Antibacterial mechanism of the Asp-Asp-Asp-Tyr peptide

Shanshan Zhuang a, 1, Yao Bao a, 1, Yaxin Zhang a, Huangyou Zhang a, Jianliang Liu b, c, Huifan Liu a, d, *

a College of Light Industry and Food, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong 510225, China
b Guangzhou Key Laboratory for Research and Development of Crop Germplasm Resources, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China
c Modern Agriculture Research Center, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong 510225, China
d Guangdong Key Laboratory of Science and Technology of Lingnan Specialty Food, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong 510225, China

ARTICLE INFO

Keywords:
Antibacterial mechanism
Peptide
Dendrobium aphyllum
DDDY
Multi omics
Molecular dynamics
Pseudomonas aeruginosa

ABSTRACT

Previously, we found that ASP-ASP-TYR (DDDY) from Dendrobium aphyllum has a minimum inhibitory concentration of 36.15 mg/ml against Pseudomonas aeruginosa. Here, we explored the antibacterial mechanism of DDDY and its potential preservation applications. Metabolomic and transcriptomic analyses revealed that DDDY mainly affects genes involved in P. aeruginosa membrane transport and amino acid metabolism pathways. Molecular dynamics simulation revealed that DDDY had a stronger effect on 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine phospholipid membranes than on 1-palmitoyl-2-oleoyl-lecithin or 1-palmitoyl-2-oleoyl phosphatidylglycerol membranes, with high DDDY concentrations displaying stronger efficacy on 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine. Mechanistically, the N-terminal of DDDY first bound to the phospholipid head group, while its C-terminal amino acid residue bound the hydrophobic tail, thereby creating a gap in the membrane when the phospholipids were clustered by hydrogen bonding. Finally, DDDY inhibited the growth of food microorganisms inoculated onto chestnut kernels, suggesting that DDDY is a promising antibacterial agent against multidrug-resistant gram-negative bacteria.

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen of humans, other animals, plants, and lower eukaryotes (Small, Chang, Toghrol, & Bentley, 2007), whose pathogenic mechanism generally involves the formation of reactive oxygen species (ROS), which cause DNA and lipid damage (Chang, Small, Toghrol, & Bentley, 2005). Due to its strong adaptability, rapid reproduction, and wide distribution in the environment, P. aeruginosa can also cause food safety problems (Xu, Xie, Soteyome, Peters, Shirtliff, Liu, et al., 2019); therefore, it is important to identify molecules with antibacterial activity against P. aeruginosa. David et al. found that sodium hypochlorite can exert antibacterial effects against P. aeruginosa through oxidative stress, oxidative phosphorylation, and organic sulfur metabolism (Small, Chang, Toghrol, & Bentley, 2007). Similarly, Chang et al. reported that peracetic acid can affect genes, amino acids, fatty acids, central intermediates, energy metabolism, membrane proteins, and the cell protection processes of P. aeruginosa (Chang, Small, Toghrol, & Bentley, 2005).

Dendrobium aphyllum is an orchid variety that is used as a herb in both medicinal and food preparations (Zi, Sheng, Goodale, Shao, & Gao, 2014). Indeed, studies have shown that Dendrobium plants contain diverse bioactive substances, including alkaloids, polysaccharides, proteins, peptides, and sesquiterpenoids, which can exert antioxidant, immunomodulatory, anti-inflammatory, and anti-cancer effects (Huifan Liu, Ma, Gong, Wei, Zhang, & Wu, 2018; Wang, Liang, Brennan, Ma, Li, Lin, et al., 2019). Previously, a co-culture system was established in which Dendrobium aphyllum was fermented by Lactobacillus amylyticus L6 and produced highly concentrated peptide fractions, from which the peptides DDDY (Asp-Asp-Asp-Tyr) and DYDD (Asp-Tyr-Asp-Asp) were isolated and identified (Huifan Liu, Ma, & Wu, 2017). Interestingly, these peptides were found to have antioxidant-related immune functions and were completely degraded by Caco-2 cells (Huifan Liu, Wu, & Wang, 2017).
2. Materials and methods

2.1. Materials

_Pseudomonas aeruginosa_ (ATCC 9027) was obtained from the Laboratory of Food Science and Engineering at the South China University of Technology (Guangzhou, China). DDDY was synthesized by the Shanghai Science Peptide Biological Technology Co., Ltd (Shanghai, China). Trypsin soy broth (TSB) was purchased from Guangdong Technology (Guangzhou, China). Phosphate buffered saline (PBS) was purchased from Beijing Huankai Microbial Science and Technology Co., Ltd (Guangzhou, China). Trypsin soy broth (TSB) was purchased from Guangdong Technology (Guangzhou, China). Phosphate buffered saline (PBS) was purchased from Beijing Leigen Biotechnology Co., Ltd (Beijing, China).

2.2. Sample preparation

_P. aeruginosa_ was incubated in TSB at 37 °C for 8 h (Wang, Cui, Zhang, Yang, & Zhang, 2018). During the logarithmic phase, 200 μL of bacterial solution was added to 200 mL of liquid medium, 200 μL of sterile water (control group; P_CK), or 50 mg/mL of DDDY solution (treatment group; P_DDDY). The bacteria were then cultured on a shaking table at 150 R/min and 37 °C for 24 h, centrifuged at 6000 R/min and 4 °C for 10 min, washed with PBS three times, and then collected and stored at −80°C.

2.3. Omics analysis

2.3.1. Metabolite preparation and analysis

Metabolites were extracted using methanol to precipitate protein overnight, followed by centrifugation 4000g for 20 min and preservation at −80°C. The samples were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a TripleTOF5600plus high-resolution tandem mass spectrometer (SCIEX). Detailed information regarding sample preparation and analysis can be found in Supplementary Text S1.

2.3.2. Transcriptome analysis

Total RNA was extracted from the cells using TRIzol Reagent according to the manufacturer’s instructions (Invitrogen, New York, USA) and genomic DNA was removed using DNase I (TaKara, San Francisco, USA). Strand-specific RNA-seq libraries were prepared with 5 μg of total RNA using a TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA). Paired-end libraries were sequenced using an Illumina NovaSeq 6000 (150 bp*2). Detailed information regarding library construction, sequencing, and analysis can be found in Supplementary Text S2.

2.4. Interaction analysis between DDDY and phospholipid membranes

POPC, POPG, and POPE phospholipid bilayers were generated using VMD1.9.2 visual molecular dynamics software. The structures and force fields of the phospholipid membranes were obtained and an initial simulation system was established as described previously (Liu, et al., 2021).

2.5. Effect of DYDD on the quality of cooked chestnut kernels

2.5.1. Preparation of DYDD preservative

A 2 mg/mL solution of DDDY was prepared using sterile water in a sterile spray bottle and a nozzle bottle for use as a spray and immersion preservative, respectively.

2.5.2. Preparation of bacterial suspension

Logarithmic phase _P. aeruginosa_ solution was centrifuged at 4000 R/min and 20 °C for 10 min and then the supernatant was discarded and resuspended with 100 mL of sterile water. The initial concentration of the suspension was determined using the plate counting method.

2.5.3. Sample preparation and bacterial inoculation

Two types of cooked chestnut kernel samples were obtained by boiling fresh chestnut shells. Samples with good appearance and similar size were sterilized under ultraviolet light for 20 min and soaked in the _P. aeruginosa_ suspension for 30 s. After 40 min the samples were placed on a sterile surgical table and sprayed or soaked in the DYDD preservative for 30 s. The samples were then ventilated and dried in a sterile console for 40 min. Chestnut kernels without DDDY treatment were used as a blank group (control boiled). The samples were stored in a sterile polyethylene preservation bag in an incubator (BPS-50CL, Shanghai Yiheng Scientific Instrument Co., Ltd, Shanghai, China) at a constant temperature and humidity of 37 °C and 80%, respectively. Samples were removed at 0, 4, 8, 12, and 16 h respectively.

Bacteria on the surface of the chestnut kernels were counted according to the method of Fan et al., with some modifications (Fan, Zhang, Bhandari, & Jiang, 2019). Briefly, each chestnut kernel was placed into a sterile sampling bag with 10 mL sterile water and shaken in a vortex mixer (QL-906, Beijing Tianchuang Shangbang Instrument Equipment Co., Ltd, Beijing, China) for 1 min. After the solution had been serially diluted (10 × ) in PBS, 1 mL was plated on the surface of petri dishes containing plate count agar, incubated at 37 °C for 48 h, and the total number of colonies was counted (Cao, Hu, Pang, Wang, Xie, & Wu, 2010).

2.5.4. Changes in chestnut kernel quality, nutrients, and enzyme activity

Color and weight were measured as described previously (Lu, Wang, Cai, Zhou, & Zhu, 2010). Briefly, the chromatic characteristics of each sample were estimated using a colorimeter (CR-400, Konica Minolta, Japan) and they were weighed every 4 h. Results were expressed as a percentage. Water migration in each chestnut kernel was detected using a low-field pulsed nuclear magnetic resonance (NMR) analyzer (Mesor23-040H-1, Suzhou Niumag Co., Ltd., China) as described previously (Bao, Kang, Li, Zhang, & Lin, 2020) with some modifications. The basic parameters of the instrument were as follows: magnet strength, 0.5 T; permanent magnet temperature, 32 °C; main frequency, 23.3127 MHz. The relaxation analysis parameters were: main frequency, 21 MHz; repetition time, 1000 ms; 90° pulse time, 10.00 μs; 180° pulse time, 19.04 μs; radio frequency delay(RFD), 0.3 ms; analog gain, 20; digital gain, 1; preamplifier gain, 1; accumulation time (ns) =
4; echo time, 0.10 ms; echo number, 5000. The imaging parameters were: field of view (FOV) read, 100 mm; FOV phase, 100 mm; repeat waiting time, 1500 ms; echo time, 20 ms; slices, 7; slice width, 3 mm; average, 4.

The starch content of the chestnut kernel was determined using a starch content kit (Solarbio, USA), while the protein content was determined using the Coomassie brilliant blue method (Chen, Zhu, & Han, 2011). Peroxidase (POD) and polyphenol oxidase (PPO) activity were determined as described previously (Fan, Zhang, Bhandari, & Jiang, 2019).

2.6. Data processing and analysis

All experimental results were expressed as the mean ± standard deviation (SD). Significant differences between two groups were indicated using the descriptors a, b, c, and d (p < 0.5). Data were analyzed using one-way ANOVA tests in SPSS v19.0 (IBM, USA). Charts were prepared using Origin 2018 (OriginLab, USA). Multi-dimensional statistical analysis was performed using MetaboAnalyst software (USA). Data were also analyzed using supervised partial least squares discriminant analysis (PLS-DA) methods.

3. Results

3.1. Effect of DYDD on P. aeruginosa metabolism

It is well known that treatment with drugs can alter the metabolic activity of bacteria (Shu, Zhang, Yun, Chen, Zhong, Hu, et al., 2020). Here, mass spectrometry-based metabolomic analyses revealed that DYDD treatment significantly affected the metabolism of P. aeruginosa (Fig. 1). Pseudomonas aeruginosa samples treated with or without DYDD were divided into two groups based on their distinctive PLS-DA plot profiles (Fig. 1A). Multivariate and univariate statistical analyses identified 217 molecular features that differed significantly between the two groups (p < 0.1 and VIP > 1; Fig. 1B), among which 116 were upregulated and 101 were downregulated (Fig. 1C). These metabolites were predominantly involved in alpha-linolenic acid, porphyrin, chlorophyll, and galactose metabolism, as well as the biosynthesis of neomycin, kanamycin, gentamicin, carotenoid, ansamycins, terpenoids, steroids, phenylpropanoids, secondary metabolites, and antibiotics (Fig. 1D).

3.2. Effect of DDDY on the P. aeruginosa transcriptome

To determine the effect of DDDY on the transcriptome of P. aeruginosa, we performed transcriptome sequencing on 10 cDNA
libraries using total RNA extracted from the control group and the group treated with DDDY. After the raw data had been filtered and had passed quality control, the clean data for each sample (n>2.77 Gb) had a GC content of > 60.47% and a Q30 base of > 96.60% (Table 1). These high-quality reads were selected for further analysis, which revealed that DDDY treatment altered the expression of 143 genes by at least twofold (57 upregulated and 86 downregulated; Fig. 2A). All differentially expressed genes (DEGs) between the P-DDDY and P-CK groups were subjected to Gene Ontology (GO) functional analysis. The DEGs were enriched for cellular components, molecular functions, and biological processes in P. aeruginosa that were subdivided into seven functional groups (Fig. 2B): cellular processes, metabolic process, cellular anatomical entity, biological regulation, intracellular, catalytic activity, and binding. Further Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis assigned 23 DEGs to 20 KEGG pathways that were mainly associated with “metabolic pathways”, “two-component systems”, and “ABC transporters” (Fig. 2C). The majority of the DEGs were involved in metabolic pathways, including cysteine and methionine metabolism (one upregulated DEG and one downregulated DEG), arginine and proline metabolism (three upregulated DEGs), tyrosine metabolism (two upregulated DEGs), and phenylalanine metabolism (one upregulated DEG). Together, these results indicate that DDDY alters amino acid metabolism.

3.3. Effects of DDDY on different phospholipid membranes

To exert their antibacterial activity, peptides must first interact with the external membrane of the bacterial cell (Papo & Shai, 2003). Here, we analyzed the effects of DDDY on different phospholipid membranes using molecular simulation. At the beginning of the simulation, DDDY molecules adhered to the membrane and caused the phospholipid molecules to become slightly disordered (Fig. 3A). As the simulation progressed, DDDY became closely combined with the POPE membrane and its Asp residues inserted into the phospholipid molecular layer (PML), causing the phospholipids to become even more disordered. In POPC membranes, DDDY inserted Tyr residues into the PML at 100 ns. Conversely, DDDY molecules did not insert themselves into the PML of POPG membranes but were still able to disorder the phospholipid molecules.

After DDDY treatment, the single molecular area of the POPC and POPG membranes fluctuated by 0.7–0.8 nm², whereas that of the POPE membrane only fluctuated by 0.6–0.7 nm² (Fig. 3B). Similarly, the MSD was higher in the POPE membrane (66.51 nm²) than in the POPC or POPG membranes (Fig. 3C). The hydrogen bond ratio formed by the interaction between DDDY and the POPC membrane was higher than for either the POPG or POPE membranes (Fig. 3D). In addition, when DYDD interacted with the POPC membrane, the Coulomb force (CF) and VDW forces ranged from 25 ~ 400 kJ/mol and 10 ~ 50 kJ/mol, respectively. When interacting with the POPE membrane, DYDD had little effect on VDW forces (-10 ~ 10 kJ/mol) but the CF had a large fluctuation range of 25 ~ -1250 kJ/mol. Similarly, DDDY had no obvious effect on VDW forces when interacting with the POPG membrane, yet a CF was observed during the later stages of its interaction with DDDY (Fig. 3E).

Taken together, these results indicate that DDDY exerts different effects on different phospholipid membranes, with a particularly weak effect on POPC and POPG membranes and a stronger effect on POPE membranes.

3.4. Preservation effect of DDDY on cooked chestnut kernels

Next, we investigated the effect of DDDY as an immersive or sprayed preservative agent against bacterial infections on the surface of cooked chestnut kernels

3.4.1. Total bacterial count

First, we determined the effect of different DDDY treatment methods on the total number of colonies on chestnut kernels inoculated with P. aeruginosa and stored for 16 h (Fig. 4A). Although the number of colonies on the surface of chestnuts inoculated with P. aeruginosa increased significantly with time (p < 0.05), the number first decreased and then increased on chestnuts treated with DDDY, indicating that DDDY can kill bacteria.

3.4.2. Wet, loss and color

As seen in Fig. 4B, chestnut kernels treated with DDDY displayed a significantly lower rate of weight loss than the control kernels (p < 0.05). In particular, those kernels sprayed with DDDY had higher weight loss than those soaked with DDDY, indicating that soaking was better than spraying. The score changes for color during storage are shown Table 2. Chestnut kernels in the control group darkened, turned red, and then gradually turned blue with prolonged storage, whereas those treated with DDDY showed no significant change, indicating that DDDY can prevent chestnut browning during storage. In addition, a film layer formed on the surface chestnuts in the control group after storage for 4 h but only appeared after 12 h on those treated with DDDY (Fig. 4E), indicating that bacteria grew rapidly on the surface of chestnuts in the control group and that their growth was effectively inhibited by DDDY.

3.4.3. Starch and protein content

During storage, the starch (Fig. 4C) and protein (Fig. 4D) contents of the control chestnut kernels decreased significantly by 85.22% and 81.91%, respectively (p < 0.05). However, the rate of starch loss was much lower in kernels soaked with DDDY (63.65%), as were the rates of protein loss rates in chestnuts that had been sprayed and soaked with DDDY (75.95% and 66.85% respectively). Thus, DDDY may alleviate protein and starch degradation in chestnut kernels.

3.4.3. Water migration

In the control group, we observed significant differences in the free and bound water contents of chestnut kernels before and after storage (Fig. 4F-H). In particular, free water appeared to become bound water by migrating from the whole fruit to its core, thereby increasing the internal decay area (Fig. 4I). However, the water content of chestnut kernels treated with DDDY did not change significantly and nor did the internal decay area. Taken together, these results suggest that DDDY could inhibit the internal decay of chestnuts contaminated with bacteria and prolong their shelf life.

4. Conclusion

In recent years, multi-omics technology has emerged as a powerful tool for studying bacterial metabolism (Rinschen, Ivanisevic, Gieri, & Siuzdak, 2019). Here, we used metabolome and transcriptome analyses to reveal that DDDY can cause numerous metabolic disorders in P. aeruginosa that are mainly related to pathways involved in membrane transport and amino acid metabolism (Fig. 5). On the extracellular membrane, LhpP and amino acid ABC transporter substrate-binding protein lhpN, which are related to hydroxyproline (Li & Lu, 2016),

Table 1. Sample sequencing data statistics.

| Samples   | Total_Reads | Total_Bases | Q20% | Q30% | GC%  |
|-----------|-------------|-------------|------|------|------|
| P-CK1     | 29588038    | 4108527374  | 99.11 | 96.72 | 61.25 |
| P-CK2     | 22927736    | 3165453856  | 99.06 | 96.57 | 60.52 |
| P-CK3     | 2373416     | 32499066281 | 99.08 | 96.62 | 61.11 |
| P-CK4     | 20918716    | 2900892733  | 99.07 | 96.60 | 61.19 |
| P-DDDY1   | 22497930    | 3074434026  | 99.13 | 96.76 | 61.27 |
| P-DDDY2   | 20526164    | 2815221237  | 99.12 | 96.74 | 60.47 |
| P-DDDY3   | 29780918    | 4105570514  | 99.15 | 96.85 | 61.08 |
| P-DDDY4   | 36077400    | 5109166131  | 99.16 | 96.73 | 61.01 |
| P-DDDY5   | 3270008     | 4491507324  | 99.12 | 96.76 | 60.52 |
| P-DDDY5   | 20032902    | 2770894852  | 99.07 | 96.61 | 60.98 |
were downregulated, whereas livF, which is related to branched-chain amino acids, and PotF, which is related to putrescine, were upregulated. In addition, the TolC gene was downregulated, which may reduce RTX toxin release as the protein expressed by TolC is an important carrier that transports intracellular RTX to the extracellular environment (Linhartova, Osicka, Bumba, Masin, & Sebo, 2018). Thus, DDDY may reduce the toxicity of \textit{P. aeruginosa} and prevent it from causing infection. In addition, KdpC, a signal transduction gene that regulates potassium transport and is essential for ATP hydrolysis, was also downregulated, indicating that DDDY can alter the potential

![Fig. 2.](image)

**Fig. 2.** Differential transcriptome analysis of \textit{Pseudomonas aeruginosa} treated with DDDY. (A) Volcano maps of DEGs in \textit{P. aeruginosa} treated with DDDY. (B) GO analysis of DEGs. (C) KEGG enrichment analysis of DEGs.

![Fig. 3.](image)

**Fig. 3.** Molecular dynamics analysis of DDDY-membrane interactions. (A) Simulated action track of DDDY (indicated by red (Asp) and green (Tyr) molecules) on POPC-, POPE-, and POPG-based lipid membranes (indicated by red spheres and gray chains). (B) Changes in the area of single phospholipid molecules during DDDY interactions with the different membranes. (C) Changes in MSD values during the simulation. (D) Number of hydrogen bonds formed between DDDY and the different membranes. (E) Changes in the interaction energies between DDDY and the different membranes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
balance of the cell membrane (Liu, et al., 2021; Ali, Li, Tang, Liu, Chen, Xiao, et al., 2017). However, the upregulation of TctB may promote the inhibition of *P. aeruginosa* degradation products as *P. aeruginosa* prefers non-fermentable carbon sources with low catabolic energy, such as succinate (McGill, Yung, Hunt, Henson, Hanley, & Carlson, 2021). These carbon sources act as intermediates in the tricarboxylic acid cycle and thus disrupt metabolic balance. Furthermore, we reported that DDDY upregulated the MCP gene in the cell membrane, which is related to

---

**Fig. 4.** Changes in the quality of ripe chestnut kernels inoculated with *Pseudomonas aeruginosa* and treated with DDDY during storage. (A) Number of bacterial colonies formed on the chestnut surface. (B) Changes in the weight of the chestnuts. (C&D) Changes in the (C) starch and (D) protein contents of the chestnuts. (E) Changes in the appearance of the chestnuts. (F-I) Changes in water migration in the chestnuts.
Food Chemistry: X 13 (2022) 100229

Effects of DDDY on the color of cooked chestnut kernel.

|      | L     | a     | b     | ΔE   |
|------|-------|-------|-------|------|
| CK-P (Cook)                  |       |       |       |      |
| 0 h  | 45.81 ± | 4.65 ± | 22.31 ± | 52.64 ± |
| 4 h   | 42.33 ± | 5.87 ± | 21.97 ± | 55.86 ± |
| 8 h   | 44.22 ± | 8.82 ± | 9.79 ± | 3.95 ± |
| 12 h  | 39.47 ± | 7.70 ± | 17.41 ± | 56.63 ± |
| 16 h  | 36.90 ± | 0.76 ± | 1.90 ± | 4.61 ± |
| 20 h  | 36.94 ± | 6.32 ± | 16.30 ± | 59.54 ± |
| 24 h  | 5.15 ± | 0.95 ± | 1.19 ± | 4.81 ± |
| DDDY (Spray)                  |       |       |       |      |
| 0 h  | 54.65 ± | 9.18 ± | 39.14 ± | 55.19 ± |
| 4 h   | 52.41 ± | 9.37 ± | 35.88 ± | 55.01 ± |
| 8 h   | 51.25 ± | 9.09 ± | 25.38 ± | 54.99 ± |
| 12 h  | 49.79 ± | 9.58 ± | 33.25 ± | 55.46 ± |
| 16 h  | 45.95 ± | 9.31 ± | 31.22 ± | 57.84 ± |
| 20 h  | 7.74a | 0.86a | 11.03a | 21.4a |
| DDDY (Soak)                  |       |       |       |      |
| 0 h  | 50.76 ± | 8.92 ± | 32.37 ± | 53.81 ± |
| 4 h   | 46.54 ± | 8.80 ± | 28.66 ± | 55.64 ± |
| 8 h   | 41.52 ± | 9.49 ± | 25.11 ± | 58.62 ± |
| 12 h  | 41.14 ± | 9.20 ± | 24.33 ± | 58.88 ± |
| 16 h  | 6.75a | 3.39a | 8.50a | 2.9b |
| 24 h  | 40.78 ± | 9.74 ± | 22.56 ± | 58.41 ± |
| 48 h  | 4.64a | 2.53a | 6.69a | 1.59b |

Data are expressed as means as ± standard deviations (SD) of triplicate measurements. Different letters in the same column (a, b and c) are significantly different (P < 0.05) according to Tukey’s Multiple Range Test.

Fig. 5. Primary effects of DDDY on the metabolic pathways of Pseudomonas aeruginosa.

At low concentrations, DDDY had similar effects on POPC and POPG membranes, but had a strong effect on POPE membranes, suggesting that DDDY is more lethal against gram-negative bacteria than against gram-positive bacteria and fungi (Mason, Marquette, & Bechinger, 2007). To further explore the antibacterial mechanism of DDDY, we examined the effects of different concentrations of DDDY on POPE membranes. Contrary to previous reports (H. Liu, et al., 2021), we found that high concentrations of DDDY did not lead to significant fluctuations in the molecular area of the POPE membrane, but did decrease the MSD value as it has a stronger lateral diffusion limit (Ghafari, Rasooli, Khajeh, Dabirmanesh, & Owlia, 2020). However, we did confirm that the antibacterial activity of DDDY was better at a high concentration than at a low concentration (Jafari, Mehrnejad, & Doustdar, 2017). At high concentrations, DDDY mainly interacted with the membrane via VDW forces, whereas these interactions were mainly mediated by electrostatic forces at a low concentration. Throughout the simulation, we clearly observed that the N-terminal of DDDY binds to the phospholipid head group first, and then its C-terminal amino acid residue binds to the hydrophobic tail after 40 and 60 ns. Subsequently, the phospholipid molecules cluster due to hydrogen bonding, resulting in a gap in the membrane.

Pseudomonas aeruginosa is a globally-distributed bacterium that is...
often found in food (T. R. Chen, Wei, & Chen, 2011; McGill, Yung, Hunt, Henson, Hanley, & Carlson, 2021) and can pose a critical risk to public health (Shu, et al., 2020). To verify the antibacterial activity of DDDY, we applied the polypeptide to cooked chestnut kernels contaminated with P. aeruginosa and evaluated their quality during storage. As expected, DDDY had good antibacterial activity and was able to effectively prolong the storage time of the chestnuts. In addition, DDDY had good bacteriostatic effects and inhibited the growth of biofilms, whose roles in food safety are generally underestimated and poorly understood (She, Wang, Liu, Tan, Chen, Luo, et al., 2019; Xu, et al., 2019). An important index of chestnut preservation is the prevention of browning (Hwang, 2011). Here, we found that DDDY could preserve the color of chestnut kernels and improve their shelf life. In addition, DDDY was able to alleviate protein and starch degradation, as well as water evaporation and water fluidity.

In conclusion, we found that the antibacterial mechanism of the small molecular peptide DDDY from D. aphyllum against P. aeruginosa mainly involves the destruction of the normal physiological function of the cell membrane. In particular, transcriptome and metabolome analyses showed that DDDY affects the expression of P. aeruginosa genes related to membrane transport and amino acid metabolism pathways. Molecular dynamics analyses further demonstrated that DDDY affects the cell membrane by clustering phospholipid molecules to create gaps in the membrane. Finally, we found that DDDY exerts antibacterial effects against microorganisms growing on chestnut kernels. Thus, DDDY may be able to achieve considerable economic benefits by prolonging the shelf life of food.

Funding sources
The authors are grateful for the support of the Guangdong Key Laboratory of Science and Technology of Lingnan Specialty Food (2021B1212040013), Guangdong Heyuan National Agricultural Science and Technology Park Intensive Processing of agricultural and sideline products key technology transformation project (2019002) and Basic and applied research project of Guangzhou Basic Research Program in 2020 (20200203083).

Declaration of Competing Interest
The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Huifan Liu reports equipment, drugs, or supplies was products key technology transformation project. Jianliang Liu reports Technology Park Intensive Processing of agricultural and sideline provided by Guangdong Heyuan National Agricultural Science and Technology, 2019 (11), 1–7.

Li, G., & Lu, C. D. (2016). Molecular characterization of LhpR in control of hydroxyproline catabolism and transport in Pseudomonas aeruginosa PA01. Microbiology (Reading), 162(7), 1232–1242.

Linhardt, R. O., Stoka, R., Bamba, L., Masin, J., & Sebo, P. (2018). Repeats-in-Toxin (RTX) Toxins: A Review. Toxicology, 353–381.

Liu, H., Ma, J., Gong, F., Wei, F., Zhang, X., & Wu, H. (2018). Structural characterization and immunomodulatory effects of polysaccharides isolated from Dendrobium aphyllum. International Journal of Food Science & Technology, 53(5), 1185–1194.

Liu, H., Ma, J., & Wu, H. (2017). Detoxifying effects of ultrafiltration fractions of Dendrobium aphyllum peptides on chemical and AAPH-induced oxidative stress. Food Chemistry, 77(77), 48913–48924.

Liu, H., Wu, H., & Wang, Q. (2018). The Variations, Including Structures and Attenuation to Hemolysis, of Peptide Purified from Dendrobium aphyllum During In Vitro Gastro-Intestinal Digestion and Caco-2 Uptake and Transportation. International Journal of Peptide Research and Therapeutics, 24(4), 1316–1331.

Liu, H., Zhang, H., Wang, Q., Li, S., Liu, Y., Ma, L., … Sun, I. (2021). Mechanisms underlying the antimicrobial actions of the antimicrobial peptides Asp-Tyr-Asp and Asp-Asp-Tyr on Pseudomonas aeruginosa as a model microorganism for investigation of chemotactic behaviors in ecosystem. Journal of Bioscience and Bioengineering, 106(1), 1–7.

McGill, S. L., Yung, Y., Hunt, K. A., Henson, M. A., Hanley, L., & Carlson, R. P. (2021). Pseudomonas aeruginosa as a model microorganism for investigation of chemotactic behaviors in ecosystem. Journal of Bioscience and Bioengineering, 106(1), 1–7.
environment investigated by solid-state NMR and MD simulations. Biochimica et Biophysica Acta, Reviews on Biomembranes, 1828(2), 824-833.

Xu, Z., Xie, J., Soteyome, T., Peters, B. M., Shirtliff, M. E., Liu, J., & Harro, J. M. (2019). Polymicrobial interaction and biofilms between Staphylococcus aureus and Pseudomonas aeruginosa: An underestimated concern in food safety. Current Opinion in Food Science, 26, 57–64.

Zhu, W., Wang, R. F., Khalifa, I., & Li, C. M. (2019). Understanding toward the Biophysical Interaction of Polymeric Proanthocyanidins (Persimmon Condensed Tannins) with Biomembranes- Relevance for Biological Effects. Journal of Agriculture and Food Chemistry, 67(40), 11044-11052.

Zi, X.-M., Sheng, C.-L., Goodale, U. M., Shao, S.-C., & Gao, J.-Y. (2014). In situ seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, Dendrobium aphyllum(Orchidaceae). Mycorrhiza, 24(7), 487.