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## The Effect of Oral Administration of the Antibacterial Peptide MPX on Intestinal Inflammation of Mice in Experimental Infection with *Escherichia Coli* Strain O157: H7

Xueqin Zhao<sup>1,2</sup>, Hanna Fotina<sup>2</sup>, Tetiana Fotina<sup>2</sup>, Jianhe Hu<sup>1</sup>, Lei Wang<sup>1</sup>

<sup>1</sup>Henan Institute of Science and Technology  
453003, Eastern HuaLan Ave., Xinxiang, China

<sup>2</sup>Sumy National Agrarian University  
40000, 160 Herasym Kondratiev Str., Sumy, Ukraine

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**Abstract.** *Escherichia coli* is a gram-negative bacterium, an intestinal pathogen that can cause intestinal inflammation. Antimicrobial peptides are a class of small molecule peptides, which has good antibacterial activity against a variety of gram-positive and negative bacteria. In this regard, the authors aimed to study the effect of the antimicrobial peptide MPX, which was administered orally, on the intestinal wall of mice infected with the intestinal barrier function, which were infected with *E. coli*. Synthesis and purification of the antimicrobial peptide MPX (H-INWKGIAAMAKKLL-NH<sub>2</sub>) was performed by Jier Sheng Hua (Shanghai, China). Mass spectrometry and liquid chromatography (HPLC) were used for this purpose. *Escherichia Coli* (O157: H7 ATCC 43889) was isolated from human faeces in which haemolytic uremic syndrome was reported. Solid LB agar (Solarbio, China) was used to isolate enterohemorrhagic *E. coli*. The culture was obtained from the Chinese Institute of Veterinary Drug Control (Beijing, China). The results of necropsy found that using of an orally administered MPX could alleviate the damage of *E. coli* to the liver, spleen, and the lungs were less affected. According to H&E results in case of an orally administered MPX group considerably relieved duodenum and organs on day 7 and day 28. qRT-PCR results showed orally administered MPX could reduce the inflammation-related factors in the mRNA expression of IL-2 and IL-6 and TNF- $\alpha$  on day 7 and day 28. In addition, orally administered MPX could significantly increase them RNA expression of tight junction proteins Occludin and Zo-1 on day 7 and day 28. The results of immune histochemistry further showed that an orally administered MPX could increase the mRNA expression of MUC2 in jejunum. The above results showed that orally administered MPX could alleviate the attack of *E. coli* on the intestinal tract of mice, relieve intestinal inflammation, and improve the intestinal barrier function. This study lays a theoretical foundation for adding antimicrobial peptides to food. In orally administered MPX authors can see reducing the mRNA expression of inflammation-related factors, thereby alleviating the intestinal inflammation caused by *E. coli* infection in mice. Authors can add that orally administered MPX shows an increase in mRNA expression of tight junction protein in intestines and improves the intestinal barrier function. This study lays the foundation for adding antimicrobial peptides to food to relieve inflammation and improve barrier function in clinical practices

**Keywords:** antimicrobial peptide MPX, enterohemorrhagic *E. coli*, inflammatory process, intestinal barrier, white mice



## INTRODUCTION

*Escherichia coli* is a bacterium whose colonies are registered in the lower intestine of mammals and birds. Under certain conditions, they are pathogenic and cause disease (Tran *et al.*, 2021). Some bacteria can move with the help of flagella. On the surface of the cells there are fimbriae, saws. There are stationary strains. Disputes do not form (Nunayon *et al.*, 2022). *Escherichia coli* is heat-resistant and can survive in the wild for several months (Lim *et al.*, 2021). More than a hundred pathogenic serotypes of *Escherichia coli* that cause diseases in humans, animals, including birds have been systematised. *Escherichia coli* is resistant to antibiotics (Li *et al.*, 2022). The emergence of antibiotic-resistant strains of *Escherichia coli* has caused economic losses to livestock (Khawaskar *et al.*, 2021). At present, the resistance of *E. coli* to antibacterial drugs has become an increasingly serious global problem. Therefore, it is urgent to find drugs that can fight against *E. coli* infection and are not easy to develop drug resistance. Antimicrobial peptides have become a research hotspot and considered to be one of the most potential antibiotic substitutes.

Antimicrobial peptides are molecules made up of amino acids produced by the body, they are an important part of the body's immune system (Vimberg *et al.*, 2022). Peptides have low molecular weight, broad antibacterial spectrum, and endurance to resistance (Lin *et al.*, 2021). Peptide molecules consist of 12 to 100 amino acids (Sudaech *et al.*, 2021). Both gram-positive (G+) and gram-negative (G-) bacteria have an inhibitory effect, most antimicrobial peptides have bactericidal activity, and their mode of action is specific to cellular targets (Peng *et al.*, 2019). With the expansion of research, people gradually discovered antimicrobial peptide have a wide range of effects and have attracted much attention in anti-inflammatory and intestinal barrier function. The earlier study has found that intraperitoneal injection MPX could alleviate the damage of *E. coli* to the intestines of mice, reduce the mRNA expression of inflammation-related factors and improve the intestinal barrier function of mice (Zhao *et al.*, 2021). However, whether orally administered MPX can also relieve intestinal inflammation and improve intestinal barrier function in mice infected with *E. coli* is still unknown.

The purpose of this study was to explore the effect of orally administered MPX on intestinal inflammation and intestinal barrier function in mice infected with *E. coli*.

## MATERIAL AND METHODS

**Ethics statement.** BALB/c mice (6 to 8 weeks old, female) were bought from Zhengzhou University. Animal studies were conducted per the principles of humane treatment of animals according to the International Guidelines for Biotic Standards and the requirements of the International Science Committee and the Animal Ethics Committee of the Henan Institute of Science and Technology (June 1, 2021).

**Antimicrobial peptide synthesis.** MPX (H-INWK-GIAAMAKLL-NH<sub>2</sub>) was synthesised and purified by Jiershenghua (Shanghai, China). High-performance liquid

chromatography (HPLC) and mass spectrometry were used to purify MPX. The purity of MPX was greater than 98%. MPX was dissolved in dd H<sub>2</sub>O and stored at -20°C.

***E. coli* strain culture.** *Escherichia coli* (enterohemorrhagic *Escherichia coli* O157:H7ATCC43889) were isolated from faeces of patients who with symptoms of diarrhoea and intestinal inflammation and obtained from the China Institute of Veterinary Drug Control (Beijing, China). LB (Solarbio, China) solid agar was used to obtain isolated pure *E. coli* colonies. Single *E. coli* colonies were seeded in 5 mL LB liquid medium with inoculation rings in an aseptic operation room. Next, the cultures were placed on a shake (Thermo, USA) at 180 rpm for 10 h at 37°C. Then, 1 mL of *E. coli*-containing liquid was replaced in a 1.5 mL centrifuge tube, then centrifuged at 8000 rpm for 5 min, and then resuspended in phosphate buffer (pH=7.4). After diluting, the bacteria were smeared on an LB solid plate for colony counting. Then, the LB plates were placed in a 37°C incubator for 12 h and counted after the growth of visible single bacterial colonies. Authors first set up three *E. coli* gradients of 4.5×10<sup>8</sup> CFU/mice; 4.5×10<sup>7</sup> CFU/mice and 4.5×10<sup>6</sup> CFU/mice for preliminary experiments. According to the results, authors ultimately found that the dose of 4.5×10<sup>6</sup> CFU/mice of *E. coli* infection. Finally, the bacteria were administered to mice at 4.5×10<sup>6</sup> CFU/mice in the experiment.

**Animal experiment.** A total of 48 BALB/c mice (aged 6-8 weeks, 18-20 g, female) were randomly divided into 4 experimental groups (control, MPX, *E. coli*, and MPX+*E. coli*; 12 mice per group, 6 mice at separate times). The mice had ad libitum access to food and water. The MPX group and MPX+*E. coli* group mice orally administered MPX at a concentration of 100 µg/mL (200 µL/mouse) every day for 7 consecutive days. The control group was intragastrically administered 200 µL of normal saline for 7 days. Authors challenged mice with *E. coli* at 24 h after an orally administered MPX. The *E. coli* and MPX+*E. coli* groups were processed with *E. coli* via oral administration at 4.5×10<sup>6</sup> CFU/mouse. Mice showed diarrhoea, decreased appetite, and were prone to gathering after *E. coli* infection.

**Necropsy observation.** Mice were euthanised via CO<sub>2</sub> inhalation at different time points. After the mice were killed by anaesthesia, the liver, lungs, and spleens of mice were collected aseptically with forceps, and pathological observations were performed. Aseptic operation was performed to observe the pathological changes of the liver, spleen and lungs of the mice after *E. coli* infection. The main pathological changes after *E. coli* infection in mice.

**H&E staining observation.** Autopsy was performed on dead mice after infection to observe the pathological changes of mice organs. The lung, liver, spleen, and intestines of mice were taken out with scissors, wipe them clean with alcohol cotton, and put them in a 15 mL centrifuge tube. Selected organs and intestines were fixed in 4% paraformaldehyde, then paraffin-embedded, sections were made, H&E was stained, and pathological changes were determined (Su *et al.*, 2021).

**qRT-PCR.** 0.1 g of jejunum tissue was weighed

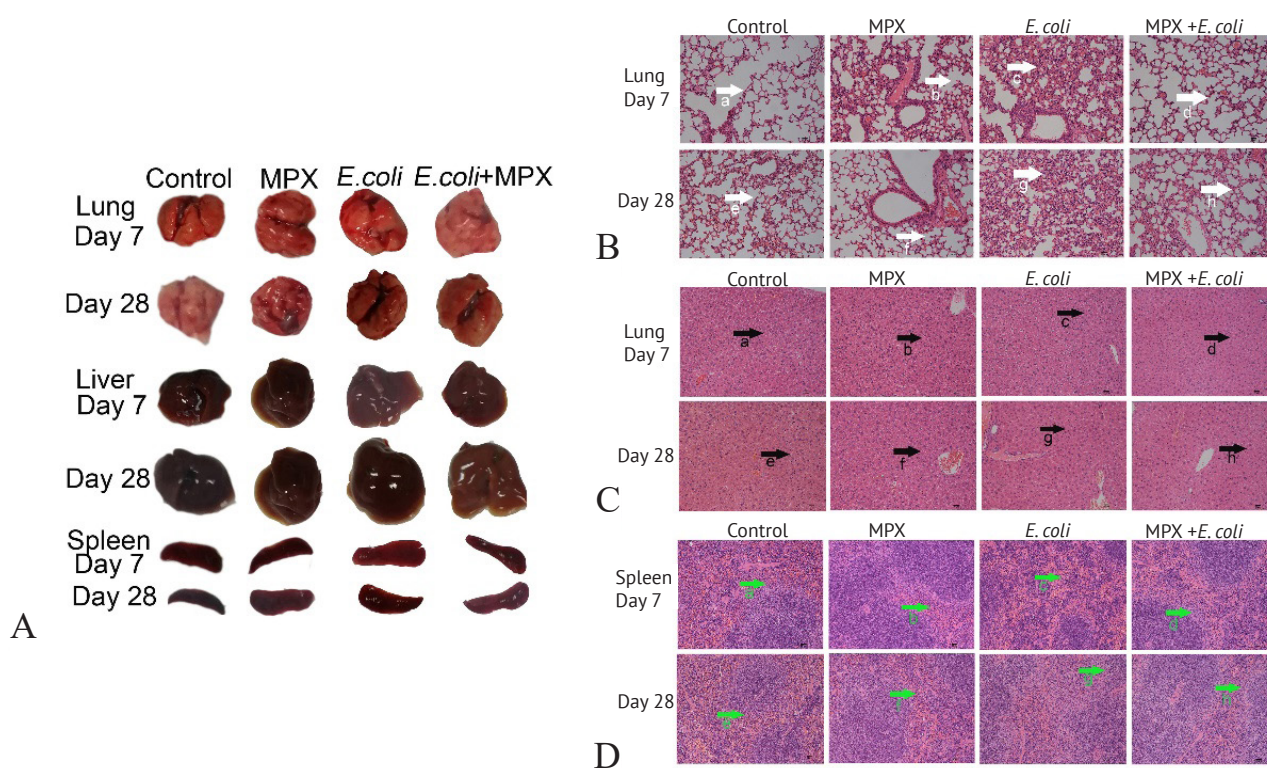
and placed in liquid nitrogen for grinding. The ground jejunum powder was placed in a 1.5 mL centrifuge tube. 1 mL RNA was added to each well for 10 min, 200  $\mu$ L of chloroform was added to each well. Shaked on the instrument for 15 s, placed it on ice for 2~3 min, centrifuged at 12000 rpm, 4°C for 10 min, slowly aspirated the supernatant, added 500  $\mu$ L of isopropanol and mixed well, centrifuged at 12000 rpm, 4°C for 10 min, discarded the supernatant, added 1 mL of 75 % ethanol to each tube, centrifuged at 12000 rpm, 4°C for 5 min, slowly discarded the supernatant, and allowed the RNA to dry naturally for 10 min. Added a proper amount of DEPC water, generally 20~30  $\mu$ L, and the RNA concentration was determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, United States). 2  $\mu$ g of total RNA was converted into cDNA. A reverse transcription kit (Thermo Scientific, USA) was used for this purpose. The reaction volume was 10  $\mu$ L and included 5  $\mu$ L of SYBR Green Master Mix (QuantiNova, China), 0.5  $\mu$ L of reverse primer (10  $\mu$ M), 0.5  $\mu$ L of direct primer (10  $\mu$ M), 0.5  $\mu$ L of cDNA and 3, 5  $\mu$ L of ddH<sub>2</sub>O. The reaction of the thermocycler took place for 2 min at 95°C and included 40 cycles at 95°C for 20 s and at 60°C for 30 s, considering the melt curves by fluorescence quantitative instrument (ABI7900, America). The primer sequences of IL-2, IL-6, TNF- $\alpha$ , ZO-1, Claudin-1, Occludin as bellow: IL-2, F:5'-CCTGAG-CAGGATGGAGAATTACA-3', R:5'-TCCAGAACATGCCGCAGAG-3'; IL-6, F:5'-CTCTGGCGGAGCTATTGAGA-3', R:5'-AAGTCTCTCGTGGAGAAA-3'; TNF- $\alpha$ ,

F:5'-CTCATGCACCACCATCAAGG-3', R:5'-ACCTGACCACTCTCCCTTTG-3'; ZO-1, F:5'-ACCCGAAACTGATGCTGTGGATAG-3', R:5'-AAATGGCCGGGCAGAACTTGTGTA-3'; Claudin-1, F:5'-AGCTGCCTGTTCCATGTACT-3', R:5'-CTCCATTTGTCTGCTGCTC-3'; Occludin, F:5'-ACGGACCCTGACCACTATGA-3', R:5'-TCAGCAGCAGCCATGTACTC-3'.

## RESULTS

### MPX relieves the pathological damage of *E. coli* to the organs of mice

The results of necropsy found that *E. coli* infection caused pathological changes in the liver and spleen of the mice, such as congestion and necrosis, and the pathological changes on day 7 were more serious than that on day 28 (Fig. 1A). There was no obvious pathological change in the lungs. Compared to *E. coli* group, the liver, lungs, and spleen of the negative control mice did not show the above adverse symptoms and orally administered MPX can effectively alleviate the above symptoms. Furthermore, H&E results found that the liver, spleen, and lungs of the mice in the control group had no lesions (Fig. 1B, C, D). *E. coli* infection leads to necrosis in the liver, caseous necrosis in the spleen, and insignificant pathological changes in the lungs. Orally administered MPX could relieve liver and spleen damage. The above results showed that orally administered MPX can effectively alleviate the pathological damage of *E. coli* to the organs of mice.

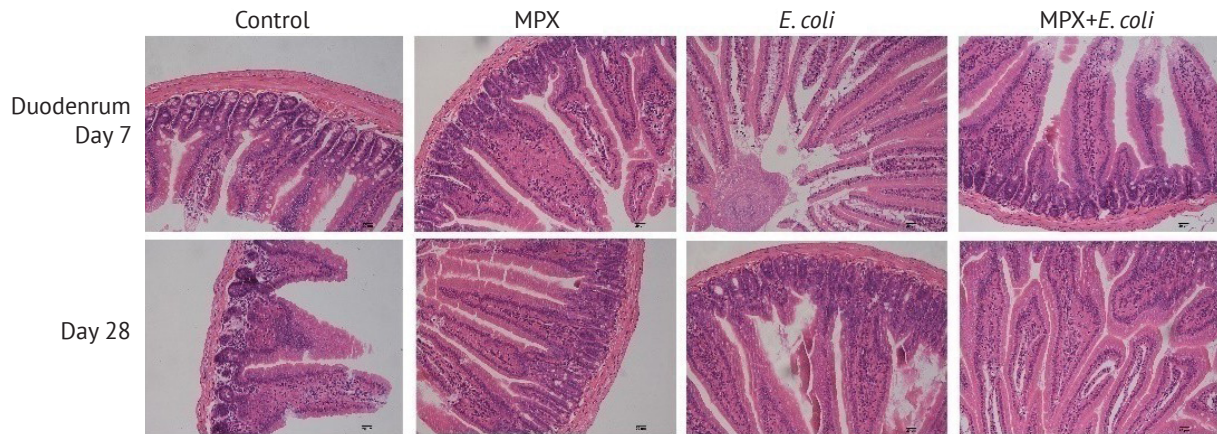


**Figure 1.** The results of necropsy and H&E staining of mice infected with *E. coli* after orally administered MPX. A: Observation of autopsy results of liver, lung, and spleen of mice; B-D: Observation the results of H&E staining of liver, lung, and spleen of mice. Lung, liver, and spleen are highlighted respectively by white arrows, black arrows and green arrows to indicate lesions or normal parts. In addition, separate groups are indicated by different lowercase letters.

### MPX can effectively alleviate the intestinal morphology

As shown in Fig. 2, H&E staining of the duodenum showed that the control group intestinal villi were arranged neatly and there was no villi loss. *E. coli* infection in mice causes damage to the structure of the duodenum, the intestinal villi fall off, the arrangement is uneven, the

depth of the intestinal crypts increases, and the intestinal villi become shorter. The orally administered MPX group significantly relieved the above symptoms on day 7 and day 28. The above results show that orally administered MPX can effectively alleviate the intestinal damage caused by *E. coli* infection.

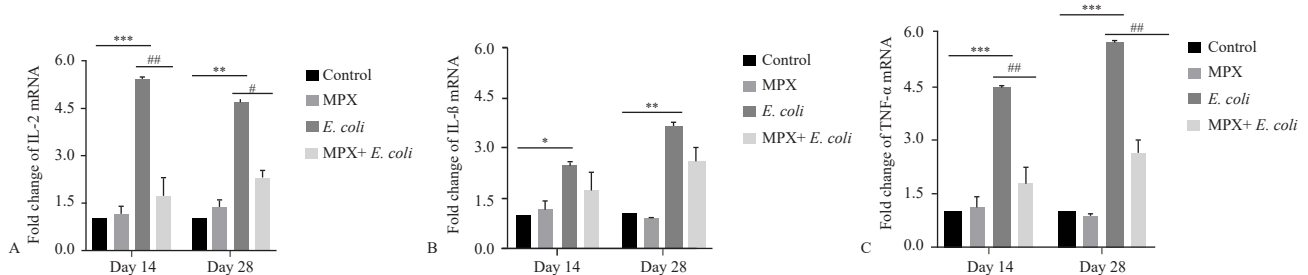


**Figure 2.** Observation of the H&E staining pathological results in duodenum of *E. coli* infected mice after orally administered MPX

### Orally administered MPX reduces the expression of inflammation-related factors in duodenum

To study the effect of an orally administered MPX on duodenum inflammation-related factors in mice, RT-qPCR was used for detection of the effect of MPX on the mRNA expression of IL-2, IL-6, and TNF- $\alpha$  in duodenum (Fig. 3A, B, C). The results showed that the mRNA levels of

inflammation-related factors IL-2, IL-6, and TNF- $\alpha$  increased significantly on day 7 and day 28 after *E. coli* infection. Orally administered MPX can inhibit the mRNA levels of inflammation-related factors in the jejunum infected with *E. coli*. This result shows that orally administered MPX can reduce the mRNA expression of inflammation-related factors and relieve intestinal inflammation.

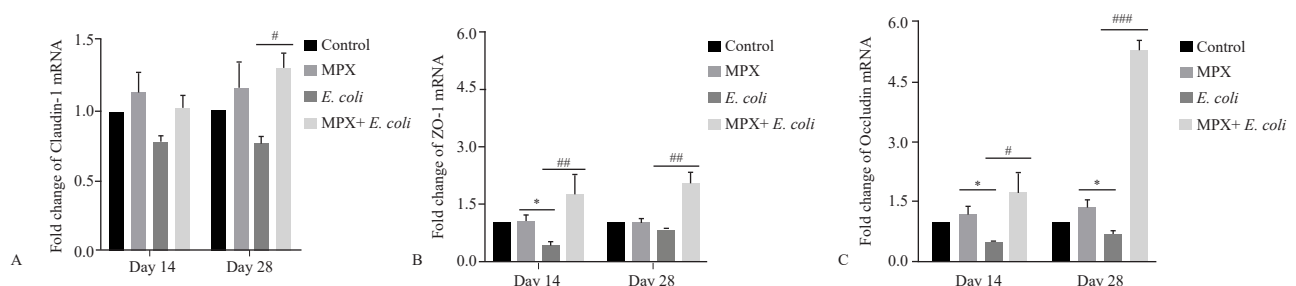


**Figure 3.** The effect of an orally administered MPX on the mRNA expression of inflammatory factor in the duodenum infected with *E. coli*

### Orally administered MPX improves intestinal barrier function

To study the effect of orally administered MPX on the intestinal barrier function of mice. The mRNA expression of tight junction proteins Occludin, Claudin-1, ZO-1 were detected by RT-qPCR (Fig. 4A, B, C). Compared to

control group, *E. coli* infection resulted in decreased mRNA expression of tight junction protein in the duodenum of mice. While orally administered MPX could significantly increase the mRNA level of intestinal tight junction proteins, thereby improving the intestinal barrier function of mice.

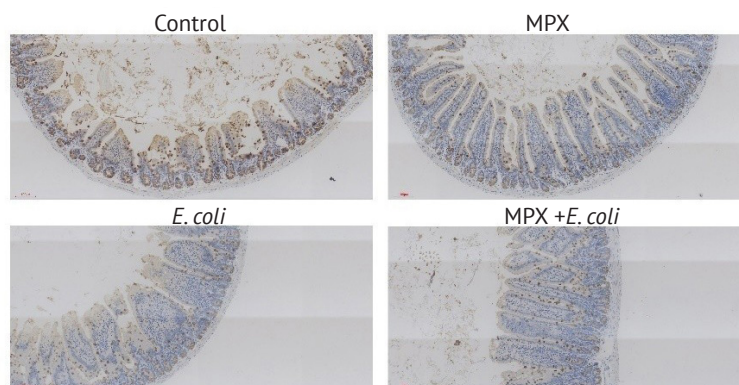


**Figure 4.** The effect of orally administered MPX on the mRNA expression of tight junction protein of mice infected with *E. coli*

### Orally administered MPX increases MUC2 protein in mouse jejunum

In case of study the effect of an orally administered MPX on MUC2 in jejunum, authors have used immunohistochemistry for detection of the effect of an orally administered MPX on the intestinal barrier function of mice. As shown in Figure 5, compared to control group, the

mRNA expression of MUC2 decreased in the jejunum after *E. coli* infection, and orally administered MPX can effectively alleviate the decreased MUC2 mRNA expression caused by *E. coli* infection. Authors' results showed that via an orally administered MPX can considerably increase MUC2 mRNA expression caused by *E. coli* infection in mice.



**Figure 5.** Immunohistochemical detection of MUC2 protein level in jejunum of mice

## DISCUSSION

This study found that after infecting mice with *E. coli*, it caused organ damage, and orally administered MPX could effectively alleviate the above symptoms. *E. coli* infection in mice leads to increased expression of inflammation-related factors, and orally administered MPX could effectively reduce jejunum inflammation-related factors. In addition, *E. coli*-infected mice disrupted the mouse's intestinal barrier function and reduced tight junction protein and mucin levels, while orally administered MPX can increase tight junction protein and mucin levels. This study lays the foundation for the use of antimicrobial peptides as feed additives to prevent the occurrence of diseases in the clinic.

Many studies have reported that antimicrobial peptides are closely related to inflammation. The antimicrobial peptides Cathelicidin have been shown to reduce the increased expression of anti-inflammatory cytokines in the intestines of mice caused by LPS, comparable results were obtained in experiments on porcine macrophages (Fijalkowska *et al.*, 2021; Ting *et al.*, 2021). The anti-inflammatory effect of LL-37 peptide has been most thoroughly studied (Vera-Cruz *et al.*, 2021). LL-37 can alleviate the inflammation caused by pathogenic bacteria, LPS or other TLR agonists through a variety of signalling pathways. Studies have found that antimicrobial peptide LL-37 not only has anti-inflammatory effects, but also increases the expression of pro-inflammatory factors under certain conditions. For example, the expression of IL-8 in macrophages and IL-6 in dendritic cells may be enhanced by the peptide LL-37 (Mohanty *et al.*, 2021). Peptides such as defensins, LFP-20 inhibits the NF- $\kappa$ B signalling pathway and reduces inflammation (Rodriguez-Carlos *et al.*, 2021; Zong *et al.*, 2015). Therefore, antimicrobial peptides may have the function of selectively regulating inflammation, thereby supporting the balance of the body's immune system. The results

of this study found that the orally administered MPX can effectively reduce the mRNA expression of inflammation-related IL-2, IL-6 and TNF- $\alpha$  in the intestinal, thereby alleviating the inflammation of the jejunum caused by *E. coli* infection in mice.

Antimicrobial peptides have abilities to increase the expression of tight junction of proteins. Antimicrobial peptides C-BF and LFP-20 reduce damage to dense compounds, increase protein expression and protect the physical barrier function of the intestine (Feng *et al.*, 2020; Han *et al.*, 2013). Snake-derived antimicrobial peptide C-BF may increase the expression of tight junction proteins Occludin and ZO-1 in porcine jejunal epithelial cells IPEC-J2 through the MAPK signalling pathway, and enhance the epithelial barrier function (Feng *et al.*, 2020). At the same time, studies have found that the porcine antimicrobial peptide PR-39 enhances the transmembrane resistance of small intestinal epithelial cells, increases the expression of tight junction proteins, and enhances the intestinal physical barrier function. The mechanism may be through up-regulation of the Rho-Rac 1 signalling pathway (Haiwen *et al.*, 2019). Authors' results found that via an orally administered MPX expression of tight junction of protein can increase in the intestine after *E. coli* infection in mice. The above results showed that antimicrobial peptides can regulate the intestinal barrier function.

## CONCLUSIONS

In summary, in this study, necropsy and H&E staining results showed that oral antimicrobial peptide MPX could effectively alleviate the pathological damage of *E. coli* in the intestine, liver, spleen and lung of mice. RT-qPCR results showed that orally administered MPX can reduce the expression levels of inflammation-related factors IL-2, IL-6 and TNF- $\alpha$  mRNA, thereby alleviating the intestinal inflammation caused by *E. coli* infection

in mice. In addition, orally administered MPX could increase the expression levels of tight junction protein Claudin-1, ZO-1 and Occludin in mRNA and improve the barrier function in the intestine. Furthermore, jejunum immune histochemistry results showed that oral administration antimicrobial peptide MPX could effectively increase the mucin MUC2 protein expression levels in the jejunum. The above results show that oral antimicrobial peptide MPX could effectively alleviate intestinal inflammation and enhance the intestinal barrier function of mice caused by *E. coli* infection. This study lays the foundation for adding antimicrobial peptides in feed to relieve inflammation and improve barrier function in clinical.

## ACKNOWLEDGMENTS

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## **Вплив орального застосування антибактеріального пептиду МРХ на запалення кишечника мишей при експериментальній інфекції *Escherichia coli* штаму O157:H7**

**Сюєцінь Чжао<sup>1,2</sup>, Ганна Анатоліївна Фотіна<sup>2</sup>, Тетяна Іванівна Фотіна<sup>2</sup>, Янг Ху<sup>1</sup>, Лі Вонг<sup>1</sup>**

<sup>1</sup>Хенанський інститут науки і техніки  
453003, Східний ХуанЛан просп., м. Сінсян, Китай

<sup>2</sup>Сумський національний аграрний університет  
40000, вул. Г. Кондратьєва, 26, м. Суми, Україна

**Анотація.** *Escherichia coli* – це грамнегативна бактерія, кишковий патоген, який може викликати запалення кишечника. Антимікробні пептиди – це клас пептидів з невеликою молекулою, які мають хорошу антибактеріальну активність проти різноманітних грампозитивних і негативних бактерій. Метою цієї роботи було дослідити вплив перорального введення антимікробного пептиду МРХ на запалення кишечника та функцію кишкового бар'єру у мишей, інфікованих *E.coli*. МРХ(H-INWKGIAAMAKLL-NH2) був синтезований та очищений компанією Ji er Sheng Hua (Шанхай, Китай). Для очищення МРХ використовували вискоелективну рідинну хроматографію (ВЕРХ) і мас-спектрометрію. Кишкова паличка (ентерогеморагічна *Escherichia coli* O157:H7ATCC43889) була виділена з фекалій пацієнта з гемолітико-уремічним синдромом та отримана з Китайського інституту ветеринарного контролю над лікарськими засобами (Пекін, Китай). Для отримання ізольованих чистих *E.coli* культур використовували агар LB (Solarbio, Китай). Результати розтину показали, що пероральне введення МРХ може полегшити ураження кишковою паличкою печінки, селезінки та легень, ці органи менш постраждали. Результати Н&Е показали, що застосування МРХ при пероральному введенні значно полегшує вплив *E. coli* на дванадцятпалу кишку та органи на 7-й та 28-й день. qRT-PCR результати показали, що пероральне введення МРХ може зменшити фактори, пов'язані із запаленням, експресію мРНК IL-2, IL-6 і TNF-α на 7 і 28 день. Крім того, пероральне введення МРХ може значно збільшити експресію мРНК білків щільного з'єднання Occludin і Zo-1 на 7 і 28 день. Результати імуногістохімії також показали, що пероральне введення МРХ може збільшити експресію мРНК MUC2 в порожній кишці. Наведені вище результати показали, що пероральний прийом МРХ може послабити вплив кишкової палички на кишковий тракт мишей, зменшити кишкове запалення та покращити функцію кишкового бар'єру. Це дослідження закладає теоретичну основу для додавання антимікробних пептидів до кормів. Пероральний прийом МРХ може зменшити експресію мРНК факторів, пов'язаних із запаленням, тим самим полегшуючи кишкове запалення, викликане інфекцією *E. coli* у мишей. Крім того, пероральне введення МРХ може збільшити експресію мРНК білка щільного з'єднання в кишечнику та покращити бар'єрну функцію кишечника. Це дослідження закладає основу для додавання антимікробних пептидів у корм для зняття запалення та покращення бар'єрної функції в клінічній практиці

**Ключові слова:** антимікробний пептид МРХ, ентерогеморагічна *E. coli*, процес запалення, кишковий бар'єр, білі миші