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PHD THESIS

ADVANCES IN RESEARCH ON MECHANISM AND FUNCTION OF ANTIMICROBIAL PEPTIDES

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The dissertation contains the results of own research. The use of ideas, results and texts of other authors have references to the relevant source ZHAO XUEQIN **Xueqin Zhao**

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ANNOTATION

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In the dissertation work on the basis of research are substantiated the bactericidal mechanism, barrier protection, anti-inflammatory effect and the relationship between the anti-inflammatory effect and intestinal flora of antimicrobial peptide to develop and applicant drugs for prevention inflammation, maintain intestinal flora balance and improve growth performance.

Though antibiotics have good antibacterial and anti-inflammatory effects, they may damage the intestinal barrier function, disrupt the intestinal microbial balance, and disrupt the body's intestinal homeostasis. The problems caused by the long-term large-scale use of antibiotics are becoming more and more serious, and the development of efficient and safe antibiotic substitutes is imminent. Antimicrobial peptides have attracted attention due to their broad-spectrum antimicrobial activity, not have drug resistance, and extensive biological functions.

During our resserch we investigated mechanism of the antimicrobial peptide mastoparan X in killing Gram-negative bacteria *in vitro* and its anti-inflammatory and barrier repair functions in pneumonia and enteritis diseases *in vivo*, and to further study the relationship between anti-inflammatory and intestinal microbes of MPX. Finally, we explored the anti-inflammatory and barrier repair functions mechanism of MPX in the intestinal epithelial cells, laying a foundation for reducing the use of antibiotics in livestock and poultry breeding, it will help provide certain theoretical and practical value for future applications in livestock and poultry.

Our research has found that antimicrobial peptides not only relieve intestinal inflammation, but also have the function of protecting the intestinal barrier.

Studies have revealed that MPX had good bactericidal activity in vitro, and the main bactericidal mechanism was to produce bactericidal effect by destroying the integrity of the bacterial cell membrane. MPX had good antibacterial and anti-inflammatory effects in mice. MPX had better effect of killing *Actinobacillus pleuropneumoniae* and alleviating the symptoms of pneumonia causedby *A. pleuropneumoniae* which mainly by reducing the level of inflammatory factors to alleviate the symptoms of pneumonia. In addition, the study further found that MPX also could alleviate the intestinal inflammation and barrier function damage infection with *E. coli* by reducing inflammatory factors and increasing tight junction proteins, and explorethe mechanism of MPX relieve inflammation and maintain intestinal barrier function in intestinal epithelial cells. Finally, the relationship between MPX to relieve intestinal inflammation and intestinal microbes was studied. The results found that MPX alleviated the intestinal inflammation by changing the diversity of intestinal flora.

The results of the studies did not reveal MPX regulate the mechanism of microbial diversity, but intestinal microbial diversity and intestinal flora homeostasis were indeed related to intestinal inflammation, and MPX alleviated the intestinal inflammation was indeed closely related to intestinal flora.

A. pleuropneumoniae is the causative agent of highly contagious and fatal respiratory infections, causing substantial economic losses to the global pig industry. Due to increased antibiotic resistance, there is an urgent need to find new antibiotic alternatives for treating *A. pleuropneumoniae* infections. MPX is obtained from wasp venom and has a killing effect on various bacteria. This study found that MPX had a good killing effect on *A.pleuropneumoniae* and that the minimum inhibitory concentration (MIC) was 16 μg/mL. The bacterial density of *A. pleuropneumoniae* decreased 1000 times after MPX (1×MIC) treatment for 1 h, and the antibacterial activity was not affected by pH or temperature. Fluorescence microscopy showed that MPX (1×MIC) destroyed the bacterial cell membrane

after treatment for 0.5 h, increasing membrane permeability and releasing bacterial proteins and Ca^{2+}, Na^{+} and other cations. In addition, MPX (1×MIC) treatment significantly reduced the formation of bacterial biofilms. Quantitative RT-PCR results showed that MPX treatment significantly upregulated the expression of the PurC virulence gene and downregulated that of ApxI, ApxII, and Apa1.In addition, the Sap A gene was found to play an important role in the tolerance of *A. pleuropneumoniae* to antimicrobial peptides.Therapeutic evaluation in a murine model showed that MPX protects mice from a lethal dose of *A. pleuropneumoniae* and relieves lung inflammation.This study reports the use of MPX to treat *A.pleuropneumonia* infections, laying the foundation for the development of new drugs for bacterial infections.

Escherichia coli is a facultative anaerobic bacterium that exists in the gastrointestinal tract of humans and animals. It can cause diarrhea, enteritis, destruction of the host's intestinal barrier, and intestinal microecological disturbances. In recent years, due to the abuse of traditional antibiotics, a variety of drug-resistant strains and super bacteria have emerged in an endless stream. Therefore, there is an urgent need to find new alternatives to antibiotics. To explore the effect of MPX against *E. coli*. The function of MPX against *E. coli* was detected by MIC, plate count, propidium iodide, NPN and DiSC3(5) permeability testing, immunofluorescence microscope observation, and the impact of MPX stability by temperature, pH, ion. In this study, the results found that MPX has good antibacterial activity against *E. coli*, and the minimum inhibitory concentration (MIC) was 31.25 ug/mL. MPX bactericidal kinetics study found that MPX had good bactericidal activity within 6 hours. Bacterial permeability studies have shown that MPX could increase the permeability of bacteria, leading to an increase in the protein content of the bacterial supernatant. In addition, NPN, PI and DiSC3(5) results showed that the fluorescence value was positively correlated with MPX. The stability test of MPX found that salt ions, temperature, pH, etc. have a slight influence on its effect. In addition, scanning electron microscopy results showed that the bacteria became smaller and the contents leaked after the

action of MPX. The above results showed that MPX has a good bactericidal activity in vitro, laying the foundation for the development of new drugs for the treatment of bacterial infections.

To investigate whether the antimicrobial peptide mastoparan X (MPX) was effective against *E. coli* infection. BALB/c mice infected with *E. coli* by intraperitoneal injection, which represents a sepsis model. The therapeutic effect of MPX was evaluated in a murine model, revealing that it protected mice from lethal *E. coli* infection. Furthermore, MPX increased the length of villi and reduced the infiltration of inflammatory cells intothe jejunum. SEM and TEM analyses showed that MPX effectively ameliorated the jejunum damage caused by *E.coli* and increased the number and length of microvilli. In addition, MPX decreased the expression of IL-2, IL-6, TNF-α, p-p38 and p-p65 in the jejunum and colon. Moreover, MPX increased the expression of ZO-1, occludin and MUC2 in the jejunum and colon, improved the function of the intestinal barrier and promoted the absorption of nutrients. This study suggests that MPX is an effective therapeutic agent for *E. coli* infection and other intestinal diseases, laying the foundation for the development of new drugs for bacterial infections.

We investigated whether the antimicrobial peptide mastoparan X (MPX) was effective against *E. coli* infection. BALB/c mice infected with *E. coli* by intraperitoneal injection, which represents a sepsis model. In this study, MPX exhibited no toxicity in IPEC-J2 cells and notably suppressed the levels of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), myeloperoxidase (MPO) and lactate dehydrogenase (LDH) induced by *E. coli*. In addition, MPX improved the expression of ZO-1, occludin, and claudin and enhanced the wound healing of IPEC-J2 cells.

We have proved that antimicrobial peptides can regulate the intestinal flora and maintain intestinal homeostasis. Previous studies have shown that MPX had good therapeutic effect on intestinal inflammation caused by *E. coli* infection. However, the relationship between the alleviation of intestinal inflammation and the intestinal microbiota by MPX is still unknown.

We investigated the correlation between the antiinflammatory effects of MPX and the regulation of the gut microbiota. In this study, *E. coli* was used to induce intestinal inflammation, and the results showed that MPX alleviated weight loss and intestinal pathological changes in necropsy specimens of *E. coli*-infected mice. MPX reduced the serum levels of the inflammation-related proteins IL-2, IL-6, TNF-α, MPO and LDH on day 7 and day 28. Furthermore, H&E results showed that MPX increased the length of villi and reduced the infiltration of inflammatory cells intothe jejunum and colon. SEM and TEM results showed that MPX could improve the morphology of jejunum villi and microvilli and increase tight junction protein levels. The16S rRNA sequencing analysis of caecal content showed that species diversity and richness were lower in the *E. coli*-infected group. At the phylum and genus levels, higher abundances of pathogenic Firmicutes, Lachnospiraceae-NK4A-136 and Alistipes bacteria were observed. MPX treatment decreased pathogenic bacterial counts in the *E. coli*-infected group. Furthermore, MPX increased the abundance of Muribaculaceae. Alpha and Beta analysis showed that there was no significant difference inbacterial community structure between the MPX andcontrol groups. In addition, alterations in theintestinal microbiome of mice affected physiological functions and metabolic pathways. Overall, this study is the first to investigate the correlation of the effects of MPX on intestinal inflammation and the intestinal microbiota, which alleviate intestinal inflammation by restoring the diversity and functions of the gut microbiota, providing a new perspective regarding thetreatment of enteritis.

Thus, the findings of these studies were aimed at clarifying MPX had good antibacterial activity against *A. pleuropneumoniae* and *E. coli* infections*in vitro* and *in vivo*. MPXcould resist the pathological damage to the lung and intestine of mice, and relieve the pneumonia, enteritis and intestinal barrierof mice caused bybacteria and further clarify the mechanism of MPX to relieve intestinal inflammation and enhance the intestinal barrier function atintestinal epithelial cells. Finally, this study found that MPX relieved intestinal inflammation by regulating the diversity of intestinal flora.

Based on the materials of the dissertation, methodological recommendations «**Prevention of antibiotic resistance through the use of antimicrobial peptides»** was developed and approved by the Academic Council of SNAU (Protocol № 5, dated 29.12.2021).

We recommend using the materials of the dissertation work when studying the courses "Veterinary microbiology", "Veterinary pharmacology" for masters of the Faculty of Veterinary Medicine of Sumy NAU.

And for the courses "Veterinary microbiology" and "Veterinary pharmacology" for masters of the Henan Institute of Science and Technology (HIST).

Keywords: antimicrobial peptide MPX, bacteria, intestinal epithelial cells, inflammation, intestinal barrier, gut microbiota, microflora, digestion, immunity, E. coli, pathogenic microflora, spectroscopy, efficiency, resistance, Salmonella enterica, Staphylococcaceae, surveillance, PCR, bactericidal action, microscopy.

АНОТАЦІЯ

Чжао Сюєцінь «Удосконалення методів досліджень та функції антимікробних пептидів» – Кваліфікаційна наукова праця на правах рукопису. Дисертація на здобуття наукового ступеня доктора філософії за спеціальністю 211 «Ветеринарна медицина» − Сумський національний аграрний університет, м. Суми, 2022

У дисертаційній роботі, на основі проведених досліджень, обґрунтовано механізм бактерицидної та протизапальної дій, бар'єрний захист, та взаємозв'язок між протизапальною дією та кишковою флорою антимікробного пептиду для подальших розробок препаратів з метою профілактики запалення, підтримки балансу кишкової флори і покращення продуктивність.

Незважаючи на те, що антибіотики мають високу антибактеріальну та протизапальну дію, в той же час вони можуть пошкодити функцію кишкового бар'єру, порушити кишковий мікробний баланс і порушити кишковий гомеостаз організму. Також існує проблема виникнення резистентних штамів мікроорганізмів. Проблеми, спричинені тривалим широкомасштабним використанням антибіотиків, стають дедалі серйознішими, а розробка ефективних і безпечних замінників антибіотиків вкрай актуальна і неминуча. Антимікр обні пептиди привернули нашу увагу завдяки своїй антимікробній дії широкого спектру, відсутності резистентності та широкими біологічними функціям.

Під час дослідження було вивчено механізм антимікробного пептиду мастопаран Х його здатність знищувати грам негативні бактерії *in vitro* та його протизапальну та бар'єрну функції відновлення при пневмонійних та ентеритних захворюваннях *in vivo*, а також для подальшого вивчення зв'язку між мікроорганізмами протизапальної та кишкової дії. Також було досліджено механізм протизапальної та бар'єрної репарації МРХ у кишкових епітеліальних клітинах, заклавши основу для зменшення використання антибіотиків у тваринництві та птахівництві, це допоможе забезпечити певну теоретичну та практичну цінність для майбутнього застосування МРХ для профілактики хвороб тварин та птиці.

Наші дослідження виявили, що антимікробні пептиди не тільки знімають запалення кишечника, але й виконують функцію захисту кишкового бар'єру.

Дослідження показали, що МРХ має високу бактерицидну активність *in vitro,* і його основним бактерицидним механізмом було створення бактерицидного ефекту шляхом руйнування цілісності мембрани бактеріальної клітини. Даний ефект було доведено в дослідах на білих мишах. МРХ ефективний щодо знищення *Actinobacillus pleuropneumoniae* та полегшення симптомів пневмонії, спричиненої *A. pleuropneumoniae*, що, головним чином, зменшує рівень факторів запалення з метою полегшення

симптомів пневмонії. Крім того, дослідженнями виявлено, що МРХ полегшує кишкове запалення та пошкодження бар'єрної функції кишковою паличкою шляхом зменшення факторів запалення та збільшення білків щільного з'єднання. Також було досліджено механізм МРХ, який знімає запалення та підтримує функцію кишкового бар'єру в епітеліальних клітинах кишечника. Було доведено зв'язок між дією МРХ при зниження рівня запалення та мікроорганізмами кишковика. Цей ефект досягається шляхом заміни патогенної мікрофлори нормальну мікрофлори кишковика.

A. pleuropneumoniae є збудником висококонтагіозних та смертельних респіраторних інфекцій, що завдає значних економічних збитків світовій галузі свинарства. Через підвищену резистентність до антибіотиків існує нагальна потреба в пошуку нових альтернативних засобів для лікування інфекцій, викликаних *A. pleuropneumoniae*.

МРХ отримують з отрути оси і він впливає на різні бактерії пригнічуючи їх ріст. Дослідженнями було встановлено, що МРХ має виражений ефект знищення *A.pleuropneumoniae* при цьому мінімальна інгібуюча концентрація (MIC) становила 16 мкг/мл. Бактеріальна щільність *A. pleuropneumoniae* зменшилася в 1000 разів після обробки МРХ (1 × MIC) протягом 1 години, при чому на антибактеріальну активність не вплинули pH та температура. Флуоресцентна мікроскопія показала, що МРХ (1 × MIC) руйнує мембрану бактеріальної клітини після обробки протягом 0,5 год, підвищуючи проникненість мембрани та вивільняючи бактеріальні білки та Ca2+, Na+ а також інші катіони. Крім того, обробка МРХ (1×MIC) значно зменшила утворення бактеріальних біоплівок. Кількісні результати RT-PCR показали, що лікування МРХ значно посилювало експресію гена вірулентності PurC і знижувало експресію ApxI, ApxII і Apa1. Крім того, було виявлено, що ген Sap A відіграє важливу роль у переносимості *A. pleuropneumoniae* до антимікробних пептидів. Терапевтична оцінка на моделях мишей показала, що МРХ захищає мишей від смертельної дози *A. pleuropneumoniae* і знімає запалення легенів. Це свідчить, що МРХ можна використовувати для

лікування інфекцій викликаних *A.pleuropneumonia*, це дає підставу для розробки нових ліків для профілактики бактеріальних інфекцій.

Escherichia coli — це факультативно-анаеробна бактерія, яка існує в шлунково-кишковому тракті людини і тварин. Ця бактерія може спричинити діарею, ентерит, руйнування кишкового бар'єру та мікроекологічні порушення кишечника. В останні роки через зловживання традиційними антибіотиками з'явилися різноманітні стійкі до ліків штами мікроорганізмів та супербактерії.

Тому існує нагальна потреба в пошуку нових альтернатив антибіотикам. В зв'язку з цим було досліджено анитибактеріальний ефект МРХ по відношенню до *E. coli*.

Антимікробну дію МРХ проти *E. coli* було виявлено за допомогою МІК, підрахунку чашок, йодиду пропідію, тесту на проникність NPN і DiSC3(5), спостереження під імунофлуоресцентним мікроскопом і впливу стабільності МРХ за температурою, pH, іонами. Результати цього дослідження показали, що МРХ має виражену антибактеріальну активність проти *E. coli*, а мінімальна інгібуюча концентрація (MIC) становила 31,25 мкг/мл. Дослідження кінетики бактерицидності МРХ довело, що МРХ має високу бактерицидну активність протягом 6 годин. Дослідження бактеріальної проникності показали, що МРХ може збільшити проникність бактерій, що призведе до збільшення вмісту білка в супернатанті бактерій. Крім того, результати NPN, PI та DiSC3(5) показали, що значення флуоресценції позитивно корелювало з МРХ.

Тест на стабільність МРХ виявив, що іони солі, температура, pH тощо мають незначний вплив на його дію. Крім того, результати скануючої електронної мікроскопії показали, що після дії МРХ бактерії різко зменшилися в розмірі та порушилася клітинна оболонка. Отримані результати показали, що МРХ має високу бактерицидну активність *in vitro*, заклавши основу для розробки нових препаратів для лікування бактеріальних інфекцій.

Також нами було досліджено, чи був антимікробний пептид мастопаран X (MPX) ефективним проти інфекції *E. coli*. Використовували мишей BALB/c, інфікованих кишковою паличкою шляхом внутрішньочеревної ін'єкції, що представляло модель сепсису. Терапевтичний ефект MPX оцінювали на моделі мишей, при цьому виявили, що він захищає мишей від смертельної інфекції обумовленою *E. coli*. Крім того, MPX збільшив довжину ворсинок і зменшив інфільтрацію запальних клітин у тонкій кишці. Аналіз SEM і TEM показав, що MPX ефективно зменшує пошкодження порожньої кишки, викликане *E. coli*, і збільшує кількість і довжину мікроворсинок.

Крім того, MPX знижував експресію IL-2, IL-6, TNF-α, p-p38 і p-p65 у тонкій та товстій кишці. Доведено, що MPX підвищував експресію ZO-1, оклюдину та MUC2 у тонкій та товстій кишці, покращував функцію кишкового бар’єру та сприяв засвоєнню поживних речовин. Це дослідження свідчить про те, що MPX є ефективним терапевтичним засобом для лікування ешерихіозу та інших кишкових захворювань. MPX не виявляв токсичності в клітинах IPEC-J2 і помітно пригнічував індуковані рівні інтерлейкіну-6 (IL-6), фактора некрозу пухлини-альфа (TNF-α), мієлопероксидази (MPO) і лактатдегідрогенази (LDH). Крім того, MPX покращив експресію ZO-1, оклюдину та клаудину та покращив загоєння ран клітин IPEC-J2.

Дослідженнями доведено, що антимікробні пептиди можуть регулювати кишкову флору та підтримувати кишковий гомеостаз. Результати досліджень показали, що MPX має високу терапевтичну дію на запалення кишечника, спричинене інфекцією *E. coli*. Однак зв’язок між зниженням запалення кишечника та кишковою мікробіотою MPX досі невідомий і потребує додаткових досліджень. При проведенні експериментів ми довели кореляцію між протизапальними ефектами MPX і регуляцією кишкової мікробіоти.

E. coli використовували для індукції кишкового запалення, і результати показали, що MPX полегшує втрату ваги та кишкові патологічні зміни у зразках аутопсії *E. coli*. інфікованих мишей. MPX знижував сироваткові рівні

пов’язаних із запаленням білків IL-2, IL-6, TNF-α, MPO та LDH на 7 і 28 день.

Крім того, результати H&E показали, що МРХ збільшив довжину ворсинок і зменшив інфільтрацію запальних клітин тонкої та товстої кишки. Результати SEM і TEM показали, що МРХ може покращити морфологію ворсинок тонкої кишки та мікроворсинок і підвищити рівень білка щільного з’єднання. Аналіз секвенування 16S рРНК зразків вмісту сліпої кишки показав, що видова різноманітність мікрофлори були нижче в групі, інфікованій *E. coli.* На рівнях типу та роду спостерігалася більша чисельність патогенних бактерій *Firmicutes, Lachnospiraceae*-NK4A-136 та *Alistipes.* При використанні МРХ для лікування дало можливість зменшити кількість патогенних бактерій у групі, інфікованій *E. coli.* Крім того, МРХ збільшив чисельність *Muribaculaceae.*

Альфа- і бета-аналіз показав, що не було суттєвої різниці в структурі бактеріальної асоціації між МРХ і контрольними групами. Крім того, зміни в кишковому мікробіомі мишей вплинули на фізіологічні функції та метаболічні шляхи. Загалом це дослідження є першим у дослідженні кореляції впливу МРХ на кишкове запалення та кишкову мікробіоту, які знижують кишкове запалення шляхом відновлення різноманітності та функцій кишкової мікробіоти, забезпечуючи новий погляд на лікування бактеріального ентериту.

Таким чином, результати цих досліджень довели, що МРХ має високу антибактеріальну активність по відношенню до *A. pleuropneumoniae* та *E. coli* як *in vitro* так і *in vivo*.

МРХ може профілактувати патологічне запалення легенів і кишечника мишей, а також має лікувальний ефект при пневмоніях, ентеритах, підвищувати кишковий бар’єр у мишей.

Дослідженнями доведено, що МРХ полегшує запалення кишечника, регулюючи спектр кишкової мікрофлори.

За матеріалами дисертації розроблено методичні рекомендації «Профілактика антибіотикорезистентності шляхом застосування антимікробних пептидів», затверджені Вченою радою СНАУ (протокол № 5 від 29.03.2021).

Матеріали дисертаційної роботи використовуються при вивченні курсів «Ветеринарна мікробіологія», «Ветеринарна фармакологія» для магістрів факультету ветеринарної медицини Сумського НАУ.

А також для курсів «Ветеринарна мікробіологія» та «Ветеринарна фармакологія» для магістрів Хенанського інституту науки та технологій (HIST).

Ключові слова: антимікробний пептид МРХ, бактерії, кишкові епітеліальні клітини, запалення, кишковий бар'єр, кишкова мікробіота, мікрофлора, травлення, імунітет, *E. coli*, патогенна мікрофлора, спектроскопія, ефективність, резистентність, *Salmonella enterica*, *Staphylococcaceae*, спостереження, ПЛР, бактерицидна дія, мікроскопія.

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CONTENT

p.

LIST OF CONDITIONAL ABBREVIATIONS

A. pleuropneumoniae- Actinobacillus pleuropneumoniae

CIP -Ciprofloxacin

DNA - Deoxyribonucleic acid

E. coli- Enterohemorrhagic Escherichia coli O157:H7 ATCC43889

ELISA - enzyme linked immunosorbent assay

Enro - nrofloxacin

H&E - hematoxylin and eosin

IL-2 - interleukin-2

IL-6 - interleukin-6

IPEC-J2 - Porcine intestinal epithelial cells

LB - Lurla-Bertani both

LDH - lactate dehydrogenase

MIC - Minimum inhibitory concentration

MPO - myeloperoxidase

MPX - antimicrobial peptide MPX

MUC2 - mucin 2

OD - Optical Density

PI - Propidium iodide

qRT-PCR - Real-time Quantitative Polymerase Chain Reaction

SEM -scanning electron microscopy

TEM -transmission electron microscopy

TNF-α - tumor necrosis factor-α

TSB - Tryptic Soy Broth

INTRODUCTION

Actuality of theme. Due to the long-term unreasonable use of antibiotics, bacterial resistance has increased, and the anti-inflammatory effect in inflammation-related diseases such as pneumonia, enteritis and other inflammation-related diseases is not significant, and antibiotic treatment disturbs the normal homeostasis of intestinal flora. Therefore, it is urgent need to find alternatives to antibiotics. Antimicrobial peptides are a class of small molecular peptides produced by the body against pathogen infections. They are an important part of the body's innate immune system and have many biological functions such as antibacterial, anti-inflammatory, immune regulation, and maintenance of the intestinal tract. To reduce the use of antibiotics in clinical, the aim of this study is to explore the bactericidal effect of MPX on *A. pleuropneumoniae* and *E. coli*, the anti-inflammatory activity of the pneumonia and intestinal inflammation caused by *A. pleuropneumoniae* and *E. coli*, and its effect on the homeostasis of the intestinal flora, providinga reference for the clinical substitution of antibiotics for antibacterial and anti-inflammatory drugs.

Relationship with academic programs, plans, themes. The dissertation is a fragment of scientific programs of research work of the National Natural Science Foundation of China(No. 31702259 and 31520103917), Young Talent Lifting Project in Henan Province (2020HYTP041), Key Scientific Research Projects of Colleges and University in Henan Province (21A230004), Youth Backbone Teacher Project of Colleges and Universities of Henan Province (2020GGJS162), Climbing Project of Henan Institute of Science and Technology (2018JY02) and Program for Innovative Research Teams (in Science and Technology) at the University of Henan Province (20IRTSTHN025). The materials of the dissertation work are part of comprehensive scientific research of the Department of Veterinary Expertise, Microbiology, Zoohygiene and Safety and Quality of Livestock Products of the Sumy National Agrarian University according to the following thematic plans of research works: "System of monitoring methods of control and

veterinary and sanitary measures, regarding the quality and safety of livestock products in diseases of infectious etiology" (state registration No. 0114U005551, 2014-2019); "Forecasting the risks of cross-border introduction and spread of particularly dangerous animal diseases and the development of scientifically based disinfection systems based on innovative import-substitutable highly effective means" (state registration No. 0115U001342, 2018-2023).

The aim and objectives of the study. To investigate mechanism of the antimicrobial peptide mastoparan X in killing Gram-negative bacteria *in vitro* and its anti-inflammatory and barrier repair functions in pneumonia and enteritis diseases *in vivo*, and to further study the relationship between anti-inflammatory and intestinal microbes of MPX. Finally, to explore the anti-inflammatory and barrier repair functions mechanism of MPX in the intestinal epithelial cells, laying a foundation for reducing the use of antibiotics in livestock and poultry breeding, it will help provide certain theoretical and practical value for future applications in livestock and poultry.

To achive this goal it was necessary to solve the following tasks:

1. To investigate the mechanism of MPX in killing *A. pleuropneumoniae* in vitro, and the anti-inflammatory effect of MPX against pneumonia caused by *A. pleuropneumoniae* infection *in vivo*.

2. To determine the bactericidal mechanism and effect of MPX in killing *E. coli in vitro*.

3. To investigate the effect of MPX against intestinal inflammation caused by *E. coli* infection and protecting the intestinal barrier *in vivo*.

4. To determine the mechanism of anti-inflammatory and protective barrier functions of MPX at the cellular level.

5. To explore the relationship between the anti-inflammation effect and the intestinal flora of MPX further analyzed in mice.

Object of study – pharmacolo-toxicological, antibacterial and anti-inflammatory assessment of MPX.

Subject of study –The antibacterial, anti-inflammatory and intestinal barrier

protective functions, and its influence on intestinal microbes of MPX.

Research methods – microbiological (microscopic, PCR examination biological), bacteriological (the antibacterial efficiency of MPX.), pharmacological, toxicological (degree of toxicity and harmlessness of MPX, acute and chronic toxicity, pharmacokpnetics, pharmacodynamics), statistical (processing of research results).

Scientific novelty of obtained results. The scientific novelty of our research result is that antibacterial mechanism, anti-pneumonia and enteritis function were studied in detail for the first time, anti-inflammatory and protection barrier mechanisms protection were explored at the cellular level, and the relationship between the anti-inflammatory function and the intestinal flora of MPX was further studied.

The practical significance of the results. The results of this study lay the foundation for the development and use of clinical antibacterial drugs against *A. pleuropneumoniae* and *E. coli*. It also lays the foundation for the selection of anti-inflammatory and intestinal microflora drugs. In addition, it provides theoretical support for reducing clinical antibiotic resistance against *A. pleuropneumoniae* and *E. coli* infection.

The main provisions of the PhD thesis were included in the Guidelines according to Prevention of antibiotic resistance through the use of antimicrobial peptides, approved by the Academic Council of SNAU (Protocol № 5, dated 29.12.2021).

The dissertanion materials are included in the silabus, work program of courses "Veterinary microbiology", "Veterinary pharmacology" for masters of the Faculty of Veterinary Medicine of Sumy NAU, and are used in distance learning of students based on the platform «Moodle».

And for the courses "Veterinary microbiology" and " Veterinary pharmacology " for masters of the Henan Institute of Science and Technology (HIST).

Personal contribution of PhD. The author took part in the implementation of scientific programs, which are the basis of PhD thesis; develohed schemes and methods of conducting experiments in laboratory. Setting objectives, discussing the results, forming conclusions were conducted together with tutors. PhD student analyzed literature and patent search on the topic of the dissertation; conducted experimental research using modern methods with co-authors of scientific papers. The applicant wrote dissertation and published articles in wich the main material of the PhD thesis stated. The author thanks to Heards and staff of laboratories for help.

Testing the results of the thesis. The main provisions of the dissertation were reported and discussed and approved at a meeting of:

- Annual scientific-practical conferences of teachers, graduate students and students of Sume National Agrarian University, Sumy, 2018-2021;
- BTRP Ukraine Regional One Health Research Symposium (20-24 May 2019, Kyiv, Ukraine);
- Chinese Society of Microbiology Veterinary Microbiology, 2021 Academic Forum (June 19-21, 2021. Zhengzhou, China);
- One Health Student International Conference (24 - 27 November, 2021, Bucharest, România).

Publications. According to the results of research, 21 scientific papers were published, including: 4 articles in professional editions of Ukraine, 5 articles in scopuse journals, 2 articles in scientific professional poublications of China, 8 theses of scientific reports, 1 – Patent, 1 methodological recommendations.

Structure and scope of the thesis. The dissertation is set out on 180 pages of computer text. The work is illustrated with 6 tables, 39 figures, and consists of annotation, introduction, review of literature, materials and methods, results of own research, generalization, analysis and discussion of research results, conclusions, proposals, list used sources, applications. The list of used sources of literature includes 230 names.

CHAPTER 1.

LITERATURE REVIEW ON THE TOPIC AND CHOICE OF RESEARCH DIRECTIONS

1.1 Overview of antimicrobial peptides

Since the discovery of penicillin by British Fleming [1] in the last century, antibiotics have been a powerful weapon for clinical treatment of pathogenic infections. With the widespread and long-term use and abuse of antibiotics, coupled with the rapid propagation of microorganisms and their life cycles short, highly mutated, easy to produce variants that evade antibiotic attack and stably inherited, leading to the emergence of resistant and super resistant strains in recent years. Antibiotic resistance is a normal ecological phenomenon that prevails in the environment. It has quickly become one of the major issues that need to be solved globally. According to conservative estimates, the global antibiotic consumption in food animal production was 63,151 tons in 2010 [2]. It is expected to reach 10 million antibiotic-resistant infections by 2050, and antibiotic resistance has become an increasingly serious cause of human death worldwide and more than the current death toll associated with various cancers [3]. More and more bacteria are becoming resistant to common antibiotics, which has caused increasing attention to public health. The emergence of multi-drug resistant microorganisms in 1950s such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus*, which is mainly due to antibiotic abuse [4, 5]. In addition, the number of new antibiotics approved has been significant reduced in the past three decades. Small-scale outbreaks of NDM-1 in 16 countries including Britain, the United States, and India in 2010, which is resistant to most antibiotics [6]. With drug resistant pathogens causing increased morbidity, mortality and overall healthcare costs in both human and veterinary medicine, rising prevalence of antibiotic resistance in bacteria

leading healthcare priority worldwide [7]. Although it is difficult to estimate, bacterial antimicrobial resistance has been reported to cost the European Union economy approximately € 1.5 billion and the US health-care system between $ 21 billion to $ 34 billion per year [8]. Therefore, it is an urgent need to find new type of antibacterial agent with broad spectrum and high efficiency, not easy to produce drug resistance, and good safety to replace antibiotics. Antimicrobial peptides rely on their own advantages have gradually become a research hotspot in recent years.

Antimicrobial peptides (AMPs) are one of the alternatives to conventional antibiotics currently being explored and have aroused great interest in the scientific community. Antimicrobial peptides are a class of small molecule peptides produced by the body to resist the invasion of pathogenic microorganisms when pathogenic microorganisms invade. They constitute the first line of defense of the body's innate immune system and are resistant to most resistant bacteria and viruses, fungi, etc. [9]. Antimicrobial peptides come from a wide range of sources, which are found in most organisms, and can be isolated from insects, invertebrates, mammals, and even humans. Antimicrobial peptides are small-molecule peptides with short amino acid sequences and a composition of no more than 100 amino acids. They are hydrophilic and hydrophobic. Most antimicrobial peptides have a positive charge and therefore have stronger antibacterial activity [10]. Antimicrobial peptides have a small molecular weight, are easily soluble in water, resistant to high temperatures, resist protease hydrolysis, have a broad antibacterial spectrum and the antibacterial mechanism different from traditional antibiotics [11]. The APD3 database currently lists more than 2,600 antimicrobial peptides from all organisms: bacteria, archaea and eukaryotes (including plants, animals, fungi and protozoa) [12]. In addition, the number of mimetic peptides synthesized using natural antimicrobial peptides as templates has reached thousands, and this number continues to grow. In summary, antimicrobial peptides have a wide range of sources, are easily isolated, have low cost, and have unique mechanisms of action. They are expected to be the best drugs to replace antibiotics.

1.2 Antimicrobial peptide length

Most AMPs display variable sequence lengths, ranging from 10 to 60 amino acid residues. However, shorter AMPs are more conducive to reducing production costs, and many short AMPs have similar antibacterial efficacy to clinical isolates compared to longer AMPs; for example, hexapeptide MP196 (RWRWRW-NH2) shows strong antibacterial activity against *E. coli* and *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) of 5 μg/mL [13]. In addition, long chain linear peptides are generally more hemolytic and cytotoxic, but their truncated N- or C-terminated sequences have lower cytotoxicity and still retain strong activity [14]. However, the peptide length is too short to reduce the tendency to form amphiphilic secondary structures, which is related to reduced membrane disruption and antibacterial efficacy. It indicates that AMPs with a certain length threshold can form a spiral structure when combined with a membrane with high affinity [15].

1.3 Amino acid composition

Although the structure of AMPs varies greatly, it mainly includes two types of amino acid residues: cationic residues and hydrophobic residues [16]. In naturally AMPs, cations reside in Arg, Lys, and His, whereas hydrophobic and mainly aliphatic and aromatic amino acids [17]. In addition, Cys and Pro residues are conserved in natural AMPs. It is generally accepted that positively charged residues of AMPs directly interact with the negatively charged components of bacterial cells. Then, the hydrophobic residues get incorporated into lipid bilayers to mediate membrane permeabilization and disruption, which lead to rapid cell death.

1.3.1 Characteristics of the secondary structure

According to the three-dimensional structures of AMPs can be classified into four major classes: α-helix, β-sheet, extended and loop peptides.

α-Helix

According to the updated database (APD: http://aps.unmc.edu/AP/main.php), natural α-helix peptides are the highest in AMP [18]. They are mainly from different species, including insects, fish, amphibians, mammals and plants. In addition, studies have shown that most peptides exhibit membrane-like properties or membrane interactions that are converted into alpha helix structures. This transformation results in the separation of the hydrophilic/charged amino acids from the hydrophobic residues in the space, thereby forming an amphiphilic structure, which is considered a prerequisite for AMPs to act on the cell membrane [16, 19]. The antimicrobial peptides of Cathelicidin family discovered for the first time is the antimicrobial peptide Cathelicidin-BF (C-BF) snake-derived in reptiles. It has a spectral antibacterial activity and a strong bactericidal activity against gram-negative bacteria. Its secondary structure is α-helix conformation [20]. The antimicrobial peptide BF15, which consists of 15 amino acids intercepted by the C-terminal phthalamide portion of the antimicrobial peptide C-BF, has similar functions to the antimicrobial peptide C-BF and also has broad-spectrum antibacterial activity. The amphiphilic α-helix structure enhances the bacterial cell plasma membrane [21]. The electrostatic interaction between the positively charged residues of AMPs and the negatively charged components of the bacterial membrane has been widely reported as the main antibacterial mechanism of most AMPs, including melittin, mylanin and antibacterial peptides, and the non-polar surfaces of these peptides can further penetrate cell membranes [22-24].

β-sheet

In addition to the α-helix, β-sheet is another major secondary structure of membrane-inducible environment-induced AMPs. In addition, most β-sheet peptides change their conformation from unstructured peptides to β-sheet

structures in a membrane mimicry environment in aqueous solutions [25, 26]. Generally, β-sheet peptides consist of 2 to 10 Cys residues, forming 1 to 5 disulfide bonds, and serve as conformational constraints to stabilize their biologically active conformation. In addition, disulfide-containing peptides often have a cyclic β-hairpin conformation, such as tachykinin and protegrin 1 [27].

1.3.2 Randomly extended antimicrobial peptides

Randomly extending antibacterial peptides have a linear morphology without a typical structure (Figure 1.1). They are common in antibacterial peptides rich in glycine or proline, and they do not undergo conformational changes like α-helical antibacterial peptides when exerting antibacterial activity [28]. Glycine-rich antibacterial peptides are most commonly found in insect-derived antibacterial peptides, and can inhibit the formation of outer membrane proteins by interfering with the bacterial outer membrane protein transcription or binding with lipopolysaccharides, such as Saroloxin II and Attacins C [29]. Proline-rich antibacterial peptides usually have intracellular sites and can exhibit antibacterial activity through a variety of pathways including functional proteases and geneticmaterial.

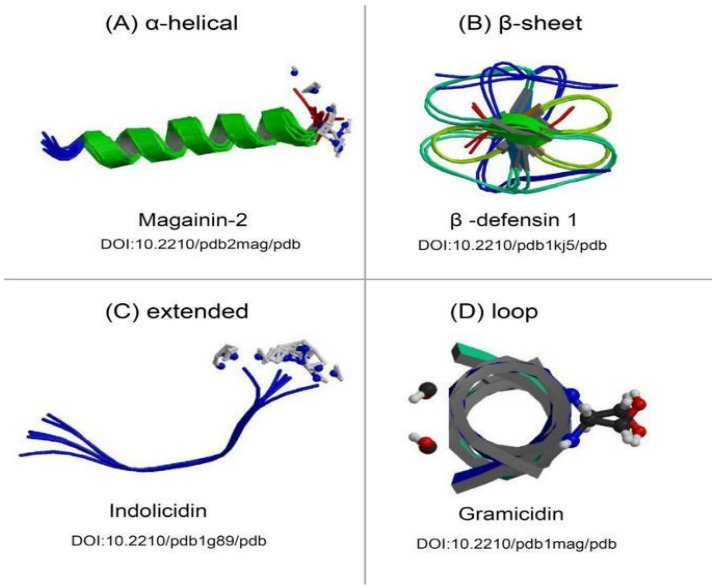

Figure 1.1. Protein models representing the structural differences of the four classes of antimicrobial peptides [30]. Antimicrobial peptides can be grouped

into four major classes based on their secondary structures, including the (A) alfa-helical peptides, (B) beta-sheets peptides, (C) peptides that adopt unconventional structures, such as extended helices, and (D) peptides that assemble into loops.

1.4 The functions of antimicrobial peptides

As the problem of antibiotic resistance caused by the abuse of antibiotics has become more and more serious, which has brought great harm to the aquaculture industry and human health, people have begun to look for drugs that can replace antibiotics. Antimicrobial peptide has broad antibacterial spectrum, regulatory effect on immune function, and inhibitory effect on the growth of bacteria, fungi, viruses and so on. With the continuous research, people found that antibacterial peptides not only have unique bactericidal mechanism, but also have anti-inflammatory and promote wound healing functions.

1.4.1 Antibacterial activity

Magainin isolated from the skin of Xenopus laevis is a family of peptides with broad-spectrum antibacterial activity. Magainin 2 (synthetic) has good antibacterial activity against a variety of Gram-negative and Gram-positive bacteria such as *E. coli*, *Staphylococcus aureus* and *Klebsiella* pneumonia [31]. Limnochariin (synthetic) is new antibacterial peptide derived from the skin of amphibians and has antibacterial activity against 4 gram-positive bacteria and 11 gram-negative bacteria [32]. Hylaranins is a new amphibious antibacterial peptide in the skin secretion of the oriental folding frog Hylarana latouchii [33]. Defense and cathelicidins are the two main classes of antibacterial peptides identified in mammals, and defensins exhibit broad antibacterial activity against antibiotic-resistant bacteria [34]. Cathlicidin peptide LL-37 isolated from humans shows strong bactericidal activity against *Pseudomonas aeruginosa*, *Salmonella*

typhimurium, *E. coli*, *Listeria monocytogenes* and *Staphylococcus aureus* [35]. Wang et al found that antimicrobial peptide JH-3 has good antibacterial activity against *Salmonella* CVCC541, and could relieve intestinal inflammation in mice and protect mice from lethal attack by *Salmonella* CVCC541 [36].

1.4.2 Antiviral activity

Many AMPs are reported to be viral inhibitors. Currently the most studied is the antimicrobial peptides against enveloped viruses such as herpes simplex virus (HSV), A immunodeficiency virus (HIV) and influenza A virus (IAV). Antimicrobial peptides have antiviral activity and are closely related to positively charged and highly hydrophobic antibacterial peptides. Antimicrobial peptides destroy the virus by binding to the envelope structure of the virus, which is similar to the mechanism of bacterial cell membranes in the antibacterial mechanism of antimicrobial peptides [37]. Magainin family antimicrobial peptides in amphibians have been studied more in antiviral activity. Studies have found that Magainin1 and Magainin2 have high specificity, obvious inhibitory effect on HSV virus, and have almost no effect on normal cells [38]. Morbidity and mortality associated with viral infectious diseases is an escalating issue, especially with the emergence of resistant virus strains. Therefore, it is important to develop new and alternative antiviral agents [10]. Natural and safe antibacterial peptide subtilosin has goodantiviral activity against herpes simplex virus 1 (HSV-1) [39]. Human cathelicidin LL-37 and murine cathelicidin mCRAMP have significant antiviral activity against influenza virus in vivo and vitro [35]. Antimicrobial peptide dermaseptin S4 prevents human immunodeficiency virus (HIV) infection by destroying the integrity of the virion [40]. Similarly, LL-37 can directly interact with the virus to inhibit multiple influenza A viruses strain infection [41]. In addition to directly inactivating virus particles, AMPs prevent viruses from entering cells or antagonize viral proteins fused to target cells through specific cellular receptor interactions involved in virus internalization [42, 43]. The activity

of antiviral AMPs appears to be related to direct interaction with the virion, or indirect effects through interaction with potential target cells.

1.4.3 Antifungal activity

Antifungal peptide has strong antifungal activity are mainly manifested on the fungal cell wall and the activity of inducing reactive oxygen spries (ROS), such as indolicidin, LL-37, BMAP, etc. ROS-mediated oxidation-reduction reaction leads to fungal apoptosis and is often used to study the antifungal activity of drugs. Wang et al. found that the antimicrobial peptide polybia-CP could induce the increase of ROS in fungal cells and affect the function of mitochondria [44]. LL-37 can not only form holes in fungal cell walls [45], but also penetrate the fungal cell membrane to induce ROS production and induce apoptosis by affecting mitochondrial function [42].

1.4.4 Anti-inflammatory

Bacterial infection of the body often produces inflammatory response. Due to the abuse of antibiotics, the anti-inflammatory effect of many antibiotics is poor. People find that antimicrobial peptides have good anti-inflammatory effects in the process of searching for alternatives to antibiotics. Studies found that antimicrobial peptides myxinidin2 and myxinidin3 inhibited the secretion of IL-6, IL-8 and TNF-α in mouse skin wound models infected with bacteria, reduced the skin inflammation and promoted wound healing in mice [43]. Wang Q et al. used a mouse endotoxemia model to detect the levels of IL-1β, IL-6 and TNF-α in the serum by ELISA method, and found that the antimicrobial peptide AWRK6 had good protective effect on mouse endotoxemia and reduce the levels of IL-1β, IL-6 and TNF-α in serum, and inflammatory response in mice [46]. Studies found that synthetic anti-lipopolysaccharide peptides (SALP) Pep19-2.5 and Pep19-4LF could alleviate the inflammatory response in keratinocytes, Langerhans cells, and

dendritic cells, and Pep19-2.5 significantly reduced LPS-induced human single nuclear cell inflammatory response [47]. Banaschewski et al. found that the combined use of the antimicrobial peptide CATH-2 and the exogenous surfactant BLES could reduce the recruitment of inflammatory cells and regulate the production of proinflammatory cytokines after mice infected with Pseudomonas aeruginosa, and antimicrobial peptide CATH-2 in the early stage of inflammation could significantly reduce the recruit ability of inflammatory cells [48]. Studies have found that antimicrobial peptides such as human β-defensin and LL-37 could inhibit the phosphorylation of ERK and p38, activate mast cells, and then suppress the inflammatory response [49].

1.4.5 Antimicrobial peptides regulate barrier function

The intestinal barrier is not only the main place for the digestion and absorption of nutrients in the intestine of animals, but also an important line of defense for the body to prevent the invasion of harmful substances such as pathogens and toxins in the intestinal cavity [50]. The intestinal barrier is composed of the intestinal epithelial barrier, immune barrier, biological barrier and chemical barrier. These four barriers are organically combined through their respective signal pathways to maintain the homeostasis of the intestine and the health of the body [51]. The epithelial cells of animal intestines, respiratory tract and urinary tract tissues secrete antimicrobial peptides. Antimicrobial peptides not only play a bactericidal and immunomodulatory function, but also enhance the body's resistance to bacterial infections by enhancing the barrier function of epithelial tissues. LL-37 selectively increases the expression of Claudins and Occludin through the Rho-Rac 1 pathway, increases cell transmembrane resistance (TER), and enhances cell barrier function [52]. Antimicrobial peptide hBD3 increases the phosphorylation of MLCK2 and the accumulation of F-actin by activating Rho stimulating the migration of intestinal epithelial cells, protects the integrity of the intestinal barrier, and relieves mouse enterocolitis [53].

Antimicrobial peptides C-BF and LFP-20 can increase the expression of intestinal tight junction proteins, alleviate the damage of tight junction structures, and protect the physical barrier function of the intestine [54, 55]. Snake-derived antimicrobial peptide C-BF increase the expression of the tight junction proteins Occludin and ZO-1 of IPEC-J2 in porcine through MAPK signaling pathway, and enhance the epithelial barrier function [56]. Studies have shown that porcine β-defensin 1 (pBD1) and porcine β-defensin 2 (pBD2) can enhance the gene expression of tight junction protein and mucin in porcine intestinal epithelial cells [57]. In addition, antimicrobial peptide PR-39 enhances the transmembrane resistance of small intestinal epithelial cells, increases the expression of tight junction proteins, and enhances the physical barrier function of the intestine may be through upregulation of Rho-Rac 1 Pathways signal. The above research indicates that antimicrobial peptides can regulate the intestinal epithelial barrier function.

1.4.6 Antimicrobial peptides improve animal performance

The application of antimicrobial peptides in production can improve production performance and animal immune function. Antimicrobial peptide Catesbeiania-1 obtained from the skin of bullfrogs after adding in Hailan brown laying hens found that the antimicrobial peptide Catesbeiania-1 significantly improved the immunity of the late laying hens. The egg production rate of chicken was increased by about 3.00% after 10 mL/kg antimicrobial peptide added group, the feed-to-egg ratio decreased by about 2.23%, the total protein was significantly increased compared with the control group, and the conversion rate of chicken spleen lymphocytes increased significantly [58]. Study found that antimicrobial peptides improved the growth performance of swine is closely related to the digestibility of swine nutrients. Antimicrobial peptides isolated from potatoes could increase the apparent digestibility of dry matter in weaned piglets [59]. Antimicrobial peptides A3 and P5 have important functions in increasing dry matter, crude protein and total energy digestion of weaned piglets [60, 61]. After

adding 400mg/kg cecropin AD to the piglet diet, the feed conversion rate and nitrogen content in the feed were improved [62]. The antimicrobial peptide tilapia piscidin 4 (TP4) was derived from Oreochromis niloticus, possesses antimicrobial activities and immunomodulatory properties, promotes intestinal health improve growth performance and prevent pathogen infection in Gallus gallus domesticus [63]. Chicken NK-lysin peptide 2 (cNK-2) is a natural lytic peptide with direct cytotoxicity against many apicomplexan parasites, the results found that oral treatment of young chickens with cNK-2 improved growth performance, enhanced gut integrity, and reduced fecal oocyst shedding [64]. Antimicrobial peptides (ABPs) could improve economic benefits by promoting growth, preventing disease, and reducing the rate of death [65]. The results show that antimicrobial peptides have important function in improving production performance.

1.4.7 Antimicrobial peptides maintain intestinal microbiome homeostasis

As a symbiotic organism of the host, gut microbes can improve digestion, absorption, synthesis of vitamins and inhibit the growth of pathogenic bacteria [66]. The antibacterial defense system and diversified microorganisms have evolved over a long period of time, and a variety of defense mechanisms have been established to ensure the mutually beneficial coexistence of microorganisms and host intestinal cells [67]. The most representative antimicrobial peptides in the intestines are defensins, cathelicidins (such as LL-37), C-type lectins, ribonuclease (RNases) and S100 protein (such as calprotectin), which play an important role in regulating the dynamic balance of intestinal microflora structure and intestinal mucosal barrier [68-70]. Previous studies found that antimicrobial peptides were mainly reducing the number of harmful bacteria in swine intestines and increasing the number of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* to maintain intestinal health [71]. Compared with the control group, lactoferrin significantly reduced the total viable count of *E. coli* and *Salmonella* in swine small intestine, and increased the number of *Lactobacilli* and *Bifidobacteria* [72].

Adding antimicrobial peptide CWA to the diarrhea piglet diet significantly reduced the ratio of *E. coli* to total bacteria in the feces, and increased the ratio of *Lactobacilli* to total bacteria, indicating that antimicrobial peptide CWA had a positive effect on the regulation of intestinal microbiota [73]. Adding potato protein to the diet of weaned piglets can significantly reduce the number of *E. coli* and bacteria in the contents of feces, cecum, colon and rectum [74]. In addition, antimicrobial peptides have the effect of plasticizing microbial communities in the intestinal tract. Wu et al. found that antimicrobial peptides used in pigs also could increase the number of cecal *Lactobacillus* and total anaerobic bacteria, and reduce the number of *E. coli* and total aerobic bacteria, adding to the pig feed gradually increased, the total anaerobic *E. coli*, *Coliforms*, and *Clostridia* in the ileum, cecum, and feces decreased linearly [75]. Studied on tilapia showed that antimicrobial peptides could effectively prevent the colonization of pathogenic bacteria in the intestine by changing the adhesion of pathogenic bacteria, and achieve the purpose of inhibiting the growth of pathogenic bacteria [76]. The above results indicate that antimicrobial peptides have the function of regulating the composition of intestinal microbes and maintaining intestinal homeostasis.

1.4.8 Immunomodulatory activity

AMPs are effectors of innate and adaptive immunity, regulating pro-inflammatory and anti-inflammatory responses, chemotaxis, and directly affect adaptive immunity [77]. Natural antimicrobial peptides as part of the innate immune system, most antimicrobial peptides are directly involved in resisting the invasion of foreign microorganisms while actively participating in the body's own immune regulation, such as defensins found in insects and host defense peptides in mammals. Some antimicrobial peptides are secreted by natural immune cells, which can regulate the immune system in multiple pathways and activate the NAPDH pathway, causing mutations or invasion of the nuclear and cell membranes of cells [78]. Antimicrobial peptides induce T lymphocytes, dendritic

cells, monocytes and other immune cells to release chemokines, mediate anti-inflammatory effects, and promote wound healing [32]. Antimicrobial peptide Cathelicidin-BF inhibited endotoxin-induced secretory protein inflammatory mediators, effectively prevented endotoxemia, and improved endotoxin-induced apoptosis [79]. Antimicrobial peptide LL-37 could inhibit the response of proinflammatory cytokines to bacterial lipopolysaccharide and lipoteic acid, prevent these bacterial components from activating macrophages, up-regulate the production of chemokines and chemokine receptors, and promote angiogenesis and wound healing [80]. Antimicrobial peptide IDR-1 protected mice from methicillin-resistant *Staphylococcus aureus*, *Vancomycin-resistant Enterococcus* and *Salmonella* infection, and affects several signaling pathways in human monocytesm, inhibiting the production of chemokines and suppresses the inflammatory response of immune cells [81]. In addition, the antimicrobial peptide LFP-20 alleviated the increase in LPS-induced proinflammatory factor release may be related to MyD88/nuclear factor-κB (NF-κB) and MyD88/ mitogen-activated protein kinase (MAPK) signaling pathways [82]. Antimicrobial peptide LL-37 formed a complex with its own DNA, activates DC cells of dendritic cells through the TLR9 signaling pathway, produced interferon and activated its own T cells [83].

1.5 Sterilization mechanism

The most successful antibiotics only hit three targets or pathways: the ribosome (affecting protein synthesis), DNA gyrase or DNA topoisomerase (affecting DNA synthesis), or cell-wall synthesis [84]. Other antibiotics target RNA synthesis, folic acid metabolism pathways and the bacterial cell membrane. Studies have found that most antimicrobial peptides have broad-spectrum antibacterial activity [85]. Because of AMP amino acid composition, amphiphilicity, cationic charge, molecular weight and antibacterial spectrum are different. Therefore, the mechanism of action of antimicrobial peptides is also different. The four mechanisms of antimicrobial peptides are mainly summarized in Figure 1.2. AMP

can directly interact with the membrane to form a transmembrane pore, or induce membrane depolarization [86]. In addition, AMPs could also interact with some important foreign proteins and transporters [87]. Antimicrobial peptides could form transmembrane potentials after interacting with bacterial cell membranes, thereby disrupting the acid-base balance of cell membranes, affecting bacterial osmotic pressure, and inhibiting bacterial respiration [88]. Bacterial membrane and antimicrobial peptides combined by electrostatic action, and antimicrobial peptides are adsorbed to the surface of the bacteria, such as magainin 2 and ceeropin A. After antimicrobial peptide are combined with the surface of the microorganism, and antimicrobial peptides further contacts the lipid bilayer to play the bactericidal function [89]. Su et al. found that antimicrobial peptide macropin combined with peptidoglycan and LPS by destroying the cell membrane, leading to bacterial death [90] (Figure 1.2A). Antimicrobial peptides interact with bacterial membranes and cause bacterial death.

Although antimicrobial peptides can form transmembrane pores and eventually cause membrane destruction and bacterial lysis and death. However, it is not the only mechanism of antimicrobial peptides kill microorganisms. In addition, AMPs block the synthesis of the cell wall by inhibiting the biosynthesis inside and outside of cell, such as proteins, nucleic acid lipoteichoic acids, causing abnormal metabolism of bacteria, leading to bacterial death [91]. Study found that antimicrobial peptide HNP-1 prevent the absorption and biological macromolecules synthesis of *E. coli*, indicating that antimicrobial peptides HNP-1 kill *E. coli* by inhibiting DNA, RNA and protein synthesis [92] (Figure 1.2 B). Antimicrobial peptides increase the K^+ efflux and the accumulation of Ca^{2+} in the cytoplasm and mitochondria, change the Ca^{2+} ion balance in the bacteria, and induce the production of ROS, which leads to bacteria death. Wang et al. found that antimicrobial peptide MPX has good bactericidal effect on *A. pleuropneumoniae* though promoting K^+ efflux and Ca^{2+} aggregation [93] (Figure 1.2 C). In addition, antimicrobial peptides also cause bacteria death by inhibiting ATP synthase activity, destroying ATP synthesis, reducing bacterial energy

metabolism and damaging enzymatic activity. Yang et al found that antimicrobial peptides could inhibit mycobacterium tuberculosis respiration, depolarize the membrane and consume ATP, leading to bacterial death [94]. Hassan Mahmood Jindal et al. found that the antimicrobial peptide RN7-IN8 released ATP after damaging bacterial cell membranes and inhibiting bacterial DNA synthesis, leading to bacterial death [95] (Figure1.2 D).

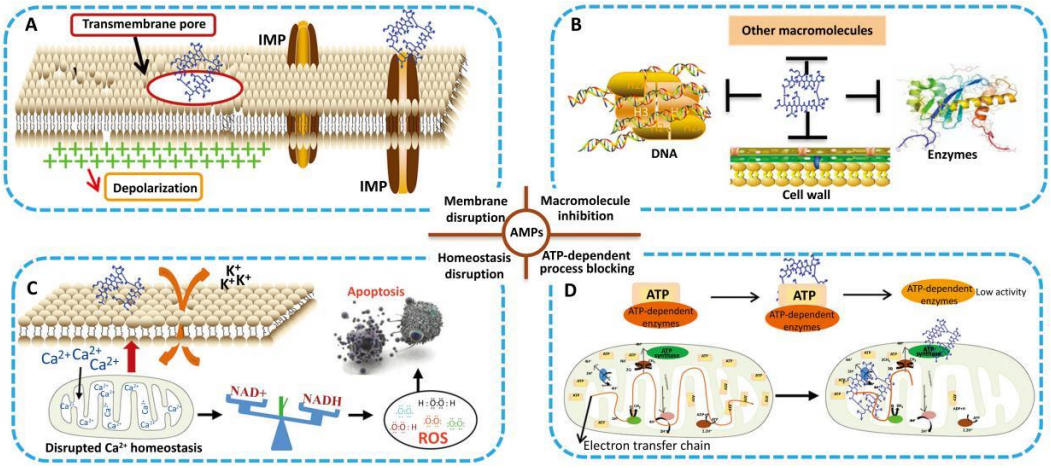

Figure 1.2. Action mechanisms of AMPs targeting microbial pathogens [96]. (A) AMPs target or bind cell membrane, and lead to membrane fluctuation, depolarization, structure disturbance or big transmembrane pores. AMPs can also interact with the important membrane proteins. +: enriched positive charge, IMP: important membrane protein. (B) AMPs inhibit intracellular or extracellular macromolecules, including synthesis of DNA, enzymes, peptidoglycan, which is the precursor of cell wall, and other macromolecules. (C) AMPs increase the concentration of Ca^{2+} in cytoplasm and mitochondria to disrupt Ca^{2+} homeostasis, then increase the ratio of NAD+/NADH, subsequently induce the generation of ROS and apoptosis. (D) AMPs interact with ATP directly and then decrease the activity of ATP-dependent enzymes. On the other hand, AMPs block ATP syntheses by interacting with ATP synthase or blocking the electron transfer chain. All these result in ATP-dependent processes blocking.

1.6 Inflammation

The inflammation caused by bacterial infections is always a threat to human health, although it is widely believed that existing antibiotics have an inhibitory effect on most inflammatory reactions caused by bacterial infections. With the irrational use of antibiotics leading to increased bacterial resistance, mild inflammatory reactions are beneficial to the body itself. However, excessive inflammatory reactions can cause diseases, such as sepsis and cause systemic inflammation. The mechanism of LPS-induced inflammation. First, LPS falls off from the cell wall. The binding protein LBP recognizes LPS and combines with lipid A in the blood circulation system to form the LPS-LBP complex. LBP transports LPS to the surface of monocytes or macrophages, and simultaneously binds to soluble CD 14 (sCD 14) on the surface of the cell membrane, forming the LPS-LBP-sCD 14 triple complex, which transduces LPS into the cell by Toll-like receptor (TLR4) transduces stimulus signals and causes inflammation [97]. Inflammation is a part of the tissue defense response of the vertebrate body to external stimuli and is an important part of the innate immune system. The body first recognizes pathogen-associated molecular pattern (PAMP) through the pattern recognition receptor (PRR) of the host cell, recruits activated monocytes, macrophages and other immune cells to migrate immune response in infected tissue sites, release inflammatory factors [98]. It has some harmful effects on the body although inflammation is the body's defense-adaptive immune response. Moderate inflammatory response is beneficial to the body's tissues. The body can quickly remove foreign damage factors and repair damaged tissues through inflammatory reactions. Excessive inflammation leads to serious diseases such as systemic inflammatory response syndrome (SIRS) and sepsis [99-101]. Inflammatory reactions cause pathological phenomena such as metamorphism, exudation and proliferation of body tissues, which leads to increased osmotic pressure of cells in the inflammatory area, degeneration and necrosis of cells, accumulation of metabolites and acidosis or alkalosis of local tissues, and

permeability of blood vessel walls [102, 103]. The factors that cause inflammation include biological factors, mechanical factors, immune factors, and physical and chemical factors. Bacterial infection is particularly common among biological factors.

1.7 The mechanism of antimicrobial peptides inhibits LPS-induced inflammation

(1) Inhibit the binding of LPS and LBP

After the bacteria die or break down, endotoxins are released into the blood, and these swimming molecules are recognized by LBP (LPS binding protein), which stimulates monocytes. The first step of the LPS-triggered inflammatory signaling pathway is recognition by LBP and binding to LPS to further transduce the inflammatory signal. Some antimicrobial peptide can occupy LBP binding sites on LPS, preventing LBP from binding LPS to prevent inflammatory signals from being transduced to cells, thereby inhibiting the inflammatory response. For example, antimicrobial peptide can inhibit the level of inflammatory factors produced by LPS in this way [104]. Studies have shown that the synthetic antimicrobial peptide HDLs can bind to LPS, thereby alleviating the inflammatory response caused by gram-negative bacteria infection. In addition, HDLs can also reduce the cellular inflammation response induced by LPS by down-regulating the expression of CD 14 in monocytes. The fragment peptides of LBP such as LBP-14, effectively inhibit LPS-induced inflammatory responses.

(2) Inhibit the binding of LPS and CD14

CD14 is an important inflammatory signaling pathway molecule. LPS binds to CD 14 and is anchored on the surface of immune cells through CD14 glycosylphosphatidylinositol. Some antimicrobial peptide can cover the area that recognizes CD14 on LPS, thereby blocking the transmission of LPS inflammation signals.

For example, the antimicrbial peptides CAP-11, CAP-18, and LL-37 derived

from the cathelicidin family can specifically bind to CD14 and occupy the recognition and binding site of LPS [105, 106]. Studies have found that Cathelicidins can interact with LPS and hinder their binding to CD14 molecules.

In addition, the Cathelicidins can also degrade LPS, reduce its affinity with LBP protein, and clear the surface LPS of monocytes and macrophages, inhibiting the production of proinflammatory cytokines. It shows that antimicrobial peptides play the role of scavenger in the innate immune system [107]. In addition, some antimicrobial peptides can also inhibit the expression of CD14, such as HDL, down-regulate the expression of CD14 on macrophages, thereby inhibiting the LPS-induced inflammatory response [108].

(3) Other pathway related proteins

Studies have found that LL-37 inhibits the translocation of p50 and p65 subunits of NF-kB into the nucleus, which significantly reduces the mRNA expression of protein 2, and further reduces the release of TNF-α and IL-6 [109].

Antimicrobial peptides interfere with the local membrane environment of the receptors to adjust the activation status of the receptors. Studies have found that the antimicrobial peptides Cathelicidins inhibit the activation of DC cells by inhibiting the up-regulation of co-stimulatory molecules such as CD40, CD80 and CD86, and the TLR4 induction effect is terminated. Cathelicidins prevent the release of cytokines by changing the structure of cell membranes [110].

In addition, Carratelli et al. found that TLR4 can induce the expression of β-defensin2, indicating that TLRs can not only induce the expression of antimicrobial peptides, but also recognize antimicrobial peptides [111].

The target of action of antimicrobial peptide PP-2 is IKB, which inhibits its phosphorylation, thereby blocking the inflammatory pathway; the anti-inflammatory mechanism of antimicrobial CLP-19 acts on tubulin to affect the normal function of TLR4, thereby inhibiting inflammatory signaling, and ultimately inhibiting the inflammatory response [112].

1.8 The role of antimicrobial peptides in inflammation diseases

In 1990s, antimicrobial peptides have entered people's field of vision and have gradually become a research hotspot. Many studies have found that the expression of antimicrobial peptides is increased in inflammatory diseases. The clinical application of antimicrobial peptides has the advantages of fast effect, not easy to produce drug resistance, and good anti-inflammatory effect. Next, this study mainly discussed the research progress of antimicrobial peptides in pneumonia, skin inflammation and enteritis.

1.8.1 The function of antibacterial peptides in pneumonia

In the respiratory system, the innate immune system is essential for the lungs to resist the invasion of external pathogens. The innate immune system can remove foreign bodies entering the airway, including dust, particles and pathogenic bacteria. Antimicrobial peptides are the main components of the innate immune system in the lungs. In addition to having antibacterial effects in the airways, antimicrobial peptides are also involved in anti-inflammation, immune activation and damage repair.

The lung is an organ that communicates directly with the outside world. Acute respiratory infections such as pneumonia are one of the most common diseases in the world. AMPs are located at the mucosal interface and the expression of AMPs can be stimulated by a variety of microorganisms. The recruitment and activation of inflammatory cells are mostly affected by inflammatory cytokines such as chemokines. The concentration of defensin is significantly increased in the lung infectious diseases of newborns and adults [113]. Yang et al. found that CSP1-E1A-cyc (Dap6E10) can attenuate mouse mortality during acute pneumonia caused by bacterial [114]. Defensins 3 and 4 secreted by respiratory epithelial cells increased rapidly during the initial period in mouse animal models of mycobacterial infection [115]. Kovach MA et al found that cathelicidin-related

antimicrobial peptide (CRAMP) can alleviate symptoms of pneumonia caused by gram-negative pneumonia infected mice [116]. Martínez-Guitián M, et al. found the peptide nucleic acids (PNAs) can reduce *A. baumannii* ATCC 17978 strain in a murine pneumonia model [117]. Xie et al. found that antimicrobial peptide PR-39 can relieve pneumonia symptoms caused by *A. Pleuropneumoniae* in a mouse model [118]. The expression of PR39 in pig polymorphonuclear neutrophils was elevated after *A. pleuropneumoniae* infection, and PR39 was found in healthy lung tissue, indicating that PR39 plays an important role in maintaining pig health [119]. The above research show that antimicrobial peptides have better antibacterial and anti-inflammatory effects in respiratory diseases caused by bacterial infection.

1.8.2 The function of antimicrobial peptide in skin inflammation

S. aureus is a common pathogen that can cause deep infections caused by superficial skin infections, severe invasive infections such as sepsis and endocarditis and metastatic infections in specific tissues. LL-37 and β-defensin synergistically inhibit Staphylococcus aureus, which is a predisposing factor for human skin infections [120]. *S. aureus* infection can induce high levels of HNPs expression in skin folliculitis lesions, antimicrobial peptide HNP1 and HNP2 have good bactericidal activity against *S. aureus* infection in skin [121]. Psoriasis is a common non-infectious chronic inflammatory skin disease. Studies have found that the expression of antimicrobial peptides in patients with psoriasis is elevated and can alleviate the body's inflammatory response [122]. Antimicrobial peptides (Camp) alleviates skin inflammation in *S. aureus* infected mice by reducing IL-6 expression and infiltration of neutrophils [123]. In addition, the study found that the expression of antimicrobial peptide LL-37 in patients with psoriasis is significantly higher than that of normal people. Studies have shown that antobial peptide LL-37 is closely related to psoriasis [124]. Ramos R et al. found that LL-37 promotes angiogenesis, epithelial cell proliferation and migration to promote re-epithelialization of damaged skin and repair skin damage. The above results show

that LL-37 can be used for skin damage repair [125]. Jin T et al. found that the serum antimicrobial peptide HBD-2 content of 18 psoriasis patients was related to the degree of psoriasis, indicating that the serum antimicrobial peptide HBD-2 level can be used as a biological monitoring agent for psoriasis treatment, and can distinguish patients with mild and severe psoriasis [126].

1.8.3 The function of antimicrobial peptides in inflammatory bowel diseases

Inflammatory bowel disease (IBD) is a collective term for a group of intestinal inflammatory diseases, which can be divided into: Crohn's disease (CD) and ulcerative colitis (UC) according to their pathological manifestations, and a few undefined colitis [127]. The expression of antimicrobial peptides is closely related to the inflammatory response. LL-37 is the main antimicrobial peptide of the non-specific innate immune system in the human intestine. The changes in the content of LL-37 are related to gastrointestinal diseases. The anti-inflammatory effect of antimicrobial peptide LL-37 is the most widely studied [128]. Studies have found that the expression of antimicrobial peptides HBD2, HBD3 and HBD4 is up-regulated in patients with ulcerative enteritis [129].

Antibacterial peptides Cathelicidin, such as C-BF can regulate intestinal inflammation and alleviate the increased expression of proinflammatory cytokines in the intestine of mice caused by LPS [130]. Sheng et al. found that the antimicrobial peptide hBD3 stimulated intestinal epithelial cell migration by activating Rho, increasing the phosphorylation of MLCK2 and the accumulation of F-actin, protecting the integrity of the intestinal barrier, and alleviating enterocolitis in mice [131].

The antimicrobial peptide coprisin was isolated from the beetle of Korean beetle, which consists of only 9 amino acids and prevents intestinal inflammation and mucosal damage in mice caused by *C.difficile* infection [132]. Jozefiak et al. found that the addition of antimicrobial peptide divercin AS7 to the diet can

alleviate necrotic enteritis in broiler chickens caused by clostridium perfringens challenge, improve production performance, increase the apparent metabolic energy of the diet, and protect the intestinal villi [133]. Yi et al. found that the expression of IL-6, IL-8, IL-12 and IL-22 was significantly increased in the jejunum of piglets on day 7 after weaning, and the expression of the inhibitory factor TGF-β, IL-10 and intestinal inflammatory factors was significantly reduced after antimicroial peptide cathelicidin-WA treatment [134].

1.8.4 Other inflammatory diseases

Periodontitis is chronic inflammatory disease caused by dental plaque. It can destroy the integrity of tooth tissues, interfere with normal bone metabolism, leading to the loss of alveolar bone. Studies have found that human β-defensin 3 can effectively relieve inflammatory symptoms of periodontitis [135]. Vasoactive intestinal peptide (VIP) can effectively relieve arthritis symptoms and bone tissue damage in mice by increasing anti-inflammatory factors [136].

1.9 Chanllenges and strategy of antimicrobial peptide

Due to antimicrobial peptides biological functional diversity, broad-spectrum antibacterial, anti-inflammatory and action mechanism is different from antibiotics, which has become a research hotspot. However, the development of antimicrobial peptides as clinical therapeutic drugs still faces great challenges. 1) Expression and recombinant expression of natural antimicrobial peptides are low, difficult separation and purification, and high cost. Based on these challenges, explore inducers such as vitamin D or butyrate to stimulate AMP production and the development of new synthetic peptides are considered as good strategy [137, 138]; 2) Although antimicrobial peptides have broad-spectrum antibacterial activity, bactericidal ability is weak, and antibacterial activities of different types antimicrobial peptides are varies; 3) The safety of antimicrobial peptides. Direct

antibacterial activity of antimicrobial peptides requires high concentration, which results in stronger cytotoxicity and high hemolytic, limiting the use of antimicrobial peptides in vivo; 4) The stability and low bioavailability of antimicrobial peptides. Various proteases and peptide enzymes in animal intestines can degrade antimicrobial peptides, which limits the use of antimicrobial peptides.

The formulation of nanoparticles, carbon nanotubes and other materials delivery systems can solve this problem [139]; 5) With the long-term application of antimicrobial peptides, drug resistance is inevitable. Overcoming pathogen resistance can use combination of drugs, which can reduce the minimum inhibitory concentration of drugs and thus reduce bacteria resistant to antimicrobial peptides.

1.10 Prospect

In summary, with the adverse consequences of antibiotics, we urgently need to find new antibiotic alternatives. Antimicrobial peptides are considered to be one of the best alternatives to antibiotics due to their superiorities. It is expected to fill the gaps in antibiotics and become a research hotspot in recent years. Antimicrobial peptides have their unique advantages: small molecular weight, simple structure, broad-spectrum antibacterial activity and anti-inflammatory and immunomodulatory activities. Antimicrobial peptides play an important role in different diseases, especially in inflammatory diseases. In-depth study on the mechanism of antimicrobial peptides participating in inflammatory response can promote the development of antimicrobial peptide and lay the foundation for the development of new antibacterial and anti-inflammatory drugs. Antimicrobial peptides have good function of promoting growth and maintaining intestinal homeostasis because of barrier repairs and regulates the function of intestinal microbial diversity. Antimicrobial peptides are easily metabolized by the body and are not easy to remain in animal products. A series of advantages will play an important role in promoting the healthy and sustainable development of animal husbandry. Under the general trend of non-antibiotic aquaculture, relevant

companies and abroad have promoted and researched antimicrobial peptide products, and the application effect have also been confirmed. However, the mature development of antimicrobial peptide products still requires further cooperation and exploration by scientific researchers and manufacturers in order to play an important role in the use of alternative antibiotics in the future. With the continuous exploration of antimicrobial peptides which will definitely benefit humanity and society with their own unique advantages.

1.11 Conclusions from literature review

A review of the literature shows that with the unreasonable use of antibiotics, drug-resistant strains emerge in an endless stream, and there is an urgent need to find alternatives to antibiotics. Antimicrobial peptides are a type of small molecule polypeptide produced by the body against the invasion of pathogenic microorganisms. It has antibacterial,anti-inflammatory, regulate the composition of the intestinal flora and other biological functions. With the continuous in-depth research on antimicrobial peptides, people believe that antimicrobial peptides are one of the best alternatives to antibiotics, laying a foundation for reducing the clinical application of antibiotics.

CHAPTER 2
OBJECTS AND METHODS

2.1 Research materials

The dissertation work is carried out in accordance with the programs of research work of Sumy National Agrarian University: "System of monitoring methods of control and veterinary and sanitary measures, regarding the quality and safety of livestock products in diseases of infectious etiology" (state registration No. 0114U005551, 2014-2019); "Forecasting the risks of cross-border introduction and spread of particularly dangerous animal diseases and the development of scientifically based disinfection systems based on innovative import-substitutable highly effective means" (state registration No. 0115U001342, 2018-2023). The dissertation is a fragment of scientific programs of research work of the National Natural Science Foundation of China (No. 31702259 and 31520103917), Young Talent Lifting Project in Henan Province (2020HYTP041), Key Scientific Research Projects of Colleges and University in Henan Province (21A230004), Youth Backbone Teacher Project of Colleges and Universities of Henan Province (2020GGJS162), Climbing Project of Henan Institute of Science and Technology (2018JY02) and Program for Innovative Research Teams (in Science and Technology) at the University of Henan Province (20IRTSTHN025). The work was carried out for the period from 2018 to 2022 at the departments of microbiologyof Sumy National Agrarian University. In addition, Conduct relevant experimental research in Henan Institute of Science and Technology, Xinxiang, China.

The work was carried out for the period from 2018 to 2022 at the departments of veterinary examination, microbiology, zooghygiene and safety and quality of animals products, the department of epizootology and parazitology and the department of virology, pathanatomy and bird diseases of Sumy National Agrarian University. In addition, veterinary studies were conducted at Henan, China.

Object of study – pharmacolo-toxicological, antibacterial and anti-inflammatory assessment of MPX.

Subject of study –The antibacterial, anti-inflammatory and intestinal barrier protective functions, and its influence on intestinal microbes of MPX.

2.2 Research methods

During the study following methods were used: microbiological (microscopic, PCR examination biological), bacteriological (the antibacterial efficiency of MPX), pharmacological, toxicological (degree of toxicity and harmlessness of MPX, acute and chronic toxicity, pharmacokpnetics, pharmacodynamics), statistical (processing of research results).

Consumables – gloves, syringes and needles, cotton wool, methyl alcohol, EDTA test tubes, test tubes, 2 ml cryogenic tubes, disposable petri dishes, tweezers, scissors, inoculating loops, laser confocal dishes, medical tape, polystyrene 96-well micro titer plate, 6-well plates, 12-well plates, squirrel cage, pipette. T*ransScript*® Uni One-Step gDNA Removal and cDNA Synthesis SuperMix (TRAN, China)，SYBR Green Master Mix (Quanti Nova, China)，TNF-α,IL-2, IL-6ELISA kit (Biolegend, USA)，LIVE/DEAD BacLight Bacterial Viability L-7012 Kit (Invitrogen, US)， rabbit anti-p-p38 (Cell Signaling Technology， USA)， rabbit anti-p-pERK (Cell Signaling Technology,USA), rabbit and anti-p-pJNK antibodies (Cell Signaling Technology, USA), HRP-conjugated secondary antibodies (Cell Signaling Technology, USA), BCA protein concentration determination kit (Beyotime, China), 0.25% pancreatin (Solarbio, China), BI serum (Bioind, Israel), Lactate Dehydrogenase Kit (Nanjing Jiancheng, China), Myeloperoxidase kit (Multi Science, China), CCK-8 kit (Meilunbio, China), RNA extraction kit reagent (Solarbio, China), RIPA lysate buffer (Key GEN, China), anti-β-actin monoclonal antibody (Cell Signaling Technology, USA), ECL (Solarbio, China)，anti-p-p65 (Cell Signaling Technology, USA), anti-TLR4

(Cell Signaling Technology, USA), Alexa Fluor® 488-anti-rabbit (ZSGB-BIO, China), Alexa Fluor® 594-anti-rabbit (ZSGB-BIO, China) MUC2 (Servicebio, China), ZO-1 (Servicebio, China), Claudin-1 (Servicebio, China), Occludin (Servicebio, China), goat anti-rabbit label CY3 (Servicebio, China).

Equipment. Microplate reader (Thermo Scientific, USA), Philips Model SU8010 FASEM (HITACHI, Japan), Transmission electron microscope (HITACHI，Japan), confocal laser microscope (EVOS M7000, USA)，High-speed centrifuge (BECKMAN COULTER, USA), Biological safety cabinet (BIOBASE，China), 37°C constant temperature incubator (BIOBASE，China), Shaker(CIMO, China), Electronic analytical balance(Sartorius,Germany), Pressure cooker (CIMO, China), world precision instruments ($EVOM^2$, USA),NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA)，DM3000 microscope (PHASE CONTRAST, Japan)，PCR instrument (BIO-RAD, USA), Water bath (JINGHONG, China).

Chemicals and solvents. Methanol (Hengxing, China), EDTA (MACKLIN， China), TAE (Hengxing, China), Absolute ethanol (Xinhua，China), Hydrochloric acid (MACKLIN, China), Sulfuric acid (MACKLIN, China), DMSO (Solarbio, China), Double antibody (Solarbio, China), NaH_2PO_4 (Shuang huan, China), Na_2HPO_4 (Shuang huan, China), $MgCl_2$ (Shuang huan, China), Tryptone (AOBOX, China)，Yeast (AOBOX, China), Agar powder (AOBOX, China), NaCl (TIAN LI, China), Chloroform (Deen, China), Isopropanol (Deen, China), Coomassie Brilliant Blue R-250 (Solarbio, China)，Tris (Solarbio, China), Glycine (Solarbio, China), SDS (Solarbio, China), Twee-20 (Baiebio, China), Bovine serum albumin (Solarbio, China).

Preparation of 200 mL solid LB medium: weigh and took 2g peptone, 1g yeast, 2g sodium chloride, 3g agar with an electronic balance, added 200mL sterile distilled water and stir evenly, put it in a pressure cooker at 120°Cfor 20 min, and waited until the temperature was reduced to 60°C and took it out.

Preparation of 200 mL solid LB medium: weigh and took 2g peptone, 1g yeast, 2g sodium chloride with an electronic balance, added 200mL sterile distilled water and stir evenly, put it in a pressure cooker at 120°C for 20 min, and waited until the temperature was reduced to 60°C and took it out.

Preparation of 200mL liquid brain heart infusion medium: weigh and took 5g yeast and 3g brain heart infusion with an electronic balance, added 200mL sterile distilled water and stir evenly, put it in a pressure cooker at 120°C for 20 min, and waited until the temperature droped to 60°C andtook it out.

Preparation of 2.5% glutaraldehyde fixative solution: first prepared 0.2M phosphate buffer solution, weigh 2.6g sodium dihydrogen phosphate ($NaH_2PO_4H_2O$), 29g disodium hydrogen phosphate ($Na_2HPO_412H_2O$) with an electronic balance, added double distilled water to 500mL, and adjusted the pH to 7.4. Next, draw 1 mL of 25% glutaraldehyde, 4 mL of double-distilled water, and 5 mL of 0.2 mol/L phosphate buffer mixed well, and the pH was 7.3-7.4.

1 M Tris-HCl preparation: weigh and took 24.22g Tris using an electronic balance and added 160 mL deionized water, stired to dissolve, added concentrated hydrochloric acid (HCl, about 8.4 mL) to pH=8.0, and diluted to 200 mL.Stored at room temperature after autoclaving.

5xTris-Glycine Buffer: weigh and took 15.1g Tris, 94g Glycine, 5 g SDS using an electronic balance and added 1L distilled water fully dissolving, stored at room temperature.

Transfer buffer: weigh and took 1.45g Glycine, 2.91g Tris, and 0.185g SDS using an electronic balance to, added 400mL of water, stir to dissolve, added 100mL of methanol, and store at room temperature.

TBST: use an electronic balance to weigh and took 8.8g of sodium chloride, measure 20mL of Tris-Hcl (pH=8.0) in a graduated cylinder, add ddH_2O to a constant volume of 1000mL, and added 0.05% Tween-20 and mixed.

2.2.1 Ethics statement

One hundred BALB/c mice (6 to 8 weeks old, body weights of 18 to 20 g, female) were purchased from the Animal Center of Zhengzhou University (No. 41003100024648). All animal studies were conducted according to the experimental practices and standards of the Animal Welfare and Research Ethics Committee at Zhengzhou University. The study was also approved by the Animal Centre of Zhengzhou University.

2.2.2 Peptide synthesis

Antimicrobial peptide sequence details are shown in Table 2.1.

Table 2.1

The sequence of antibacterial peptides

	Sequences	Reference
MPX	H-INWKGIAAMAKKLL-NH_2	(Henriksen et al., 2014)
GI24	GRFRRLRKKTRKRLKKIGKVLKWI-NH_2	(Lv et al., 2014)
PMAP-36	GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG-NH_2	(Lv et al., 2014)
Arenicin-1	RWCVYAYVRVRGVLVRYRRCW	(Kang et al., 2015)
Fow-3	KRFWPLVPVAINTVAAGINLYKAIRRK-NH_2	(Qu et al., 2016)
AeaAp2a Pexiganan	FLFKLIPKAIKGLVKAIRK GIGKFLKKAKKFGKAFVKILKK	(Du Q et al., 2015)
IDR-1002	VQRWLIVWRIRK-NH_2	(Haney et al., 2015)
BSN-37	FRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPP	
BSN-37(34)	FRPIRRPPIRPPFYPPFRPPIRPPPPIRPPFRPP	
BSN-37(25)	FRPRRPPPPFPPRPPPPIPPRPPPP	
BSN-37(27)	FRPIRRPRPPFPPPPIRPFPPIRPRPP	
JH-3	RRFKLLSHSLLVTLASHL	(Wang et al., 2019)

Antimicrobial peptides were purified by Shanghai Gil Biochemical Co., Ltd (China), using a solid-phase N-9-fluoromethoxycarbonyl (Fmoc) strategy and high-performance liquid chromatography (HPLC); the purity was as high as 98%. *A. pleuropneumoniae* serovar 5bL20 strain was used in this study.

2.2.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The method for determining the MIC of MPX was described previously [140], according to the Experimental Guide of Clinical and Laboratory Standards Institute (CLSI), the MIC of antimicrobial peptides against *A. pleuropneumoniae* was determined by the modified microbroth dilution method. TSB with 4% newborn bovine serum and 1% 1 mg/mL NAD was used to determine the MICs of *A. pleuropneumoniae*. The concentration of *A. pleuropneumoniae*, *E. coli* and *S. aureus* was $2x10^5$ CFU/mL. A 96-well plate was placed in a 37°C incubator for 16-18 h after dilution, and each dilution was replicated 3 times. The MIC was the concentration at which there was no visible bacterial growth. Then, 20 μL of each bacterial peptide suspension in the 96-well microtiter plates was plated onto BHI agar plates and incubated for 20 h at 37°C. The MBC value was determined as the lowest concentration of MPX that showed no visible growth on the plates [118]. Three replicates per sample were assessed.

2.2.4 Killing curve

A. pleuropneumoniae was cultured in BHI+NAD liquid medium to log phase (OD_{600}=1.0); a final concentration of MPX (16 μg/mL), gentamicin (50 μg/mL, Solarbio, China), PR39 (100 μg/mL, San Jose, CA), and PG (100 μg/mL, Dipexium Pharmaceuticals, Inc., New York, NY) was added to the samples; and ddH_2O was added to the negative control. After incubation at 37°C for 0 min, 20 min, 40 min, 60 min, 120 min, 240 min, 360 min, and 480 min, the OD_{600} was determined. The bacteria were diluted by decimal serial dilutions every 1 h, and then the plates were assessed [141].

2.2.5 The sensitivity of MPX to pH, salt and temperature

The effect of temperature on the antibacterial activity of MPX was determined. The solution was incubated in a 0, 20, 40, 60, 80, and 100℃ water bath for 1 h after MPX was dissolved in ddH_2O. To determine the effect of pH on the antibacterial activity of MPX, ddH_2O was prepared with 1 mol/L HCl and 5 mol/L NaOH to give pH values of 3, 4, 5, 6, 7, 8, 9, 10 and 11. DdH_2O with different pH values was used to dissolve MPX. To determine the effect of salt ions on the antibacterial activity of MPX, salt solutions of NaCl, KCl, and $CaCl_2$ at concentrations of 50, 100, 150, and 200 mmol/mL were prepared and dissolved in MPX. The activity of MPX was determined using an agarose diffusion assay. Then, 10 μL of antimicrobial peptide MPX treated at different pH values, temperatures and salt concentrations was added to a plate containing $5x10^5$ CFU/mL *A. pleuropneumoniae*; this plate was incubated in a 37°C incubator overnight. The inhibition zone of MPX was measured, and the effect of different treatments on the antibacterial activity of MPX was observed [142]. The initial diameter (Do) of all samples (*A. pleuropneumoniae*) was measured using Vernier callipers immediately before adding the spread agarose. After 24 h incubation, the diameter (Di) of any zone of growth inhibition was measured using Vernier callipers. The inhibition ratio (Di–Do) reflects the mostability of MPX [143]. Three replicates per sample were assessed.

2.2.6 Permeability analysis

A. pleuropneumoniae was cultured to logarithmic phase, MPX (1×MIC) was incubated for 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h and centrifuged at 8000 rpm for 5 min, and the bacterial supernatant was collected. A BCA protein concentration determination kit was used to detect the change in total protein content in the supernatant of the bacterial culture. In addition, the LIVE/DEAD BacLight Bacterial Viability L-7012 Kit (Invitrogen, US) was used to observe the effect of

MPX (1×MIC) under a fluorescence microscope for 0.5 h. The test operation was carried out in strict accordance with the instructions.

2.2.7 Cytoplasmic outer membrane permeabilisation assay

Mid-log phase and stationary phase cultures of *A. pleuropneumoniae* were independently harvested, washed with a 1:1 mixture of 5 mM HEPES buffer and glucose and resuspended with the same. The working concentration of mid-log phase and stationary phase bacteria was 10^8 CFU/mL. This study was performed in a black Corning 96-well plate with a clear bottom containing 10 μM N-phenyl-naphthylamine (NPN) dye and 190 μL of bacterial suspension. Then, the fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. After that, bacterial suspensions with dye in each well were treated with 10 μL of MPX at concentrations of 16 μg/mL, 32 μg/mL and 64 μg/mL. The same volume of water without compound was used as the control for this experiment. The increase in fluorescence intensity was monitored for another 10 min with an INFINITE M PLEX microplate reader [144].

2.2.8 Cytoplasmic inner membrane permeabilisation assay

Briefly, the mid-log phase and stationary phase of *A. pleuropneumoniae* were separately centrifuged (3500 rpm, 5 min), washed and resuspended in a 1:1 ratio of 5 mM glucose and HEPES buffer (pH = 7.4). The working concentration of *A. pleuropneumoniae* was 10^8 CFU/mL. After that, 190 μL of bacterial suspension containing 10 μM propidium iodide (PI) was added to the well of a black Corning 96-well plate with a clear bottom. An excitation wavelength of 535 nm and emission wavelength of 617 nm were used to monitor the PI fluorescence for 4 min. Next, 10 μL of MPX (at working concentrations of 16 μg/mL, 32 μg/mL, and 64 μg/mL) was added to the wells containing dye and bacterial suspension. The same

volume of water without compound was used as the control for this experiment. The increase in the fluorescence intensity of PI was monitored as a measure of membrane permeabilisation for another 25 min using an INFINITE M PLEX microplate reader [144].

2.2.9 Ion release detection

Ion release was measured based on ion atomic absorption spectroscopy, and the test method was described previously [145]. *A. pleuropneumoniae* ($1x10^5$ CFU/mL) was incubated with MPX (1×MIC) for different times.

The bacterial culture supernatant was filtered through a 0.22 μm filter, and the bacteria were treated with deionised water as a negative control using an Optical Emission Spectrometer Optima 2100 DV. T represents ion concentration detection.

2.2.10 Quantitative RT-PCR

A. pleuropneumoniae was cultured to logarithmic phase, MPX (1×MIC) was added to the bacterial culture, and the cells were collected after an incubation of 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h. After extracting bacterial RNA, the mRNA was reverse transcribed into cDNA with the cloned AMV FirstStrand cDNA Synthesis Kit (Invitrogen), and qRT-PCR was carried out in a total volume of 10 μL. Trimeric autotransporter adhesion (TAA) is an important virulence factor discovered in recent years that can cause bacterial infection.

Previous research in our laboratory confirmed the presence of TAA on the *A. pleuropneumoniae* serovar 5b L20 strain, named Apa1. Among the RTX toxins, Apx I, Apx II, and Apx IV toxins have been identified for *A. pleuropneumoniae* serovar 5b L20 strain. PurC is a key enzyme in the de novo purine biosynthetic pathway of bacteria, which is an ideal target pathway for the discovery of

antimicrobials. Clp can regulate the formation of various pathogenic bacterial biofilms. These genes were important for *A. pleuropneumonia*.

The 16S rRNA of *A. pleuropneumoniae* was used as the housekeeping gene, and all samples were analysed in triplicate and normalised against 16S rRNA expression [146].

For detailed instructions, refer to the Fast Start Universal SYBR Green Master (ROX) (Recho, USA) instructions. The primer sequences and cycling conditions were reported previously [141]. Three replicates per sample were assessed.

2.2.11 Detection of biofilm formation by crystal violet staining

An overnight culture of the APP 5b L20 strain in a 96-well polystyrene microplate was inoculated with 100 μL of 1% BHI+NAD liquid medium per well. Different concentrations of MPX (final concentrations of 16 μg/mL, 32 μg/mL, and 64 μg/mL) were added to each well of the test group; gentamicin, PR39, PG, and ddH_2O were used as controls. The plates were placed in a 37°C incubator for 12 h, and the culture supernatants were aspirated.

Each well was washed 3 times with 200 μL of sterile PBS; 70% methanol was fixed for 30 min; the fixative was aspirated and dried in a 37°C incubator for 30 min; 100 μL of 1% Hucker crystal violet staining solution was added to each well and stained for 5 min at room temperature; the staining solution was removed, and the plate was rinsed under a slow flow of water.

The effluent water was colourless, dried at 37°C in a thermostat and observed under a microscope. Then, 100 μL of 70% ethanol solution was added to each well for decolourisation, vortexed to mix, and quickly placed in a multiplate reader to determine the OD_{570} value [147].

2.2.12 Detection of biofilm formation by scanning electron microscopy

A. pleuropneumoniae (mid-log phase) was diluted to $1x10^5$ CFU/mL in BHI+NAD liquid medium and transferred to a 6-well cell plate with sterile slides; MPX (1×MIC final concentration) was added. PR39 was used as an antimicrobial peptide control, and the negative control did not have an antimicrobial peptide added. After incubation in a 37℃ incubator for 24 h, *A. pleuropneumoniae* was washed 3 times with sterile physiological saline to wash away floating bacteria. Then, 300 μL of a 2.5% glutaraldehyde solution was added to each well, and the coverslips were fixed at room temperature for 30 min and washed 3 times with pH=7.4 phosphate buffer for 10 min each time. Coverslips were dehydrated with an ethanol gradient (30%, 50%, 70%, 80%, 90%, 95%, 100%) for 15 min each time. After the coverslips were dried, biofilm formation was observed by scanning electron microscopy [148].

2.2.13 Observation of biofilm formation by confocal laser microscopy

The method of cultivating an *A. pleuropneumoniae* biofilm was as described above. MPX (0.5×MIC, 1×MIC, or 2×MIC final concentration) was added to the sample.

The negative control did not have any added peptide. After culturing in a 37℃ incubator for 24 h, *A. pleuropneumoniae* was washed 3 times with 0.85% NaCl to remove nucleic acids and other media components.

The LIVE/DEAD BacLight Bacterial Viability L-7012 Kit (Molecular Probes, Eugene, OR, USA), containing two component dyes (SYTO 9 and PI in a 1:1 mixture) in solution, was used for microscopy and quantitative assays according to the test instructions.

A total of 3 μL of the dye mixture was added to each well, the wells were incubated at room temperature in the dark for 15 min, and the bacterial survival in the biofilm was observed by confocal laser microscopy.

2.2.14 Bactericidal assays

Bactericidal assays were performed as described previously [118]. The *A. pleuropneumoniae* serovar 5b L20 strain, △sapA, and P△sapA were grown in BHI medium to an OD_{600} of 0.8. Cells from the broth cultures of each strain were harvested and diluted in PBS (pH 7.4) to a concentration of 10^6 CFU/mL. The wells of a sterile, polystyrene 96-well microtiter plate (Costar 3599, U.S.A.) were filled with 90 μL of PBS. MPX was serially diluted in the wells, and each well retained 90 μL of MPX (0.5 MBC). Gentamicin (25 μg/mL) and PR39 (50 μg/mL) were used as controls. A blank was used as a negative control. Ten microliters of the bacterial suspension were added to each well, and the plate was incubated for 0.5-3 h at 37°C. Bacteria incubated with PBS served as the control. Serial dilutions of the bacteria were plated on BHI agar. The bactericidal effect was expressed as the percentage of surviving cells, with the counts obtained for bacteria incubated in PBS representing 100%. Three replicates per sample were assessed.

2.2.15 Animal infection experiment

The *A. pleuropneumoniae* serotype 5b L20 strain was cultured to the logarithmic growth phase, centrifuged at 8000 rpm for 5 min, and washed with pH=7.4 phosphate buffer 3 times. One hundred BALB/c mice (6 to 8 weeks old, body weights of 18 to 20 g, female) were used as expected. The animal experiments were divided into 5 groups, which were blank, 5b L20, 5b+MPX, 5b+PR-39 and 5b+gentamicin, with 20 mice per group. BALB/c mice were anaesthetised for bacterial infection. BALB/c mice in the infected group were inoculated intranasally with a bacterial suspension of *A. pleuropneumoniae* containing approximately 10^8 CFU/mL [149]. The infection dose of each mouse was $1x10^8$ CFU/mL, and infectious dose administration was performed as previously described [150]. After infection for 2 h, MPX (20 mg/kg) was injected intraperitoneally. In addition, to evaluate the effect of MPX (20 mg/kg),

intraperitoneal injection was performed on sterile mice. The clinical signs of all mice in each group, including the state of activity, hair, appetite, mental state, body temperature, and body weight were recorded after challenge. The animals were scored as follows: no clinical signs as 0; slight as 1; moderate as 2; severe as 3. These standards were used to score the mice in the experimental and control groups. The clinical symptoms were recorded every day. The mice were sacrificed at 6 h, 12 h, 24 h, and 48 h after bacterial infection. After the lungs were weighed, part of the lungs was homogenised. The TNF-α and IL-6 levels of lung homogenates were detected with a double-antibody sandwich ELISA kit, and 4% paraformaldehyde was used to fix residual lungs and sections for H&E staining to observe pathological changes in the mouse lungs.

The bactericidal activity and its killing mechanism of MPX in vitro mainly adopts the following experimental steps.

2.2.16 Preparation of the *E. coli* strain

E.coli (Enterohemorrhagic Escherichia coli O157:H7ATCC43889) wereisolated from feces of patien twith hemolytic uremic syndromeand obtained from the China Institute of Veterinary Drug Control (Beijing, China). The *E. coli* strain was seeded on LB (Solarbio, China) agar to obtain isolated pure colonies, and a single colony was inoculated into LB broth and incubated overnight at 37℃ while shaking at 180 rpm (75003442, Thermo, USA) for 10 h. In order to get the bacteria to the logarithmic growth phase, one hundred microliters was transferred into 10mL of fresh LB broth and incubated at 37℃ while shaking at 180 rpm for 4 h. Then, 1mL of *E.coli* bacteria liquid in a 1.5mL centrifuge tube, centrifuged at 8000rpm (6010 g) for 5min,and resuspend the bacterial pelletwith phosphate buffer (pH=7.4). The *E.coli* bacteria liquid was then serially diluted ten times, seeded onto LB plates and placed the LB plates in a 37℃ incubator for 12 h, and counted the number of *E. coli* per milliliter after growing visible single bacteria. The result

was 4.5×10^8 CFU/mL. The bacteria were diluted by decimal serial dilutions one time, and 4.5×10^7 CFU/mL was obtained.

E. coli was cultured in LB liquid medium to the logarithmic phase (OD_{600}=1.0), and the final concentrations of MPX (31.25 ug/mL), PR39 (50 ug/mL), andEnro (50 ug/mL) were added to the bacterial solution, added dd H_2O as the negative control, incubatedat 37℃ for 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, then measured the OD_{600} of the bacterial solution and dilute the bacterial solution every 1 h. Putiton the LBplateandin cubatedina 37℃ degree incubator, andincubatefor 12 huntilasing lecolonyis clearly visible, and then counted heplates [93].

2.2.17 MPX for pH, salt and temperature sensitivity detection

In order to detect the influence of pH, temperature and salt on the antibacterial activity of MPX, ddH_2O was prepared with 1 mol/L HCl and 5 mol/L NaOH, and the pH values were 3, 4, 5, 6, 7, 8, 9, 10, 11 dissolve ddH_2O with different pH values in MPX; dissolve ddH_2O in MPX and incubate in a water bath at 0°C, 20°C, 40°C, 60°C, 80°C and 100°C for 1 h; respectively prepare concentrations of 50 mmol/mL, 100 mmol/mL, 150 mmol/mL, 200 mmol/mL and 250 mmol/mL salt solution of NaCl, KCl, $CaCl_2$, $MgCl_2$ and dissolved the prepared salt solution in MPX. Pipette more than 10 uL of different processed MPX and add it to a plate containing $5x10^5$CFU/mL, placed it in a 37℃ incubator overnight, measured the MPX antibacterial radius, and observe the effect of different treatments on the antibacterial activity of MPX [93].

2.2.18 The effect of MPX on the permeability of *E. coli*

5 μL of *E. coli* in LB liquid medium under aseptic conditions, put it on a shaker at 37℃ and shaked at 180 rpm for about 10 h, centrifuged the bacterial liquid at 8000 rpm for 2 min, discard the supernatant, and added fresh LB liquid medium to adjust the OD_{600} to 1.0, MPX was added to the bacterial solution with

1MIC, 2MIC and 4MIC, respectively. The negative control was ddH_2O. After treatment with MPX for 2 h, the BCA protein concentration was detected using BCA protein content determination kit. The absorbance of A562 wavelength was obtained with a microplate reader, and calculated the protein content of the sample according to the standard curve. At the same time, immunofluorescence microscopy was used to observe the killing effect of the antimicrobial peptide MPX (0.5 MIC, 1MIC, 2 MIC, 4MIC) on *E. coli*. The bacteria stained was using the kit (Bacterial Viablility Kit), and the detailed experimental procedures were strictly in accordance with the kit [141].

2.2.19 The effect of MPX on the outer membrane of *E. coli*

E.coli were washed with 1:1 mixture of 5 mM HEPES buffer and resuspended with the same. The concentration of *E.coli* were $1x10^8$ CFU/mL. This study was performed in a Corning 96 black well plate with clear bottom containing 10 μM of N-Phenyl naphthylamine (NPN) dye and 190 μL of bacterial suspension.

After that, bacterial suspensions with dye in each well were treated with 10 μL of MPX at concentrations of 1 MIC, 2 MIC, 4 MIC. Then, the fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The increase in fluorescence intensity was monitored for another 10 min with an INFINITE M PLEX microplate reader [151].

2.2.20 The effect of MPX on cell plasma membrane

E.coli were collected separately washed with 1:1 ratio of 5 mM glucose and HEPES buffer (pH = 7.4). Next, the bacterial plate was resuspended in 1:1:1 ratio of 5 mM HEPES buffer, 100 mM KCl solution supplemented with 0.2 mM EDTA and 5 mM glucose.

For this study EDTA was used to allow the dye uptake by permeabilizing outer membrane of *E.coli*. This study was performed in a Corning 96 black well

plate with clear bottom containing 2 μM of 3,3′- dipropylthiadicarbocyanine iodide [DiSC3(5)] and 190 μL of bacterial suspension. After that, 10 μL of MPX (1 MIC, 2 MIC，4 MIC) was mixed with the suspension of bacteria and dye of each well. In this experiment, ddH_2O was used as the control.

Fluorescence intensity was measured at 622 nm excitation wavelength and 670 nm emission wavelength for 10 min using an INFINITE M PLEX microplate reader [152].

2.2.21 The effect of MPX on the inner membrane of *E. coli*

Briefly, the mid-log phase of *E.coli* were separately centrifuged (8000rpm, 5 min), washed and resuspended in a 1:1 ratio of 5 mM glucose and HEPES buffer (pH = 7.4). The working concentration of *E.coli* was $1x10^8$ CFU/mL. After that, 190 μL of bacterial suspension containing 10 μM propidium iodide (PI) was added to the well of a black Corning 96-well plate with a clear bottom. Next, 10 μL of MPX (1 MIC, 2 MIC, 4 MIC) was added to the wells containing dye and bacterial suspension. The control was ddH_2O.

An excitation wavelength of 535 nm and emission wavelength of 617 nm were used to monitor the PI fluorescence for 10 min using an INFINITE M PLEX microplate reader [153].

2.2.22 Detection of MPX to inhibit *E. coli* from forming biofilms

The ability of MPX inhibited *E. coli* biofilm formation was tested according to the reference [154]. In a 96-well polystyrene microtiter plate, the overnight cultured *E. coli* was inoculated into 100 uL of LB liquid medium according to the amount of 1%, and different concentrations of MPX (0.5 MIC, 1 MIC, 2 MIC, 4 MIC); ddH_2O is used as a negative control.

Place the culture plate in a 37°C incubator for 24 h. Aspirated and discarded the culture supernatant. Washed each well with 200 uL sterile PBS 3 times; fixed

with 70% methanol for 30 min; aspirated and discarded the fixative at 37°C dry in the incubator for 30 min; added 100 uL of 1% Hucker crystal violet staining solution to each well, and stained for 5 min at room temperature; removed the staining solution, and rinsed the culture plate under a slow stream of water until the flowing water was colorless;

After the oven was dried, placed it under a microscope for observation; then added 100 uL of 70% ethanol solution to each well for decolorization, vortex and mix, and quickly placed it in a multifunctional microplate reader to determine the absorbance value of OD_{570} [154].

2.2.23 Scanning electron microscope observed the formation of biofilm

After culturing the *E. coli* overnight, diluted it with fresh LB liquid medium by 100 times, transfer it to a 6-well cell plate with sterile glass slides, add 500 uL bacterial solution to each well, added MPX (1 MIC), ddH_2O as negative control. After 24 h of incubation in a constant temperature incubator at 37°C, slowly removed the cell culture solution and rinsed with sterile saline for 3 times to wash away floating bacteria.

Add 300 uL of 2.5% glutaraldehyde solution to each well. After fixation at room temperature for 30 min, rinsed with pH=7.4 phosphate buffer for 3 times, with an interval of 10 min each time. 30%, 50%, 70%, 80%, 90%, 95%, 100% alcohol gradient dehydration respectively, each time interval of 15 min.

After the slides are dry, observe the formation of biofilm under scanning electron microscope [93].

2.2.24 Clinical symptoms and observation of necropsy lesions

A total of 20 BALB/c mice (6 to 8 weeks old, 18 to 20 g, female) were purchased from Zhengzhou University.

After the cages were cleaned and disinfected, all mice were housed in individual cages at a constant humidity (40-70%) and temperature (21 ± 1°C) under a 12 h light/dark cycle for 3 d to acclimate to the environment.

The mice had ad libitum access to food and water before the start of the experiment. BALB/c mice were randomly divided into 4 groups, namely control group, *E. coli*, *E. coli* + MPX, *E. coli* + enrofloxacin, and the dose of *E. coli* infected BALB/c mice was $4.5x10^7$ CFU /mice [155], MPX (20 mg/kg) and Enro (20 mg/kg) were treated by intraperitoneal injection after infection with *E. coli* for 2 h, and treatment was continued for 3 days.

Observed the clinical manifestations and necropsy of the mice after *E. coli* infection, took out the mouse lungs, liver, spleen and intestines with scissors and toothless forceps, observed the pathological changes of the mouse intestines and organs, and took pictures. The clinical symptoms of the mice, including their fur state, body weight changes, mental state, and appetite, were recorded every day.

The specific scoring criteria were as follows: no clinical signs, 0; slight clinical signs, 1; moderate clinical signs, 2; severe clinical signs, 3.

The mouse sera were used for inflammatory factor analysis, and their livers, spleens, lungs and intestines were collected and fixed with 4% paraformaldehyde for H&E staining, immunohistochemistry and immunofluorescence analysis.

The jejunum samples were fixed with glutaraldehyde (2.5%) to observe changes in the intestinal villi and microvilli by SEM and TEM.

2.2.25 Fecal and tissue microbiota counts

The mice were euthanized, and their feces and tissues were collected aseptically. The fecal and tissue microbiota counts were determined and calculated by the dilution counting method in a sterile room. The fresh fecal samples were resuspended in sterile PBS and vortexed (0.5 g of fresh feces in 4.5 mL of sterile saline), and appropriate 100 μL dilutions of fecal tissues were spread over MacConkey agar plates to count the colonies. In addition, different volumes of

PBS were added according to the tissue weights, and appropriate 100 μL dilutions of tissues were spread over MacConkey agar plates to count the colonies. The plates were incubated in a 37°C incubator for 12 h under anaerobic conditions, and the results are shown as CFU/g feces and tissues. The assays were repeated three times.

2.2.26 Histopathology and immunohistochemistry

The jejunum and colon tissues of the mice were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin.

Hematoxylin and eosin (H&E) staining was used to stain the jejunum [156], and images were obtained using a DM3000 microscope (PHASE CONTRAST, Japan). Image-Pro software (ipwin32 software) (Media Cybernetics, USA) was used to measure the villous height and crypt depth, and the ratio of villous height to crypt depth was then calculated [141].

For immunohistochemistry (IHC), 5-μm-thick sections of the jejunum and colon tissues were embedded in paraffin, incubated in sodium citrate buffer (pH=6.0) to repair the antigens and placed in a 3% hydrogen peroxide solution to block endogenous peroxidase after dewaxing and rehydration.

To block nonspecific binding sites, the sections were incubated with 3% BSA for 30 min and then incubated with rabbit anti-p-p38 (1:200 dilution, #4511, Cell Signaling Technology), rabbit anti-p-pERK (1:200 dilution, #4370, Cell Signaling Technology), rabbit and anti-p-pJNK antibodies (1:200 dilution, #4668, Cell Signaling Technology) overnight at 4°C.

Then, the sections were incubated with HRP-conjugated secondary antibodies (1:200 dilution, #7074, Cell Signaling Technology) for 50 min and counterstained with hematoxylin after development with DAB buffer [157].

2.2.27 Transmission Electron Microscopy

The TJs and microvillus morphologies of mouse intestinal epithelial cells were observed by transmission electron microscopy (TEM) [158]. Mouse jejunum specimens were obtained with a scalpel and fixed in 2.5% glutaraldehyde for 12 h at 4℃.

Then, the jejunum samples were treated with osmic acid and embedded in Epon, and ultrathin sections were acquired using a diamond knife and then stained with uranyl acetate and lead citrate before being observed by TEM (7610plus/FEI Apreo, Japan).

2.2.28 Scanning Electron Microscopy

The morphologies of the mouse jejunum villi and microvilli were observed by scanning electron microscopy (SEM) [134]. Jejunum tissues from the mice were fixed with 2.5% glutaraldehyde overnight at 4℃ and then incubated with 1% OsO4 for 1 h.

The jejunum specimens were then dehydrated with an ethanol gradient (30%, 50%, 70%, 80%, 90%, 95% and 100%) for 15 min at each step and treated with a mixture of alcohol and isoamyl acetate (v:v=1:1) for 30 min.

Then, isoamyl acetate was added for 1 h, and the dehydrated specimens were coated with gold-palladium and visualized with a Philips Model SU8010 FASEM (HITACHI, Japan).

2.2.29 qRT-PCR

The primer sequences for real-time PCR are shown in Table 2.2. Total RNA was extracted using RNA extraction kit reagent (Solarbio, China).

Table 2.2

The primer sequences for real-time PCR

Genes	Sequence
MUC2 (Mouse)	F:5'-CTGCTCCGGGTCCTGTGGGA-3'
	R:5'-CCCGCTGGCTGGTGCGATAC-3'
TNF-α (Mouse)	F:5'-CTCATGCACCACCATCAAGG-3'
	R:5'-ACCTGACCACTCTCCCTTTG-3'
IL-6 (Mouse)	F:5'-CTCTGGCGGAGCTATTGAGA-3'
	R:5'-AAGTCTCCTGCGTGGAGAAA-3'
IL-2 (Mouse)	F:5'-CCTGAGCAGGATGGAGAATTACA-3'
	R:5'-TCCAGAACATGCCGCAGAG-3'
Occludin (Mouse)	F:5'-ACGGACCCTGACCACTATGA-3'
	R:5'-TCAGCAGCAGCCATGTACTC-3'
Claudin-1 (Mouse)	F:5'-AGCTGCCTGTTCCATGTACT-3'
	R:5'-CTCCCATTTGTCTGCTGCTC-3'
ZO-1 (Mouse)	F:5'-ACCCGAAACTGATGCTGTGGATAG-3'
	R:5'-AAATGGCCGGGCAGAACTTGTGTA-3'
Reg3γ (Mouse)	F:5'-CCCGACACTGGGCTATGAAC-3'
	R:5'-GGTACCACAGTGATTGCCTGA-3'
Relmβ (Mouse)	F:5'–CTGATAGTCCCAGGGAACGC-3'
	R:5'-GTCTGCCAGAAGACGTGACA-3'
TFF3(Mouse)	F:5' -CCTGGTTGCTGGGTCCTCTG-3'
	R:5'-GCCACGGTTGTTACACTGCTC-3'
GAPDH (Mouse)	F:5'-TGGAGAAACCTGCCAAGTATGA-3'
	R:5'-TGGAAGAATGGGAGTTGCTGT-3'
IL-6 (Pig)	F:5'-TGGCTACTGCCTTCCCTACC-3'
	R:5'-CAGAGATTTTGCCGAGGATG-3'
TNF-α(Pig)	F:5'-ATGGATGGGTGGATGAGAAA-3'
	R:5'-TGGAAACTGTTGGGGAGAAG-3
Claudin-1 (Pig)	F:5'-CCATCGTCAGCACCGCACTG-3'
	R:5'-CGACACGCAGGACATCCACAG-3'
Occludin (Pig)	F:5'-GACAGACTACACAACTGGCGG-3'
	R:5'-TGTACTCCTGCAGGCCACTG-3'
ZO-1 (Pig)	F:5'-ATGAGCAGGTCCCGTCCCAAG-3'
	R:5'-GGCGGAGGCAGCGGTTTG-3'
GAPDH (Pig)	F:5'-ACTCACTCTTCCACTTTTGATGCT-3'
	R:5'-TGTTGCTGTAGCCAAATTCA-3'

RNA concentration and OD260/OD280 were measured by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

The integrity of RNA was verified by visualization in an agarose gel. In addition, 2 μg of total RNA was converted to cDNA. cDNA was obtained using a reverse transcription kit (Thermo Scientific, USA). Each reaction (10 μL) volume included 5 μL of SYBR Green Master Mix (QuantiNova, China), 0.5 μL of the

forward primer (10 μM), 0.5 μL of the reverse primer (10 μM), 0.5 μL of cDNA and 3.5 μL of ddH_2O. The thermocycler reaction included 2 min at 95℃ and 40 cycles of 20 s at 95℃ and 30 s at 60℃, and melt curves were added; GAPDH served as the housekeeping gene. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression levels [159].

2.2.30 Cell culture

The porcine intestinal epithelial cell line IPEC-J2 is intestinal columnar epithelial cells, which were isolated from neonatal piglet midjejunum, and donated by professor Liancheng Lei of Jilin University. IPEC-J2 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin) and incubated at 37℃ and 5% CO2. When they reached 100% confluence, the cells were cultured in DMEM without 1% antibiotics and then treated with MPX (10 μg/mL) for 2 h. Then, the cells were further cultured with *E. coli* (MOI=10) for 12 h.

2.2.31 Cytotoxicity studies

Cell viability was determined by the CCK-8 kit (Meilunbio, China) in accordance with the manufacturer's instructions. First, IPEC-J2 cells were cultured in 96-well plates at 1×10^4 cells/well and treated with the indicated concentration (2-512 μg/mL) of MPX for 24 h. Then, the IPEC-J2 cells were incubated with 10 μL of CCK-8 per well in a cell culture incubator for 2 h, and the absorbance of each well was detected at 450 nm with a microplate reader (Dynatech Laboratories, USA) [160].

The lactose dehydrogenase (LDH) release assay (Nanjing Jiancheng, China) was performed to determine whether MPX damaged the IPEC-J2 cell membrane in accordance with the manufacturer's instructions. Briefly, IPEC-J2 cells were cultured in 96-well plates at 1×10^4 cells/well and treated with the indicated

concentration (2-512 μg/mL) of MPX at 37℃ and 5% CO2 for 24 h. In addition, the IPEC-J2 cells were pretreated with MPX (10 μg/mL) for 2 h and infected with *E. coli* (MOI=10) for different amounts of time (3 h, 6 h, 12 h, 24 h). The optical densities were measured at 450 nm using a microplate reader (VARIOSKAN FLASH, USA) [161].

2.2.32 Wound healing assay

IPEC-J2 cells were cultured in a 6-well plates, and the cell monolayer was scraped with a 200 μL pipette tip. The recovered IPEC-J2 cells were incubated with medium only or with 10 μg/mL MPX for different amounts of time, and images were acquired at 0 h, 48 h and 96 h by Nikon (Ti-E 531235, JAPAN). The wound width was measured at 48 h by ImageJ2x (Rawak Software, Inc.Germany)：Rawak Software, Inc. Germany, and each sample was analyzed in triplicate [162].

2.2.33 Trans-Epithelium Electrical Resistance Measurements

IPEC-J2 cells were seeded onto polycarbonate membrane filters (0.4-μm pore size, 6.5 mm Diameter Inserts) inside Transwell® cell culture chambers (Corning Incorporated costar, USA) at a density of 1 x 10^5 cells/cm^2. The culture medium was changed every day and trans-epithelium electrical resistance (TEER) values were measured every other day using the world precision instruments (EVOM2, USA). When a monolayer of cells was considered to be completely differentiated, cells were treated with PBS, *E. coli*, MPX, *E. coli* + MPX (cells were treated with *E.coli* (MOI=1), followed by MPX) and the TEER was measured at 0 h, 4 h, 8 h, 12 h, 16 h, 20 h and 24 h, and there were 3 replicates for each experiment [163].

2.2.34 Scanning electron microscope

Place an autoclaved 6mm round glass slide on the bottom of the 12-well plate.

When the IPEC-J2 cells grow into a single layer on the 12-well plate, infect the cells with *E. coli* at MOI=10, and add MPX (10 ug/mL) for 2 h, after aspirating DMEM, add 500 μL 2.5% glutaraldehyde to each well, fixed overnight at 4°C. After the 2.5% glutaraldehyde is aspirated, 30%, 50%, 70%, 80%, 90%, 95%, 100%, and 100% alcohol gradient dehydration, with an interval of 15 minutes each time. After the dehydration is completed, the sample is sprayed with gold and observed with a scanning electron microscope SU8010 FASEM (HITACHI, Japan) [164].

2.2.35 Laser confocal detection

After the IPEC-J2 cells grew into a single layer, they are spread on a laser confocal dish with $2x10^5$ cells per well. After overnight culture, the DMEM1640 cell culture medium is changed to DMEM 1640 without dual-antibody serum and the MOI =10 and MPX action for 2.5 h, preheat PBS and wash 3 times. Add 1 mL 2.5% glutaraldehyde to each well and fixed overnight at 4°C. Room temperature for 30 min, discard the fixative solution and washed with PBS at 3 times for 5 min every time, 5% PBS skim milk, block for 1 h at room temperature, and acted on the primary antibody for 1 h. It can be gently shaken on a shaker and washed 3 times with PBS for 5 min every time. Goat anti-rabbit fluorescent secondary antibody 594 (Zhongshan Jianqiao) for 1 h in dark, and shaken gently on a shaker. Washed with PBS at 3 times for 5 min every time in dark. DAPI was stained for 15 min in the dark, washed with PBS at 3 times for 5 min every time, and the results were observed with a laser confocal microscope [165].

2.2.36 qRT-PCR

After IPEC cells were cultured into a monolayer in a 6-well plate, the group were IPEC+*E.coli*, IPEC+*E.coli*+MPX, IPEC+*E.coli*+MPX+NSC 23766, IPEC+*E.coli*+ NSC 23766. Infection was carried out at MOI=10. IPEC-J2 cells

were collected for the detection of tight junction protein ZO-1, Occludin, Claudin-1 mRNA expression after 12 h, and 1 mL RNAio plus was added to each well to extract total cell RNA. 200 μL of chloroform to each well, centrifuged at 12000 rpm, 4℃ for 10 min, slowly aspirated the supernatant, added 500 μL of isopropanol to mix centrifuged at 12000 rpm at 4°C for 10 min. Added 1 mL of 75% ethanol to each tube centrifuged at 12000 rpm at 4°C for 5 min. Added 20~30 μL DEPC water.

The primer sequences as shown in Table 2.3

Table 2.3

The primer sequences for qRT-PCR

Genes	Sequence
Occludin	F:5'-GACAGACTACACAACTGGCGG-3' R:5'-TGTACTCCTGCAGGCCACTG-3'
Claudin-1	F:5'-CCATCGTCAGCACCGCACTG-3'
	R:5'-CGACACGCAGGACATCCACAG-3'
ZO-1	F:5'-ATGAGCAGGTCCCGTCCCAAG-3'
	R:5'-GGCGGAGGCAGCGGTTTG-3'
GAPDH	F:5'-ACTCACTCTTCCACTTTTGATGCT-3'
	R:5'-TGTTGCTGTAGCCAAATTCA-3'

Reversed transcription adopts Takala kit (Cat. No. DRR047A), the reaction conditions are: 95℃ 5 min, 95℃ 30 s, 55℃ 30 s, 72℃ 20 s, total 40 cycles. The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method and overexpression efficiency was also calculated using the $2^{-\Delta\Delta CT}$ method [166].

2.2.37 IPEC-J2 cells adhesion and invasion

IPEC-J2 cells were cultured in a 6-well plate, $1x10^6$ cells were added to each well, after 24 hours of culture, replaced with fresh DMEM: F 12 blank medium, added 50 uM Rac1 inhibitor NSC 23766 or the same volume of 0.1% DMSO, and

then added 10 ug/mL MPX, or sterile water, put it in the cell incubator and continue culturing for 12 h, washed it with PBS at 3 times, and then added it at MOI =10. Incubated *E. coli* suspension at 37°C for 1 h, washed away unadhered and invaded bacteria with PBS; added 200 uL 0.5% Triton X-100 to each well for 5 min, added 800 uL of pre-cooled PBS, collected the cells and proceed multiply dilution and LB medium coating, inverted culture in a biochemical incubator at 37°C for 16 h [167].

2.2.38 Animal experiment

A total of 48 BALB/c mice were purchased from Zhengzhou University. The mice had ad libitum access to food and water. The animals were randomly divided into 4 experimental groups (control, MPX, *E. coli*, and MPX+ *E. coli*; 12 mice per group, 6 mice at different times). The MPX group and MPX+*E. coli* group mice were gavaged with MPX at a concentration of 100 μg/mL (200 μL/mouse) every day for 7 consecutive days. The *E. coli* and MPX+*E. coli* groups were challenged with *E. coli* via oral (4.5×10^6 CFU/mouse) [73]. The mental state and the faeces of mice were observed every day. Stool consistency was classified according to the following visual criteria: (1) formed, brown, score 1; (2) soft, yellow, score 2; and (3) liquid, yellow, score 3.

2.2.39 Sample collection

The serum was collected to detect inflammatory protein and MPO, LDH. The jejunum samples were fixed with glutaraldehyde (2.5%) to observe changes in the intestinal villi and microvilli by SEM and TEM. The jejunum and colon of the mice were collected and fixed in a 4% paraformaldehyde solution for H&E to observe pathological changes. The contents of the caeca of the mice were collected and placed in liquid nitrogen for the analysis of the gut microbiota of the mice.

2.2.40 DNA extraction

A DNA extraction kit was used to extract total genomic DNA following the manufacturer's instructions. A NanoDrop spectrophotometer and agarose gel electrophoresis were used to verify the concentration of DNA. For bacterial diversity analysis, the V3-V4 variable regions of the 16S rRNA gene were amplified with the universal primers 343 F and 798 R.

2.2.41 Library construction

A Qubit dsDNA assay kit was used to quantify the final amplicon after purification with AMPure XP beads again. Equal amounts of the purified amplicons were pooled for subsequent sequencing.

2.2.42 Magnetic bead purification

A pipette gun was used to blow on the beads 10 times, after which they were stored at room temperature for 5 min. A 200 μL aliquot of fresh 80% ethanol was added to the beads at room temperature for 30 s, and the supernatant was then discarded. The previous step was repeated, and the beads were washed twice. The beads were blown upon 10 times after adding 25 μL H_2O for elution and were then thoroughly mixed at room temperature for 2 min. The supernatant was placed on a magnetic rack for 5 min until it was transparent, and 20 μL of the supernatant was transferred to a new PCR tube.

2.2.43 Bioinformatic analysis

The raw sequencing data were in FASTQ format. Trimmomatic software [168] was used to preprocess paired-end reads and trim ambiguous bases (N). A sliding window trimming approach was used to trim low-quality sequences with an

average quality score below 20. After trimming, the paired-end reads were assembled using FLASH software [169]. QIIME software [170](version 1.8.0) was used to detect and remove reads with chimeric sequences and retain reads in which 75% of bases showed values above Q20.

Vsearch software was used to remove the primer sequences of clean reads and to perform clustering to generate operational taxonomic units (OTUs) with a 97% similarity cut-off [171]. RDP Classifier was employed to annotate and perform blastall searches of representative reads against the Silva database Version 123 with a confidence threshold of 70% [172]. In addition, the Unite database (ITSrDNA) was used for BLAST, annotation and blastall analyses of representative reads [173].

2.2.44 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0, La Jolla, CA, USA). All the results are expressed as the mean ± S.E.M. Group comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was expressed as $P < 0.05$ as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

CHAPTER 3
RESULTS OF OWN RESEARCH

3.1 The antimicrobial peptide MPX kills *Actinobacillus pleuropneumoniae* and reduces its pathogenicity in mice

Due to increased antibiotic resistance, there is an urgent need to find new antibiotic alternatives for treating *A. pleuropneumoniae* infections. The MIC and MBC of peptides for different serotypes of *A. pleuropneumoniae* in Table 3.1

Table 3.1

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of peptides for different serotypes of *A. pleuropneumoniae*

Number	Name	APP Serotype 1		APP Serotype 3		APP Serotype 5b		APP Serotype 7	
		MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MIC (μg/mL)
1	GI24	32	32	32	64	64	128	32	64
2	PMAP-36	>256	>256	>256	>256	>256	>256	>256	>256
3	Arenicin-1	>256	>256	>256	>256	>256	>256	>256	>256
4	JH-3	>256	>256	>256	>256	>256	>256	>256	>256
5	IDR-1002	>256	>256	>256	>256	>256	>256	>256	>256
6	Pexigan an	>256	>256	>256	>256	>256	>256	>256	>256
7	Fow-3	>256	>256	>256	>256	>256	>256	>256	>256
8	AeaAA p2a	32	32	32	64	>256	>256	>256	>256
9	MPX	16	32	16	32	32	32	32	32
10	BSN-37	128	>256	>256	>256	16	16	16	16
11	BSN-37(25)	>256	>256	>256	>256	>256	>256	>256	>256
12	BSN-37(27)	>256	>256	>256	>256	>256	>256	>256	>256
13	BSN-37(34)	128	>256	>256	>256	>256	>256	>256	>256

This study found that MPX had a good killing effect on *A. pleuropneumoniae* and that the minimum inhibitory concentration (MIC) was 16 μg/mL. The bacterial density of *A. pleuropneumoniae* decreased 1000 times after MPX (1×MIC) treatment for 1 h, and the antibacterial activity was not affected by pH or temperature. Fluorescence microscopy showed that MPX (1×MIC) destroyed the bacterial cell membrane after treatment for 0.5 h, increasing membrane permeability and releasing bacterial proteins and Ca^{2+}, Na^{+} and other cations.

In addition, MPX (1×MIC) treatment significantly reduced the formation of bacterial biofilms.

Quantitative RT-PCR results showed that MPX treatment significantly upregulated the expression of the PurC virulence gene and downregulated that of ApxI, ApxII, and Apa1.

In addition, the Sap A gene was found to play an important role in the tolerance of *A. pleuropneumoniae* to antimicrobial peptides.

Therapeutic evaluation in a murine model showed that MPX protects mice from a lethal dose of *A. pleuropneumoniae* and relieves lung inflammation.

This study reports the use of MPX to treat *A. pleuropneumonia* infections, laying the foundation for the development of new drugs for bacterial infections.

3.1.1 MPX has a good killing effect on *A. Pleuropneumoniae*

According to the literature results, the 13 antimicrobial peptides have a significant effect on anti-enterobacteriaceae gram-negative bacteria. To screen antimicrobial peptides that have better effects against different serotypes of *A. pleuropneumoniae*, we selected 13 antimicrobial peptides for MIC and MBC detection. The results are shown in Table 3.1. Compared with other antimicrobial peptides, GI24 and AeaAAp2a had good antibacterial activity against *A. pleuropneumoniae*, and the MIC was 32 μg/mL. MPX had the best antibacterial activity against *A. pleuropneumoniae*, with an MIC of 16 μg/mL, and the

minimum bactericidalconcentration was 32 μg/mL, which is the MIC of antimicrobial peptides reported against *A. pleuropneumoniae*.

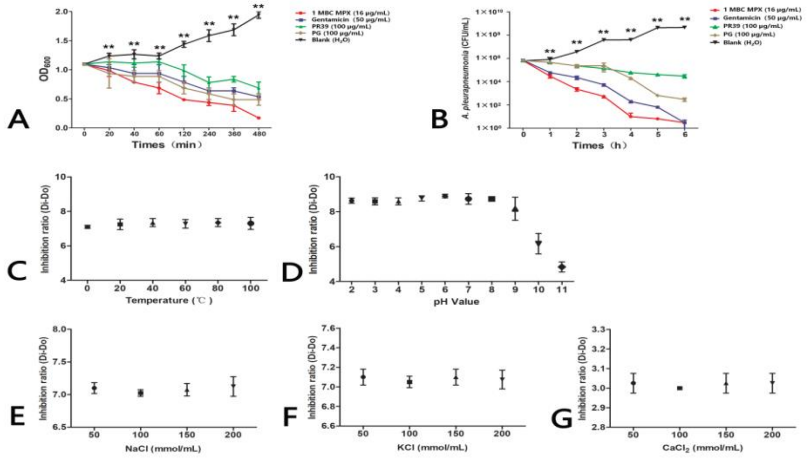

Fig. 3.1 MPX rapidly kills *A. pleuropneumoniae* and has good stability

(A) OD_{600} values of *A. pleuropneumoniae* after treatment with MPX at different time points; (B) The number of viable *A. pleuropneumoniae* after treatment with MPX at different time points; (C) The effect of different acid-base environments on the antibacterial activity of MPX (The initial diameter (Do) of all samples (*A. pleuropneumoniae*) was measured using Vernier callipers immediately before adding the spread agarose. The initial diameter (Do) of all samples (*A. pleuropneumoniae*) was measured using Vernier callipers immediately before adding the spread agarose. The inhibition ratio (Di–Do) reflects the thermostability of MPX); (D) The effect of different temperatures on the antibacterial activity of MPX; (E-G) The effects of different ion concentrations (NaCl, KCl, $CaCl_2$) on the antibacterial activity of MPX.

We examined the change in the bacterial OD_{600} value after MPX (1×MBC) treatment. Compared with that of the control group, the OD_{600} of bacteria decreased significantly after MPX treatment for 20 min, and the OD_{600} decreased to 0.1 at 480 min (Fig. 3.1 A, $P<0.01$). The number of viable bacteria significantly decreased with time after treatment with MPX for 1 h (Fig. 3.1B, $P<0.01$). The above results indicate that MPX has a good bactericidal effect on *A.pleuropneumoniae*. To study the effects of different acid-base environments and

salt ion concentrations on the antibacterial activity of MPX, the antibacterial activity of MPX at different pH values, temperatures and salt concentrations against A. *pleuropneumoniae* was determined by the bacteriostatic radius method. MPX was treated at different temperatures, with a maximum temperature of 100℃, and the temperature had no effect on the antibacterial activity of MPX, indicating that MPX has good thermal stability (Fig. 3.1C). As shown in Fig. 1, a pH of 2-8 had no effect on MPX activity (Fig. 3.1D, $P>0.05$). MPX bacteriostatic activity decreased at pH>9, indicating that MPX is most stable in acidic and weakly alkaline environments. After MPX treatment with different concentrations of Na^+, K^+, and Ca^{2+}, it was found that Na^+ and K^+ had no effect on the antibacterial activity of MPX, while Ca^{2+} significantly decreased the antibacterial activity of MPX (Fig. 3.1E, F, G), presumably due to the presence of cationic Ca^{2+} leading to changes in the secondary structure of MPX and thereby affecting its antibacterial activity.The above results indicated that the antibacterial activity of MPX was not affected by temperature but was affected by an alkaline environment and Ca^{2+}.

3.1.2 MPX increases membrane permeability and changes bacterial ion concentration distribution

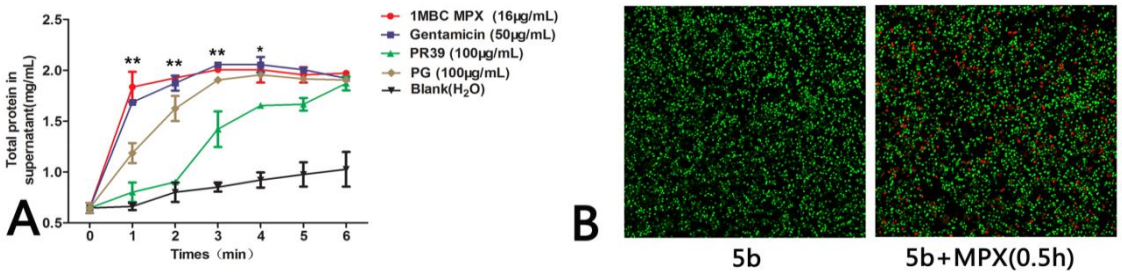

Fig. 3.2 The effect of MPX on the membrane permeability of *A.pleuropneumoniae*.

(A) Total protein content in the bacterial supernatant; (B) The results of staining *A.pleuropneumoniae* with SYTO 9/propidium iodide (red represent dead bacteria stained by propidium iodide; green represent live bacteria stained by SYTO 9).

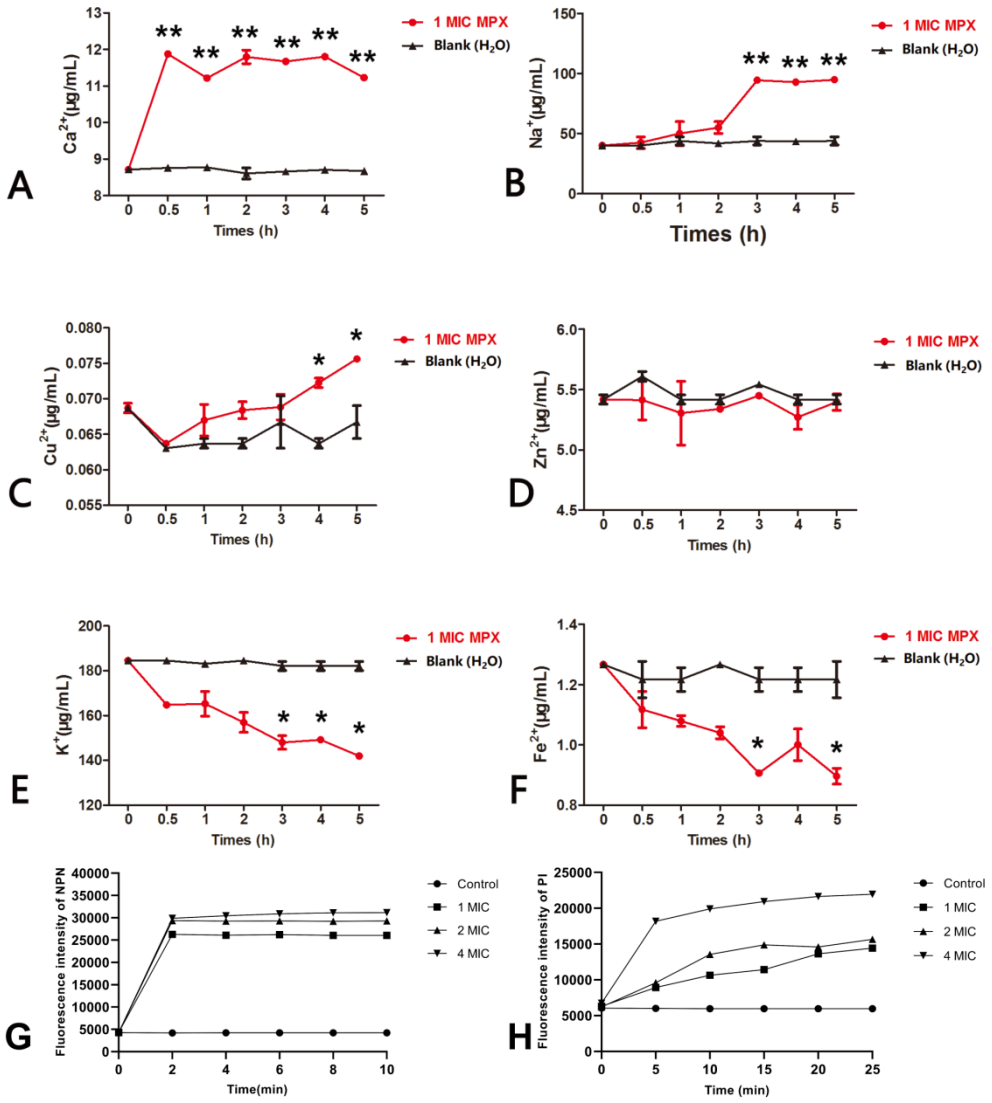

Fig. 3.3. MPX increases membrane permeability and changes bacterial ion concentration distribution

(A-F) The results of cation (Ca^{2+}, Na^{+}, Cu^{2+}, Zn^{2+}, K^{+}, Fe^{2+}) release after treatment of *A. pleuropneumoniae* with MPX (1MIC, 16ug/mL) at different time points. The bacterial culture supernatant using an Optical Emission Spectrometer Optima 2100 DV. T for ion concentration detection; (G) Outer membrane permeabilization of MPX was measured by detecting the fluorescence intensity of NPNin *A. pleuropneumoniae*; (H) Innermembrane permeabilization of MPX was measured by detecting the fluorescence intensity of PIin *A. pleuropneumoniae.*

Previous studies have found that MPX breaks membrane integrity by targeting the cell membrane to form an annular hole. To explore the effect of MPX on the membrane permeability of *A. pleuropneumoniae*, a BCA protein content kit was used to detect total protein content changes after MPX treatment at different times. Compared with the control treatment, *A. pleuropneumoniae* treatment with MPX (1×MIC) for 10 min showed a significantly increased total protein content of the supernatant (Fig. 3.2 A, $P < 0.01$), and the effect was comparable to that for gentamicin, while PR39 and PG had little effect on the total protein content of the supernatant. The total protein content of *A. pleuropneumoniae* supernatant increased significantly after MPX treatment for 1-3 min, and the protein content became stable by 6 min. The effect was comparable to that for gentamicin, which was significantly different from the control group (Fig. 3.2 A, $P<0.01$). The total protein content of *A. pleuropneumoniae* supernatant treated with PR39 and PG increased slowly, reaching the highest by 6 min.

Fluorescence microscopy was performed with SYTO 9/PI (Molecular Probes, Eugene, OR, USA) for double staining of *A.pleuropneumoniae*. (With an appropriate mixture of SYTO 9 and PI stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red.) Compared with that of the control group, the cell membrane integrity was damaged after treatment with MPX (1×MIC) for 0.5 h, PI entered the inside of the bacterial cells, and the number of dead bacteria was significantly increased (Fig. 3.2 B).The results indicated that MPX could cause bacterial death by destroying the cell membrane and increasing membrane permeability.

The cation concentration of the bacterial supernatant after treatment with MPX (1×MIC) was detected by an Optical Emission Spectrometer Optima 2100 DV. The results showed that the concentration of Ca^{2+} ions in the supernatant was significantly increased after treatment of *A. pleuropneumoniae* with MPX for 0.5 h, and the concentration of Ca^{2+} ions was higher than that inthe control group within 5 h (Fig. 3.3 A, $P<0.01$).

The concentration of Na^+ ions released increased after treatment with MPX, and the concentration of Na^+ ions reached the highest level after 3 h (Fig. 3.3B, $P<0.01$). The concentration of Cu^{2+} ions released increased with time and significantly increased after 4 h (Fig. 3.3C, $P<0.05$).

The concentrations of Zn^{2+} ions were not significantly different (Fig. 3.3 D).

with those of the control group, the concentrations of K^+ and Fe^{2+} were significantly decreased after treatment with MPX (1×MIC) for 3 h (Fig. 3.3 E, F, $P<0.05$). It is speculated that K^+ and Fe^{2+} ions flow into the interior of the bacterial cells.

The results indicate that MPX leads toan increase in membrane protein and ion permeability, thereby altering the distribution of ions inside and outside the bacterial cells.

The outer membrane permeabilisation of *A. pleuropneumoniae* was determined by using the NPN uptake assay. NPN, a neutral hydrophobic fluorescent probe, is normally excluded by the outer membrane but exhibits increased fluorescence intensity when it partitions into the outer membrane.

As shown in Fig. 3.3 G, MPX rapidly permeabilised the outer membrane of *A. pleuropneumoniae* in a concentration-dependent manner, as observed by an increase in NPN fluorescence.

MPX was able to permeabilise the outer membrane of *A. pleuropneumoniae* even at a concentration of 16 μg/mL (Fig. 3.3 G).

The inner membrane permeabilising ability of MPX was determined by using PI dye. PI shows enhanced fluorescence upon entering a compromised bacterial cell due to the strong binding with bacterial DNA.

Upon treatment with MPX, even at 16 μg/mL, a significant increase in fluorescence intensity was observed.

At the higher concentrations of 32 μg/mL and 64 μg/mL, the enhanced fluorescence signals followed concentration dependency (Fig. 3.3 H).

3.1.3 MPX significantly reduced the biofilm formation of *A. pleuropneumoniae*

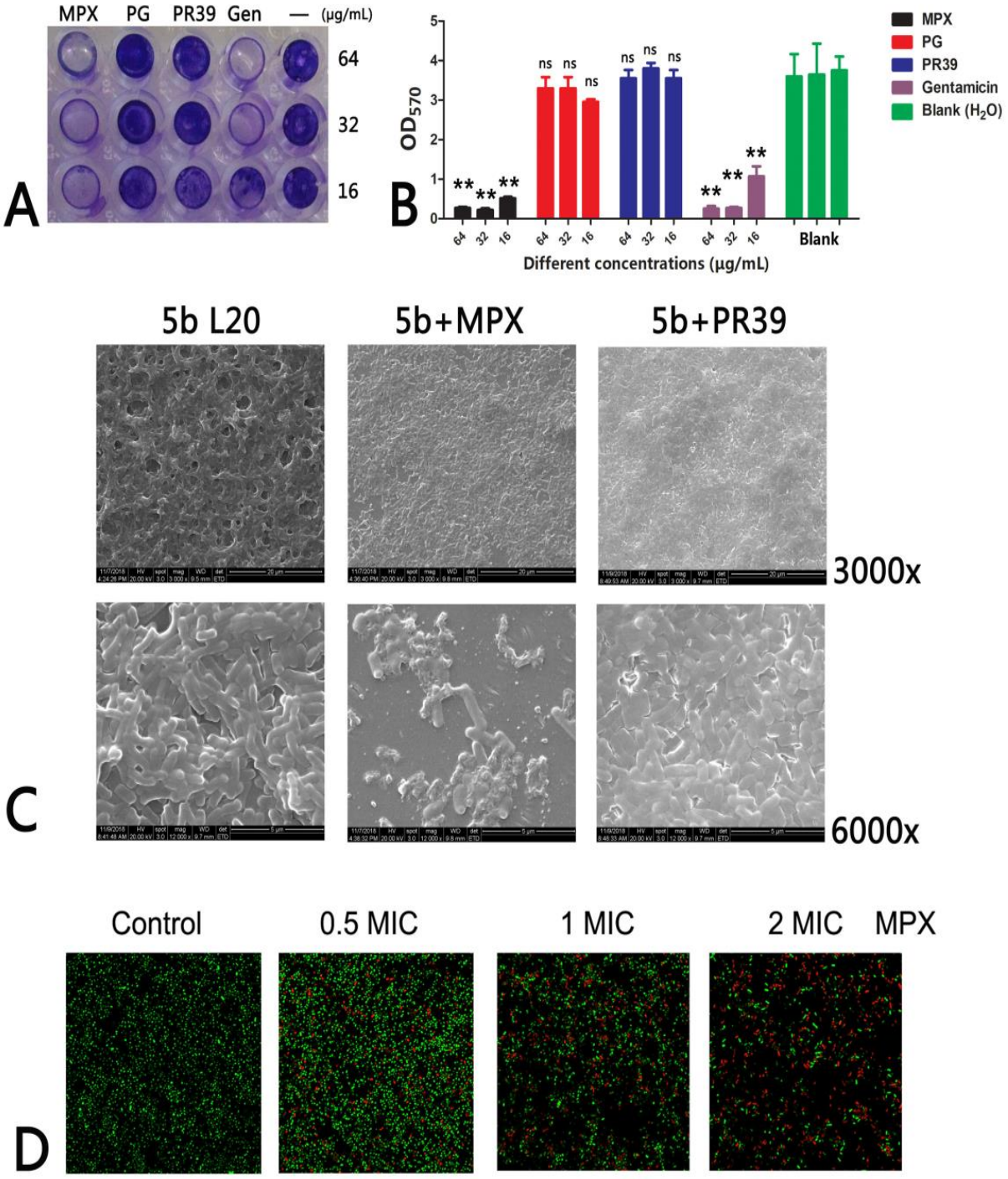

Fig. 3.4 MPX significantly affects biofilm formation of *A. pleuropneumoniae.*

(A) Crystal violet staining results of *A.pleuropneumoniae* biofilm formation; (B) OD_{570} evaluationof 70% ethanol-dissolved crystal violet using a spectrophotometer; (C) SEM results of *A. pleuropneumoniae* biofilm formation («5b» and «L20» represent the abbreviation of *A. pleuropneumoniae* serotype 5b

L20 strain);(D)The results of staining *A.pleuropneumoniae* with SYTO 9/propidium iodide.

To study the effect of MPX on *A.pleuropneumoniae* biofilm formation, MPX, PG, PR39 and Gen (16 μg/mL, 32 μg/mL, 64 μg/mL) were coincubated with *A.pleuropneumonia* for 24 h. Biofilm formation was evaluated using crystal violet staining.

Compared with the control treatment, MPX (16 μg/mL) significantly reduced *A. pleuropneumoniae* biofilm formation, indicatingthat the antimicrobial peptide MPX inhibited the growth of *A. pleuropneumoniae* to some extent.

The effect was better than that for Gen, while the same concentration of the antimicrobial peptides PG and PR39 had no effect on *A. pleuropneumoniae* biofilm formation (Fig. 3.4 A).

In addition, after dissolution in 70% alcohol, crystal violet was detected at OD_{570} using a spectrophotometer, which showed that MPX treatment significantly reduced *A. pleuropneumoniae* biofilm formation (Fig. 3.4B, $P<0.01$).

This result is consistent with previous studies (Fig. 3.4A).

Scanning electron microscopy showed that MPX (1×MIC) significantly reduced *A. pleuropneumoniae* biofilm formation and caused a looser structure accompanied by decreased bacterial adhesion, resulting in increased interstitial space between the bacteria. The negative control bacteria formed a dense biofilm, and the bacterial gap was small, while PR39 had no effect on *A. pleuropneumoniae* biofilm formation (Fig. 3.4 C), which was consistent with a previous study.

The confocal laser microscopy results showed that with increasing MPX concentration, the number of dead bacteria in the *A. pleuropneumoniae* biofilm was significantly increased, and the number of viable bacteria was significantly reduced (Fig. 3.4D).

The above results indicated that MPX could significantly inhibit *A. pleuropneumoniae* biofilm formation.

3.1.4 MPX reduces the pathogenicity of A. pleuropneumonia by down regulating virulence gene expression

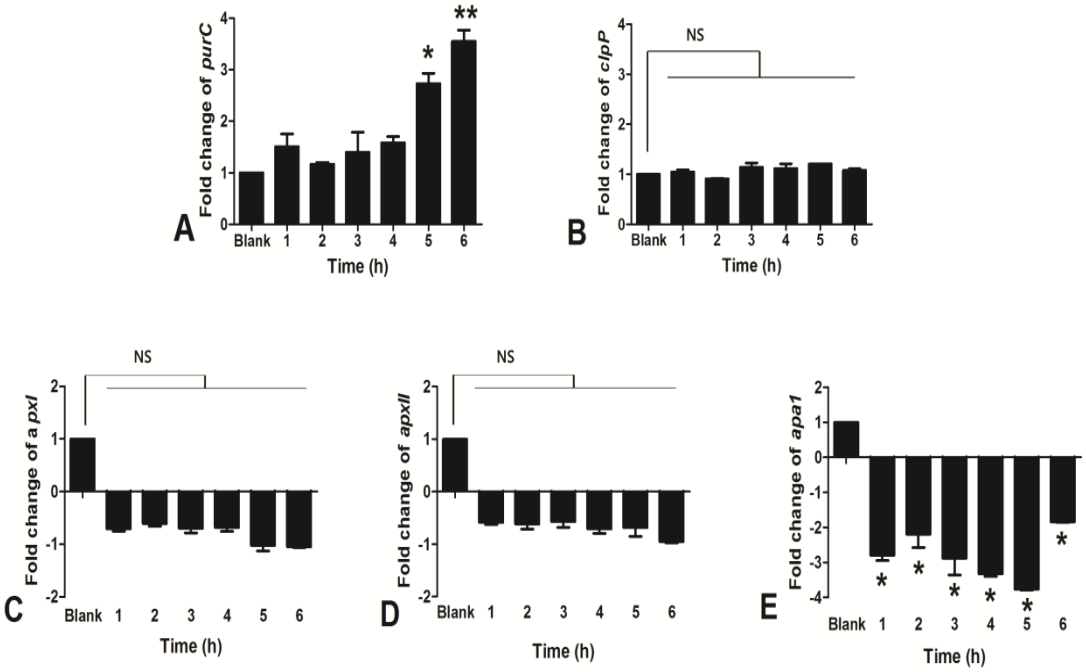

Fig. 3.5 qRT-PCR results of the expression of virulence-related genes (*pur C, clp P, apx I, apx II, apa 1*) in MPX-treated *A. pleuropneumoniae*

The *pur C*, *clp P*, *apx I*, *apx II*, and *apa 1* genes are related to the virulence of *A. pleuropneumoniae*. Therefore, the mRNA expression of these genes was detected by qRT-PCR after MPX treatment.

The results showed that MPX led to the upregulation of *pur C* (Fig. 3.5 A, $P<0.01$). It is speculated that MPX can destroy the cell membrane and damage the extracellular structure. MPX had no effect on the expression of the *clp P* gene in *A. pleuropneumoniae* (Fig. 3.5 B, $P>0.05$). *Apx I and apx II* gene expression was down regulated after treatment with MPX (Fig. 3.5 C, D).

This study found that MPX significantly down regulated *apa 1* gene expression after treatment for 1 h (Fig. 3.5 E, $P<0.05$), suggesting that MPX could regulate early infection of *A. pleuropneumonia* by down regulating *apa 1* gene expression.

3.1.5 Sap A plays an important role in *A. pleuropneumoniae* resistance to MPX

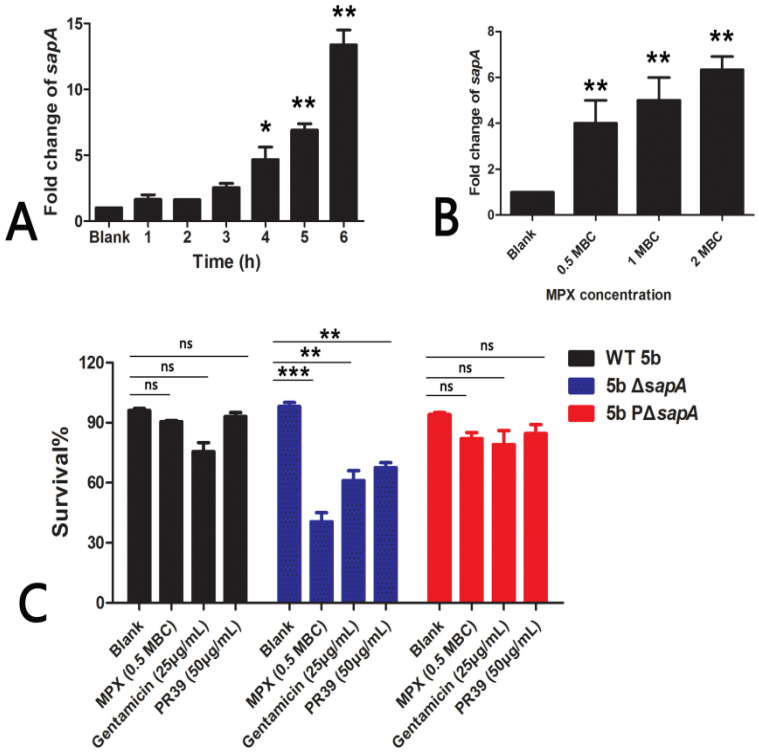

Fig. 3.6 The role of *sap A* in resistance to MPX

(A) qRT-PCR results of s*ap A* gene expression in *A. pleuropneumoniae* after treatment with MPX for different amounts of time; (B) qRT-PCR results of *sap A* gene expression in *A. pleuropneumoniae* after treatment with MPX at differentconcentrations; (C) The effect of *Δ sap A* gene deletion on the survival rate of *A. pleuropneumoniae* after MPX treatment.

This study found that MPX significantly upregulated *sap A* gene expression after treatment for 4 h (Fig. 3.6 A, $P < 0.01$), suggesting that *sap A* plays an important role in blocking MPX function. In addition, the expression of the *sap A* gene was significantly upregulated after treatment with different concentrations of MPX (Fig. 3.6B, $P < 0.01$). To further investigate the role of *sap A* in *A. pleuropneumoniae* resistance to MPX, the *sap A* gene was knocked down, and the survival rate of the wild-type strain and complemented strain P Δ *sap A* was compared after treatment with MPX. The survival rate of Δ *sap A A.*

pleuropneumoniae was significantly reduced compared to that of the control group after treatment with MPX (0.5×MBC) (Fig. 3.6C, P<0.001), and the effect of MPX was better than that of gentamicin and PR39. However, MPX had relatively little effect on the survival rate of the WT5b and PΔ *sap A* strains. This study suggesting that *sap A* played an important role in *A. pleuropneumoniae* resistance to MPX.

3.1.6 MPX protects mice against a fatal infection with *A. pleuropneumoniae*

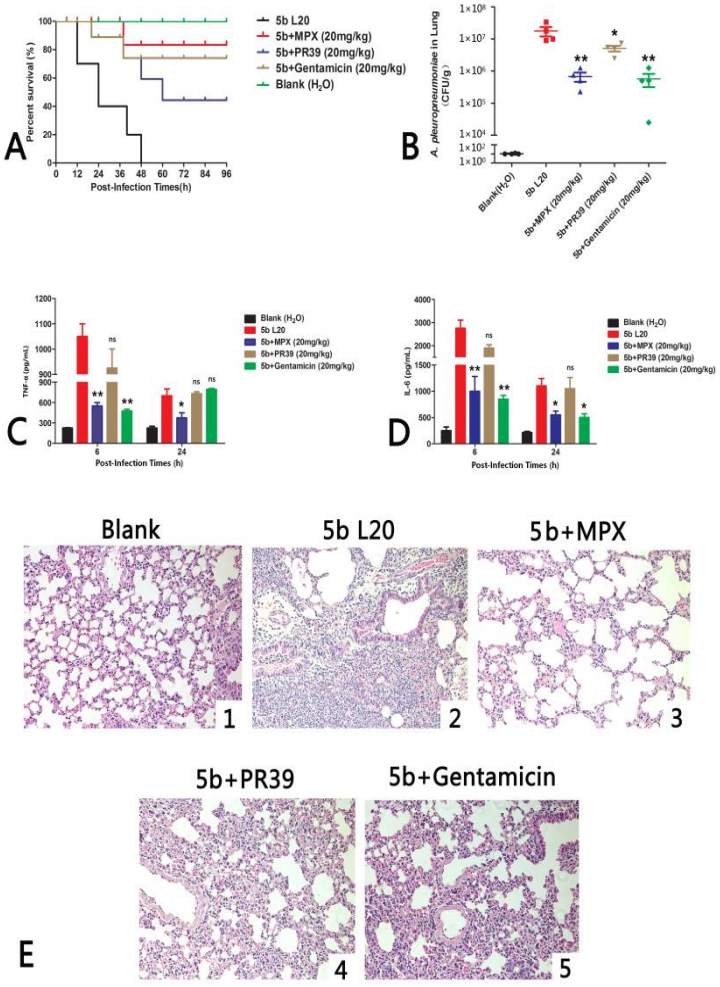

Fig. 3.7 MPX protects mice against a lethal dose of *A. pleuropneumoniae*

(A) The survival rate of mice infected with *A. pleuropneumoniae* after MPX treatment; (B) Lung bacterial colonization results of mice infected with *A. pleuropneumoniae* after MPX treatment; (C-D)

The levels of TNF-α and IL-6 in the lung homogenate of *A. pleuropneumoniae*-infected mice after treatment with MPX; (E) The lung HE staining results of *A. pleuropneumoniae*-infected mice after treatment with MPX. 5b and L20 are the abbreviations of *A. pleuropneumoniae* serotype 5b L20 strain. 20 BALB/c mice in per group.

The therapeutic effect of MPX on pneumonia was evaluated in a BALB/c mouse model.

Compared with the untreated *A. pleuropneumoniae* infection group, MPX (20 mg/kg)-treated mice showed mild symptoms and no shortness of breath, lack of energy or other clinical symptoms.

MPX protects mice against a lethal dose of *A. pleuropneumoniae*, and the protection rate was 80%, which is superior to the effects of the same dose of gentamicin or PR39.

In the untreated group, *A. pleuropneumonia* caused all infected mice to die within 48 h (Fig. 3.7A).

The number of bacteria colonising the lung in mice was significantly lower in the *A. pleuropneumoniae* infection group after MPX treatment than in the untreated group (Fig. 3.7 B, $P<0.01$).

The effect was comparable to that of gentamicin and better than that of PR39. The levels of TNF-αand IL-6 in lung homogenate were detected using ELISA.

The levels of TNF-α and IL-6 were significantly decreased after MPX treatment for 6 h compared with those of the group infected with *A. pleuropneumonia* alone (Fig. 3.7 C, D, $P <0.01$).

However, the levels of TNF-α and IL-6 were not affected by PR39 treatment. Pathological analysis of the lung showed that *A. pleuropneumoniae* infection caused typical pneumonia symptoms in mice, a large number of inflammatory cells

infiltrating into the lung and alveolar cavity, severe lung haemorrhage, and alveolar wall fusion.

MPX treatment alleviated the above symptoms, reducing lung damage and recruiting neutrophils to the alveoli of the treated mice compared with the control mice. Similarly, gentamicin treatment with IP also relieved pneumonia symptoms in mice, while PR39 had no effect on pneumonia symptoms (Fig. 3.7 E).

The above results indicated that MPX could protect mice against a lethal dose of *A. pleuropneumoniae* and alleviate the symptoms of pneumonia in mice.

3.2 The mechanism of antimicrobial peptide MPX against enterohemorrhagic *Escherichia coli in vitro*

In this study, the results found that MPX has good antibacterial activity against *E. coli*, and the minimum inhibitory concentration (MIC) was 31.25 ug/mL. MPX bactericidal kinetics study found that MPX had good bactericidal activity within 6 hours.

Bacterial permeability studies have shown that MPX could increase the permeability of bacteria, leading to an increase in the protein content of the bacterial supernatant.

In addition, NPN, PI and DiSC3 (5) results showed that the fluorescence value was positively correlated with MPX.

The stability test of MPX found that salt ions, temperature, pH, etc. have a slight influence on its effect.

In addition, scanning electron microscopy results showed that the bacteria became smaller and the contents leaked after the action of MPX.

The above results showed that MPX has a good bactericidal activity in vitro, laying the foundation for the development of new drugs for the treatment of bacterial infections.

3.2.1 MPX has good bactericidal activity

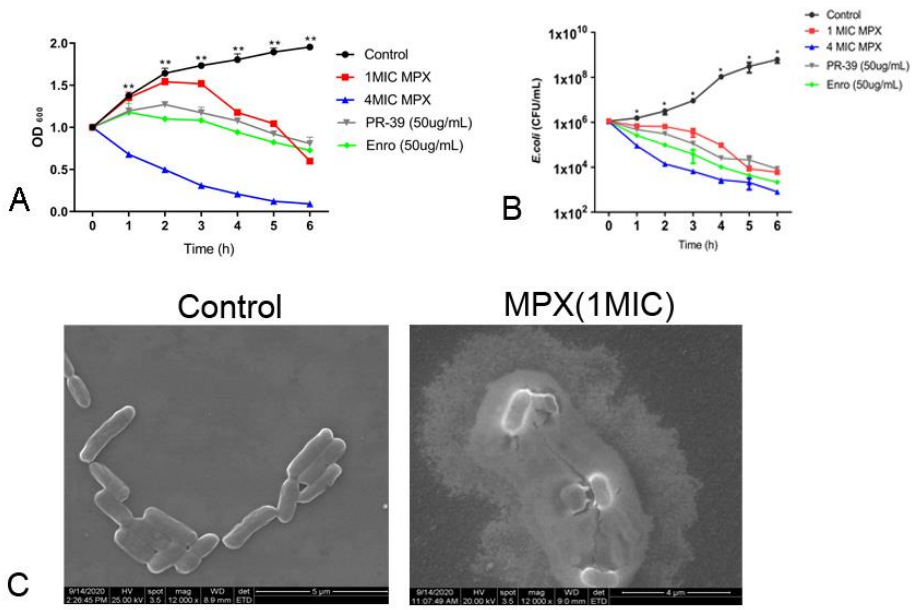

Fig. 3.8 The bactericidal activity detection of MPX in vitro

(A) The *E. coli* OD_{600} detection after treatment with MPX; (B) The *E. coli* plate count detection after treatment with MPX; (C) The effect of MPX on *E. coli* was detected by scanning electron microscope.

The antibacterial activity of MPX against *E. coli* was detected by double-layer agarose amplification. It was found that MPX could effectively inhibit the growth of *E. coli* at 1 mg/mL, and the size of the antibacterial ring was equivalent to that of Enro (1 mg/mL).The negative control has no antibacterial activity. In addition, a modified micro broth dilution method was used to determine the minimum inhibitory concentration of MPX against *E. coli* 31.25 ug/mL. In order to test the bactericidal activity of the antimicrobial peptide MPX against *E. coli*, the OD_{600} value of the bacterial culture was measured, and it was found that the OD_{600} of the bacterial culture solution decreased to 0.1 after the antimicrobial peptide MPX acted on for 6 h, which was significantly lower than that of the control group (Fig.3.8A, $p<0.01$). The bactericidal activity of MPX against *E. coli* was measured by a plate counting method. It was found that MPX showed an MIC-dependent

increase in the bactericidal efficiency of *E. coli*. The number of bacteria was significantly reduced (Fig.3.8B).

Scanning electron microscope wasused to observe the effect of MPX on the morphology of *E. coli*. The results showed that the negative control *E. coli* has a full morphology, large cells, and a smooth surface. After MPX (1 MIC) treatment for 2 h, the cells became significantly smaller, resulting in leakage of bacterial contents (Fig.3.8 C). The above results all indicate that MPX can effectively kill *E. coli* in vitro.

3.2.2 The effect of pH and temperature on the antibacterial activity of MPX

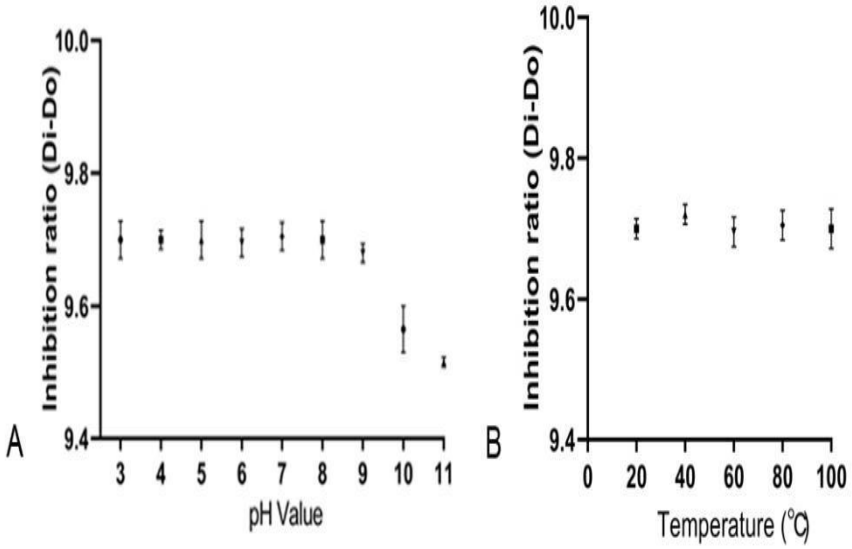

Fig. 3.9 The effect of pH and temperature on MPX

(A) The effect of pH on MPX; (B) The effect of temperature on MPX.

In order to study the effect of pH and temperature on the antibacterial activity of MPX, the antibacterial radius method was used to determine the antibacterial activity of MPX on *E. coli* at different pH values and temperatures. As shown in Fig.3.9A, the pH in the range of 2-9 has no effect on MPX activity. When pH>10, the antibacterial activity of MPX decreases, indicating that the activity of MPX wasstable compared in acidic and weakly alkaline environments.

The MPX was subjected to different temperature treatments, and the highest temperature reached 100°C. It was found that the temperature had no effect on the antibacterial activity of MPX, indicating that MPX has good thermal stability (Fig.3.9 B).

3.2.3 The effect of ions on the antibacterial activity of MPX

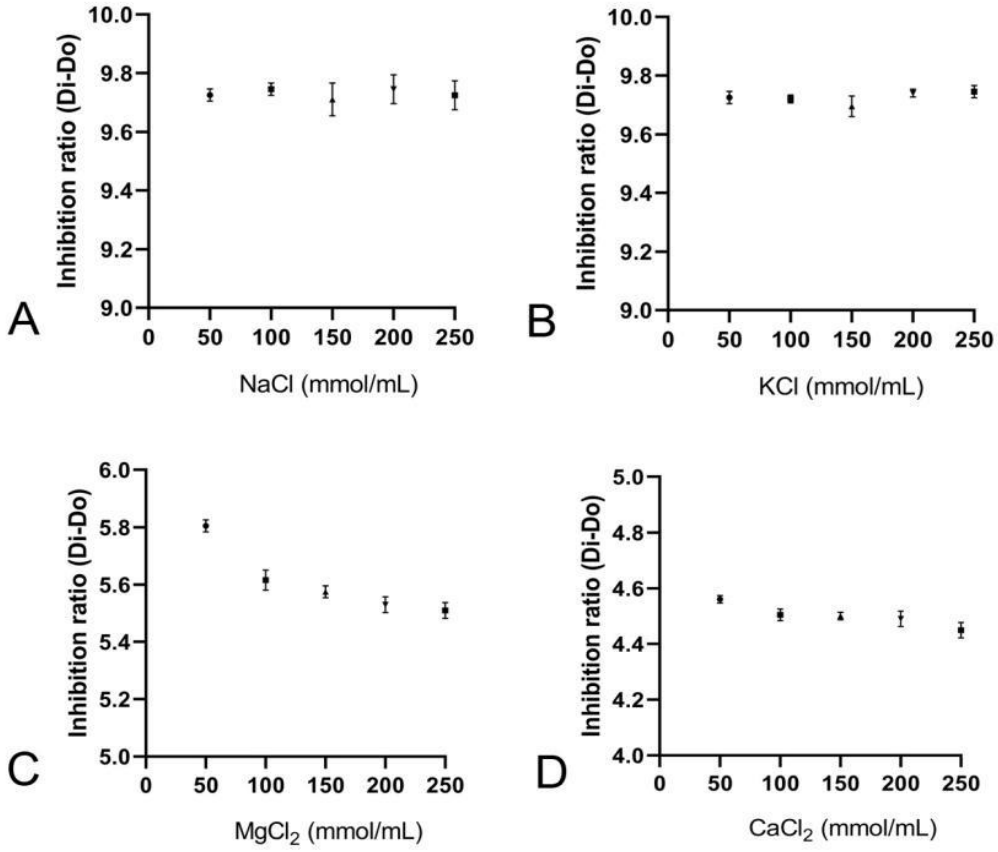

Fig. 3.10 The effect of cations Na^+, K^+, Mg^{2+}, Ca^{2+} on the activity of MPX

(A) The effect of cations Na^+ on the activity of MPX; (B) The effect of cations K^+ on the activity of MPX; (C) The effect of cations Mg^{2+} on the activity of MPX; (D) The effect of cationsCa^{2+} on the activity of MPX.

In order to study the effect of different salt ions on the activity of MPX after treating with MPX at different concentrations of cations Na^+, K^+, Mg^{2+}, Ca^{2+}, it was found that cations Na^+, K^+ had no effect on the antibacterial activity of MPX, while cationic Mg^{2+} and Ca^{2+} had effect on MPX activity (Fig.3.10 A-D). It is

speculated that the presence of cationic Ca^{2+} leads to changes in the secondary structure of MPX, thereby affecting its antibacterial activity.

3.2.4 Bacterial permeability of MPX

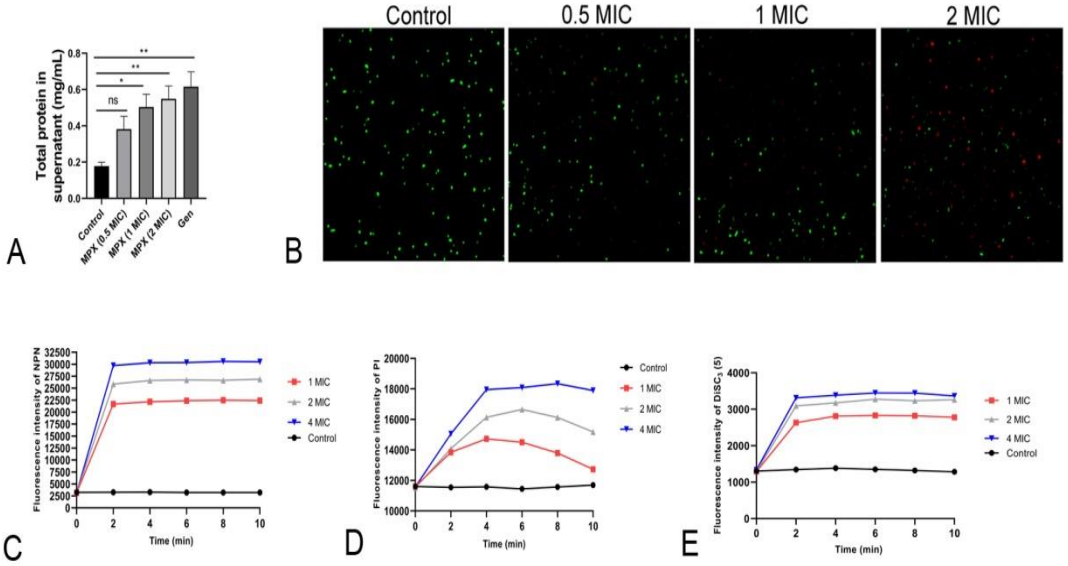

Fig. 3.11 The effect of MPX on the permeability of *E. coli.*

(A) The results of total protein in *E. coli* bacterial supernatant after MPX treatment; (B) The effect of MPX on the permeability of *E. coli* was observed by immunofluorescence; (C) Detection of NPN fluorescence after MPX treatment with *E. coli*; (D)Detection PI of fluorescence after MPX treatment with *E. coli*;(E) Detection of DISC3(5) fluorescence after MPX treatment with *E. coli*.

In order to study the permeability of antimicrobial peptide MPX to *E. coli* strains, BCA protein content determination and immunofluorescence observation was used in this studyand found that the protein content in supernatant of *E.coli*was significantly higher than that of the control groupafter the action of MPX treatment for 3 hours(Fig. 3.11A), The protein content is significantly higher than MPX (1 MIC) after treatment with MPX (2MIC), and the effect of MPX on *E. coli* is positively correlated with the concentration. Fluorescence microscope used SYTO 9/PI (Properly mix SYTO 9 and PI staining, bacteria with intact cell membranes are stained fluorescent green, while bacteria with damaged cell

membranes are stained fluorescent red). Compared with the control group, the integrity of the cell membrane was damaged after MPX (2 MIC) treatment, PI entered into the bacterial cells, and the number of dead bacteria increased significantly. The effect was significantly better than that of MPX 1MIC, and it was consistent with the BCA protein content determination results. MPX exerts its bactericidal function by changing the permeability of bacteria.

The permeability of MPX to the outer membrane of *E. coli* was measured by NPN uptake assay. NPN is a neutral hydrophobic fluorescent probe, which is usually excluded by the outer membrane, but the fluorescence intensity increases when it enters the outer membrane. As shown in Fig. 3.11 C, MPX rapidly penetrated the outer membrane of *E. coli* in a concentration-dependent manner, which could be observed by the increase in NPN fluorescence. MPX could penetrate the outer membrane of *E. coli* even at a concentration of 1 MIC in a dose-dependent manner.

PI was used to determine the permeability of MPX to the inner membrane of *E. coli*. Due to its strong binding to bacterial DNA, the fluorescence of PI increases when it enters the damaged bacterial cell. After *E. coli* treatment with MPX, the PI fluorescence intensity was significantly increased, and the fluorescence signal intensity increased in a concentration-dependent manner (Fig. 3.11 D).

The effect of MPX on the depolarization of *E. coli* cytoplasmic membrane was observed. Membrane-potential sensitive dye 3,3′-dipropylthiadicarbocyanine iodide [DiSC3(5)] was used for this study. Under the normal potential on the cell membrane, DiSC3 (5) dye is distributed inside and outside the bacterial cell. Therefore, the initial fluorescence intensity of the dye decreases due to its "self-quenching" within the bacterial cell. After treatment with drugs that affect the normal membrane potential, the release of the dye in the external medium increases, which in turn leads to an increase in fluorescence intensity. After adding different concentrations of MPX (1MIC, 2MIC, 4MIC), it was found that the fluorescence intensity of *E. coli* increased with the increase of the concentration of

MPX (Fig. 3.11E). This result showed that MPX had a significant effect on the normal membrane potential of *E. coli.*

3.2.5 The effect of MPX on *E. coli* biofilm formation

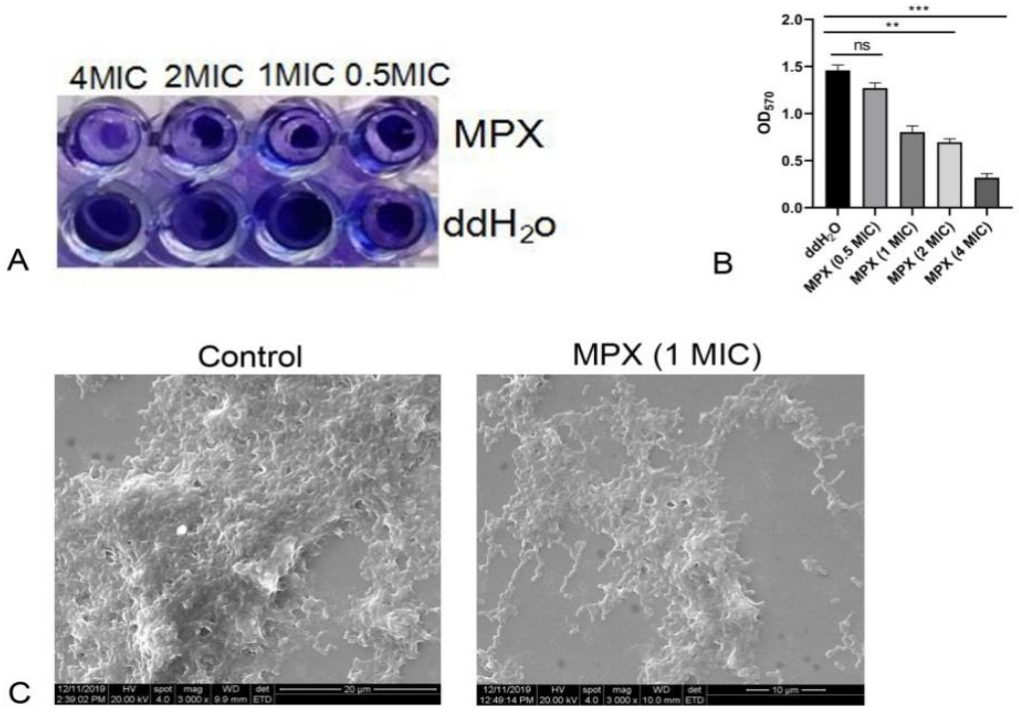

Fig. 3.12 The effect of MPX on the formation of *E. coli* biofilm.

(A) The effect of MPX on *E. coli* biofilm by crystal violet staining; (B) OD_{570} detected the effect of MPX on *E. coli* biofilm; (C) The effect of MPX on the biofilm formation of *E. coli* by scanning electron microscope.

The formation of *E. coli* biofilm plays an important role in its virulence and drug resistance. Therefore, this study first adopted the crystal violet method to study the effect of MPX on the biofilm formation of *E. coli.* The results showed that compared with the control group, MPX (1 MIC) could reduce the formation of *E. coli* biofilm in a dose-dependent manner, and MPX (4 MIC) had the best effect (Fig. 3.12 A). Furthermore, after using 70% alcohol to dissolve the crystal violet, the absorbance value was measured with an OD_{570} spectrophotometer, compared

with the control group, MPX significantly reduced the absorbance value of the *E. coli* biofilm (Fig. 3.12 B).

Scanning electron microscope was used to observe the effect of MPX (1 MIC) on the biofilm of *E. coli*, which resulted in a loose structure, reduced bacterial adhesion, and an increase in the gap between bacteria. While control group formed dense biofilm with small gaps between bacteria (Fig. 3.12 C). The above results indicate that MPX can significantly inhibit the formation of *E. coli* biofilm.

3.3 The antimicrobial peptide MPX protects against Enterohemorrhagic Escherichia coli O157:H7 infection, inhibits inflammation

Studies have found that MPX had better bactericial activity against *E. coli* in vitro. However, whether MPX also has better bactericidal activity in mice still unknown. In this study, the results found that *E. coli* infected mice loss of appetite, diarrhea, and grouping together, while MPX treatment significantly alleviated these symptoms. The results of autopsy found that the intestinal congestion, bleeding, thinning of the intestinal wall, yellow viscous fluid in the intestinal cavity, congestion of the lungs, necrosis in the liver, congestion and bleeding of the spleen, and MPX treatment effectively relieved the above symptoms. The qRT-PCR results found that MPX could increase the mRNA expression of the antibacterial protein TFF3 in the jejunum and colon, and reduce the expression of the antibacterial protein Reml β and REG3 γ in the jejunum and colon. H&E staining results further found that MPX could alleviate the pathological damage of mouse intestines and organs caused by *E. coli* infection. SEM and TEM analyses showed that MPX effectively ameliorated the jejunum damage caused by *E. coli*and increased the number and length of microvilli. In addition, MPX decreased the expression of IL-2, IL-6, TNF-α, p-p38 and p-p 65 in the jejunum and colon. Moreover, MPX increased the expression of ZO-1, occludin and MUC2 in the jejunum and colon, improved the function of the intestinal barrier and promoted

the absorption of nutrients. This study suggests that MPX is an effective therapeutic agent for *E. coli* infection and other intestinal diseases, laying the foundation for the development of new drugs for bacterial infections.

3.3.1 MPX alleviates the clinical manifestations of mice

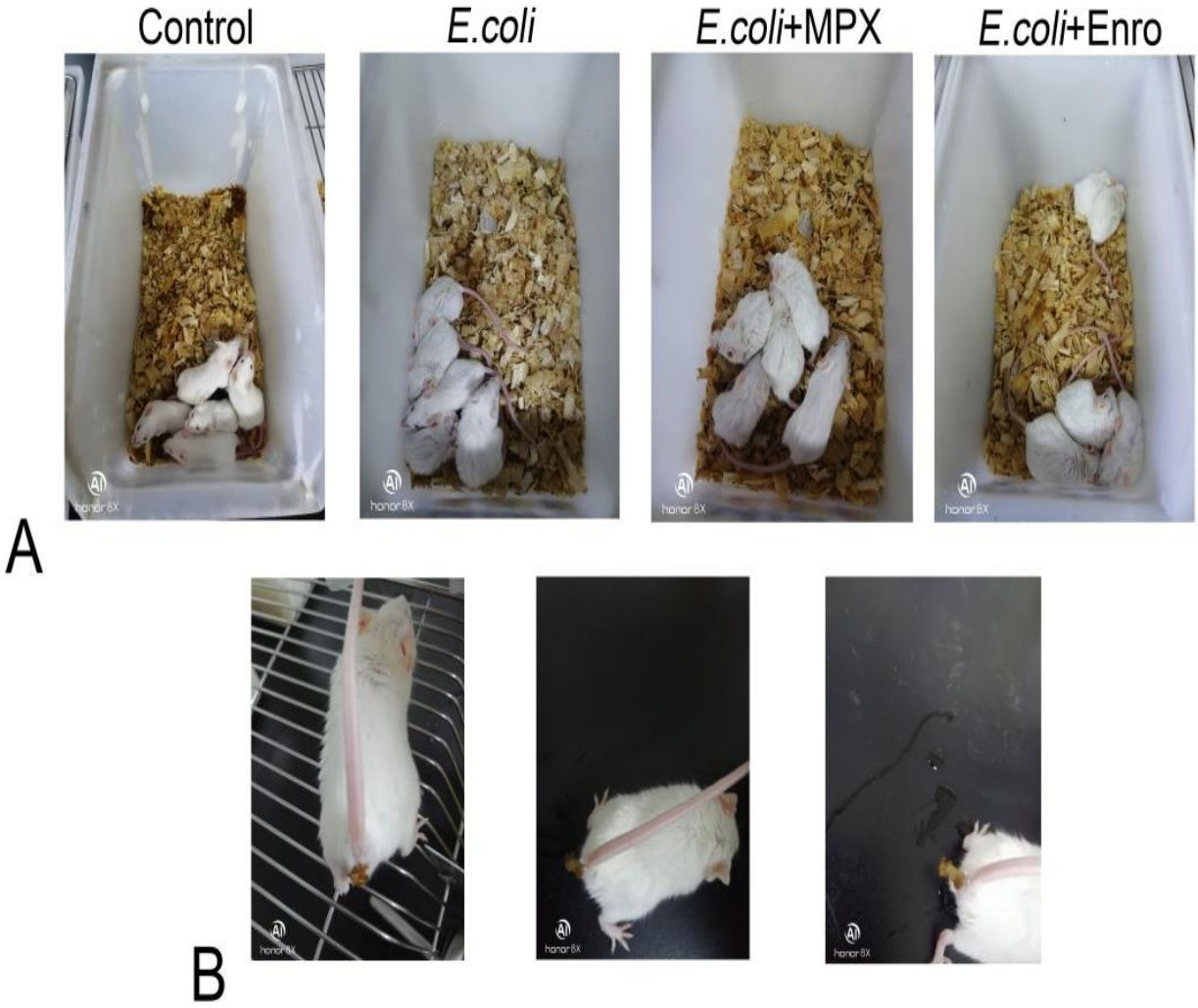

Fig. 3.13 Observation of clinical symptoms of *E. coli* infection with BALB/c mice (A, B).

Observation of clinical symptoms after infection of *E. coli* in mice was shown in Fig. 3.13A and B: mice infected with *E. coli* alone showed loss of appetite, rapid heartbeat, body tremor, loose hair, bunching up, arched back, anal prolapse, feces

clinical manifestations such as irregularities, while MPX treatment significantly alleviated the adverse reactions caused by *E. coli* infection.

Mice increased appetite, smooth coat, and the effect was better than enrofloxacin treatment. The control group did not show any adverse reactions.

3.3.2 MPX alleviates the pathological changes of mice by necropsy

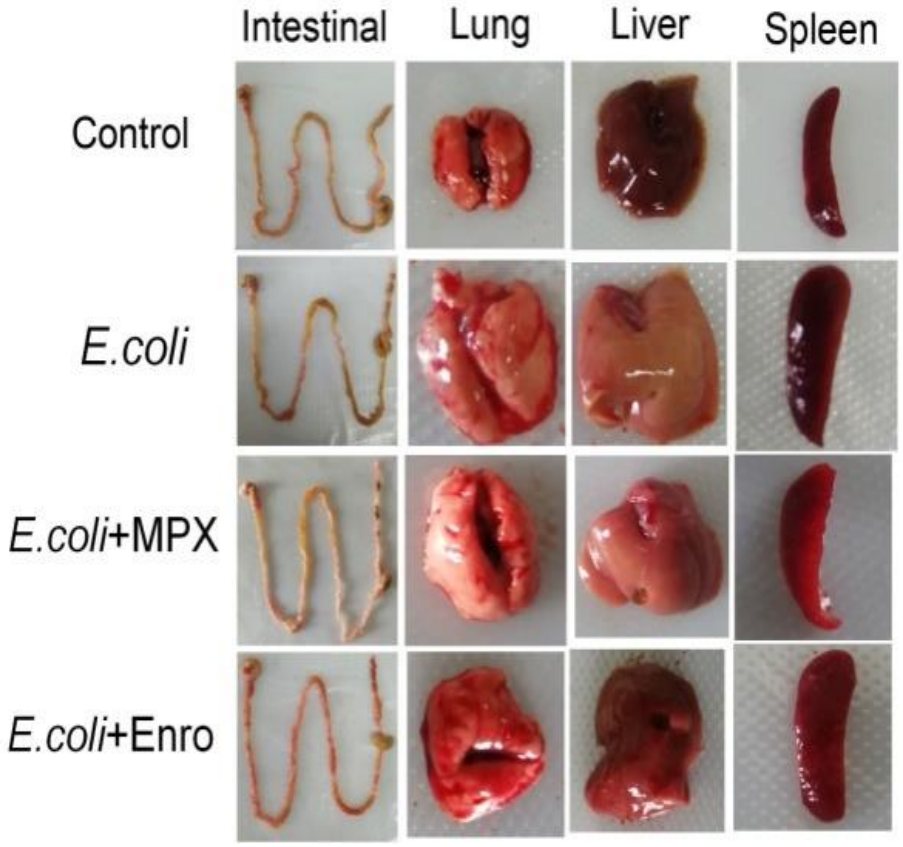

Fig. 3.14 Autopsy results of mouse intestines and organs after *E. coli* infection.

The results of the necropsy were shown in Fig. 3.14, the intestines of mice in the control group were normal, with thick and flexible intestinal walls, and no pathological changes were seen in the liver, spleen, and lungs.

Mice infected with *E. coli* had intestinal congestion, hemorrhage, intestinal wall thinning and easy to rupture, the intestinal cavity was filled with yellow viscous liquid, the jejunum was severely congested, and the lungs, liver, and spleen were congested and bleeding.

While MPX could effectively alleviate the intestinal inflammatory response and organ pathological damage caused by *E. coli* infection, and its effect was equivalent to that of the antibiotic Enro.

3.3.3 MPX protects mice fromfatal *E. coli* infection

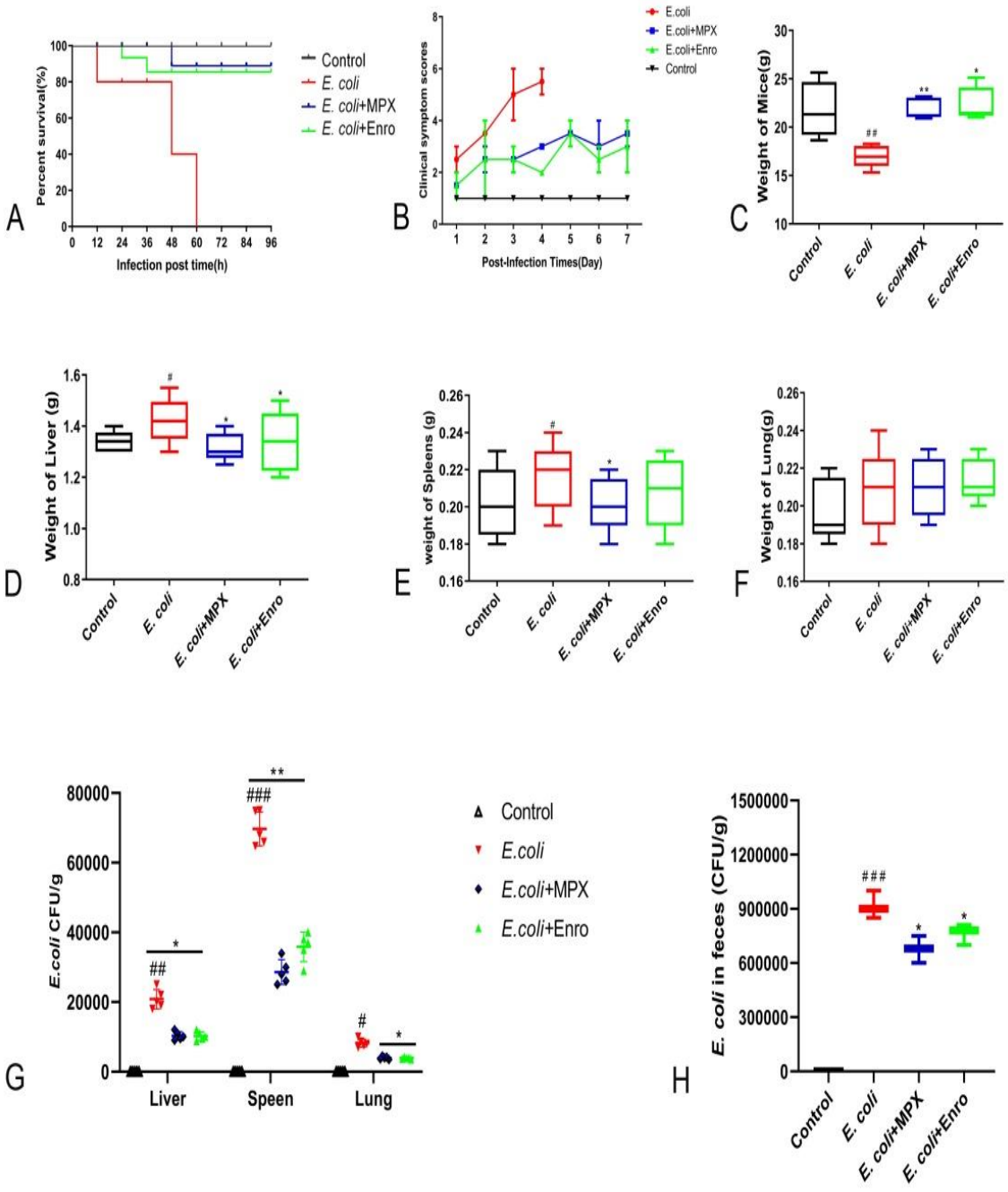

Fig. 3.14 MPX protects mice against infection with a lethal dose of *E. coli*.

(A) The survival rate of mice infected with *E. coli* after MPX treatment. (B) The clinical symptom score of mice infected with *E. coli* after MPX treatment. (C)

The weight of mice infected with *E. coli* after MPX treatment. (D-F) The weight of the liver, spleen and lung of mice infected with *E. coli* after MPX treatment. (G) The number of bacteria in the liver, spleen and lung of mice infected with *E. coli* after MPX treatment. (H) The number of bacteria in thefeces of mice infected with *E. coli* after MPX treatment.

The the rapeutic effect of MPX onintestinal inflammation and the intestinalbarrierwas evaluated in a BALB/c mouse model. The results showed that MPX could protect the mice froma lethal dose of *E.coli*, and the survival rate of the mice was90%; this effect of MPX was better than that of Enro. However, all of the mice infected with *E. coli* without MPX treatment within 60 h (Fig. 3.14 A).

The observation of clinical symptoms revealed that*E.coli* infection caused severe diarrhea, lack of energy, loss of appetite, clustering, and messy back hair, while these symptoms were significantly alleviated after MPX treatment; these effects of MPX were superior to those of the same dose of Enro (Fig. 3.14 B). The weights of the mice infected with *E. coli* were notably reduced (Fig. 3.14 C, $p<0.01$) but were significantly increased after MPX and Enro treatment (Fig. 3.14 C, $p<0.01$) and were not significantly different from those of control mice (Fig. 3.14 C, $p>0.05$).

The weights of the livers and spleens in the *E. coli* infection group were notably high (Fig. 3.14 D**,** E, $p<0.05$) and were significantly decreased after MPX treatment; after MPX treatment, these weights were not significantly different from those in the control group (Fig. 3.14 D, E, $P>0.05$). The lung weights were not significantly different after *E. coli* infection (Fig. 3.14F, $P>0.05$). The colonization of *E. coli* in the mouse liver, spleen, lung and feces was examined by counting on LB agar plates.

The number of bacteria colonizing the spleens of mice in the *E. coli* group was greater than those colonizing the liver and lung, and this number was significantly decreased after MPX and Enro treatment (Fig. 3.14 G, $P<0.05$).

The number of bacteria colonizing the feces was significantly lower after MPX and Enro treatment than after infection with *E. coli* alone (Fig. 3.14H, P>0.05).

These results indicated that MPX exerted good antibacterial effects in vivo and protected against lethal infection with *E. coli* in mice.

3.3.4 MPX reduces inflammatory cytokineexpression and improves intestinal morphology

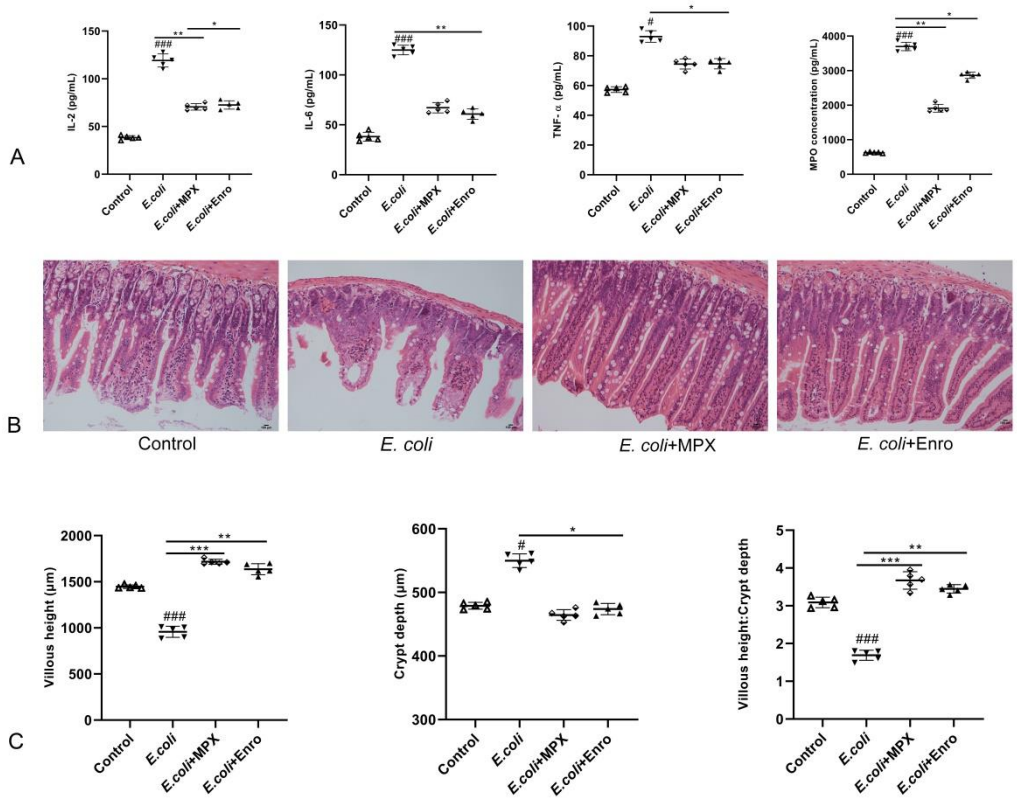

Fig. 3.15MPX inhibits inflammatory cytokine expression and improves intestinal morphology

(A) The levels of inflammatory cytokines (IL-2, IL-6 and TNF-α) and MPO in mouse serum were detected using ELISA; (B) The jejunum of mice was stained with H&E (bars, 100 μm); images were obtained at 200× magnification; (C) The length of jejunal villi, the depth of crypts, and the ratios of villi length to crypt depth were detected by ipw in 32 software.

To evaluate the effect of the MPX-mediated anti-inflammatory response after *E. coli* infection, the levels of IL-2, IL-6, TNF-α and MPO were detected by ELISA. As shown in Fig. 3.15 A, the levels of the inflammatory factors IL-2, IL-6, TNF-α and MPO were significantly increased after *E. coli* infection, while MPX reduced the serum levels of IL-6 ($p<0.01$), IL-2, TNF-α and MPO ($p<0.05$). H&E staining was used to explore the effect of MPX on the intestinal morphology of the jejunum in mice infected with *E. coli*; infection with *E. coli* caused typical intestinal inflammation and barrier damage, shortened villi, reduced mucosal thickness, necrosis, large amounts of inflammatory cell infiltration into the jejunum and disruption of intestinal villi.MPX treatment increased the villous height and goblet cell counts and decreased the infiltration of leukocytes into the jejunum, and these levels were not significantly different from those in the control group (Fig. 3.15 B). Moreover, compared with *E. coli* infection alone, MPX treatment increased the villus length in the mouse jejunum, decreased the crypt depth, and increased the ratio of villus height to crypt depth, and these effects of MPX were better than those of Enro (Fig. 3.15C, $p<0.05$). These results suggested that MPX effectively reduced the inflammatory factor secretion and improved the intestinal morphology and integrity in mice infected with *E. coli*.

3.3.5 MPX relieves intestine pathological damage

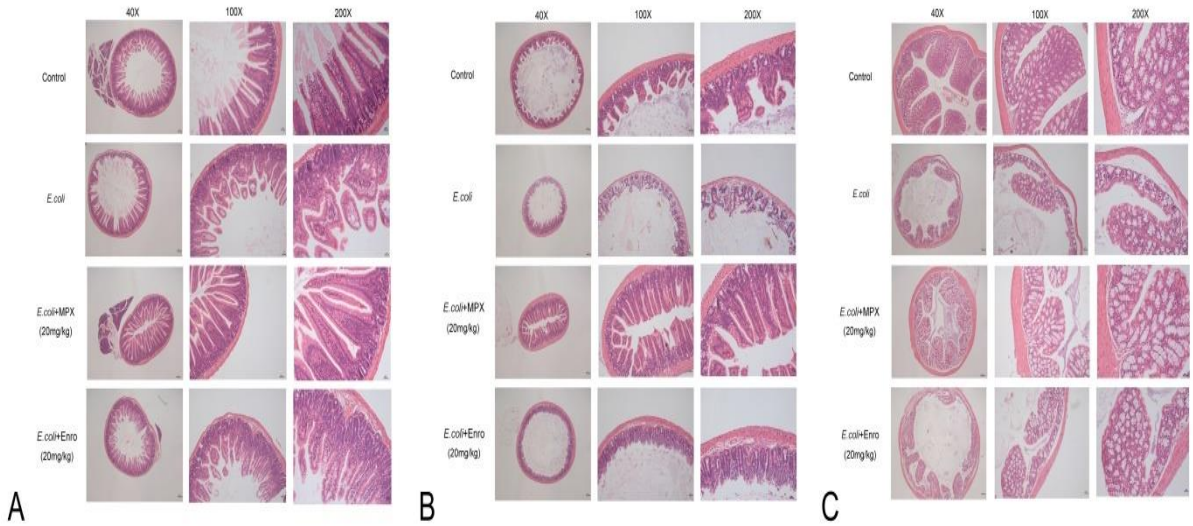

Fig. 3.16 H&E staining of intestines after *E. coli* infection in mice

(A) H&E staining of duodenum after *E. coli* infectionin mice; (B) H&E staining of ileum after *E. coli* infection in mice; (C) H&E staining of colon after *E. coli* infectionin mice.

Further H&E staining was used to observe the pathological changes of the duodenum, ileum and colon after *E. coli* infection. As shown in Fig. 3.16, the duodenum, ileum, and colon of mice infected with *E. coli* showed intestinal villi shedding, breaking and falling into the intestinal lumen, catarrhal enteritis, degeneration, necrosis, shedding of intestinal mucosal epithelial cells, congestion of the lamina propria and a large number of neutrophil infiltration, showing the pathology of necrotizing enteritis and fibrinous necrotizing enteritis Changes (Fig. 3.16 A, B, C). While the pathological changes of each bowel segment were significantly alleviated after treatment with MPX. The intestinal villi of the control were neatly arranged without the above-mentioned pathological changes.

3.3.6 MPX relieves pathological damage of organs in mice

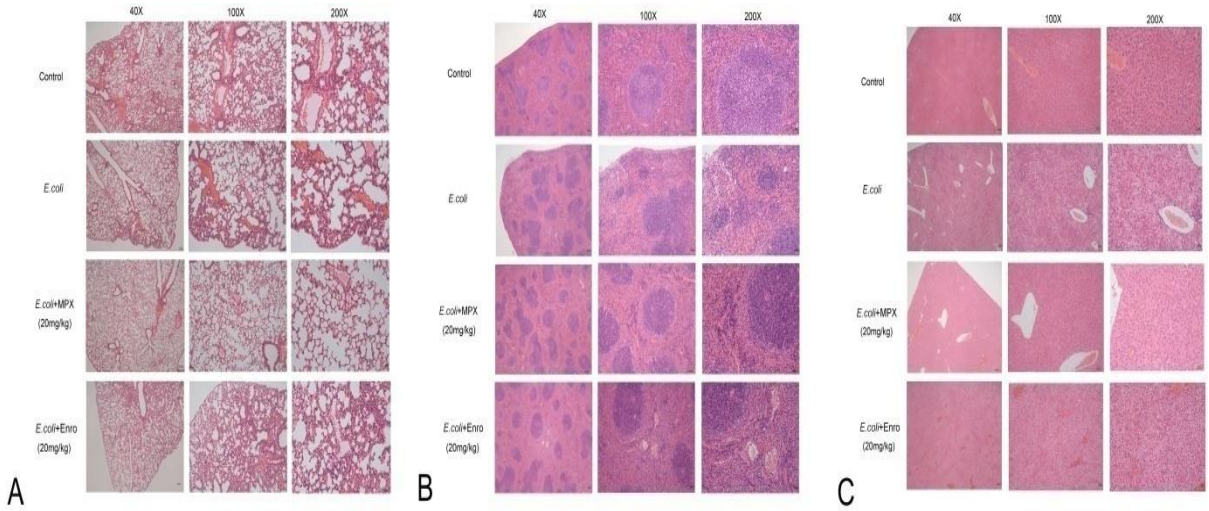

Fig. 3.17 H&E staining of the organs infected with *E.coli* in mice

(A) H&E staining of lung infected with *E. coli* in mice; (B) H&E staining of spleen infected with *E. coli* in mice; (C) H&E staining of liver infected with *E. coli* in mice.

E. coli-infected mice developed acute interstitial pneumonia, widened alveolar septum, ruptured alveoli, neutrophil infiltration, and mild lung disease, showing local pulmonary congestion and a small amount of red blood cell and inflammatory cell infiltration (Fig.3.17 A). Symptoms of hemorrhagic splenitis, congestion, local necrosis, small splenic corpuscles appear in the spleen, a large number of neutrophil infiltration in the splenic sinus (Fig.3.17 B), degeneration and necrosis of hepatocytes, and acute necrosis in the liver, disintegration of liver cells, congestion, liver congestion, dilation of liver sinusoids, infiltration of red blood cells and neutrophils (Fig.3.17 C). The above symptoms were significantly alleviated after treatment with MPX, indicating that MPX can protect mice against the damage of *E. coli* to the organs.

3.3.7 MPX improves intestinal villi and microvilli

Previous H&E staining studies have shown that *E. coli* infection can damage the intestinal morphology in mice. SEM and TEM were used to further evaluate the effects of MPX on the intestinal morphological changes induced by *E.coli*. The SEM results showed that *E. coli* infection severely damaged the morphology and integrity of intestinal villi, while MPX treatment obviously alleviated the injury to the jejunum villi and microvilli, as observed at high (200×) and low (30.000×) magnifications (Fig.3.18 A). We furthere valuated the effect of MPX on the microvilli and TJ proteins of intestinal epithelial cells by TEM. *E. coli* infection caused microvilli to fall off, decreased the number of microvilli, and damaged the TJ structure of the intestinal epithelial cells, while MPX treatment increased the number of the microvilli in intestinal epithelial cells; this effect of MPX was better than that of Enro (Fig.3.18 B).

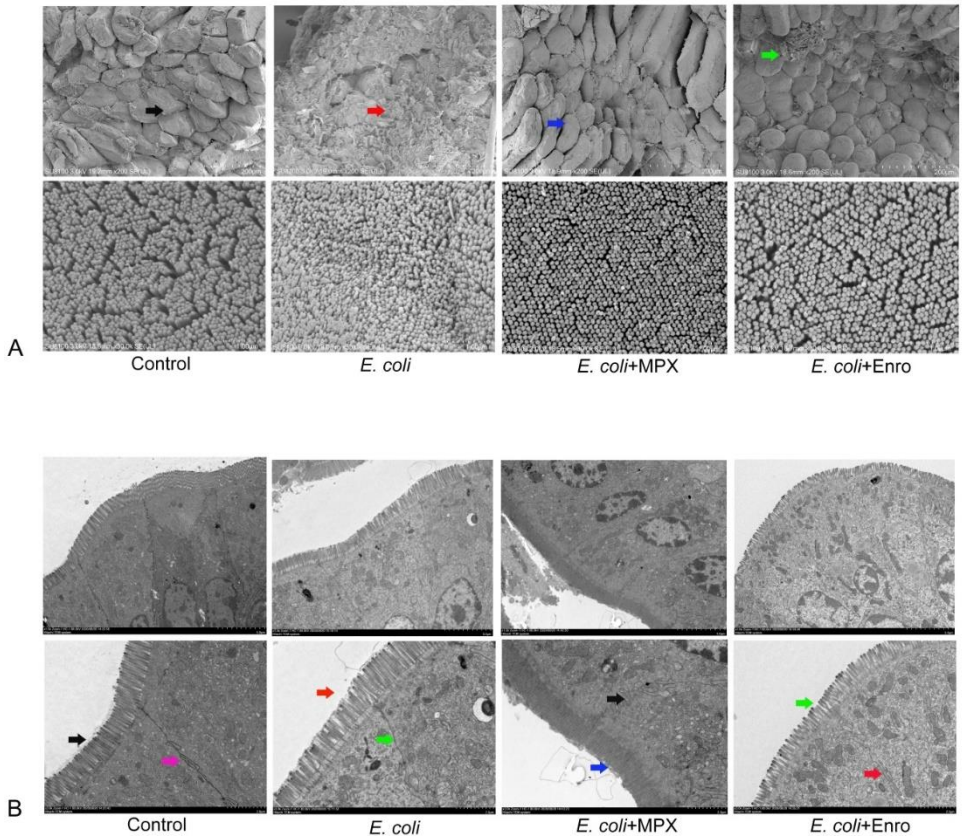

Fig 3.18 MPX improves the intestinal morphology of the jejunum and the microvilli of intestinal epithelial cells

(A) Morphological changes in the jejunum villi were observed by SEM (upper, 200×; lower, 30,000×); (B) Morphological changes in the microvilli and tight junction proteins in intestinal epithelial cells were observed by TEM (upper, 1500×; lower, 3000×).

These results indicate that MPX can protect against *E. coli*-induced damage to jejunal villi and microvilli in intestinal epithelial cells.

3.3.8 MPX increases the expression of intestinal antimicrobial peptide protein

The mRNA expression of intestinal antibacterial related proteins REG3 γ, Reml β, and TFF3 by qRT-PCR. In the jejunum (Fig. 3.19 A, B, C), compared with the control group, the TFF3 gene expression level in the jejunum of the *E. coli* group was increased ($P<0.05$); while the TFF3 gene expression in the jejunum of *E.*

coli + MPX significantly lower than the *E. coli* group (P <0.05), and no significant difference from the control group.

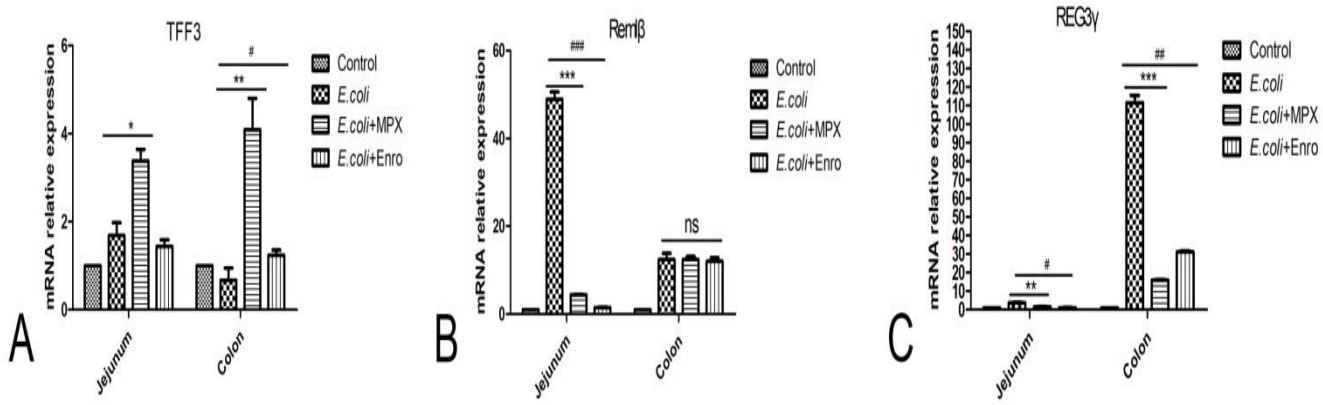

Fig. 3.19 The mRNA expression of antibacterial protein in mouse intestine

(A) The mRNA expression of TFF3 in mouse jejunum and colon; (B) The mRNA expression of Remlβ in mouse jejunum and colon; (C) The mRNA expression of REG3γ in mouse jejunum and colon.

Compared with the control group, the mRNA expression level of Reml β in the jejunum tissue of *E. coli* infected mice was significantly increased (P<0.001). MPX significantly reduced the mRNA expression level of Remlβ, which was equivalent to the effect of Enro; while the expression level of Remlβ in mouse colon was not significantly different in other group. In addition, *E. coli* infection leads to increased REG3γ expression in mouse jejunum and colon, and MPX could significantly reduce REG3γ mRNA expression caused by *E. coli* infection.

3.3.9 MPX suppresses intestinalin flammation by down regulatingthe expression of p-p38 and p-p65

Previous studies showed that MPX treatment could reduce the serum levels of inflammatory factors after *E.coli* infection.

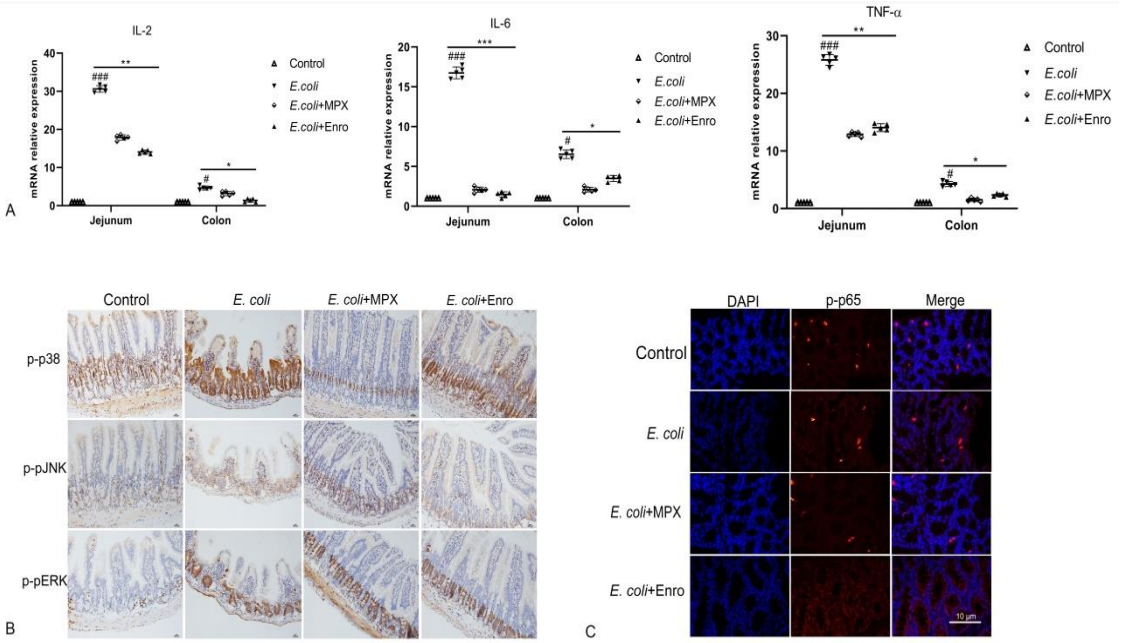

Fig. 3.20 MPX suppresses intestinal inflammation by inhibiting the activation of the MAPK and P65 signaling pathways

(A) The mRNA expression of IL-2, IL-6, and TNF-α in the jejunum and colon after MPX treatment was measured by real-time PCR; (B) The expression of p-p38, p-pJNK and p-pERKin the jejunumafter MPX treatment was determinedby immunohistochemistry (bars, 100 μm); (C)The effect of MPX on the expression of p-p65 in the colon was assessed by immunofluorescence.

To further investigate the anti-inflammatory effect of MPX on the intestine, the mRNA expression of IL-2, IL-6 and TNF-α in the jejunum and colon was detected by real-time PCR.

E.coli infection significantly increased the expression of the inflammatory factors IL-2, IL-6 and TNF-α in the jejunum and colon (Fig. 3.20A, $p<0.01$), while MPX and Enro treatment significantly inhibited their mRNA expression in the jejunum ($p<0.01$) and colon ($p<0.05$); after MPX and Enro treatment, the levels were not significantly different from those in the control group ($P>0.05$).

Immunohistochemistry and immunofluorescence were used to further explore the mechanism by which MPX inhibits the secretion of inflammatory factors.

The immunohistochemistry results showed that MPX notably reduced the expression of p-p38 in the crypts of the jejunum, and this effect was superior to those of Enro at the same dose (Fig. 3.20 B).

However, the expression of p-pJNK and p-pERK in the jejunum was not significantly changed after treatment with MPX and Enro (Fig. 3.20 B).

In addition, the activation of p65 was analyzed by immunofluorescence, revealing that MPX significantly decreased the phosphorylation of p65 compared with that in the group infected with only *E. coli* (Fig. 3.20 C).

3.3.10 MPX enhances theexpression of intestinal tight junction proteins and mucin

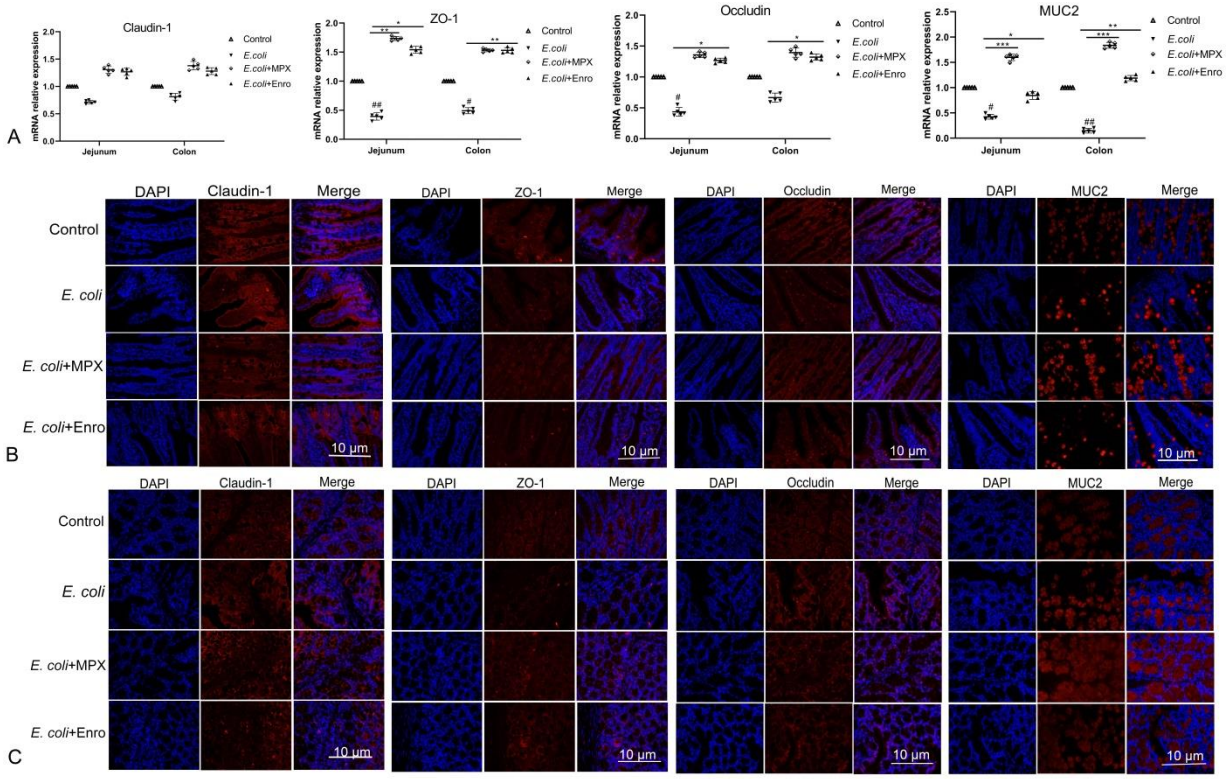

Fig. 3.21 MPX improves the expression of tight junction proteins and mucin in the jejunum and colon

(A) The mRNA expression of claudin-1, ZO-1, occludin and MUC2 in the jejunum and colon; (B) The protein expression of claudin-1, occludin, ZO-1 and

MUC2 (red)and DAPI (blue) in the jejunum;(C) The protein expression of claudin-1, occludin, ZO-1 and MUC2 (red) and DAPI (blue). Scale bar=10 μm.

Immunofluorescence and real-time PCR were used to further investigate the effects of MPX on *E.coli*-induced TJ protein and MUC2 expression in the jejunum and colon. *E.coli* infection decreased the expression of ZO-1, occludin and MUC2, while MPX treatment significantly increased their expression in the jejunum and colon; this effect of MPX was superior to that of Enro (Fig. 3.21A, $P<0.05$). However, none of the groups showed a significant effect on the expression of the TJ protein claudin-1 (Fig. 3.21A, $P>0.05$). Immunofluorescence was used to further study the effect of MPX on TJ protein and MUC2 expression after *E.coli* infection. *E.coli* infection reduced the expression of ZO-1, occludin and MUC2 in the mouse jejunum and colon, while the expression of ZO-1, occludin and MUC2 was improved after treatment with MPX. The effect of MPX was better than that of Enro, and the expression levels were not significantly different from those in the control group (Fig. 3.21 B, C). Collectively, these results indicate that MPX treatment could significantly improve the expression of TJ proteins and mucin in the jejunum and colon.

3.4 Exploring the mechanism of antimicrobial peptide MPX at the cellular level to relieve inflammation and barrier protection

IPEC-J2 cells have differentiated characteristics and exhibit strong similarities to primary intestinal epithelial cells, which in turn might serve as a good model for humans, demonstrating that IPEC-J2 cells represent a better model of normal intestinal epithelial cells than transformed cell lines [179]. Pigs as a good gastrointestinal (GI) model for humans which have the high similarity between pigs andhumans, the advantages of using IPEC-J2 cells as in vitro model of the GI tract are the high resemblance between humans and pigs [180].In this study, MPX exhibited no toxicity in IPEC-J2 cells and notably suppressed the levels of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), myeloperoxidase (MPO) and lactate dehydrogenase (LDH) induced by *E. coli*. In addition, MPX

improved the expression of ZO-1, occludin, and claudin and enhanced the wound healing of IPEC-J2 cells. scanning electron microscopy results found that *E. coli* infection caused cell apoptosis and destroyed cell membranes of IPEC-J2 cell. MPX effectively alleviated apoptosis of IPEC-J2 cells. The laser confocal results further found that MPX prevented cell apoptosis by inhibiting caspase-3 and caspase-9 activation. In addition, it was found that MPX regulated the expression of tight junction proteins ZO-1, Occludin, and Claudin-1 in IPEC-J2 cells and is closely related to Rac1 by adding a Rac1 inhibitor.

3.4.1 MPX reduces LDH release and inhibits inflammatory cytokine expression

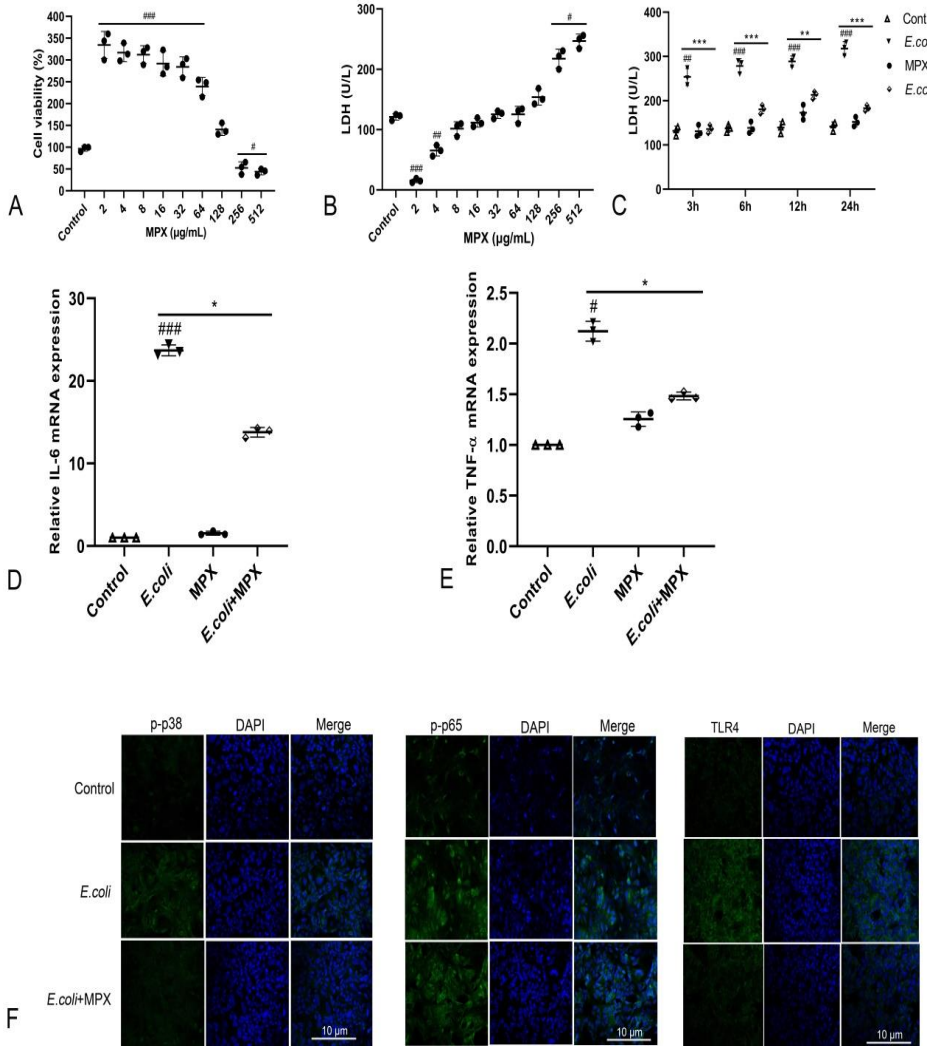

Fig. 3.22 MPX does not induce cytotoxicity and alleviates inflammation in IPEC-J2 cells

(A) Cell viability was measured using the CCK-8 assay, IPEC-J2 cells were cultured with different concentrations (2-512 μg/mL) of MPX for 24 h; (B) The release of LDH from IPEC-J2 cells after treatment with different concentrations (2-512 μg/mL) of MPX for 24 h; (C) MPX decreased the *E.coli*-induced release of LDH from IPEC-J2 cells (MOI=10) at different times;(D-F) The mRNA expression of IL-6 and TNF-α after MPX treatment. (G) The expression of p-p38, p-p65 and TLR4 in IPEC-J2 cells assessed by confocal laser microscopy.

The cytotoxicity of MPX was assessed since this study aimed to develop this peptide as a safe alternative to antibiotics. A CCK-8 kit was used to determine the viability of IPEC-J2 cells after treatment with MPX at different concentrations (2-512 μg/mL) for 24 h.

Compared with the control treatment, the results showed that cell viability analyzed by the CCK-8 assay was not affected, but it might have increased celleluar metabolism when cells were supplemented with MPX (Fig. 3.22 A). The effect on cultured cells was not significant, even at a high concentration of 128 μg/mL. The release of LDH was examined to further determine the toxicity of MPX. Compared with the control group, treatment with different concentrations (2-512 μg/mL) of MPX for 24 h did not significantly increase the release of LDH, even at a concentration of 128 μg/mL (Fig. 3.22 B).

Moreover, the LDH release from IPEC-J2 cells was notably reduced after pretreatment with MPX for 2 h prior to infection with *E. coli* (Fig. 3.22C, $p<0.05$). These results indicated that MPX maintained the cellular membrane integrity of IPEC-J2 cells.

To evaluate the anti-inflammatory effects of MPX after *E.coli* infection, the expression of IL-6 and TNF-α was determined by real-time PCR.

Compared with *E. coli* alone, treatment with MPX significantly inhibited the *E. coli*-induced mRNA expression of IL-6 and TNF-α (Fig. 3.22D, E, F, $p<0.05$).

In addition, the confocal laser microscopy results showed that *E.coli* infection significantly increased the expression of p-p38, p-p65 and TLR4, while pretreatment with MPX significantly decreased the expression of p-p38, p-p65 and

TLR4 (Fig. 3.22 G), indicating that MPX could inhibit the release of inflammatory cytokines by reducing the phosphorylation of p38 and the activation of p65 and TLR4.

3.4.2 MPX inhibits *E. coli*-induced tight junction damage in IPEC-J2 cells

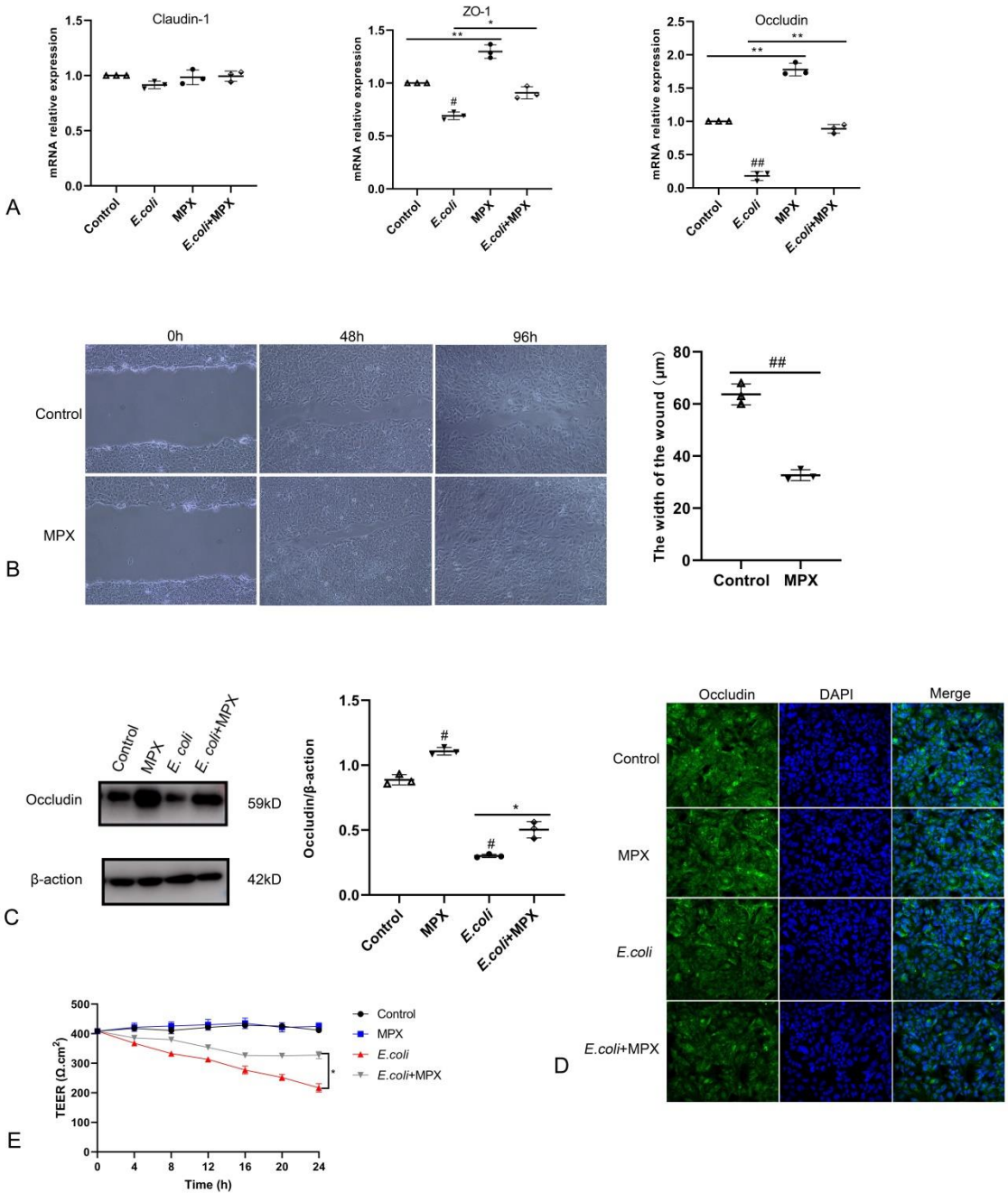

Fig.3.23 MPX enhances IPEC-J2 cell barrier function

(A) The *E. coli*-induced mRNA expression of ZO-1, occludin and claudin-1 in IPEC-J2 cells after treatment with MPX; (B) IPEC-J2 cells were incubated with medium alone or MPX (10μg/mL) in a wound healing assay. Images were obtained at 0 h, 48 h and 96 h, and the wound width was measured at 48 h; (C) The *E. coli*-induced protein level of occludin in IPEC-J2 cells after pretreatment with MPX was determined by western blotting; (D) The effect of MPX on the expression of occludin in IPEC-J2 cells was assessed by confocal laser microscopy; (E) MPX increases TEER of differentiated IPEC-J2 cells monolayers subjected to *E.coli* stimulus.

To evaluate the effects of MPX after *E.coli* infection, the expression of ZO-1, occludin, and claudin-1 was determined by real-time PCR. As shown in Figure 2 A, the *E. coli*-induced mRNA expression of ZO-1 and occludin in IPEC-J2 cells was significantly increased after MPX treatment ($p<0.05$), while the expression of claudin-1 was not significantly altered ($p>0.05$).

Interestingly, in a wound healing assay, the wound width was significantly reduced at 48 h in IPEC-J2 cells treated with MPX (Fig.3.23 B, $p<0.01$), indicating that MPX is beneficial for healing intestinal epithelial cell damage. Furthermore, western blot and immunofluorescence analyses were used to investigate the effects of MPX on TJ proteins in IPEC-J2 cells after *E.coli* infection. As shown in Fig.3.23 C, D, compared with the control treatment, MPX significantly increased the expression of occludin in IPEC-J2 cells.

Moreover, the expression of occludin induced by *E.coli* was significantly increased after treatment with MPX.

These results suggested that MPX could significantly increase the TJ protein expression inhibited by *E.coli* in IPEC-J2 cells.TEER is a key parameter of epithelial and used as a measure of cell monolayer integrity. The effect of MPX on *E.coli*-induced intestinal permeability was detected by TEER.

As shown in Fig.3.23E, *E.coli* induced significantly the decrease of TEER continuing to 24 hours. In contrast, 10 μg/mLMPX alone increased TEER, indicating that MPX reduced intestinal epithelium permeability. Co-treatment of

MPX and *E.coli* demonstrated that MPX repaired *E.coli*-induced intestinal tight junction permeability in IPEC-J2 cells. Thus, MPX is able to protect *E.coli*-induced intestinal permeability.

3.4.3 MPX reduces IPEC-J2 cells apoptosis

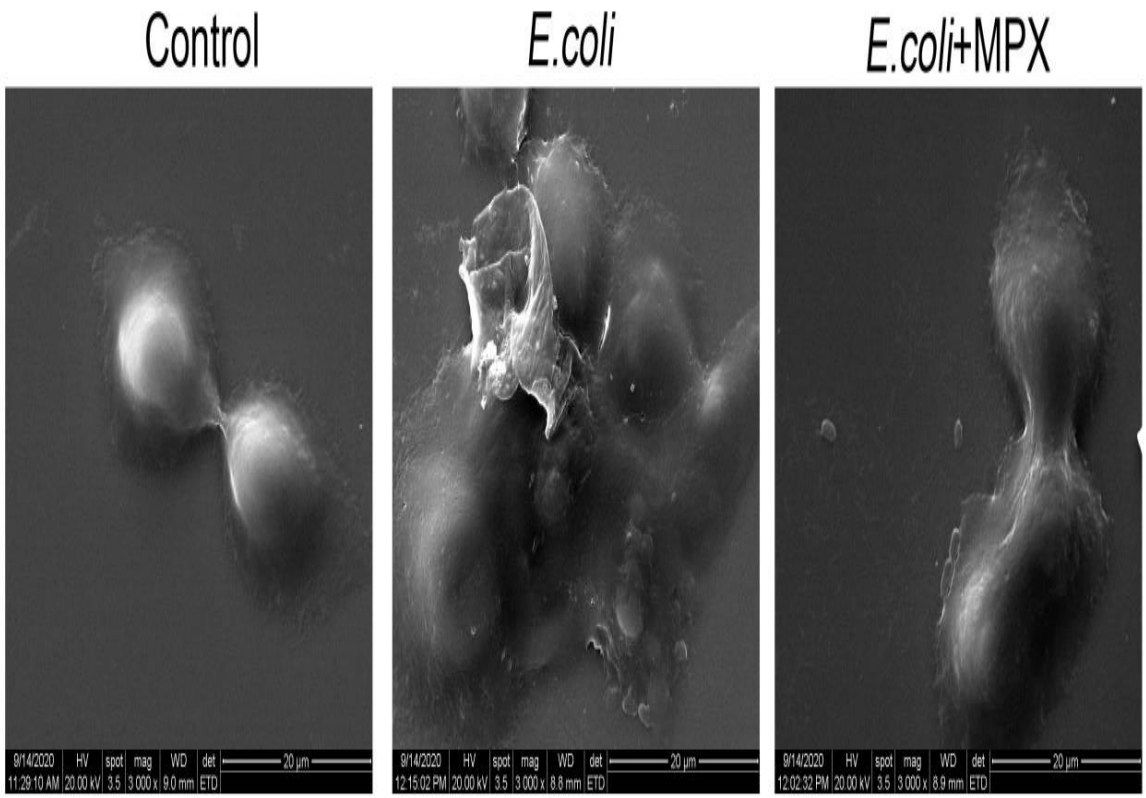

Fig. 3.24 The results of IPEC-J2 cells infected with *E. coli* observed by scanning electron microscope.

After *E. coli* infected IPEC-J2 cells with MOI=10, the morphology of the infected cells was observed by scanning electron microscope, as shown in Fig.3.24. The results showed that the surface of IPEC-J2 cells alone was smooth and round, and the cell morphology was intact.

Phenomenon such as swelling and collapse of the cytoskeleton. After *E. coli* infection with IPEC-J2 cells, the cells appear obvious apoptosis, the cells swell, there are apoptotic vesicles around, and the cytoskeleton collapses and collapses.

IPEC-J2 cells did not appear to collapse, or apoptotic vesicles after the action of MPX.

3.4.4 MPX inhibits Caspase-3 and Caspase-9 activation

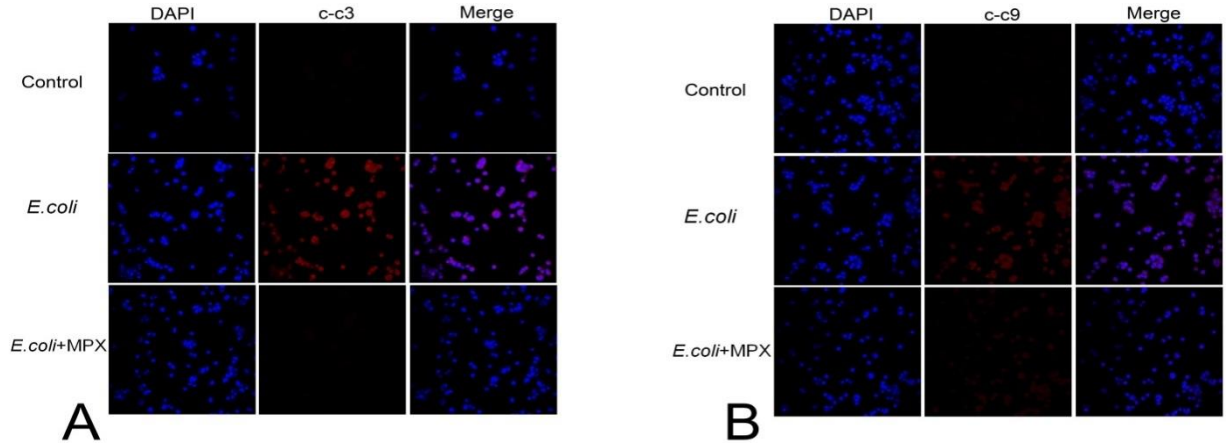

Fig. 3.25 The results of IPEC-J2 cells infected with *E.coli* observated by Laser confocal

(A)The activation result of Caspase-3 of IPEC-J2 cells after *E.coli* infection; (B) The activation result of Caspase-9 of IPEC-J2 cells after *E.coli* infection. Scanning electron microscopy results showed that MPX could alleviate the apoptosis of IPEC-J2 cells caused by *E. coli.* The effect of MPX on the activation of Caspase-3 and Caspase-9 was further detected by laser confocal microscopy. As shown in Fig.3.25, the results showed that IPEC-J2 cells activated Caspase-3 and Caspase-9 after *E. coli* infection, while MPX significantly decrease the activation of Caspase-3 and Caspase-9, thereby inhibiting the apoptosis of IPEC-J2 cells.

3.4.5 MPX regulates tight junction protein expression by Rac1

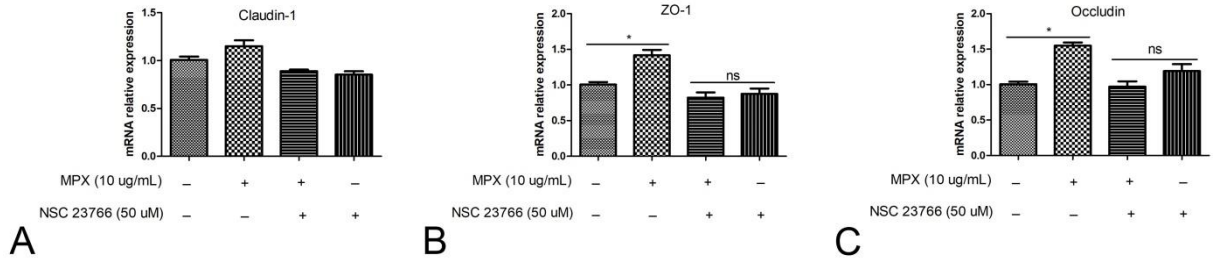

Fig.3.26 The mRNA expression of tight junction protein after *E. coli* infection

(A) The mRNA expression of Claudin-1 after *E. coli* infection; (B) The mRNA expression of ZO-1 after *E. coli* infection; (C) The mRNA expression of Occludin after *E. coli* infection.

Rac 1 inhibitor NSC 23766 was used to study the regulation mechanism of MPX on tight junction proteins (Fig. 3.26 A, B, C). The results showed that compared with IPEC-J2+*E.coli* group, MPX pretreatment could increase the mRNA expression of ZO-1 and Occludin in IPEC-J2 cells, While the mRNA expression of Claudin-1 was not significant. However, the effect of MPX on ZO-1 and Occludin was inhibited after adding inhibitor NSC 23766. The above results suggest that MPX may regulate the tight junction protein of intestinal epithelial cells and enhance the barrier function through the Racl pathway.

3.4.6 MPX inhibits *E. coli* adhesion and invasion

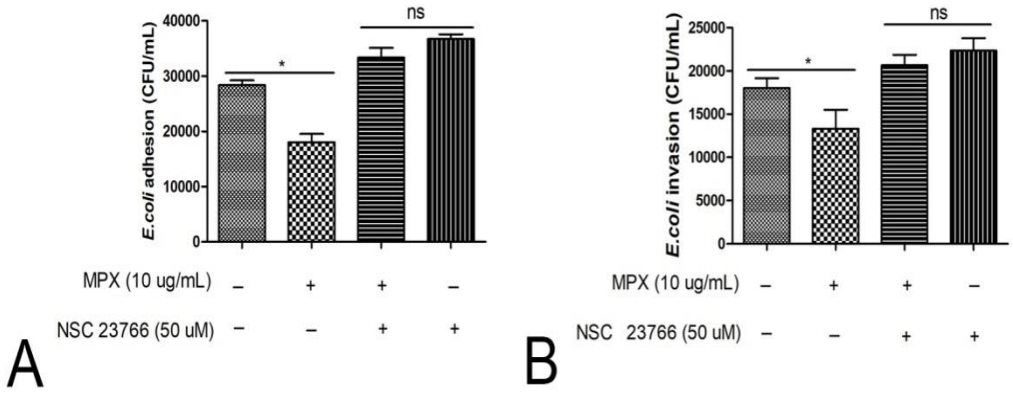

Fig. 3.27 The results of *E.coli* adhesion and invasion in IPEC-J2 cells

(A)The results of *E.coli* adhesion in IPEC-J2 cells; (B) The results of *E.coli* invasion in IPEC-J2 cells

Then, we tested the effect of MPX on the adhesion and invasion of IPEC-J2 cells by adding Rac1 inhibitor. As shown in Figure 4, the results found that *E. coli* adhered and invaded more in the IPEC-J2+*E. coli* group. While pretreatment with MPX could significantly alleviated the adhesion and invasion of *E. coli* in IPEC-J2 cells. In the presence of NSC 23766, MPX failed to reduce *E. coli* adhesion and invasion in IPEC-J2 cells.

The above results indicate that Rac1 is related to the cell barrier of IPEC-J2 cells, and MPX enhances the cell barrier function of IPEC-J2 cells by regulating Rac1, thereby reducing *E. coli* adhesion and invasion in IPEC-J2 cells.

3.4.7 The mechanism of action of antimicrobial peptide MPX

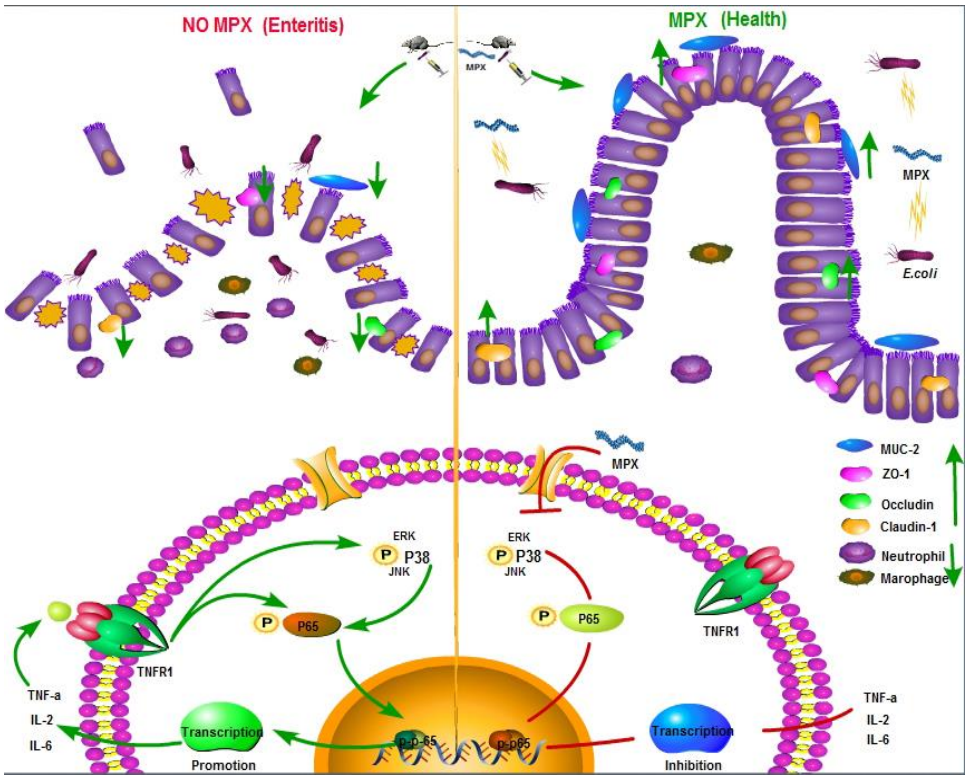

Fig. 3.28 MPX regulates epithelial cells and in vivo signaling pathways.

In summary, as shown in Fig. 3.28, we demonstrated that MPX could reduce *E.coli* growth, attenuate the inflammatory response and intestinal damage, inhibit *E.coli*-induced TLR4 expression, and decrease theIL-2, IL-6 and TNF-α levels by blocking the activation of the p65 and p38 inflammatory pathways in vitro and in vivo. In addition, MPX improved the intestinal barrier function and increased the expression of the TJ proteins ZO-1, occludin and mucin.

3.5 Oral administration antimicrobial peptide Mastoparan X alleviates enterohemorrhagic *Escherichia coli*-induced intestinal inflammation and regulates gut microbiota

The importance of the gut microbiota to host health has been the focus of research in the past decade. The intestinal microbiota is a complex and diverse system that is sensitively affected by many factors, such as environment, age, diet, dietary additives, and hygiene levels [181].

The composition of the microbiota in the intestine plays a vital role in the health and functions of the host and may be related to serious intestine-related obesity and inflammatory bowel disease [182]. In this study, *E. coli* was used to induce intestinal inflammation, and the results showed that MPX alleviated weight loss and intestinal pathological changes in necropsy specimens of *E. coli*-infected mice. MPX reduced the serum levels of the inflammation-related proteins interleukin-2, interleukin-6, tumor necrosis factor-α, myeloperoxidase and lactate dehydrogenaseon day 7 and day 28.

Furthermore, haematoxylin and eosin staining results showed that MPX increased the length of villi and reduced the infiltration of inflammatory cells in to the jejunum and colon. Scanning electron microscope and transmission electron microscope results showed that MPX could improve the morphology of jejunum villi and microvilli and increase tight junction protein levels.

The 16S rRNA sequencing analysis of caecal content samples showed that species diversity and richness were lower in the *E. coli*-infected group. At the phylum and genus levels, higher abundances of pathogenic Firmicutes, Lachnospiraceae-NK4A-136 and Alistipes bacteria were observed.

Furthermore, MPX increased the abundance of Muribaculaceae. Alpha and Beta analysis showed that there was no significant difference in bacterial community structure between the MPX and control groups.

In addition, alterations in the intestinal microbiome of mice affected physiological functions and metabolic pathways.

3.5.1 MPX alleviates intestinal inflammation

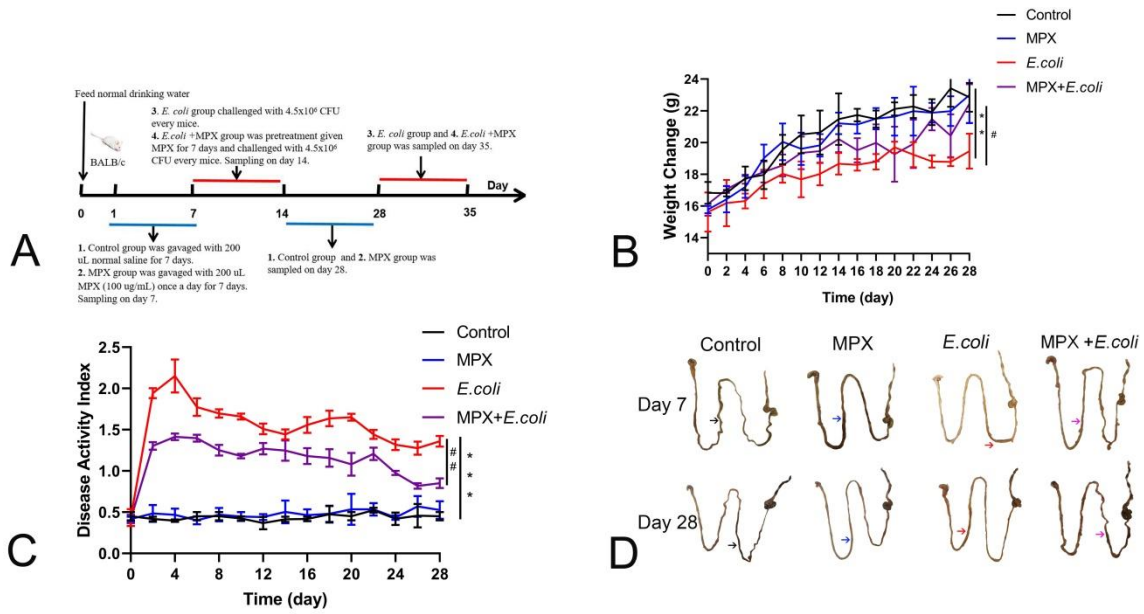

Fig. 3.29 Effect of MPX on intestinal inflammation induced by *E. coli*

(A) Schedule of the experiment and various experimental groups; (B) weight change of BALB/c mice; (C) Disease activity index of mice; (D) pathological observations of intestinal necropsy specimens of mice.

The detailed experimental design of this study is shown in Fig. 3.29 A. The control group was intragastrically administered 200 μL of normal saline, the MPX group was intragastrically administered 200 μL of 100 μg/mL MPX for 7 days. The *E. coli* group and the MPX+*E. coli* group were treated with *E. coli* at $4.5x10^6$CFU/mouse and killed on days 7 and 28. As shown in Fig. 3.29 B, the results demonstrated that mice infected with *E. coli* exhibited a decrease in appetite and a significant decrease in body weight, while MPX significantly alleviated the weight loss induced by *E. coli*. The weight change ofthe MPX alone and control groups was not significant. The disease activity index showed that the *E.coli* infection of the mice caused diarrhoea, malaise, anorexia, bunching and other phenomena, and these differences from the control and MPX groups were significant (Fig. 3.29C, $P<0.001$). MPX significantly reduced the disease activity index resulting from *E. coli* infection (Fig. 3.29C, $P<0.01$). The pathological

changes in the intestine are shown in Fig. 3.29 D. Compared to the control and MPX groups, intestinal haemorrhage and congestion were observed, the intestinal wall became thinner and lost elasticity, and the intestine was filled with yellow viscous liquid in the *E. coli*-infected group. In addition, the intestinal pathology was more severe on day 7 than on day 28. MPX treatment significantly alleviated the symptoms observed in the mice after *E. coli* infection. The above results indicated that MPX could effectively alleviate the intestinal inflammation and have better function of preventing intestinal inflammation induced by *E. coli*.

3.5.2 MPX reduces the level of inflammatory proteins in the serum

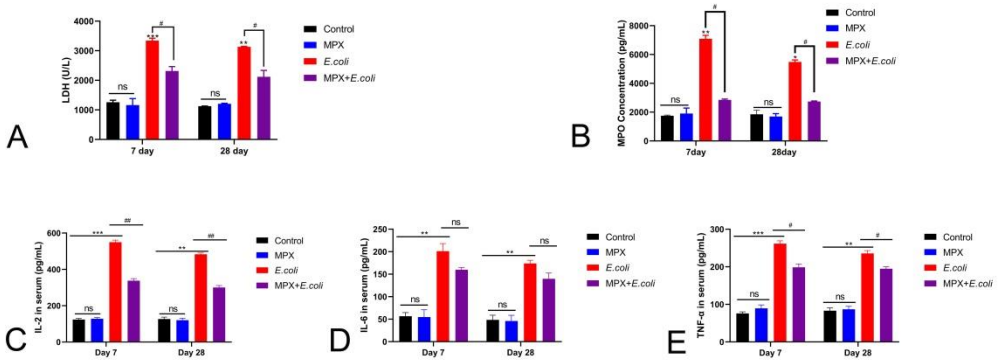

Fig. 3.30 MPX treatment reduces inflammation-related protein levels in serum on day 7 and day 28

(A) The levels of LDH in serum; (B) the levels of MPO in serum;(C) the levels of IL-2 in serum determined by ELISA;(D) the levels of IL-6 in serum determined by ELISA; (E) the levels of TNF-α in serum determined by ELISA.

To evaluate the effect of the MPX-mediated anti-inflammatory response after *E. coli* infection, the levels of LDH, MPO, IL-2, IL-6 and TNF-α in serum were detected by ELISA. As shown in Fig. 3.30, the level of LDH in serum was increased significantly in the *E. coli*-infected group (Fig. 3.30 A, $P<0.01$), while MPX significantly reduced LDH release in serum (Fig. 3.30 A, $P<0.05$). The levels of MPO in serum were significantly increased in the *E. coli*-infected group

relative to the MPX and control groups (Fig. 3.30 B, P<0.01). The levels of IL-2, IL-6, and TNF-α in serum showed that the levels of IL-2, IL-6 and TNF-α in serum increased after *E. coli* infection, and MPX effectively reduced the levels of IL-2 and TNF-α in the serum (Fig. 3.30C, E, P<0.05), but had no significant effect on the level of IL-6 (Fig. 3.30 D, P>0.05).

The results indicated that MPX could reduce inflammation-related protein levels in serum.

3.5.3 MPX alleviates pathological damage in the intestine

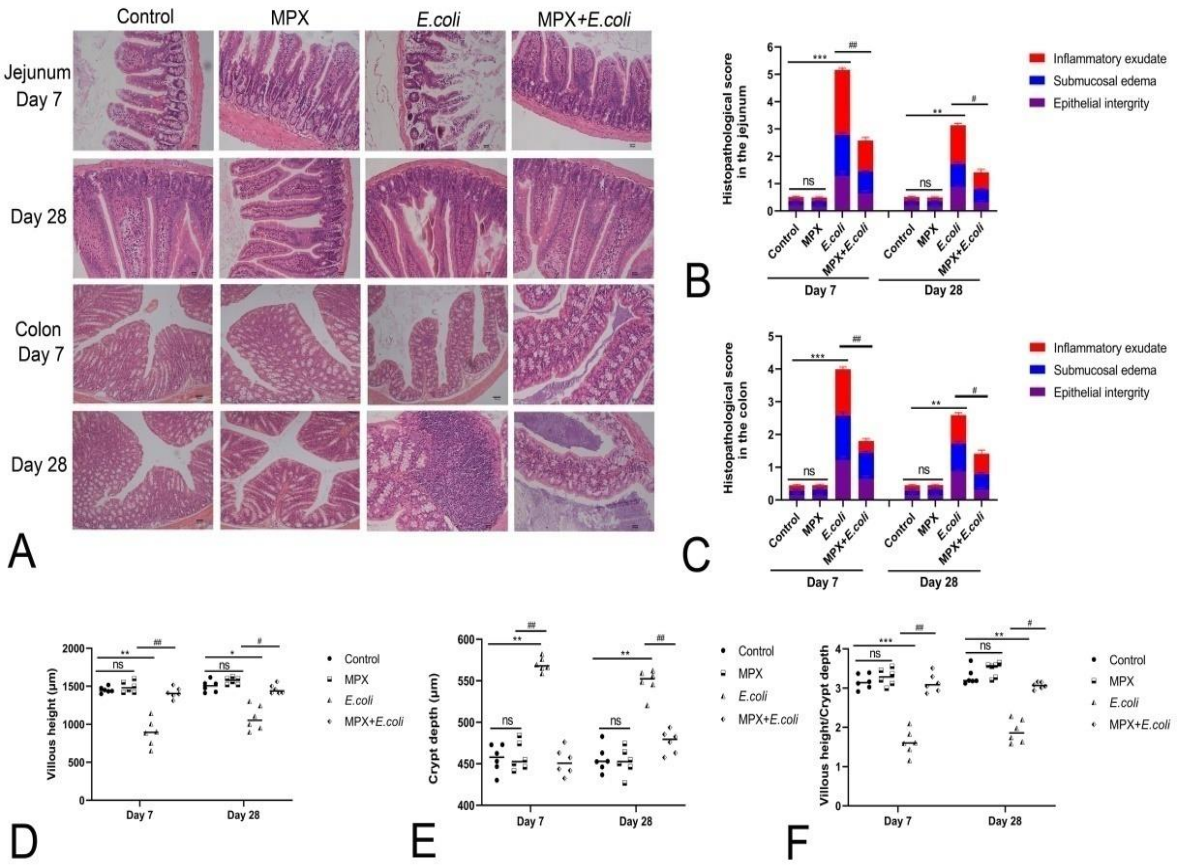

Fig.3.31MPX improves intestinal morphology in the jejunum and colon on day 7 and day 28

(A) The jejunum and colon of mice were stained with H&E (bars, 20 μm) on day 7 and day 28; images were obtained at 200× magnification; (B) histopathological score of the jejunum on day 7 and day 28; (C) histopathological

score of the colon on day 7 and day 28; (D-F) The length of jejunum villi, the depth of crypts, and the ratios of villi length to crypt depth were detected with ipwin32 software.

Next, H&E staining was used to further explore the effect of MPX on the intestinal morphology of the jejunum and colonof mice after *E. coli* infection on day 7 and day 28; *E. coli* infection mainly led to damage to the jejunal epithelium and intestinal inflammation.

The jejuna of the *E. coli*-infected mice showed typical intestinal inflammation and barrier damage, shortened villi, reduced mucosal thickness, necrosis, large amounts of inflammatory cell infiltration and disruption of intestinal villi, and MPX resulted inless severe intestinal injury than was observed in the *E. coli*-infected groupon day 7 and day 28 (Fig. 3.31 A). In addition, the colonic pathological damage inthe *E. coli*-infected group was more severe on day 7 than on day 28, which resulted in necrosis (Fig. 3.31 A). The jejunum pathology scoresare shown in Figure 3 B. The *E. coli*-infected group had the highest score, while the MPX-treated group had a lower jejunum pathology score after *E. coli* infection (Fig.3.31 B, $P<0.05$).

In addition, the colon pathology scoresare shown in Fig. 3.31 C. Necrosis appeared in the colons of mice in the *E. coli*-infected group on day 28, and severe pathological damage was observed (Fig. 3.31 A).

The jejunum villus heights and crypt depths of the mice in the *E. coli*-infected groupwere decreased, resulting in a decrease in the ratio of villus height to crypt depth, which was a significantly different relative tothe control group (Fig. 3.31 D-F, $P<0.01$).

In contrast, MPX increased villous height, reduced crypt depth and increased the ratio of villus height to crypt depth inthe jejunum, resulting in no significance difference from the control group (Fig. 3.31 D-F, $P>0.05$).

3.5.4 MPX alleviates damage to intestinal villi and microvilli

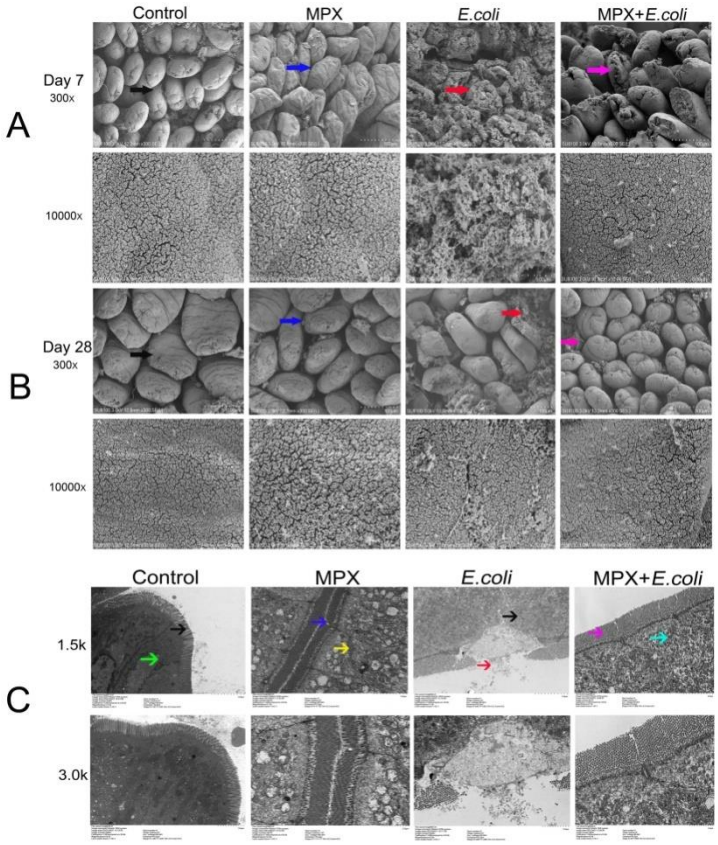

Fig. 3.32 MPX improves the intestinal morphology and microvilli of intestinal epithelial cellsof the jejunum

(A), (B) Morphological changes in the jejunum villi after MPX treatment on day; (C) Microvilli and tight junction proteins in intestinal epithelial cells were observed on day 28 after MPX treatment by TEM (upper,1500×; lower, 3000×). Previous H&E results have confirmed that MPX can effectively alleviate the pathological damage to intestines caused by *E. coli*. SEM was used to further study whether MPX could affectthe intestinal morphology induced by *E. coli*. Compared with the MPX and control groups, *E. coli* infection severely damaged the morphology and integrity of intestinal villi in thejejunum on day 7 and alleviated this damage on day 28 (Fig. 3.32 A). As observed at high (300×) and low (10.000×) magnifications, MPX treatment obviously alleviated the injury to the jejunum villi and microvilli on day 7 and day 28 after *E. coli* infection (Fig. 3.32 B). Transmission electron microscopy was carried out to further observe the effects of MPX on the intestinal microvilli and tight junction proteins of mice after

E. coli infection. Relative to the control group, *E. coli* infection caused the shedding of microvilli, thus decreasing their numbers, and damaged the tight junction proteinstructure of intestinal epithelial cells (Fig. 3.32 C), while MPX increased the number of microvilli, reduced epithelial

3.5.5 Species correlation analysis

The tags stat_summary on day are listed in the tables 3.2, 3.3

Table 3.2

The tags _stat_summary on day 7

Sample_ID	clean_tags	valid_tags	valid_percent	valid minLength	valid meanLength	valid maxLength	OTU_counts	Total_OTUs
Control.1	77902	69919	89.75%	258	413.77	437	1836	4458
Control.2	74759	68437	91.54%	258	412.06	440	1613	4458
Control.3	75019	67261	89.66%	251	412.96	436	1822	4458
Control.4	61032	52375	85.82%	252	411.95	441	1987	4458
Control.5	76008	66623	87.65%	228	413.01	440	1282	4458
Control.6	74296	66760	89.86%	256	409.2	441	1697	4458
MPX.1	77869	68672	88.19%	229	412.18	440	2143	4458
MPX.2	71655	61639	86.02%	256	413.4	449	2243	4458
MPX.3	76315	66965	87.75%	258	407.25	440	1785	4458
MPX.4	72799	61042	83.85%	258	409.12	449	1690	4458
MPX.5	76269	66291	86.92%	258	410.8	441	2055	4458
MPX.6	74024	63608	85.93%	258	407.66	435	1928	4458
E.coli.1	76350	64820	84.90%	258	418.11	437	1774	4458
E.coli.2	77041	67941	88.19%	258	413.89	441	1436	4458
E.coli.3	77839	66738	85.74%	256	414.93	442	1724	4458
E.coli.4	71347	55950	78.42%	240	416.36	437	1751	4458
E.coli.5	69321	61535	88.77%	241	415.13	437	1635	4458
E.coli.6	66117	57385	86.79%	256	418.51	437	1905	4458
E.coli.MPX.1	69176	61477	88.87%	253	413.46	440	1998	4458
E.coli.MPX.2	72532	64494	88.92%	256	415.21	439	1814	4458
E.coli.MPX.3	72742	63878	87.81%	256	418.07	441	1717	4458
E.coli.MPX.4	67310	60283	89.56%	228	417.27	441	2065	4458
E.coli.MPX.5	72563	60783	83.77%	258	416.77	441	1726	4458
E.coli.MPX.6	66606	54787	82.26%	253	414.91	439	1724	4458

Table 3.3

The tags _stat_summary on day 28

Sample_ID	clean_tags	valid_tags	valid_percent	valid minLength	valid meanLength	valid maxLength	OTU_counts	Total_OTUs
Control.1	77902	69822	89.63%	258	413.84	437	1755	4562
Control.2	74759	68194	91.22%	258	412.09	440	1558	4562
Control.3	75019	66986	89.29%	256	413.01	436	1700	4562
Control.4	61032	52127	85.41%	252	411.98	441	1870	4562
Control.5	76008	66506	87.50%	228	413	440	1220	4562
Control.6	74296	66605	89.65%	256	409.22	441	1617	4562
MPX.1	75843	68440	90.24%	258	418.39	439	2045	4562
MPX.2	76227	70415	92.38%	257	416.6	442	2036	4562

Table continuation 3.3

MPX.3	74550	68268	91.57%	258	418.39	441	1773	4562
MPX.4	76643	71115	92.79%	258	418.08	448	1974	4562
MPX.5	75508	69315	91.80%	258	416.99	452	2171	4562
MPX.6	75825	70074	92.42%	258	417.11	441	2087	4562
E.coli.1	77294	69355	89.73%	258	414.96	441	2074	4562
E.coli.2	77709	69259	89.13%	259	411.08	441	1620	4562
E.coli.3	76754	70802	92.25%	258	414.81	441	1836	4562
E.coli.4	75147	69379	92.32%	256	415.21	441	2087	4562
E.coli.5	75859	69856	92.09%	258	417.02	439	1807	4562
E.coli.6	74794	68157	91.13%	259	417.63	441	2177	4562
E.coli.MPX.1	76181	68177	89.49%	258	418.01	440	1629	4562
E.coli.MPX.2	76072	68612	90.19%	259	417.49	441	2000	4562
E.coli.MPX.3	78004	73083	93.69%	229	416.41	441	1619	4562
E.coli.MPX.4	77420	70691	91.31%	259	418.19	440	2282	4562
E.coli.MPX.5	74746	67524	90.34%	259	417.6	443	1879	4562
E.coli.MPX.6	76876	69398	90.27%	258	415.62	441	2303	4562

7 and day 28 were observed by SEM (upper, 300×; lower, 10,000×).

The microbial community structure of each analysed group is introduce on Fig. 3.33.

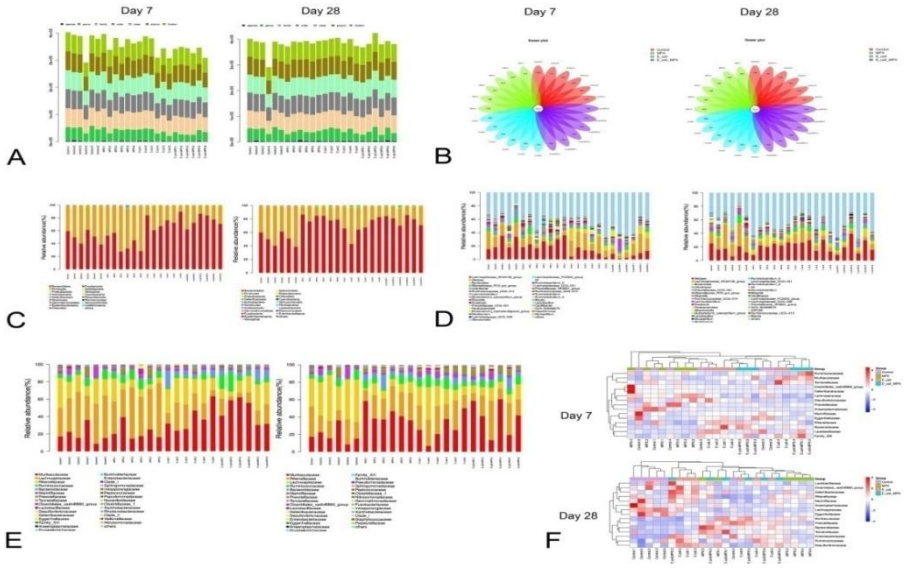

Fig. 3.33 The microbial community structure of each group was analysed

(A) Bar plots of operational taxonomic unit (OTU) numbers. The name of each sample and the total number of OTU tags in each class are shown on the X-axis and the Y-axis, respectively; (B) the number of OTUs in each sample according to flower plot analysis. The exact OTU numbers of each sample are shown as values on petals. The sequences are in the centre of the circle; (C) analysis of the relative abundance of phyla. Sample was shown in each column.

The different bacteria in phyla are shown as different colours. Samples and relative abundance in phyla are displayed on the X-axis and Y-axis, respectively, respectively; (D) Analysis of the relative abundance of genera. Sample was shown on each column. Samples and relative abundance in genera are displayed on the X-axis and Y-axis, respectively; (E) Analysis of the relative abundance of families. One sample is shown in each column. The different bacteria in families are shown in different colours. Samples and relative abundance in families are displayed on the X-axis and Y-axis, respectively;(F) Relative abundance of families in the samples analysed in heat maps. The clustering of families is shown in a cluster tree on the left. Different sample groups are shown as cluster branches above the figure. Higher and lower relative abundances are indicated by orange and blue, respectively. To determine whether MPX regulated the gut microbiota and intestinal inflammation and characterize the changes in the intestinal microbiota composition during *E.coli* infection, the total number of tags per OTU was collected to produce a bar plot of the OTU numbers in each sample, and the results are shown in Tables 3.2 and 3.3 and Fig. 3.33 A. Approximately 77,902 clean tags were obtained after quality control. Next, chimeric sequences were removed to analyse the valid tags obtained. The valid mean length ranged from 407.25–418.51 bp on day 7 and 409.22–418.39 bp on day 28. The OTU number in each sample was determined from the OTU counts by subtracting the representative sequences; it ranged from 1113–2027 bp on day 7 and 1041–2124 bp on day 28, and the score was 169 on day 7 and 179 on day 28 (Fig. 3.33 B). The differencesin abundance between each group at the phylum level are shown in Fig. 3.33 C. The top 30 representative phyla were detected, and the results showed that the most prevalent members of the gut microflora in the groups were Firmicutes (Gram-positive) and Bacteroidetes (Gram-negative). The gut microbiota of mice mainly consists of Bacteroidetes and Firmicutes. There was no significant difference in Firmicutes between the control and MPX groups. In a healthy intestine, the Firmicutes and Bacteroidetes phyla predominate and contribute to the production of epithelial metabolic substrates. However, the *E. coli*-infected group showed a reduced

abundance of Firmicutes on day 7. Firmicutes abundance in the MPX group was also lower than that in the control group, and MPX treatment effectively reduced the abundance of Firmicutes relative to that in the *E. coli*-infected group on day 28 (Fig. 3.33C). Studies have found that the presence of Firmicutes is a sign of intestinal flora imbalance. In this study, the results showed that MPX could reduce the abundance of Firmicutes, indicating that MPX could regulate flora imbalances and maintain intestinal homeostasis. We further studied the abundance of each group at the genus and family levels. At the genus level, there was no significant difference in the abundance of Lachnospiraceae-NK4A-136 between the control and MPX groups. Compared to the *E. coli*-infected group, MPX treatment significantly reduced the increase in the abundance of Lachnospiraceae-NK4A-136 induced by *E. coli*. In addition, compared to the *E. coli*-infected group on day 7, MPX treatment significantly reduced the abundance of Alistipes. However, there was a significant difference between the control and MPX groups on day 28. MPX significantly reduced the abundance of Lachnospiraceae-NK4A-136 (Fig. 3.33D). In contrast, Muribaculaceae abundance was reduced in *E. coli*-infected mice, while the MPX treatment group showed increased Muribaculace aeabundance correction of the disorder on day 7 and day 28 (Fig. 3.33D). At the family level, relative tothe control and *E. coli*-infected groups, MPX increased the abundance of Muribaculaceae and decreased the abundance of Lachnospiraceae on days 7 and 28 (Fig. 3.33E). Finally, the average values of the data of each group were calculated, and a heatmap illustrating the top 15 speciesat the family level was generated. The heatmap showed that Tannerellaceae and Clostridiales_vadin BB60 levels were increased in the *E. coli*-infected group but decreased after MPX treatment on days 7 and 28 (Fig. 3.33F).

3.5.6 The diversity of species in the microbiological environment

The sequencing depth was corrected by alpha diversity index calculation statistics and introduce in Fig. 3.34

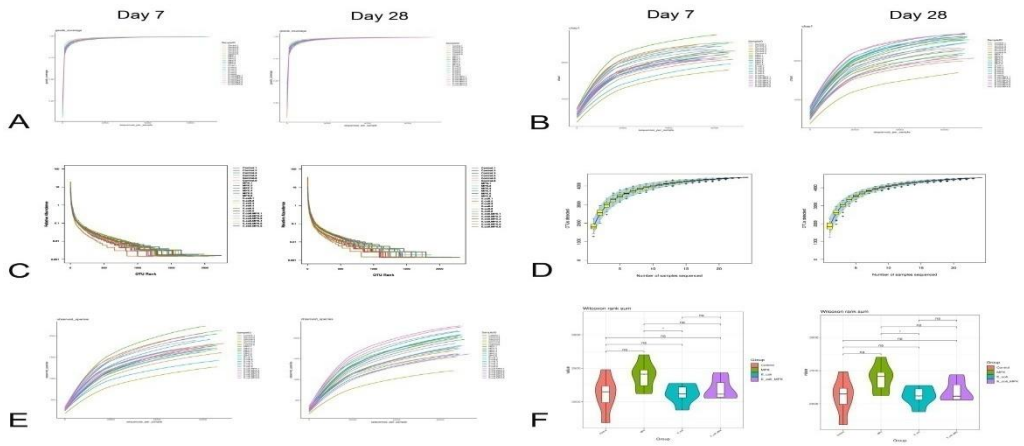

Fig. 3.34 The sequencing depth was corrected by alpha diversity index calculation statistics

(A) Good's coverage analysis of each sample; each curve represents a sample. The depth of random sampling is shown on the X-axis, and the exponential value is shown on the Y-axis. The amount of sequencing data was reasonable, with an increase in the number of extracted sequences and a gradual flattening curve; (B) Chao1 analysis. The total number of species is related to Chao1. The depth of random sampling is shown onthe X-axis, and the number of OTUs is shown on the Y-axis; (C) The evenness and species richnessof each group. OTUsare sorted according to the number of sequences on the X-axis, and the relative abundance of OTUs is shown on the Y-axis; (D) Species accumulation curve. The X-axis and the Y-axis represent the numbers of samples and detected operational taxonomic unit (OTU) numbers, respectively. The flat curve represents sufficient sampling; (E) The average OTU numbers of observed species in each group. The depth of random sampling and the exponential value are shown on the X-axis and the Y-axis, respectively. The amount of sequencing data was large enough to reflect most of the microbial species information in the sample when the curve tended to be flat; (F) Alpha diversity index values shown as a violin diagram. Different colours represent different groups and index values on the X-axis and the Y-axis, respectively.

Species diversity in each individual sample was determined via alpha diversity analysis. Alpha diversity was used to analyse the diversity of the microbial community in the sample, which can reflect the richness and uniformity

of the microbial community in the sample. The differences in species richness were observed by constructing a dilution curve of the diversity index, and the goodscoverage shown in Fig. 3.34 A proved the rationality of this analysis, in which the sequencing depth had an index value close to 1. The Chao1 index was calculated as the species richness index (Fig. 3.34 B). *E.coli*-infected mice showed a lower richness index, suggesting that *E.coli* disrupted the microbiological structure, while MPX treatment increased the Chao1 index, indicating that MPX regulated the microbial structural composition on days 7 and 28 (Fig. 3.34 B). The species richness and evenness of each group were determined by rank abundance. The results showed that the balance of the gut microbiota composition was disrupted by *E.coli* infection. Higher species richness and evenness were observed in the MPX-treated group than in the *E. coli*-infected group on days 7 and 28 (Fig. 3.34C). Furthermore, the specaccum species accumulation curve was used to evaluate whether the number of samples was sufficient. As shown in Fig. 3.34D, the number of species increased with an increasing number of samples, and the gentle curve indicates sufficient sampling on days 7 and 28.

To verify the results, an observed species curve was obtained from the average results in each group. The results showed that relative to the control group, *E.coli* infection reduced microbiome diversity, and MPX treatment increased the diversity of microbiome species on days 7 and 28 (Fig. 3.34 E). To further observe microbial diversity in mice from different groups, a violin diagram was obtained by Wilcoxon rank-sum analysis.

As shown in Fig. 3.34 F, we confirmed that *E.coli* infection destroyed the microbiological structure in mice and that MPX treatment slightly alleviated this phenomenon. In addition, relative to the *E. coli*-infected group, MPX treatment significantly increased the microbial diversity of mice (Fig. 3.34F, $P<0.05$).

3.5.7 Microorganisms analysed according to the correlation of sequencing depth and multivariate statistical analysis

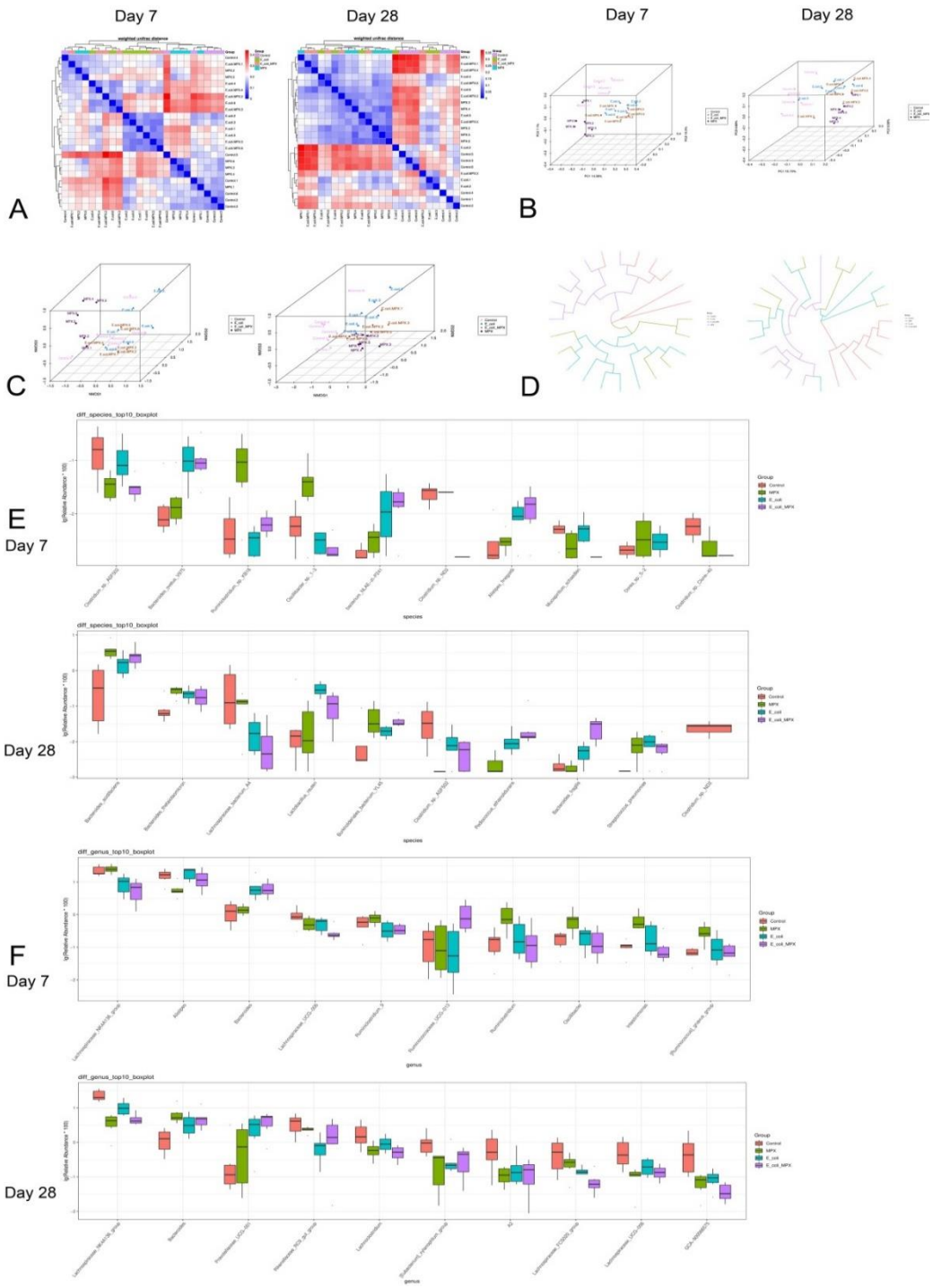

Fig. 3.35 Multivariate statistical analysis of microorganisms and sequencing depth correction

(A)The variability of each group assessed by beta diversity analysis. The clustering branches represent samples from different groups in the heat map. A deeper colour represents two groups, and red indicates the opposite; (B)

Differences between groups assessed by PCoA. The first and second principal coordinates are shown on the horizontal and vertical axes, respectively. The grouping information of the samples is displayed in dots with different colours/shapes. The distances between the sampling points in the same group represent the repeatability of the samples, and the distances between the sampling groups in different groups reflect the differences in sampling distance between the groups. The more heterogeneous the samples are, the greater the distance; (C) NMDS analysis results. The dots in the graph represent the samples, and the different colours/shapes represent the information of the groups to which the samples belong. The distances between the sampling points in the same group indicate the repeatability of the samples, and the distances between the samples in different groups reflect the differences in the ranks of the sampling distances between the groups; (D) UPGMA difference analysis between samples. Different colours represent different samples. The shorter the distance, the more similar the samples; (E) Analysis of relative species abundancesusing bar boxes; (F) Analysis of genus abundance correlations using bar boxes.

Beta diversity refers to the comparison of biodiversity among different samples, which reflects the diversity among habitats. Beta diversity analysis usually starts by calculating the distance matrix between environmental samples, decomposing the community data structure naturally, and sorting the samples (ordination) to observe the differences between them. In this study, the beta diversity index results showed that microbial diversity in the *E. coli*-infected group treated with MPX was similar to that inthe control and MPX groups on days 7 and 28, while the *E. coli*-infected group showed low microbial diversity, indicating that MPX could regulate intestinal microbial diversity (Fig.3.35 A). A 3 D diagram was used to further compare the degrees of variation between different samples according to a principal coordinate analysis (PCoA) of the microbial community. The results showed similar microbial evolution inthe control and MPX groups. In addition, the MPX treatment group was more similar to the control on day 7. However, microbial evolution in the MPX treatment group was significantly

different from that in the control group on day 28 (Fig.3.35 B). The MPX+*E. coli* treatment group was more similar to the control on day 7. However, the microbial evolution of the MPX, *E. coli*, MPX+*E. coli* groups was significantly different from that of the control on day 28 (Fig.3.35 B). Nonmetric multidimensional scaling (NMDS) is based on evolutionary relationships or quantitative distance matrices and is often used to compare differences between sample groups. As shown in a 3DNMDS diagram (Fig.3.35 C), the results indicated that the microbial evolution of the MPX treatment group was similar to that of the control on day 7 (Fig.3.35 C), while the MPX, *E. coli* and MPX+*E. coli* treatment groups were more similar on day 28 (Fig.3.35 C). In addition, a circular hierarchical clustering tree was obtained via unweighted pair-group method with arithmetic mean (UPGMA) statistical analysis. The differences between two samples are indicated by the distance between two branches. As shown in Fig.3.35 D, the results indicated that the MPX and control groups were more similar on day 7 and that the evolution of microorganisms in the MPX+*E. coli*, control, and MPX groups were relatively similar on day 28. The top 10 most abundant microbiomes at the genus level and species level were counted by analysis of variance (ANOVA). The results showed that the microbiomes were generally similar in these groups, but there were also some differences in the four groups. At the species level, relative to the *E. coli* and MPX+*E. coli* groups, Oscillibacter_sp._1-3 relative was sharply increased abundance in the MPX group. The relative abundance of Alistipes_finegoldii increased in the *E. coli*-infected group on day 7 (Fig.3.35 E). In addition, the relative abundance of Lachnospiraceae bacterium_A4 in the MPX group was similar to that in the control, while MPX treatment decreased the abundance of Lachnospiraceae bacterium_A4 in the *E. coli*-infected group on day 28 (Fig.3.35 E). At the genus level, MPX treatment decreased the abundance of Lachnospiraceae_NK4A136, Alistipes, and Lachnospiraceae_UGG-006 relative to the *E. coli*-infected group. In addition, E.coli infection decreased the abundance of Ruminococcaceae_UCG- 013, while MPX treatment increased the abundance of Ruminococcaceae_UCG- 013 on day 7, resulting in a higher abundance than in the

control on day 7 (Fig.3.35F). The abundances of Lachnospiraceae_NK4A136, Lachnoclostridium, Lachnospiraceae_FCS020, and Lachnospiraceae_UCG-006 were decreased after MPX treatment on day 28 (Fig.3.35 F).

3.5.8 Analysis of species and phylogenetic correlations

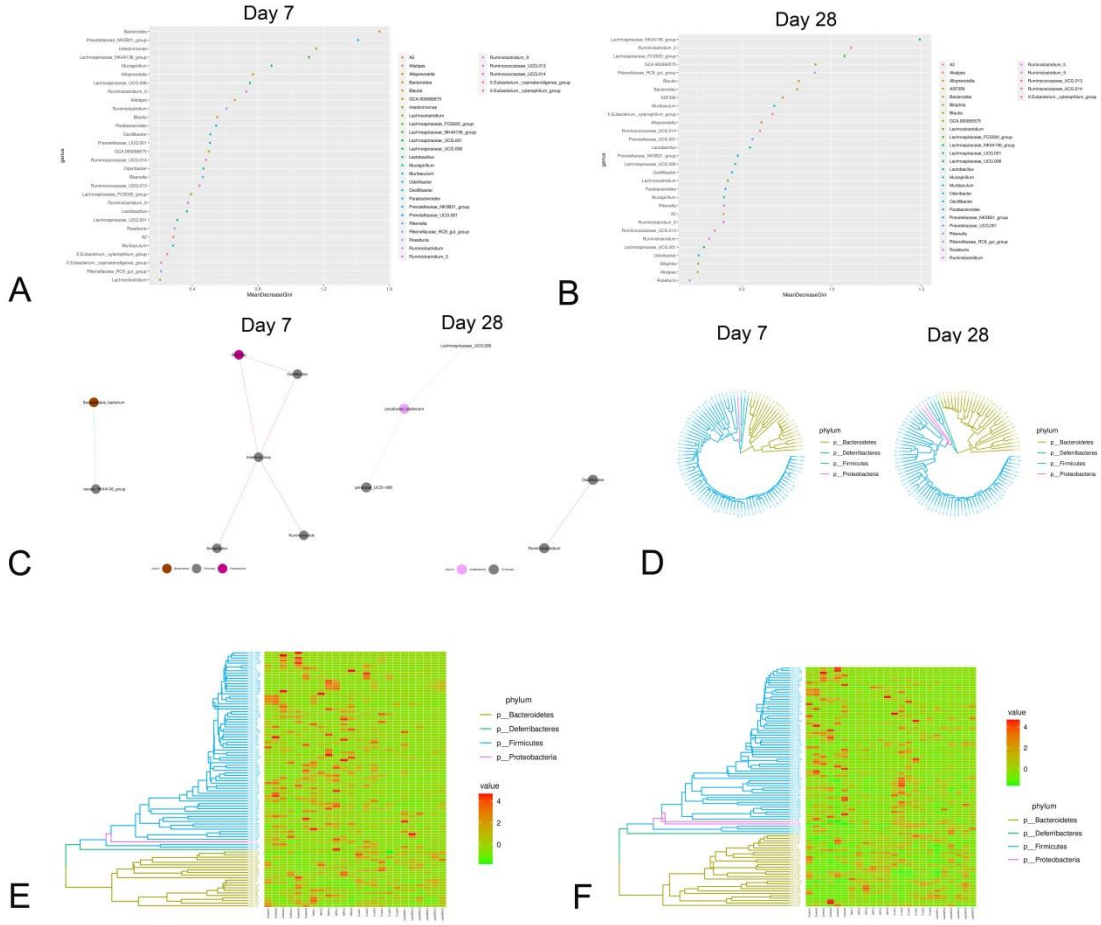

Fig. 3.36 Sample classification of the microbiome community and phylogenetic analysis of phyla

(A) (B)The importance point map of species on day 7 and day 28. The importance measurement standard was on the abscissa, and the importance of species names was sorted on the ordinate; (C) Correlation network diagram of species. The abundance of species is displayed by the size of the nodes, and different species are shown in different colours; the positive and negative correlations depend on the colour of the line, where red indicates a positive

correlation, and green indicates a negative correlation; the size of Pearson's correlation coefficient is indicated by the thickness of the line, where a higher correlation between species is indicated by a thicker line. The abundance of OTUs in each sample is shown; (D) and (E) (F) A combined diagram is obtained from the phylogenetic tree and species abundance on day 7 and day 28. Different bacterial phyla with clustering branches. The abundance of operational taxonomic units (OTUs) in each sample on the left corresponds to the abundance graph on the right.

To effectively and accurately classify microbial community samples and identify different key components (OTUs or species) between regions, a random forest machine learning algorithm was used. A random forest diagram was drawn to predict the outstanding species.

As shown in Fig. 3.36 A, the relative abundance between species samples was used to calculate the spearman correlation coefficient. We obtained the inter relationships between species within the sample or group of samples, and the inter relationships between species and used visual software to construct the network of species interactions on day 7 and day 28.

To show the species correlations of various classification levels under certain environmental conditions, a network map of species is plotted in Fig. 3.36 B. To calculate the abundance of each OTU, a phylogenetic tree was constructed by selecting the TOP100 OTUs with the most tags shown in Fig. 3.36 C and the heatmap shown in Fig. 3.36 D.

The results showed that the MPX and control groups had similar abundances of bacteria such as Firmicutes and Bacteroidetes. MPX treatment decreased the abundance of bacteria such as Firmicutes and Bacteroidetes relative to that in the *E. coli*-infected group on day 7 (Fig. 3.36 E). In addition, relative tothe control, MPX decreased the abundance of Firmicutes bacteria, and MPX treatment decreased the abundance of Firmicutes bacteria on day 28 (Fig. 3.36 E).

3.5.9 Gut microbiome disorder results in metabolic dysfunction

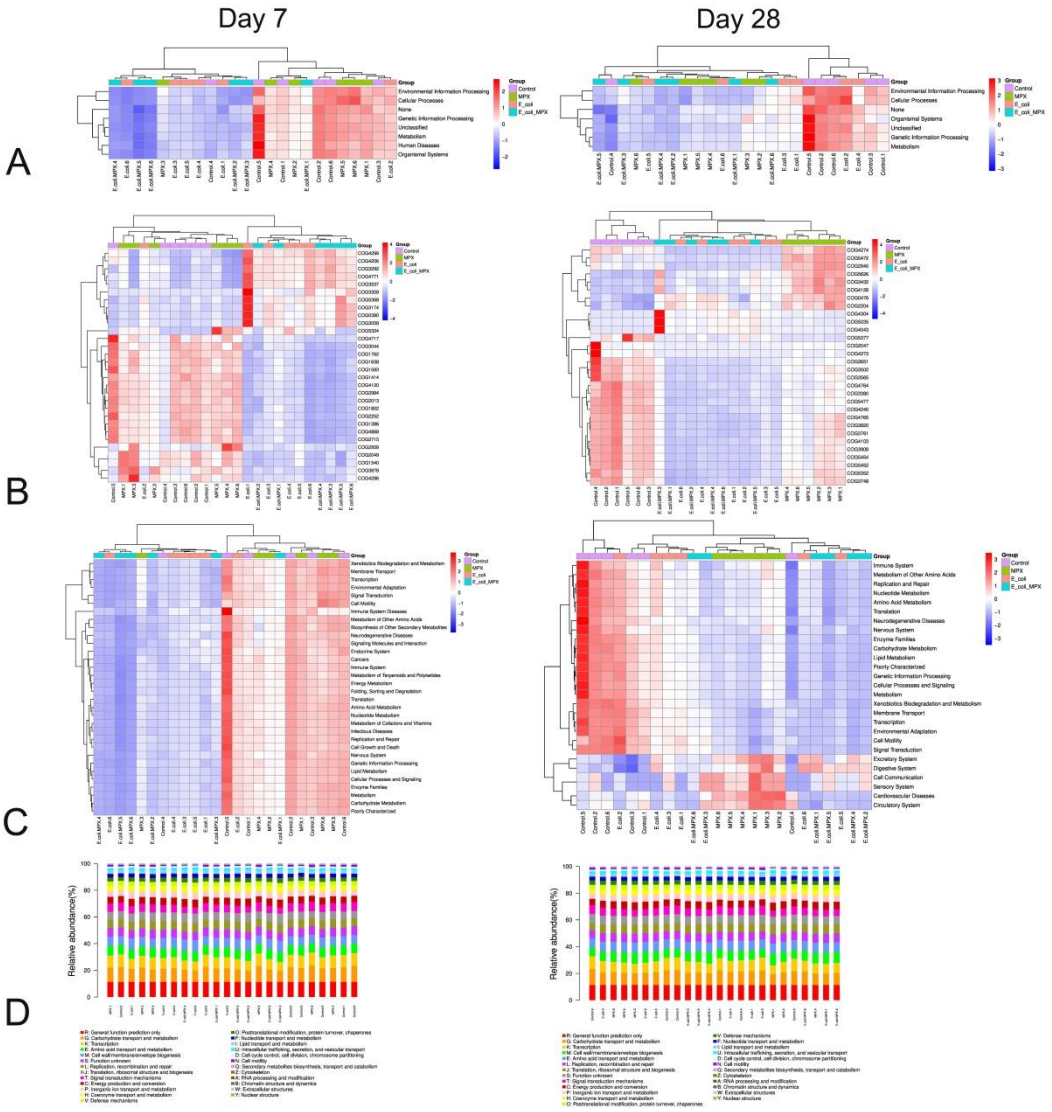

Fig. 3.37 The composition and functionof the gut microbiome was predicted

(A)The functions were determined by Kruskal-Wallis analysis and are shown in the heatmap; (B) Prediction of Clusters of Orthologous Groups of proteins. The results are shown in the heatmap. The clustering of different metabolic pathwaysis displayed in the cluster tree on the left, and samples from different groups are shown in clustering branches; (C) Pathways wereassessed by Kruskal-Wallis analysis and are shown in the heatmap; (D) Microbiome functions were predicted by the COG bar plot. The sample name is on thex-axis, and the relative abundance of the predicted COG_categoryis onthe Y-axis.

The changes in the intestinal flora in different groups wereanalysed byWilcoxon analysis. The results showed that the most prominent functions were focused on environmental information processing, cellular processes, organismal systems, genetic information processing, metabolism and human diseases on day 7, which was not related to human diseases on day 28 (Fig. 3.37 A). The Clusters of Orthologous Groups (COG) database is a database for the homologous classification of gene products. It was a relatively early database for identifying orthologous genes derived from a large number of comparisons of protein sequences of various organisms. In this study, cluster analysis of differences in four groups were performed in the COG database, and the top 30 COGs related to different species are shown in the heatmap in Fig. 3.37 B. According to the COG results, the main proteins identified on day 7 in the MPX group were an uncharacterized haem biosynthesis enzyme (COG2959) and an uncharacterized protein conserved in bacteria (COG4296); that in the control group was an uncharacterized conserved protein (COG4717); and that in the *E. coli* group was an uncharacterized virulence-associated protein D (COG3309). On day 28 the main identified proteins were an uncharacterized protein (ATP-grasp superfamily) (COG2047) in the control anda cell cycle control protein (COG 5035) in the MPX+*E. coli* group (Fig. 3.37B). The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a knowledge base for the systematic analysis of gene function and for linking genome information and functional information. KEGG analysis identified 30 pathways related to different flora. The results showed that most of these pathways were related to metabolism, including xenobiotic biodegradation and metabolism, metabolism of other amino acids, biosynthesis of other secondary metabolites, the immune system, genetic information processing, translation, energy metabolism, and replication and repair on day 7 and day 28 (Fig. 3.37 C). A bar blot was generated to further predict the potential functions of the microbiome. As shown in Fig. 3.37 D, only two functions, carbohydrate transport and metabolism and transcription, were slightly related to the microbiome.

CHAPTER 4

SUMMARY AND ANALYSIS OF RESULTS

Antimicrobial peptides (AMPs) have the advantages of good water solubility, strong thermal stability, a broad antibacterial spectrum and a wide range of sources [183]. MPX can destroy cell membranes and increase membrane permeability and has good antibacterial and antitumour effects [184]. Therefore, MPX has good development and application prospects as a therapeutic drug. For the first time, we report that MPX has good bactericidal activity against *A. pleuropneumoniae*, increasing cell membrane permeability; promoting cation leakage of Ca^{2+}, Na^{+}, etc.; significantly reducing *A. pleuropneumoniae* biofilm formation; and regulating the expression of virulence factors. In addition, for the first time, the Sap A gene is shown to play an important role in MPX resistance. Moreover, MPX can protect mice from lethal doses of *A. pleuropneumoniae* and relieve lung inflammation. This study systematically evaluated the antibacterial and anti-inflammatory effects of MPX on *A. pleuropneumoniae* in vivo and in vitro, laying the foundation for MPX treatment of *A. pleuropneumonia* infections.

The problem of bacterial resistance is becoming increasingly serious. Archambault et al. isolated 43 strains of *A. pleuropneumoniae* from Canadian pigs and found that they were highly resistant to chlortetracycline (88.4%) and oxytetracycline (90.7%); 5 strains exhibited multidrug resistance to penicillin, streptomycin, sulfonamide and tetracycline antibiotics, and 3 strains had multidrug resistance to streptomycin, sulfonamide and tetracycline antibiotics [185]. Monalessa Fábia Pereira et al. isolated 21 strains of *A. pleuropneumoniae* from infected pigs and found that 33% were resistant to β-lactams, 81% to aminophenols, 95% to aminoglycosides, 14% to quinolones and 76% to sulfonamides; the results showed that *A. pleuropneumoniae* has multidrug resistance [148]. Widespread florfenicol use has caused resistance among *A. pleuropneumoniae* isolates as high as 34% [186]. Epidemiological cut-off value

results showed that the sensitivity of *A. pleuropneumonia* to amoxicillin was 0.5 μg/mL, but susceptibility to amoxicillin was reduced in 11.3% of isolated strains [187]. Thus, there is an urgent need to develop new drugs that replace antibiotics.

Antimicrobial peptides are considered some of the best potential alternatives to antibiotics. Zeng et al. found that the expression of the antimicrobial peptide PR39 in transgenic mice could inhibit *A. pleuropneumoniae* infection, increasing the survival rate of mice, reducing the bacterial load of tissues and alleviating the symptoms of pneumonia [188]. Previous studies have shown that the expression of the antimicrobial peptide PR39 is increased in bronchoalveolar lavage fluid and respiratory tissues of pigs after *A. pleuropneumonia* infection [189]. Porcine β-defensin 2 (PBD-2) provides the first line of defence against porcine bacterial infection. When PBD-2 was overexpressed by porcine somatic cell cloning, *A. pleuropneumoniae* infection was inhibited; PBD-2 was overexpressed in multiple tissues of transgenic pigs, such as the heart, liver, spleen, lung and kidney; the number of *A. pleuropneumoniae* colonising the lung was significantly reduced; and lung pathological damage was alleviated [190]. Hennig-Pauka et al. found that the expression of PR39 in pig polymorphonuclear neutrophils was elevated after *A. pleuropneumoniae* infection, and PR39 was found in healthy lung tissue, indicating that PR39 plays an important role in maintaining pig health [191]. In this study, we found that the antimicrobial peptide MPX protected mice from a lethal dose of *A. pleuropneumoniae*, reduced the colonisation of *A. pleuropneumoniae* in the lungs and the levels of TNF-α and IL-6, and relieved pneumonia symptoms. The results showed that MPX was superior to PR39 both in vitro and in vivo, suggesting that the clinical application of the antimicrobial peptide MPX is promising.

Leite et al. found that MPX had strong antibacterial activity against gram-positive and gram-negative pathogens. The MIC value against *E. coli* was 6-62 μM, and that against *Staphylococcus aureus* was 4-31 μM [192]. Da Silva et al. found that MPX had a strong anticancer effect on human pleomorphic glioblastoma malignant brain tumours. The mechanism is membrane lysis, which leads to

tumour necrosis. MPX is expected to become a new type of anticancer drug [193]. Similar to other peptides extracted from wasp venom, MPX has degranulation activity on rat mast cells and reduces the haemolysis rate and chemotaxis in rat blood cells [192]. Henriksen et al. found that MPX puts unnatural amino acids into residues 1, 8, and 14 and that octyl side chain formation by amino acid substitutions can enhance the bactericidal effect of MPX on *E. coli* and *Lactococcus*, indicating that changing the side chain hydrophobicity of MPX could achieve the best membrane selectivity or bactericidal effect [194]. Carter et al. found that the survival rate of malaria parasites was significantly reduced after MPX treatment (100 μM) for 30 min *in vitro*. The survival rate of malaria parasites was reduced to 2% after mixing MPX (25 μM) with other peptides, indicating that MPX had better antimalaria parasite effects [195]. For the first time, we report that MPX had good bactericidal activity against *A. pleuropneumoniae*; increased cell membrane permeability; promoted cation leakage of Ca^{2+}, Na^{+}, etc.; significantly reduced *A. pleuropneumoniae* biofilm formation; and regulated the expression of virulence factors. The above results indicated that MPX has the potential to be developed into an antibacterial drug.

In recent years, the unreasonable use of antibiotics has led to an increase in the resistance of *E. coli*. Therefore, there is an urgent need to find alternatives to antibiotics. Amphaiphan C and others isolated *E. coli* from dogs and cats suffering from urinary system diseases, and tested for drug sensitivity, and found that the resistance of *E. coli* was 16.7% [196]. Fayemi OEFrom et al from 180 samples of fresh beef and meat products detected that 61 samples contained different serotypes of *E. coli*. The resistance analysis of the isolated *E. coli* O157:H7 showed that 23.6% resistance of STEC serotype [197]. Sarjana Safain K et al determined the spectrum of AMR and associated genes encoding aminoglycoside, macrolide and β-lactam classes of antimicrobials in bacteria isolated from hospitalized patients in Bangladesh, found that 53% of isolates were multidrug-resistant (MDR), including 97% of *E. coli* [198]. Shin H et al isolated high level carbapenem and extensively drug resistant (XDR) strain N7 of *E.coli*, which

produces a variant of New Delhi metallo-β-lactamase (NDM-5) from the influent of the Jungnang wastewater treatment plant located on Han River, Seoul, South Korea N7, which harbors the gene, showed high level of carbapenem resistance at concentrations of doripenem (512 mg/L) and meropenem (256 mg/L), and XDR to 15 antibiotics [199]. The above results indicate that *E. coli* has high resistant to antibiotics, and there is an urgent need to find the best alternative to antibiotics against *E. coli* infection. This study found that MPX has good antibacterial activity against *E. coli*, with a minimum inhibitory concentration of 31.25 ug/mL and has no drug resistance, indicating that MPX is expected to become one of the antibiotic alternatives for the treatment of *E. coli* infections.

The formation of bacteria biofilm leads to an increase in bacterial resistance. Morroni G et al. found that the antimicrobial peptide LL-37 has good antibacterial activity against multi-drug resistant *E. coli*, and MIC and sub-MIC concentrations of LL-37 were able to reduce *E. coli* biofilm formation [200]. Vergis J et al found that the antimicrobial peptide Lactoferricin (17-30) has good antibacterial and anti-biofilm activity against multi-drug-resistant Enteroaggregative *Escherichia coli*, and Lactoferricin (17-30) significantly reduced the formation of *E. coli* biofilm [201]. Mishra BE et al found that antimicrobial peptide WW298 could effectively inhibit the MRSA attachment and disrupt its preformed biofilms more effectively than daptomycin [202]. Liu Ye et al found that tryptophan-rich amphiphilic peptide termed WRK-12 significantly inhibited the formation of biofilm in a dose-dependent manner, especially multidrug-resistant (MDR) bacteria particularly Gram-negative bacteria [203]. This study found that MPX (1 MIC) significantly inhibited the formation of *E. coli* biofilm, indicating that antibacterial peptides have a good antibacterial biofilm formation effect, which lays the foundation for the development of antibacterial biofilm formation drugs.

Intestine is in direct contact with the external environment and colonizes a large number of microorganisms. Antimicrobial proteins secreted by intestinal epithelial cells play an important role in maintaining the homeostasis of intestinal epithelium and normal microbial flora [204]. REG3γ is mainly expressed in the

small intestine tissues of mice and humans. In addition, REG3γ also conditionally expressed when pathogen infection or inflammation occurs in the large intestine tissues [205]. Study showed that REG3γ was almost not expressed in the intestinal tract of sterile mice, and the expression of REG3 γ was significantly increased after the normal flora was colonized [206]. The expression of RemLp is mainly regulated by Th2 cytokines, which playsan important role in the process of innate immunity and host defense [207].TFF3 is produced by mucous secreting cells, which plays an important role in the function of the intestinal mucus layer and mucosal repair function [208].In this study, the results found that MPX can increase the mRNA expression of the antimicrobial protein TFF3 in the jejunum and colon, and reduce the expression of the antimicrobial protein Remlβ and REG3γ in the jejunum and colon.

Previous studies have found that the morphological integrity of villi and microvilli, which play key roles in the absorption of intestinal nutrients, is an important indicator of the performance and health of the host [209]. Daneshmand et al investigated the effect of the addition of the antimicrobial peptide cLFchimera (20 mg/kg) to the diet of broiler chickens in thecontext of necrotic enteritis (NE) challenge and found that cLFchimera ameliorated intestinal lesions and changes to the villus morphology in the jejunum [210]. Liang et al found that bovine antimicrobial peptide-13 (APB-13) had good antiviral activity against transmissible gastroenteritis virus (TGEV) and significantly reduced the piglet diarrhea induced by TGEV, thereby improving the intestinal villus morphology [211]. Previous studies demonstrated that the antimicrobial peptide MccJ25 could protect against enterotoxigenic *E. coli* (ETEC) infection and significantly alleviate the destruction of intestinal morphology and changes in villus morphology in mice infected with ETEC [212]. Wang et al investigated the effect of the antimicrobial peptide JH-3 on the intestinal inflammation induced by *Salmonella* CVCC541 and found that it could effectively alleviate the pathological damage to the duodenum and jejunum, reduce the loss of intestinal villi and improve the morphology of intestinal villi [36]. In this study, MPX significantly improved the pathological

damage to the intestine caused by *E. coli*, reduced the loss of intestinal villi, and maintained the morphology of intestinal villi. The effects of MPX on the intestinal epithelial cell microvilli were further confirmed by TEM, revealing that MPX not only improved the morphology of intestinal villi but also increased the number of microvilli, thereby increasing nutrient absorption in the intestine. These results indicated that MPX could effectively alleviate intestinal damage and maintain villi and microvilli morphology, thereby promoting nutrient absorption in the intestine.

Antimicrobial peptides, as an important part of the natural immune system, possess good anti-inflammatory activity [93]. Long-term and excessive production of proinflammatory cytokines may lead to intestinal damage and high energy requirements [213]. Wubulikasimu et al found that the antimicrobial peptide AKK8 possessed good antibacterial activity against drug-resistant strains of *C. albicans*, significantly reducing the levels of IL-6, IL-1β andTNF-α in the serum of mice infected with *C. albicans* [214]. Ding et al evaluated the effect of the antimicrobial peptide microcin J25 on ETEC infections in a murine model and found that microcin J25 decreased the secretion of inflammatory factors by inhibiting the activation of the MAPK and NF-κB signaling pathways, thereby alleviating the intestinal inflammatory response inducedby ETEC [212]. Shin et al investigated the effect of the antimicrobial peptide Lycotoxin-Pa4a on the lipopolysaccharide (LPS)-induced inflammatory response in RAW264.7 cells and found that it significantly reduced the expression of the inflammatory cytokines IL-1β and TNF-α by inhibiting the activation of the MAPK pathway, thereby inhibiting the LPS-induced inflammatory response in RAW264.7 cells [215]. In this study, MPX significantly reduced the *E. coli*-mediated expression of the inflammatory factors IL-2, IL-6 and TNF-α by inhibiting the activation of p-p38 and p-p65 in vitro and in vivo, thereby attenuating intestinal inflammation. These results indicated that MPX exerts good anti-inflammatory effects and can be beneficial as an alternative to antibiotics.

The intestinal barrier mainly includes the intestinal epithelial barrier, immune barrier, chemical barrier and biological barrier. In addition, the intestinal epithelial

barrier is the first barrier for preventing bacteria, antigens and other toxic and harmful substances from entering the submucosa of the intestine and blood [216]. The TJ structure is composed of TJ proteins, such as ZO-1, occludin and claudins, which are an important part of the intestinal epithelial barrier and play important roles in intestinalepithelial cells [217]. Lin et al investigated the effect of the antimicrobial peptide gloverin A2 (BMGlvA2) on ETEC-induced intestinal barrier disruption in mice and found that it clearly improved the expression of the TJ protein ZO-1 in the intestine after ETEC infection [218]. Zhang et al found that the hybrid peptide LL-37-Tα1 (LTA) could increase the LPS-induced expression of ZO-1 and occludin in the jejunum of mice, thereby improving intestinal barrier function [157]. Yu et al explored the therapeutic effects of the recombinant antimicrobial peptide microcin J25 on epithelial barrier dysfunction in a murine model and found that it could enhance the expression of TJ proteins, thereby attenuating ETEC-induced intestinal barrier dysfunction [219]. In this study, MPX improved the expression of the TJ proteins ZO-1 and occludin in IPEC-J2 cells and enhanced the expression of ZO-1, occludin and MUC2 in the mouse jejunum and colon, indicating that it attenuates intestinal barrier dysfunction by improving TJ protein and mucin expression. Surprisingly, MPX was more effective in increasing TJ protein and mucin expression than Enro, which was potentially due to broad antibacterial activity of MPX and antibiotics, which control the balance of microorganisms in the intestine. However, this study of *E. coli* infection by intraperitoneal injection has certain drawbacks and can not fully represent intestinal infection with *E. coli* strain.

The regulatory mechanism of tight junction proteins is very complicated, and the signal pathways involved include: Rho, MAPK, PI3K/Akt, protein kinase C (PKC), MLCK and other pathways [220]. In recent years, studies have found that Rho signaling pathway may play an important role in the regulation of tight junction proteins by antimicrobial peptides [221]. At present, more than 20 Rho family members have been discovered and mainly included RhoA. Rac1, Cdc42. In addition, many studies have shown that activation of Rac 1 enhanced cell barrier

function [73]. Studies have shown that the antimicrobial peptide hBD3 improves the barrier function of tight junctions of epithelial cells by increasing the activation level of Rac I [222]. This study found that the effect of MPX on tight junction proteins ZO-1 and Occludin was inhibited after adding inhibitor NSC 23766, and inhibited the function of MPX against *E. coli* adhesion and invasion in IPEC-J2 cells, suggesting that MPX may regulate IPEC-J2 cells tight junction proteins through the Racl pathway, and enhance the intestinal epithelial barrier function.

At the genus level, we discovered that the relative abundances of Lachnospiraceae_NK4A136, Alistipes and Lachnospiraceae_UGG-006 were significantly increased in the caecal microbiota of *E. coli*-infected mice compared to those in MPX-treated mice, whereas the relative abundance of the genus Ruminococcaceae_UCG-013 was significantly reduced after MPX treatment. The development of nonalcoholic fatty liver disease is correlated with Ruminococcaceae_UCG-013 [223]. In addition, we found that the abundance of Bacteroides was increased after MPX treatment, and a decreased abundance of Bacteroides was associated with several diseases, such as obesity [224] and diabetes [225], which has been verified by previous studies.Furthermore, previous studies have revealed a high abundance of Alistipes in patients with gastrointestinal complications [226] and chronic fatigue syndrome [227]. Alistipes is sufficient to induce colitis and tumours in IL10-/- mouse models [228]. Notably, Alistipes species influence the availability of tryptophan, which is also the precursor of serotonin, and an increased abundance of Alistipes might disrupt the balance inthe intestinal serotonergic system [229]. Irritable bowel syndrome patients show a greater frequency of abdominal pain, which is associated with higher levels of Alistipes, and it is speculated that Alistipes is associated with inflammation [230]. In this study, the symptoms of intestinal inflammation were more serious after *E. coli* infection, which was speculated to be related to the increased abundance of Alistipes. MPX addition significantly alleviated intestinal inflammation and reduced the abundance of Alistipes, and the mechanism by which MPX relieves intestinal inflammation associated with changes in Alistipes

needs to be further studied. In addition, the contribution of MPX to modulating the composition of the gut microbiota needs to be further investigated in the healthy and inflamed gut.

CONCLUSION

In the dissertation work, based on research, it is investigated mechanism of the antimicrobial peptide mastoparan X in killing Gram-negative bacteria *in vitro* and its anti-inflammatory and barrier repair functions in pneumonia and enteritis diseases *in vivo*, and relationship between anti-inflammatory and intestinal microbes of MPX. Finally, explored the anti-inflammatory and barrier repair functions mechanism of MPX in the intestinal epithelial cells, laying a foundation for reducing the use of antibiotics in livestock and poultry breeding.

Based on the results of the research, the following conclusions are justified:

1. MPX significantly reduced *A.pleuropneumoniae* biofilm formation and regulated the virulence factor expression of *A.pleuropneumoniae* in vitro and protected mice from lethal doses of *A.pleuropneumoniae* in vivo.

2. MPX had good antibacterial effect on *E. coli* and the stability was less affected by temperature, pH, ions and significantly inhibited the formation of *E. coli* biofilms.

3. MPX could reduce *E.coli* growth, attenuate the inflammatory response and intestinal damage in vivo.In addition, MPXimproved the intestinal barrier function and increased the expression of the TJ proteins ZO-1, occludin and mucin.

4. MPX notably suppressed the levels of MPO and LDH and improved the expression of ZO-1, occludin induced by *E. coli*. In addition, MPX inhibited *E. coli*-induced TLR4 expression, and decreased the IL-2, IL-6 and TNF-α levels by blocking the activation of the p65 and p38 inflammatory pathways.

5. MPX altered intestinal microbiome composition the of *E. coli*-infected mice, increasing the abundance of beneficial bacteria and reducing the abundance of harmful bacteria.

PRODUCTION PROPOSALS

1. On the basis of our recearsh Methodological Recommendations «Prevention of antibiotic resistance through the use of antimicrobial peptides», for laboratory, practical classes and independent work for master's students of veterinary department from disciplines "Veterinary Microbiology"and «Veterinary Zoohygiene», specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise" was development. (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021).

2. We recommend using the materials of the dissertation work when studying the courses "Veterinary microbiology", "Veterinary pharmacology" for masters of the Faculty of Veterinary Medicine of Sumy NAU. And for the courses "Veterinary microbiology" and " Veterinary pharmacology " for masters of the Henan Institute of Science and Technology (HIST).

3. We can recommend that antimicrobial peptide can develop as new antibacterial and anti-inflammatory therapy drug at farm and new oral drug to regulate gut bacteria in production in the future.

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APPLICATIONS

Appendix A

List of works published on the topic of the dissertation

LIST OF WORKS PUBLISHED ON THE THEME OF THE DISSERTATION

Articles in scientific professional publications of Ukraine:

1. **Zhao, X.**, Fotina, H., Wang, L., & Hu, J. (2020). Antimicrobial peptides as novel alternatives to antibiotics. Scientific Messenger of LNU of Veterinary Medicine and Biotechnologies. Series: Veterinary Sciences, 22(98), 74-78. https://doi.org/10.32718/nvlvet9813 https://nvlvet.com.ua/index.php/journal/article/view/3979 (The applicant participated in research, analysis of the results and writing the article).

2. **Zhao, X**. (2021). The function of antimicrobial peptide MPX on the apoptosis and barrier of IPEC-J2 cells. Scientific Messenger of Lviv National University of Veterinary Medicine and Biotechnologies. Series: Veterinary sciences, 23(102), 125–129. https://nvlvet.com.ua/index.php/journal/article/view/4189

https://doi.org/10.32718/nvlvet10219 (The applicant participated in research, analysis of the results and writing the article).

3. **Xueqin ZHAO**, Hanna FOTINA, Lei WANG, Jianhe HU. (2020). The mechanism of antimicrobial peptide MPX against Enterohemorrhagic *Escherichia coli* in vitro. Вісник Сумського національного аграрного університету Серія «Ветеринарна медицина», випуск 4(52), 18-25. http://repo.snau.edu.ua:8080/xmlui/handle/123456789/9511 (The applicant participated in research, analysis of the results and writing the article).

4. **Zhao, X**., Fotina, H., Wang, L., & Hu, J. (2021). Antimicrobial peptide MPX alleviates the lethal attack of Escherichia coli in mice. Bulletin of Sumy National Agrarian University. The series: Veterinary Medicine, No. 3 (54), 54-59. https://snaubulletin.com.ua/index.php/vm/issue/view/39
DOI: https://doi.org/10.32845/bsnau.vet.2021.3.8 (The applicant participated in research, analysis of the results and writing the article).

Articles in scopuse journals:

5. **Zhao, X.,** Wang, L., Zhu, C., Xia, X., Zhang, S., Wang, Y., Zhang, H., Xu, Y., Chen, S., Jiang, J., Liu, S., Wu, Y., Wu, X., Zhang, G., Bai, Y., Fotina, H., & Hu, J. (2021). The Antimicrobial Peptide Mastoparan X Protects Against Enterohemorrhagic *Escherichia coli* O157:H7 Infection, Inhibits Inflammation, and Enhances the Intestinal Epithelial Barrier. *Frontiers in microbiology*, *12*, 644887. https://doi.org/10.3389/fmicb.2021.644887* *https://www.scopus.com/record/display.uri?eid=2-s2.0-85108665431&origin=resultslist&sort=plf-f (The applicant participated in research, analysis of the results and writing the article).*

6. Chunling Zhu, Yaya Zhao, **Xueqin Zhao**, Shanqin Liu, Xiaojing Xia,Jinqing Jiang,Yundi Wu, Xilong Wu, Gaiping Zhang,Jianhe Hu, Hanna Fotina, Lei Wang (2022). The Antimicrobial Peptide MPX Can Kill *Staphylococcus aureus*, Reduce Biofilm Formation, and Effectively Treat Bacterial Skin Infections in Mice. Front. Vet. Sci., 29 March 2022 Sec. Veterinary Infectious Diseases https://doi.org/10.3389/fvets.2022.819921 https://www.frontiersin.org/articles/10.3389/fvets.2022.819921/full *(The applicant participated in research, analysis of the results and writing the article).*

7. Lei Wang, **Xueqin Zhao**, Chunling Zhu, Yaya Zhao, Shuangshuang Liu, Xiaojing Xia, Xin Liu, Huihui Zhang, Yanzhao Xu, Bolin Hang, Yawei Sun, Shijun Chen, Jinqing Jiang, Yueyu Bai, Gaiping Zhang, Liancheng Lei, Langford Paul Richard, Hanna Fotina, Jianhe Hu (2020). The antimicrobial peptide MPX kills Actinobacillus pleuropneumoniae and reduces its pathogenicity in mice, Veterinary Microbiology, Vol 243, 108634, ISSN 0378-1135, https://doi.org/10.1016/j.vetmic.2020.108634. https://www.sciencedirect.com/science/article/pii/S0378113519309617?pes=vor *(The applicant participated in research, analysis of the results and writing the article).*

8. Wang, L., **Zhao, X**., Xia, X. *et al.* (2019). Antimicrobial Peptide JH-3 Effectively Kills *Salmonella enterica* Serovar Typhimurium Strain CVCC541 and Reduces Its Pathogenicity in Mice. *Probiotics & Antimicro. Prot.* **11,** 1379–1390 https://doi.org/10.1007/s12602-019-09533-w https://link.springer.com/article/10.1007/s12602-019-09533-

9. **Zhao, X.,** Fotina, H., Fotina, T., Hu, J.,& Wang, L. (2022). The effect of oral administration of the antibacterial peptide MPX on intestinal inflammation of mice in experimental infection with Escherichia coli strain O157: H7. Scientific Horizons, 25 (2), 9-15. https://www.scopus.com/record/display.uri?eid=2-s2.0-85135872101&origin=resultslist&sort=plf-f *(The applicant participated in research, analysis of the results and writing the article).*

***Articles in scientific professional poublications of Chine*:**

10. ZHAO Ya-ya, LIU Xin-xin, **ZHAO Xue-qin**, LIU Shuang-shuang, ZHU Chun-ling, WANG Heng, XIA Xiao-jing, ZHANG Hui-hui, WANG Qing, XU Yan-zhao, HANG Bo-lin, SUN Ya-wei,CHEN Shi-jun, HU Jian-he, WANG Lei*. (2020). Study on the bactericidal activity of JH-3 against MRSA (Methicilin-resistant Staphyloccocus aureus) and its inhibitoryeffect on MRSA-induced inflammatory response in A549 cells. Chinese Journal of Preventive Veterinary Medicine, Vol. 42 No.7. https://kns.cnki.net/kcms/detail/detail.aspx?dbcode=CJFD&dbname=CJFDLAST2020&filename=ZGXQ202007014&uniplatform=NZKPT&v=F5EZwCw7TrR0MzRpss0pcsbmS-MKvGsC2Z4XG0lSuv1vu8HwzBfJqD6HESFrhrIj *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

11. HU Bin, ZHANG Yanhong, **ZHAO Xueqin**, DONG Mengmeng, GUO Feng, HU Jianhe. (2019). Application of Antimicrobial Peptides on Broiler Production. China Poultry, Vol.41， No.16. https://doi: 10.16372/j.issn.1004-

6364.2019.16.009 https://www.cabdirect.org/cabdirect/abstract/20203021762 *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

Theses of scientific reports:

12. **Xueqin Zhao,** Fotina Hanna. **(**2018).The mechanism of antimicrobial peptides to regulate local immunity and host defense against the pathogenic intestinal bacteria. Proceedings of the All-Ukrainian Student Scientific Conference Dedicated to Student`s Day. (12-16 October 2018.) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

13. **Xueqin Zhao,** Fotina Hanna. **(**2019). Antibacterial peptide JH-3 effectively reduces the fatal attack of *Salmonella enterica* serovar typhimurium strain CVCC541 and relieves pathogenicity in mice. BTRP Ukraine Regional One Health Research Symposium(20-24 May 2019, Kyiv, Ukraine). *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

14. **Xueqin Zhao,** Fotina Hanna . **(**2019). The mechanism of antimicrobial peptide sterilization. Proceedings of the All-Ukrainian Student Scientific Conference Dedicated to Student`s Day. (11-15 October 2019.) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

15. **ZHAO Xue-qin**，ZHU Chun-ling，WANG Lei，FOTINA Hanna，HU Jian-he. **(**2021). Antimicrobial peptide MPX relieves Enterohemorrhagic Escherichia coli-induced intestinal inflammation by improving the diversity of intestinal flora.Chinese Society of Microbiology Veterinary Microbiology, 2021 Academic Forum (19-21June, 2021.Zhengzhou, China). *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

16. **ZHAO Xue-qin**，ZHU Chun-ling，WANG Lei，FOTINA Hanna，HU Jian-he. **(**2021). The antimicrobial peptide MPX protects against Enterohemorrhagic Escherichia coli O157:H7 infection, inhibits inflammation, and enhances the intestinal epithelial barrier. Chinese Society of Microbiology Veterinary Microbiology, 2021 Academic Forum(19-21June, 2021.Zhengzhou, China). *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

17**. ZHAO Xue-qin**，ZHU Chun-ling，WANG Lei，FOTINA Hanna，HU Jian-he. (2021). The mechanism of antimicrobial peptide MPX against Enterohemorrhagic *Escherichia coli* in vitro. Chinese Society of Microbiology Veterinary Microbiology, 2021 Academic Forum (19-21June, 2021. Zhengzhou, China). *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

18. **Xueqin Zhao**, Hanna Fotina, Lei Wang, Jianhe Hu. (2021). Antimicrobial peptide MPX against *Escherichia coli* infection in IPEC cells. One Health Student International Conference (24 - 27 November, 2021, Bucharest, România) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

19. ZHAO Ya-ya, ZHU Chun-ling, **ZHAO Xue-qin,** LIU Shuang-shuang, XIA Xiao-jing, HU Jian-he, WANG Lei. (2021). The bactericidal mechanism of antimicrobial peptide MPX against methicillin-resistant Staphylococcus aureus. Chinese Society of Microbiology Veterinary Microbiology, 2021 Academic Forum (19-21June, 2021. Zhengzhou, China). *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

Patent:

20. ZHAO Ya-ya, **ZHAO Xue-qin,** LIU Shuang-shuang, XIA Xiao-jing, HU Jian-he, WANG Lei. (2020). *Paking Box B*ainong Peptide. Registration 09.29, registration № ZL 2020 30179216.5

Methodological recommendations:

21. **Xueqin Zhao**, Hanna Fotina, Lei Wang, Jianhe Hu. «Prevention of antibiotic resistance through the use of antimicrobial peptides»35 pp. approved by the Academic Council of SNAU (Protocol № 5, dated 29.11.2021).

Appendix B

Methodical Guidelines

Xueqin Zhao, Hanna Fotina, Lei Wang, Jianhe Hu. «Prevention of antibiotic resistance through the use of antimicrobial peptides»35 pp. approved by the Academic Council of SNAU (Protocol № 5, dated 29.11.2021).

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MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE

SUMY NATIONAL AGRARIAN UNIVERSITY

Methodical guidelines

"Prevention of antibiotic resistance through the use of antimicrobial peptides"

Sumy- 2021

Compilers:

Xueqin Zhao, postgraduate student of Department Of Veterinary Examination, Microbiology, Zoohygiene And Safety And Quality Of Livestock Products

Fotina T.I., doctor of veterinary science, professor, head of the Department of Veterinary Expertise, Microbiology, Zoohygiene and Safety and Quality of Animal Husbandry Products

Lei Wang, College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang, China

Jianhe Hu, College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang, China

Methodical guidelines "Modern methods of cow mastitis diagnostic and prevention", for laboratory, practical classes and independent work for master's students of veterinary department from disciplines "Veterinary Microbiology"and "Veterinary Zoohygiene", specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". 35p

Reviewers:

R.V. Petrov, Doctor of Veterinary Medicine, Professor, Head of the Department of Department Of Virusology, Patanatomy And Bird Diseases Named After Professor I.I.Panicar of Sumy NAU;

O.I. Shkromada, Doctor of Veterinary Medicine, Professor, Head of the Department of Obstetrics and Surgery of the Sumy National University of Science.

Recommended for publication by the educational and methodical council of the Faculty of Veterinary Medicine of SNAU, protocol No.5 dated "29" 12. 2021.

Appendix C

Patent:

ZHAO Ya-ya, ZHAO Xue-qin, LIU Shuang-shuang, XIA Xiao-jing, HU Jian-he, WANG Lei. (2020). Paking Box Bainong Peptide. Registration 09.29, registration № ZL 2020 30179216.5

证书号第6098664号

外观设计专利证书

外观设计名称：包装盒（百农肽克）

设　计　人：王磊;刘善芹;刘欣欣;赵雪芹;夏小静;张守平;赵娅娅
罗维玉;徐彦召;孙亚伟;杭柏林;王彦华;安志兴;白跃宇

专　利　号：ZL 2020 3 0179216.5

专利申请日：2020年04月26日

专利权人：河南科技学院

地　　址：453003 河南省新乡市红旗区华兰大道东段

授权公告日：2020年09月29日　　授权公告号：CN 306077142 S

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局长
申长雨

国家知识产权局
2020年09月29日

其他事项参见续页

证书号第6098664号

国家税务总局北京市海淀区税务局 印花税代扣专用章 国家知识产权局

专利权人应当依照专利法及其实施细则规定缴纳年费。本专利的年费应当在每年04月26日前缴纳。未按照规定缴纳年费的，专利权自应当缴纳年费期满之日起终止。

申请日时本专利记载的申请人、设计人信息如下：

申请人：

河南科技学院

设计人：

王磊；刘善芹；赵雪芹；夏小静；张守平；赵娅娅；罗维玉；徐彦召；孙亚伟；杭柏林；王彦华；白跃宇；胡建和

证书号第6098664号

外观设计专利证书

设　计　人：胡建和

2020年09月29日

Appendix D

Laboratory Biosafety Certificate

实验室生物安全证书

我校博士生赵雪芹，在开展研究：抗菌肽作用机制与功能优势过程中，涉及病原微生物的实验。该生在实验过程中，严格遵守国家及学校生物安全相关规定，并根据《人间传染的病原微生物名录》（卫科教发：2006]15 号） 规定的生物安全防护和标准操作规程开展实验研究，并确保实验室生物安全。

Laboratory Biosafety Certificate

Xueqin Zhao, PhD student who involved in the use of pathogenic microorganisms in the research on Advances in research on mechanism and function of antimicrobial peptides. During the experiment, she strictly abide by the relevant national and school biosafety regulations, and carry out experimental activities in accordance with the standard operating procedures in the biosafety protection level laboratory stipulated in the "List of Pathogenic Microorganisms Infected Between Humans" (Wei Ke Jiao Fa: 2006] No. 15). And ensure laboratory biosafety.

Henan institute of Science and Technology

Date: September 4, 2019

河南省教育厅

教科外〔2009〕80号

河南省教育厅
关于公布2008年度河南省高校工程技术研究中心建设项目的通知

各高等学校：

根据《河南省教育厅关于开展2008年度河南省高校工程技术研究中心立项建设的通知》（教科外〔2008〕737号）精神，经学校申报、中心负责人答辩、专家评审、现场考察，教育厅审核并公示，决定依托河南科技大学等学校建设12个河南省高校工程技术研究中心（以下简称工程中心）。现将工程中心及依托单位名单予以公布（附件1），有关事宜通知如下：

附件1

2008年度河南省高校工程技术研究中心建设项目名单

序号	中心名称	依托学校	技术领域	类别
1	轴承工程技术	河南科技大学	制造业	认定
2	动物疫病和残留物防控	河南科技学院	农业与生物	认定
3	生态化工	郑州大学	资源与环境	立项
4	作物抗逆改良	河南大学	农业与生物	立项
5	农业资源与环境	河南农业大学	资源与环境	立项
6	道地中药材保育及利用	河南师范大学	农业与生物	立项
7	矿物加工与矿用材料	河南理工大学	新材料	立项
8	粮食信息与检测技术	河南工业大学	现代服务业	立项
9	高性能土木工程材料与环境	郑州航空工业管理学院	新材料	立项
10	生物质降解与气化	商丘师范学院	资源与环境	立项
11	信阳毛尖茶产业	信阳农业高专	农业与生物	立项
12	怀药提取分离	焦作大学	化学化工	立项

Appendix E

Conclusion of the commission on bioethics in English

Laboratory Animal Welfare and Ethical review of Henan Institute of Science and Technology

Application Number: 201909-025 Application Date: September 4, 2019 Issue No.

<table>
<tr><td colspan="3">Program and No.: Advances in research on mechanism and function of antimicrobial peptides</td></tr>
<tr><td rowspan="2">Applicant</td><td>Name: Xueqin Zhao</td><td>Organization: College of animal science and veterinary medicine</td></tr>
<tr><td>E-mail: zxueqin0708 @163.com</td><td>Telephone Number: 13525077791</td></tr>
<tr><td rowspan="2">Animals</td><td colspan="2">Animal Source: Henan Animal Experiment Center</td></tr>
<tr><td colspan="2">Animal Grade（Normal、SPF or Others）: Normal</td></tr>
<tr><td colspan="3">（Experimental objective, necessity and significance and how the program has been designed to achieve the objectives of the research）:
Objective: To study antimicrobial peptide on anti-inflammatory and regulating gut microbiota.
Necessity and significance: This study can lay the foundation for antimicrobial peptides to relieve inflammation and regulate gut microbiota.
experimental steps:
1. The mechanism of antimicrobial peptide MPX against Actinobacillus pleuropneumoniae and its effect on relieving the symptoms of pneumonia in mice.
2. The effect of antimicrobial peptide MPX on intestinal inflammation and gut microbiota in mice infected with E. coli.</td></tr>
<tr><td>Animal Care</td><td colspan="2">The nursing standards of ordinary piglets were adopted</td></tr>
<tr><td rowspan="2">Animal Disposition</td><td colspan="2">Death conduct:
□ CO_2 suffocated ■ Exsanguinations with anesthesia
□ Cervical dislocation □ Anesthesia overdose
□Others, detailed description</td></tr>
<tr><td colspan="2">Not for the death of the animal disposition:
□ Continue to use □ Save in the agency
□ Release to the wild
□ Others, detailed description:</td></tr>
<tr><td colspan="3">Poisonous (harmful) material (infection, radiate, chemical poison and other) being used
□ Yes ■ no
Declare:</td></tr>
<tr><td colspan="3">Supplementary instruction for investigate
No</td></tr>
<tr><td colspan="3">Declaration for the information disclosure and confidentiality requirements, declaring the information need to be kept secret, the information can be disclosed
No</td></tr>
<tr><td colspan="3">Claiming jurors for being debarb
No</td></tr>
</table>

Applicant's Declaration:

1. I will abide by the law and regulation stipulation, and accept the supervision and inspection by the committee and laboratory animal department.
2. The information I have given is accurate, detailed and comprehensive.

Applicant Signature (Seal): Jianhe Yu

September 04, 2019

Approval opinions:

■ Approval □ Not approve

Authorized Personnel Signature (Stamp):

September 10, 2019