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Antimicrobial peptide Mastoparan X has good activity against *Escherichia coli* in vitro and alleviates its pathogenicity in mice

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Abstract

Background *Escherichia coli* is a facultative anaerobic bacterium that normally resides in the gastrointestinal tract of humans and animals but can cause diarrhea and enteritis. MPX is an antimicrobial peptide extracted from wasp venom and has potent bactericidal effects against many bacteria. This study aimed to investigate the mechanism of MPX's bactericidal activity against *E. coli* in vitro, its effect on IPEC-J2 cell apoptosis and barrier function, and its therapeutic efficacy against *E. coli* infection in mice.

Methods The effects of MPX on *E. coli* were investigated in vitro, at the cellular level, and in vivo.

Results The study found that the MIC of MPX against *E. coli* was 31.25 μg/mL, and scanning electron microscopy showed that MPX caused the bacteria to become smaller in size with leaked contents. Additionally, NPN, PI, and DiSC3(5) results showed that MPX positively correlated with the fluorescence intensity. MPX significantly inhibited *E. coli* biofilm formation. Furthermore, MPX effectively reduced IPEC-J2 cell apoptosis, regulated ZO-1, Occludin, and Claudin-1 expression through the Rac1 pathway, and alleviated the pathological damage in the intestine, as shown by H&E staining results. qRT-PCR results indicated that MPX increased TFF3 mRNA expression in the jejunum and colon.

Conclusions This study is the first to explore the mechanism of MPX's bactericidal activity against *E. coli* in vitro, providing a foundation for developing new drugs to treat bacterial infections.

Keywords Antimicrobial peptide MPX, *Escherichia coli*, IPEC-J2 cells, Pathogenicity, Mice

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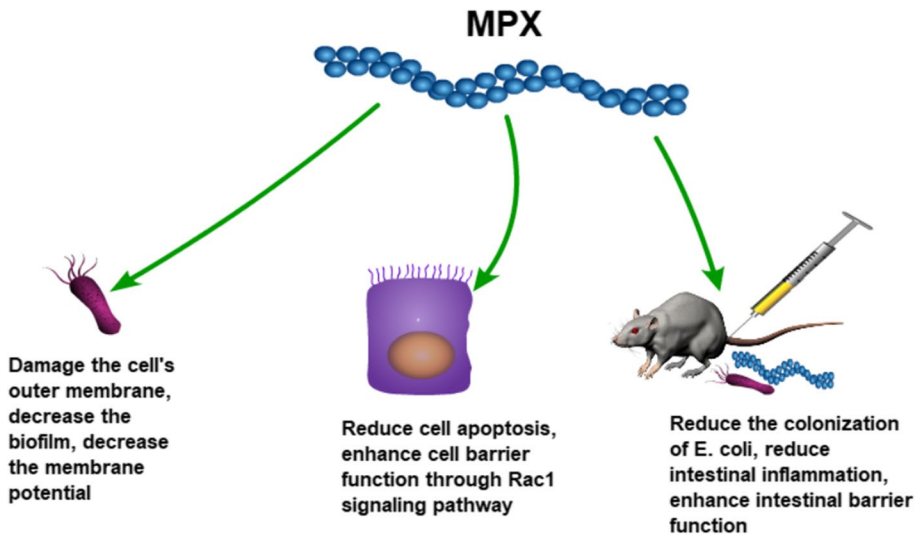
Graphical Abstract



Introduction

Escherichia coli is facultative anaerobic bacterium that mainly exists in the gastrointestinal tract of humans and animals, colonizes the small intestine, and is usually associated with acute secretory diarrhoea [1]. It can cause diarrhoea, enteritis, destruction of the host's intestinal barrier, and intestinal microecological disturbances [2]. Enterotoxigenic *Escherichia coli* (ETEC) is classically associated with acute secretory diarrhoea, which induces 2 million deaths in developing countries per year, predominantly in children in the first years of life [3]. In addition, in recent years, due to the widespread and excessive use of antibiotics, the resistance of *E. coli* has increased. The tetracycline, sulfonamide and penicillin antibodies, which are most commonly used in animal production, have the highest resistance rates [4]. Therefore, there is an urgent need to find alternative antibacterial drugs against *E. coli* infection.

Antimicrobial peptides are class of small defensive peptides produced when organisms resist the invasion of foreign microorganisms. They are important part of the body's innate immune system [5]. Both peptides and antibiotics are drugs used to treat bacterial infections, but peptides have advantages in certain aspects. Unlike many antibiotics, most peptides have low toxicity in the body, meaning that peptide drugs may cause fewer side effects and be safer [6]. Many peptides exhibit high selectivity towards bacteria, which can reduce the risk of damage to beneficial bacteria in the body and lower the rate of infection recurrence after treatment [7]. For example, some peptides target the cell membrane mechanism involved in bacterial growth inhibition, while antibiotics primarily target bacterial metabolic pathways, thus reducing dependence on antibiotics and lowering the risk of antibiotic resistance [8]. Antimicrobial peptides also have broad-spectrum antibacterial activity, as they can act against multiple types of bacteria rather than targeting a single bacterial species [9, 10]. Compared with traditional antibiotics, antimicrobial peptides have the characteristics of small molecular weight, good water solubility, good thermal stability, unique antibacterial mechanism, broad-spectrum antibacterial activity against clinically resistant strains, and robustness to bacterial resistance [11]. MPX (H-INWKGIAAMAKKLL-NH2) belongs to the family of bee venom antimicrobial peptides. MPX has high concentration in bee venom, it is an amino acid peptide with a net positive charge of 4 [12]. Previous research by our group found that MPX has good antibacterial activity against *Actinobacillus pleuropneumoniae* [13]. However, the bactericidal mechanism and effect of MPX against *E. coli* infection in vitro and the in vivo are still unknown.

In this study, the mechanism of MPX against *E. coli* infection was explored in vitro, and it was found that MPX mainly destroyed the membrane of bacteria, increased the permeability of the membrane, and led to the leakage of bacterial content, thereby exerting a bactericidal effect. In addition, MPX could significantly inhibit *E. coli* biofilm formation. Furthermore, MPX could significantly inhibit the apoptosis and the number of adhesions of IPEC-J2 cells and regulate the cell barrier function through the Rac 1 pathway caused by *E.*

coli infection. Finally, the effect of MPX against *E. coli* infection was further evaluated in vivo, and the results showed that MPX could alleviate the pathological damage of *E. coli* to the intestine of mice and increase the mRNA expression of antimicrobial proteins. This study laid a theoretical foundation for screening antimicrobial peptides to find drugs for the clinical treatment of *E. coli* infection.

Methods

Ethics statement

BALB/c mice (6–8 weeks old, 18–20 g, female) were purchased from Zhengzhou University. All animal experiments were performed in accordance with the guidelines of the Animal Welfare and Research Ethics Committee and were approved by the Animal Ethics Committee at Henan Institute of Science and Technology.

Peptide synthesis

MPX (H-INWKGIAAMAKKLL-NH2) was obtained from Shanghai Jier Biochemical Company (China) using a solid-phase N-9-fluoromethoxycarbonyl (Fmoc) strategy and high-performance liquid chromatography (HPLC) purification, and its purity was 98%.

Bactericidal activity test

E. coli was cultured in LB liquid medium to the logarithmic phase ($OD_{600}=1.0$), and the final concentrations of MPX (31.25 μg/mL), PR39 (50 μg/mL), and Enro (50 μg/mL) were added to the bacterial solution. ddH_2O was added as the negative control and incubated at 37 °C for 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h. Then, the OD_{600} of the bacterial solution was measured, and the bacterial solution was diluted every 1 h. Each plate was placed on the LB plate and incubated in a 37 °C incubator for 12 h until a single colony was clearly visible, and then the plates were counted [14]. The assays were repeated three times.

Determination of BCA protein content

BCA method for protein concentration measurement principle: In an alkaline environment, proteins will form complexes with Cu^{2+} ions and reduce them to Cu^{1+} ions (biuret reaction). BCA will then react with Cu^{1+} to form a stable purple-blue complex, which has a high absorbance value at 562 nm and is proportional to the protein concentration. Proteins are one of the most important nitrogen-containing biomolecules in cells and are involved in various biological functions. Briefly, 25 μL of each sample and 200 μL of BCA working reagent were added to each well. The microplate was shaken for 15 s and then incubated for 30 min at 37 °C using a BioTek® Cytation 5 Cell Imaging Multi-Mode Reader. Absorbance was measured at 562 nm [15]. The assays were repeated three times.

Detection of live and dead bacteria

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.

MPX on the outer membrane of *E. coli*

E. coli were washed with a 1:1 mixture of 5 mM HEPES buffer and resuspended with the same solution. The concentration of *E. coli* was 1×10^8 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, and 4 MIC. Then, the fluorescence was monitored at excitation wavelength of 350 nm and emission wavelength of 420 nm. The increase in fluorescence intensity was monitored for another 10 min with an INFINITE M PLEX microplate reader [16]. The assays were repeated three times.

MPX on the cell membrane potential

E. coli were collected and separately washed with a 1:1 ratio of 5 mM glucose and HEPES buffer (pH = 7.4). Next, the bacterial plate was resuspended in a 1:1:1 ratio of 5 mM HEPES buffer and 100 mM KCl solution supplemented with 0.2 mM EDTA and 5 mM glucose. For this study, EDTA was used to enable dye uptake by permeabilizing the outer membrane of *E. coli*. This study was performed in a Corning 96 black well plate with a clear bottom containing 2 μM 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 from each well. 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 [17]. The assays were repeated three times.

MPX on the inner membrane of *E. coli*

Briefly, the mid-log phase of *E. coli* was separately centrifuged (8000 rpm/min, 5 min), washed and resuspended in 1:1 ratio of 5 mM glucose and HEPES buffer (pH = 7.4). The working concentration of *E. coli* was 1×10^8 CFU/mL. Next, 190 μL of bacterial suspension containing

10 μM propidium iodide (PI) was added to the well of black Corning 96-well plate with 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 PI fluorescence for 10 min using an INFINITE M PLEX microplate reader [18]. The assays were repeated three times.

Crystal violet staining

The ability of MPX to inhibit *E. coli* biofilm formation was tested according to the reference. In a 96-well polystyrene microtiter plate, an overnight culture of *E. coli* was inoculated into 100 μL of LB liquid medium at a proportion of 1%, and different concentrations of MPX (0.5 MIC, 1 MIC, 2 MIC, 4 MIC) were applied; ddH_2O was used as a negative control. The culture plate was placed in a 37 °C incubator for 24 h. The culture supernatant was aspirated and discarded. Each well was washed 3 times with 200 μL of sterile PBS and fixed with 70% methanol for 30 min. The fixative was aspirated and discarded, and the wells were dried at 37 °C in the incubator for 30 min. Then, 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 culture plate was rinsed under a slow stream of water until the flowing water was colourless. After the oven was dried, the sample was placed under a microscope for observation. Then, 100 μL of 70% ethanol solution was added to each well for decolorization, vortexed and mixed, and quickly placed in a multifunctional microplate reader to determine the absorbance value of OD_{570} [19].

Scanning electron microscopy observation of biofilm formation

After culturing the *E. coli* overnight, it was diluted by a factor of 100 with fresh LB liquid medium and transferred to 6-well cell plate with sterile glass slides. Then, 500 μL of bacterial solution was added to each well, and MPX (1 MIC) and ddH_2O were added as negative control. After 24 h of incubation in a constant-temperature incubator at 37 °C, the cell culture solution was slowly removed and rinsed with sterile saline 3 times to wash away floating bacteria. Then, 300 μL of 2.5% glutaraldehyde solution was added to each well. After fixation at room temperature for 30 min, the samples were rinsed with pH = 7.4 phosphate buffer 3 times, with an interval of 10 min each time. Next, 30%, 50%, 70%, 80%, 90%, 95%, and 100% alcohol were used for gradient dehydration, and each time interval was 15 min. After the slides were dried, the formation of biofilms was observed under a scanning electron microscope [13].

IPEC-J2 cells adhesion and invasion detection

IPEC-J2 cells were cultured in a 6-well plate and 1×10^6 cells were added to each well. After 24 h of culture, the medium was replaced with fresh DMEM:F12 blank medium, 50 μM Rac1 inhibitor NSC23766 or the same volume of 0.1% DMSO was added, and then 10 μg/mL MPX or sterile water was added. The cells were placed in the cell incubator and cultured for 12 h, washed with PBS 3 times, and then added at an MOI of 10. The *E. coli* suspension was incubated at 37 °C for 1 h, and then unadhered and contaminant bacteria were washed away with PBS, 200 μL of 0.5% Triton X-100 was added to each well for 5 min, followed by 800 μL of precooled PBS. The cells were collected and subjected to multiple dilution and LB medium coating, with inverted culture in a biochemical incubator at 37 °C for 16 h. The assays were repeated three times.

Animal experiments

A total of 20 BALB/c mice (6–8 weeks old, 18–20 g, female) were purchased from Zhengzhou University. 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 days to acclimate to the environment. The animals were randomly divided into four experimental groups (control, *E. coli*, *E. coli* + MPX, and *E. coli* + enrofloxacin (Enro); five mice per group) and challenged with an intraperitoneal injection of *E. coli* (4.5×10^7 CFU/mL). The mice were treated with an intraperitoneal injection of MPX (20 mg/kg) or Enro (20 mg/kg) for 3 days after *E. coli* infection for 2 h. Ethics Approval and Consent to Participate is Henan Institute of Science and Technology. The ethical approval No. 2022HIST022. The assays were repeated three times.

qRT–PCR detection

IPEC cells were cultured to form a monolayer in a 6-well plate and then divided into four groups: IPEC + *E. coli*, IPEC + *E. coli* + MPX, IPEC + *E. coli* + MPX + NSC 23766, and IPEC + *E. coli* + NSC 23766. Infection was carried out at an MOI of 10. After 12 h, IPEC-J2 cells were collected for mRNA expression detection of the tight junction proteins ZO-1, Occludin, and Claudin-1. Total cell RNA was extracted by adding 1 mL of RNAio Plus to each well, followed by the addition of 200 μL of chloroform and centrifugation at 12,000 rpm, 4℃ for 10 min. The supernatant was slowly aspirated, and 500 μL of isopropanol was added and mixed, followed by centrifugation at 12,000 rpm at 4 °C for 10 min. Then, 1 mL of 75% ethanol was added to each tube, which was then centrifuged

at 12,000 rpm/min at 4 °C for 5 min. Finally, 20–30 μL of DEPC water was added for reverse transcription using a Takara kit (Cat. No. DRR047A) under the following reaction conditions: 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s, for a total of 40 cycles. The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method [20]. The primer sequences were shown in Table 1. The assays were repeated three times.

H&E staining

After being sterilized with alcohol wipes, mouse organs and intestines were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with H&E to observe the pathological changes in the duodenum, ileum, colon, liver, spleen, and lungs. The specific procedures were carried out following the previously described methods [21].

Statistical analyses

GraphPad Prism 8.0 software was used to perform statistical analysis of the experimental results and identify significant differences (one-way ANOVA). $P<0.05$ was considered a significant difference (*$P<0.05$; **$P<0.01$; *** $P<0.001$).

Results

MPX has good antibacterial activity against *E. coli*

The antibacterial activity of MPX against *E. coli* was evaluated using the double-layer agar diffusion assay (Fig. 1A). Results showed that MPX effectively inhibited the growth of *E. coli* at 1 mg/mL, and the size of the inhibition zone was comparable to that of Enro (1 mg/mL). The negative control exhibited no antibacterial activity. Furthermore, a modified microbroth dilution method was employed to determine the minimum inhibitory concentration (MIC) of MPX against *E. coli*, which was found to be 31.25 μg/mL (Fig. 1B). To test the bactericidal activity of MPX against *E. coli*, the OD_{600} value of the bacterial culture was measured, and it was observed that after 6 h of exposure to MPX, the OD_{600} of the bacterial culture decreased to 0.1, which was significantly lower than that of the control group (Fig. 1C, $p<0.01$). The bactericidal activity of MPX against *E. coli* was also evaluated using the plate counting method, and a dose-dependent increase in bactericidal efficacy against *E. coli* was observed (Fig. 1D).

Scanning electron microscopy was used to examine the effect of MPX on the morphology of *E. coli*. Results showed that the negative control *E. coli* had intact morphology, with large cells and smooth surfaces. However, cells treated with MPX (2 MIC) for 2 h exhibited significantly smaller size and leakage of bacterial contents (Fig. 1E, 12000x). These findings collectively demonstrate that MPX can effectively kill *E. coli* in vitro, indicating its potential as an antibacterial agent.

Table 1 The primers for this study

Genes	Sequence
Occludin (pig)	F:5′- GACAGACTACACAACTGGCGG-3′
	R:5′-TGTACTCCTGCAGGCCACTG-3′
Claudin-1 (pig)	F: 5′-CCATCGTCAGCACCGCACTG-3′
	R: 5′-CGACACGCAGGACATCCACAG-3′
ZO-1 (pig)	F: 5′-ATGAGCAGGTCCCGTCCCAAG-3′
	R: 5′-GGCGGAGGCAGCGGTTTG-3′
GAPDH (pig)	F: 5′-ACTCACTCTTCCACTTTTGATGCT-3′
	R: 5′-TGTTGCTGTAGCCAAATTCA-3
Reg3γ (mice)	F:5′-CCCGACACTGGGCTATGAAC-3′
	R:5′-GGTACCACAGTGATTGCCTGA-3′
Relmβ (mice)	F:5′ –CTGATAGTCCCAGGGAACGC-3′
	R:5′-GTCTGCCAGAAGACGTGACA-3′
TFF3 (mice)	F:5′-CCTGGTTGCTGGGTCCTCTG-3′
	R:5′-GCCACGGTTGTTACACTGCTC-3′
GAPDH (mice)	F:5′-GAGAAACCTGCCAAGTATGATGAC-3′
	R:5′-TAGCCGTATTCATTGTCATACCAG-3′

The antibacterial activity of MPX is relatively unaffected by changes in pH and temperature

To investigate the impact of pH and temperature on the activity of MPX, we used the antibacterial radius method to assess the antibacterial activity of MPX on *E. coli* under different pH values and temperatures. Our results (Fig. 2A) indicate that pH values within the range of 2–9 have no significant effect on the antibacterial activity of MPX. However, when pH exceeds 10, the antibacterial activity of MPX decreases. These findings suggest that MPX maintains stability in acidic and weakly alkaline environments. We also evaluated the impact of different temperature treatments on the antibacterial activity of MPX, with the highest temperature reaching 100 °C. Interestingly, we found that temperature did not affect the antibacterial activity of MPX, indicating that MPX has good thermal stability (Fig. 2B). Furthermore, we investigated the effect of different salt ions on the activity of MPX, after treatment with various concentrations of Na^+, K^+, Mg^{2+}, and Ca^{2+} cations. Our results (Fig. 2C-F) showed that the monovalent cations Na^+ and K^+ did not affect the antibacterial activity of MPX, while the divalent cations Mg^{2+} and Ca^{2+} had an impact on the activity of MPX. It is speculated that the presence of Ca^{2+} may cause changes in the secondary structure of MPX, leading to altered antibacterial activity. Overall, our findings suggest that while the antibacterial activity of MPX is not significantly influenced by temperature and monovalent cations, it is greatly affected by divalent cations.

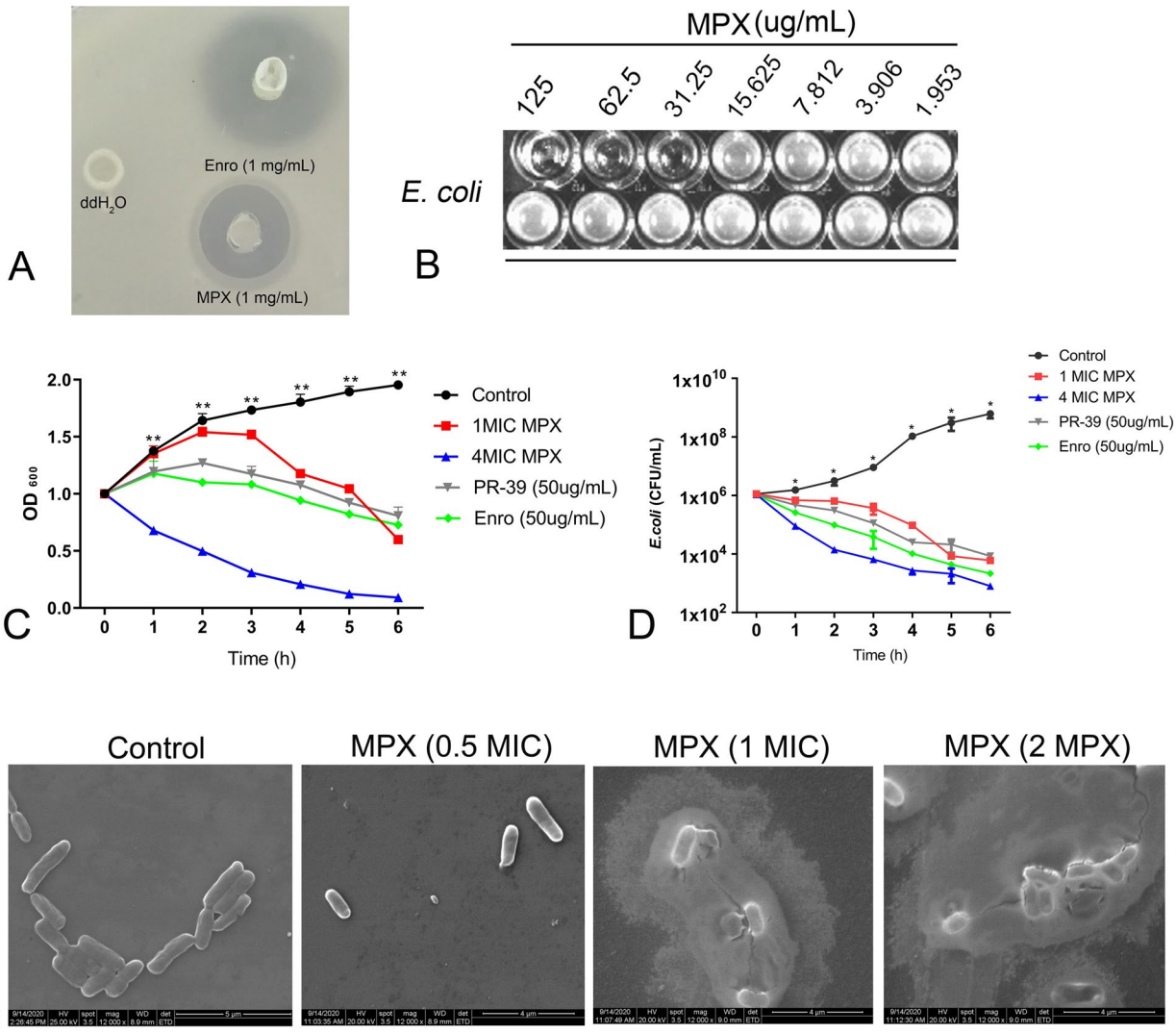


Fig. 1 Bactericidal activity detection of MPX in vitro. **A** Double-layer agar diffusion test results. **B** Minimum inhibitory concentration test results. **C** *E. coli* OD_{600} detection after treatment with MPX. **D** *E. coli* plate count detection after treatment with MPX. **E** The effect of MPX on *E. coli* was detected by scanning electron microscopy

MPX increases bacterial membrane permeability

In this study, we used BCA protein content determination and immunofluorescence observation to investigate the permeability of MPX on *E. coli* strains. Our results showed that after treatment with MPX for 3 h, the protein content in the supernatant of *E. coli* was significantly higher than that of the control group (Fig. 3A). Moreover, the protein content was significantly higher in the group treated with MPX at a concentration of 2 MIC compared to 1 MIC, indicating a positive correlation between the effect of MPX on *E. coli* and its concentration. To visualize the bacterial morphology, SYTO 9/PI was used in fluorescence microscopy. After treatment with MPX at a concentration of 2 MIC, the integrity of the cell membrane was compromised, leading to the entry of PI into bacterial cells and a significant increase in the number of dead bacteria (as shown in Fig. 3B). This effect was found to be significantly superior to that observed with MPX at a concentration of 1 MIC, and the results were consistent with the protein content determination using BCA assay.

To assess the permeability of MPX to the outer membrane of *E. coli*, an NPN uptake assay was performed. NPN is a neutral hydrophobic fluorescent probe that is typically excluded by the outer membrane, but its fluorescence intensity increases upon entry into the outer membrane. As shown in Fig. 3C, MPX exhibited a concentration-dependent penetration of the

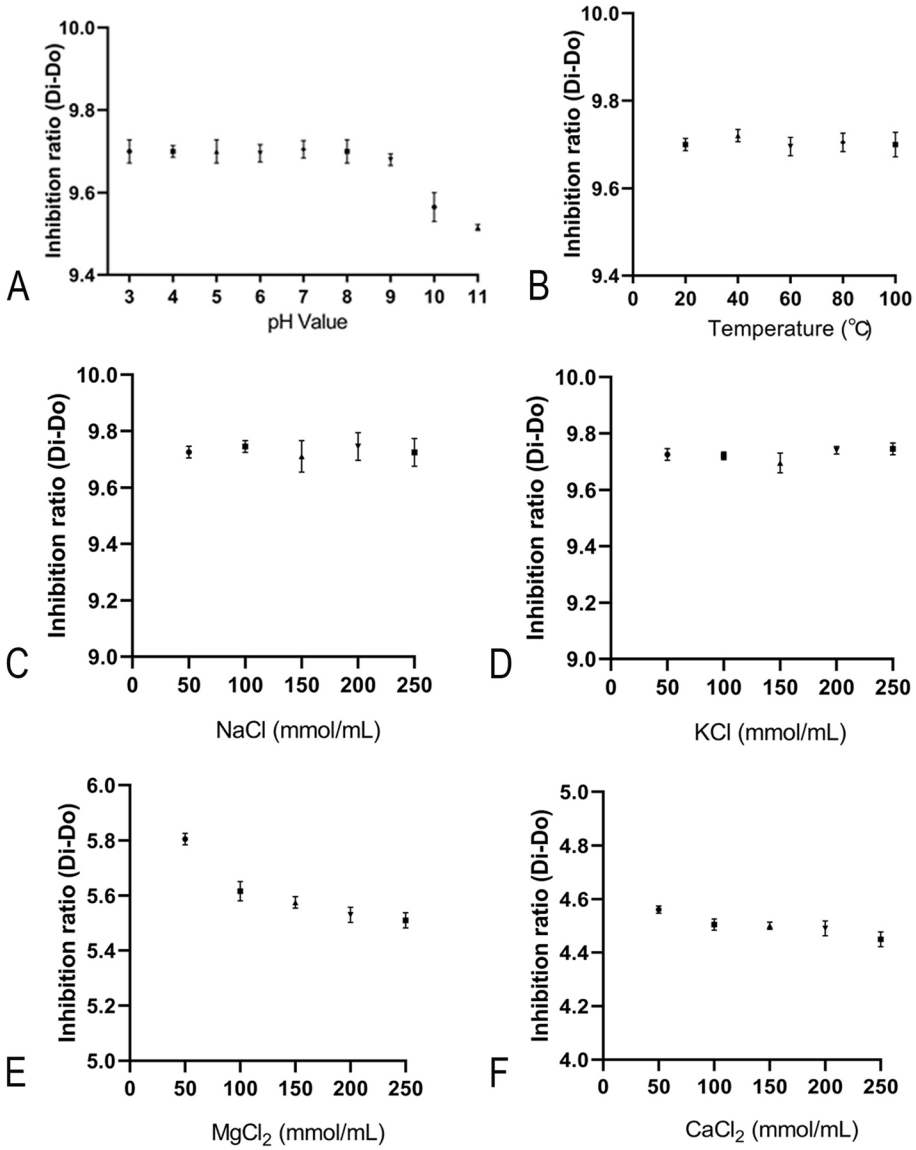


Fig. 2 The effects of pH, temperature and cations on MPX antibacterial activity. **A** The effect of pH on MPX. **B** The effect of temperature on MPX. **C** The effect of Na^+ cations on the activity of MPX. **D** The effect of K^+ cations on the activity of MPX. **E** The effect of Mg^{2+} cations on the activity of MPX. **F** The effect of Ca^{2+} cations on the activity of MPX

outer membrane of *E. coli*, which was evident from the increase in NPN fluorescence. Notably, even at a concentration of 1 MIC, MPX could penetrate the outer membrane of *E. coli* in a dose-dependent manner.

To assess the permeability of MPX to the inner membrane of *E. coli*, PI was utilized. PI has a strong affinity for bacterial DNA, and its fluorescence intensity increases upon entry into damaged bacterial cells. As observed in Fig. 3D, treatment of *E. coli* with MPX resulted in a significant increase in PI fluorescence intensity, which further increased in a concentration-dependent.

The impact of MPX on the depolarization of the *E. coli* cytoplasmic membrane was investigated using the membrane potential-sensitive dye 3,3'-dipropylthiadicarbocyanine iodide [DiSC3(5)]. Under normal membrane potential conditions, the DiSC3(5) dye is distributed both inside and outside the bacterial cell, resulting in a decrease in the initial fluorescence intensity of the dye due to its "self-quenching" within the bacterial cell. However, after exposure to drugs that alter the normal membrane potential, the release of the dye in the external medium increases, leading to an increase in fluorescence intensity. In the present study, following treatment with

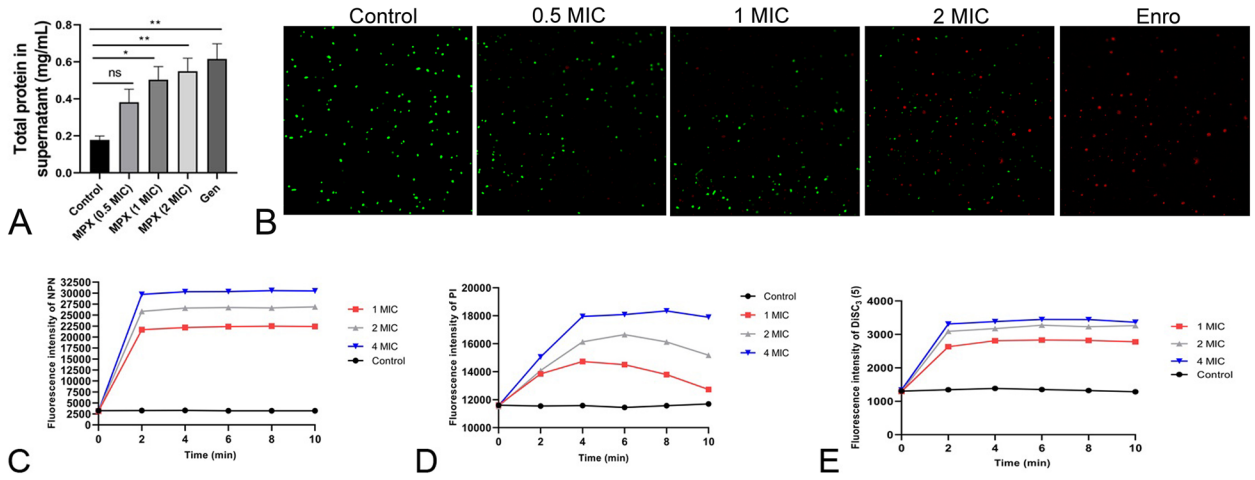


Fig. 3 Effect of MPX on the permeability of *E. coli*. **A** The results of total protein in *E. coli* bacterial supernatant after MPX treatment. **B** The effect of MPX on the permeability of *E. coli* was observed by immunofluorescence. **C** Detection of NPN fluorescence after MPX treatment with *E. coli*. **D** Detection PI of fluorescence after MPX treatment with *E. coli*. **E** Detection of DISC3(5) fluorescence after MPX treatment with *E. coli*

varying concentrations of MPX (1 MIC, 2 MIC, and 4 MIC), the fluorescence intensity of *E. coli* was found to increase with increasing concentrations of MPX (Fig. 3E). These results suggest that MPX exerts its bactericidal effect by increasing the permeability of bacteria.

MPX inhibits *E. coli* biofilm formation

The formation of biofilms by *E. coli* plays a crucial role in its virulence and drug resistance. Hence, this study initially employed the crystal violet method to investigate the impact of MPX on *E. coli* biofilm formation. The findings demonstrated that MPX (1 MIC) dose-dependently reduced the formation of *E. coli* biofilms compared to the control group, with MPX (4 MIC) having the most substantial effect (as shown in Fig. 4A). Additionally, after dissolving the crystal violet using 70% alcohol, the absorbance value of the *E. coli* biofilm was measured using an OD_{570} spectrophotometer. Compared to the control group, MPX significantly reduced the absorbance value of the *E. coli* biofilm (Fig. 4B).

Scanning electron microscopy was utilized to investigate the effect of MPX (1 MIC) on *E. coli* biofilms, which

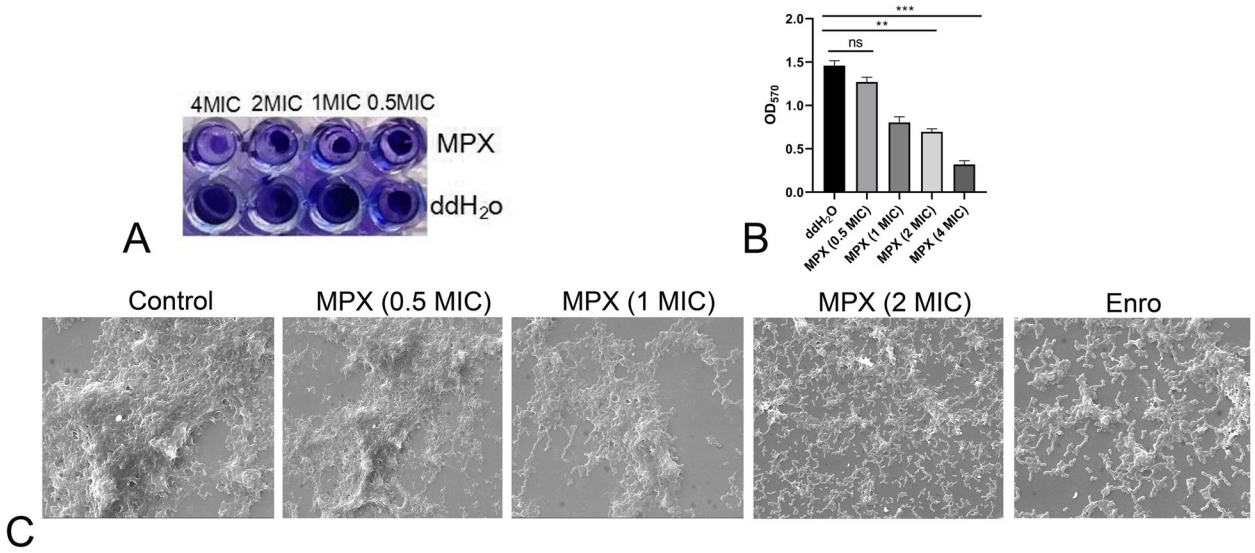


Fig. 4 The effect of MPX on the formation of *E. coli* biofilms. **A** The effect of MPX on *E. coli* biofilm by crystal violet staining. **B** OD_{570} detected the effect of MPX on *E. coli* biofilm. **C** The effect of MPX on the biofilm formation of *E. coli,* observed by scanning electron microscopy

revealed a loose structure, reduced bacterial adhesion, and an increase in the gaps between bacteria. In contrast, the control group formed dense biofilms with minimal gaps between bacteria (Fig. 4C). Collectively, these results suggest that MPX can substantially inhibit the formation of *E. coli* biofilms.

MPX inhibits *E. coli*-induced apoptosis, adhesion and regulates tight junction proteins

IPEC-J2 cells were infected with *E. coli* at a MOI of 10, and their morphology was observed using scanning electron microscopy (SEM), as shown in Fig. 5A. The results indicated that the surface of untreated IPEC-J2 cells was smooth and round, and the cell morphology was intact, despite some cytoskeletal swelling and collapse. In contrast, when infected with *E. coli*, IPEC-J2 cells showed signs of apoptosis, such as cell swelling, apoptotic vesicles, and cytoskeletal collapse. Treatment with MPX did not cause any collapse or apoptotic vesicles in the IPEC-J2 cells. Next, the effect of MPX on the adhesion and invasion of IPEC-J2 cells was tested by adding a Rac1 inhibitor. The results, shown in Fig. 5B and C, revealed that *E. coli* adhesion and invasion were significantly reduced in the MPX-treated group compared to the IPEC-J2+*E. coli* group. However, pre-treatment with NSC 23766, a Rac1 inhibitor, abolished the protective effect of MPX on IPEC-J2 cells, suggesting that Rac1 is involved in the cell barrier function of IPEC-J2 cells, and that MPX enhances this function through Rac1. Moreover, the regulatory mechanism of MPX on tight junction proteins was studied using the Rac1 inhibitor NSC23766 (Fig. 5C, D, E). The results showed that MPX

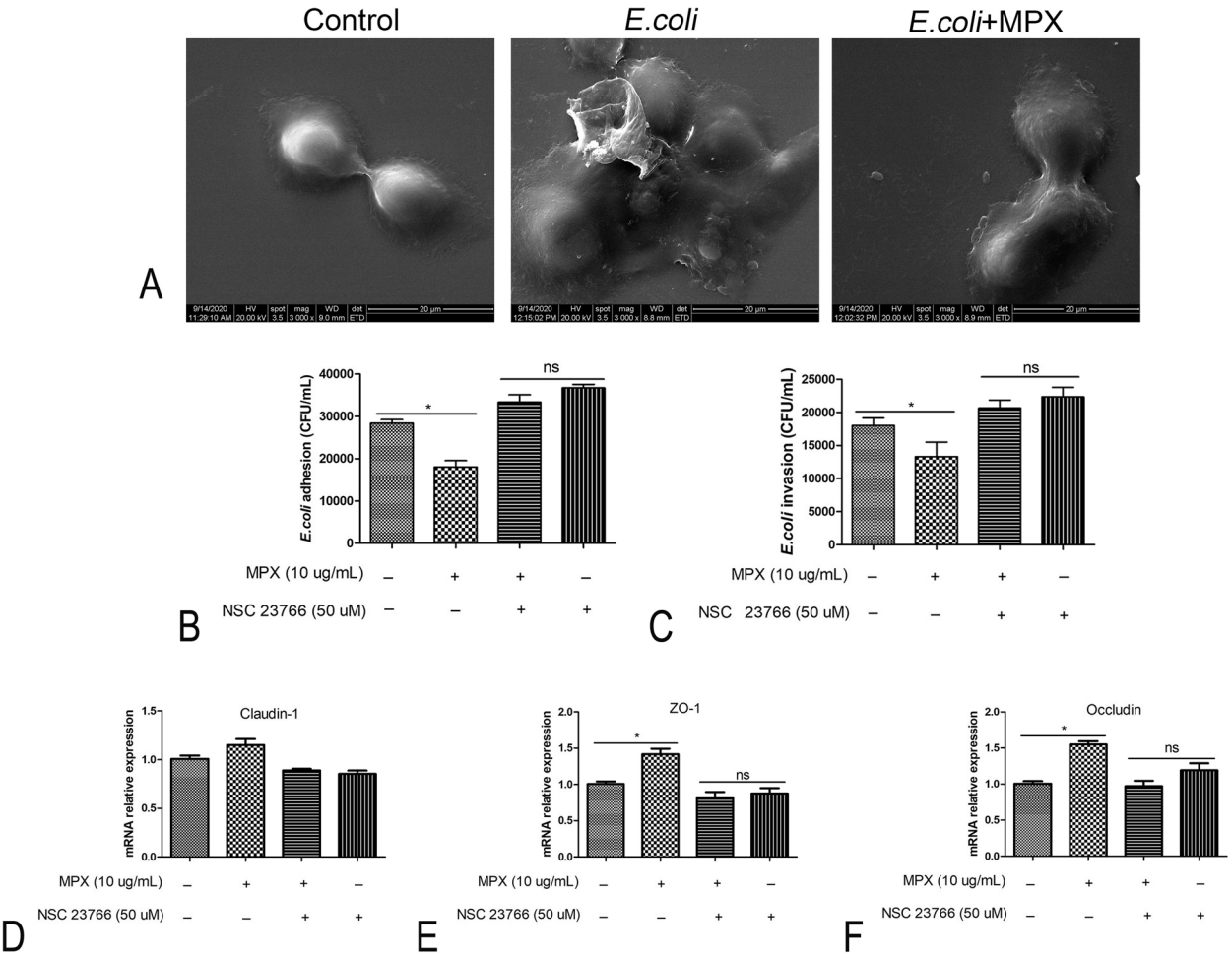


Fig. 5 The effect of MPX on invasion and barrier function of IPEC-J2 cells after *E. coli* infection. **A** The results of IPEC-J2 cells infected with *E. coli* observed by scanning electron microscopy. **B** The results of *E. coli* adhesion in IPEC-J2 cells. **C** The results of *E. coli* invasion in IPEC-J2 cells. **D** The mRNA expression of Claudin-1 after *E. coli* infection. **E** The mRNA expression of ZO-1 after *E. coli* infection. **F** The mRNA expression of Occludin after *E. coli* infection

pretreatment increased the mRNA expression of ZO-1 and Occludin in IPEC-J2 cells, while the mRNA expression of Claudin-1 was not significantly affected. However, the effect of MPX on ZO-1 and Occludin was inhibited after adding the inhibitor NSC23766. These findings suggest that MPX may regulate the tight junction proteins of intestinal epithelial cells and enhance barrier function through the Rac1 pathway.

MPX relieves the pathological damage to the intestines

H&E staining was conducted to observe the pathological changes in the duodenum and ileum after E. coli infection. As illustrated in Fig. 6A, B, mice infected with *E. coli* exhibited shedding of intestinal villi, as well as catarrhal enteritis, degeneration, necrosis, shedding of intestinal mucosal epithelial cells, and congestion of the lamina propria, with a large amount of neutrophil infiltration. These changes indicated the presence of necrotizing enteritis and fibrinous necrotizing enteritis (Fig. 6A, B). However, treatment with MPX significantly alleviated the pathological changes in each bowel segment. The intestinal villi of the control group were arranged neatly without any pathological changes mentioned above. Additionally, the liver, spleen, and lung of *E. coli*-infected mice showed pathological changes after MPX treatment, as shown in Supplementary Fig. 1A, B, C. The mRNA expression of intestinal antibacterial-related proteins REG3γ, Remlβ, and TFF3 was analysed using qRT–PCR. In the jejunum, compared with the control group, the TFF3 gene expression level was increased in the *E. coli* group ($P<0.05$), while the TFF3 gene expression level in the jejunum of the *E. coli* + MPX group was significantly lower than that in the jejunum of the *E. coli* group ($P<0.05$), with no significant difference from the control group. The mRNA expression level of Remlβ in the jejunum tissue of *E. coli*-infected mice was significantly increased ($P<0.001$) compared with the control group. MPX significantly reduced the mRNA expression level of Remlβ, which was equivalent to the effect of Enro, while the expression level of Remlβ in the mouse colon was not significantly different in the other groups. Furthermore, *E. coli* infection increased the expression of REG3γ in the mouse jejunum and colon, and MPX significantly reduced REG3γ mRNA expression caused by *E. coli* infection (Fig. 6C, D, E).

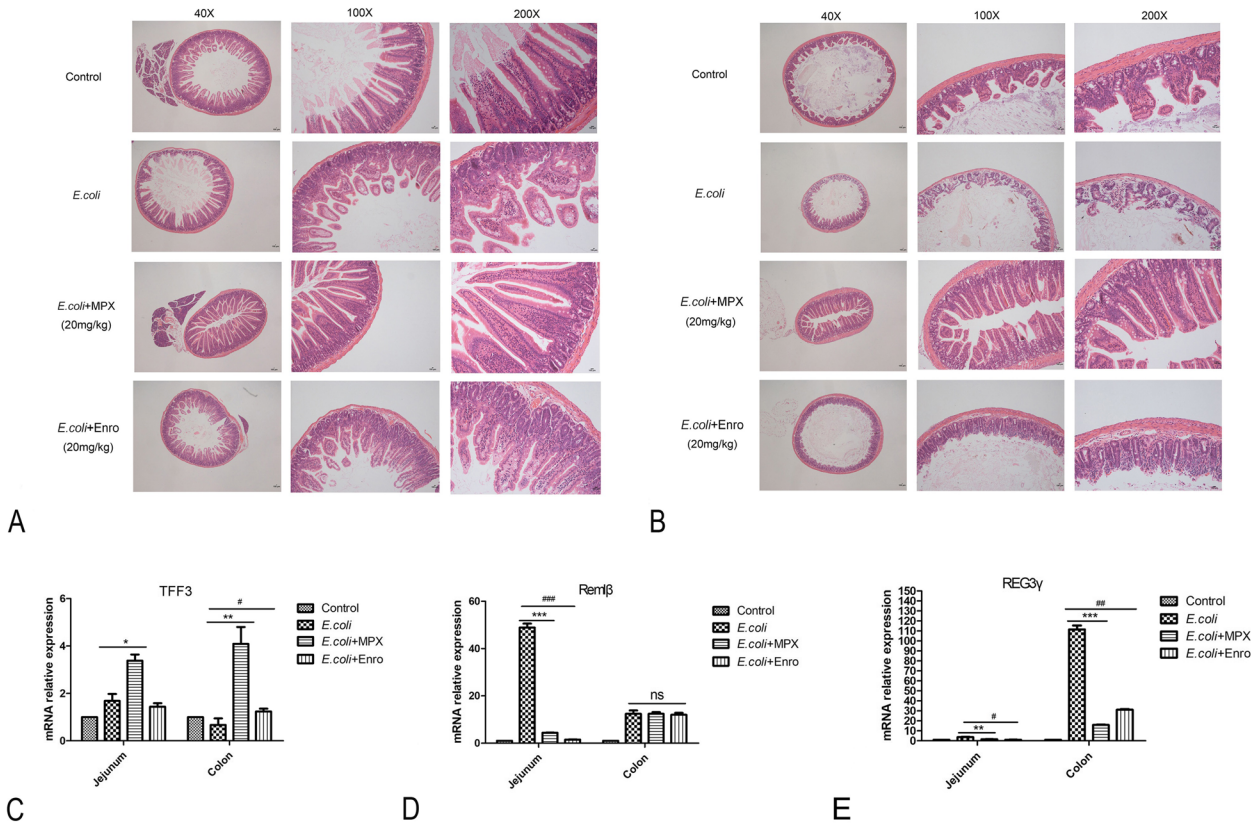


Fig. 6 The effects of MPX on intestinal pathology and the mRNA expression of antimicrobial proteins. **A** H&E staining of the duodenum after *E. coli* infection in mice. **B** H&E staining of the ileum after *E. coli* infection in mice. **C** The mRNA expression of TFF3 in the mouse jejunum and colon. **D** The mRNA expression of Remlβ in the mouse jejunum and colon. **E** The mRNA expression of REG3γ in the mouse jejunum and colon

These results indicate that MPX can effectively alleviate intestinal pathological damage caused by *E. coli* infection and regulate the expression of antimicrobial proteins in the jejunum and colon.

Discussion

In recent years, the excessive 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, tested for drug sensitivity, and found that the resistance of E. coli was 16.7% [22]. Fayemi OE et al. tested 180 samples of fresh beef and meat products and detected that 61 samples contained different serotypes of *E. coli*. Resistance analysis of the isolated *E. coli* O157:H7 showed 23.6% resistance of the STEC serotype [23]. Sarjana Safain K et al. determined the spectrum of AMR and associated genes encoding resistance to the aminoglycoside, macrolide and β-lactam classes of antimicrobials in bacteria isolated from hospitalized patients in Bangladesh and found that 53% of isolates were multidrug-resistant (MDR), including 97% of *E. coli* [24]. Shin H et al. isolated the high-level carbapenem-resistant 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 wastewater treatment plant located on Han River, Seoul, South Korea, which harbours the gene, showed a high level of carbapenem resistance todoripenem (512 mg/L) and meropenem (256 mg/L) and XDR to 15 antibiotics [25].

The formation of bacterial biofilms leads to increased bacterial resistance. Morroni G et al. found that the antimicrobial peptide LL-37 has good antibacterial activity against multidrug-resistant *E. coli*, and MIC and sub-MIC concentrations of LL-37 were able to reduce *E. coli* biofilm formation [26]. Vergis J et al. found that the antimicrobial peptide lactoferricin (17–30) has good antibacterial and antibiofilm activity against multidrug-resistant enteroaggregative *E. coli*, and lactoferricin (17–30) significantly reduced the formation of *E. coli* biofilms [27]. Mishra BE et al. found that the antimicrobial peptide WW298 could effectively inhibit MRSA attachment and disrupt its preformed biofilms more effectively than daptomycin [28]. Liu Ye et al. found that a tryptophan-rich amphiphilic peptide termed WRK-12 significantly inhibited the formation of biofilms in a dose-dependent manner, especially in multidrug-resistant (MDR) bacteria, particularly gram-negative bacteria [29].

The intestinal barrier is composed of four parts: the intestinal epithelial barrier, immune barrier, chemical barrier and biological barrier. The intestinal epithelial barrier is the first barrier in animals to resist the invasion of pathogens. It is particularly important in protecting the body from pathogenic bacteria and other microorganisms.

The tight junction structure is the most critical part of the intestinal epithelial barrier [30]. ZO-1, Occludin and Claudin-1 are important proteins in the tight junction structure [31]. Yi et al. found that the antimicrobial peptide CWA increased the expression of the tight junction proteins ZO-1 and Occludin in the intestinal tract of weaned piglets with diarrhoea and enhanced intestinal barrier function [32]. LL-37 also increases the expression of the tight junction proteins Claudins and Occludin, increases cell transmembrane resistance (TER), and enhances cell barrier function [33]. Han et al. found that defensin pBD2 could increase the gene expression of ZO-1 and Occludin in the mouse colon and relieve the colon inflammation induced by DSS in mice [34]. Lin et al. found that *E. coli* K88 led to a decrease in the expression of the tight junction protein ZO-1 in IPEC-J2 cells, and the antimicrobial peptide porcine NK-Lysine (PNKL) significantly increased the expression of the tight junction protein ZO-1 [35].

The intestine is the largest digestion and absorption organ of animals and the most important immune organ of the body. Zhang et al. found that adding the antimicrobial peptide plectasin to chicken diets could improve chicken performance, immune function and intestinal health and increase the length of intestinal villi [36]. Roque-Borda CA et al. found that the antimicrobial peptide Ctx(Ile)-Ha could effectively alleviate intestinal pathological damage [37]. Shang et al. found that the antimicrobial peptide Microcin J25 could alleviate DSS-induced intestinal inflammation and improve intestinal morphology [38]. Xiong et al. found that oral antimicrobial peptide-defensin-1 (DEFB1) could improve intestinal function and enhance intestinal barrier function [39].

The results of the study indicate that *E. coli* has a high resistance to antibiotics, highlighting the urgent need to find alternatives. MPX was found to have good antibacterial activity against *E. coli*, with no observed drug resistance, making it a potential alternative treatment. MPX was also found to significantly inhibit the formation of *E. coli* biofilms, which could pave the way for the development of drugs that hinder biofilm formation. Furthermore, MPX was found to increase the expression of intestinal tight junction proteins, enhancing the intestinal epithelial barrier, and effectively reducing intestinal damage caused by *E. coli* infection in mice. Overall, the findings suggest that MPX has great potential as an alternative to antibiotics for the treatment of *E. coli* infections.

Conclusion

In summary, MPX demonstrated potent antibacterial activity against *E. coli* in vitro by disrupting the bacterial cell membrane, leading to the release of bacterial contents. Moreover, MPX mitigated *E. coli*-induced apoptosis in IPEC-J2 cells, modulated the expression of tight junction proteins at the mRNA level via the Rac1 pathway, ameliorated intestinal pathological damage, and enhanced the mRNA expression of antimicrobial proteins in response to *E. coli* infection.

Abbreviations

MPX	Antimicrobial peptide Mastoparan X
E. coli	Enterohemorrhagic Escherichia coli O157:H7 ATCC43889
SEM	Scanning electron microscopy
H&E	Hematoxylin and eosin

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41231-023-00145-9.

Additional file 1: S Fig. 1. The effects of MPX on intestinal pathology. **A**: H&E staining of the lung after *E. coli* infection in mice. **B**: H&E staining of the liver after *E. coli* infection in mice. **C**: H&E staining of the spleen after *E. coli* infection in mice.

Authors' contributions

XZ performed the experiments, analyzed, interpreted the data and write the manuscript. LW and JH conceived the idea for this study. CZ, XX and YW involved in the conception and design of the study. YB, XX and XW involved in the drafting and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

All data used during the study are available from the corresponding author by request.

Declarations

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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