ORIGINAL ARTICLE

Veterinary Research Forum. 2026; 17 (2): 81 - 88
doi: 10.30466/vrf.2025.2056782.4711
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

In silico molecular design of narrow-spectrum antimicrobial peptide XMK-8 and analysis of factors influencing its antibacterial activity

Hang Bolin[1], Zhu Qianlei[1], Zhou Yuqi[1], Mustalimu Mustafa[1], Wang Yuxin[1], Yan Yuting[1], Yu Man[1], Zhang Guoshuai[1], Zhang Jinrong[1], Li Jie[2], He Hongxuan[1,3], Chen Lin[4], Xia Xiaojing[1]*, Liu Baoguo[1]*

[1] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang, China; [2] Product Quality Inspection and Testing Center in Xinxiang, Xinxiang, China; [3] Institute of Zoology, Chinese Academy of Sciences, Beijing, China; [4] Agricultural Comprehensive Administrative Law Enforcement Brigade of Jinpu New Area, Dalian, China.

Article Info

Article history:

Received: 28 March 2025
Accepted: 27 August 2025
Available online: 15 February 2026

Keywords:

Antimicrobial activity
Antimicrobial peptide
Hemolysis
Molecular design
Narrow-spectrum antibiotics

Abstract

This study aimed to obtain a narrow-spectrum antimicrobial peptide. A peptide XMK-8 was designed based on the amino acid sequence of goose MyHC1 protein from positions 1919 to 1936 (some parameters do not meet the requirements of antimicrobial peptides through bioinformatics analysis) using bioinformatics tools and amino acid substitution method. The minimum inhibitory concentration was determined using liquid double dilution method, the hemolysis rate was determined using dilution method, and the effects of temperature, acid-base, enzyme, and salt ions on its antimicrobial activity were evaluated using liquid double dilution method. The results showed that the designed peptide was a cationic hydrophilic peptide with high amphiphilicity and low hemolytic activity on mouse red blood cells. It had no antimicrobial activity against *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, and *Aeromonas hydrophila*. The minimum inhibitory concentration against *Pasteurella multocida* was 250 μg mL^{-1}, and the minimum inhibitory concentration against *Haemophilus parasuis* was 1.00 mg mL^{-1}. The antimicrobial activity of the narrow-spectrum antimicrobial peptide XMK-8 can still be detected after treatment with temperature (0.00 - 100 ℃), salt ions (sodium ions and potassium ions; 50.00 - 200 mmol L^{-1}), pH (4.00 - 10.00), and protease K (20.00 - 100 μg mL^{-1}). Antimicrobial peptide XMK-8 was expected to become a new alternative to antibiotics and would have good application prospects in the prevention and treatment of *P. multocida* and *H. parasuis* infections.

Introduction

Currently, the issue of bacterial resistance poses a significant threat to the health of both humans and animals, and in some cases, leads to the alarming scenario of "no available treatment options".[1] The rate of iteration of new antibiotics has lagged far behind the emergence of bacterial resistance.[2] However, bacterial diseases in humans and animals still occur frequently and need to be prevented and treated. Thus, the search for alternatives to antibiotics has become an inevitable choice. Antimicrobial peptides (AMPs) have become the leader in the antibiotic replacement family because of their characteristics, such as no-easy resistance, simple structure, small molecular weight, diverse biological activities, and green residue-free.[3,4]

The AMPs primarily exert their bactericidal effects through unique mechanisms of membrane disruption and intra-cellular killing.[5,6] This bactericidal mechanism is different from antibiotics, making AMPs interesting alternatives to antibiotics. Natural AMPs (nAMPs) are one of the important components of the innate immune system of organisms, and play an important role in the process of counteracting pathogen infection.[7,8] However, many nAMPs have some defects, such as low antibacterial activity, low safety, poor stability, and immunogenicity.[8,9] New AMPs can be obtained by artificial modification of nAMPs. Artificial modification of AMPs is to modify the amino acid sequence of existing AMPs according to the structure-activity relationship of AMPs and then, perform functional verification. Based on this, AMPs can also be artificially

***Correspondences:**

Xia Xiaojing. PhD
College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang, China
E-mail: xjxia@hist.edu.cn
Liu Baoguo. PhD
College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang, China
E-mail: liubaoguo@hist.edu.cn

designed according to the structure-activity relationship of AMPs.[9,10] The modified or designed AMPs are all non-nAMPs. Most AMPs, including nAMPs and non-nAMPs, often have a common feature; so, they have broad-spectrum antibacterial activity and can be called broad-spectrum AMPs. This property makes AMPs bactericidal activity against both beneficial and harmful bacteria. Bacteria that are good for the host should not be killed. Therefore, it is necessary to design AMPs with narrow-spectrum AMPs (nsAMPs).

The nsAMPs should have antibacterial activity against only a few types of bacteria or only one type of target bacteria.[11,12] The clinical application of nsAMPs will have a very strong pertinence or specificity, which can be used as a special drug for the prevention and treatment of corresponding pathogens and a special reagent for diagnosis and identification of corresponding pathogens, as well as a specific sterilization reagent or disinfectant in the preparation of inactivated vaccines. Therefore, nsAMPs have stronger selectivity and better application prospects, and the development of nsAMPs is also of great significance for clinical application.

Materials and Methods

Design and synthesis of polypeptide XMK-8. By scanning the amino acid sequence of goose MyHC1 protein (Accession No. KM675469.1) with HeliQuest tool, the polypeptide (SQVNKLRVKSREFHSKKI) from positions 1919 to 1936 had five positive charges, but the hydrostatic moment value was low. Based on the amino acid sequences from positions 1919 - 1936 of goose MyHC1 protein, AMPs (XMK-8) were designed by amino acid substitution method. The amino acid sequence of XMK-8 was designed as RLLPKLPRKVPRFPRKIP. The XMK-8 was synthesized by Wuxi YaTai Biotechnology Co. Yibin, China, purified by high performance liquid chromatography, and determined by mass spectrometry with a purity of > 95.00%.

Test strain. *Staphylococcus aureus* ATCC49525 was purchased from Hangzhou Baosai Biotechnology Co. (Hangzhou, China). *Escherichia coli* ATCC25922 was purchased from Qingdao High-tech Industrial Park Hope Biotechnology Co. (Qingdao, China), and *Salmonella Typhimurium* CVCC541 was obtained from China Institute of Veterinary Drug Control (Beijing, China). *Pasteurella multocida* EO630 strain was isolated and identified in the laboratory from a commercially available triple live vaccine used for the prevention of swine fever, swine erysipelas, and pasteurellosis in pigs. The *Aeromonas hydrophila* strain was donated by the Associate Professor Wang Li of Henan Institute of Science and Technology, Xinxiang, China, and the *Haemophilus paracuis* strain was isolated and identified from infected pigs by the research team.[13]

Reagents and instruments. Sodium chloride, potassium chloride, sodium citrate, protease K, nicotinamide adenine dinucleotide, and Triton X-100 were purchased from Beijing Solarbio Science and Technology Co., Beijing, China. Concentrated hydrochloric acid was purchased from Shanghai Endian Chemical Co., Shanghai, China. Sodium hydroxide was purchased from Chengdu United Chemical Reagent Research Institute, Chengdu, China. Pancreatic cheese soybean peptone liquid (TSB) culture medium was purchased from Qingdao High-tech Industrial Park Hope Biotechnology Co., and other reagents were laboratory routine reagents. Electronic balance was purchased from Shenzhen Boway Electronic Technology Co., Shenzhen, China. Pure water instrument was purchased from Sichuan Zhuoyue Water Treatment Equipment Co., Sichuan, China. Super-clean workbench was purchased from Suzhou Purification Equipment Co., Suzhou, China. Constant temperature incubator was purchased from Shanghai Jinghong Experimental Equipment Co., Shanghai, China. High pressure steam sterilizer was purchased from Shanghai ShenAn Medical Equipment Factory, Suzhou, China. Water bath and electric thermal constant temperature blower drying chamber were purchased from Shanghai Yiheng Scientific Instrument Co., Shanghai China. Enzyme blotting analyzer was purchased from Tecan Austria GmbH., Grödig, Austria. The centrifuge was purchased from Eppendorf Company, Hamburg, Germany.

Bioinformatics analysis of XMK-8. The physical and chemical characteristics of XMK-8 were analyzed using the ProParam tool in ExPaSy (https://www.expasy.org/) and APD6 (https://aps.unmc.edu/) database. The hydrophobicity and hydrophobic moment of the XMK-8 were calculated by the HeliQuest tool. The spiral wheel structure model of the XMK-8 was drawn by the DNASTAR Lasergene (version X.X; DNASTAR Inc., Madison, USA). The three-dimensional model of the XMK-8 was drawn by the PEP FOLD3 (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/). The potential of the XMK-8 to become an AMP was predicted by the computational method in CAMPR4 (https://ngdc.cncb.ac.cn/database commons/database/id/304).

Analysis of antibacterial activity of XMK-8. The antimicrobial activity of XMK-8 was tested by broth microdilution method. Single colonies of the tested strains were selected and placed in 5.00 mL TSB medium (0.50% nicotinamide adenine dinucleotide should also be added for the culture of *H. paracuis*) and oscillated for about 4 - 5 hr at 37.00 ˚C and 180 revolutions *per* min until the concentration of the bacterial solution was approximately 1.00×10^8 colony-forming unit *per* mL, and the bacterial solution was diluted with TSB medium to 1.00×10^5 colony-forming unit *per* mL. The 50.00 μL of TSB medium was added into a row of wells in a 96-well bacterial culture plate. The 50.00 μL of XMK-8 (4.00 mg mL^{-1}) was added into the first well. The liquid from the 1st well was mixed thoroughly. Then, 50.00 μL of mixed liquid in the 1st well

was taken and placed in the 2nd well, and the liquid in the 2nd well was mixed thoroughly again. This process was continued until 10th well. The 50.00 µL of the mixed liquid from the 10th well was withdrawn and discarded. The 11th well was served as a control with bacteria but without peptides. The 12th well was served as a control without bacteria and peptide. The 50.00 µL of diluted bacterial suspension was added to 1 - 11 wells. The 96-well culture plate was turned gently to mix the liquid. The culture plate was placed in a constant temperature incubator at 37.00 ˚C for 18 - 24 hr and then, the optical density $(OD)_{600}$ value was read. The concentration of XMK-8 corresponding to the well with sudden change of OD_{600} value was taken as a minimum inhibitory concentration (MIC) for the tested bacterial strain.[5,6]

Hemolytic analysis of XMK-8. Blood samples collected from mouse with 3.80% sodium citrate as an anti-coagulant were centrifuged at 3,000 rpm for 10 min. The precipitates were collected and washed three times with 0.90% physiological saline solution (PSS). Finally, 2.00% of mouse erythrocyte suspension was prepared with PSS. The 100 µL of PSS was sequentially added to a row of wells in the 96-well bacterial culture plate. Subsequently, 100 µL of XMK-8 (2.00 mg mL^{-1}) was added into the 1st well and serially diluted with PSS in a double ratio according to the method described in the section of "analysis of antibacterial activity of XMK-8". The 11th well was served as a negative control without peptide, while the 12th well was served as a positive control with 100 µL of Triton X-100 (1.00%). Then, 100 µL of 2.00% mouse erythrocyte suspension was added to each well, and the plate was gently shaken to ensure thorough mixing. The plate was incubated at 37.00 ˚C for 1 hr. The contents of each well were gently mixed, and transferred to the 1.50 mL centrifuge tube. The samples were centrifuged at 3,000 rpm for 5 min. The 150 µL of the supernatant was transferred to another row of wells in the 96-well plate. The absorbance values of the samples were measured at a wavelength of 540 nm. Finally, the hemolysis rate of XMK-8 was calculated using the formula:

$$Hemolytic\ rate\ (\%) = \frac{OD_{sample} - OD_{negative}}{OD_{positive} - OD_{negative}} \times 100$$

Analysis of factors influencing the antibacterial activity of XMK-8. The solution of XMK-8 (2.00 mg mL^{-1}) was prepared and divided into five equal aliquots (50.00 µL each). These aliquots were subsequently treated at 0.00, 25.00, 50.00, 75.00, and 100 ˚C for 30 min, respectively. This procedure was conducted to systematically evaluate the influence of temperature on the antibacterial activity of XMK-8. Solutions with pH values of 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, and 10.00 were prepared using hydrochloric acid (1.00 mol L^{-1}) and sodium hydroxide solutions (1.00 mol L^{-1}). The solution of the XMK-8 (2.00 mg mL^{-1}) was divided into seven equal portions (50.00 µL each), being subsequently mixed with an equal volume of the respective pH solutions. The mixtures were incubated at 37.00 ˚C for 30 min. The effects of varying acidic and basic conditions on the antibacterial activity of XMK-8 was then evaluated. Protease K solutions (0.00, 20.00, 40.00, 60.00, 80.00, and 100 µg mL^{-1}) were prepared with sterilized water. The solution of the XMK-8 (2.00 mg mL^{-1}) was divided into six equal portions (50.00 µL each). Each portion was subsequently mixed with an equal volume of protease K solution at varying concentrations. The mixtures were incubated at 37.00 ˚C for 30 min, followed by a 5-min heat inactivation step in a boiling water bath to terminate enzymatic activity. This experimental procedure was designed to evaluate the impact of protease K on the antibacterial activity of XMK-8. Sodium chloride and potassium chloride solutions at concentrations of 50.00, 100, 150, and 200 mmol L^{-1} were prepared separately. The solution of XMK-8 (2.00 mg mL^{-1}) was divided into eight equal portions (50.00 µL *per* portion) and individually mixed with salt ion solutions of equal volume but varying concentrations. The mixtures were then incubated at 37.00 ˚C for 30 min to evaluate the impact of salt ions on the antibacterial activity of the XMK-8. After the completion of various treatments, the MICs of AMPs after different treatments were determined according to the methods described in the section of "analysis of antibacterial activity of XMK-8", and the tested bacteria were *P. multocida* strains.

Results

Analysis of physicochemical properties of XMK-8. The APD3 database and analysis software provided by the ExPaSy website were utilized to evaluate the physico-chemical properties of the designed XMK-8, and the results are presented in Table 1. The designed XMK-8 is a cationic alkaline polypeptide consisting of 18 amino acid residues. The instability index of XMK-8 is below 40.00, indicating good stability. Its aliphatic index exceeds 80.00, suggesting strong thermo-stability.

Table 1. Physicochemical properties of XMK-8.

Items		Results
Number of amino acids		18
Molecular weight		2,211.82 Da
Number of positively charged residues		7
Theoretical isoelectric point (pI)		12.48
Instability index		23.93
Aliphatic index		102.78
Grand average of hydropathicity		– 0.822
Hydrophobic ratio (%)		33.00
Hydrophobicity (H)		0.361
Hydrophobic moment (µH)		0.704
Estimated half-life	**Mammalian reticulocytes***	60 min
	Yeast†	2 min
	***Escherichia coli*†**	2 min

*: *in vitro*, and †: *in vivo.*

The grand average of hydropathicity value is less than 0.00, with both hydrophobicity and hydrophilicity values being low, confirming that XMK-8 is a hydrophilic polypeptide. The high hydrophobic moment further demonstrates that XMK-8 exhibits significant amphiphilicity, implying potential for effective antibacterial activity. Additionally, the half-life of XMK-8 is moderate in mammalian cells but shorter in yeast cells and *E. coli*.

Similarity analysis of XMK-8. Homology analysis was conducted to compare the amino acid sequence of the designed XMK-8 with the AMPs listed in the AMP database APD3. The information regarding the top five AMPs ranked by similarity is presented in Table 2. The highest similarity value is only 50.00%, suggesting that the designed XMK-8 represents a novel peptide.

Analysis of spiral wheel model and three-dimensional model for XMK-8. The spiral wheel model analysis tool in the DNAStar Software was employed to evaluate the design of XMK-8, with the results presented in Figure 1A. The positively charged basic amino acid residues (four arginine and three lysine residues, denoted as R and K) are positioned on one side of the spiral wheel model. This arrangement suggests that the designed XMK-8 exhibits favorable amphiphilic properties and is hypothesized to possess antibacterial activity. The three-dimensional model was constructed using the PEP FOLD3 tool, and the resulting structure is presented in Figure 1B. The designed XMK-8 exhibits an incomplete helical structure, featuring a curled conformation.

The XMK-8 emerges as promising AMPs. Three independent machine-learning algorithms from the CRAMP4 database, including random forest, support vector machine, and artificial neural network models, were employed to evaluate the anti-microbial potential of the designed peptide XMK-8. All three prediction models yielded high probability scores (> 0.50), with random forest scores of 0.87 (natural) and 0.99 (synthetic), support vector machine scores of 0.99 for both forms, and artificial neural network scores of 0.98 (natural) and 0.99 (synthetic), indicating a strong likelihood that XMK-8 possesses antimicrobial properties.

Analysis of the antimicrobial activity exhibited by the designed XMK-8. The MIC of XMK-8 against six different bacterial strains was determined using the liquid ratio dilution method, and the results are presented in Table 3. The MIC of XMK-8 against *P. multocida* was 250 μg mL^{-1}, while the MIC against *H. parasuis* was 1.00 mg mL^{-1}.

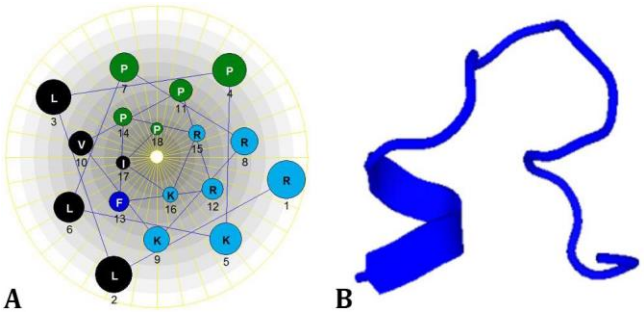

Fig. 1. Analysis of **A)** Spiral wheel model and **B)** Three-dimensional (3D) model for designed XMK-8. In the diagram of spiral wheel of XMK-8, black circle represents non-polar hydrophobic amino acid of the fatty family, blue circle represents non-polar hydrophobic amino acid of the aromatic family, light blue circle represents polar positively charged amino acid residues, and green circle represents non-polar hydrophobic amino acid of the amino acid family. In the 3D model of XMK-8, the parts of thick lines represent α-helix, and the parts of thin lines represent other 2nd structure.

Notably, XMK-8 exhibited no inhibitory activity against *E coli*, *S. Typhimurium*, *S. aureus*, and *A. hydrophila*. These findings suggest that the designed XMK-8 is an nsAMPs.

Hemolytic analysis of XMK-8. The hemolysis rate of the XMK-8 on mouse red blood cells was determined using the double dilution method, and the results are presented in Figure 2. The hemolysis rate was below 20.00% even at a high concentration of 500 μg mL^{-1}, indicating that the hemolysis activity of XMK-8 is minimal.

Effect of temperature on antibacterial activity of XMK-8. The XMK-8 was incubated at various temperatures for 30 min, after which its antibacterial activity was assessed, as presented in Figure 3. The antibacterial activity of XMK-8 remained unchanged following exposure to temperatures ranging from 0.00 to 100 ˚C. These results indicate that XMK-8 exhibits excellent thermal stability.

Table 3. Bacteriostatic activity analysis of designed XMK-8.

Bacterial strains	MIC*
***Pasteurella multocida* EO630**	250 μg mL^{-1}
***Escherichia coli* ATCC25922**	> 1.00 mg mL^{-1}
***Salmonella Typhimurium* CVCC541**	> 1.00 mg mL^{-1}
***Staphylococcus aureus* ATCC49525**	> 1.00 mg mL^{-1}
Aeromonas hydrophila	> 1.00 mg mL^{-1}
Haemophilus parahauis	1.00 mg mL^{-1}

* When the minimum inhibitory concentration (MIC) > 1.00 mg mL^{-1}, it was considered that the peptide had no antibacterial activity against the bacteria.

Table 2. Homology analysis of the designed XMK-8.

Antimicrobial peptides (AMPs)	Number in database	Sources	Similarity (%)
mini-ChBac7.5N beta	AP02221	Natural AMPs	50.00
mini-ChBac7.5N alpha	AP03615	Natural AMPs	47.83
PR-39 (20-39)	AP04455	Synthetic AMPs	47.62
PR-39 (24-39)	AP04456	Synthetic AMPs	45.00
Bac7 (1-16)	AP03754	Synthetic AMPs	45.00

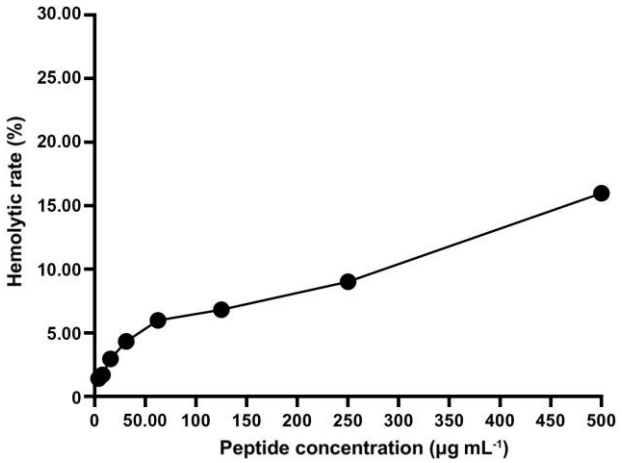

Fig. 2. Hemolysis rate of mouse red blood cells induced by XMK-8. The hemoglobin released from mouse red blood cells was monitored with a microplate auto-reader at 540 nm.

Effect of chemical factors on antibacterial activity of XMK-8. The XMK-8 was incubated in solutions with varying pH values for 30 min, after which the change in its MIC was evaluated, as presented in Figure 4A. The antibacterial activity of the XMK-8 remained unchanged following exposure to different acid-base solutions. These findings suggest that the XMK-8 exhibits excellent acid-base stability. The XMK-8 was incubated with varying concentrations of protease K for 30 min to determine its MIC, as illustrated in Figure 4B. The antibacterial activity of the XMK-8 remained unchanged following exposure to different concentrations of protease K. This suggests that the XMK-8 exhibits excellent stability against protease K.

The antibacterial activity of the XMK-8 was evaluated following exposure to varying concentrations of salt ions, with the results presented in Figures 4C and 4D. Neither sodium nor potassium ions exerted any significant influence on the antibacterial activity of the XMK-8. These findings suggest that the XMK-8 exhibits excellent stability in the presence of salt ions.

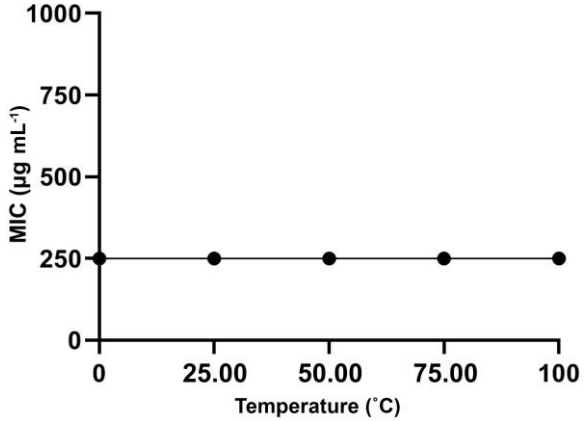

Fig. 3. Effect of temperature on antimicrobial activity of XMK-8. MIC: Minimum inhibitory concentration.

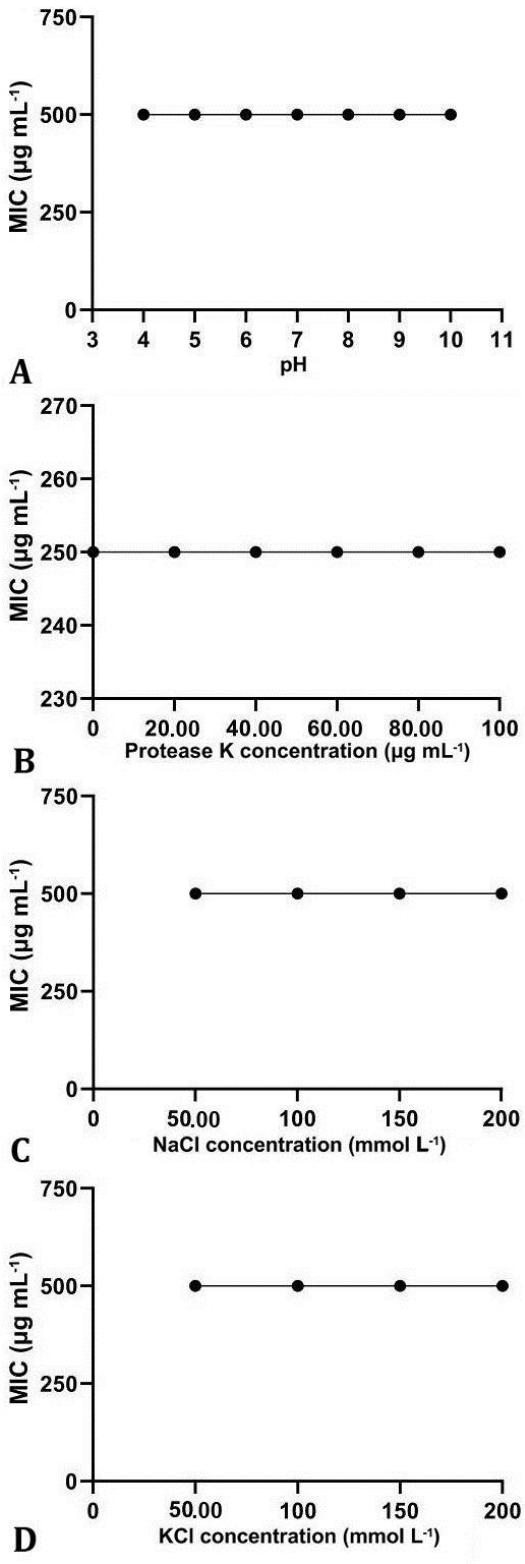

Fig. 4. Effect of chemical factors on antimicrobial activity of XMK-8. **A)** Effect of different pH on the minimum inhibitory concentration (MIC) of XMK-8; **B)** Effect of protease K on the MIC of XMK-8; **C)** Effect of NaCl on the MIC of XMK-8; **D)** Effect of KCl on the MIC of XMK-8.

Discussion

The widespread clinical use of broad-spectrum antibiotics has significantly contributed to the health and well-being of both humans and animals. However, the rise in drug resistance attributable to this factor is also increasing, posing a significant threat to both human and animal health. There is thus no room for delay in tackling the drug resistance crisis. The development and deployment of narrow-spectrum antibiotics, which target specific bacterial strains or genera, represent one of the critical strategies for addressing the issue of drug resistance.[14] The utilization of narrow-spectrum antimicrobial agents can decrease the incidence of drug resistance in clinical settings and may serve as a potential alternative to highly specific antibiotics.[11,14,15] Narrow spectrum antibiotics with high affinity and targeted efficacy towards specific bacteria have been shown to develop resistance at a slower rate than broad-spectrum antibiotics with varying efficacies against different pathogens.[11,14,16,17] Narrow-spectrum antibiotics can be effectively utilized in clinical practice by adopting a descending ladder strategy.[18]

The selection of antibiotic alternatives represents a crucial strategy in addressing the bacterial resistance crisis. Antimicrobial peptides represent a promising alternative to traditional antibiotics. Based on their antimicrobial spectrum, these peptides can be further classified into broad-spectrum and narrow-spectrum AMPs. Most broad-spectrum AMPs exhibit antibacterial or bactericidal effects not only on pathogens but also on beneficial bacteria within the host. Therefore, it is imperative to develop nsAMPs capable of selectively inhibiting specific pathogens. The bactericidal spectrum of nsAMPs is significantly more limited compared to that of broad-spectrum AMPs.[19] The nsAMPs are capable of minimizing the impact on the host microbiome and regarded as having significant potential for application in microbiome restoration therapy.[11,20] The nsAMPs can also be utilized to differentiate or identify specific micro-organisms.[15] As an nsAMP, nisin has been widely used in food preservation.[21] Based on the concept of developing and applying narrow-spectrum antibiotics, the research, development, and application of nsAMPs are expected to exhibit a significant potential and promising application prospect in various fields.

Currently, the design of AMPs primarily relies on their structure-activity relationships, resulting in AMPs that often exhibit broad-spectrum activity. The targeted AMP represents a highly promising narrow-spectrum anti-microbial agent, and its design principles have now been largely established. However, the identification and acquisition of nsAMPs under natural conditions remain challenging, often relying on serendipitous discovery. The moroNC-NH2 is an nsAMP that was serendipitously discovered.[19] Nisin G is an nsAMP obtained from *Streptococcus salivarius* DPC6487 strain by whole genome sequencing.[22] The XMK-8 obtained in this study is a non-nAMP derived through amino acid substitution based on the structure-activity relationship of AMPs. Although our initial objective was to develop AMPs with a high antibacterial activity and broad spectrum, the results of the antibacterial assays revealed that the peptides exhibited narrow-spectrum activity. The unintentionally discovered XMK-8 has been added to the family of nsAMPs. Given the advantages associated with nsAMPs, further research and development of XMK-8 are worth.

Any AMP must undergo rigorous testing for safety and stability prior to its application. The hemolysis rate is frequently employed as a key indicator to evaluate the safety of AMPs. Although certain AMPs with high hemolysis rates are utilized in some applications, during the process of AMP mining and design, it is generally desirable to identify AMPs with low or negligible hemolytic activity. Antimicrobial peptides exhibiting a low hemolysis rate are frequently regarded as possessing higher safety profiles.[23,24] The AMP moroNC-NH2 exhibits a remarkably low hemolysis rate when tested on both sheep and horse red blood cells, demonstrating its high safety profile.[19] In this study, the hemolysis rate of the XMK-8 was evaluated. The results indicated that even at a high concentration of 500 μg mL^{-1}, the hemolysis rate of XMK-8 remained below 20.00%. This suggests that the XMK-8 exhibits promising safety profiles for potential applications.

In practical applications, various environmental factors, including temperature, pH levels, enzymes, and salt ions, as well as other physicochemical conditions, can influence the antibacterial activity of AMPs. Following exposure to these physicochemical factors, the anti-bacterial activity of AMPs may potentially diminish or be completely lost. With the increasing sodium concentration, the antibacterial activity of moroNC-NH2 exhibited a gradual decrease.[19] Therefore, these factors are frequently assessed during the evaluation of utility and applicability of AMPs. In this study, it was demonstrated that the XMK-8 exhibited excellent stability against heat and protease K. Despite a slight reduction in antibacterial activity caused by acidic or basic conditions, as well as sodium and potassium ions, the peptide retained significant anti-bacterial efficacy. This indicates that the XMK-8 has good stability to some factors, but not high stability to some factors. In fact, most AMPs exhibit this common limitation. Subsequently, the XMK-8 can be modified or optimized to obtain an improved AMP with enhanced efficacy.

The XMK-8 is a cationic, hydrophilic polypeptide with a high degree of amphiphilicity. It exhibits no antibacterial activity against *E. coli*, *Salmonella*, *S. aureus*, and *A. hydrophila*, but demonstrates antibacterial activity against *P. multocida* and *H. parasuis*. Additionally, it shows low hemolytic activity and exhibits excellent stability under

heat and protease K exposure. The XMK-8 is expected to play a significant role in the treatment and prevention of corresponding pathogens, as well as development of inactivated vaccines.

Acknowledgments

This work was funded by the Natural Science Foundation of Henan (232300421031), the joint fund of science and technology research and development plan in Henan province (225200810044),the National Natural Science Foundation of China (32172876 and 32473070), and Central Plains Thousand Talents Program-Central Plains Science and Technology Innovation youth top talent.

Conflict of interest

The authors declare no conflict of interest.

References

1. Ho CS, Wong CTH, Aung TT, et al. Antimicrobial resistance: a concise update. Lancet Microbe 2025; 6(1): 100947. doi: 10.1016/j.lanmic.2024.07.010.
2. Feng J, Zheng Y, Ma W, et al. Multitarget antibacterial drugs: an effective strategy to combat bacterial resistance. Pharmacol Ther 2023; 2023: 108550. doi: 10.1016/j.pharmthera.2023.108550.
3. Tong J, Liu Z, Zhou K, et al. Thermosensitive bovine lactoferricin-loaded chitosan hydrogels for sustained antibacterial release: an alternative to antibiotics for treating bovine mastitis. Int J Biol Macromol 2025; 303: 140673. doi: 10.1016/j.ijbiomac.2025.140673.
4. Ji S, An F, Zhang T, et al. Antimicrobial peptides: an alternative to traditional antibiotics. Eur J Med Chem 2024; 265: 116072. doi: 10.1016/j.ejmech.2023. 116072.
5. Lei Y, Lyu A, Pan M, et al. Efficacy and mechanisms of antimicrobial peptide BP15 and its lipopeptides against citrus blue mold. LWT 2025; 217: 117378. doi: 10.1016/j.lwt.2025.117378.
6. Dashtbin S, Razavi S, Ganjali Koli M, et al. Intracellular bactericidal activity and action mechanism of MDP1 antimicrobial peptide against VRSA and MRSA in human endothelial cells. Front Microbiol 2024; 15: 1416995. doi: 10.3389/fmicb.2024.1416995.
7. Hu J, Hang B, Xu Y, et al. Animal-derived antimicrobial peptides [Chinese]. 1st ed. Beijing, China: Science Press 2015; 1-35.
8. He Y, Wang X, He S, et al. Enhancing antibiotic-resistant bacterial infection therapy: self-assembling gemini quaternary ammonium-functionalized peptide nano-assemblies with multiple antibacterial mechanisms. ACS Nano 2025; 19(7): 6977-6992.
9. Shao C, Wang M, Wang Y, et al. Research progress in anti-enzymatic antimicrobial peptides [Chinese]. Sheng Wu Gong Cheng Xue Bao 2024, 40(12): 4396-4407.
10. Mwangi J, Kamau PM, Thuku RC, et al. Design methods for antimicrobial peptides with improved performance. Zool Res 2023; 44(6): 1095-1114.
11. Shepperson OA, Harris PWR, Brimble MA, et al. Thanatin and vinyl sulfide analogues as narrow spectrum antimicrobial peptides that synergise with polymyxin B. Front Pharmacol 2024; 15: 1487338. doi: 10.3389/fphar.2024.1487338.
12. Hang B, Xu J, Li J, et al. Screening of narrow-spectrum antimicrobial peptides and analysis of hemolytic activity [Chinese]. J Henan Inst Sci Technol (Nat Sci Ed). 2017; 45(4): 37-40.
13. Wang C, Wang Q, Wang X, et al. Isolation and identification of *Haemophilus parasuis* serotype 14 and evaluation of its pathogenicity [Chinese]. Chin J Prev Vet Med 2020; 42(8):766-771.
14. Melander RJ, Zurawski DV, Melander C. Narrow-spectrum antibacterial agents. Medchemcomm 2018, 9(1): 12-21.
15. Hayashi K, Misawa T, Goto C, et al. The effects of magainin 2-derived and rationally designed anti-microbial peptides on *Mycoplasma pneumoniae*. PloS One 2022; 17(1): e0261893. doi: 10.1371/ journal.pone.0261893.
16. Ueda T, Takesue Y, Nakajima K, et al. Correlation between antimicrobial resistance and the hospital-wide diverse use of broad-spectrum antibiotics by the antimicrobial stewardship program in Japan. Pharmaceutics 2023; 15(2): 518. doi: 10.3390/ pharmaceutics15020518.
17. Alm RA, Lahiri SD. Narrow-spectrum antibacterial agents-benefits and challenges. Antibiotics (Basel) 2020; 9(7): 418. doi: 10.3390/antibiotics9070418.
18. Ma Y, Ceng J, Feng W. Application of narrow-spectrum antibiotics in step-down (de-escalation) antimicrobial therapy [Chinese].China Pract Med 2012; 7(1): 248-249.
19. Shin SC, Ahn IH, Ahn DH, et al. Characterization of two antimicrobial peptides from Antarctic fishes (*Notothenia coriiceps* and *Parachaenichthys charcoti*). PloS One 2017; 12(1): e0170821. doi: 10.1371/journal. pone.0170821.
20. Lakshmaiah Narayana J, Mechesso AF, Rather IIG, et al. Origami of KR-12 designed antimicrobial peptides and their potential applications. Antibiotics (Basel) 2024; 13(9): 816. doi: 10.3390/antibiotics13090816.
21. Liu B, Xie Q, Liu Y, et al. Effects of nisin loaded chitosan-pectin nanoparticles on shelf life and storage stability of room temperature stored processed cheese. Food Chem 2025; 473: 143103. doi: 10.1016/j.foodchem. 2025.143103.
22. Lawrence GW, Garcia-Gutierrez E, O'Mahony AK, et al. A gut-derived Streptococcus salivarius produces the

novel nisin variant designated nisin G and inhibits *Fusobacterium nucleatum* in a model of the human distal colon microbiome. mBio 2025; 16(2): e0157324. doi: 10.1128/mbio.01573-24.
23. Xu Y, Wang Q, Dong M, et al. Evaluation of the efficacy of the antimicrobial peptide HJH-3 in chickens infected with *Salmonella pullorum*. Front Microbiol 2023; 14: 1102789. doi: 10.3389/fmicb.2023.1102789.
24. Zhao D, Tang M, Hu P, et al. Antimicrobial peptide Hs02 with rapid bactericidal, anti-biofilm, and anti-inflammatory activity against carbapenem-resistant *Klebsiella pneumoniae* and *Escherichia coli*. Microbiol Spectr 2025; 13(1): e105024. doi: 10.1128/spectrum.01050-24.