Polish Journal of Veterinary Sciences Vol. 21, No. 3 (2018), 533–542

DOI 10.24425/124287

Original article

Biological characteristics of a new antibacterial peptide and its antibacterial mechanisms against Gram-negative bacteria

Z. Pei[1], X. Ying[1], Y. Tang[2], L. Liu[3], H. Zhang[4], S. Liu[1], D. Zhang[1], K. Wang[1], D. Zhang[1], L. Kong[1], Y. Gao[1], H. Ma[1*]

[1] College of Animal Science and Technology, Jilin Agricultural University, Xincheng Street 2888, Changchun 130118, PR China
[2] Institute of Animal Husbandry and Veterinary Medicine, Academy of Agricultural Science of Guizhou Province, Laolipo 1, Guiyang 550005, PR China
[3] Jilin Medical University, Jilin Street 5, Jilin 132013, PR China
[4] Animal Husbandry Bureau of Yongji County, Tianjin Street, Jilin,132100, Jilin Province, PR China

Abstract

MDAP-2 is a new antibacterial peptide with a unique structure that was isolated from houseflies. However, its biological characteristics and antibacterial mechanisms against bacteria are still poorly understood. To study the biological characteristics, antibacterial activity, hemolytic activity, cytotoxicity to mammalian cells, and the secondary structure of MDAP-2 were detected; the results showed that MDAP-2 displayed high antibacterial activity against all of the tested Gram-negative bacteria. MDAP-2 had lower hemolytic activity to rabbit red blood cells; only 3.4% hemolytic activity was observed at a concentration of 800μg/ml. MDAP-2 also had lower cytotoxicity to mammalian cells; IC50 values for HEK-293 cells, VERO cells, and IPEC-J2 cells were greater than 1000 μg/ml. The circular dichroism (CD) spectra showed that the peptide mostly has α-helical properties and some β-fold structure in water and in membrane-like conditions. MDAP-2 is therefore a promising antibacterial agent against Gram-negative bacteria. To determine the antibacterial mechanism(s) of action, fluorescent probes, flow cytometry, and transmission electron microscopy (TEM) were used to study the effects of MDAP-2 on membrane permeability, polarization ability, and integrity of Gram-negative bacteria. The results indicated that the peptide caused membrane depolarization, increased membrane permeability, and destroyed membrane integrity. In conclusion, MDAP-2 is a broad-spectrum, lower hemolytic activity, and lower cytotoxicity antibacterial peptide, which is mainly effective on Gram-negative bacteria. It exerts its antimicrobial effects by causing bacterial cytoplasm membrane depolarization, increasing cell membrane permeability and disturbing the membrane integrity of Gram-negative bacteria. MDAP-2 may offer a new strategy to for defense against Gram-negative bacteria.

Key words: antimicrobial peptide, biological characteristics, antibacterial mechanism, Gram-negative bacteria

Correspondence to: H. Ma, email: hongxia0731001@163.com, tel/fax: +86 043184532810

Introduction

For many decades, Gram-negative bacteria (GNB) have been the main pathogenic bacteria that trigger infections in hospitals and communities, and drug resistance in clinical isolates is increasing each day (Cantôn et al. 2012, Patriarca et al. 2017). The majority of these bacteria are conditional pathogenic bacteria, and they easily mutate into superbugs that are resistant to almost all antibiotics, creating a serious threat to human and animal health (Falagas et al. 2014) Therefore, it has become a top priority to increase the pace of research on new antimicrobial agents for the treatment of infections caused by Gram-negative bacteria.

Antimicrobial peptides (AMPs) are important components of innate immunity, and have highly effective and broad-spectrum antimicrobial activities. AMPs have strong inhibitory effects on bacteria (Mai et al. 2014, Zhuang et al. 2014, Meloni et al. 2015), fungi (Lee et al. 2015), viruses including Lent virus (Wachinger et al. 1998, Fernandes et al. 2002), mycoplasma (Tassanakajon et al. 2015), etc. AMPs can also effectively inhibit the proliferation of tumor cells in vitro. In addition, AMPs have the advantages of not causing injury anima to cells, and of having no immunogenicity and a low possibility of incurring resistance (Lazarev 2009). Thus, AMPs are a promising alternative antibiotic drug.

Houseflies live in a wet, organic matter-enriched environment, they carry many types of pathogens (bacteria, fungi, viruses, and parasites), and transmit these pathogens to people, domestic animals, and poultry. However, houseflies rarely show signs of disease. Researchers have predicted that the houseflies' stronger protection against infections of pathogens being largely due to the antimicrobial peptides produced by their bodies. In recent years, many antimicrobial peptides have been discovered from houseflies, such as attacin, defensin, cecropin, and deptericin. Antimicrobial peptides isolated from Musca domestica have a wide spectrum of antimicrobial activity against bacteria, fungi, parasites, and viruses, and therefore have broad research prospects and great research value.

MDAP-2 is a new antibacterial peptide (7.5 kDa, highly soluble in water) with a unique structure that was isolated from houseflies. Compared with other antimicrobial peptides, MDAP-2 had high antibacterial activity against all of the tested Gram-negative bacteria strains, but the antibacterial activity against Gram-positive bacteria was not detected (Pei et al. 2014). As a promising antibacterial agent against Gram-negative bacteria, the hemolytic activity, the cytotoxicity, and the action mechanisms of MDAP-2 against bacterial membranes are still poorly understood.

In this study, the antibacterial activity, hemolytic activity, and the cytotoxicity was determined, and the secondary structure of MDAP-2 was then determined by circular dichroism (CD) spectrophotometer. Fluorescent probes, flow cytometry, and transmission electron microscopy (TEM) were also used to determine how MDAP-2 interacts with the G-negative bacterial cell membrane. The aim of this study was to provide effective data for development of new antimicrobial agents against Gram-negative bacteria.

Materials and Methods

Bacterial strains and cell lines

Salmonella pullorum NCTC5776, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Vibrio parahaemolyticus* ATCC 27969 were purchased from the China Institute of Veterinary Drugs Control; One strain of *Salmonella gallinarum*/*pullorum* and one strain of *Pseudomonas aeruginosa* were isolated from chickens; One strain of *Pasteurella multocida* and one strain of *Klebsiella pneumoniae* were isolated from cattle; One strain of *Escherichia coli* was isolated from pigs; One strain of *Proteus vulgaris* were isolated from sheep. *HEK-293* cells, *VERO* cells and *IPEC-J2* cells were kindly provided by the College of Animal Sciences and Technology, Jilin Agricultural University.

Peptides and main reagents

Antimicrobial peptide MDAP-2 was synthesized by Shanghai Biotech Bioscience & Technology Co., Ltd.; Melittin was purchased from Sigma (USA); polymyxin B was purchased from Amresco (USA); O-nitrophenyl--β-D-galactopyranoside (ONPG) was purchased from Amresco (USA); N-phenyl-1-naphthylamine (NPN), bis-(1, 3-dibutylbarbituric acid) pentamethine oxonol (DiBAC4 (3)), propidium iodide (PI) and Triton X-100 were purchased from Sigma (USA).

Circular dichroism spectroscopy

CD spectra were acquired at 25°C on a Bio-Logic MOS-500 circular dichroism spectrometer (FRA).

Scan parameters: the wavelength range was 190-270 nm, the acquisition duration was 1 s, the bandwidth was 2 nm, and the scanning speed was 100 nm/min. Reaction system: PBS 5 mM (pH 7.4), 50% TFE (pH 7.4) solution, and 30% SDS (pH 7.4) solution. The concentration of MDAP-2 in the three solutions was 320 g/ml. A blank spectrum, a buffer solution lacking peptide, was also recorded and subtracted in the final analysis. Three spectra were accumulated and

averaged to generate the final spectra. The mean residue ellipticity was calculated according to Wallace and Janes (Wallace and Janes 2001)

Assays of antibacterial activity

The minimal inhibitory concentration (MIC) of MDAP-2 required for antibacterial activity was detected using the tube broth dilution method. Briefly, single colonies of bacteria were inoculated into nutrient broth supplemented with fetal bovine serum and cultured overnight at 37°C, and the concentrations of bacteria were then adjusted to 2-6×10^5 CFU/ml. 100 μl of bacterial culture (2-6 ×10^5 CFU/ml) were mixed with 20 μl of MDAP-2 solution (PBS 5 mM, pH 7.4), and the final concentrations of MDAP-2 were between 0.0125 and 0.8 mg/ml, the mixtures were then incubated at 37°C for 16-20 h. A Multiskan Spectrum spectrophotometer (Model 1500; Thermo Scientific, Nyon, Switzerland) was used to measure the bacterial growth by monitoring the optical density at 600 nm, and the MIC of MDAP-2 was determined by comparing the cell densities with the cell densities in cultures treated with sterile saline (negative control) or with gentamicin (0.008 mg/ml) (positive control).

Hemolytic activity and cytotoxicity assay

The hemolytic activity of the peptide to rabbit red blood cells was assayed using the method described by Sang et al. (Sang et al. 2017). Erythrocytes were suspended with PBS buffer, dispensed into 96-well plates as 100 μl samples of 5% (v/v) Erythrocytes in PBS, and were incubated with 100 μl of two-fold serial diluted MDAP-2 (final concentrations were 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, 800 μg/ml and 1600 μg/ml) for 120 min at 37°C. The absorbance of the supernatant was measured using a Multiscan MK3 microplate reader at 540 nm. Cells incubated with PBS were used as a negative control, and cells treated with 2% Triton X-100 were used as a positive control. Hemolytic (%) = (A_{test}-$A_{negative}$)/ ($A_{positive}$ -$A_{negative}$)*100. Treatment with 100 μl of melittin (purity >95%; Sigma) solution (50 μg/ml, dissolved in PBS) was used as the control. The final results were recorded by averaging at least three experiments.

The cytotoxicity of the antibacterial peptide MDAP-2 was determined by a TransDetect® Cell Counting Kit (CCK). *HEK*-293 cells, *VERO* cells, as well as *IPEC-J2* cells, were seeded at 5.0×10^3 cells/well in a 96-well plate, after 4-6 h incubation, the cells were treated with 0, 15.625, 31.25, 62.5, 125, 250, 500, or 1000 μg/ml of the peptides, after a 24 h incubation with the different concentrations of peptides, 20 μl of 5 mg/ml CCK solution was added to each well and incubated for 4-8 h. The absorbance was measured with a Multiscan MK3 microplate reader at a test wavelength of 450 nm. The final results were recorded by averaging at least three experiments and the viability of the control cells was set as 100% cell survival. The IC_{50} value for each cell line was defined as the dose of peptide causing a 50% decrease in absorbance compared to the control. Treatment with 50 μg/ml melittin (purity >95%; Sigma) was used as the control.

Transmission electron microscopy

Mid-growth-phase Salmonella pullorum were resuspended at 1×10^8 CFU/ml in sodium-phosphate buffer (pH: 7.4) and then incubated with 25 or 50 μg/ml MDAP-2, or 25 μg/ml melittin (positive control) at 25°C for 1 h. A bacterial solution with no peptide served as the negative control. The cells were centrifuged at 8000×g for 5 min, the supernatant was discarded, and 500 μl glutaraldehyde [2.5% (v/v)] was added to fix the cells and incubated at 4°C for 12 h. The liquid was removed, and the samples were rinsed 3 times, centrifuged at 8000×g for 5 min, and fixed for 2 h with 1% osmic acid solution. The samples were then dehydrated with ethanol at gradually increasing concentrations (50%, 70%, 80%, 90% and 95%). Sections were sliced using a Reichert Ultramicrotome and observed with a transmission electron microscope (JEM-1230, JPN).

Membrane permeability assay

Outer membrane (OM) permeability assay

Single colonies of *Salmonella pullorum* were inoculated into nutrient broth supplemented with fetal bovine serum and cultured overnight at 37°C. Aliquots of the culture were transferred to 50 ml of fresh medium and incubated at 37°C for 3-6 h. The bacteria were collected at OD_{600}=0.4-0.5 and centrifuged at 8000×g for 5 min, and the supernatant was discarded. The bacterial cells were resuspended in buffer (5 mM HEPES, pH 7.2, 5 mM KCN) and adjusted to 1 × 10^8 CFU/ml. 2 ml of *Salmonella pullorum* cells were then added to a fluorescent cup, and the fluorescence intensity was measured for 50 s (Ex=350 nm/Em=420 nm). 20 μl NPN solutions (0.4 mM) were then added, and the fluorescence intensity was again measured for 50 s. A series of MDAP-2 solutions was added with a final concentration of 4, 8, or 16 μg/ml, followed by measurement of the fluorescence intensity for 10 min. Negative control: *Salmonella pullorum* with NPN; positive control: *Salmonella pullorum* cells with NPN and polymyxin B (32 μg/ml). The curve of fluorescence intensity versus time was drawn.

Table 1. MICs of MDAP-2 against ten Gram-negative bacteria.

Bacterial strains	Concentrations of MDAP-2 (mg/ml)								MIC[a] (mg/ml)
	0.4	0.2	0.1	0.05	0.025	0.0125	0.00625	0.003125	
Salmonella pullorum NCTC5776	-	-	-	-	+	+	+	+	0.05
Salmonella pullorum clinical isolate	-	-	-	-	+	+	+	+	0.05
E. coli NTCC 25922	-	-	-	+	+	+	+	+	0.1
E.coli clinical isolate	-	-	-	+	+	+	+	+	0.1
Pseudomonas aeruginosa ATCC 27853	-	-	+	+	+	+	+	+	0.2
Pseudomonas aeruginosa clinical isolate	-	-	-	+	+	+	+	+	0.1
Klebsiella pneumoniae clinical isolate	-	-	-	+	+	+	+	+	0.1
Vibrio parahaemolyticus ATCC 27969	-	-	+	+	+	+	+	+	0.2
Pasteurella multocida clinical isolate	-	-	+	+	+	+	+	+	0.2
proteus vulgaris clinical isolate	-	-	-	+	+	+	+	+	0.1
Positive control[b]	-	-	-	-	-	-	-	-	/
Negative control[c]	+	+	+	+	+	+	+	+	/

[+] There was bacterial growth in the tube.
- There was no bacterial growth in the tube.
[a] Minimal inhibitory concentration, the minimal concentration of peptide that inhibited microbial growth.
[b] Positive control: gentamicin was added to the sample.
[c] Negative control: only sterile saline (no recombinant peptide) was added to the sample.

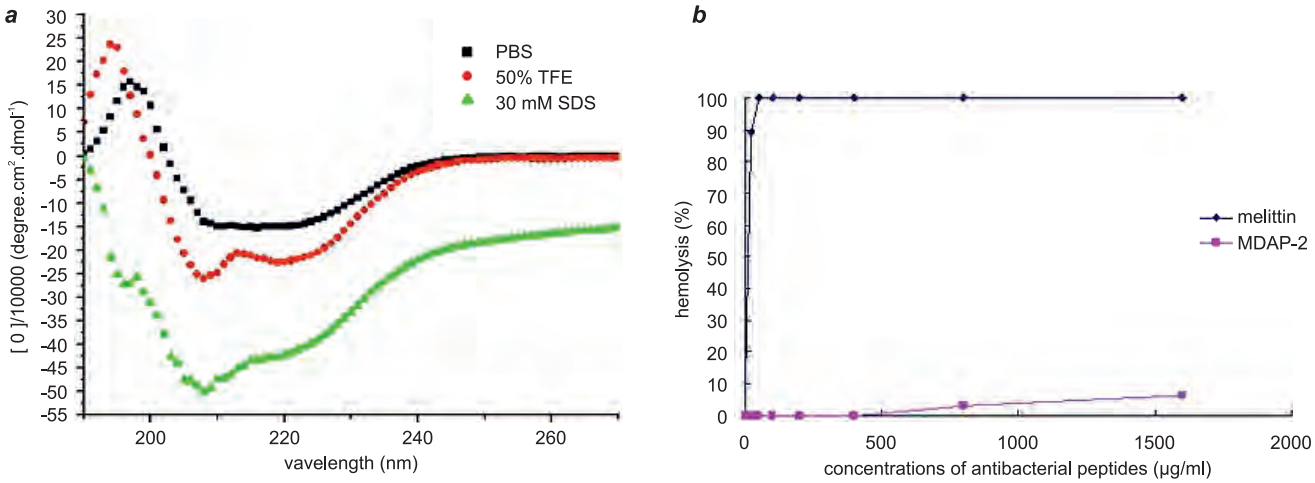

Fig. 1. Secondary structures and hemolytic activity detection results of MDAP-2
a: CD spectra of MDAP-2 in PBS, 30 mM SDS or 50%TFE; b: hemolytic activity results in vitro of different concentrations of MDAP-2.

Membrane permeability assay

Inner membrane (IM) permeability assay

Single colonies of *Salmonella pullorum* were inoculated into nutrient broth supplemented with fetal bovine serum and cultured overnight at 37°C. Aliquots were transferred to 20 ml of fresh medium and incubated at 37°C for 3-6 h. The bacteria were centrifuged at 8000×g for 5 min, and the supernatant was discarded. The cell pellet was resuspended in sodium–phosphate buffer (pH 7.4), and the concentration of bacteria was adjusted to 1×10^8 CFU/ml. ONPG and MDAP-2 were then added with the final concentrations of 1.5 mmol/L and 50 μg/ml, respectively. The mixture was incubated at 37°C and continuously sampled every 15 min for 140 min. The samples were centrifuged at 8000×*g* for 5 min to collect the supernatants for determination of absorbance at 405 nm and compared with the positive control (bacteria treated with 0.2% Triton

X-100) and negative control (bacteria treated with sterile saline). The curve of A_{405} versus time was drawn.

Depolarization of the bacterial membrane assay

Single colonies of *Salmonella pullorum* were inoculated into nutrient broth supplemented with fetal bovine serum, and cultured overnight at 37°C. Aliquots of culture were transferred to 20 ml of fresh medium and incubated at 37°C for 3-6 h. The bacteria solution was collected at OD_{600}=0.5 and centrifuged at 8000×g for 5 min; the supernatant was discarded. Bacterial cells were resuspended in sodium–phosphate buffer (pH 7.4), and adjusted to 1×10^6 CFU/ml. MDAP-2 was then added to a final concentration of 50 μg/ml, incubated at 37°C for 2 h, and compared with the bacteria treated with sterile saline (negative control) and bacteria treated with melittin (50 μg/ml) (positive control). DiBAC4(3) was added to a final concentration of 50 μg/ml and incubated at 37°C for 2 h protected from light. the bacterial cells were then detected using a flow cytometer (Ex=493 nm/Em=516 nm). The results were analyzed using FlowJo 6.2.1 and GraphPad Prism 5 software.

Bacteria cell membrane integrity assay

During the logarithmic phase of cultivated *Salmonella pullorum*, the bacterial cells were washed 3 times by PBS (pH 7.4), resuspended in MH liquid medium, and adjusted to 1×10^6 CFU/ml. MDAP-2 was then added with a final concentration of 50 μg/ml, incubated at 37°C for 0.5 h in shaker incubator (150 r/min), and compared with the bacteria treated with sterile saline (negative control) and bacteria treated with melittin (25 μg/ml) (positive control). Propidium iodide (PI) solution was added to a final concentration of 10 μg/mL and incubated while protected from light at 25°C for 15 min. The samples were centrifuged at 8000×*g* for 5 min, the supernatant was discarded, and the pellet was washed with PBS (pH 7.4), the bacterial cells were then detected by a flow cytometer (Ex=535 nm/Em=615 nm). The results were analyzed by FlowJo 6.2.1 and GraphPad Prism 5 software.

Results

Circular dichroism spectroscopy

The CD spectra of MDAP-2 (Fig. 1a) display a strong positive band at 200 nm and a strong negative band near 218 nm in 5 mm PBS (hydrophilic environment), indicating that MDAP-2 contains the secondary structures of alpha helices and beta folds in a hydrophilic environment. In contrast, in solutions containing 50% TFE (simulating a cell membrane) and 30 mM SDS (hydrophobic environment), a strong positive band near 192 nm, and two intense negative bands at approximately 208 nm and 220 nm were observed, indicating that MDAP-2 has α-helical properties in membrane-like conditions.

Assays of antibacterial activity

The results showed that MDAP-2 displayed broad-spectrum antibacterial activity against all of the tested Gram-negative bacteria, including *Salmonella pullorum*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Pasteurella multocida, Klebsiella pneumoniae* and *proteus vulgaris*. The MIC value of MDAP-2 against Salmonella pullorum was 0.05 mg/mL, the lowest in all tested bacteria. The MIC value of MDAP-2 against E. coli, *proteus vulgaris*, Pasteurella multocida, *Klebsiella pneumoniae,* Pseudomonas aeruginosa and Vibrio parahaemolyticus were 0.1, 0.1, 0.1, 0.2, 0.2, and 0.2 mg/ml, respectively (Table 1).

Haemolytic activity

In vitro haemolytic assays showed that incubation of rabbit red blood cells with MDAP-2 (800 μg/ml) at 37°C for 120 min, only 3.4% hemolysis was observed at a concentration of 800 μg/ml (Fig. 1b), while melittin exhibited 100% hemolysis at a concentration of only 50 μg/ml. These results strongly suggest that MDAP-2 only weakly disrupts rabbit red blood cells.

The cytotoxicity assay

The cytotoxicity assay showed that the IC_{50} value for *HEK*-293 cells, *VERO* cells, and *IPEC-J2* cells were greater than 1000 μg/ml. In contrast, melittin showed 100% cytotoxicity in the three mammalian cells. These results suggest that MDAP-2 has very low cytotoxicity to mammalian cells.

Transmission electron microscopy examination of bacteria

Transmission electron microscopy images showed that MDAP-2 killed bacteria by damaging their cell membranes and caused leakage of the cytoplasm. *Salmonella pullorum,* without treatment of any antibacterial peptides, was round or oval in shape, the cellular membrane was intact, and the electron density of cytoplasm was homogeneous (Fig. 2a). *Salmonella pullorum* showed deformation, damaged cell membranes, evidence of leaked cell contents, and the electronic cloud density in some regions of cytoplasm were reduced when treated with 24 μg/ml (0.5×MIC) MDAP-2 (Fig. 2b). When the concentration of MDAP-2

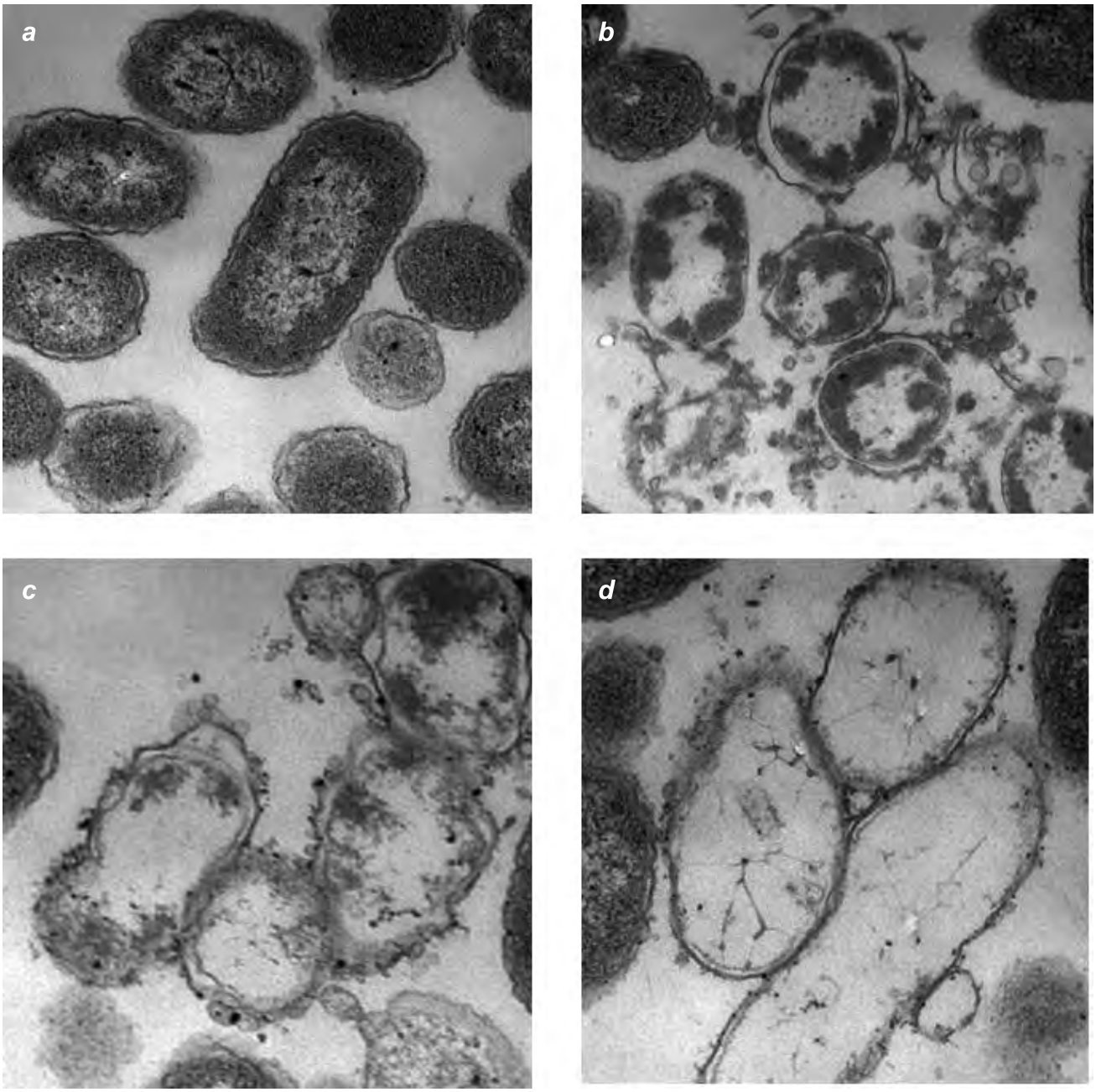

Fig.2. Bacterial morphology of *Salmonella pullorum* treated with MDAP-2 under transmission electron microscope, x7000
a: normal bacterial morphology of *Salmonella pullorum* (negative control); b: bacterial morphology of *Salmonella pullorum* treated with MDAP-2 at 0.5×MIC; c: bacterial morphology of *Salmonella pullorum* treated with MDAP-2 at 1×MIC; d: bacterial morphology of *Salmonella pullorum* treated with melittin at 1×MIC (positive control).

was increased to 48 μg/ml (1×MIC), the cellular morphology was similar to those treated with melittin: the cell membranes were notably damaged and caused leakage of the cytoplasm, and the bacterial cells were completely cracked (Fig. 2c, 2d).

Outer membrane (OM) permeability assay

N-Phenyl-1-naphthylamine (NPN) is a hydrophobic fluorescent agent, that emits faint fluorescence in aqueous solution. However, when in a hydrophobic medium, it will emit strong fluorescence. The integrated outer membrane can stop the NPN from entering into bacterial cells, but when the outer membrane is damaged, the permeability of the cells is increased, and NPN will enter the bacterial cells and fluoresce. Compared with the negative control, the fluorescence intensity in the experimental groups treated with MDAP-2 markedly increased (Fig. 3a). As the concentration of MDAP-2 increased, NPN fluorescence intensity also gradually increased. The results indicate that MDAP-2

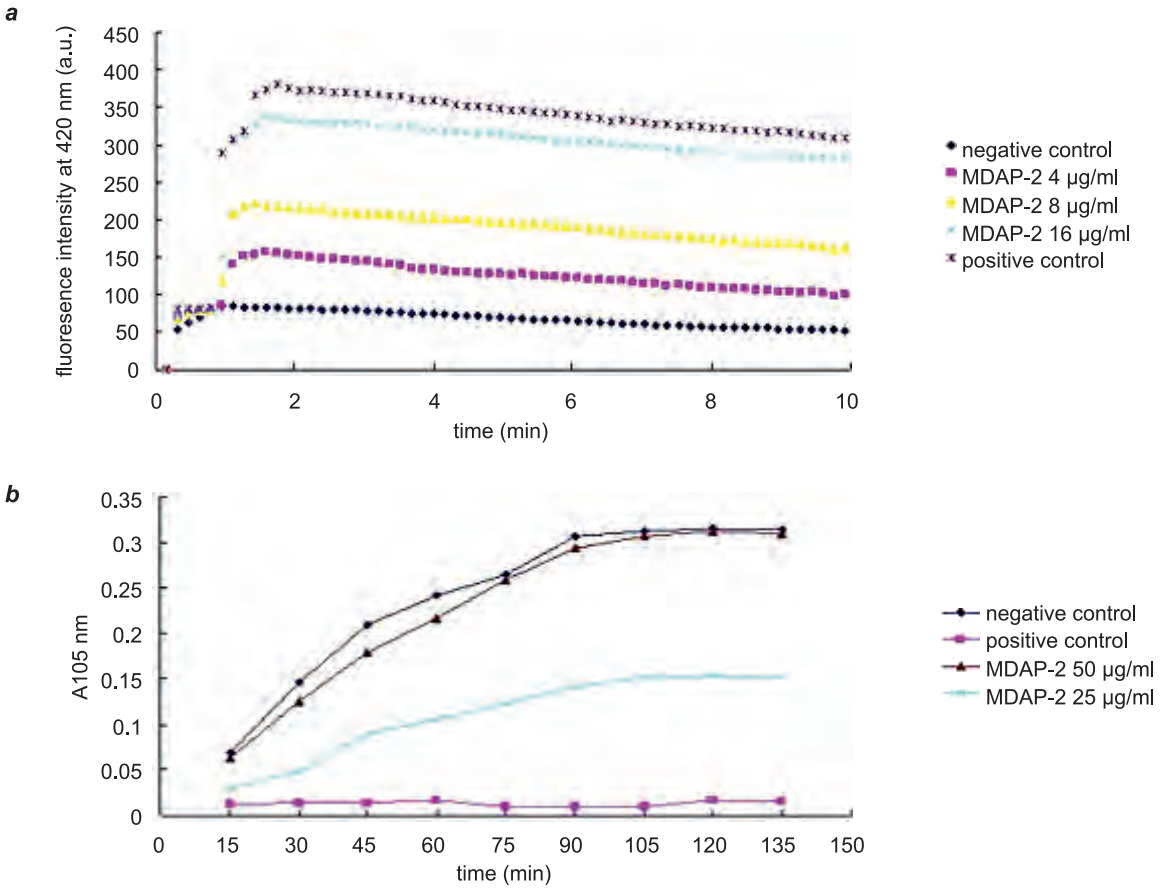

Fig. 3. Membrane permeability assay results of MDAP-2
a: time course of NPN uptake in the outer membrane of *Salmonella pullorum NCTC5776*; b: effects of MDAP-2 on inner membrane permeability of *Salmonella pullorum NCTC5776.*

permeabilized the outer membrane of *Salmonella pullorum*.

Inner membrane (IM) permeability assay

Compared with the negative control, the A_{405} in the experimental groups treated with MDAP-2 (25 μg/ml) was significantly increased, and the increase in A_{405} in the groups treated with MDAP-2 (50 μg/ml) was similar to that of the positive control (Fig. 3b). The results indicated that MDAP-2 increased the inner membrane permeability of *Salmonella pullorum*, and beta-galactosidase was released from the cells or ONPG entered the cells, resulting in the increase of A_{405} in the experimental groups.

Depolarization of the bacterial membrane assay

To determine whether MDAP-2 could cause depolarization of the bacterial cytoplasmic membrane, *Salmonella pullorum* cells treated with MDAP-2 were stained with DiBAC4(3), and the changes of fluorescence intensity were detected by flow cytometry. Compared with the negative control, the fluorescence intensity of *Salmonella pullorum NCTC5776* cells treated with MDAP-2 increased by 35.3% ($p<0.01$) (Fig. 4a), and the result showed that MDAP-2 could depolarize the bacterial cytoplasmic membrane.

Bacteria cell membrane integrity assay

Compared with the negative control, the fluorescence intensity of *Salmonella pullorum* cells treated with MDAP-2 increased by 66.5% ($p<0.01$) (Fig. 4b). The results showed that MDAP-2 can destroy the cell membrane integrity of bacteria, allowing PI to enter the cells and embed in the DNA, thus emitting red fluorescence.

Discussion

Antibacterial peptides from various organisms have been reported (Diamond 2001, Haug et al. 2007). Some of them have antibacterial activity against both Gram-positive and Gram-negative bacteria, such as cecropin and melittin. Some of them have antibacterial activity only against Gram-positive bacteria, such as

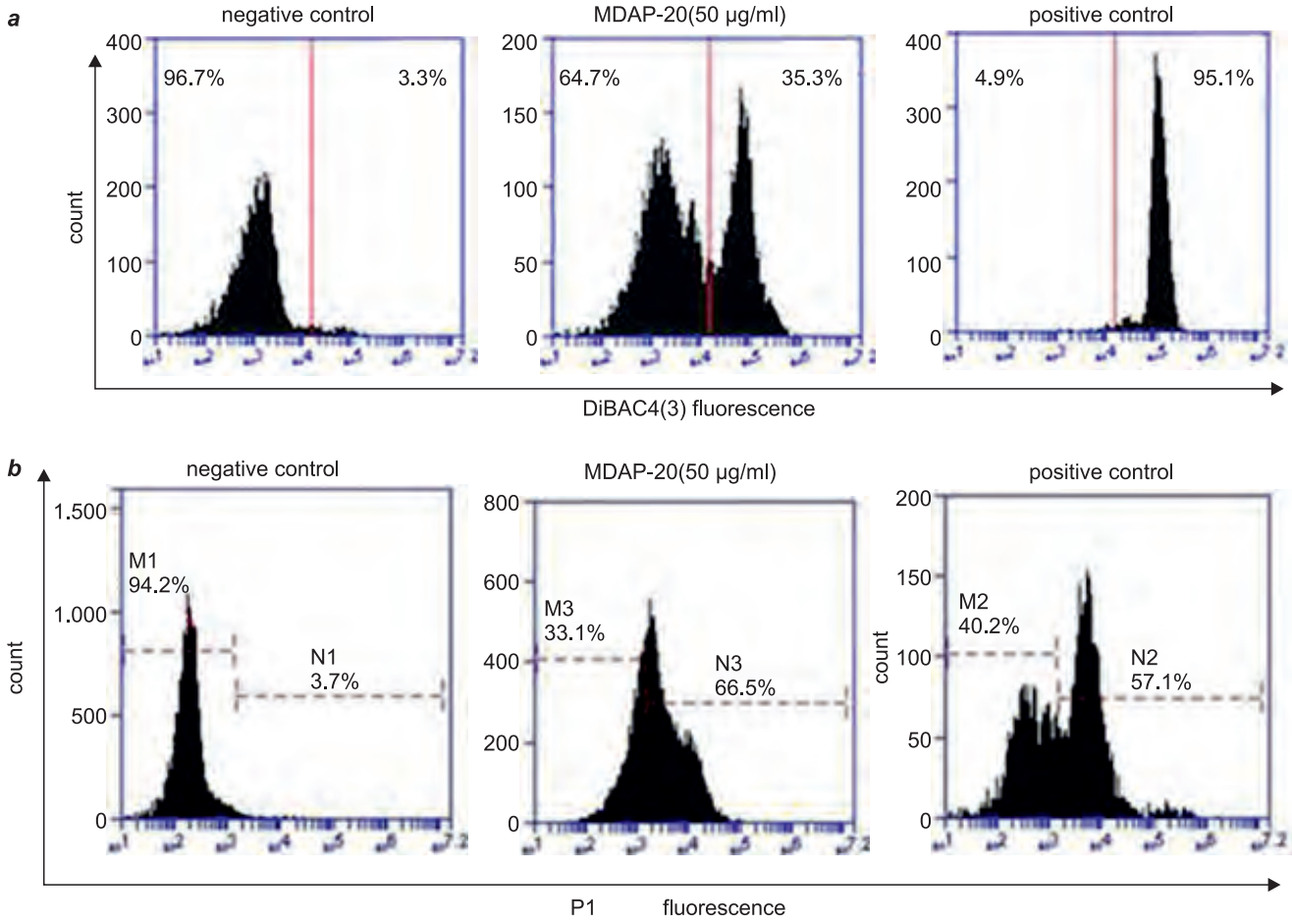

Fig. 4. Depolarization ability of MDAP-2 and its affection on *Salmonella pullorum* membrane integrity
a: depolarization ability of MDAP-2 on *Salmonella pullorum* plasma membrane by flow cytometry; b: effects of MDAP-2 on membrane integrity of *Salmonella pullorum* assayed by flow cytometry.
M: proportion of *Salmonella pullorum* in which fluorescence was not detected; N: proportion of *Salmonella pullorum* in which fluorescence was detected.

Plectasin. And some antibacterial peptides have high antibacterial activity only against Gram-negative bacteria, such as microcin. MDAP-2 is a new antibacterial peptide, with a unique structure, that was isolated from houseflies. It has high antibacterial activity against all of the tested Gram-negative clinical isolates, inferring that MDAP-2 has a higher specificity, and it may also have unique antibacterial mechanisms. In this study, the antibacterial activity assay results showed that MDAP-2 has the highest antibacterial activity towards *Salmonella pullorum* among the 6 tested Gram-negative bacteria; therefore, *Salmonella pullorum* NCTC5776 was selected as the model bacteria to study the antibacterial mechanisms in this study.

Properties of antibacterial peptides such as amino acid composition, secondary structure, and size allowed them to bind to or insert themselves into bacteria, thus bringing about antibacterial action through various mechanisms (Abiraj et al. 2004, Sun et al. 2014). Before exploring the antibacterial mechanisms of MDAP-2, we examined its secondary structure. The CD spectra showed that MDAP-2 has α-helical properties in membrane-like conditions, which was consistent with bioinformatics prediction results. The properties provide some suggestions for antibacterial mechanistic studies of the new antibacterial peptide.

Most antimicrobial peptides have toxicity to eukaryotic cells, and this is one of the most important factors restrict their application in clinical contexts. Hemolytic test and cytotoxicity assay were often used to to detect and measure the toxicity of antimicrobial peptides to eukaryotic cells. In this study, the haemolytic activity of MDAP-2 was far lower than that of melittin, and only 3.4% hemolysis of rabbit red blood cells was observed at a concentration of 800μg/ml. The cytotoxicity assay showed that the IC_{50} value for *HEK*-293 cells, *VERO* cells, and *IPEC-J2* cells was greater than 1000 μg/ml. The results suggest that MDAP-2 has lower cytotoxicity to mammalian cells. The above experiment results indicate that MDAP-2 has very low toxicity to eukaryotic

cells, and could become a new type of antibacterial agent by structure modification.

Most antimicrobial peptides interact with bacterial cell membranes through electrostatic or receptor--mediated interactions, causing increased membrane permeability, altering the efflux of intracellular ions and the influx of water molecules, and eventually killing the bacteria (Sugawara et al. 2010, Fernandez et al. 2013, Torcato et al. 2013). Gram-negative bacteria have a two-layer membrane, the outer membrane and an inner cytoplasmic membrane (Michela et al. 2015); the outer membrane and the underlying peptidoglycan layer constitute the cell wall. In this study, the hydrophobic fluorescent probe (NPN) and the inner membrane probe (ONPG) were used to study the effects of MDAP-2 on the outer/inner membrane permeability, respectively. Under normal circumstances, the integrated outer membrane can prevent NPN from entering bacterial cells. In this study, the outer membrane was destroyed and its permeability increased, and NPN entered the bacterial cells and emitted fluorescence, which was detected by a fluorescence spectrophotometer. Similarly, ONPG cannot enter bacteria cells when the inner membrane is intact. But if the permeability of the inner membrane is increased or disrupted, ONPG can enter and be hydrolyzed by β-galactosidase into galactose and the yellow compound o-nitrophenol, which can be detected by spectrophotometry. Membrane permeability results indicated that MDAP-2 increased the membrane permeability of *Salmonella pullorum*, and the increase was in a concentration-dependent manner.

In addition to the effect on membrane permeability, antimicrobial peptides can also cause depolarization of the bacterial cytoplasmic membrane or destroy cell membrane integrity, eventually resulting in the death of the bacteria. In this study, transmission electron microscopy and flow cytometry were used to detect the effect of MDAP-2 on the membrane integrity of bacteria, TEM images and the flow cytometry results showed that MDAP-2 destroyed the integrity of *Salmonella pullorum* membranes, and caused the leakage of cytoplasm; the level of destruction was slightly lower than that of melittin.

Acknowledgements

This research was funded by the National Natural Science Foundation of China (Grant Nos. 31502121 and 31572574), the Science and Technology Development Plan in Jilin province (Grant No. 20170101021JC), and the key scientific and technological project of Jilin province (Grant No. 20160204002NY).

References

Abiraj K, Prasad HS, Gowda AS, Gowda DC **(2004)** Design, synthesis and antibacterial activity studies of model peptides of proline/arginine-rich region in bactenecin7. Protein Pept Lett 11: 291-300.

Cantôn R, Akôva M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, Livermore D M, Miriagou V, Naas T, Rossolini GM, Samuelsen Ø, Seifert H, Woodford N, Nordmann P **(2012)** Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect 18: 413-431.

Diamond G **(2001)** Natures antibiotics: the potential of antimicrobial peptides as new drugs. Biologist (London) 48: 209-212.

Falagas ME, Tansarli GS, Karageorgopoulos DE, Vardakas KZ **(2014)** Deaths attributable to carbapenem-resistant Enterobacteriaceae infections. Emerg Infect Dis 20: 1170-1175.

Fernandes JM, Smith V J **(2002)** A novel antimicrobial function for a ribosomal peptide from rainbow trout skin. Biochem Biophys Res Commun 296: 167-171.

Fernandez DI, Sani MA, Miles AJ, Wallace B A, Separovic F **(2013)** Membrane defects enhance the interaction of antimicrobial peptides, aurein1.2 versus caerin 1.1. Biochimica et Biophysica Acta - Biomembranes 1828: 1863-1872.

Haug BE, Strom MB, Svendsen JS **(2007)** The medicinal chemistry of short lactoferricin-based antibacterial peptides. Curr Med Chem 14: 1-18.

Lazarev VN **(2009)** The genes of antimicrobial peptides for the therapy of intracellular infections. Acta Naturae 1: 121-123.

Lee F, Lee DG **(2015)** Fungicidal mechanisms of the antimicrobial peptide Bac8c. Biochimica et Biophysica Acta 1848: 673-679.

Mai W, Liu P, Chen H, Zhou Y **(2014)** Cloning and immune characterization of the c-type lysozyme gene in red--spotted grouper, *Epinephelus akaara.* Fish Shellfish Immunology 36: 305-314.

Meloni M, Candusso S, Galeotti M, Volpatti D **(2015)** Preliminary study on expression of antimicrobial peptides in European sea bass (Dicentrarchus labrax) following in vivo infection with Vibrio anguillarum, A time course experiment. Fish Shellfish Immunology 43: 82-90.

Michel JP, Wang YX, Dé E, Fontaine P, Goldmann M, Rosilio V **(2015)** Charge and aggregation pattern govern the interaction of plasticins with LPS monolayers mimicking the external leaflet of the outer membrane of Gram-negative bacteria. Biochimica et Biophysica Acta - Biomembranes 1848: 2967-2979.

Patriarca F, Cigana C, Massimo D, Lazzarotto D, Geromin A, Isola M, Battista ML, Medeot M, Cerno M, Sperotto A, Candoni A, Crapis M, Sartor A, Scarparo C, Bassetti M, Fanin R **(2017)** Risk Factors and Outcomes of Infections by Multidrug-Resistant Gram-Negative Bacteria in Patients Undergoing Hematopoietic Stem Cell Transplantation. Biol Blood Marrow Transplantation. 23: 333-339.

Pei Z, Sun X, Tang Y, Wang K, Gao Y, Ma H **(2014)** Cloning, expression, and purification of a new antimicrobial peptide gene from Musca domestica larva, Gene 549: 41-45.

Sang M, Zhang J, Zhuge Q **(2017)** Selective cytotoxicity of the antibacterial peptide ABP-dHC-Cecropin A and its analog towards leukemia cells. Eur J Pharmacol 803: 138-147.

Sugawara M, Resende JM, Moraes CM, Marguatte A, Chich J, Metz-Boutigue M, Bechinger B **(2010)** Membrane structure and interactions of human catestatin by multidimensional solution and solidstate NMR spectroscopy. FASEB J 24: 1737-1746.

Sun J, Xia Y, Li D, Du Q, Liang D **(2014)** Relationship between peptide structure and antimicrobial activity as studied by de novo designed peptides. Biochimica et Biophysica Acta 1838: 2985-2993.

Tassanakajon A, Somboonwiwat K, Amparyup P **(2015)** Sequence diversity and evolution of antimicrobial peptides in invertebrates. Dev Comp Immunol 48: 324-341.

Torcato I M, Huang Y, Franquelim H G, Gaspar D, Graik D, Castanho MARBC, Henriques ST **(2013)** Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria. Biochimica et Biophysica Acta 1828: 944-955.

Wachinger M, Kleinschmidt A, Winder D, Pechmann N, Ludvigsen A, Neumann M, Holle R, Salmons B, Erfle V, Brack-Werner R **(1998)** Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. J Gen Virol 79: 731-740.

Wallace BA, Janes RW **(2001)** Synchrotron radiation circular dichroism spectroscopy of proteins: Secondary structure, fold recognition, and structural genomics. Curr Opin Chem Biol 5: 567-571.

Zhuang J, Coates CJ, Zhu H, Zhu P, Wu Z, Xie L **(2014)** Identification of candidate antimicrobial peptides derived from abalone hemocyanin. Dev Comp Immunol 49: 96-102.