% hemolysis, respectively. Hemolysis % values represent the mean ± SD of three independent trials, each performed in triplicate (n=3).

Concentration of histone H5 (mg/mL)	Hemolysis % [a]
1	1.97 ± 1.42 [b]
0.75	2.84 ± 1.26
0.5	2.39 ± 1.37 [b]
0.25	2.57 ± 0.63
0.125	3.18 ± 1.09
0.05	2.26 ± 0.58
0.025	1.76 ± 1.56 [b]
0.0125	0.50 ± 0.52 [b]
0.005	0.06 ± 0.44 [b]

a. Hemolysis % was calculated according to the following equation: hemolysis (%) = ((OD540 nm sample – OD540 nm no hemolysis)/(OD540 nm 100% hemolysis – OD540 nm no hemolysis)) x 100
b. No significant hemolytic activity of histone H5 was observed, compared to the PBS (no hemolysis) control. A P value ≤ 0.05 was necessary for statistical significance.

3.6 Assessing the antimicrobial properties of the histone H5-derived peptides

3.6.1 Bioinformatics analysis

A thorough bioinformatics analysis was completed based upon consideration of the general characteristics of known antimicrobial peptides. Using the results obtained from the Antimicrobial Peptide Database (APD), six histone H5-derived peptides with potential antimicrobial activity were identified (**Table 7** & **Figure 10**). These peptides have a net charge that range from +2 to +5, contain a substantial proportion of hydrophobic residues (30% to 50%), have at least 3 hydrophobic residues on the same surface of the alpha-helix structure and have a maximum similarity of 46% with other AMPs in the database. This similarity is low, considering that buforin I and hipposin, well established novel AMPs, have a 71% similarity (Wang et al. 2016) (**Figure S3**). Interestingly, all identified peptides are located between amino acid positions 15 and 100 in the histone H5 sequence (full length is 190 amino acids), potentially representing the active antimicrobial domain(s) of histone H5 (**Figure 10**).

Table 7. Characteristics of the six histone H5-derived peptides. An *in silico* analysis identified six histone H5 peptide sequences with potential antimicrobial properties. Properties such as length of the peptide and where it is located within the original H5 sequence, charge, hydrophobicity, number of hydrophobic residues on the same surface and highest similar AMP are shown in the table. All information was retrieved from the Antimicrobial Peptide Database (APD: http://aps.unmc.edu/AP/main.php).

Sequence	# of a.a. [1]	Charge	Hydrophobicity	# of hydrophobic residues on the same surface [2]	Highest similarity with AMP in database [3]
EMIAAAIRAEKSRGGSSRQS	20 (31 to 50)	+2	35%	5	35% (Pantinin-2)
EMIAAAIRAEKSRGGSSRQSIQKYIKSHYK	30 (31 to 60)	+5	30%	7	33.33% (Rugosin)
RVKASRRSASHPTYSEMIAAAIRAEKSRGG	30 (16 to 45)	+5	33%	5	35.13% (Cecropin P3)
VLKQTKGVGASGSFRLAKSD	20 (81 to 100)	+3	35%	3	40% (Caerin 4.2)
LSIRRLLAAGVLKQTKGVGA	20 (71 to 90)	+4	50%	5	46.15% (Caerin 2.1)
VGHNADLQIKLSIRRLLAAGVLKQTKGVGA	30 (61 to 90)	+4	46%	5	39.39% (Caerin 2.1)

[1] amino acid sequence length (where it is located in original H5 sequence)
[2] Each of the peptides is predicted to form an alpha-helix. The website algorithm can also predict how many hydrophobic residues are on the same surface. If there are 2 or less hydrophobic residues on the same surface, it is predicted that the peptide will not be an AMP.
[3] The website also allows us to do an alignment to find the 5 most similar peptides in the database (>2900 AMPs in the database). The highest similar AMP in the database for each peptide is shown in the column.

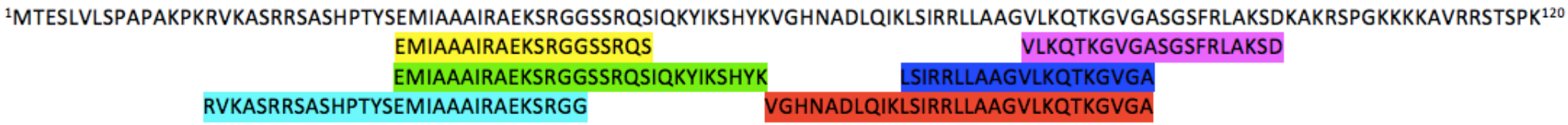


Figure 10. Alignment of the six histone H5 peptide sequences with potential antimicrobial activity with histone H5. Alignment of the six peptides with the first 120 amino acid residues of the full-length histone H5 protein (full length histone H5: 190 amino acids). All peptides are within amino acid positions 15 to 100, potentially representing the active antimicrobial domain(s) of histone H5.

3.6.2 Preliminary peptide screening for antimicrobial activity against planktonic bacteria

Approximately 5 mg of lyophilized powder (purities ranging from 50% to 81.9%) for each histone H5-derived peptide was synthesized by our colleagues, Dr. Ajoy Basak and his student Chunyu Lu at the University of Ottawa. The estimated purity of each peptide is as follows: H5(16-45) 57.7%, H5(61-90) 81.4%, H5(71-90) 80.9%, H5(81-100) 78.1%, H5(31-50) 50% and H5(31-60) 81.9%. The quantity received for each peptide was sufficient to complete a preliminary peptide screening to test their antimicrobial properties against Gram-positive (*Listeria monocytogenes*) and Gram-negative (*Pseudomonas aeruginosa*) planktonic bacteria in three independent trials, each in triplicate (up to 64 μg/mL). The MIC values of each independent trial of the broth microdilution assays are shown in **Table 8** and a summary of these results (mean ± SD, n=3) are shown in **Table 9.** The histone H5-derived peptide H5(61-90) has a MIC value of 23.1 ± 8.1 μg/mL against *Listeria monocytogenes*, while the other five peptides (H5(16-45), H5(71-90), H5(81-100), H5(31-50) and H5(31-60)) have unknown MIC values > 64 μg/mL against the Gram-positive bacterium. The H5-derived peptides were also tested against *Pseudomonas aeruginosa* and have MIC values of 16 ± 0 μg/mL (H5(61-90)), 26.7 ± 14.1 μg/mL (H5(71-90)) and > 64 μg/mL (H5(16-45), H5(81-100), H5(31-50) and H5(31-60)). The histone H5-derived peptide H5(61-90) is the only peptide which displayed antimicrobial activity against *L. monocytogenes* within the tested concentration range (1μg/mL – 64 μg/mL) and has the most potent antimicrobial activity versus *P. aeruginosa* compared to the other peptides. Furthermore, as

demonstrated by **Figure 11**, bacterial growth inhibition by the histone H5-derived peptide H5(61-90) is dose-dependent.

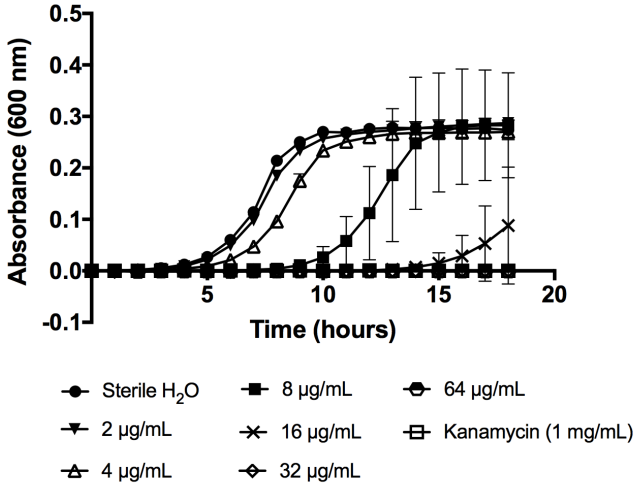
Table 8. Preliminary peptide screening - minimum inhibitory concentration (MIC) values. MIC values of histone H5-derived peptides versus *Listeria monocytogenes* and *Pseudomonas aeruginosa* determined by broth microdilution assays. Every independent trial was performed in triplicate (n=3).

Planktonic bacteria	Peptide H5 (16-45)	Peptide H5 (61-90)	Peptide H5 (71-90)	Peptide H5 (81-100)	Peptide H5 (31-50)	Peptide H5 (31-60)
Gram-positive						
Listeria monocytogenes	> 64 µg/mL	32 µg/mL	> 64 µg/mL	> 64 µg/mL	> 64 µg/mL	> 64 µg/mL
	> 64 µg/mL	21.3 µg/mL	> 64 µg/mL	> 64 µg/mL	> 64 µg/mL	> 64 µg/mL
	> 64 µg/mL	16 µg/mL	64 µg/mL	> 64 µg/mL	> 64 µg/mL	> 64 µg/mL
Gram-negative						
Pseudomonas aeruginosa	> 64 µg/mL	16 µg/mL	21.3 µg/mL	> 64 µg/mL	> 64 µg/mL	> 64 µg/mL
	> 64 µg/mL	16 µg/mL	16 µg/mL	> 64 µg/mL	> 64 µg/mL	> 64 µg/mL
	> 64 µg/mL	16 µg/mL	42.7 µg/mL	> 64 µg/mL	> 64 µg/mL	> 64 µg/mL

Table 9. Summary of MIC values of histone H5-derived peptides against planktonic bacteria. Minimum inhibitory concentration (MIC) values (mean ± SD, n=3) of the histone H5-derived peptides versus Gram-positive (*L. monocytogenes*) and Gram-negative (*P. aeruginosa*) planktonic bacteria determined by broth microdilution assays.

	MIC values (µg/mL)					
Planktonic bacteria	**Peptide H5 (16-45)**	**Peptide H5 (61-90)**	**Peptide H5 (71-90)**	**Peptide H5 (81-100)**	**Peptide H5 (31-50)**	**Peptide H5 (31-60)**
Gram-positive						
Listeria monocytogenes	> 64	23.1 ± 8.1	> 64	> 64	> 64	> 64
Gram-negative						
Pseudomonas aeruginosa	> 64	16 ± 0	26.7 ± 14.1	> 64	> 64	> 64

A. *L. monocytogenes* (MIC = 32 µg/mL)



B. *P. aeruginosa* (MIC = 16 µg/mL)

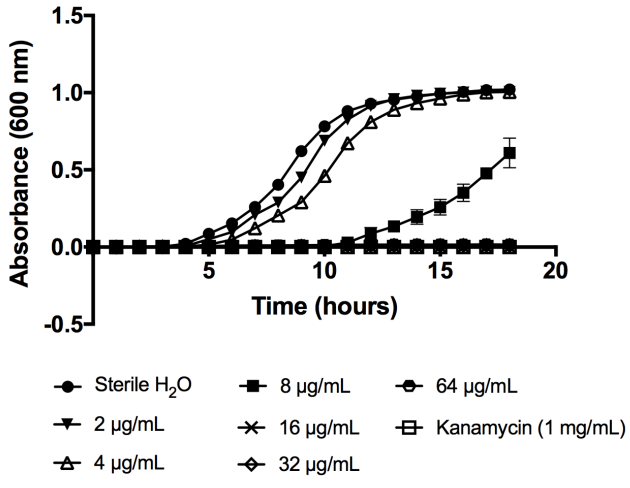


Figure 11. Dose-dependent growth inhibition of Gram-positive and Gram-negative planktonic bacteria versus histone H5-derived peptide H5(61-90). Minimum inhibitory concentrations (MICs) of peptide H5(61-90) versus *L. monocytogenes* (A) and *P. aeruginosa* (B) determined by broth microdilution assays. Sterile H_2O, pH 7.4, was the negative control, and kanamycin (1 mg/mL) was the positive control for inhibition. Results are representative of three independent trials, each performed in triplicate (n=3).

3.6.3 Antimicrobial properties of purified (>95%) histone H5-derived peptide H5(61-90)

Following the preliminary peptide screening of the six histone H5-derived peptides with potential antimicrobial activity, it was determined that peptide H5(61-90) had the most potent antimicrobial activity compared to the other peptides. Accordingly, two lots of peptide H5(61-90) were synthesized at a purity exceeding 95%: with guaranteed TFA removal (replaced with hydrochloric salt) and without TFA removal. Both versions were synthesized since it has been reported that TFA can affect the secondary structure of peptides. For example, TFA modified the structural folding of pediocin, a 44-amino acid bacteriocin, whereas HCl did not affect its conformation (Gaussier et al. 2002). The mass spectra as well as the HPLC chromatograms for each version of peptide H5(61-90) are shown in **Figure S4** – **Figure S9**. The antimicrobial activity of these peptides against Gram-positive and Gram-negative planktonic bacteria was evaluated using broth microdilution assays. The average MIC values (mean ± SD, n=3) are shown in **Table 10**. The MIC values for histone H5-derived peptide H5(61-90) V2 against *L. monocytogenes* and *P. aeruginosa* were >128 µg/mL and 33.8 ± 26.3 µg/mL, respectively. The MIC values for histone H5-derived peptide H5(61-90) V3 against *L. monocytogenes* and *P. aeruginosa* were 106.7 ± 37.0 µg/mL and 48.0 ± 32.4 µg/mL, respectively. *L. monocytogenes* was less susceptible to the histone H5-derived peptide H5(61-90) V2, requiring over 128 µg/mL which was the highest concentration tested. Although H5(61-90) V2 was unable to completely inhibit *L. monocytogenes* bacterial growth, a dose-dependent response to the increasing concentrations of the peptide was observed (**Figure 12c** and **Figure 13**). Furthermore, a significant growth

inhibition was detected at 128 µg/mL (2.0 ± 0.6 log inhibition) of H5(61-90) V2 treatment compared to the untreated control cells (0 µg/mL) (**Figure 13**, P value ≤ 0.03). The MIC values for peptides H5(61-90) V1 and H5(61-90) V3 against *L. monocytogenes* are not significantly different from each other. There is also no significant difference between the MIC values of all three versions of the peptide versus *P. aeruginosa*, which demonstrates similar susceptibilities to the peptides (**Table 10** and **Figure 12**).

Table 10. Summary of the characteristics and MIC values of histone H5-derived peptides H5(61-90). The purities, salt forms and antimicrobial properties of the three versions of peptide H5(61-90) are listed in this table. TFA = trifluoroacetic acid. HCl = hydrochloric acid. Minimum inhibitory concentration (MIC) values (mean ± SD, n=3) of the peptides H5(61-90) versus Gram-positive (*L. monocytogenes*) and Gram-negative (*P. aeruginosa*) planktonic bacteria determined by broth microdilution assays.

			MIC values (µg/mL) Mean ± SD	
Histone H5-derived peptide	**Purity (%)**	**Salt Form**	**Antimicrobial activity against *L. monocytogenes***	**Antimicrobial activity against *P. aeruginosa***
H5(61-90) V1	81.4	TFA	21.3 ± 8.2	16 ± 0
H5(61-90) V2	96.5	HCl	> 128	33.8 ± 26.3
H5(61-90) V3	95.5	TFA	106.7 ± 37.0	48.0 ± 32.4

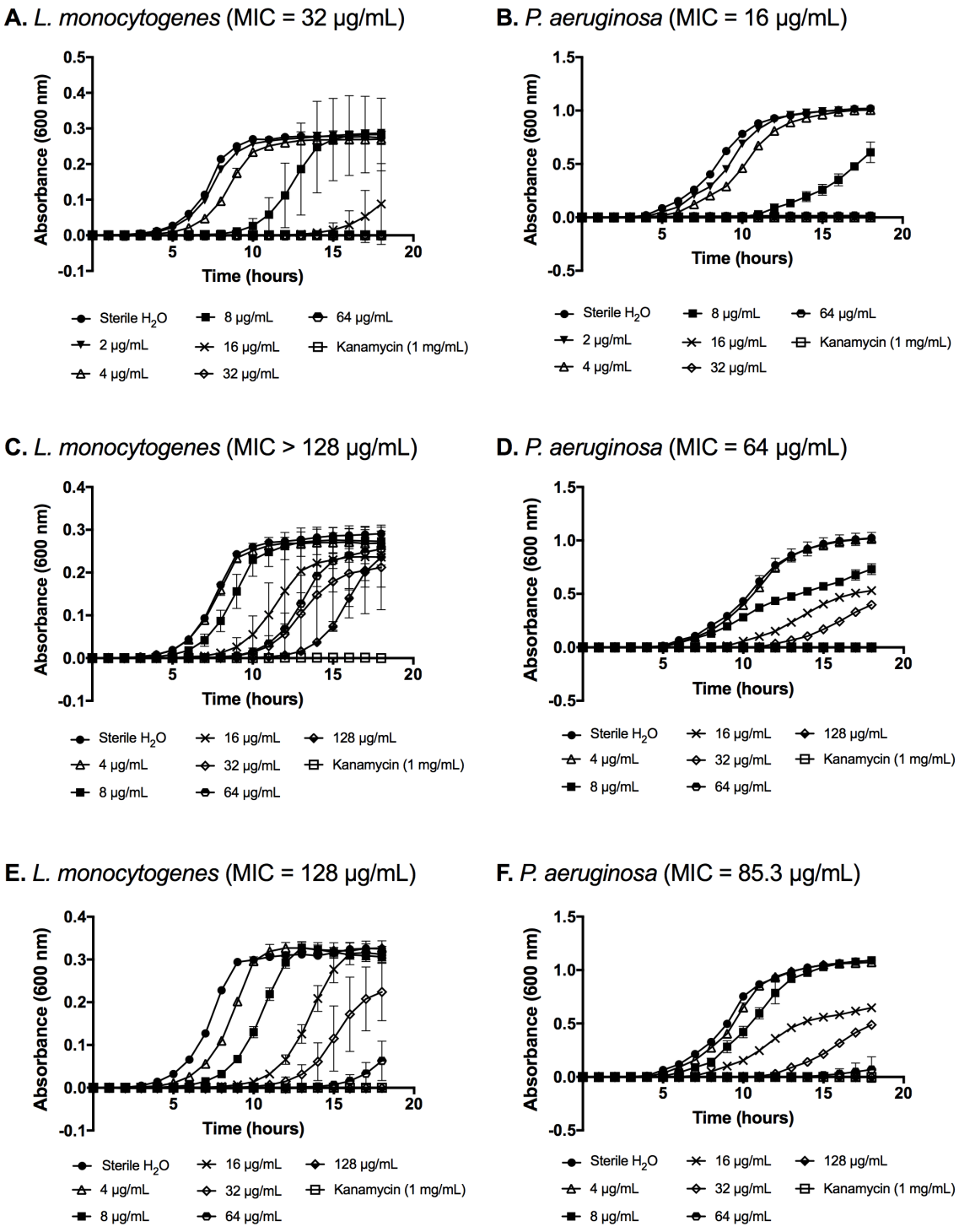


Figure 12. Dose-dependent growth inhibition of Gram-positive and Gram-negative planktonic bacteria versus histone H5-derived peptides H5(61-90) V1-V3. MICs of peptide H5(61-90) V1 versus *L. monocytogenes* (A) and *P. aeruginosa* (B), of peptide H5(61-90) V2 versus *L. monocytogenes* (C) and *P. aeruginosa* (D) and of peptide H5(61-90) V3 versus *L. monocytogenes* (E) and *P. aeruginosa* (F) determined by broth microdilution assays. Sterile H_2O, pH 7.4, was the negative control for inhibition. Kanamycin (1 mg/mL) was the positive control for inhibition. Results are representative of three independent trials, each performed in triplicate (n=3).

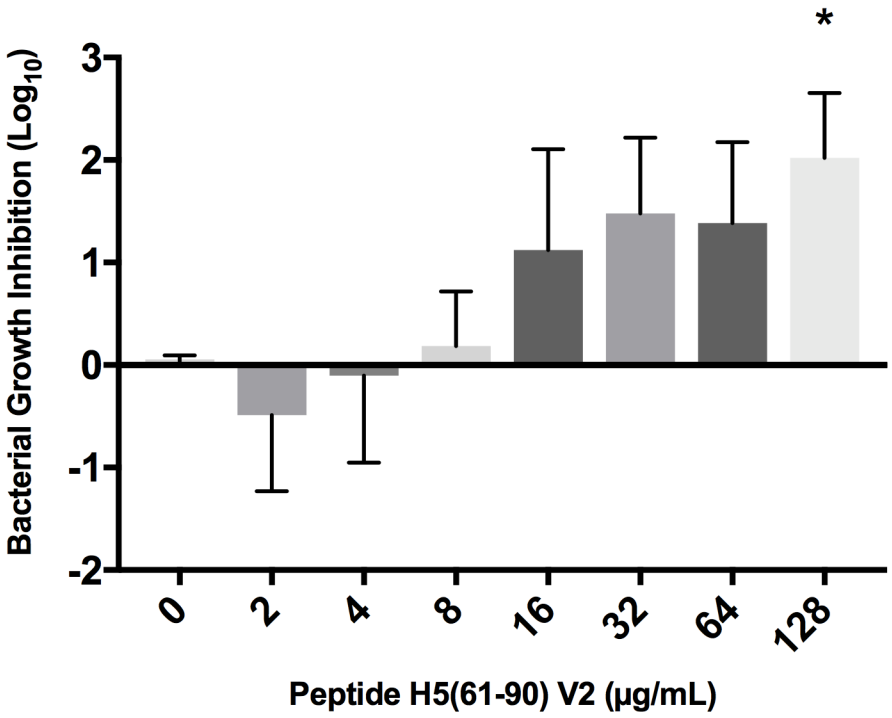


Figure 13. Dose-dependent logarithmic growth inhibition of *Listeria monocytogenes* planktonic bacteria versus concentrations of peptide H5(61-90) V2. Growth inhibition of *L. monocytogenes* by purified peptide H5(61-90) V2 was determined by broth microdilution assays. Results are a summary of three independent trials, each performed in triplicate (n=3). Statistical analysis was done by Student's T-Test, (*) indicates $P \leq 0.03$ compared with the control (0 µg/mL of peptide H5(61-90) V2).

3.7 Circular dichroism (CD) analysis of histone H5 and histone H5-derived peptides

In order to investigate if a correlation exists between the antimicrobial properties and the secondary structure of histone H5 and the histone H5-derived peptides, a circular dichroism (CD) analysis was completed. The secondary structure of histone H5 and the three preparations of histone H5-derived peptide H5(61-90) was investigated in different environments: sterile H_2O, pH 7.4 (aqueous environment) and 30 mM SDS (mimicking the negatively charged bacterial membrane environment), by circular dichroism (CD) spectroscopy. The superimposition of the CD spectrum in different environments, for each peptide, is shown in **Figure 14**. Histone H5 and the histone H5-derived peptides displayed random coil secondary structures in the aqueous environment; however, in the presence of 30 mM SDS, the spectra of the peptides displayed an α-helical conformation (evidenced by the minima at approximately 222 nm and 208 nm as well as the positive band at 193 nm). Deconvolution of the spectral data with the CDSSTR program estimated the secondary structural components (α-helix, β-sheet, turns, etc.) of each peptide (**Table 11**). The calculated α-helical component of full length histone H5 was 23.4%, while the histone H5-derived peptides had α-helical content of 47.7% (H5(61-90) V1), 41.5% (H5(61-90) V2) and 6.1%(H5(61-90) V3) in the aqueous environment. However, the α-helical proportions in the presence of 30 mM SDS were 76.9%, 84.2%, 75.5% and 84.7% for histone H5, H5(61-90) V1, H5(61-90) V2 and H5(61-90) V3, respectively.

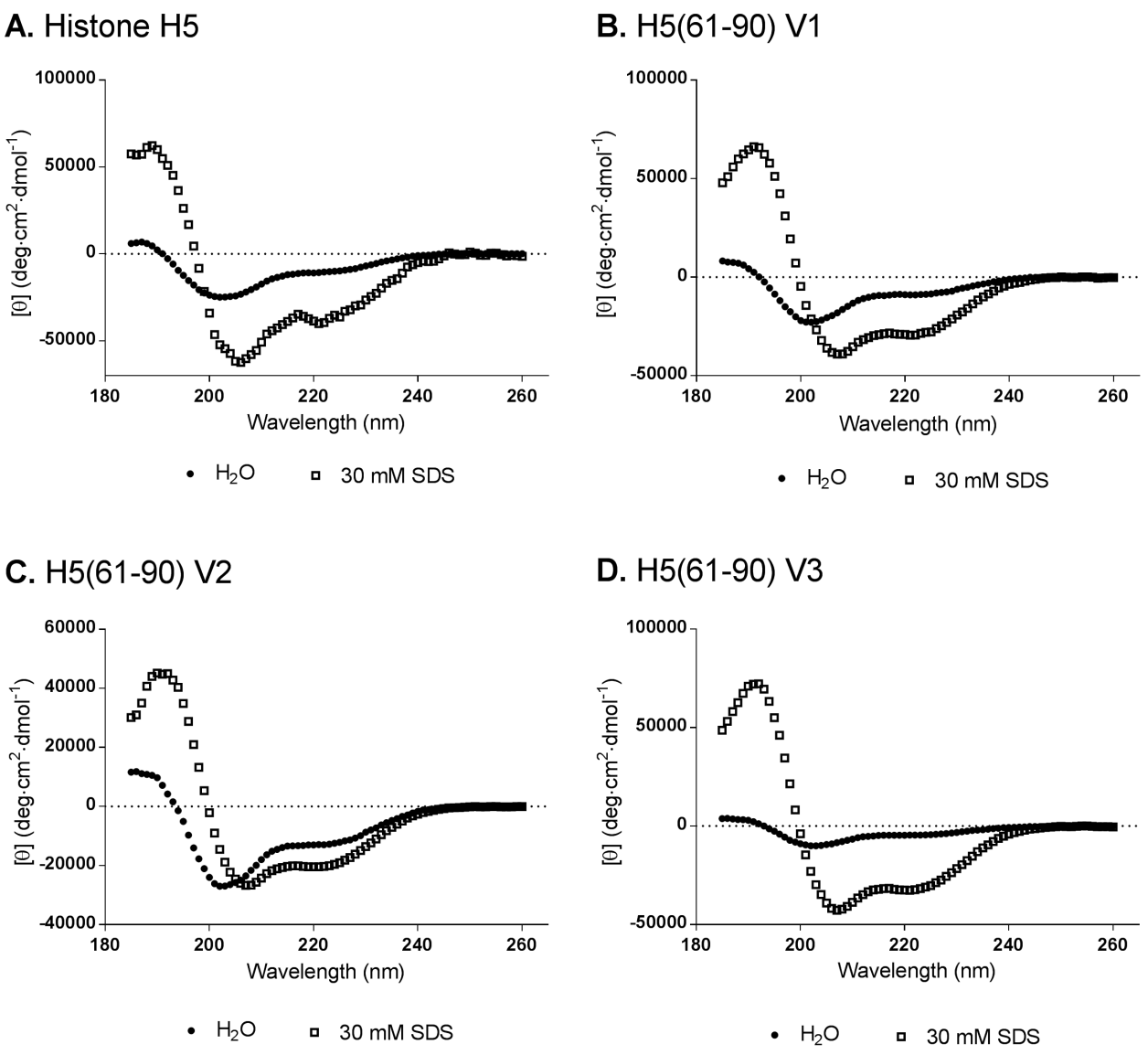


Figure 14. CD spectra of histone H5 and histone H5-derived peptides. CD spectra of histone H5 and histone H5-derived peptides dissolved in sterile ddH2O pH 7.4 (●) or 30 mM SDS (□), recorded at room temperature.

Table 11. Predicted percentages of secondary structure components of histone H5 and of histone H5-derived peptides. Deconvolution of circular dichroism data for each peptide sample was accomplished using the CDSSTR software (Johnson 1999).

	%							
	H_2O				30 mM SDS			
	Histone H5	H5(61-90) V1	H5(61-90) V2	H5(61-90) V3	Histone H5	H5(61-90) V1	H5(61-90) V2	H5(61-90) V3
α-helix	23.4	47.7	41.5	6.1	76.9	84.2	75.5	84.7
β-sheet	22.7	18.7	15.0	34.7	9.7	4.1	3.2	4.2
Turns	22.4	17.4	18.2	24.0	4.5	2.7	5.9	2.1
Random coil	32.2	16.6	25.5	34.2	8.9	8.9	14.2	8.3

4.0 DISCUSSION

The overuse and misuse of antibiotics in agriculture and medicine has led to the emergence of antibiotic-resistant bacterial pathogens. Therefore, the development of novel alternatives to treat infections is an urgent necessity. Cationic antimicrobial peptides (CAMPs) are interesting candidates as anti-infective therapeutic compounds since they show rapid bactericidal activity against a broad spectrum of microorganisms, and have been observed to induce limited bacterial resistance due to their main target being the bacterial membranes (Hancock & Sahl 2006; Mahlapuu et al. 2016). Histones are generally known as proteins that package and regulate the transcription of DNA. However, they also possess all of the essential characteristics of CAMPs necessary for antimicrobial activity (positive charge, hydrophobicity and can form α-helical amphipathic structures) (Kawasaki & Iwamuro 2008). Histone H5, an archetypal CAMP, is a nucleated-erythrocyte-specific histone that can effortlessly be extracted and purified from chicken blood, a major poultry industry waste product. In fact, Agriculture and Agri-Food Canada (AAFC) reports that 681 million chickens were slaughtered in Canada for consumption in 2016 (CFIA 2017). Considering that the blood volume range is 4.9-14.9 mL/100 g in chickens (average broiler chicken weight is 1.8 kg) (Lumeij 1987), this waste product is an ideal source of purified histones. This study documents the antimicrobial activity of histone H5 and histone H5-derived peptides, in which, to our knowledge, has not been previously investigated.

Once extracted and purified (>95%) from chicken erythrocytes, the antimicrobial activity of histone H5 was tested against six Gram-positive and four Gram-negative planktonic bacterial strains. All of the bacteria tested in this study showed similar

susceptibilities to the protein (MIC range: 1.9 ± 1.8 to 4.9 ± 1.5 µg/mL), except for *E. faecalis* which was significantly less susceptible to complete growth inhibition, requiring >32 µg/mL (**Table 2**). Human β-defensin-3, a human host defense antimicrobial peptide, has a comparable spectrum of antimicrobial activity, with MIC values of 6, 12, 13 and >512 µg/mL against *E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecalis*, respectively (Bao et al. 2012; Dhople et al. 2006). Several CAMPs, such as indolicidin, ranalexin, magainin II and LL-37 to name a few, have a broad-spectrum of antimicrobial activity against microorganisms, including Gram-positive and Gram-negative bacteria (Dürr et al. 2006; Giacometti et al. 1998). Similarly, histone H5 demonstrated potent broad-spectrum antimicrobial activity. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) are among the bacterial pathogens with equivalent sensitivities to histone H5, demonstrating that the mechanisms responsible for methicillin and vancomycin resistance differ from those by which histone H5 targets bacteria.

Our laboratory has recently demonstrated that a histone mixture (H1, H2A, H2B, H3, H4 and H5) extracted from chicken erythrocytes had antimicrobial and anti-biofilm activity against both methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* (Rose-Martel et al. 2017). In the same manuscript published in *Scientific Reports*, I demonstrated that histone H5 was equally effective against both strains of *S. aureus*. Additionally, purified histone H5 showed enhanced antimicrobial activity, by 3-4 fold, compared to the histone mixture, suggesting that histone H5 contributes significantly to the antimicrobial properties of the histone mixture in contrast to the other constituents (Rose-Martel et al. 2017).

Bactericidal activity, the capability of an agent to kill bacteria, can be recognized when the MBC value is ≤ 4 times the MIC value (Pankey & Sabath 2004). In this study, the MIC and MBC values for each bacterial strain (Gram-positive and Gram-negative) were not significantly different from each other (**Table 2** & **Table 3**; $P \leq 0.05$) indicating that the antimicrobial activity of histone H5 is bactericidal in contrast to bacteriostatic. Thus, histone H5 exerts potent broad-spectrum antimicrobial activity that ultimately leads to bacterial cell death (MBC/MIC ≤ 4). Other histones have also been shown to possess bactericidal activity, including histones extracted from calf thymus, and histones H1 and H2B extracted and purified from the chicken reproductive system (Hirsch 1958; Silphaduang et al. 2006). However, this study is the first reported assessment of the antimicrobial activity of purified histone H5.

CAMPs display bacteriolytic activity through establishing electrostatic interactions with bacterial membranes followed by membrane permeabilization and pore formation. Previous research from our laboratory has demonstrated that histones extracted from chicken erythrocytes have the ability to bind to lipopolysaccharides (LPS) and lipoteichoic acids (LTA), components of the cell wall in Gram-negative and Gram-positive bacteria, respectively (Rose-Martel & Hincke 2014). Accordingly, the effect of histone H5 on bacteria surfaces was investigated through scanning electron microscopy (SEM). *Listeria monocytogenes*, a major foodborne pathogen, and *Pseudomonas aeruginosa*, an important nosocomial and resistant pathogen, were treated with 4 μg/mL of histone H5 (*L. monocytogenes* MIC value = 4 μg/mL; *P. aeruginosa* MIC value = 2.9 ± 1 μg/mL) followed by assessment of bacterial cell morphology by SEM. After histone H5 treatment, damage of the bacterial cell surfaces, indicated by loss of cell

smoothness, pronounced deformations, pore formation and leakage of cytoplasmic content was observed (**Figure 6** & **Figure 7**). Similarly, other CAMPs including RI18, a PMAP-36-derived peptide, and sphistin, a crab histone H2A-derived peptide, also induced bacterial cell surface damage when tested against *Escherichia coli* and *Staphylococcus aureus*, respectively (Chen et al. 2015; Lyu et al. 2016). These observations are indicative of a membrane dependent mechanism of bactericidal activity.

L. monocytogenes and *P. aeruginosa* are major pathogens that cause serious illnesses through foodborne and hospital-acquired infections, respectively. Additionally, both pathogens have the ability to form biofilms, which enhances their persistence in unfavorable environments and further decreases the effectiveness of antibiotics (Davies 2003). In this study, the biofilm eradicating capabilities of histone H5 were tested against *L. monocytogenes* and *P. aeruginosa* biofilms. Histone H5 was unable to completely eradicate *P. aeruginosa* biofilms within the concentrations tested and, therefore, has an unknown MBEC value > 128 µg/mL (**Table 5**). Other CAMPs, including LL-37, CAMA, melittin, defensin and magainin II, have been tested against *P. aeruginosa* biofilms, with MBEC values of 640 µg/mL for LL-37 and CAMA (MBEC/MIC ratio of 5-80), and unknown MBEC values > 640 µg/mL for melittin, defensin and magainin II (Dosler & Karaaslan 2014). Although complete *P. aeruginosa* biofilm eradication was not obtained in this study, histone H5 induced a dose-dependent response to its increasing concentrations which significantly inhibited bacterial growth at 32, 64 and 128 µg/mL (**Figure 7**). Interestingly, previous research from our laboratory showed that the histone mixture had an unknown MBEC value > 512 µg/mL against *P.*

aeruginosa and no significant growth inhibition at any of the concentrations tested (Rose-Martel 2015). These results comply with the statement that histone H5 contributes significantly to the antimicrobial activity of the mixture, compared to the other components. Considering the MIC value of 2.9 ± 1 µg/mL against planktonic *P. aeruginosa* and the significant biofilm growth inhibition by H5 observed at concentrations as low as 32 µg/mL, we would expect complete *P. aeruginosa* biofilm eradication at histone H5 concentrations of 512 µg/mL, similar to the MBEC values of LL-37 and CAMA. This hypothesis would have to be tested in future studies that utilizes higher levels of histone H5. On the other hand, histone H5 was able to completely eradicate *L. monocytogenes* biofilms at a concentration of 19.1 ± 13.1 µg/mL, which gives an approximate MBEC/MIC ratio of 5 fold (**Table 5**). Paenibacterin, an antimicrobial lipopeptide produced by *Paenibacillus thiaminolyticus*, has also been tested against established *L. monocytogenes* biofilms. After 2 and 4 hours of treatment, paenibacterin (6.8 µg/mL) was effective in disrupting established *L. monocytogenes* biofilms developed statically at 30°C, but not effective in destroying stronger biofilms developed at 37°C (Li et al. 2017). While several CAMPs have been previously shown to inhibit *L. monocytogenes* biofilm formation, histone H5 and paenibacterin are the first antimicrobial peptides to our knowledge to possess complete *L. monocytogenes* biofilm eradication activity. Accordingly, histone H5 has the potential to be developed as an effective anti-biofilm agent aimed at minimizing *L. monocytogenes* persistence in the food industry and therefore preventing new outbreaks involving this major foodborne pathogen.

CAMPs have a non-specific mode of action and mainly target the bacterial cell wall, making the development of bacterial resistance difficult. Unfortunately, this characteristic can also lead to plasma membrane damage in mammalian cells, making many antimicrobial peptides unsuitable for clinical applications. As amphipathic structures, CAMPs have exposed hydrophobic domains that can potentially interact with RBC membrane protein constituents (cholesterol or phospholipids) leading to hemolysis (Xiong et al. 2015). Gramicidin S, piscidin I and dermaseptin S4 are examples of CAMPs that display strong hemolytic activity towards mammalian red blood cells (RBCs), which unfortunately restricts clinical use of Gramicidin S to topical applications (Jiang et al. 2014; Swierstra et al. 2016). In order to evaluate the ability of histone H5 to cause toxicity by interacting with mammalian cell membranes, the hemolytic activity of histone H5 versus rat erythrocytes was measured. In contrast to the other CAMPs mentioned above, rat RBCs incubated with increasing concentrations of histone H5 did not show significant hemolysis compared to the control, even at an H5 concentration of 1 mg/mL (**Table 6**; $P \leq 0.05$).

Due to their broad-spectrum of bactericidal activity and unique mechanism of action, CAMPs have attracted attention as potential therapeutic alternatives to antibiotics. However, a major barrier preventing the exploitation of natural peptides as therapeutics is the high cost for large scale manufacturing (Hancock & Sahl 2006). In fact, it is estimated that it costs between $50-$400 to produce one gram (estimated average daily dose) of antimicrobial peptides by solid-phase chemical synthesis, the only method that has proven commercially feasible to date (Seo et al. 2012). Therefore, an effective strategy for reducing the manufacturing cost is the use of shorter peptides

(Hancock & Sahl 2006; Lyu et al. 2016). In this study, I performed an *in silico* analysis based on the most important characteristics of antimicrobial peptides (positive charge and hydrophobicity %); six histone H5-derived peptides composed of either 20 or 30 amino acids, with potential antimicrobial activity, were identified (the full length of histone H5 is 190 a.a.). All of these peptides are located within amino acid positions 15-100, possibly representing the active antimicrobial domain(s) of histone H5 (**Figure 10**). Interestingly, amino acid positions 20-109 represents the globular domain of histone H5 which folds into a helical bundle containing three α-helices, and possesses the two DNA-binding sites which are required for nucleosome organization (Duggan & Thomas 2000; Ramakrishnan et al. 1993). Once synthesized, the antimicrobial properties of the histone H5-derived peptides were tested against *P. aeruginosa* and *L. monocytogenes,* which demonstrated that peptide H5(61-90) had the most potent antimicrobial activity (**Table 9** & **Figure 11**). Reasons for this superior antimicrobial activity could be due to the secondary structure that this peptide folds into upon contact with bacterial membranes and the high content of hydrophobic residues (46%) of this peptide, compared to the other H5-derived peptides. In fact, it has been shown that increasing the hydrophobicity of α-helical peptides to a certain degree is correlated with increased antimicrobial activity (Chen et al. 2007). Once the most potent histone H5-derived peptide was identified following the initial screen for antimicrobial activity (H5(61-90) V1), the peptide was synthesized at >95% purity with chloride (H5(61-90) V2) or trifluoroacetate (H5(61-90) V3) counterions, and tested against *P. aeruginosa* and *L. monocytogenes*. Even though *L. monocytogenes* displayed less susceptibility to H5(61-90) V2, all three versions of the peptide H5(61-90) showed similar MICs to one another

against both of the bacterial strains tested (**Table 10**). However, despite identifying histone H5-derived peptides with the most potent predicted antimicrobial activity, the full length histone H5 protein inhibited *P. aeruginosa* and *L. monocytogenes* bacterial growth at significantly lower MICs than any of these peptides ($P \leq 0.05$). For instance, histone H5 has MIC values of 0.19 ± 0 μM and 0.14 ± 0.05 μM against *L. monocytogenes* and *P. aeruginosa*, respectively; while the most potent H5-derived peptide, H5(61-90) V1, has MIC values of 7.39 ± 2.60 μM (*L. monocytogenes*) and 5.12 ± 0 μM (*P. aeruginosa*). In some cases, it has been reported that shorter peptides display more potent antimicrobial activity than their parent peptide. For example, buforin I, a 39-amino acid CAMP isolated from the stomach of the Asian toad *Bufo bufo garagrizans*, is less active against Gram-positive bacteria, Gram-negative bacteria and fungi than the 21-amino acid derived CAMP buforin II (Cho et al. 2009). In other examples, however, peptide derivatives display reduced antimicrobial activity than their parent peptides. For instance, arasin I, a 37-amino acid CAMP isolated from the spider crab, has more potent antimicrobial activity against a broad range of microorganisms compared to the arasin I-derived peptides arasin I(1-20), arasin I(1-14) and arasin I(9-23) (Paulsen et al. 2013). Correspondingly, compared to the histone H5-derived peptides tested in this study, further study with peptides of different lengths (shorter or longer) will be necessary to identify sequences with optimal antimicrobial activity.

The secondary structural components of peptides are important factors that influence their antimicrobial properties. For instance, as seen with buforin II and LL-37, an increase in α-helical content is associated with stronger antimicrobial activity (Brogden 2005). The secondary structures of histone H5 and the three versions of

histone H5-derived peptide H5(61-90) were analysed using circular dichroism (CD) spectroscopy and the majority of the peptides displayed an unordered random coil conformation when dissolved in ddH_2O (**Table 11** & **Figure 14**). This is not surprising, since many α-helical CAMPs have disordered secondary structures in aqueous solutions and fold into amphipathic α-helical conformations upon interaction with bacterial membranes (Mahlapuu et al. 2016; Yeaman & Yount 2003). For example, four cathelicidin PMAP-36-derived peptides displayed unordered conformations in 10 mM PBS (aqueous environment) and then folded into amphipathic α-helical conformations in the presence of trifluoroethanol (mimicking the hydrophobic environment of the bacterial membrane) or SDS (mimicking the negatively charged bacterial membrane) (Lyu et al. 2016). Similarly, LL-37 is converted from a random coil to an α-helix in the presence of Lipid A (Turner et al. 1998). Moreover, cecropin A changed from an unordered conformation in water to an α-helical structure upon contact with lipoteichoic acids (LTA) (North & Taitt 2015). These examples demonstrate that CAMPs have the ability to fold into amphipathic α-helical conformations in membrane-mimetic environments, which we also observed for histone H5 and the three versions of peptide H5(61-90), the histone H5-derived peptide that displayed the most potent antimicrobial activity (**Table 11** & **Figure 14**).

Therefore, with the results obtained throughout this study, it can be concluded that histone H5, extracted from chicken erythrocytes, displays amphipathic α-helical conformations in the presence of bacterial membrane environments which leads to potent broad-spectrum bacteriolytic activity (**Figure 15**).

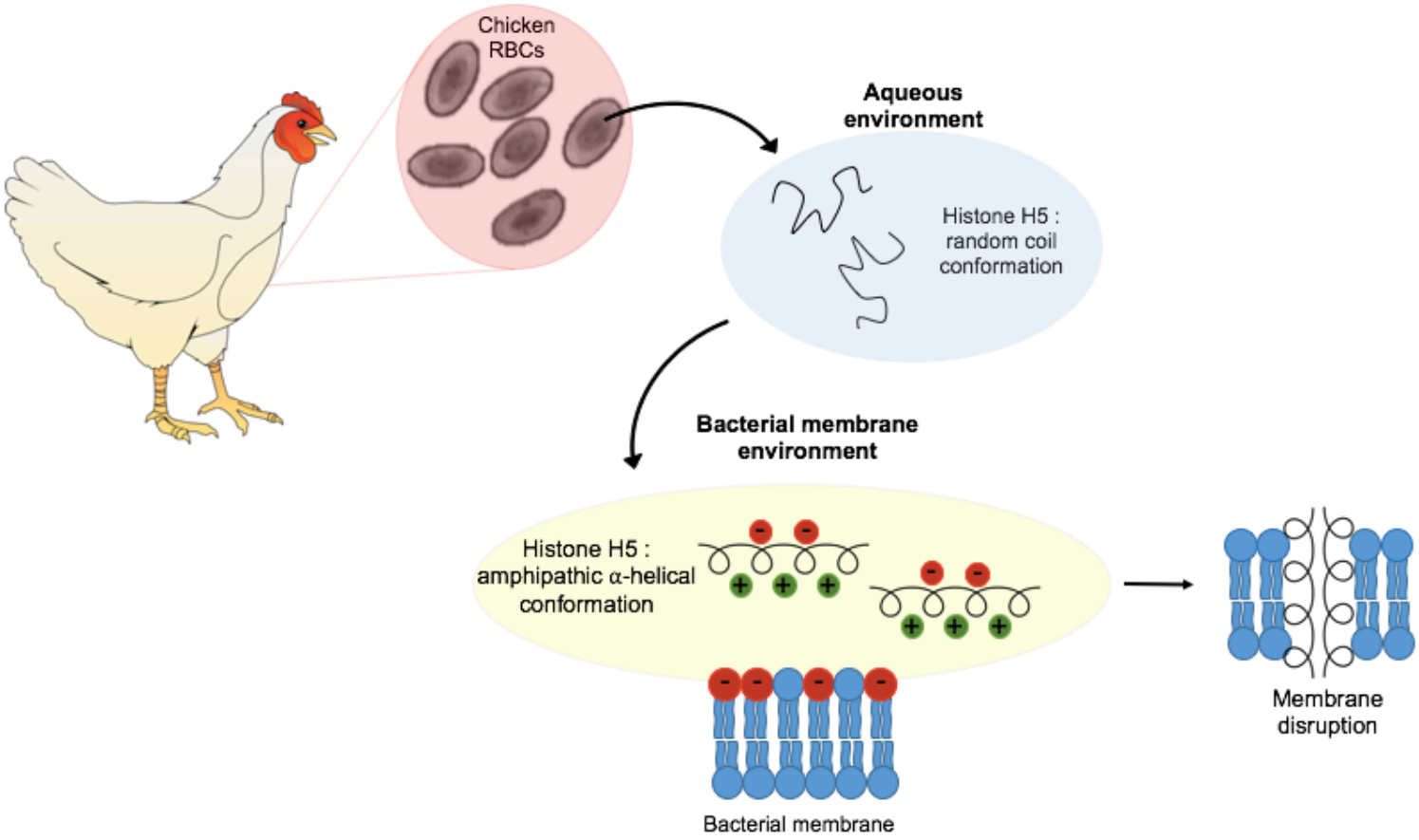


Figure 15. Summary of the proposed antimicrobial function of histone H5. Histone H5, extracted and purified from chicken erythrocytes, displays amphipathic α-helical conformations in the presence of bacterial membrane environments which leads to potent bacteriolytic activity.

5.0 FUTURE PROSPECTS & EXPERIMENTAL DIRECTIONS

Future considerations for development of this project include testing histone H5 against *P. aeruginosa* bacterial biofilms at concentrations higher than 128 µg/mL, in order to verify if complete histone H5 biofilm eradication occurs at similar MBEC values than LL-37 and CAMA (both eradicate *P. aeruginosa* biofilms at 640 µg/mL (Dosler & Karaaslan 2014)).

Although the development of CAMP bacterial resistance is difficult due to their mode of action targeting the bacterial cell wall, it does occasionally occur. Therefore, another aspect that would be important to investigate is the effect of histone H5 on the expression of genes responsible for CAMP resistance mechanisms. For example, the upregulation of the *aps* sensor/regulator system in MSSA and MRSA bacterial biofilms was previously evaluated in response to the histone mixture (Rose-Martel et al. 2017). Similar investigations with histone H5 would provide insight into the mechanisms of action leading to the antimicrobial activity of H5.

In this study, my findings demonstrate that histone H5 has potent bactericidal activity against Gram-positive and Gram-negative bacteria. However, CAMPs have also been shown to display antimicrobial activity against viruses (ex. NP-1, from rabbit neutrophils), fungi (ex. indolicidin), parasites (ex. magainin) and even cancerous cells (ex. dermaseptin-B2, from the Giant leaf frog's skin) (Bahar & Ren 2013; Wang et al. 2016). Accordingly, future directions of this project include investigating if the potent broad-spectrum antimicrobial activity of histone H5 expands to other microorganisms.

Additional future considerations of this project consist of using the potent histone H5 as a bioinspiration for the development of other novel potential antimicrobial H5-

derived peptides. In this study, 20 and 30-amino acid histone H5-derived peptides were chosen, however, an abundance of novel H5-derived peptides of different lengths could be identified within the full length 190-amino acid protein. For example, an 85-amino acid peptide covering the entire predicted antimicrobial domain of histone H5 (peptide H5(15-100)) could be synthesized and tested for antimicrobial activity.

Finally, CAMPs have considerable advantages as alternative anti-infective compounds (rapid broad-spectrum antimicrobial activity and limited emergence of bacterial resistance), however, they also possess limitations for drug development. Examples include, potential toxicity of CAMPs and proteolytic degradation. Although histone H5 was non-hemolytic towards rat RBCs, possible systematic toxicity could occur following an oral application of H5, for example. In order to overcome these obstacles, introduction of D-form amino acids, acetylation or amidation of the terminal regions, and liposome encapsulation are methods that can improve the stability and reduce potential toxicity by CAMPs (Seo et al. 2012). Consequently, in order to develop histone H5 and histone H5-derived peptides as candidates for therapeutic applications, these considerations must be investigated in future studies.

6.0 CONCLUSIONS

In this study, a successful protocol to extract and purify histone H5 from chicken erythrocytes, a major poultry industry waste product, was developed. For the first time, histone H5 has been established as a novel antimicrobial protein that is not only highly effective against Gram-positive and Gram-negative planktonic bacterial pathogens, including resistant strains such as MRSA and VRE, but also against *L. monocytogenes* and *P. aeruginosa* biofilms. While other CAMPs such as LL-37, CAMA and paenibacterin have shown anti-biofilm properties, histone H5 is the first purified histone protein, to our knowledge, to demonstrate complete biofilm eradication activity. Furthermore, the MBC/MIC ratio and the SEM results demonstrate that histone H5 targets bacterial membranes and induces significant damage leading to pronounced deformations, pore formation, leakage of cytoplasmic content, and ultimately cell death. This membrane-damaging activity, however, is limited to bacterial cells, since histone H5 was shown to be non-toxic towards mammalian erythrocytes. Considering these appealing characteristics, histone H5 is a bioinspiration for the development of novel histone H5-derived peptides. Although predicted H5-derived antimicrobial peptides were identified within the antimicrobial domain of histone H5, they did not possess more potent antimicrobial activity than the full length protein. Overall, my findings demonstrate that histone H5 is a potent, broad-spectrum antimicrobial peptide with interesting potential as a novel candidate for therapeutic applications in the clinical setting, as well as the food and agriculture industries, which is necessary as we foresee a post-antibiotic era.

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SUPPLEMENTARY DATA

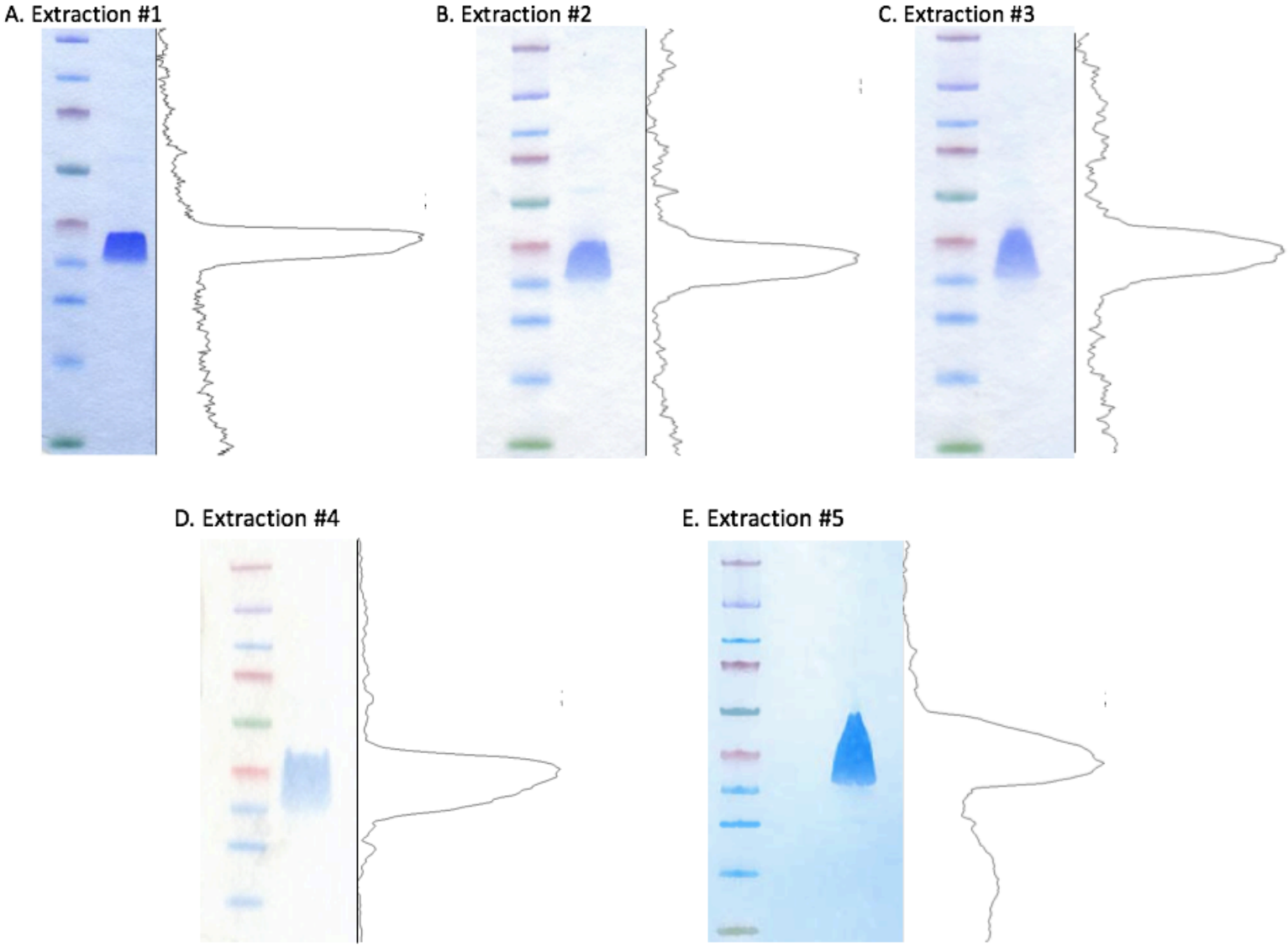


Figure S1. Densitometry analysis of histone H5 samples following extraction and purification from chicken erythrocytes. Each of the histone H5 samples extracted and purified from chicken erythrocytes (A. Extraction #1, B. Extraction #2, C. Extraction #3, D. Extraction #4 and E. Extraction #5) were were analyzed by SDS-PAGE using 4-12% NuPAGE® Bis-Tris mini gels (1.5 µg of protein). The gels were stained with Coomasie Brilliant Blue and the bands were analyzed by densitometry using ImageJ densitometry software (version 1.48, NIH, USA).

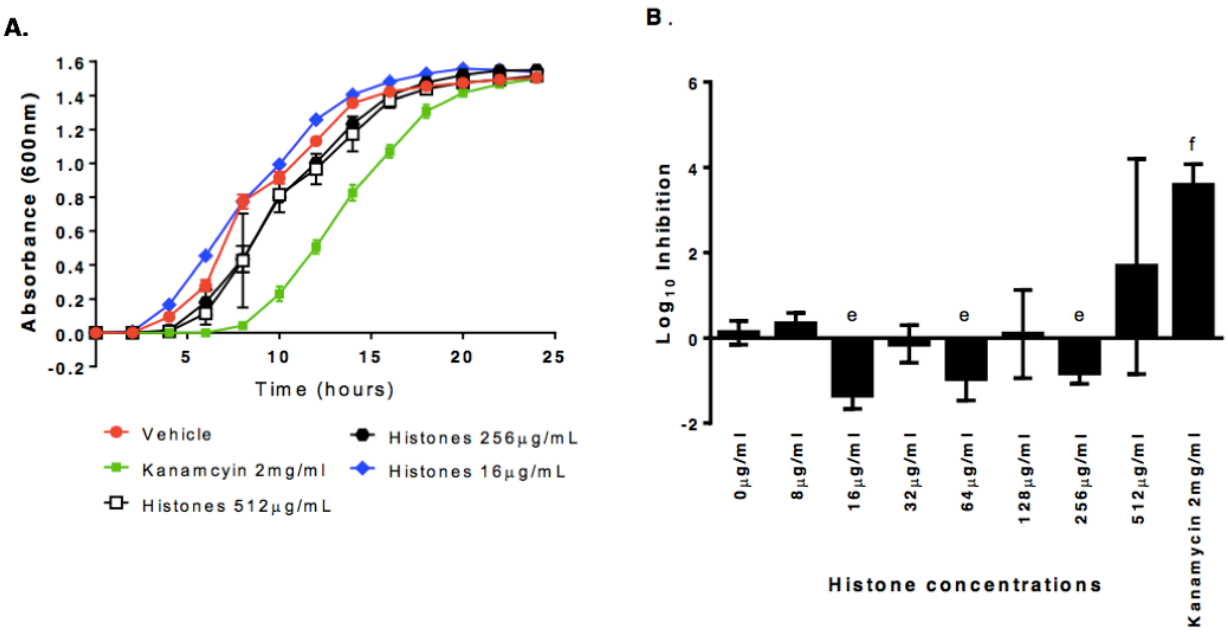


Figure S2. Histone mixture extracted from chicken erythrocytes versus *P. aeruginosa* biofilms. A) Dose-dependent growth inhibition of *P. aeruginosa* biofilms treated with histone mixture from chicken erythrocytes determined by an MBEC assay. Growth curves are representative of three independent trials, each in triplicate. B) Dose-dependent logarithmic bacterial growth inhibition of biofilms treated with histone mixture from chicken erythrocytes determined by an MBEC assay. Statistical analysis was done by Student's T-Test, (e) $p \leq 0.04$ vs. 0 µg/ml *P. aeruginosa* (biofilm growth); (f) $p \leq 0.0005$ vs. 0 µg/ml *P. aeruginosa* (biofilm inhibition). Results are of three independent trials, each in triplicate. Figure adapted with permission from: (Rose-Martel 2015).

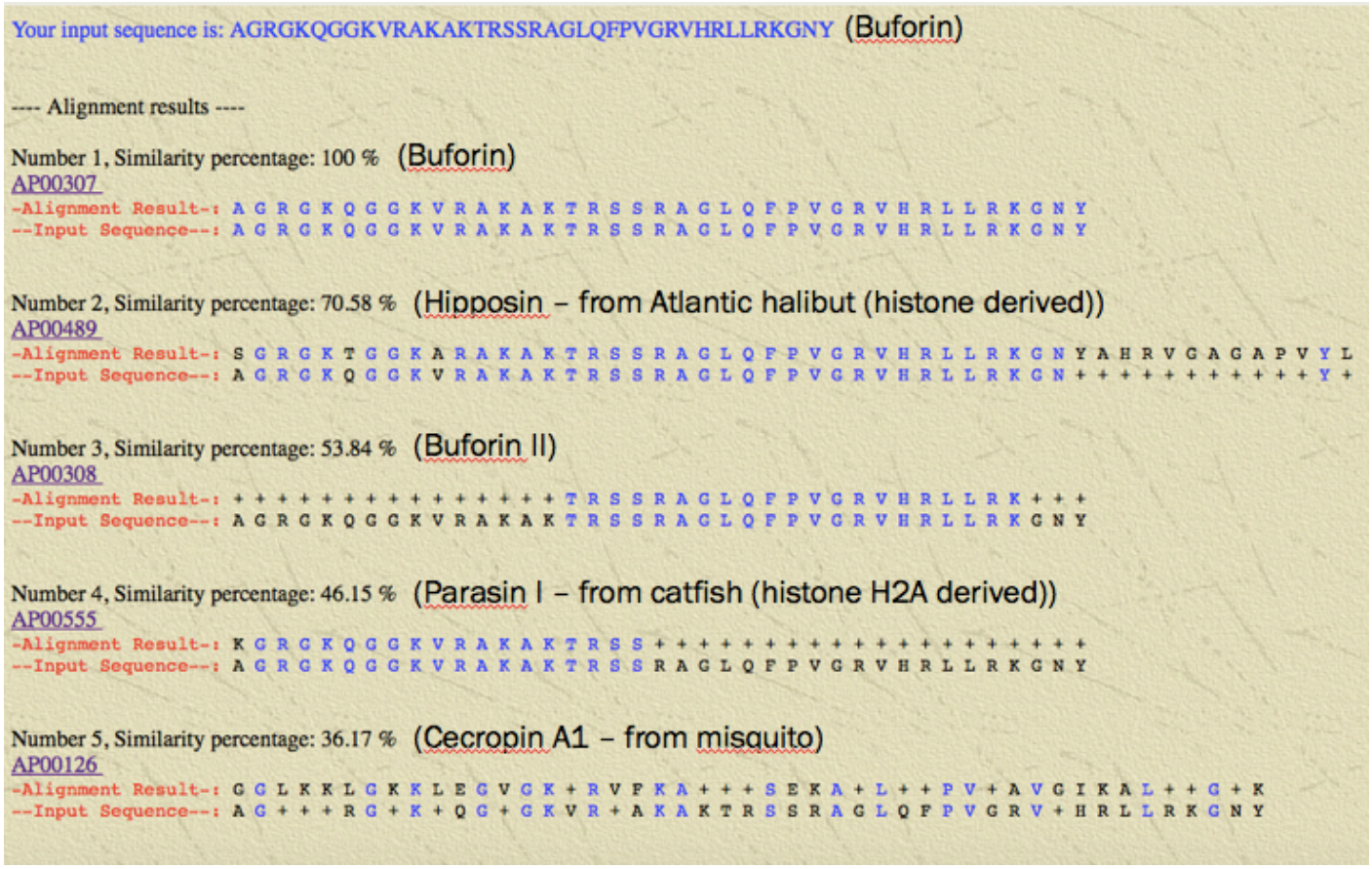


Figure S3. The similarity percentage of buforin with other AMPs in the Antimicrobial Peptide Database. Alignment of buforin I with the 5 most similar peptides in the database (> 2900 AMPs in the database). Information was retrieved from the Antimicrobial Peptide Database (APD: http://aps.unmc.edu/AP/main.php).

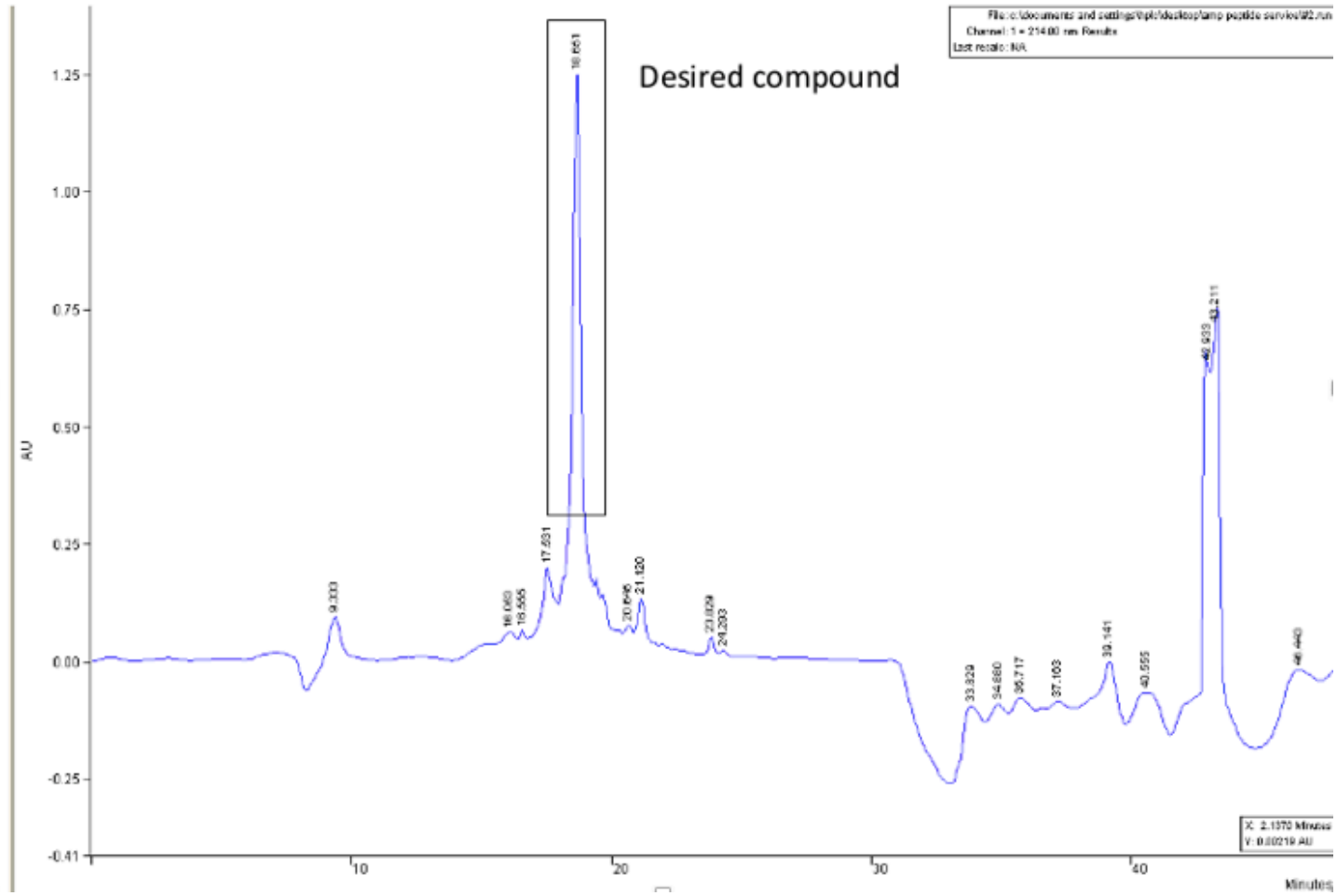


Figure S4. HPLC chromatogram of the histone H5-derived peptide H5(61-90) V1. Analysis of the peaks determined that the peptide purity is 81.4%.

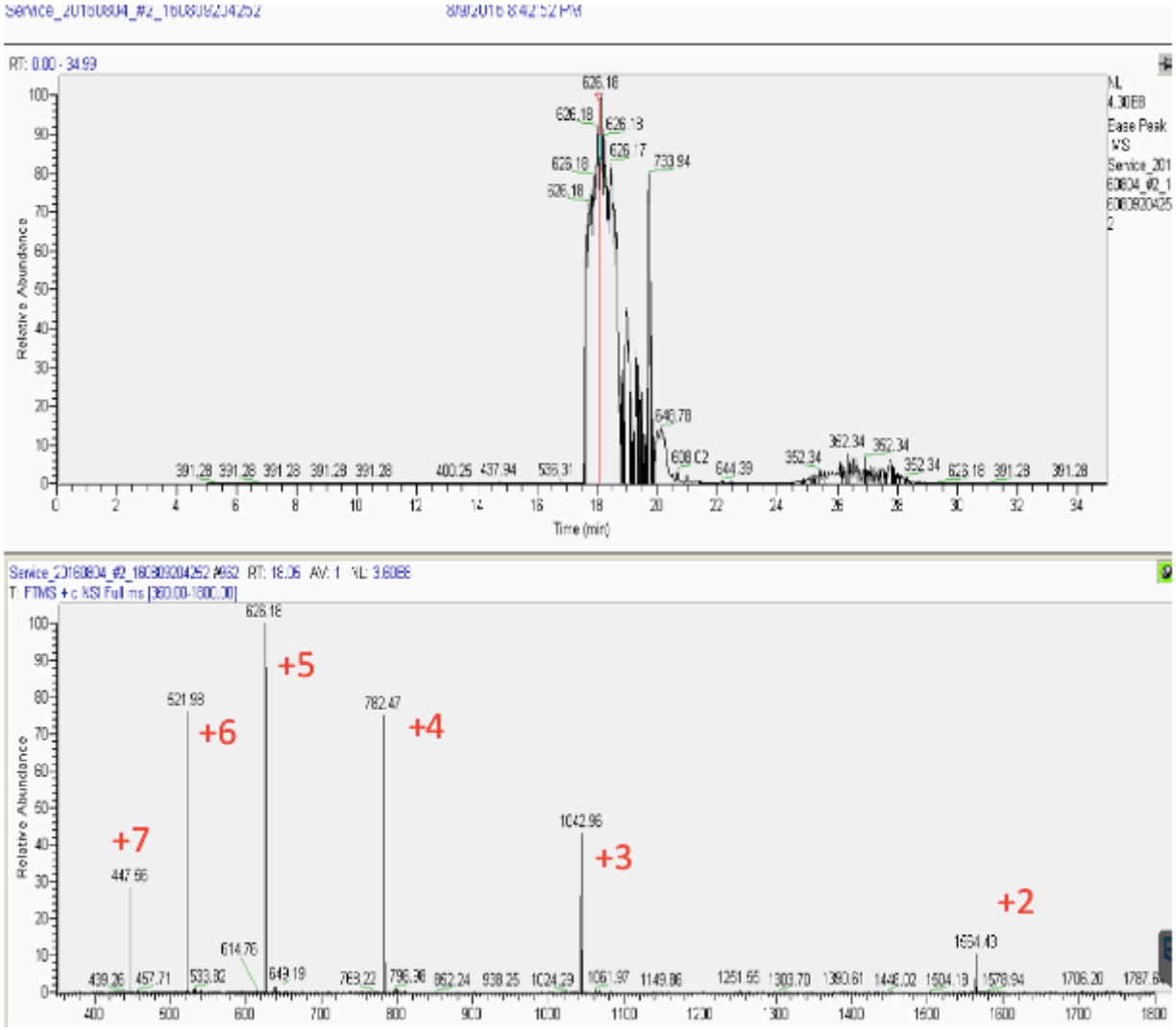
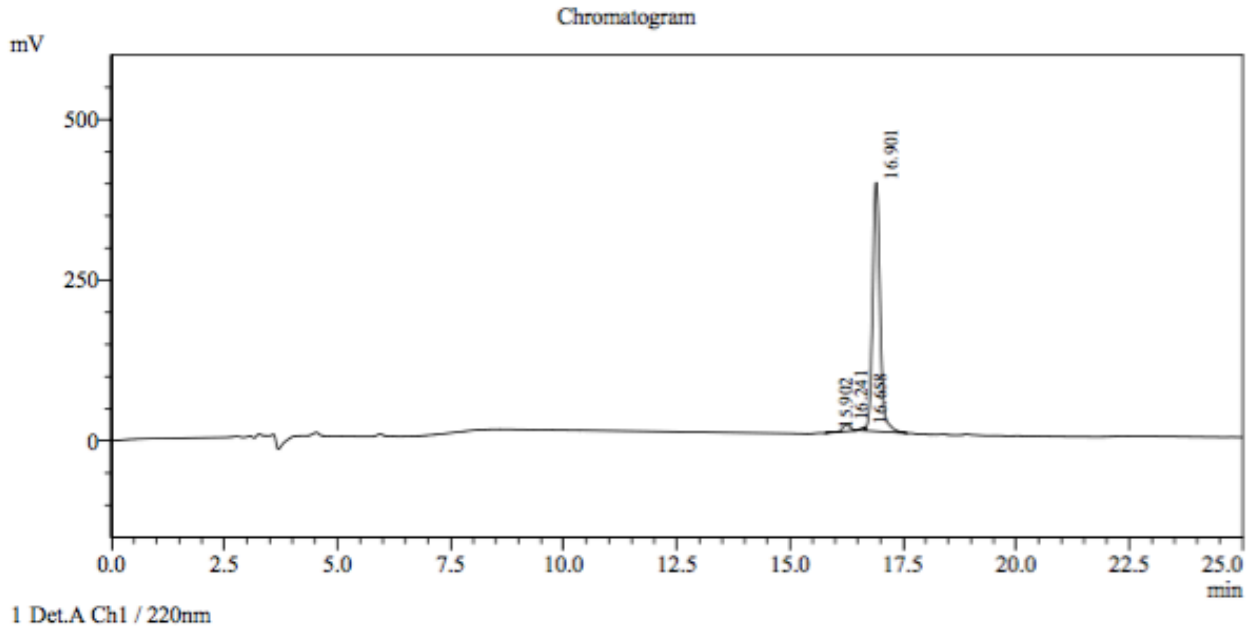


Figure S5. Mass spectrum of the peptide H5(61-90) V1 sample.



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	15.902	4728	619	0.104
2	16.241	119930	11167	2.647
3	16.658	32410	5195	0.715
4	16.901	4373510	386799	96.533
Total		4530579	403780	100.000

Figure S6. HPLC chromatogram of the histone H5-derived peptide H5(61-90) V2. Analysis of the peaks determined that the peptide purity is 96.5%.

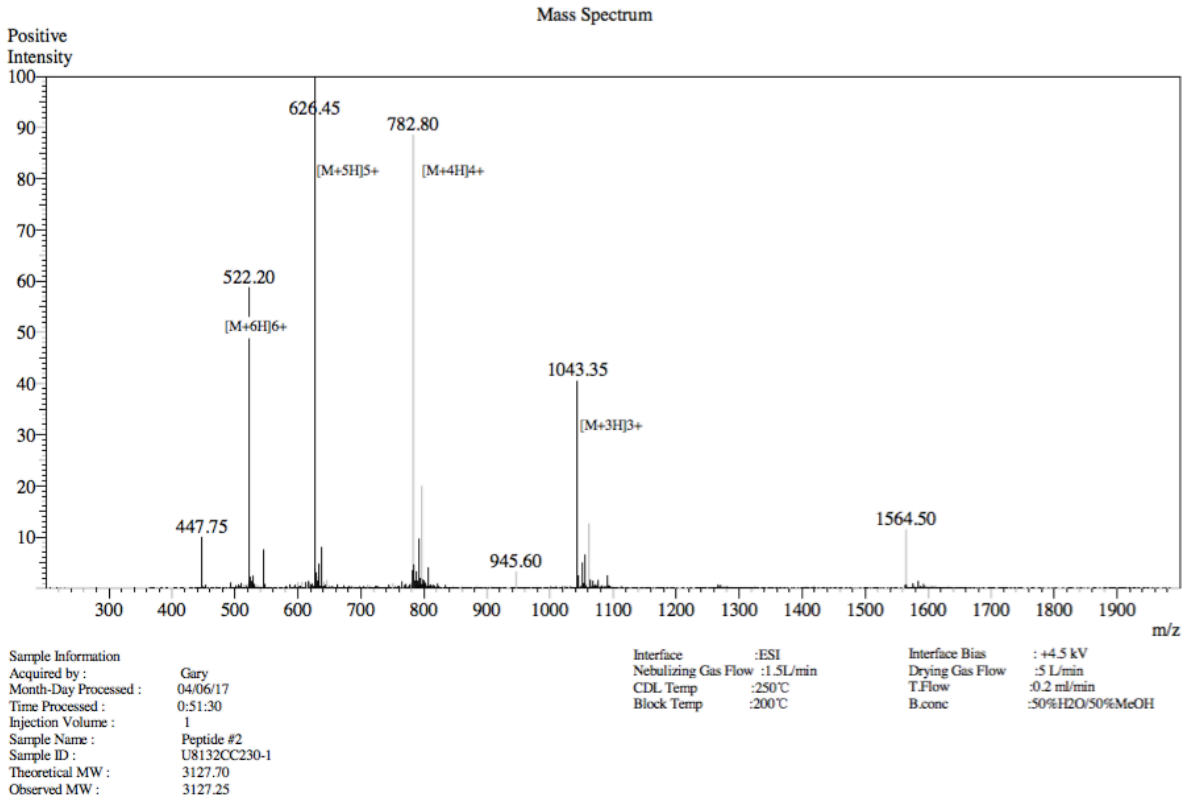
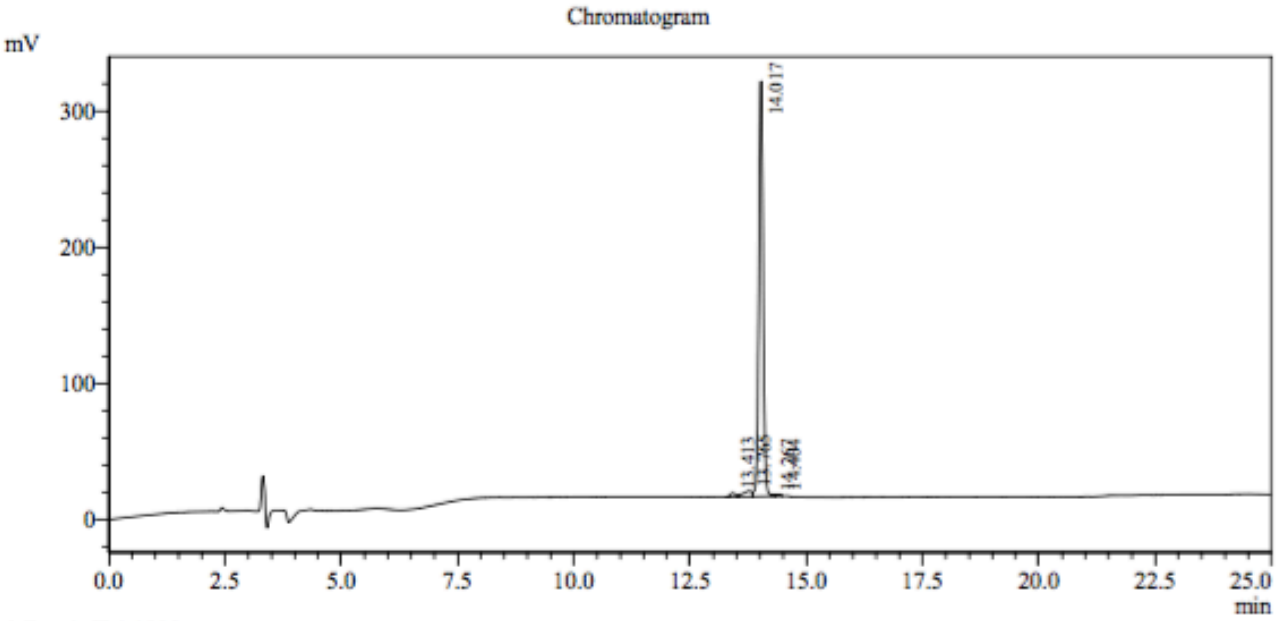


Figure S7. Mass spectrum of the peptide H5(61-90) V2 sample.



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	13.413	20810	2811	1.036
2	13.765	55332	4342	2.755
3	14.017	1920343	304700	95.598
4	14.267	4134	1087	0.206
5	14.404	8144	1387	0.405
Total		2008763	314328	100.000

Figure S8. HPLC chromatogram of the histone H5-derived peptide H5(61-90) V3. Analysis of the peaks determined that the peptide purity is 95.6%.

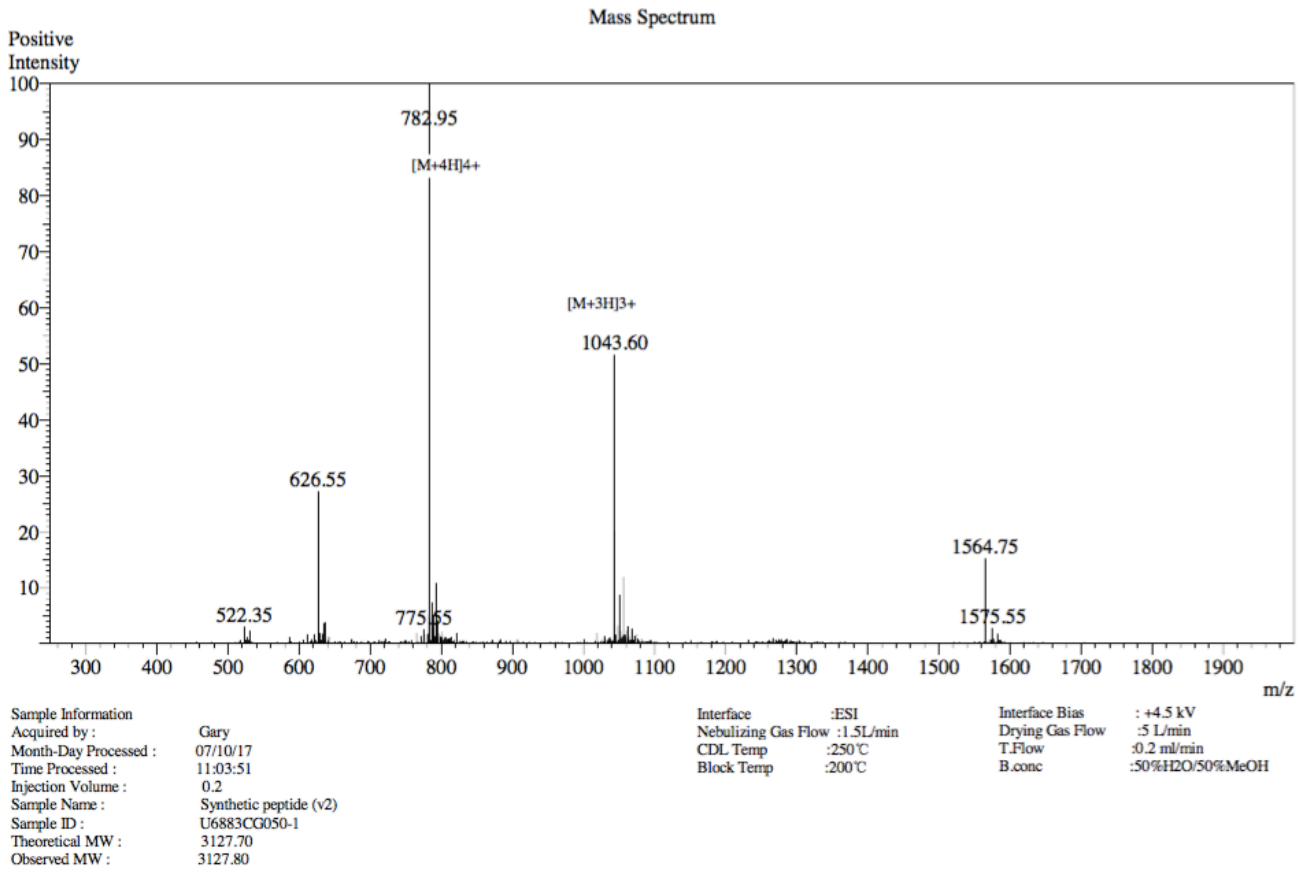


Figure S9. Mass spectrum of the peptide H5(61-90) V3 sample.