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Evaluation of the antimicrobial activities and mechanisms of synthetic antimicrobial peptide against food-borne pathogens

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ABSTRACT

Food-borne pathogens are an important challenge for the food industry. In this study, the possibility of using the previously designed and synthesized antimicrobial peptide HX-12C as a new food antimicrobial was evaluated. Bacteriostatic and bactericidal tests showed that HX-12C has strong, rapid and broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria. Outer- and inner-membrane permeabilization assays revealed that HX-12C killed food-borne pathogens by inducing membrane permeability. Further Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) also showed that HX-12C can distinctively act on the bacterial membrane leading to the leakage of cellular contents. Moreover, HX-12C also showed *anti*-biofilm ability in bacterial killing tests. In the food storage test, HX-12C exhibited significant antimicrobial function in raw pork and orange juice. Therefore, HX-12C has shown great potential as a new antimicrobial agent in food storage.

1. Introduction

Food-borne diseases are caused by food contamination and may occur at any stage of the food production, delivery and consumption chain (WHO, 2015a; 2015b). In addition, with the increasing complexity and globalization of food production, food-borne diseases have become a common public health problem all over the world (Horn & Friedrich, 2019). According to the statistics of the World Health Organization (WHO), nearly one-in-ten people in the world are sick after eating contaminated food and more than 420,000 die every year (WHO, 2016).

Unsafe foods containing harmful bacteria, chemical substances, viruses, or parasites can cause more than 200 diseases ranging from diarrhoea to cancers, with food-borne pathogens the main cause of food related diseases (Lambrecht et al., 2015). According to the WHO's estimation, out of the 600 million cases of food-borne diseases in 2010, of which 350 million were caused by pathogens (Asante et al., 2019). Therefore, food poisoning and food-borne diseases caused by food-borne pathogens have become the main problems of global concern (Badul et al., 2021; Cava-Roda et al., 2021; Y. Li et al., 2019; Tertis et al., 2021).

Most food-borne pathogens can form biofilms, which are bioactive substrates of extracellular polymers secreted by microorganisms. The microbial cells in this matrix are usually highly protected from adverse conditions, such as environmental changes, antibiotics, aggressive chemical and physical agents and disinfectants (González-Rivas et al., 2018). These could increase the nutrition and water required for their growth, reduce the risk of dehydration, and get close to other bacteria to promote genetic exchange (Flemming et al., 2016; Jefferson, 2004). Moreover, the bacteria in the biofilm enter a state of metabolic dormancy state, which further reduces the effectiveness of antimicrobial agents (Koo et al., 2017). This is because the pathways (such as cell wall synthesis, translation or topoisomerase activity) containing drug targets are inactive in dormant cells, so the antibacterial agents cannot act against bacteria even if they successfully combine with the target (Fisher et al., 2017; Zou et al., 2022). Therefore, the control or inhibition of biofilms has become an important aspect of ensuring food safety in the food processing environment (Chmielewski & Frank, 2003), and it is also one of the main reasons why food contact surfaces should be sanitized frequently (Bayoumi et al., 2012). However, the use of antimicrobial agents in the control of biofilms has safety problems because of

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Abbreviations

AMPs	Antimicrobial peptides
CV	Crystal violet
CFU	Colony forming unit
HDPs	Host defense peptides
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
MH	Mueller-Hinton
MIC	Minimal Inhibitory Concentrations
NPN	1-*N*-phenylnaphthylamine
ONPG	O-Nitrophenyl β-D-galactopyranoside
PBS	Phosphate-buffered saline
RP-HPLC	Reverse phase high performance liquid chromatography
rRBCs	Rabbit red blood cells
SEM	Scanning electron microscope
TEM	Transmission electron microscope
TSB	Tryptic soy broth
WHO	World Health Organization
w/v	Weight per volume

their repeated use can cause drug resistance (Singh et al., 2017) and introduce antimicrobial residues into food (Falowo and F. A. O, 2019). Therefore, alternative agents, such as antimicrobial peptides (AMPs) that are not easily subjected to develop drug resistance are needed (Joerger, 2003; Kang et al., 2012; Parachin & Franco, 2014).

AMPs, commonly known as host defense peptides (HDPs), are an integral part of the natural innate immune system to protect the host from the invasion of pathogenic bacteria (Fox, 2013). AMPs have a broad-spectrum of activity, being active against bacteria, viruses, fungi, and parasites (Alfei & Schito, 2020; da Cunha et al., 2017). In general, AMPs are composed of 10–50 amino acid residues, most of which are cationic peptides (Rahman et al., 2018). However, there are also several AMPs (Harris et al., 2009). Most AMPs are amphiphilic, combining cationic charges and hydrophobic components, so they can be electrostatically bound to anionic bacterial membranes or other anionic targets (Ling et al., 2015; Nguyen et al., 2011; Yeaman & Yount, 2003). AMPs act directly on cell membranes through four recognized mechanisms (toroidal, carpet, aggregate and barrel models) to destroy the integrity or function of phospholipid bilayers (J. Li et al., 2017; Wang et al., 2019). This is also the reason why AMPs have a lower likelihood of inducing drug resistance, because bacteria need to change the overall properties of their membranes to produce drug resistance characteristics, rather than simply modifying individual receptors (Costa et al., 2019; Kang et al., 2012). Altogether, AMPs can not only inhibit bacteria, but also induce rapid sterilization, and has a lower tendency of drug resistance than conventional antibiotics. Therefore, since the first discovery of AMPs in insects and animals in the 1980s, they have been considered as a promising alternative to antibiotics (Hancock & Sahl, 2006; Haney et al., 2017). Indeed, more and more AMPs have been found to have good potential applications in food preservation (Ning et al., 2021; Ucar et al., 2021; Verma et al., 2022; Zhang et al., 2021).

Our research team has a long-term commitment to the transformation and screening of natural AMPs to obtain more AMPs with different structures and functions. To date, our group has designed and screened hundreds of AMPs with strong antimicrobial activity using natural AMPs as templates. The antimicrobial peptide HX-12C (FFRKVLKLIRKIWR) is a cationic peptide, which was modified based on the template of Temporin-Pta (FFGSVLKLIPKIL). Previous study has shown that HX-12C has good antimicrobial activity against multidrug-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae*, with the possible mechanism of peptides being to act on the cell membranes according to results from transmission electron microscopy (Luo et al., 2018). Therefore, HX-12C has a great potential to be developed into an antimicrobial agent. This peptide has never been used before in food control and safety. In this study, HX-12C, synthesized and purified by our team, was for the first time tested against potent food pathogens. The antimicrobial activity and mechanism of HX-12C against food-borne pathogens, including antimicrobial and bactericidal functions, biofilm inhibitory and scavenging activities, changes in membrane permeability and cell morphology were determined. At the same time, we have also evaluated its toxicity by mammalian cell hemolysis test, and finally evaluated the antimicrobial function of HX-12C in food storage.

2. Materials and methods

2.1. Bacterial strains

The standard strains *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Listeria monocytogenes* ATCC19115 were purchased from the China Center of Industrial Culture Collection. *E. coli* EC19ZNBYY76, *E. coli* EC19ZNBYY69, *Enterococcus* 3–9, *Enterococcus* 3–10, *Enterobacter cloacae* 32, *E. cloacae* ZQF1904T001, *S. aureus* SAF01QJ082, *S. aureus* SAF01QJ083, *Salmonella* SNL07B1SX002, *Salmonella* SNL07B1SX003, *Klebsiella pneumoniae* 29-2, *K. oxytoca* 55, *E. vulneris* 29-1, *E. cancerogenus* A26, *Pantoea agglomerans* A13 and *E. cloacae* 32 were obtained from the Zhejiang Academy of Agricultural Sciences, *E. coli* 1, *E. coli* 2, *S. aureus* 1, *S. aureus* 2, *S. aureus* 3 and *S. aureus* 4 were obtained from Lanzhou Institute of Animal Husbandry. Bacteria were cultured in Mueller-Hinton broth (MH broth, HOPEBIO, China) at 37 °C.

2.2. Peptide synthesis

HX-12C was chemically synthesized by solid-phase peptide synthesis and purified by reverse phase high performance liquid chromatography (RP-HPLC) separately; the purity was as high as 98%. Peptides were stored at −20 °C after freeze-dried.

2.3. Antimicrobial activity assay

The microbroth dilution method for determining the Minimal Inhibitory Concentrations (MIC) of HX-12C against multi drug resistant bacteria was described previously (Luo et al., 2018). The bacteria were cultured to the exponential phase, and then diluted to 10^5–10^6 CFU/mL with MH medium. 160 μL bacterial suspension and 40 μL HX-12C solution of different concentrations were added to 96 well plates, and then incubated for 16–18 h at 37 °C. The MIC was the peptide concentration without visible bacterial growth.

2.4. Growth inhibition test

The time growth curves of *E. coli* ATCC25922 and *S. aureus* ATCC25923 treated with HX-12C were determined according to the absorbance at 620 nm at each time point. The test strains were co-cultured with different concentrations of HX-12C in 96 well plates in the same way as the determination of MIC.

2.5. Time-kill kinetics assay

The killing rate of HX-12C to the test bacteria was evaluated through the time-killing kinetic study at various exposure time points. This study was carried out by plate count technique at each time point. Briefly, 3,200 μL bacterial suspensions (10^8–10^9 CFU/mL) were co-cultured with 800 μL HX-12C solution at 37 °C. 200 μL co-cultured bacterial suspensions were transferred to a sterilized tube at each time point. The supernatants were removed after centrifugation at 1,000 g for 10 min, and the pellets were resuspended in 200 μL MH medium. The bacterial suspensions were placed on agar plate after serially diluted, then

incubated at 37 °C until viable colonies could be observed.

2.6. Biofilm inhibition assay

The biofilm inhibition effect of HX-12C against *S. aureus* ATCC25923 was determined by a crystal violet (CV) assay on 96-well plates as described previously (Shi et al., 2021). The bacteria were diluted to 10^7–10^8 CFU/mL with tryptic soy broth (TSB) liquid medium containing 0.2% glucose after cultured to the logarithmic growth stage. Then 160 μL diluted bacteria were co-cultured at 37 °C with 20 μL different concentrations of HX-12C in 96 well plates. The supernatants containing free bacteria were discarded carefully after incubation for 24 h, and the wells were washed three times with sterile phosphate-buffered saline (PBS). The biofilms were fixed with 100% methanol for 10 min, and the biofilms were stained for 30 min at room temperature with 0.1% CV after removing methanol. Then the wells were washed three times with sterile water to remove excess CV. Finally, the CV-stained biofilms were eluted in 200 μL 95% ethanol, and quantified by measuring the intensity of CV at 570 nm.

The percentage inhibition of the biofilm formation was calculated by the following equation:

$$\text{The inhibition of biofilm formation } \% = (1\text{-}(Ab_{\text{treatment}} / Ab_{\text{negative control}})) \times 100\%$$

where $Ab_{\text{treatment}}$ is the absorbance value of the HX-12C-treated test group; and $Ab_{\text{negative control}}$ is the absorbance value of the PBS-treated test group.

2.7. Biofilm eradication assay

The biofilm eradication effect of HX-12C against *S. aureus* ATCC25923 was also determined by CV assay. 100 μL *S. aureus* ATCC25923 (10^7–10^8 CFU/mL) were incubated in TSB liquid medium containing 0.2% glucose in 96 well plates at 37 °C for 24 h. The wells were washed three times with PBS to remove the free bacteria after removing the culture medium. And then the cultures were co-cultured with different concentrations of HX-12C at 37 °C for another 24 h. The removed biofilms were quantified in the same way as the biofilm inhibition test.

2.8. Outer-membrane permeability assay

The outer-membrane permeabilization of the Gram-negative bacteria (*E. coli* ATCC25922) was determined by the uptake of 1-*N*-phenylnaphthylamine (NPN) as previously described (Elliott et al., 2020; Mai et al., 2015; Yang et al., 2019). The logarithmic growing bacteria were washed three times and resuspended in 5 mM HEPES buffer (pH 7.4) containing 5 mM glucose. The absorbance of the final cell suspension at 600 nm was adjusted to 0.2. The fluorescence was recorded by an RF-6000 fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) for subtraction (excitation $\lambda = 350$ nm; emission $\lambda = 420$ nm). Firstly, the fluorescence of the microbial cell suspension was measured. Then, 20 μL of 5 mM NPN was added with data recording continued for 15 s, and then peptide of different concentrations was quickly added with recording continued until no further increase in fluorescence was detected.

The percent of NPN uptake was calculated by the following equation:

$$\text{NPN uptake } (\%) = (F_{\text{treatment}} - F_0)/ (F_{\text{positive control}} - F_0) \times 100\%$$

where $F_{\text{treatment}}$ is the fluorescence observed at HX-12C-treated test group, F_0 is the initial fluorescence observed at non-treated test group, and $F_{\text{positive control}}$ is the fluorescence observed at polymyxin B (10 μg/mL)-treated test group.

2.9. Inner-membrane permeability assay

The inner-membrane permeabilization of the Gram-negative bacteria (*E. coli* ATCC25922) was determined by O-Nitrophenyl β-D-galactopyranoside (ONPG) assay (Hong et al., 2018; Jia et al., 2020; Saito et al., 2019). The mid-logarithmic growing bacteria were washed three times with PBS, and then diluted to an absorbance of 0.05 at 600 nm with 5 mM HEPES buffer (pH 7.4) which containing 1.5 mM ONPG and 20 mM glucose. Subsequently, 100 μL cell suspension and 100 μL peptide solution (2–32 μM) were co-incubated at 37 °C in 96 well plates. The absorbance of the mixture was recorded every 6 min at 420 nm from 0 to 120 min.

2.10. Morphological observation of microorganisms

The morphology and structure of microorganisms were evaluated by scanning electron microscope (SEM) and transmission electron microscope (TEM) (C. Liu et al., 2020; H. Liu et al., 2021; Zhao et al., 2019). For SEM, the mid-logarithmic growing bacteria were co-incubated with the peptide (2 × MIC) for 30 min at 37°C. After centrifugation, the pellets were rinsed and fixed with 2.5% (w/v) glutaraldehyde at 4°C overnight. The samples were then washed three times with PBS and postfixed in 1% (w/v) osmium tetroxide for 1 h. Next, the samples were washed three times with PBS and dehydrated by a graded series of ethanol aqueous solutions (50, 70, 85, 95, and 100%) for 15 min at each step. Finally, the samples were dried in a critical point drying apparatus with liquid CO_2. The dehydrated samples were coated with gold palladium by an ion sputtering apparatus (E−1010 Hitachi, Tokyo, Japan) Model for 5 min and then observed by SEM (SU-8010 Hitachi, Tokyo, Japan).

The preliminary preparation procedure of the TEM samples was the same as that of the SEM samples. After prefixation with 1% (w/v) osmium tetroxide for 1 h and washing with PBS three time, the samples were dehydrated in a sequential ethanol series (30, 50, 70, and 80%) for 15 min at each step, then dehydrated in 90% and 95% acetone for 15 min at each step. At the end, the samples were dehydrated twice in absolute acetone for 20 min. The samples were then immersed in a mixture of absolute acetone and epoxy resin (1:1, v/v) for 1 h, then transferred to a mixture of absolute acetone and epoxy resin (1:3, v/v) for 3 h and to pure epoxy resin for overnight. Next, the specimens were sectioned by an ultramicrotome (EM UC7 Leica, Nussolch, Germany) and the sections were stained with uranyl acetate and lead citrate for 10 min respectively, and finally observed by TEM (H-7650 Hitachi, Tokyo, Japan).

2.11. Hemolytic activity assay

The hemolytic activity of HX-12C was determined by hemolysis against rabbit red blood cells (rRBCs) as previously described (Khodaparast et al., 2018). An equal volume of 5% rRBCs solution was co-incubated with a series of concentrations of peptide for 1 h at 37 °C. Then the samples were centrifuged at 1,000×*g* for 15 min. The release of hemoglobin was determined by measuring the absorbance of the supernatant at 570 nm. Erythrocytes in 1% Triton and PBS were used as the controls for 100 and 0% hemolysis, respectively.

2.12. Application of HX-12C in food storage

The antimicrobial function of AMP was studied in two different food substrates, including raw pork and orange juice. For the preservation test of orange juice, fresh orange juice was prepared by juicing mechanism. Peptide HX-12C was added to orange juice with final concentrations of 32, 64 and 128 μg/mL respectively. The orange juice was stored in sterile glass bottles at 25 °C for 5 days. The pH and microbial number of samples were determined every day. The positive control was potassium sorbate (128 μg/mL) and sodium benzoate (128 μg/mL)

For the preservation test of raw pork, fresh raw pork purchased was immediately brought to the laboratory from the local supermarket (Yongzhou City, Hunan Province, China) at 4 °C. Then raw pork was cut into 2 × 2 × 2 cm pieces, and then soaked in peptide solutions (1280 μg/mL) for 30 min. Next, the samples were put into sterile glass bottles and stored in a refrigerator at 4 °C for 6 days after dried naturally. Pork samples were randomly selected, homogenized and coated on MH agar plates, and the microbial colony was counted at 0, 2, 4 and 6 days. The positive control was nisin and butyl paraben of the same concentration.

2.13. Statistical analysis

Statistically, all experiments were conducted at least three times independently, and the results were expressed as mean ± standard deviation (SD). The statistical difference was determined by one-way ANOVA between the two groups, and $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Antimicrobial activity of HX-12C

The antimicrobial activity of the peptide HX-12C against food-borne pathogens was evaluated by determining the MIC *via* the broth microdilution method. Briefly, the bacteria with known density (10^5–10^6 CFU/mL) were co-cultured with a series of concentrations of peptides and the ability to inhibit bacterial growth was measured after 18 h. The inhibition of peptide HX-12C on Gram-positive and Gram-negative strains was tested in the MIC assay. The results are given in Table 1 and showed that peptide HX-12C showed broad-spectrum and strong antimicrobial activity against both Gram-positive and Gram-negative strains tested, with MIC values ranging from 2 to 16 μM. These properties of HX-12C was also confirmed in our previous study (Luo et al., 2017). Being standard strains of typical Gram-negative and Gram-positive bacteria, respectively, *E. coli* ATCC25922 and *S. aureus* ATCC25923 were considered for further investigations using this peptide.

Table 1
MICs (μM) of peptide HX-12C against food-borne pathogens.

Bacterial Species	MICs (μM)
Gram (+)	
Staphylococcus aureus ATCC25923	8
S. aureus SAF01QJ082	2
S. aureus SAF01QJ083	2
S. aureus-1	8
S. aureus-2	8
S. aureus-3	8
S. aureus-4	8
Listeria monocytogenes ATCC19115	16
Gram (−)	
Escherichia coli ATCC25922	16
E. coli EC19ZNBYY76	4
E. coli EC19ZNBYY69	4
E. coli-1	16
E. coli −2	8
Enterococcus 3-9	4
Enterococcus 3-10	4
Enterobacter cloacae 32	2
Enterobacter cloacae ZQF1904T001	2
Salmonella SNL07B1SX002	2
Salmonella SNL07B1SX003	2
Klebsiella pneumoniae 29-2	16
Klebsiella oxytoca 55	4
Escherichia vulneris 29-1	4
Enterobacter cancerogenus A26	2
Pantoea agglomerans A13	2
Enterobacter cloacae 32	2

3.2. Growth inhibitory and time-kill kinetics of HX-12C against E. coli ATCC25922 and S. aureus ATCC25923

In order to determine the antimicrobial rate of HX-12C against food-borne pathogens, standard bacterial growth inhibition and killing kinetics tests were carried out. For the growth inhibitory of HX-12C, the results shown in Fig. 1A and B, indicate that HX-12C with MIC concentration could inhibit the growth of standard strain *E. coli* ATCC25922 and *S. aureus* ATCC25923 24 h after addition. The growth of *E. coli* ATCC25922 and *S. aureus* ATCC25923 was inhibited by HX-12C at the concentration of 1/2 × MIC 6 h after addition. Therefore, it is evident that peptide HX-12C can completely inhibit the growth of *E. coli* ATCC25922 and *S. aureus* ATCC25923 *in vitro*.

The relationship between sterilization and aging with HX-12C was studied by plotting the growth of colonies treated with HX-12C (4 × MIC) at different time points. As shown in Fig. 1C and D, HX-12C can kill both *S. aureus* ATCC25923 and *E. coli* ATCC25922 very quickly in a short time. Within 15 min under the treatment of 4 × MIC of HX-12C, the unit concentration of *S. aureus* ATCC25923 decreased rapidly from 10^8 to 10^3 CFU/mL, and the unit concentration of *E. coli* ATCC25922 decreased rapidly from 10^9 to 10^4 CFU/mL. In the positive control group, the unit concentration of *S. aureus* decreased slowly, and the unit concentration of *E. coli* ATCC25922 decreased by 3 orders of magnitude. In the negative control group, both *E. coli* ATCC25922 and *S. aureus* ATCC25923 treated with normal saline showed slow growth. These results show that HX-12C can kill both *E. coli* ATCC25922 and *S. aureus* ATCC25923 by rapid sterilization. Generally, the faster the antimicrobial action, the less likely to induce bacterial resistance for antimicrobial agent (Chen et al., 2019). Therefore, HX-12C was unlikely to induce bacterial drug resistance.

3.3. Biofilm inhibition and biofilm eradication activities of HX-12C

Biofilms are one of the important causes of food cross-contamination, and induce microorganisms to develop resistance to chemical preservatives, so as to reduce the effectiveness of food processing strategies and affect the quality and safety of food (Coughlan et al., 2016; Miao et al., 2019). Therefore, the effects of HX-12C on the staphylococcal biofilm were performed, including biofilm inhibition and eradication assays. HX-12C showed significant biofilm inhibition of 58.5, 77.1 and 93.3% at the concentration of 1/4 × MIC, 1/2 × MIC and 1 × MIC respectively compared to the non-treated control (Fig. 2 A2). Besides, the CV-stained images showed the biofilm inhibition of HX-12C in a concentration-dependent manner (Fig. 2 A1). For the biofilm eradication test, HX-12C showed biofilm eradication of 13.7, 27.0 and 49.1% at the concentration of 1/4 × MIC, 1/2 × MIC and 1 × MIC respectively (Fig. 2 B2) compared to the non-treated control. In addition, the CV-stained images further illustrate how biofilms were eradicated by HX-12C (Fig. 2 B1). Taken together, the results above suggest that HX-12C not only inhibits the biofilm formation effectively, but also eliminates the biofilms formed by *S. aureus* ATCC25923, indicated that HX-12C has potential in preventing and controlling food pollution caused by bacterial biofilms.

3.4. Outer and inter-membrane permeabilization assays

After showing the significant antimicrobial and bactericidal activity of HX-12C against Gram-positive and Gram-negative bacteria, we next further clarify the potential mechanism of action of HX-12C. The effects of HX-12C on the permeability of the outer and inter-membrane of *E. coli* ATCC25922 as the model microorganism was first studied. NPN is an environment sensitive hydrophobic fluorescent dye that fluoresces strongly in a hydrophobic membrane environment, but weakly in an aqueous environment. This property is used to evaluate the permeability of the outer-membrane of Gram-negative bacteria (Elliott et al., 2020; Ma et al., 2019; MacNair et al., 2018). As can be seen from Fig. 3A.

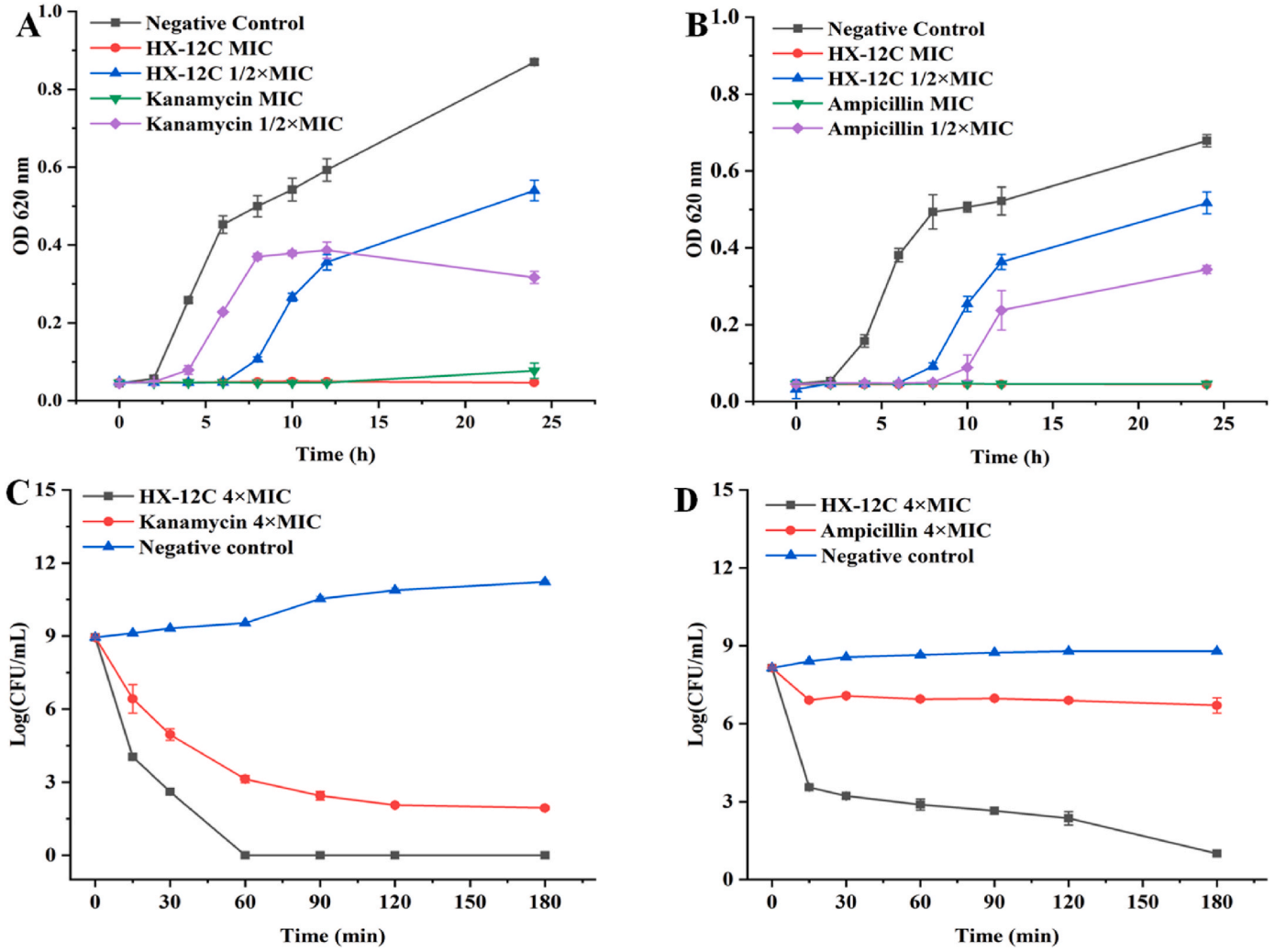

Fig. 1. Growth inhibitory and time-kill kinetics of HX-12C against *E. coli* ATCC25922 and *S. aureus* ATCC25923; (A) Growth inhibitory of HX-12C against *E. coli* ATCC25922; (B) Growth inhibitory of HX-12C against *S. aureus* ATCC25923; (C) Time-kill kinetics of HX-12C on *E. coli* ATCC25922; (D) Time-kill kinetics of HX-12C on *S. aureus* ATCC25923.

HX-12C increased the outer-membrane permeability in a concentration-dependent manner according to the absorbance of the NPN. The outer-membrane permeabilization of HX-12C were 50% and 104% at the concentrations of 1 × MIC and 4 × MIC respectively. Moreover, HX-12C increased the outer-membrane permeability within 1 min of contact with the bacteria (results not shown). Next, the inner-membrane permeabilization of HX-12C was studied by measuring the content of O-Nitrophenol (ONP), which was a degradation product of ONPG. As shown in Fig. 3B, HX-12C induced rapid increase in the permeability of the inner-membrane in a concentration-dependent manner. The above-described results suggested that HX-12C exerted a better membrane permeabilization capacity. Previous reports showed that cationic peptides could be electrostatically adsorbed to the membrane surface, and then the hydrophobic core of the peptide can be inserted into the phospholipid layer, which leads to the rupture of the outer-membrane through the transmembrane potential. Then the peptide molecules continued to interact with the inner-membrane, resulting in significant changes in the permeability of the bacterial membrane (Blasco et al., 2020; Dou et al., 2017; Yang et al., 2019).

3.5. Morphological observation of bacteria

In order to observe the effect of HX-12C on the membrane of bacterial directly, the bacteria were co-cultured with HX-12C at 2 × MIC for 1 h, then the cell morphology and ultrastructure of *E. coli* ATCC25922 and *S. aureus* ATCC25923 were observed using SEM and TEM separately (Fig. 4). The membrane surface of bacteria in the control groups was intact and smooth (Fig. 4 A1 and B1); however, after the bacterial strains were co-cultured with HX-12C, the morphology of the bacterial membrane was significantly abnormal (Fig. 4 A2, A3, B2, B3), including the presence of creping, pore formation and content leakage. In addition, the intracellular ultrastructure of bacteria was further observed by TEM (Fig. 4 C1-D3). The outer membrane and inner-membrane of *E. coli* ATCC25922 treated with HX-12C showed some clear separation, and there were obvious sparse cytoplasmic distribution and empty area in the cells (Fig. 4 C2, C3) compared to the control (Fig. 4 C1). Similarly, the surface of *S. aureus* ATCC25923 treated with HX-12C showed membrane rupture and micropore formation (Fig. 4 D2) compared to the surface of non-treated group (Fig. 4 D1). Subsequently, further leading to the leakage of cytoplasm, sparse distribution of cytoplasm and empty intracellular areas (Fig. 4 D3).

3.6. Hemolytic activity of HX-12C

In order to evaluate the toxicity of the peptide to eukaryotic cells, the hemolytic activity assays were performed by measuring hemoglobin release from rRBCs at series concentrations of HX-12C. HX-12C exhibited good hemocompatibility similar to the negative control PBS, as can

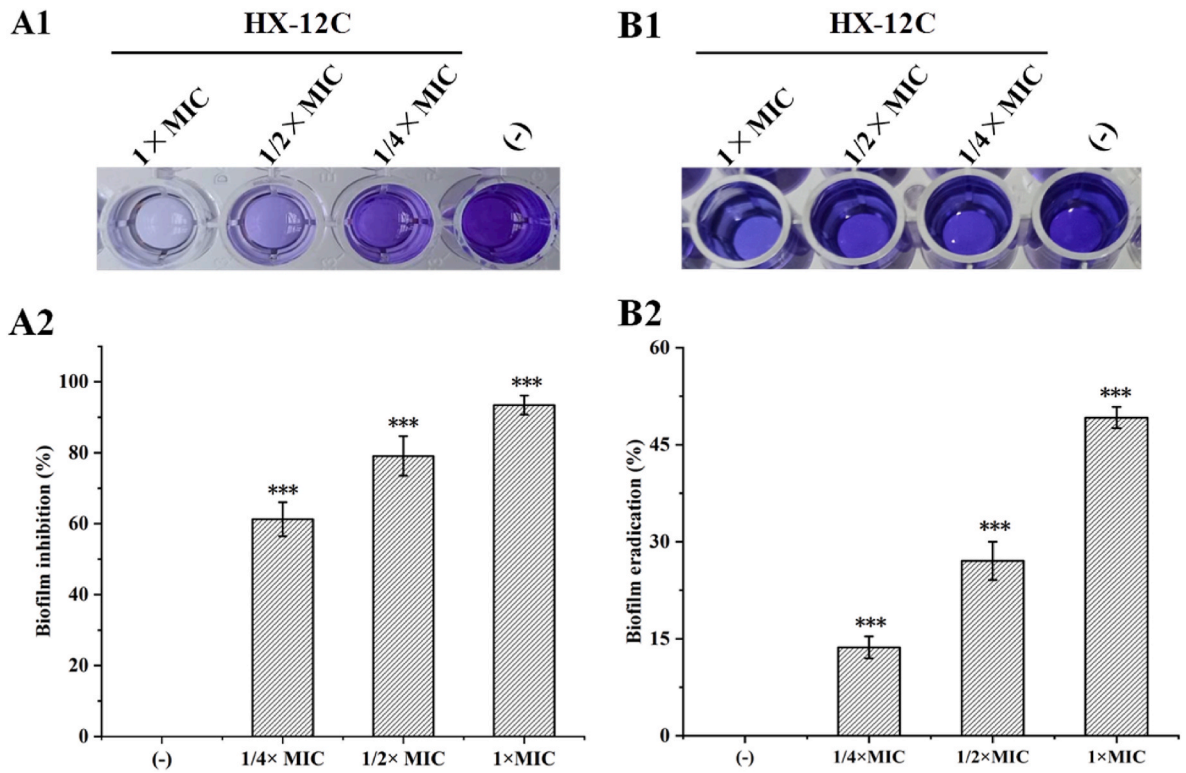

Fig. 2. The biofilm inhibition and eradication effect of HX-12C on *S. aureus* ATCC25923. (A1) CV-stained image of Biofilm inhibition; (A2) The percentage inhibition of the biofilm formation; (B1) CV-stained image of Biofilm eradication; (B2) The percentage eradication of the pre-formed biofilm.

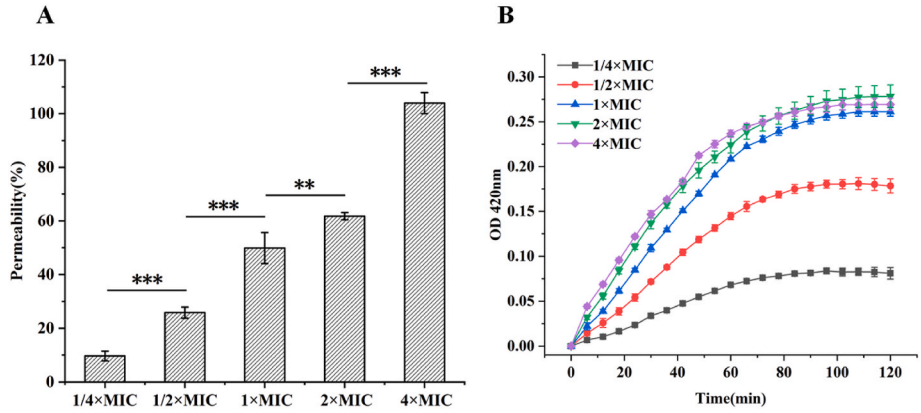

Fig. 3. Membrane permeabilization capacity of HX-12C. (A) Outer-membrane permeability induced by HX-12C. (B) Inner-membrane permeability induced by HX-12C.

be seen from the Fig. 5. Specifically, the hemolysis percentages of HX-12C were 2.6, 3.9, and 4.6 at the concentration of 1 × MIC, 2 × MIC and 4 × MIC respectively. The above results suggested that HX-12C present good hemocompatibility within the concentration of 4 × MIC according to the standard acceptance value (<5%).

3.7. Application of HX-12C in food storage

In order to evaluate the possibility of peptide HX-12C as an antimicrobial agent in food storage, its antimicrobial function was studied in two food substrates such as raw pork and orange juice. Pork is an important source of animal protein and is the most widely consumed meat in the world (McAuliffe et al., 2017). Orange juice is one of the most consumed beverages in the world because of its pleasant sensory and healthy beneficial properties (Neves et al., 2020). The high-water content and rich nutrition of pork and orange juice provide a growth environment for various microorganisms. Therefore, one of the biggest challenges during preservation of these food items is finding effective strategy to inhibit microbial growth.

In the orange juice preservation test, HX-12C showed the most effective antimicrobial function (Fig. 6A). HX-12C could significantly reduce the total number of bacteria throughout the storage period (except the second day) ($P < 0.01$) compared to the non-treated group. And the antimicrobial function of HX-12C was concentration-dependent (Fig. 6B). The antimicrobial effect of three concentration gradients of HX-12C was significantly different ($P < 0.01$) especially from the third day of storage. This may be due to the degradation of peptides. The pH value of orange juice during storage was shown in Fig. 6C. During the 5-day storage period at 25 °C, the pH value of all groups got a modest drop. However, there was no significant difference between the HX-12C-

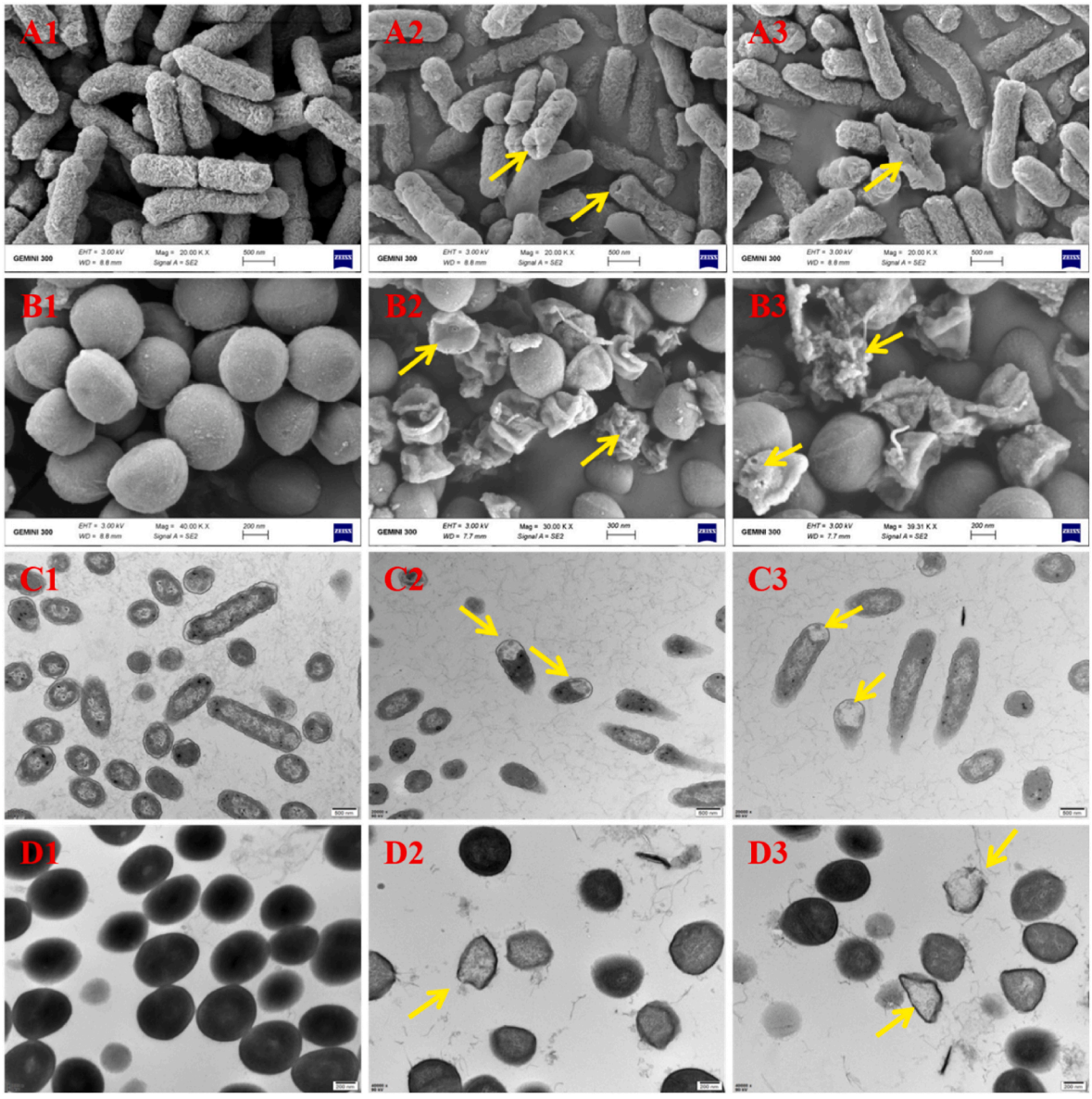

Fig. 4. SEM and TEM micrographs of *E. coli* ATCC25922 and *S. aureus* ATCC25923.(A1) SEM micrograph of PBS treated *E. coli* ATCC25922; (A2, A3) SEM micrographs of HX-12C treated *E. coli* ATCC25922; (B1) SEM micrograph of PBS treated *S. aureus* ATCC25923; (B2, B3): SEM micrographs of HX-12C treated *S. aureus* ATCC25923; (C1) TEM micrograph of PBS treated *E. coli* ATCC25922; (C2, AC): TEM micrographs of HX-12C treated *E. coli* ATCC25922; (D1) TEM micrograph of PBS treated *S. aureus* ATCC25923; (D2, D3): TEM micrographs of HX-12C treated *S. aureus* ATCC25923.

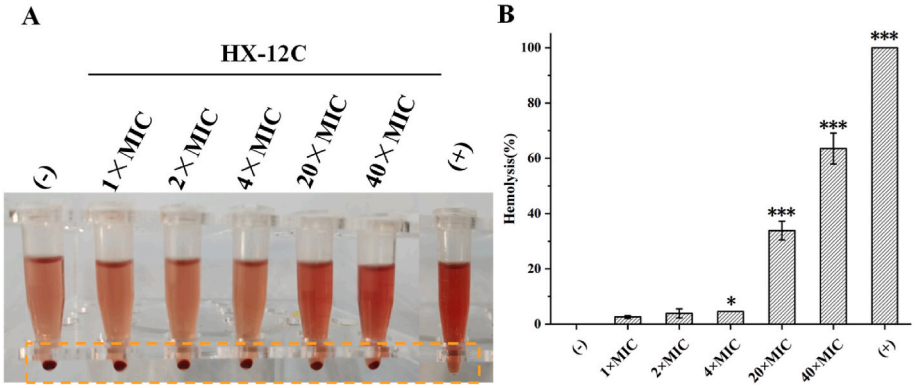

Fig. 5. Hemolytic activity of HX-12C. (A) The images of rRBCs exposed HX-12C; (B) Hemolysis percentage of rRBCs after treatment with HX-12C.

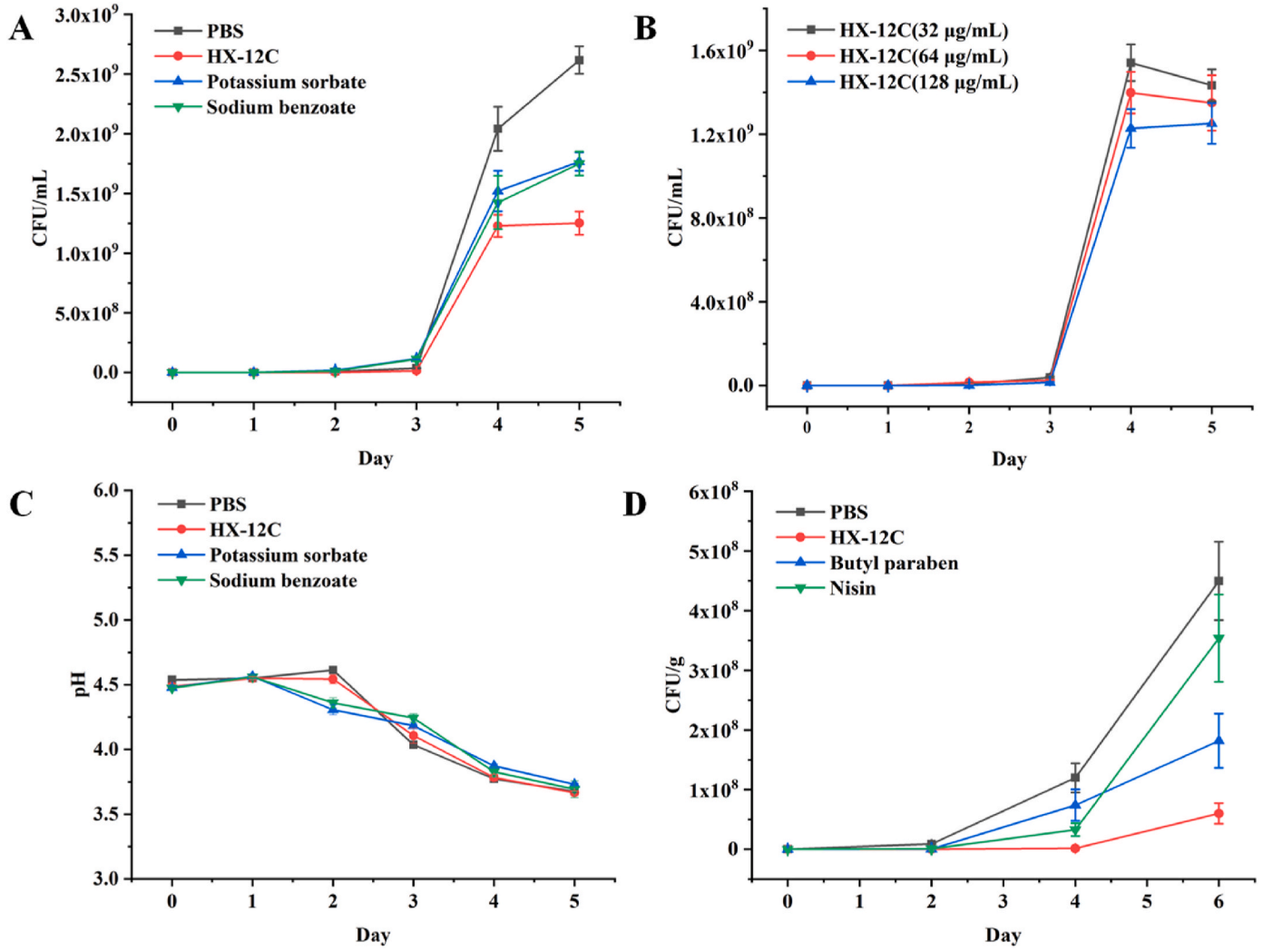

Fig. 6. Antimicrobial function of HX-12C in food storage. (A) Inhibition of microbial growth in orange juice; (B) Inhibition of microbial growth in orange juice with different concentrations of HX-12C; (C) The variation of pH value of orange juice during storage; (D) Inhibition of microbial growth in raw pork. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treated group and the common preservative group ($P > 0.05$). As demonstrated by previous studies (Hui et al., 2016; Zhang et al., 2021), PH value is not suitable for food quality evaluation.

HX-12C also showed the most effective antimicrobial function in raw pork preservation (Fig. 6D). The total number of viable bacteria between the PBS-treated group and the HX-12C-treated group showed a very significant difference in the storage period ($P < 0.001$). The positive control butyl paraben and nisin also showed effective antimicrobial effects, but lower than that of HX-12C.

4. Conclusions

HX-12C has broad-spectrum, strong and rapid antimicrobial activity against pathogenic bacteria. This AMP kills pathogenic bacteria by inducing membrane permeability. In addition to direct antimicrobial activity, it also shows strong antibiofilm ability. HX-12C exhibits effective antimicrobial function in two different food substrates: raw pork and orange juice. Overall, the results show that HX-12C has great potential as a new antimicrobial agent in food storage. However, it is necessary to further evaluate the practical application of this peptide by examining its stability in the food environment. And stronger efforts are still required to resolve challenges with the cost related with peptides synthesis.

Author contributions

Xiaofang Luo: conception and design, data collection and analysis, manuscript writing; Yannan Song and Zhengmin Cao: Data collection and mapping; Wubliker Dessie and Zongcheng Wang: Revise, format and edit the manuscript; Nongyue He: collected references; Zuodong Qin and Yimin Tan: Supervision and project administration.

Conflicts of interest

The authors have no conflicts of interest to declare.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Data availability

Data will be made available on request.

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