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Research Article

Structural Analysis and Antimicrobial Mechanism of a Protein GBSPI-A from Ginkgo Biloba Seed

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Ginkgo biloba seed has antimicrobial activity. In this study, ginkgo biloba seed protein was prepared, identified, and named GBSPI-A, finding its construction was similar to 11-S globulin. Then, the influence of GBSPI-A on the cell membrane and physiological metabolism of *K. pneumoniae* and *S. aureus* were investigated. The results showed that GBSPI-A (20 mg/mL) destroyed the cell membrane, causing leakage of intracellular material and inhibited bacterial growth with an inhibition rate of approximately 80%. In addition, the GBSPI-A (10 mg/mL) caused the decreasing activity of ATPase and respiratory rate, and the respiratory depression rate was 7.24%. Furthermore, the decreasing ATP synthesis and intracellular β-galactosidase activity led to an insufficient supply of physiological metabolic energy. Therefore, the results showed that GBSPI-A could be used as a natural bacteriostatic agent to replace related drugs and also provide a new insight into the application of GBSPI-A in food safety.

1. Introduction

Antibiotic tolerance, as an increasingly serious problem, could constitute a danger to human health. Therefore, the new substitute goods of antibiotic have been widely concerned to solve this problem. Antimicrobial protein, a micromolecule polypeptide extensively existing in the natural world, plays a crucial role in the biological immune system [1, 2].

Since the cecropin with the antimicrobial character was found [3], more antimicrobial peptides from different sources, such as cytokines, animal, and plants sources, were further discovered [4–6]. The peptides produced by the previous resource could restrain the growth of numerous microorganisms [7].

The antimicrobial protein is deemed to the promising alternative to antibiotic and has wide application prospect in medicine, food, and agriculture due to the stabilization and difficulty to produce tolerance for target bacteria [8, 9]. The research of antibacterial mechanism on protein is mainly concentrated on protein from animal origin. The antimicrobial peptides from animal have been restricted to employ on account of food safety, hence, the protein from vegetable has received more attention [10].

The antimicrobial protein separated from botany such as corn peptide and soybean peptide could inhibit the activity of pathogens including bacteria, fungus, and viruses [11, 12]. The mechanism of antimicrobial protein on bacteria is multiple due to the diversity of sources [13].

Ginkgo, the ancient living fossils, is one of the oldest trees and possesses a very strong resistance ability. In this study, the GBSPI-A separated from ginkgo biloba seeds was separated and identified, and the result of LC-MS-MS was researched in the NCBInr protein database. In our previous studies, Hai-xia et al. [14] found that GBSPI-A has antibacterial activity by evaluating the inhibition zone and measuring the minimum inhibitory concentration (MIC) values. However, the antimicrobial mechanism of GBSPI-A

was not further research. The aim of this study is to explore the antimicrobial mechanism of GBSPI-A. Firstly, ginkgo proteins were separated and identified. Secondly, the interaction between protein and bacteria was researched by studying the bacterial membrane integrity, the cell damage, the synthetic ability of DNA and protein, the content of ATP, and the activity of ATPase, β-galactosidase, and Alkaline phosphatase (AKP), which will provide a practical and theoretical basis for exploiting novel antimicrobial peptide derived from plant.

2. Materials and Methods

2.1. Bacterial Strains and Chemicals. Ginkgo biloba seeds were purchased from Jiangsu Taixing in October 2011 and stored at −80°C for nine years. Dialysis tubes and lysozyme were purchased from Golden Spike Biotechnology Co., Ltd (Shanghai, China). DEAE-Cellulose 52 was purchased from Whatman Co., Ltd (Mettlestone, England). Sephadex G-75 was obtained from Pharmacia Co., Ltd (NY, USA). The standard substances of the amino acid were obtained from Sigma Co., Ltd (MD, USA). All of the other chemicals and reagents used were of analytical grade.

The *K. pneumoniae* and *S. aureus* were obtained from the Institute of Chemical Industry of Forest Products, CAF (Nanjing, China) and inoculated in fresh sterilized nutrient broth (NB) medium (pH = 7.2) and cultivated at 37°C, with the shaking table in 130 rpm for 24 h, the bacterial concentration was reached to 10^8 cfu/mL for further experiments.

2.2. Preparation and Purification of Ginkgo Protein. The GBSPI-A with antibacterial activity was extracted and purified from ginkgo biloba seeds according to the method of our previously reported [14]. The ginkgo biloba seed proteins were extracted by phosphate buffer (0.07 mol/L, pH 7.0). The mixed proteins were seriatim purified using DEAE-cellulose 52 column and Sephadex G-75.

2.3. Structural Characterization of Protein Analysis

2.3.1. Amino Acid Composition Analysis. The amino acid composition was measured according to the method of Misurcova et al. [15] described previously. Proteins (50 mg) were dissolved with 20 mL HCl (6 mol/L) and hydrolyzed at 110°C for 24 h after being transferred to hydrolyzing tube. The volume of hydrolysate was titrated to 50 mL. Then, the amino acid composition was analyzed by the amino acid automatic analyzer (L-8900, Hitachi Limited Co., Tokyo, Japan).

2.3.2. Fourier Transformed Infrared Spectroscopy. GBSPI-A (0.5 mg) was slowly ground with 50 mg of KBr powder in an agate bowl under an infrared lamp. The uniform grinding powders were measured on the machine after being pressed into thin slice using tablet press. Finally, the slice was scanned by IR-360 fourier transform infrared spectrometer (Thermo Nicolet Co., Madison, USA) in the range of 400–4000 cm^{-1}.

2.3.3. Liquid Mass Spectrometry. The molecular weight of the protein was measured by two-dimensional liquid phase-ultra high resolution quadrupole time of flight mass spectrometry (LC-MS-MS) [16]. The analysis of two-dimensional liquid was proceeded on UltiMate 3000 (Thermo Fisher Co., Massachusetts, USA) at 200–400 nm. The detector and analytical column of the liquid chromatograph was Bruker Q-TOF and BEH C_{18} (2.1 × 100 mm, 1.7 μm), respectively. The 0.2% TFA (solvents A) and acetonitrile (solvents B) were used as mobile phases. The samples (3 μL) were injected into the column and eluted with solvents A and B at 45°C using a flow rate of 0.3 mL/min. The mass spectrometry of GBSPI-A was analyzed using Q-TOF (Bruker Co., Karlsruhe, Germany). The ion mode of the mass spectrometer was ESI+. The voltage of the capillary, cone, and detector was successively 3500 Volts, 30 Volts, and 1700 Volts. The temperature of the source block and desolvation was, respectively, 100°C and 250°C. The gas flow of desolvation and the cone was successively 500 l/h and 50 l/h. Then, the qualitative data of measured peptides were compared to the NCBInr protein data bank.

2.4. Effects on Growth of Bacteria

2.4.1. Growth Curve of Bacteria Measure. The antibacterial effect of GBSPI-A had been observed in *K. pneumoniae* and *S. aureus*, and the MIC value of the previously bacteria was 20 mg/mL as our previously reported [14]. The growth curve was measured by turbidimetry, and the growth curves of the normal cultured test bacteria and those treated with GBSPI-A were drawn. The activated bacteria solution for 24 h was, respectively, put into the nutrient broth medium and another medium contained GBSPI-A (20 mg/mL) using a sterile pipette according to 1% of the inoculation amount and mixed uniformly. The bacteria were taken into 13 labeled sterile tubes and cultivated at 130 rpm at 37°C. The OD value of bacteria was measured at 600 nm by the molecular device (SpectraMax I3X, Molecular Devices Co., Shanghai, China) per 4 hours and persistently measured for 48 h [17].

2.4.2. Electron Microscopy of Bacteria Observe. The ginkgo protein was mixed with a liquid medium making the final concentration reach to MIC, then *K. pneumoniae* and *S. aureus* were added into the medium, respectively according to 2% inoculum size, and the suspension without ginkgo protein was used as control. The samples were cultivated at 37°C in the 120 rpm shaking table for 6 h and 12 h, respectively. The samples were collected and washed thrice with 0.1 mol/L PBS (pH 7.2) every 30 min and then it was fixed with 4% glutaraldehyde at 4°C for 72 h. The fixed samples were washed thrice with 0.1 mol/L PBS (pH 7.2) every 30 min and then were dehydrated for 5 h at vacuum state. After gold-coating, the sample was observed by a Quanta-200 SEM (FEI Co., USA) with 25 kV working voltage [18].

For TEM examination, the dehydrated cell was cut into slices by ultramicrotome after infiltration and immobilization. The slices were dyed with uranium acetate and citric

acid and then observed by a JEM-1400 TEM (Seiko JEOL Co., Japan) [19].

2.4.3. The Extracellular Nucleic and Protein of K. pneumoniae and S. aureus Measure. Bacteria solutions (2 mL) in the logarithmic phase of *S. aureus* and *K. pneumoniae* were centrifuged, washed, and suspended in the 50 mL solution of phosphate buffer (pH 6.0, 0.02 mol/L). The leakage of protein and nucleic acid was measured according to Yoo et al. [20] described previously. The solution of GBSPI-A was added into 2 mL of bacterium suspension making the final concentration reach to MIC and cultivated for different times (0, 30, 60, 90, 120, and 150 min) in the shaking table at 37°C. Then, the 0.2 mL of solution was diluted to 5 mL with buffer solution, and the supernate was used to, respectively, measure absorbance at 260 nm and 280 nm with a molecular device (SpectraMax I3X, Molecular Devices Co., Shanghai, China). Meanwhile, the bacterial suspension without adding protein was served as the blank control.

2.4.4. Extracellular Na^+ Leakage from Bacteria Measure. The bacteria were inoculated into the nutrient broth medium according to the 1% of inoculation amount and cultivated in a shaking table for 12 h at 37°C. After being centrifuged at 3500 rpm, the samples were washed twice using sterile ultrapure water and made into bacterial suspension. The GBSPI-A was added into bacterial suspension (5 mL) making the concentration of sample reach to MIC. After mixed and dissolved, the samples were taken for 1 mL sample at different times (0, 30, 60, 90, and 120 min) in the process of cultivation at 37°C, and the supernate was preserved at the refrigerator after being centrifuged at 4000 rpm. The 0.1 mL of liquid supernatant was joined to 9.9 mL ultrapure water, and its Na^+ concentration was measured with PinAAcle 900 atomic absorption spectrometer (PerkinElmer, USA) [21]. There were three parallel experiments in each group.

2.5. Influence of GBSPI-A on Respiration of Bacterium. The respiratory activity of the bacterium was monitored by oxygen consumption as Zheng et al. [22] described previously. The bacterium suspension was mixed well with 3.6 times the volume of phosphate buffer (pH 7.2, 0.1 mol/L) and 0.4 times the volume of 1% glucose solution. Three typical inhibitors (iodoacetic acid, malonic acid, and sodium phosphate) and GBSPI-A were added into the tube to make the ultimate density reach 1 mg/mL and 10 mg/mL, respectively. The dissolved oxygen was measured at the hermetic system after 5 min, and the sample without inhibitor was controlled. The respiratory depression rate of *S. aureus* was evaluated using the following equation:

$$I_R = \frac{(R_0 - R_1)}{R_0} \times 100\%, \quad (1)$$

where I_R is the respiratory depression rate, R_0 is the respiratory rate of control, and R_1 is the respiratory rate of inhibitor, respectively.

2.6. Influence of GBSPI-A on DNA Content of Bacteria. The GBSPI-A was added to the bacterial suspensions making the final concentration reach to MIC and 2MIC, respectively. The bacterial suspensions were cultivated for different times (0, 30, 60, 90, 120, and 150 min) in the shaking table at 37°C. Then, the suspensions were centrifuged at 5000 rpm for 5 min and the sediments were collected. The sediment was used for the determination of intracellular DNA, protein, ATP, ATPase, and alkaline phosphatase.

The DNA was extracted by a Biospin bacterial genome DNA extraction test kit (Bofei Technology Co., Nanjing, China). The structure of the bacterial cell was destroyed by EL Buffer, RS Buffer, and PK solution, successively, causing the release of DNA. After purified, the released DNA was combined with the Biospin membrane under the effect of specific salt and pH in the binding buffer. After further washing, the DNA was eluted from the membrane with the elution buffer. Then, the DNA was diluted to 100 mg/μL, and the concentration and purity of DNA was measured using protein nucleic acid tester (Eppendorff Co., Hamburg, Germany). The 10 μL sample was mixed with bromophenol blue indicator buffer and then the mixture was added into a sample hole of agarose (1%) gel plate for an electrophoresis experiment. The voltage was set as 100–140 V and the time was 30 min.

2.7. Influence of GBSPI-A on Protein Synthesis Ability of Bacteria. The sediments were washed with normal saline and added into 500 μL TE buffer solution and then 500 μL lysozyme of 10 mg/mL after centrifugation. The sample was resuspended and incubated at 37°C for 20 min. The bacterium suspension was ice bathed for 5 min after mixed with 400 μL ice water and 500 μL ice TCA of 20%. The sediments were resuspended with 1 mL ice TCA of 10% and then the sediments were mixed with 100 μL NaCl (0.15 mol/L) and 5 mL CBB G-250 after being centrifuged. The absorbance value was measured at 595 nm [23, 24].

2.8. Influence of GBSPI-A on ATP Content and ATPase Activity of Bacteria. The sediments were washed twice with normal saline and added into 500 μL TE buffer solution and 500 μL lysozyme of 10 mg/mL after centrifugation. The sample was incubated at 37°C for 20 min after suspension and then diluted with 3 mL of normal saline. The sample was broken intermittently for 5 min using ultrasonic cell pulverizer (Xinzhi Biotechnology Co., Ningbo, China) at 200 W in ice bath condition. The ATP content of bacteria was measured using ATP content test kit (Jiancheng Institute of Biological Engineering, Nanjing, China). The ATP and creatine were catalyzed by creatine kinase to produce phosphocreatine. The content of ATP was measured by the phosphomolybdic acid colorimetric method. The activity of ATPase was measured using a high-speed Adenosine triphosphatase assay kit (Jiancheng Institute of Biological Engineering, Nanjing, China). The ATPase could promote the biolysis of ATP and generate the ADP and inorganic phosphorus, so the activity of ATPase could be measured by the content of inorganic phosphorus. The ATP content was calculated using the following equation:

$$\text{ATP concentration} = \frac{[(OD_3 - OD_2)/(OD_1 - OD_0) \times n_1 \times M]}{n_2}, \quad (2)$$

where OD_0 is the absorbance of blank, OD_1 is the absorbance of standard, OD_2 is the absorbance of control, OD_3 is the absorbance of sample, n_1 is the concentration of the standard (1×10^3 μmol/L), M is the dilution rate of sample before measured, and n_2 is the protein concentration of sample (gprot/L).

2.9. Influence of GBSPI-A to Enzyme

2.9.1. β-Galactosidase. The 1 mL of bacterial suspension induced by the M9 lactose medium was diluted 10 times. The 5 mL of diluted bacterial suspension was mixed with ginkgo protein making the ultimate density reach to MIC and 2MIC, respectively. Then, the 1 mL of bacterial suspension was transferred into a centrifuge tube and incubated at 37°C for different times (0, 30, 60, 90, and 120 min). The sample was centrifuged for 5 min at 5000 rpm, and sediment was mixed with 1000 μL lysozyme (10 mg/mL) and 1000 μL TE buffer solution. Then, the sample was incubated at 37°C for 20 min and then added to 8 mL buffer solution of β-galactosidase. The sample was broken intermittently for 5 min by ultrasonic cell pulverizer (Xinzhi Biotechnology Co., Ningbo, China) at 200 W in ice bath condition. 500 μL supernate was mixed with 1000 μL buffer solution of β-galactosidase and 500 μL ONPG (10 mg/mL). The mixture was incubated at 37°C for 2 h until presenting yellow, and the absorbance was measured at 405 nm using a spectrophotometer.

2.9.2. Alkaline Phosphatase. The activity of alkaline phosphatase is measured by an alkaline phosphatase kit (Jiancheng Institute of Biological Engineering, Nanjing, China). The phenyl disodium phosphate was by alkaline phosphatase to produce free phenol and phosphoric acid. Phenol and 4-amino-antipyrine reacts to form a red quinone derivative via the oxidizing reaction of potassium ferricyanide in an alkaline solution. The sediments were washed twice with normal saline and added into 500 μL TE buffer solution and 500 μL lysozyme of 10 mg/mL after centrifugation. The sample was incubated at 37°C for 20 min after suspension, then diluted with 3 mL of normal saline. The sample was broken intermittently for 5 min using an ultrasonic cell pulverizer (Xinzhi Biotechnology Co., Ningbo, China) at 200 W in ice bath condition. After centrifugation, the supernate (0.5 mL) was adequately mixed with 0.5 mL buffer solution and 0.5 mL basic phosphokinase matrix fluid. The mixture was heated in a water bath at 37°C for 15 min, and its absorbance was measured at 520 nm after adding a color developing agent. The ultrapure water in a blank tube was the negative control. The standard tube was positive control using phenol standard liquid (0.1 mg/mL), and the blank tube was contrast.

2.10. Statistical Analysis. In this study, data were analyzed by SPSS software (version 26; IBM Corp., Armonk, NY, USA). All experiments were performed in duplicate and repeated three times, and the results were presented as mean ± standard deviation. Graphs were created by Origin software (Origin Lad Co., Pro. 17.0, Northampton, MA, USA).

3. Results

3.1. Characterization of GBSPI-A. The purified protein was identified by the composition analysis of amino acid, infrared spectroscopy, and analysis of LC-MS-MS after purification. The analysis result of protein was shown that the percentage of hydrophobic amino acid for valine, methionine, isoleucine, leucine, and phenylalanine was 5.505%, 1.821%, 2.37%, 4.185%, and 1.932%, respectively (Table 1). The percentage of alkaline and acidic amino acid was 11.75% and 22.86%, respectively.

To detect the molecular structure, the result of infrared spectroscopy on GBSPI-A is shown in Figure 1. The result of LC-MS-MS indicated the molecular weight of the samples was 51.40 kDa. The result of the NCBInr protein data bank indicated that the GBSPI-A was similar to 11-S globulin from ginkgo and the score was 919.5.

3.2. Effect of GBSPI-A on Growth and Cytomembrane of Bacteria. To evaluate the antibacterial property of protein on *K. pneumoniae* and *S. aureus*, the grow curve was measured using turbidimetry. Optical density was reflected the concentration of bacteria. As shown in Figure 2, the growth curve of bacteria untreated with GBSPI-A shows the typical characteristic of bacteria growth. Growth of bacteria treated with GBSPI-A was restrained obviously and never arose logarithmic stage. Therefore, the GBSPI-A could restrain the growth of bacteria. To study the effect of GBSPI-A on structure, the morphology of bacteria was observed using the TEM and SEM, respectively. The control group showed a smooth surface and unbroken structure without damage and cell debris (Figure 3 a1 and b1). The bacteria treated with GBSPI-A (20 mg/mL) for 6 h showed an unsharp margin and distorted thallus (Figure 3 a2 and b2). Some thallus were broken and content was outflowed (Figure 3 a3 and b3) after 12 h. The damage to the ultramicroscopic structure of bacteria was observed using TEM. As shown in Figure 4, clear structure in the control group indicated alive and healthy bacteria. On the contrary, the cell of protein-treated bacteria showed blurry borders indicating that the cytomembrane was damaged.

3.3. The Leakage Rate of Molecule of Bacteria. To verify the influence of GBSPI-A on membrane permeability, the leakage of Na^+ and macromolecule was measured using absorption photometry. As shown in Figure 5, the absorbance at 260 nm and 280 nm in the control group was not a significant change, and that in the protein group was increased to double times compared to the control at

Table 1: Amino acid composition and content of GBSPI-A.

Name	Symbol	Content (g/100 g)
Aspartic acid	Asp	7.269
Threonine	Thr	4.89
Serine	Ser	4.65
Glutamic acid	Glu	7.086
Glycine	Gly	3.315
Alanine	Ala	2.028
Cysteine	Cys	4.032
Valine	Val*	5.505
Methionine	Met*	1.821
Isoleucine	Ile*	2.37
Leucine	Leu*	4.185
Tyrosine	Tyr	4.122
Phenylalanine	Phe*	1.932
Lysine	Lys	3.096
Histidine	His	1.143
Arginine	Arg	3.141
All	$\sum$	60.585

*means hydrophobic amino acid.

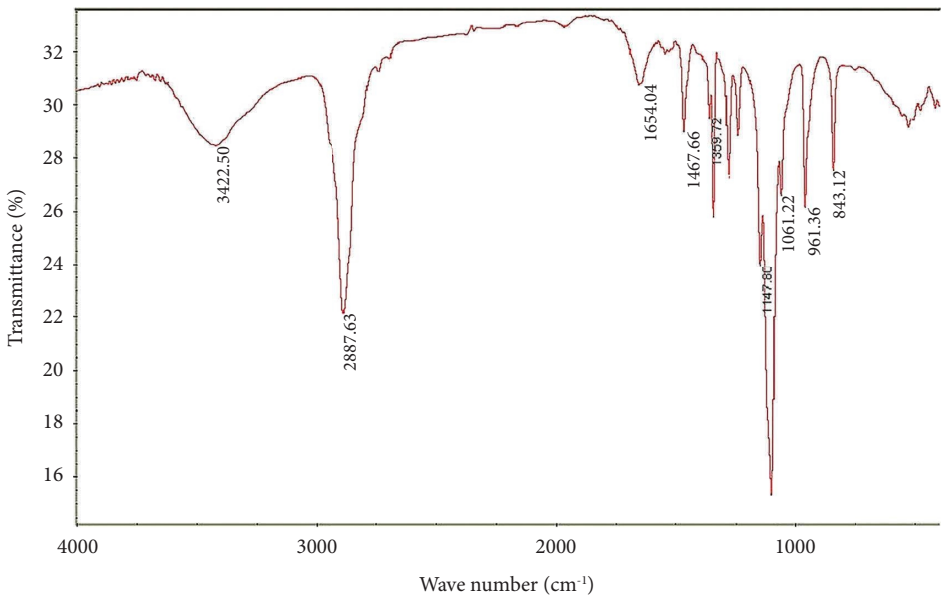

Figure 1: Infrared absorption spectrogram of GBSPI-A.

150 min, which indicated that the intracellular protein and nucleic acid outflowed the cell.

The electrolyte is one of the important substances during the bacterial metabolism, such as K^+, Na^+, and Ca^+, which plays a significant role in bacterial metabolism. The content of extracellular Na^+ rose by 60% at 150 min in comparison with the control group (Figures 5 a2 and b2). The increase in extracellular Na^+ content indicated that the integrality of the cytomembrane was destroyed by GBSPI-A.

3.4. Effect of GBSPI-A on Respiratory Rate of Bacteria. The major respiratory metabolic pathways in microorganisms exist three pathways, including Embden-Meyerhof-Parnas (EMP), Tricarboxylic acid (TCA) cycle, and Hexose monophosphate pathway (HMP) pathways. The typical inhibitor of EMP, TCA, and HMP pathways was iodoacetic acid, malonic acid, and sodium phosphate, successively [25]. In this experiment, the respiratory rate of bacteria treated with typical inhibitor and GBSPI-A was lower than that in the control group, which meant that typical inhibitor and GBSPI-A could restrain the respiration of bacteria from Table 2. Although the inhibiting effect of GBSPI-A was lower than the typical inhibitor, the inhibition rate could reach 7.24% when the concentration was 10 mg/mL.

3.5. The DNA Synthetic Ability of Bacteria. As shown in Figures 6 a1 and b1, the resultant quantity of DNA was increased in the control group, and that in the protein group

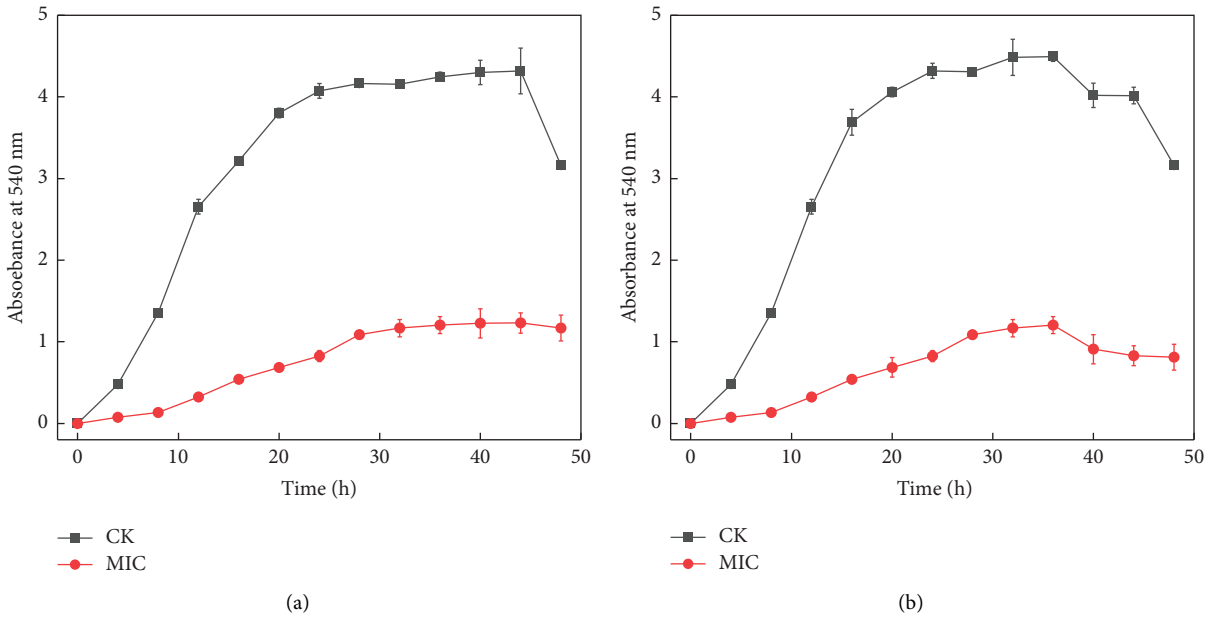

Figure 2: Growth curve of *K. pneumoniae* and *S. aureus* treated with GBSPI-A (20 mg/mL): (a) *K. pneumoniae* and (b) *S. aureus*.

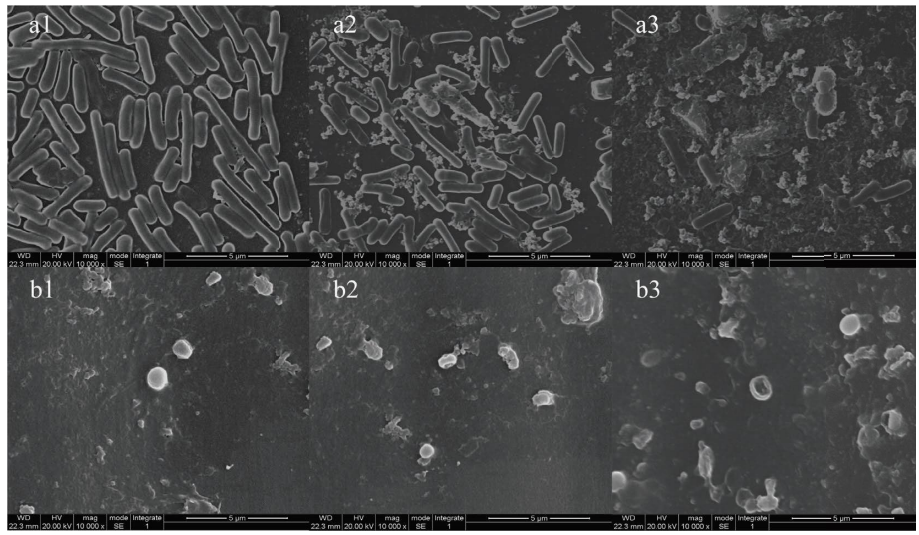

Figure 3: SEM photographs of *K. pneumoniae* and *S. aureus* treated with GBSPI-A. a1, a2, and a3 represent the *K. pneumoniae* treated with GBSPI-A for 0, 6, and 12 h, respectively. b1, b2, and b3 represent the *S. aureus* treated with GBSPI-A for 0, 6, and 12 h, respectively.

showed contrary tendency with the extension of cultured time. The DNA resultant quantity of *K. pneumoniae* and *S. aureus* treated with GBSPI-A (20 mg/mL) was, respectively, decreased by 83.96% and 75.02% compared with the control group. The result of DNA gel electrophoresis maintained clear and intact, which did not show the phenomenon of dispersion and drag. Therefore, the addition of protein did not destroy the intracellular DNA of bacteria and reduced significantly the content of DNA.

3.6. The Protein Synthetic Ability of Bacteria. As shown in Figures 6 a2 and b2, the absorbance of bacteria treated with GBSPI-A rose in the first 30 min and then reduced, while control showed an increase of the absorbance in the early stage, and had no evident changes as time prolongs. The protein content of *K. pneumoniae* and *S. aureus* treated with GBSPI-A (20 mg/mL) was, respectively, reduced to 66.34% and 75.31% compared with the control group at 120 min. The treatment of protein resulted in the content of intracellular protein was not significantly reduced in the early stage and decreased after 30 min.

3.7. Influence of GBSPI-A on ATP Content and ATPase Activity. To evaluate the effect of GBSPI-A on the respiration, the ATP content and ATPase activity was measured. The changes in the ATP content and ATPase activity of the

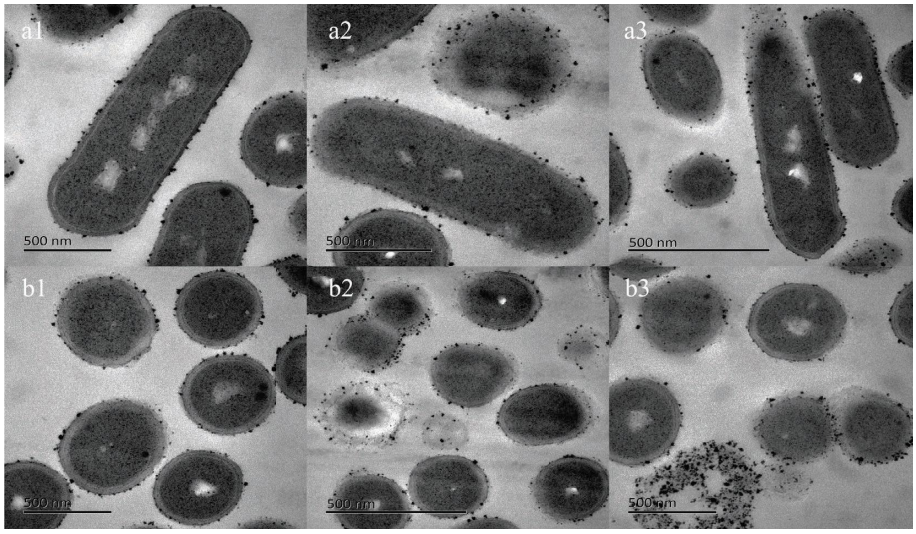

Figure 4: TEM photographs of *K. pneumoniae* and *S. aureus* treated with GBSPI-A. a1, a2, and a3 represent the *K. pneumoniae* treated with GBSPI-A for 0, 6, and 12 h, respectively. b1, b2, and b3 represent the *S. aureus* treated with *GBS*PI-A for 0, 6, and 12 h, respectively.

bacteria with time are observed in Figure 7. The addition of GBSPI-A led to a decrease in ATP content and ATPase activity over 120 min. The ATP content of the sample treated with GBSPI-A was extremely reduced in the 0–60 min and tended to be stable finally (Figures 7 a1 and b1). Meantime, the ATPase activity was reduced, especially at 0–30 min (Figures 7 a2 and b2). The ATPase activity and ATP content of *K. pneumoniae* and *S. aureus* in the protein group, respectively, was reduced by 90% and 93% compared with the control group at 120 min.

3.8. Influence of GBSPI-A on Activity of β-galactosidase and AKP. The activity of *β*-galactosidase and AKP was measured to explore the damage of GBSPI-A on bacteria. As shown in Figure 8 a1 and a2, the activity of *β*-galactosidase and AKP in control increased along with the incubation time of bacteria. The AKP activity and *β*-galactosidase of *K. pneumoniae* treated with GBSPI-A (20 mg/mL) were, respectively, reduced by 90.47% and 37.65%, and those of *S. aureus* treated with GBSPI-A (20 mg/mL) were, respectively, reduced by 94.93% and 8.23% compared with the control group at 120 min. The AKP activity of *K. pneumoniae* (2MIC) and *S. aureus* (2MIC) could not be detected at 80 min and 50 min, respectively (Figures 8 a2 and b2).

4. Discussion

Antibiotic resistance of antibiotics has a serious damage to human health, so it is important to search for natural antibacterial agent. In this study, the antimicrobial mechanism of GBSPI-A separated from ginkgo biloba seeds was explored.

From the result of amino acid composition and content, the acidic amino acid content of GBSPI-A was 87.82% in the medium (pH = 7.2) and exceed that of alkaline amino acid, which was indicated GBSPI-A was an acidic protein. The infrared spectroscopy is one of the useful methods to measure the molecular structure. The strength grade at 3420 cm^{-1} represented the stretching vibration of -OH, O-H, and N-H, which indicated exist of intramolecularly or intermolecular hydrogen bond. The stretching vibration of C-H resulted in the absorbance at 2887 cm^{-1}. The stretching vibration of C=O of carboxylate and -$NHCOCH_3$ and flexural vibration of N-H bond was observed at 1650 cm^{-1}. The strong absorption peak at 1650 cm^{-1} explained GBSPI-A contained amidogen. Strong alpha type C-H equatorial bonds variable angle vibration and pyranoside C-O-C stretching vibration signal were observed at 840 cm^{-1} and 1100 cm^{-1}, respectively. The signal of beta type pyranoside bond was observed at 960 cm^{-1}. These observations confirmed that GBSPI-A is a pyrano glycoprotein compound containing the alpha and beta type glucosidic bound. The sequence identification result of LC-MS-MS was indicated the molecular weight of GBSPI-A was 51.40 kDa. The search result of the NCBInr protein database indicated the structure of GBSPI-A was similar to that of an 11-S globulin from ginkgo biloba, and some studies indicated that the 11-S globulin could inhibit the growth of bacteria [26, 27]. The antimicrobial activity of GBSPI-A against *K. pneumoniae* and *S. aureus* was verified by Hai-xia et al. [14] and the antimicrobial mechanism was not distinct. So, it was essential to explore the antibacterial mechanism against *K. pneumoniae* and *S. aureus*.

The growth curves of GBSPI-A indicated that GBSPI-A has an obvious antibacterial effect and it could inhibit the growth and reproduction of *K. pneumoniae* and *S. aureus*. The existence of peptidoglycan and lipopolysaccharide, respectively, results the negative charge of gram-positive and negative bacterium. Because the amino acid is an amphoteric substance having isoelectric points, protein, consisted of

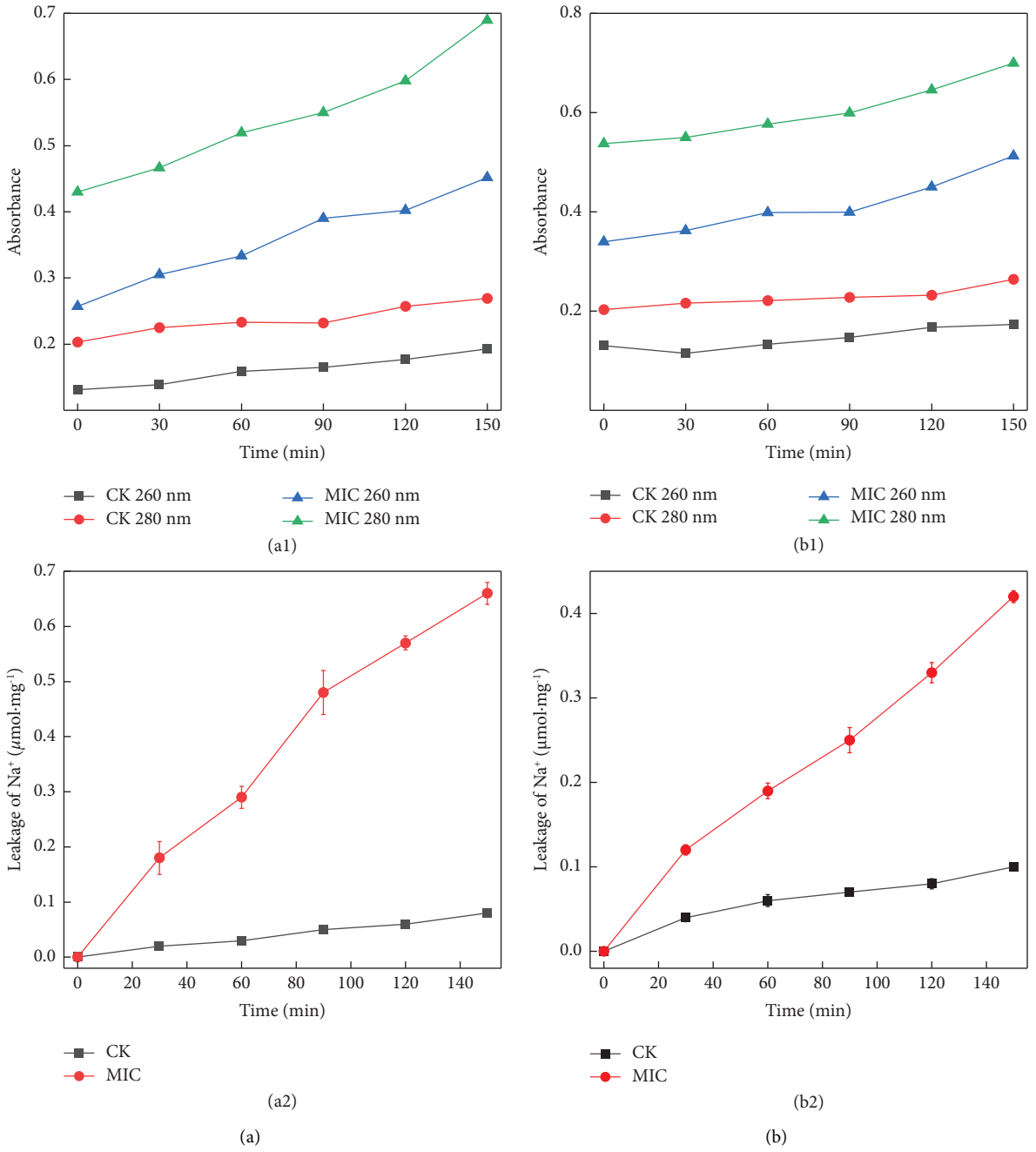

Figure 5: Macromolecular and Na^+ leakage of *K. pneumoniae* and *S. aureus* treated with GBSPI-A. a1 and b1 represent the macromolecular leakage of *K. pneumoniae* and *S. aureus*. a2 and b2 represent the Na^+ leakage of *K. pneumoniae* and *S. aureus*. CK: control group, MIC: treated with 20 mg/mL GBSPI-A, and 2MIC: treated with 40 mg/mL GBSPI-A: (a) *K. pneumoniae* and (b) *S. aureus*.

Table 2: Inhibiting rate of the representative inhibitor and GBSPI-A to *S. aureus*.

Inhibitor	Concentration (mg/mL)	R_0 (μmol O_2/g·min)	R_1 (μmol O_2/g·min)	I_R (%)
Sodium phosphate	1	0.6714	0.5397	19.61
Iodoacetic acid	1	0.6714	0.5954	11.32
Malonic acid	1	0.6714	0.4784	28.75
GBSPI-A	10	0.6714	0.6228	7.24

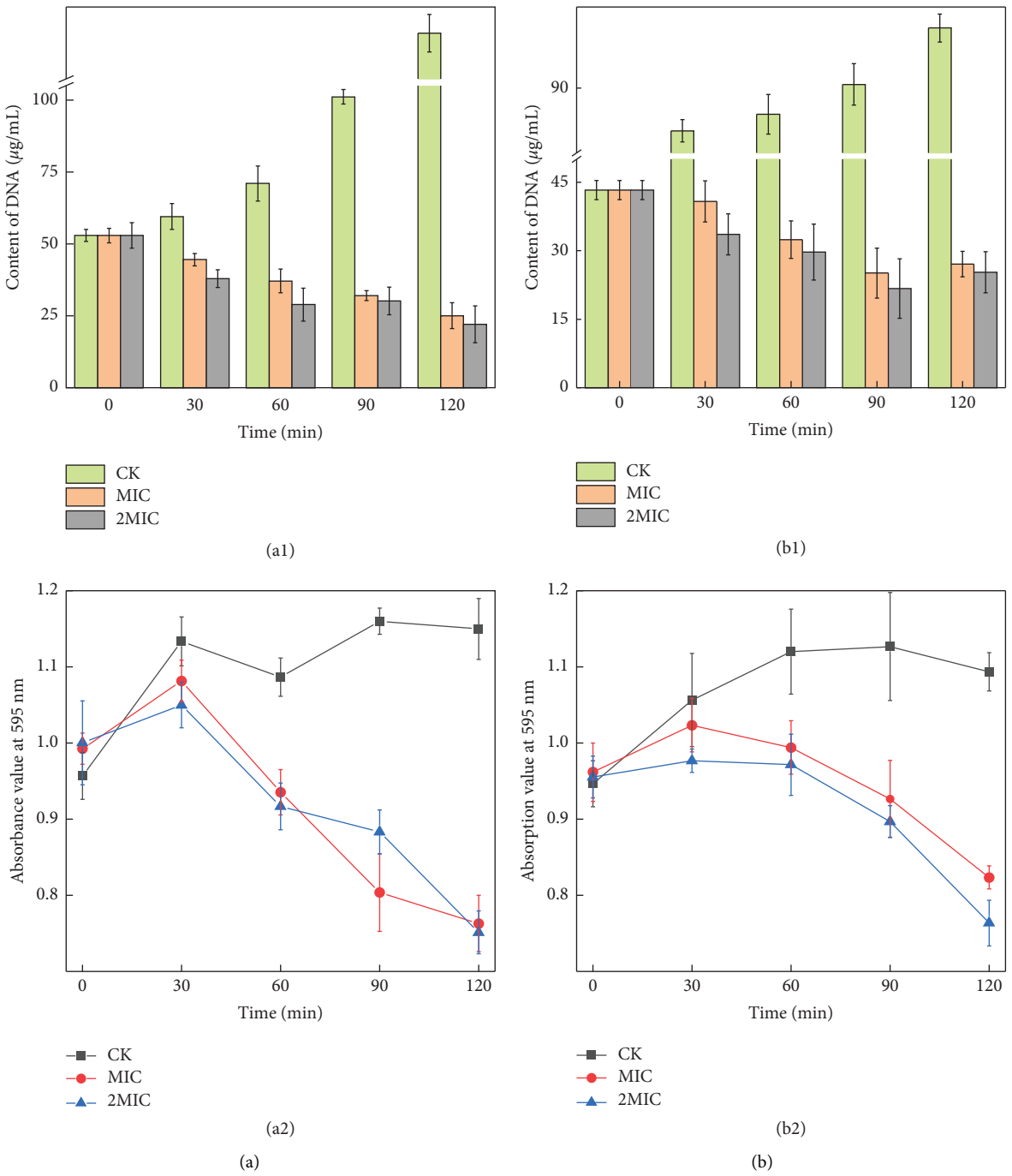

Figure 6: The influence of GBSPI-A on the DNA and protein synthesis of bacteria. a1 and b1 represent the influence of GBSPI-A on the DNA synthesis of *K. pneumoniae* and *S. aureus*. a2 and b2 represent the influence of GBSPI-A on protein synthesis of *K. pneumoniae* and *S. aureus*. CK: control group, MIC: treated with 20 mg/mL GBSPI-A, and 2MIC: treated with 40 mg/mL GBSPI-A: (a) *K. pneumoniae* and (b) *S. aureus*.

amino acid, also possess isoelectric points. Although the GBSPI-A as a whole is negatively charged, it also contain positively charged amino acids. Therefore, protein could absorb on the bacterial membrane surface by electrostatic interaction.

Antibacterial peptides could combine with the negative component of the outer membrane by electrostatic interaction in the presence of cationic amino acid residues [28]. Then, the hydrophobic parts of antimicrobial embed into the plasmalemma and disturbed the membrane structure causing the membrane structure was damaged, then the intracellular material was outflowed, for instance, extravasation of protoplasm, which generated the cell death [29]. The content of cationic amino acid residue in GBSPI-A was 12.18%, which was beneficial to the preliminary combination between GBSPI-A and surface of bacteria. The

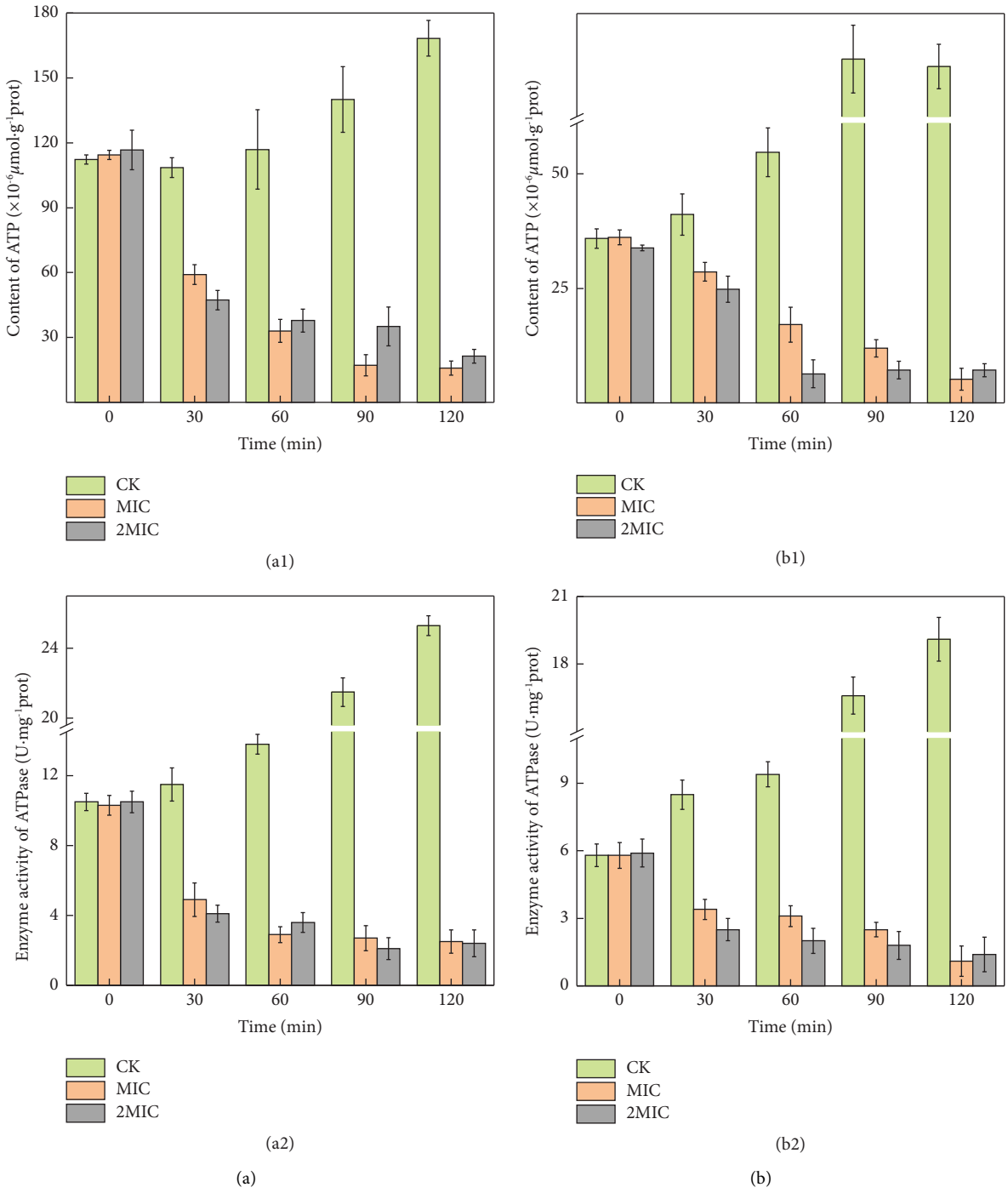

Figure 7: The effect of GBSPI-A on the ATP content and ATPase activity of bacteria. a1 and b1 represent the influence of GBSPI-A on the ATP content of *K. pneumoniae* and *S. aureus*. a2 and b2 represent the influence of GBSPI-A on ATPase activity of *K. pneumoniae* and *S. aureus*. CK: control group, MIC: treated with 20 mg/mL GBSPI-A, and 2MIC: treated with 40 mg/mL GBSPI-A: (a) *K. pneumoniae* and (b) *S. aureus*.

proportion of hydrophobic amino acid was 25.18%, which promoted the membrane destruction of GBSPI-A to bacteria. In addition, some arginine-rich antibacterial peptides could be internalized into the cytoplasm via the endocytic and direct translocation pathways [30]. The content of arginine in GBSPI-A was 3.141 g/100 g, which was beneficial to the internalization of GBSPI-A into the cytoplasm.

The cell membrane is an important protective barrier for bacteria, and the results of SEM showed that the cell wall of bacteria was damaged. The damage to cell membrane was usually accompanied by the leakage of cell contents [31], so the content of extracellular protein, nucleic acid, and Na^+ was detected. In this paper, the amount of protein, nucleic acid, and Na^+ in the extracellular was increased compared

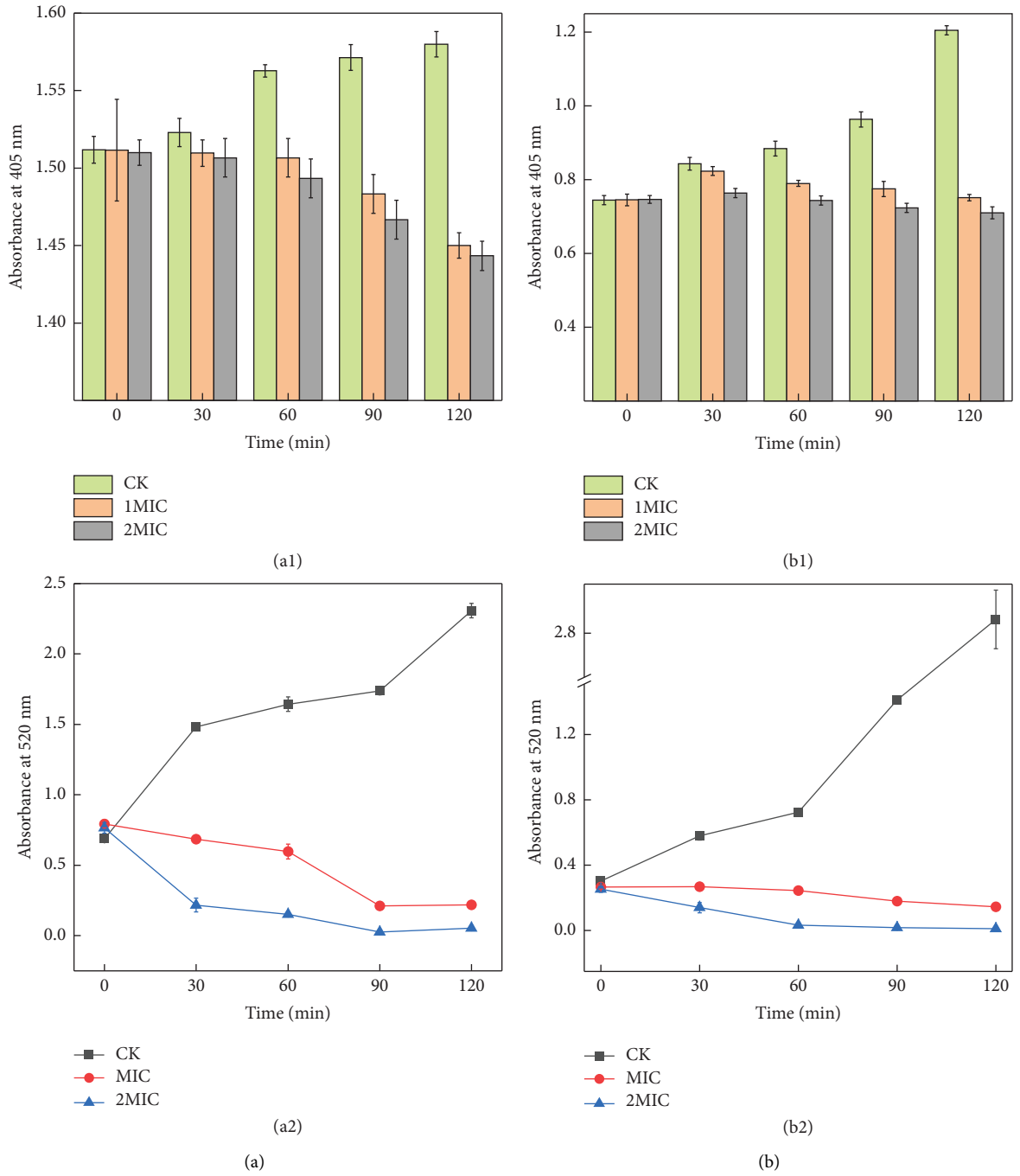

Figure 8: The influence of GBSPI-A on the β-galactosidase and AKP activity of bacteria. a1 and b1 represent the influence on the β-galactosidase activity of *K. pneumoniae* and *S. aureus*. a2 and b2 represent the influence on the intracellular AKP activity of *K. pneumoniae* and *S. aureus*. CK: control group, MIC: treated with 20 mg/mL GBSPI-A, and 2MIC: treated with 40 mg/mL GBSPI-A: (a) *K. pneumoniae* and (b) *S. aureus*.

with the control group along with incubation time, which demonstrated that the permeability of cell membrane could increase at the existence of GBSPI-A, further causing the leakage of internal material. Reference [32] studied that the addition of cottonseed protein could cause damage of cell membrane and the leakage of cytoplasmic.

The surface of bacteria treated with GBSPI-A was broken while that of the control group was intact from SEM images. The SEM image provided morphological evidence of GBSPI-A acting on the cell membranes, which was consistent with previous studies [33, 34]. The outer cell wall of bacteria treated with GBSPI-A was attached with many black objections from TEM images, and we speculated that may cause by the attach of GBSPI-A. The GBSPI-A attaches to the cell membrane of bacteria, causing the changes in the physicochemical property of the cell membrane, further

leading to the death of bacteria. The essence of respiratory metabolism is the oxidative degradation of sugar in organism, which not only provide the energy for vital movement but also supply the carbon skeleton and reducing power during the synthetic process of protein and lipid [35]. The respiratory of bacteria was inhibited to 7.24% upon the concentration of GBSPI-A reached 10 mg/mL. The restraint of respiratory metabolism could result in the abnormity of physiological metabolism, further inhibiting the growth of bacteria [36].

The changes of protein content in the protein group along with growth time indicated GBSPI-A could inhibit the synthesis of intracellular protein for bacteria, which could be caused by energy shortage produce by respiration and may also be caused by leakage of protein. Nucleic acids are a key macromolecule in the organism carrying unique genetic information. In this study, the content of DNA in the protein group decreased distinctly, while that in the control group increased gradually along with the incubation time. The DNA bands in the protein group were shallower than that in the control and not appeared trailer. Based on the results, the reason for decreasing DNA is may that the GBSPI-A could destroy the cell membrane leading to the leakage of DNA. It also may cause by the required energy of synthesis DNA being insufficient [36].

The ATPase activity is closely associated with energy metabolism and the balance of intracellular and extracellular ions [37], playing a crucial role in transmembrane transport and maintaining membrane permeability [38]. This study found that the ATPase activity in bacteria treated with GBSPI-A was significantly lower than that of the control group. ATP, as the most basic carrier of energy conversion, plays an important role in the energy metabolism of the organism and its content could reflect the survival status of bacteria [17, 39]. The content of ATP appeared similar tendency with the prolong of incubation time compared with the change of ATPase activity. This study indicated that GBSPI-A may restrain the respiration by decreasing the activity of ATPase, causing the reducing synthesis of ATP, further leading to energy shortage in the synthetic process of macromolecule.

Enzymes participate in the majority chemical reaction in the internal metabolic system and their activity are regulated by multifactor to adapt the changes of external conditions. The β-galactosidase, the main supplier of energy for microorganism, provides energy by catalyzing lactose to monosaccharide [40]. The AKP exists between the cell wall and membrane, whose activity could not detect outside the cell under normal conditions. The addition of GBSPI-A resulted in the decreasing of activity on β-galactosidase and AKP, which may be caused by the inhibition of GBSPI-A on protein synthesis. The inhibition of β-galactosidase activity could generate abnormal energy metabolism and further affect the vital movement of bacteria. The reason of decreasing activity on AKP may also be caused by that when the cell wall was damaged, AKP outflows the cell walls [41] causing the decreasing of intracellular AKP and increasing of extracellular protein, which was in agreement with the result of protein leakage. The result showed that the inhibition effect of GBSPI-A on *K. pneumoniae* was better than that of GBSPI-A on *S. aureus*, which was attributed to the thick peptidoglycan layer of *S. aureus* [42].

Finally, GBSPI-A caused the decreasing activity of ATPase and respiratory rate, leading to the reducing synthesis of ATP. Then, GBSPI-A inhibited the activity of β-galactosidase, which further made abnormal energy metabolism and not satisfied the needs of physiological metabolic, resulting in the growth inhibition of bacteria. When bacteria were attacked, bacteria did not make the normal repair mechanism causing the damaged cell membrane and the leakage of intracellular content, which led to the death of bacteria.

5. Conclusion

This study showed that GBSPI-A had an effective antibacterial activity on *K. pneumoniae* and *S. aureus*. The result from SEM and TEM indicated that GBSPI-A (20 mg/mL) damaged the cell membrane, causing the leakage of nucleic acid, protein, and Na^+ and further causing the inhibiting bacterial growth with an inhibition rate of approximately 80%. Simultaneously, GBSPI-A caused the decreasing activity of ATPase and respiratory rate, leading to the reducing synthesis of ATP. GBSPI-A also inhibited the β-galactosidase activity, which further causes the insufficient energy in the synthetic process of macromolecule and the abnormal physiological activity of bacteria. In short, this study indicated that GBSPI-A could be the natural inhibitor against *K. pneumoniae* and *S. aureus*.

Data Availability

Previously reported data were used to support this study and these prior studies (and datasets) are cited at relevant places within the text as references.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ting Xu conceptualized the study, proposed the methodology, provided software, wrote the original draft, performed data curation, and investigated the study. Haixia Wu proposed the methodology, performed data collection, and investigated the study. Caie Wu proposed the methodology, provided resources, supervised the study, was responsible for funding acquisition, and reviewed and edited the manuscript. Gongjian Fan validated the study, proposed the methodology, wrote the original draft, conceptualized the study, and investigated the study. Tingting Li was responsible for resources, supervised the study, and responsible for funding acquisition. Dongbei Shen performed data curation and provided software. Yue Pan validated and investigated the study.

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Supplementary Materials

Table 1: Mascot search results of tryptic digested purified protein determinated using LC-MS-MS. Figure 1: agarose gel electrophoresis of DNA from bacteria treated with GBSPI-A at different times. (a) *K. pneumoniae* was treated with GBSPI-A at different time and (b) *S. aureus* treated with GBSPI-A at different time. The time of DNA bands treated with GBSPI-A from left to right was 0, 30, 60, 90, and 120 min, respectively. (*Supplementary Materials*)

References

[1] E. Malik, S.-R. Dennison, F. Harris, and D.-A. Phoenix, "pH dependent antimicrobial peptides and proteins, their mechanisms of action and potential as therapeutic agents," *Pharmaceuticals*, vol. 9, no. 4, p. 67, 2016.

[2] A. Soares, K. Alexandre, and M. Etienne, "Tolerance and persistence of *Pseudomonas aeruginosa* in biofilms exposed to antibiotics: molecular mechanisms, antibiotic strategies and therapeutic perspectives," *Frontiers in Microbiology*, vol. 11, Article ID 2057, 2020.

[3] M. Wang, K.-W. Zheng, J.-L. Lin et al., "Rapid and efficient production of cecropin A antibacterial peptide in *Escherichia coli* by fusion with a self-aggregating protein," *BMC Biotechnology*, vol. 18, no. 1, p. 62, 2018.

[4] R. Bals, "Epithelial antimicrobial peptides in host defense against infection," *Respiratory Research*, vol. 1, no. 3, pp. 14–150, 2000.

[5] D. Hultmark, "*Drosophila* immunity: paths and patterns," *Current Opinion in Immunology*, vol. 15, no. 1, pp. 12–19, 2003.

[6] Z. Wang and G.-S. Wang, "APD: the antimicrobial peptide database," *Nucleic Acids Research*, vol. 32, no. 90001, pp. D590–D592, 2004.

[7] L.-N. Shwaiki, K.-M. Lynch, and E.-K. Arendt, "Future of antimicrobial peptides derived from plants in food application-A focus on synthetic peptides," *Trends in Food Science & Technology*, vol. 112, pp. 312–324, 2021.

[8] M.-L. Mangoni, "Host-defense peptides: from biology to therapeutic strategies," *Cellular and Molecular Life Sciences*, vol. 68, no. 13, pp. 2157–2159, 2011.

[9] A.-B. Schafer and M. Wenzel, "A how-to guide for mode of action analysis of antimicrobial peptides," *Frontiers in Cellular and Infection Microbiology*, vol. 10, Article ID 540898, 2020.

[10] S. Mahgoub, M. Alagawany, M. Nader et al., "Recent development in bioactive peptides from plant and animal products and their impact on the human health," *Food Reviews International*, pp. 1–26, Article ID 192307, 2021.

[11] D. Agyei, "Bioactive proteins and peptides from soybeans," *Recent Patents on Food, Nutrition & Agriculture*, vol. 7, no. 2, pp. 100–107, 2015.

[12] J. Noonan, W.-P. Williams, and X. Shan, "Investigation of antimicrobial peptide genes associated with fungus and insect resistance in maize," *International Journal of Molecular Sciences*, vol. 18, no. 9, p. 1938, 2017.

[13] R. Zhang, X.-B. Fan, X.-L. Jiang, M.-Y. Zou, H. Xiao, and G.-Q. Wu, "Multiple mechanisms of the synthesized antimicrobial peptide TS against Gram-negative bacteria for high efficacy antibacterial action in vivo," *Molecules*, vol. 26, no. 1, p. 60, 2020.

[14] W. Hai-xia, W. Cai-e, F. Gong-jian, L. Ting-ting, Y. Rui-feng, and H. Jing, "Purification and antimicrobial activity of protein from ginkgo biloba seed kernels," *Food Chemistry*, vol. 35, no. 13, pp. 108–113, 2014.

[15] L. Misurcova, F. Bunka, J. Vávra Ambrožová, L. Machu, D. Samek, and S. Kracmar, "Amino acid composition of algal products and its contribution to RDI," *Food Chemistry*, vol. 151, pp. 120–125, 2014.

[16] J. Huang, H. Liu, and F. Xu, "Two-dimensional divisor problems related to symmetric *L*-functions," *Symmetry*, vol. 13, no. 2, p. 359, 2021.

[17] Y. Han, Z. Sun, and W. Chen, "Antimicrobial susceptibility and antibacterial mechanism of limonene against *listeria monocytogenes*," *Molecules*, vol. 25, no. 1, p. 33, 2019.

[18] F. Zhang, X.-W. Cui, Y.-R. Fu et al., "Antimicrobial activity and mechanism of the human milk-sourced peptide Casein201," *Biochemical and Biophysical Research Communications*, vol. 485, no. 3, pp. 698–704, 2017.

[19] Z.-L. Sun, X.-M. Wang, X.-X. Zhang et al., "Class III bacteriocin Helveticin-M causes sublethal damage on target cells through impairment of cell wall and membrane," *Journal of Industrial Microbiology and Biotechnology*, vol. 45, no. 3, pp. 213–227, 2018.

[20] J.-H. Yoo, K.-H. Baek, Y.-S. Heo, H.-I. Yong, and C. Jo, "Synergistic bactericidal effect of clove oil and encapsulated atmospheric pressure plasma against *Escherichia coli* O157:H7 and *Staphylococcus aureus* and its mechanism of action," *Food Microbiology*, vol. 93, Article ID 103611, 2021.

[21] H.-J. Lee, G.-J. Choi, and K.-Y. Cho, "Correlation of lipid peroxidation in *Botrytis cinerea* caused by dicarboximide fungicides with their fungicidal activity," *Journal of Agricultural and Food Chemistry*, vol. 46, no. 2, pp. 737–741, 1998.

[22] S. Zheng, G. Jing, X. Wang, Q. Ouyang, L. Jia, and N. Tao, "Citral exerts its antifungal activity against *Penicillium digitatum* by affecting the mitochondrial morphology and function," *Food Chemistry*, vol. 178, no. 1, pp. 76–81, 2015.

[23] M.-M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[24] Z. Li, R. Luo, Y. Zhang, X. Yan, and Q. Pang, "Effective protein extraction from mycelium and fruiting body of *Auricularia auricula* for proteomics studies," *International Journal of Food Properties*, vol. 21, no. 1, pp. 2156–2166, 2018.

[25] G. Tong, M. Yulong, G. Peng, and X. Zirong, "Antibacterial effects of the Cu(II)-exchanged montmorillonite on *Escherichia coli* K88 and *Salmonella choleraesuis*," *Veterinary Microbiology*, vol. 105, no. 2, pp. 113–122, 2005.

[26] S. Abdel-Shafi, A.-R. Al-Mohammadi, A. Osman, G. Enan, S. Abdel-Hameid, and M. Sitohy, "Characterization and antibacterial activity of 7S and 11S globulins isolated from cowpea seed protein," *Molecules*, vol. 24, no. 6, p. 1082, 2019.

[27] S.-F. Taghizadeh, R. Rezaee, M. Mehmandoust, H. Badibostan, and G. Karimi, "Assessment of in vitro bioactivities of Pis v 1 (2S albumin) and Pis v 2.0101 (11S

globulin) proteins derived from pistachio (*Pistacia vera L.*)," *Journal of Food Measurement and Characterization*, vol. 14, no. 2, pp. 1054–1063, 2020.

[28] Y. Luo and Y. Song, "Mechanism of antimicrobial peptides: antimicrobial, anti-inflammatory and antibiofilm activities," *International Journal of Molecular Sciences*, vol. 22, no. 21, Article ID 11401, 2021.

[29] J. Wang, B. Li, Y. Li et al., "BF-30 effectively inhibits ciprofloxacin-resistant bacteria in vitro and in a rat model of vaginosis," *Archives of Pharmacal Research*, vol. 37, no. 7, pp. 927–936, 2014.

[30] A. Lorents, P. Saalik, U. Langel, and M. Pooga, "Arginine-richcell-penetrating peptides require nucleolin and cholesterol-poor subdomains for translocation across membranes," *Bioconjugate Chemistry*, vol. 29, no. 4, pp. 1168–1177, 2018.

[31] S. Pei, R. Liu, H. Gao et al., "Inhibitory effect and possible mechanism of carvacrol against *Colletotrichum fructicola*," *Postharvest Biology and Technology*, vol. 163, Article ID 111126, 2020.

[32] X. Kong, W. Song, Y. Hua et al., "Insights into the antibacterial activity of cottonseed protein-derived peptide against *Escherichia coli*," *Food & Function*, vol. 11, no. 11, Article ID 10047, 2020.

[33] Y. Lyu, Y. Yang, X. Lyu, N. Dong, and A. Shan, "Antimicrobial activity, improved cell selectivity and mode of action of short PMAP-36-derived peptides against bacteria and *Candida*," *Scientific Reports*, vol. 6, no. 1, Article ID 27258, 2016.

[34] L. Xu, S. Chou, J. Wang et al., "Antimicrobial activity and membrane-active mechanism of tryptophan zipper-likebeta-hairpin antimicrobial peptides," *Amino Acids*, vol. 47, no. 11, pp. 2385–2397, 2015.

[35] W. Jingyan, Z. Shenggeng, and X. Changfa, *Biochemistry*, Higher Education Press, Beijing, China, 3 edition, 2002.

[36] H. Cui, C. Zhang, C. Li, and L. Lin, "Antimicrobial mechanism of clove oil on Listeria monocytogenes," *Food Control*, vol. 94, pp. 140–146, 2018.

[37] G. Kragol, S. Lovas, G. Varadi, B.-A. Condie, R. Hoffmann, and L. Otvos, "The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding," *Biochemistry*, vol. 40, no. 10, pp. 3016–3026, 2001.

[38] P. Lebrun, W.-J. Malaisse, and A. Herchuelz, "Na^+--K^+ pump activity and the glucose-stimulated Ca^{2+}-sensitive K^+ permeability in the pancreatic B-cell," *Journal of Membrane Biology*, vol. 74, no. 1, pp. 67–73, 1983.

[39] D. Spari and G. Beldi, "Extracellular ATP as an inter-kingdom signaling molecule: release mechanisms by bacteria and its implication on the host," *International Journal of Molecular Sciences*, vol. 21, no. 15, p. 5590, 2020.

[40] A.-L. Watson and N.-H.-L. Chiu, "Fluorometric cell-based assay for β-galactosidase activity in probiotic gram-positive bacterial cells — *Lactobacillus helveticus*," *Journal of Microbiological Methods*, vol. 128, pp. 58–60, 2016.

[41] J.-W. Sheng, D.-M. Liu, L. Jing et al., "Striatisporolide A, a butenolide metabolite from Athyrium multidentatum (Doll.) Ching, as a potential antibacterial agent," *Molecular Medicine Reports*, vol. 20, no. 1, pp. 198–204, 2019.

[42] M. Varghese and M. Balachandran, "Antibacterial efficiency of carbon dots against Gram-positive and Gram-negative bacteria: a review," *Journal of Environmental Chemical Engineering*, vol. 9, no. 6, Article ID 106821, 2021.