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

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ABSTRACT

Previously, we found that ASP-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 amylolyticus* 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,

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2018), and exerted antimicrobial effects against *Escherichia coli*, *P. aeruginosa*, and *Monilia albicans* (Liu, Zhang, Wang, Li, Liu, Ma, et al., 2021). Since peptides are naturally occurring small molecules, they can have a broad spectrum of antibacterial activity (Nam, Yun, Rajasekaran, Kumar, Kim, Min, et al., 2018) that is usually caused by the destruction of cell membrane, which results in the leakage of intracellular components and microbial cell death (Travkova, Moehwald, & Brezesinski, 2017). Previously, we found that the anionic polypeptide DDDY exerts antibacterial effects by binding to the cell membrane surface via van der Waals (VDW) forces, thereby destroying the arrangement of the phospholipid head layer (Liu, et al., 2021). However, the specific effects of DDDY against the cell membranes of gram-positive bacteria, gram-negative bacteria, and fungi remain unclear.

In this study, we explored the potential mechanism of DDDY through transcriptomic, metabolomic, and molecular dynamics analyses using 1-palmitoyl-2-oleoyl-lecithin (POPC), 1-palmitoyl-2-oleoyl phosphatidyl-glycerol (POPG), and 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphoetha-nolamine (POPE) phospholipid bilayers to represent the cell membranes of gram-negative bacteria, gram-positive bacteria, and fungi, respectively (Murzyn, Zhao, Karttunen, Kurdziel, & Róg, 2006; Witte, Olausson, Walrant, Alves, & Vogel, 2013; Zhu, Wang, Khalifa, & Li, 2019). In addition, we explored the preservative effects of DDDY in cooked chestnut kernels.

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 Huankai Microbial Science and Technology Co., Ltd (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 4000*g* for 20 min and preservation at −80 °C. The samples were subjected to liquid chromatography- tandem mass spectrometry (LC-MS/MS) by Lianchuan Biotechnology Co., Ltd (Hangzhou, China) using an ultra-performance liquid chromatography (UPLC) system (SCIEX, UK) with 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 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, Zhang, 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 (MesoMR23-040H-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 DDDY treatment significantly affected the metabolism of *P. aeruginosa* (Fig. 1). *Pseudomonas aeruginosa* samples treated with or without DDDY 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
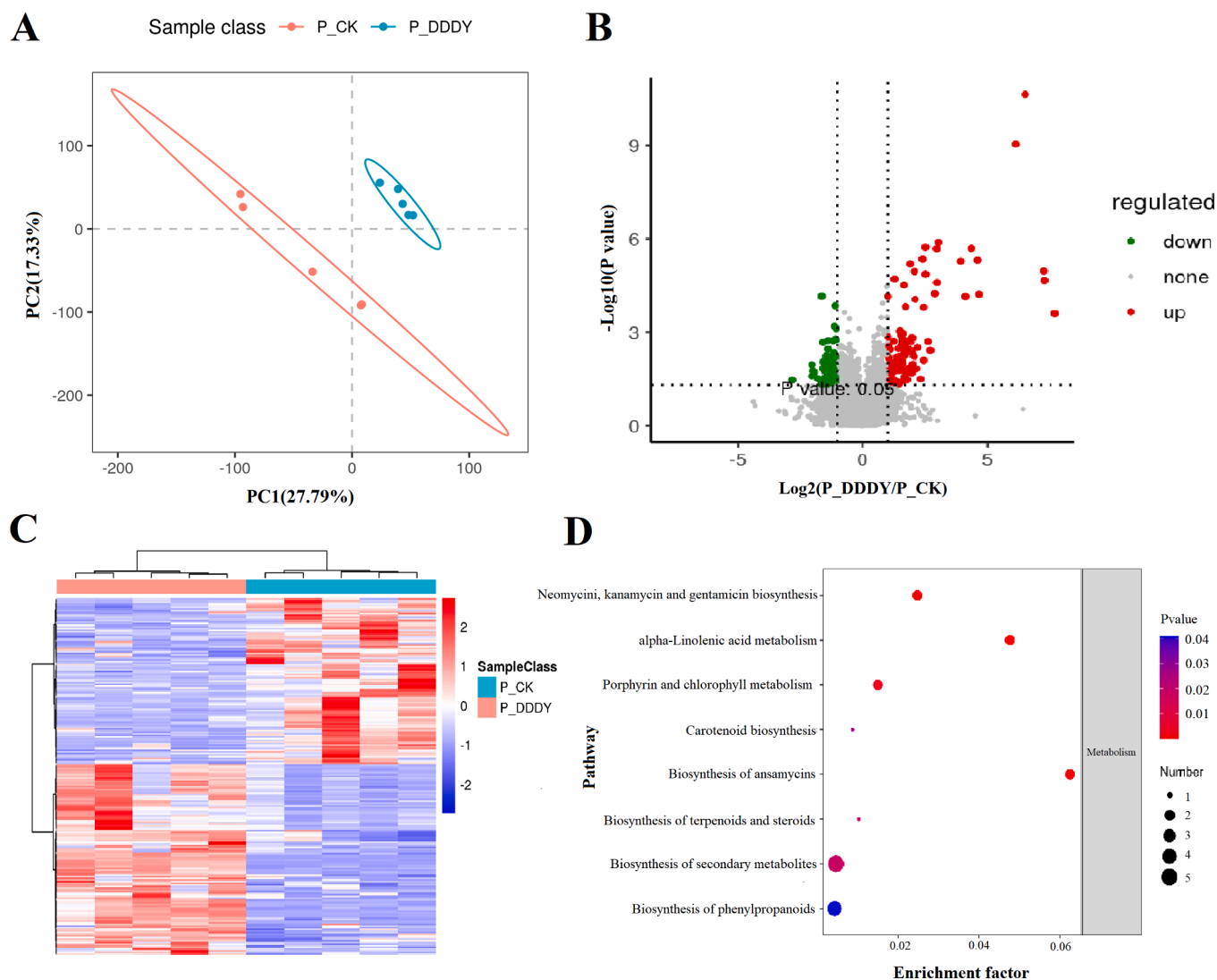

Fig. 1. Differential metabolome analysis of *Pseudomonas aeruginosa* treated with DDDY. (A) Partial least squares discriminant analysis of DDDY and control groups. (B) Volcano maps of different metabolites. (C) Heat maps of different metabolites. (D) KEGG enrichment analysis of different metabolites.

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 (no<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 POPC membranes fluctuated by 0.7–0.8 nm^2, whereas that of the POPE membrane only fluctuated by 0.6–0.7 nm^2 (Fig. 3B). Similarly, the MSD was higher in the POPE membrane (66.51 nm^2) than in the POPC or POPE membranes (Fig. 3C). The hydrogen bond ratio formed by the interaction between DDDY and the POPE membrane was higher than for either the POPG or POPC 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 wt. 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 a better form of preservation 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 rate 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, Giera, & 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	3165453056	99.06	96.57	60.52
P-CK3	23373416	3249066281	99.08	96.62	61.11
P-CK4	20918716	2900892733	99.07	96.60	61.19
P-CK5	22249730	3074434026	99.13	96.76	61.27
P-DDDY1	20526104	2815221237	99.12	96.74	60.47
P-DDDY2	29708198	4109570514	99.15	96.85	61.08
P-DDDY3	36977400	5109166131	99.10	96.71	61.01
P-DDDY4	32700808	4491507324	99.12	96.76	60.52
P-DDDY5	20032902	2770894852	99.07	96.61	60.98
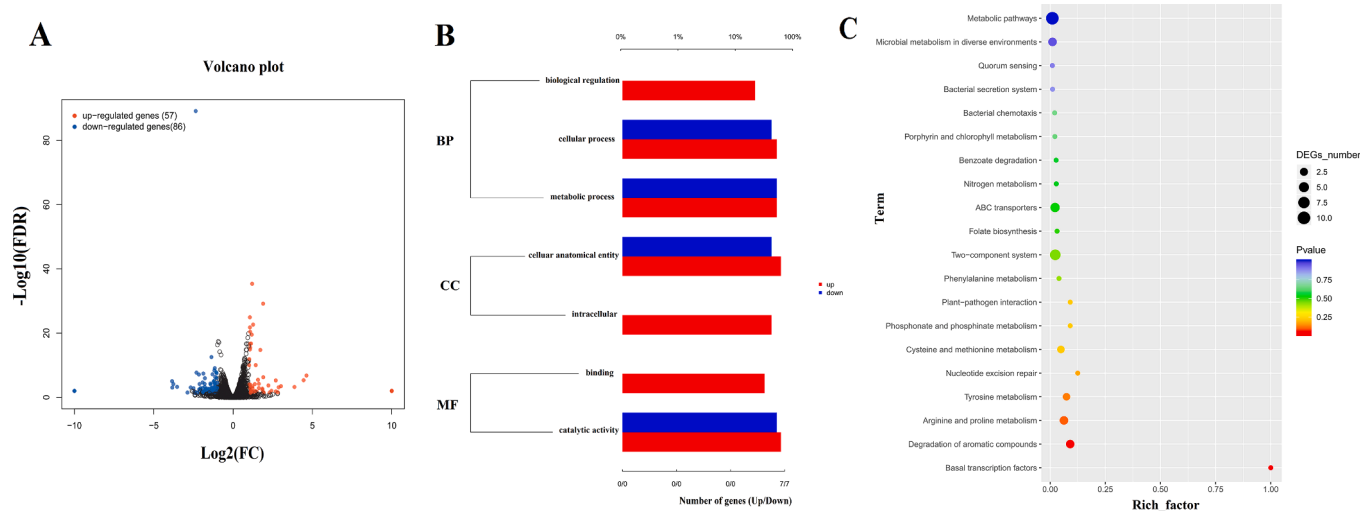
Fig. 2. Differential transcriptome analysis of *Pseudomonas aeruginosa* treated with DDDY. (A) Volcano maps of DEGs in *P. aeruginosa* treated with DDDY. (B) GO analysis of DEGs. (C) KEGG enrichment analysis of DEGs.
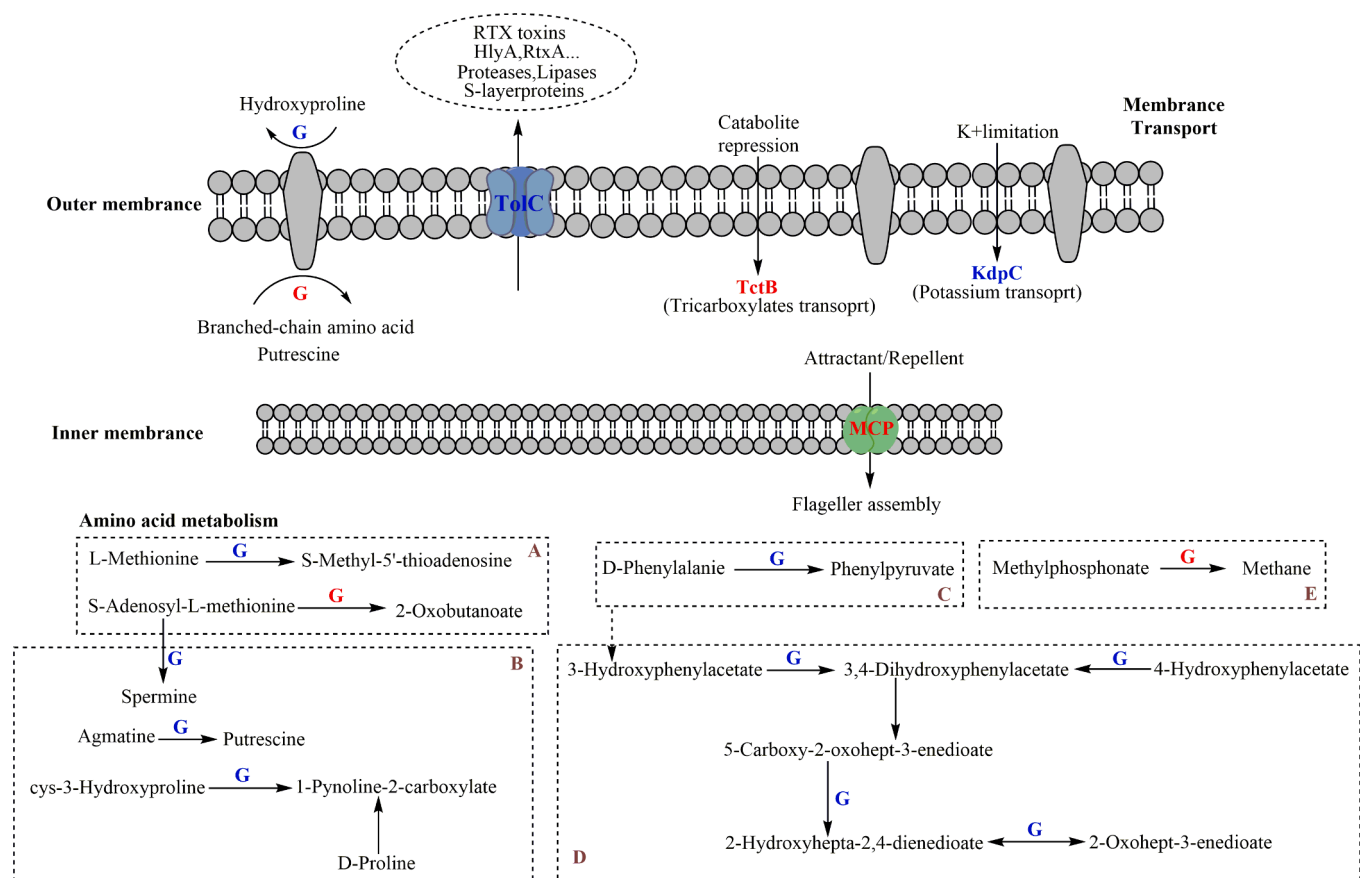

Fig. 3. Molecular dynamics analysis of DDDY-membrane interactions. (A) Simulated action track of DDDY (indicated by red (Asp) and green (Tyr) molecules) on POPC-, POPE-, and POPG-based lipid membranes (indicated by red spheres and gray chains). (B) Changes in the area of single phospholipid molecules 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.)

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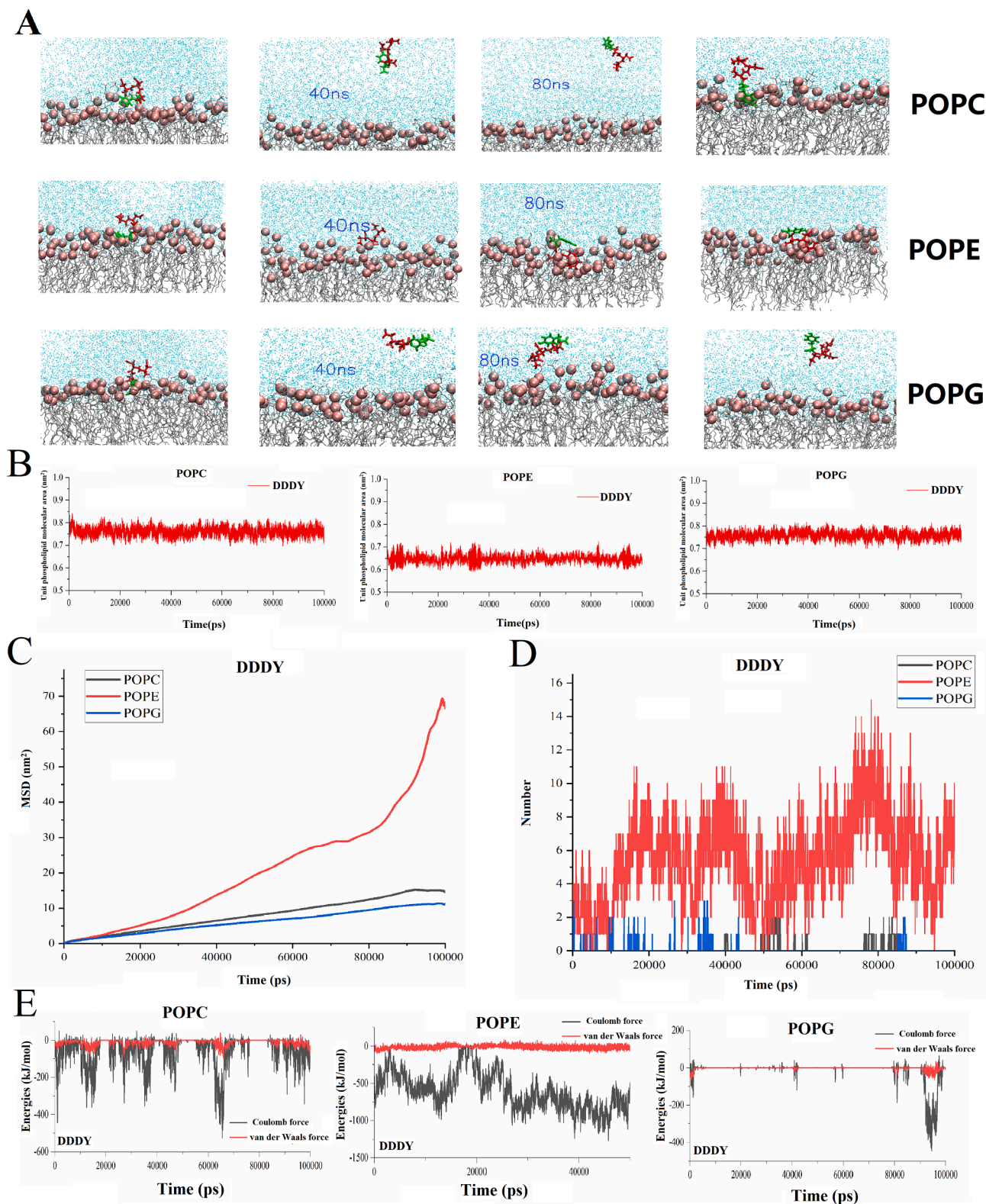
Fig. 4. Changes in the quality of ripe chestnut kernels inoculated with *Pseudomonas aeruginosa* and treated with DDDY during storage. (A) Number of bacterial colonies formed on the chestnut surface. (B) Changes in the weight of the chestnuts. (C&D) Changes in the (C) starch and (D) protein contents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

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

Table 2
Effects of DDDY on the color of cooked chestnut kernel.

		L	a	b	ΔE
CK-P (Cook)	0 h	45.81 ± 0.81b,c	4.65 ± 0.47a	22.31 ± 3.44b	52.64 ± 0.87a
	4 h	42.33 ± 4.42a,b	5.87 ± 0.82a,b	21.97 ± 0.99b	55.86 ± 3.95a
	8 h	37.47 ± 1.76a	6.71 ± 0.44b,c	18.87 ± 1.42a,b	59.66 ± 1.42a
	12 h	36.90 ± 1.85a	7.70 ± 0.76c	17.41 ± 1.09a	56.63 ± 4.61a
	16 h	36.94 ± 5.15a	6.32 ± 0.95b	16.30 ± 1.19a	59.54 ± 4.81a
DDDY (Spray)	0 h	54.65 ± 6.42a	9.18 ± 2.59a	39.14 ± 10.12a	55.19 ± 2.46a
	4 h	52.41 ± 7.16a	9.37 ± 0.75a	35.88 ± 10.52a	55.01 ± 0.55a
	8 h	51.25 ± 4.03a	9.09 ± 1.34a	35.38 ± 7.35a	54.99 ± 2.46a
	12 h	49.79 ± 5.54a	9.58 ± 1.64a	33.25 ± 9.99a	55.46 ± 1.70a
	16 h	45.95 ± 7.74a	9.31 ± 0.86a	31.22 ± 11.03a	57.84 ± 2.14a
DDDY (Soak)	0 h	50.76 ± 4.65a	8.92 ± 2.56a	32.37 ± 7.82a	53.81 ± 1.79a
	4 h	46.54 ± 6.04a	8.80 ± 2.83a	28.66 ± 7.98a	55.64 ± 1.96a,b
	8 h	41.52 ± 5.38a	9.49 ± 3.22a	25.11 ± 6.60a	58.62 ± 2.02b
	12 h	41.14 ± 6.75a	9.20 ± 3.39a	24.33 ± 8.50a	58.88 ± 2.90b
	16 h	40.78 ± 4.64a	9.74 ± 2.53a	22.56 ± 6.69a	58.41 ± 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.

chemotaxis (Kato, Kim, Takiguchi, Kuroda, & Ohtake, 2008). Thus DDDY could be used as a repellent to accelerate the activity of *P. aeruginosa*. In amino acid metabolism, the DEGs in *P. aeruginosa* treated with DDDY were mainly enriched in arginine, proline, and tyrosine metabolism, while AugB, SpeD, LhpI, HpaG, HpaB were downregulated. Since tyrosine, arginine, and proline are related to the tricarboxylic acid cycle, these changes affect the normal metabolic pathway of *P. aeruginosa*. Therefore, we speculate that the antibacterial effect of DDDY on *P. aeruginosa* is mainly related to membrane transport and amino acid metabolism pathways, and that DDDY mainly achieves bactericidal effects by affecting the normal function of the cell membrane.

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.

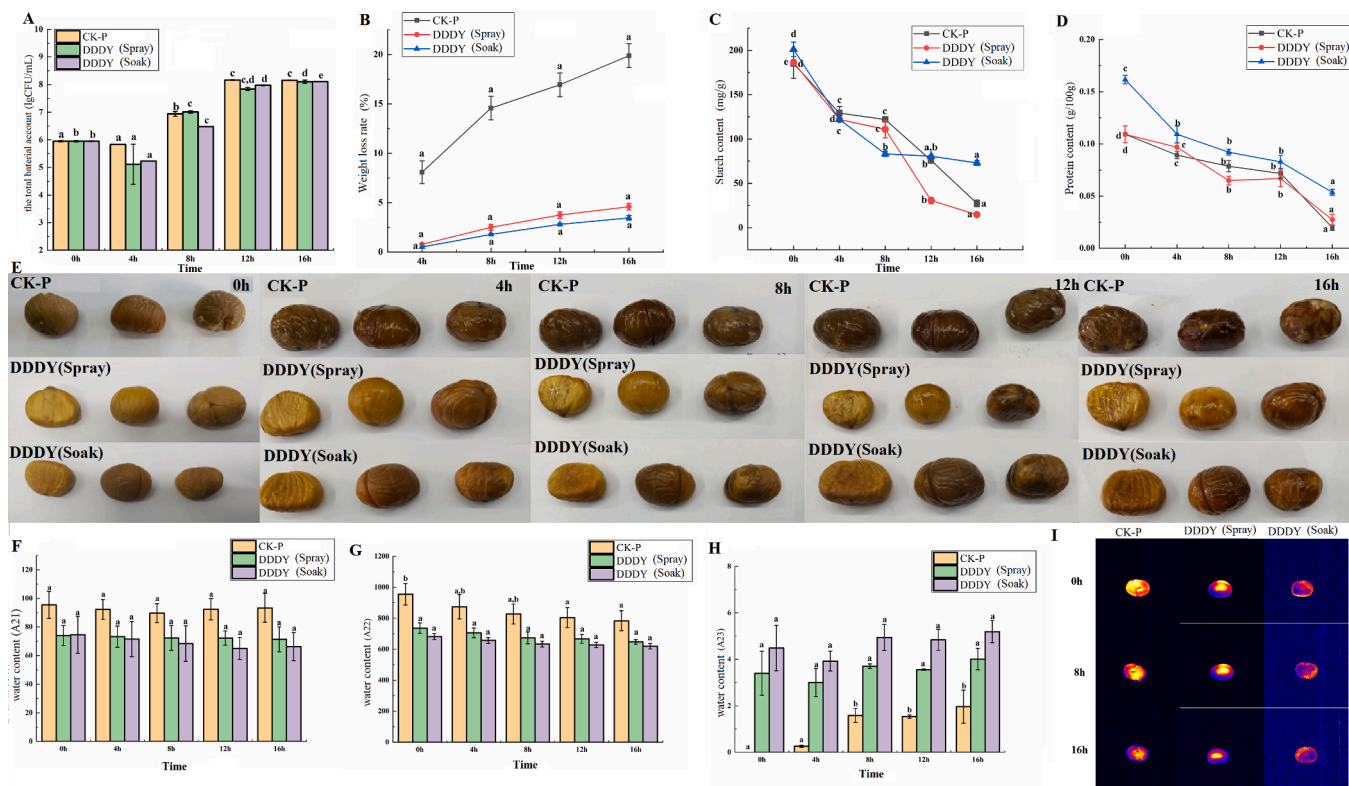Pseudomonas aeruginosa is a globally-distributed bacterium that is

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

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.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Huifan Liu reports was provided by Basic and applied research project of Guangzhou Basic Research Program in 2020. Huifan Liu reports was provided by Guangdong Heyuan National Agricultural Science and Technology Park Intensive Processing of agricultural and sideline products key technology transformation project. Jianliang Liu reports equipment, drugs, or supplies was provided by Modern Agriculture Research Center. Huifan Liu reports equipment, drugs, or supplies was provided by Guangdong Key Laboratory of Science and Technology of Lingnan Specialty Food.

Appendix A. Supplementary data

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