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Inhibition mechanism of quorum sensing and biofilm formation of *Pseudomonas fluorescens* by a novel quorum quenching bacteria *Lactiplantibacillus plantarum* YP4-1-2

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ABSTRACT

Pseudomonas fluorescens is a widespread spoilage bacterium in food, and its spoilage characteristics and biofilm formation are regulated by *N*-acyl-homoserine lactone (AHLs) quorum sensing (QS) system. Quorum quenching (QQ) is considered as an effective strategy to control the spoilage bacteria. Therefore, this study revealed a new QQ bacteria *Lactiplantibacillus plantarum* YP4-1-2. Notably, the crude cell extract (CCE) from *L. plantarum* YP4-1-2 showed strongly QQ activity against the AHLs of *P. fluorescens*. The degradation rate of AHLs (*N*-octanoyl-*L*-homoserine lactone) reached 25.90%, 78.57% and 100% at the CCE protein concentrations of 57, 86 and 114 μg/mL, respectively. In addition, the CCE could drastically reduce the formation of biofilm, bacterial motility (swimming and swarming), and the release of extracellular protease and biogenic amines of *P. fluorescens*. Real-time polymerase chain reaction (PCR) results revealed that the CCE downregulated QS-related genes (*rhlI*, *rhlR*, *aprX*, *algA*, *orm*, *flgA*, and *ldcA*). Finally, using whole-genome sequencing analysis, the active substance in the CCE was identified to be penicillin V acylase (PVA) and named *Lp*PVA. Meanwhile, homologous modeling and molecular docking analysis showed that the binding effect of *Lp*PVA on long-chain AHLs was better than that on short-chain AHLs. This study demonstrated that *L. plantarum* YP4-1-2 could be considered as a promising QS inhibitor and antibiofilm agent against foodborne spoilage bacteria.

1. Introduction

Pseudomonas fluorescens, a typical psychrophilic bacterium, is a major contributor to the spoilage of refrigerated foods, such as milk, fruits and vegetables, and meat. Studies have shown that its spoilage characteristics and biofilm formation are regulated by *N*-acyl-homoserine lactone (AHL) type quorum sensing (QS) system[1-2]. QS, a communication process, can interfere with the composition and function of bacterial communities by regulating their movement, secretion, efflux pump function, pathogenic gene expression, toxin production, and biofilm formation[3]. Therefore, disrupting the QS system of *P. fluorescens* can block the information exchange between microorganisms and reduce the expression of QS-regulated phenotypes, which has become a potential target for controlling *P. fluorescens* contamination in food[4].

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Quorum quenching (QQ) is a process that prevents and reduces the generation, releases of QS signals, impedes the "information exchange" between microorganisms, reduces the expression level of harmful factors. Substances with QQ activity are collectively referred to as QS inhibitors (QSIs). Considering the efficiency of QSIs and the selection pressure faced by bacteria, QQ enzymes (QQEs) may be the most ideal QSIs at present. QQEs targeting AHLs mainly include AHL lactonases, AHL acylases, and AHL oxidoreductases[5]. Among them, AHL lactonases and AHL acylases are the most well-studied. AHL lactonases can disrupt the ester bond in the AHL ring to generate the corresponding acyl homoserine. AHL acylases irreversibly cleave the amide bond between the acyl chain and the AHL ring, generating the corresponding free fatty acids and homoserine lactone (HSL) rings. The lactonolysis catalyzed by AHL lactonases can be reversed at an acidic pH. However, in acylase hydrolysates, active signals cannot be restored spontaneously. Thus, AHL acylases have more advantages than lactonases in biotechnological applications[5]. AHL acylases mainly belong to the *N*-terminal nucleophilic (NTN) hydrolase superfamily[6], which contains many enzymes with different activities, including amidase, penicillin G acylase (PGA), and choloylglycine hydrolases (CGHs)[7]. Studies have shown that penicillin V acylases (PVAs), which belong to the CGH family, have strong AHL degradation ability. At present, *Pa*PVA and *At*PVA have been obtained from *Pectobacterium atrosepticum* and *Agrobacterium tumefaciens*, respectively, and have been identified as having good AHLs degrading activity[8].

Recent studies have confirmed that the genes encoding PVAs are closely related to *Lactobacillus* species from a wider variety of environments[9]. Lactic acid bacteria (LAB) are important probiotics and are safe for humans and animals. Thus, they have been widely used in foods[10]. Some studies have reported that LAB can act as promising QSIs against spoilage bacteria. A study demonstrated that the cell-free culture supernatant of *Lactobacillus fermentum* (LF: MTCC 5898) downregulates the expression of *lasI* and *rhlI* and reduces elastase activity in *Pseudomonas aeruginosa* PAO1[11]. Additionally, secondary metabolites extracted from *Pediococcus pentosaceus* BS-2 and *L. fermentum* BM-2 have strong QQ activity against *P. aeruginosa* JUPG01 and can prevent biofilm formation in these bacteria[12]. However, few studies have focused on developing and utilizing PVAs from LAB to control foodborne spoilage bacteria, and the QSI-related mechanism of PVAs is not entirely clear.

In our previous study, the LAB with degradation activity against *P. fluorescens* PF08 AHLs was screened using the agar plate diffusion method, in which *Chromobacterium violaceum* CV026 was used as a biosensor report strain. *Lactiplantibacillus plantarum* YP4-1-2, exhibiting strong AHLs degradation activity, was isolated from the intestine of *Paralichthys olivaceus* (Jinzhou, Liaoning, China). This study aimed: i) to investigate the type and location of QQ substances in *L. plantarum* YP4-1-2 against the AHLs of *P. fluorescens*; ii) to analyse the action mechanism of the active QQ substances on biofilm formation and phenotypes in *P. fluorescens* at the cellular and molecular levels; and iii) to identify the active QQ substances from *L. plantarum* YP4-1-2 using whole-genome sequencing and homologous modeling. This study could provide additional theoretical support for the application of *L. plantarum* YP4-1-2 biologics in controlling the food spoilage caused by *P. fluorescens*.

2. Materials and methods

2.1 Strains and culture conditions

L. plantarum YP4-1-2 (GDMCC62035) was originally isolated from the intestine of *P. olivaceus* (Jinzhou, Liaoning, China) and preserved in the Guangdong Microbial Culture Collection Center (GDMCC). It was grown in de Man, Rogosa and Sharpe (MRS) broth at 37 ℃ for 24 h. Meanwhile, *P. fluorescens* PF08 (GenBank accession number: CP032618) was isolated from the spoiled turbot. It was cultured in Luria-Bertani (LB) broth at 30 ℃ for 24 h. *C. violaceum* CV026 was used as a QS bioreporter strain and incubated in LB medium supplemented with 20 μg/mL kanamycin at 30 ℃ for 24 h. All strains were sub-cultured for three generations to ensure their activity.

2.2 Type and location of the QQ substances of L. plantarum YP4-1-2

To investigate the site of the QQ substances of *L. plantarum* YP4-1-2, 30 mL of *L. plantarum* YP4-1-2 culture was centrifuged at 8 000 × *g* for 10 min at 4 ℃. The supernatant was filtered through a 0.22-μm filter to obtain a cell-free supernatant (CFS). Bacterial cells were washed three times with 20 mL phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.0) and then resuspended in 10 mL PBS (0.01 mol/L, pH 7.0). Subsequently, the cell suspension was intermittently sonicated in a cold water bath for 5 min (1 s on, 2 s off; 33 kHz) using an ultrasonic cell disruptor (JY92-IIDN, Scientz, Zhejiang, China). After centrifugation (8 000 × *g* for 45 min at 4 ℃), the supernatant was filtered through a 0.22-μm filter to obtain the crude cell extract (CCE) of strain YP4-1-2. Afterwards, *N*-octanoyl-*L*-homoserine lactone (C_8-HSL; Sigma, USA) at a final concentration of 1.76 μmol/L was added to the CFS (pH adjusted to 7.0 with 1.0 mol/L NaOH) and CCE. The co-culture was incubated at 30 ℃ for 24 h, followed by centrifugation at 10 000 × *g* for 5 min to obtain the supernatant. PBS (0.01 mol/L, pH 7.0) was used as the control. The remaining C_8-HSL in the supernatant was examined using the agar well diffusion method[13].

After preparing the co-culture as described above, the supernatant from the co-culture was adjusted to pH 2.0 with 1.0 mol/L HCl and incubated for 24 h at 30 ℃. The residual AHL content was detected using the agar plate diffusion method.

2.3 Determination of the minimum inhibitory concentration (MIC) of the CCE

The protein concentration in the CCE was detected using Bradford's method. The CCE was serially diluted two-fold in PBS to achieve a protein concentration range of 0 to 228 μg/mL[14]. The Clinical and Laboratory Standards Institute (CLSI) guidelines were used to detect the MIC of the CCE with an inoculum of 10^6 CFU/mL *P. fluorescens* suspension. The growth of *P. fluorescens* was monitored at $OD_{595\ nm}$ every 2 h for 24 h using an automatic growth curve analyzer (Bioscreen C, Shanghai, China). The MIC was defined as the lowest concentration of the CCE that could completely inhibit the growth of the target strain. Meanwhile, the MIC of the CCE against *C. violaceum* CV026 was also evaluated using this method.

2.4 Determination of AHL levels

N-Butanoyl-*L*-homoserine lactone (C_4-HSL), *N*-hexanoyl-*L*-homoserine lactone (C_6-HSL), and C_8-HSL (Sigma, USA) at a final concentration of 1.76 μmol/L were added to the CCE (57, 86, and 114 μg/mL, respectively) and then incubated at 30 ℃ for 24 h. PBS (0.01 mol/L, pH 7.0) was used as the control. The co-culture was centrifuged at 10 000 × *g* for 5 min, and the AHLs remaining in the supernatant were detected using the agar well diffusion method.

The enzyme activity of the CCE for C_8-HSL degradation was determined using *C. violaceum* CV026 as the reporter strain with the agar well diffusion method[15]. The total volume of the enzyme activity determination reaction system was 200 μL: C_8-HSL 10 μL (0.781 25, 1.562 5, 3.125, 6.25, 12.5, 25, 50 and 100 μmol/L) and CCE (114 μg/mL) 190 μL. The system was incubated at 30 ℃ for 45 min before termination via the addition of 50 μL 10 g/100 mL SDS. One unit (U) was defined as the amount of enzyme required to hydrolyze 1 μmol/L of C_8-HSL per hour under the conditions described.

2.5 Analysis of inhibitory effect of the CCE on *P. fluorescens* biofilms

The inhibitory effect of the CCE on *P. fluorescens* biofilm formation was evaluated as previously described by Zhou et al.[16] with slight modifications. Briefly, the CCE was added to *P. fluorescens* suspensions (10^6 CFU/mL, 200 μL) at final concentration of 57, 86, and 114 μg/mL, and then cultured at 30 ℃ for 12 h in 96-well cell culture plates. LB broth was used as the control. The planktonic cells were removed for the planktonic cell survival assay. The biofilms were washed 3–5 times with PBS and stained with crystal violet (0.4%) for 15 min; residual crystal violet was rinsed off with sterile water. After drying for 10 min, 200 μL of 33% glacial acetic acid was added to each well, and $OD_{570\,nm}$ was measured using a microplate reader (Bio-Rad, USA).

The ability of the CCE to scavenge preformed *P. fluorescens* biofilms was also examined. After 12 h of cultivation, *P. fluorescens* formed a mature biofilm, and the suspension cultures were removed for the cell survival test. The mature biofilm was washed 3–5 times with sterile PBS to remove any planktonic cells. Fresh LB medium and the CCE (57, 86, and 114 μg/mL) were added to the wells and then incubated at 30 ℃ for 12 h. The amount of biofilm formation was quantified using crystal violet staining as described above.

Scanning electron microscopy (SEM; Hitachi, Japan) and confocal laser scanning microscopy (CLSM; Leica, Germany) were used to evaluate the interference effect of the CCE against biofilm formation and preformed biofilms. A biofilm inhibition assay was established in 24-well cell culture plates (Costar 3524, Corning, USA). The silicon slice (1 cm × 1 cm × 0.6 mm) was immersed in 2 mL of LB broth containing the CCE at concentrations of 57, 86, and 114 μg/mL. Subsequently, 5 μL of *P. fluorescens* (10^6 CFU/mL) were added, and the cultures were incubated at 30 ℃ for 12 h to facilitate biofilm formation. Biofilms on silicon slices were washed thrice with PBS and fixed with 2.5% glutaraldehyde (Solarbio, Beijing, China) for 12 h at 4 ℃. Graded dehydration was performed using 40%, 70%, 90%, and 100% ethanol. After drying, gold was sprayed and the biofilms were observed using SEM.

For CLSM, the bacteria were treated with the CCE, as described the SEM, and the silicon slice was substituted with the coverslip (5 mm × 5 mm × 0.15 mm) in CLSM analysis. Biofilms on the coverslips were washed thrice with PBS and fixed with 2.5% glutaraldehyde for 30 min. After drying, the coverslips were stained with 40 μg/mL acridine orange (AO) for 30 min, followed by 5 μg/mL ethidium bromide (EB) for 15 min. Then, the slides were washed twice, gently, using PBS and dried for 30 min. The dry coverslips were treated with 10 μL sealant and observed at excitation wavelengths of 488 nm and 561 nm using a 10× objective lens and CLSM.

In order to intuitively observe the elimination effect of the CCE on *P. fluorescens* preformed biofilms, preformed biofilms were established in 24-well cell culture plates. Then, LB medium and CCE were added to the wells and incubated at 30 ℃ for 12 h. Samples were prepared for SEM and CLSM according to the methods described above.

2.6 Motility inhibition assays

Swarming and swimming motilities were detected using swarming agar (1% tryptone, 0.5% NaCl, and 0.5% agar) and swimming agar (1% tryptone, 0.5% NaCl, and 0.3% agar), respectively[17]. Briefly, the CCE (57, 86, and 114 μg/mL) was mixed with the swarming agar and swimming agar, and 5 μL of *P. fluorescens* suspension (10^6 CFU/mL) was inoculated in the center of the solidified agar plate. The plates were cultured at 30 ℃ for 24 h to record the migration diameter.

2.7 Analysis of QS-regulated phenotypes of *P. fluorescens*

The determination of extracellular proteases was performed using the agar well diffusion method[18]. Briefly, *P. fluorescens* was treated with the CCE (57, 86, and 114 μg/mL) and cultured overnight. PBS was used as the control. The co-culture was centrifuged at 10 000 × *g* for 5 min and the supernatant was collected. The supernatant was filtered through a 0.22-μm filter and stored at 4 ℃.

LB plates containing 1.5% skim milk were perforated with Oxford cups. The filtered cell-free supernatant (100 μL) was added to each well and then incubated at 30 ℃ for 24 h. The level of extracellular protease was measured based on the area of the transparent zone around the hole in the milk plates.

Biogenic amines (BAs) screening medium (tryptone 5.0 g/L, yeast extract 5.0 g/L, NaCl 5.0 g/L, $CaCO_3$ 1 g/L, agar 20 g/L, bromocresol violet 0.06 g/L, *L*-histidine 2 g/L, tryptophan 2 g/L, and tyrosine 2 g/L; adjust the pH to 5.3 ± 0.2 with 1 mol/L HCl) was poured into an agar plate with an Oxford cup. After cooling, the Oxford cup was removed, and 180 μL of the above supernatant was added into the well and cultured at 30 ℃ for 24 h. The diameter of hydrolytic circles was measured using a colony counter.

2.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

P. fluorescens (10^6 CFU/mL, 100 μL) was inoculated to the LB broth (10 mL) with or without the CCE (114 μg/mL), and incubated at 30 ℃ for 24 h. After cultivation, cells were obtained after centrifugation at 8 000 × *g* for 10 min. Total RNA was extracted using an RNA extraction

kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The qRT-PCR was conducted following the protocol described by Li et al.[19] in the Gene 9600 Quantitative PCR Instrument (Bori, Hangzhou, China), using the primers listed in Table S1. The 16S rRNA was used as the internal control, and the fold change in the target genes was calculated according to the $2^{-\Delta\Delta Ct}$ method[20].

2.9 Sequence identification and phylogenetic tree of the QQE

L. plantarum YP4-1-2 was sent to Beijing Baimaike Biotechnology Co., Ltd. (Beijing, China) for genomic DNA extraction, library construction and inspection, and bacterial genome assembly. The potential QQE-encoding gene was successfully identified. Based on the search results, the multi-sequence homology of the obtained sequences was analyzed using the NCBI GeneBank database. The phylogenetic tree was constructed with MEGA 7.0 software[21].

2.10 Homology modeling, model evaluation, and interaction with the AHLs of LpPVA

The amino acid sequence obtained for the target gene was submitted to SWISS-MODEL (https://swissmodel.expasy.org) to construct the three-dimensional structure[22]. After sequence alignment, the sequence with high sequence similarity was selected (similarity > 30%) as the template for homology modeling, and the best template was selected for quality evaluation. The constructed three-dimensional structure was obtained with SAVESv6.0 (https://saves.mbi.ucla.edu/) and its quality was evaluated using Ramachandran plots.

AHL small molecules with different carbon chains were obtained from the PDB database (https://www.rcsb.org/). The models were used as receptor proteins. Molecular docking analysis was performed using Discovery Studio 2017 R2 Client in CDOCKER program[22].

2.11 Statistical analysis

All assays were repeated at least 3 times and data were represented as the means ± standard deviation (SD). Statistical analysis with one-way analysis of variance (ANOVA) was performed using SPSS 19.0 software. Origin Pro 10 software was used to construct graphs. $P < 0.05$ was considered statistically significant in all experiments.

3. Results and discussion

3.1 Type and site of the QQ substances in L. plantarum YP4-1-2

To ascertain the site of the QQ active substances of *L. plantarum* YP4-1-2, C_8-HSL was used as an exogenous AHL signal. *C. violaceum* CV026 was used as a biosensor, producing violacein pigment in the presence of AHLs with short and medium fatty-acid chains[23]. As shown in Fig. 1a, the biosensor *C. violaceum* CV026 was activated and produced purple pigment on plates treated with MRS and the CFS of *L. plantarum* YP4-1-2. In contrast, co-culture of C_8-HSL with the CCE of *L. plantarum* YP4-1-2 did not activate *C. violaceum* CV026, indicating that the C_8-HSL had been degraded by the CCE. The results showed that the CCE of *L. plantarum* YP4-1-2 had AHL degradation activity, but the CFS did not.

To determine if the QQ activity was due to a lactonase-type enzyme, the CCE of *L. plantarum* YP4-1-2 was acidified after incubation with C_8-HSL. This acidification would cause the lactone ring to restructure itself if it had previously been opened by a lactonase[13]. As shown in Fig. 1a, the recovery of AHLs concentration after culture acidification was insignificant when compared with the control. This indicated that the degradation effect of *L. plantarum* YP4-1-2 could be due to an enzyme that was not a lactonase. A similar result was also obtained with *Alteromonas stellipolaris* PQQ-42, such that the lactone ring of the AHLs was not restructured after treatment with the CCE of strain PQQ-42 after acidification. The degradation enzyme in strain PQQ-42 was found to be an AHLs acylase[13].

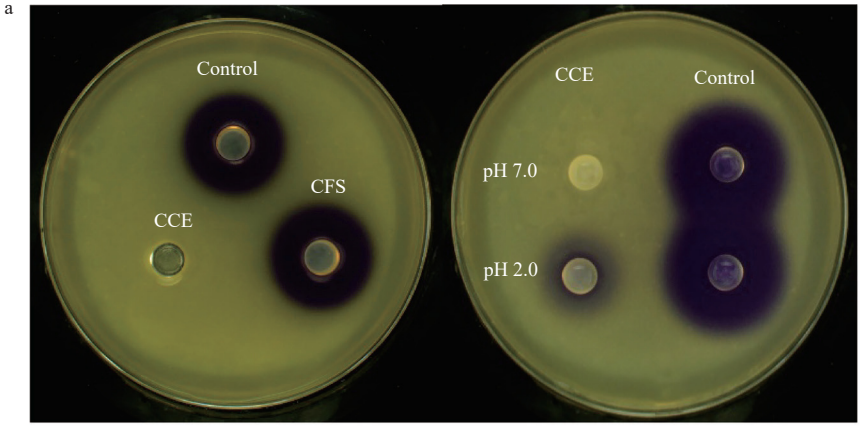


Fig. 1 (a) Location and types of QQ active substances in *L. plantarum* YP4-1-2. (b) Effect of the crude cell extract (CCE) from *L. plantarum* YP4-1-2 on the growth curves of *P. fluorescens*, with PBS (pH 7.2) used as the negative control. Error bars represent the standard deviations of three independent experiments. (c) Effect of the CCE from *L. plantarum* YP4-1-2 on the growth curves of *C. violaceum* CV026, with PBS (pH 7.2) used as the negative control. Error bars represent the standard deviations of three independent experiments. (d) Degradation activity of the CCE from *L. plantarum* YP4-1-2 on C_4-HSL, C_6-HSL, and C_8-HSL. After treatment with the CCE from *L. plantarum* YP4-1-2 for 24 h, the remaining C_4-HSL, C_6-HSL, and C_8-HSL were detected using the well agar diffusion method. PBS (pH 7.2) was used as the negative control. Error bars represent the standard deviations of three independent experiments. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.000\ 1$ versus control.

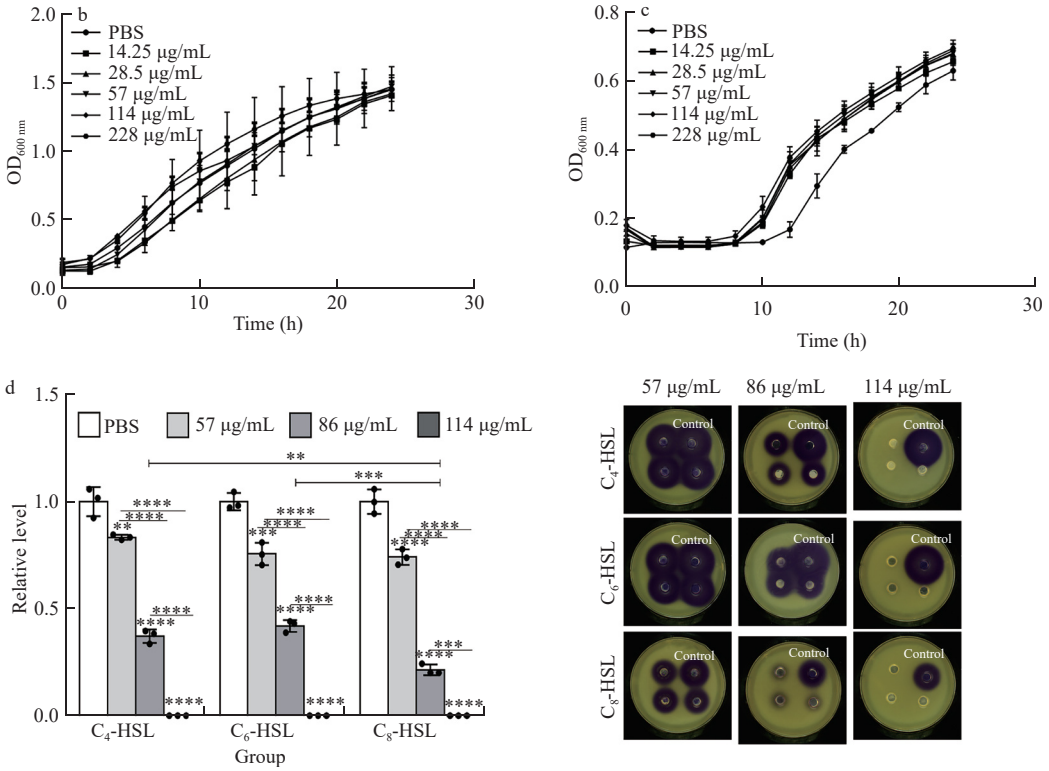


Fig. 1 (*Continued*)

3.2 MIC of the CCE from L. plantarum YP4-1-2

The value of the new QSIs lies in the fact that they not only inhibit the production of QS-regulate phenotypes and biofilms in target organisms, but also maintain the survival of target microorganisms. Hence, we preliminarily determined the MIC of the CCE from *L. plantarum* YP4-1-2. As shown in Figs. 1b–c, compared with the control group, *P. fluorescens* and *C. violaceum* CV026 treated with the CCE at concentrations of 14.25, 28.5, 57, 114, and 228 μg/mL showed a characteristic S-type growth curve. The CCE did not inhibit the growth of *P. fluorescens* and *C. violaceum* CV026 at a concentration range of 14.25 to 228 μg/mL. Thus, CCE concentrations of 57, 114, and 228 μg/mL were selected for follow-up experiments.

3.3 Effect of the CCE on AHL levels in P. fluorescens

To verify that QQ activity of the CCE was due to the action of enzyme, the enzyme activity in the CCE from *L. plantarum* YP4-1-2 was quantified in the study. The corresponding enzyme activities in the CCE at protein concentrations 57, 86, and 114 μg/mL were found to be 0.207, 0.313, and 0.415 U/mL, respectively. Although this study did not purify the enzyme, the activity of the enzyme in CCE indicated its efficiency in degrading AHLs.

P. fluorescens exhibits diversity, and the QS system is not universally present in all strains[24]. So to avoid this problem, the *P. fluorescens* PF08 used in this study has been confirmed to have rhlI/R system[25]. *P. fluorescens* mainly secretes AHL signaling molecules, including C_4-HSL, C_6-HSL, C_8-HSL, *N*-[(*RS*)3-hydroxybutyryl]-*L*-homoserine lactone (3-HO-C_4-HSL), *N*-(3-hydroxyoctanoyl)-homoserine lactone (3-hydroxy-C_8-HSL), and *N*-(3-hydroxydodecanoyl) homoserine lactone (3-hydroxy-C_{12}-HSL) [1-2]. The AHLs acyl side chain with 4 to 8 carbon atoms are more abundant in *P. fluorescens*[1,26]. Thus, the degradation activity of the CCE (57, 86, and 114 μg/mL) against C_4-HSL, C_6-HSL, and C_8-HSL was analyzed in the study. The degradation rates of C_4-HSL, C_6-HSL, and C_8-HSL treated with the CCE were 16.64%–100%, 24.42%–100%, and 25.90%–100%, respectively (Fig. 1d). The degradation rate for C_8-HSL was higher than that for C_4-HSL and C_6-HSL. Meanwhile, the degradation activity of the CCE was dose-dependent. These results indicated that the CCE had significant degradation ability against AHLs, and the degradation activity against long-chain AHLs was greater than that against short-chain AHLs. An analysis by Torres et al.[27] also showed that strain *Thalassomonas* sp. PP2-459, with the ability to quench short-chain AHLs, had a more significant quenching effect on long-chain AHLs. Our findings were consistent with these previous results.

3.4 Effect of the CCE on biofilm formation in P. fluorescens

The formation of biofilms involves complex dynamic interactions between host and abiotic surfaces, pathogens in biofilms, the extracellular polymeric substance matrix, and surrounding biological fluid[28]. After biofilm formation, the adhesion strength and viscoelasticity of the biofilms make it difficult to remove them from the surface[29]. This enhancement of resistance also makes microorganisms resistant to antibacterial agents. The biological complexity and antibiotic tolerance of biofilms make them less vulnerable to traditional treatments. Additionally, many studies have shown that the QS system plays an important role in the regulation of biofilm formation[30-32]. Therefore, interfering with or blocking the QS system could effectively reduce bacterial biofilm formation.

The CCE-mediated degradation of AHLs inhibited the biofilm formation of *P. fluorescens* in the present study. The crystal violet assay showed that the inhibition rate of the CCE (57, 86, and 114 μg/mL) against *P. fluorescens* biofilms ranged from 19.76% to 61.89% (Fig. 2a). Meanwhile, the CCE did not affect the survival of planktonic *P. fluorescens* cells (Fig. 2b). This confirmed that the CCE could effectively inhibit the formation of *P. fluorescens* biofilms while maintaining the survival of planktonic cells.

SEM intuitively confirmed the inhibitory effect of the CCE on the formation of *P. fluorescens* biofilms (Fig. 2c). In the control group, *P. fluorescens* cells were tightly wrapped by extracellular polymers and formed a thick and dense biofilm. However, in the CCE treatment group, although a large number of bacteria were present, the structure of the biofilms was destroyed and their thickness was reduced. In particular, bacteria existed independently following 114 μg/mL CCE treatment, and no biofilm formation was observed. Therefore, SEM results further verified the inhibitory effect of the CCE on *P. fluorescens* biofilms.

Microbes usually form multilayer biofilms when they adhere to the surface of objects and to each other[33]. The effect of the CCE on the three-dimensional structure of *P. fluorescens* biofilms was further observed using CLSM. Living and dead cells of *P. fluorescens* were stained with specific fluorescent markers. Under CLSM, the living cells emitted green fluorescence and the dead or damaged cells

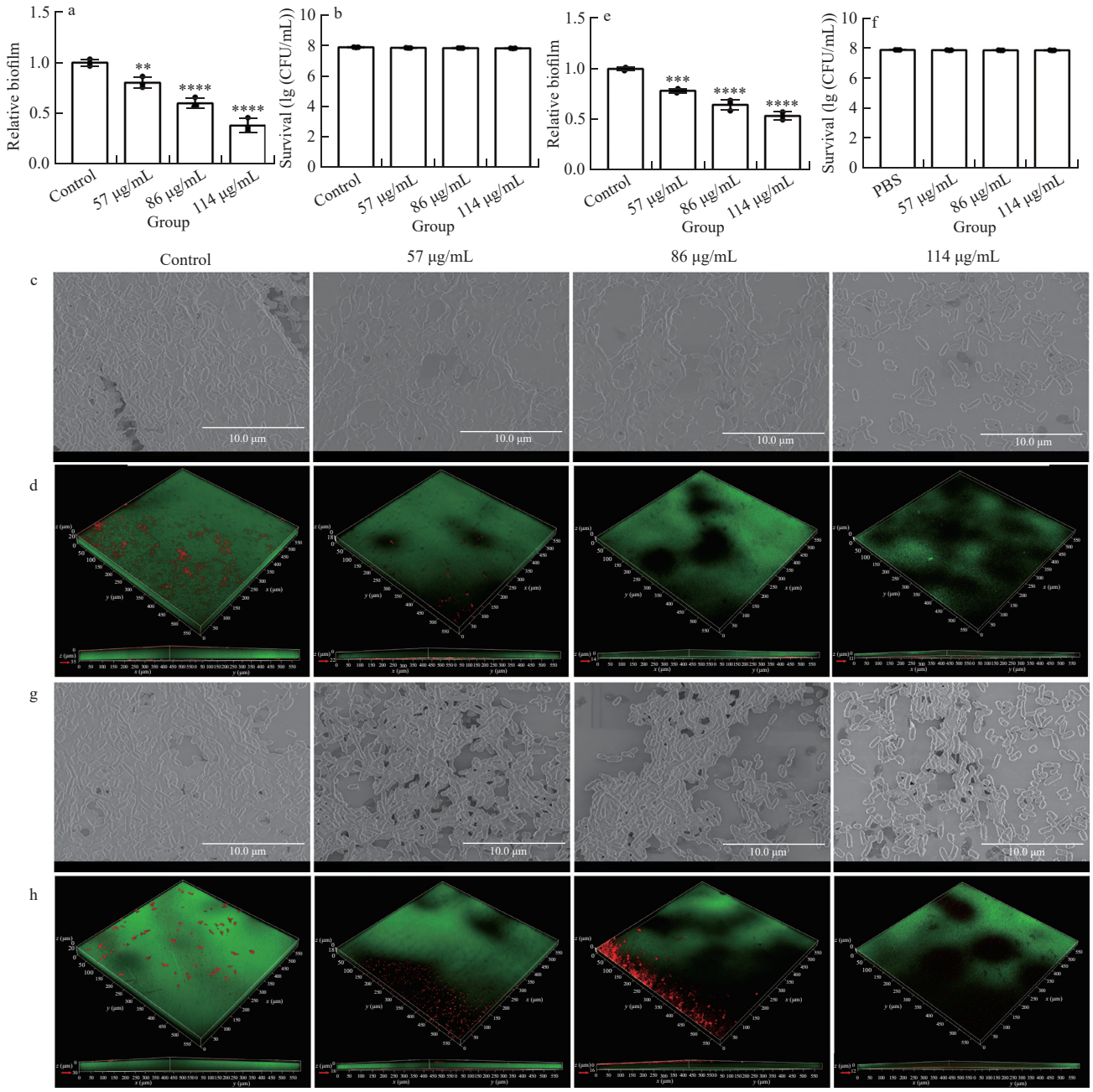


Fig. 2 Inhibition (a–d) and removal (e–h) effect of the crude cell extract (CCE) at protein concentrations of 57, 86, and 114 μg/mL against biofilms of *P. fluorescens*. (a, e) Relative biofilm formation using the 96-well plate method. (b, f) Planktonic cell survival assay. (c, g) SEM images of *P. fluorescens* biofilms. (d, h) CLSM images of *P. fluorescens* biofilms. Error bars represent the standard deviations of three independent experiments. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.000\ 1$ versus control.

emitted red fluorescence. CLSM images showed that the thickness of *P. fluorescens* biofilms was reduced after CCE treatment (Fig. 2d). After CCE treatment (57, 86, and 114 μg/mL), the colony density of *P. fluorescens* decreased significantly, the cell clusters in the biofilm became sparse and dispersed, and the thickness of the biofilms was reduced from 35 to 22, 14, and 11 μm, respectively. CLSM images further proved that after treatment, the thickness of *P. fluorescens* biofilms decreased and the gap between cells increased. These results were consistent with SEM findings, indicating that the CCE has an inhibitory effect on the formation of *P. fluorescens* biofilms. Weiland-Bräuer et al.[34] found that the thickness of *Staphylococcus epidermidis* biofilms decreases by (88 ± 3)% and (56 ± 4)% after treatment with the QQ enzymes MBP-QQ-5 and MBP-QQ-7, respectively. These drastic effects on biofilm maturation were confirmed by SEM and were similar with those seen in our experiment.

3.5 Inhibitory effects of the CCE on preformed *P. fluorescens* biofilms

The effect of the CCE on the preformed biofilm structure of *P. fluorescens* is shown in Figs. 2e–h. The clearance rates of the CCE at concentrations of 57, 86, and 114 μg/mL against preformed biofilms of *P. fluorescens* were 21.73%, 35.71%, and 46.67%, respectively (Fig. 2e). This indicated that the CCE also had a scavenging capacity for preformed biofilms of *P. fluorescens*. Meanwhile, cell survival assays indicated that the CCE had no effect on the survival of planktonic cells (Fig. 2f).

The biofilm scavenging activity of the CCE was also confirmed using SEM and CLSM. The removal of preformed biofilms was observed using SEM after treatment with the CCE (Fig. 2g). In the control group, the *P. fluorescens* cells were embedded within and wrapped in the extracellular matrix, forming dense and concentrated biofilms. When treated with 57 μg/mL CCE, the biofilms began to break and disintegrate, creating gaps. When the concentration of CCE increased to 114 μg/mL, only a few dispersed rod-shaped *P. fluorescens* cells remained. This phenomenon confirmed the scavenging effect of the CCE on preformed *P. fluorescens* biofilms.

Fig. 2h shows the CLSM images of *P. fluorescens* biofilms cleared by the CCE. The green fluorescence signal gradually decreased with an increase in CCE concentration (57, 86, and 114 μg/mL). The pores formed on the biofilm became larger, and the thickness gradually decreased. Under CCE treatment at 57, 86, and 114 μg/mL, the thickness of the biofilms reduced from 30 to 18, 16, and 12 μm, respectively. These changes were consistent with SEM observations, showing that the CCE had a certain scavenging ability against preformed biofilms of *P. fluorescens*. Yu et al.[35] used CLSM and SEM to demonstrate that andrographolide has a certain scavenging effect against *Listeria monocytogenes* biofilms without affecting the activity of planktonic cells, but its scavenging ability is weaker than its inhibitory ability, consistent with our findings. *L. plantarum* YP4-1-2 CCE has a limited action against preformed biofilms, since its penetration is reduced due to the compact nature of the biofilm structure.

3.6 Effect of the CCE on *P. fluorescens* motilities

Swarming and swimming are the two main modes of bacterial migration mediated by flagella. The movement characteristics of bacteria help them colonize the host surface and form microcolonies[36]. The capacity for movement plays a key role in the subsequent formation of bacterial biofilms. Figs. 3a–d shows the inhibitory effect of the CCE on *P. fluorescens* swarming and swimming. Treatment with CCE at concentrations of 57, 86, and 114 μg/mL led to swimming inhibition rates of 34.17%, 48.22%, and 58.42%, respectively. Meanwhile, the corresponding swarming inhibition rates were 82.46%, 87.87%, and 89.77%, respectively. These results indicated that the motilities of *P. fluorescens* were significantly inhibited in the presence of the CCE. This further suggests that the CCE may inhibit the formation of *P. fluorescens* biofilms by interfering with the adhesion of their flagella to contact surfaces.

3.7 Effect of the CCE on the QS-regulated phenotypes of *P. fluorescens*

In skim milk plates (Fig. 3e), a large transparent circle was formed in the control group, which indicated that *P. fluorescens* released a large amount of extracellular protease and decomposed the proteins in the medium. Compared with the control group, the 57, 86, and 114 μg/mL CCE treatment groups showed extracellular protease

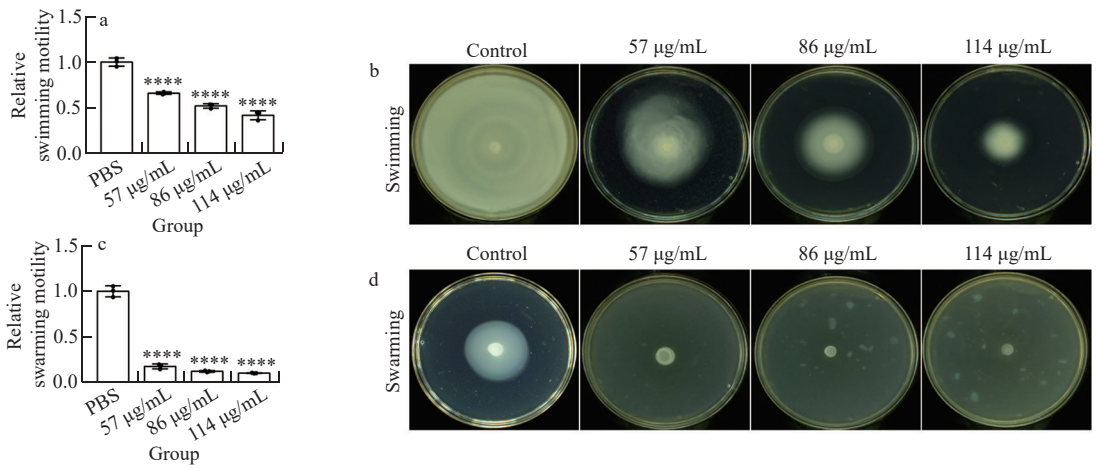


Fig. 3 Effect of the crude cell extract (CCE) at protein concentrations of 57, 86, and 114 μg/mL on the swimming (a, b) and swarming (c, d) motility of *P. fluorescens*. Effect of the CCE at protein concentrations of 57, 86, and 114 μg/mL on the (e) extracellular enzymes and (f) BAs of *P. fluorescens*. Error bars represent the standard deviations of three independent experiments. **** $P < 0.000\ 1$ versus control.

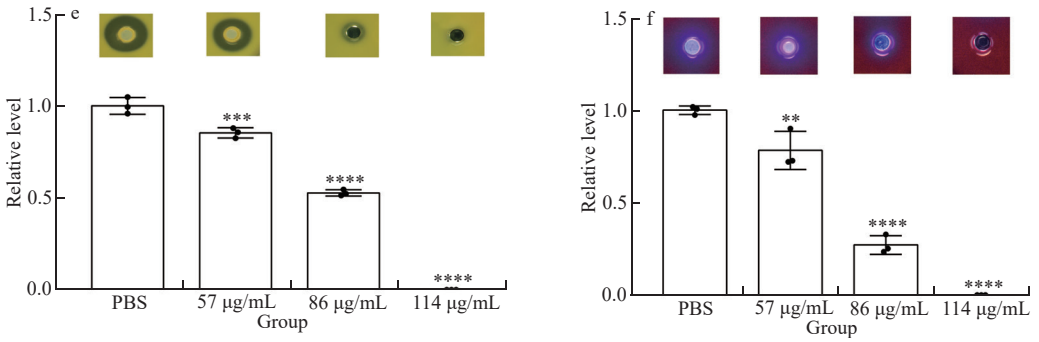


Fig. 3 (*Continued*)

inhibition rates of 14.65%, 47.27%, and 100%, respectively. This indicated that the CCE had a significant inhibitory effect on the release of extracellular protease in *P. fluorescens*. Shen et al.[37] found that the AHL acylase PF-1240 could inhibit the expression of *Hafnia alvei* extracellular protease. Furthermore, as the enzyme concentration increased, the size of the clear circle gradually decreased. When 25 μg/mL of enzyme was added, the activity of the extracellular protease was completely inhibited. These previous findings were consistent with our results.

Similarly, in the BAs test plate (Fig. 3f), a purple area appeared in the control group, indicating that *P. fluorescens* produced a large amount of BAs through amino acid decomposition. Studies have shown that high concentrations of BAs (histamine and putrescine) can be produced from fish and fish products in the presence of microorganisms such as intestinal bacteria and *P. fluorescens*. The BAs such as putrescine, histamine, and cadaverine with unpleasant and unacceptable off-flavors are produced[38]. Dai et al.[39] found that exogenous AHLs can significantly increase the production of BAs in *P. koreensis* PS1. QS plays an important role in the spoilage potential of *P. fluorescens* by controlling the expression of coding sequences related to amino acid and BA metabolism[38]. After treatment with the CCE at concentrations of 57, 86, and 114 μg/mL, the purple region became smaller, and the inhibition rates for BAs were 21.74%, 72.87%, and 100%, respectively. The results were in agreement with that of Wang et al.[40], who demonstrated that anthocyanins inhibit BAs and downregulate the expression of the *odc* gene (responsible for putrescine) in *Shewanella baltica* by interfering with QS. Gene *speA* (encode arginine decarboxylase), *potA* (encode polyamine transporter ATP-binding protein), *potC* (encode polyamine ABC transporter permease), and *arcA* (encode arginine deiminase) are involved in BAs production in *Pseudomonas*[38]. But, the inhibition effect of QSIs on BA related-gene in *Pseudomonas* has not been reported in previous study. Based on predictions of the degradation effect of the YP4-1-2 CCE, AHL inhibited the production of BAs by *P. fluorescens*.

3.8 Effect of the CCE on QS-related gene expression

In order to further explore anti-QS mechanism of the YP4-1-2 CCE against *P. fluorescens*, the transcriptional response of *P. fluorescens* after treatment with the CCE was detected. As shown in Fig. 4, the expression levels of all tested genes critical for the regulation of the QS system in *P. fluorescens* (*rhlI*, *rhlR*, *aprX*, *algA*, *orm*, *flgA*, *ldcA*, and HZ99-RS00670) were down-regulated after CCE treatment.

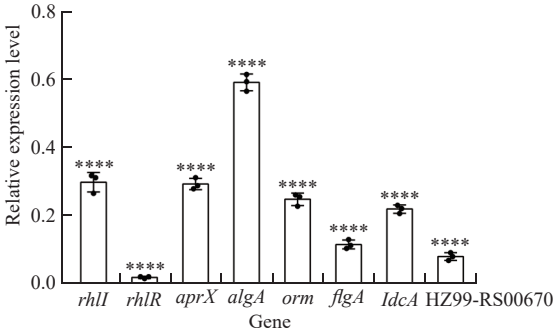


Fig. 4 Effect of the CCE at a protein concentration of 114 μg/mL on the expression of QS-related genes in *P. fluorescens*. Error bars represent the standard deviations of three independent experiments. **** $P < 0.000\ 1$ versus control.

The AHL synthetase gene (*rhlI*) and QS signaling molecule receptor gene (*rhlR*) are responsible for regulating the synthesis and reception of AHLs in the QS system of *P. fluorescens*[25]. After treatment with 114 μg/mL CCE, the expression levels of *rhlI* and *rhlR* in *P. fluorescens* decreased by 70.18% and 98.15%, respectively. The decrease in *rhlI*/*R* gene expression confirmed our finding that the AHLs of *P. fluorescens* were degraded after CCE treatment. Bacterial motility is achieved by the rotation of flagella, which consist of a polymer complex that allows bacterial cells to swim in a liquid environment[41]. The *flgA* gene is responsible for regulating the synthesis of periplasmic flagellar proteins in *P. fluorescens*[42]. Compared with the control group, the CCE treatment group showed an 88.48% lower expression of this gene. Meanwhile, gene *algA*, an important component of biofilms, is involved in the synthesis of extracellular polysaccharides[25]. The expression of this gene was downregulated by 40.92%, which consistent with the inhibition effect of the CCE on biofilm and extracellular polysaccharides.

The extracellular alkaline metal proteinase, encoded by *aprX* gene, is identified as the primary factor contributing to spoilage in refrigerated milk[43]. The QS-regulated gene *aprX* plays an important role in regulating the spoilage ability of *P. fluorescens*[44]. The expression of *aprX* was downregulated by 70.72% after CCE treatment. Siderophores are low molecular weight (200–2 000 Da) ferric ion-specific chelating agents secreted by *P. fluorescens* to obtain the iron required for their growth. Ornithine monooxygenase (encoded by *orm*) catalyzes the synthesis of siderophore precursors. The expression of the *orm* gene was downregulated by 75.14% following CCE treatment. The gene lysine decarboxylase (*ldcA*) is responsible for cadaverine production and is involved in the release of QS-regulated phenotypes in spoilage bacteria[45]. This gene was downregulated by 78.06% after

treatment with the CCE. Tyramine is considered to be one of the most toxic BAs, and it is particularly relevant for food safety. HZ99-RS00670 encodes a synthetic tyrosine decarboxylase, which can catalyze the production of tyramine[38]. Compared with the control, the expression of this gene was downregulated by 92.04% in the CCE treatment group.

Collectively, qRT-PCR data suggested that the CCE could downregulate the genes related to AHL synthetase, QS signaling receptor, biofilm-related flagellar, and the key phenotypes genes of *P. fluorescens*. The highest reduction recorded was in *rhlR* gene expression. The anti-QS mechanism of the YP4-1-2 CCE might be that the CCE-mediated degradation of AHLs prevented AHLs bind to the receptor protein rhlR, resulting in decline *rhlR* gene expression, which in turn decreases the expression of downstream phenotypes genes.

3.9 Identification of putative QQE-coding genes in L. plantarum YP4-1-2

Based on the functional annotation of the genome sequence, the gene numbered GE001915 (YP1915) in *L. plantarum* YP4-1-2 was identified as the target gene encoding the putative QQE. Sequence analysis of YP1915 revealed that it encoded a precursor protein with 338 amino acids. The phylogenetic tree showed that the YP1915 sequence was 100% homologous to the sequence encoded a CGH family protein from *Lactiplantibacillus* WP 011100864.1 (Fig. 5a). This result indicated that YP1915 belonged to the Ntn hydrolase super family. Thus, the QQE encoded by YP1915 was a typical PVA and was named *Lp*PVA.

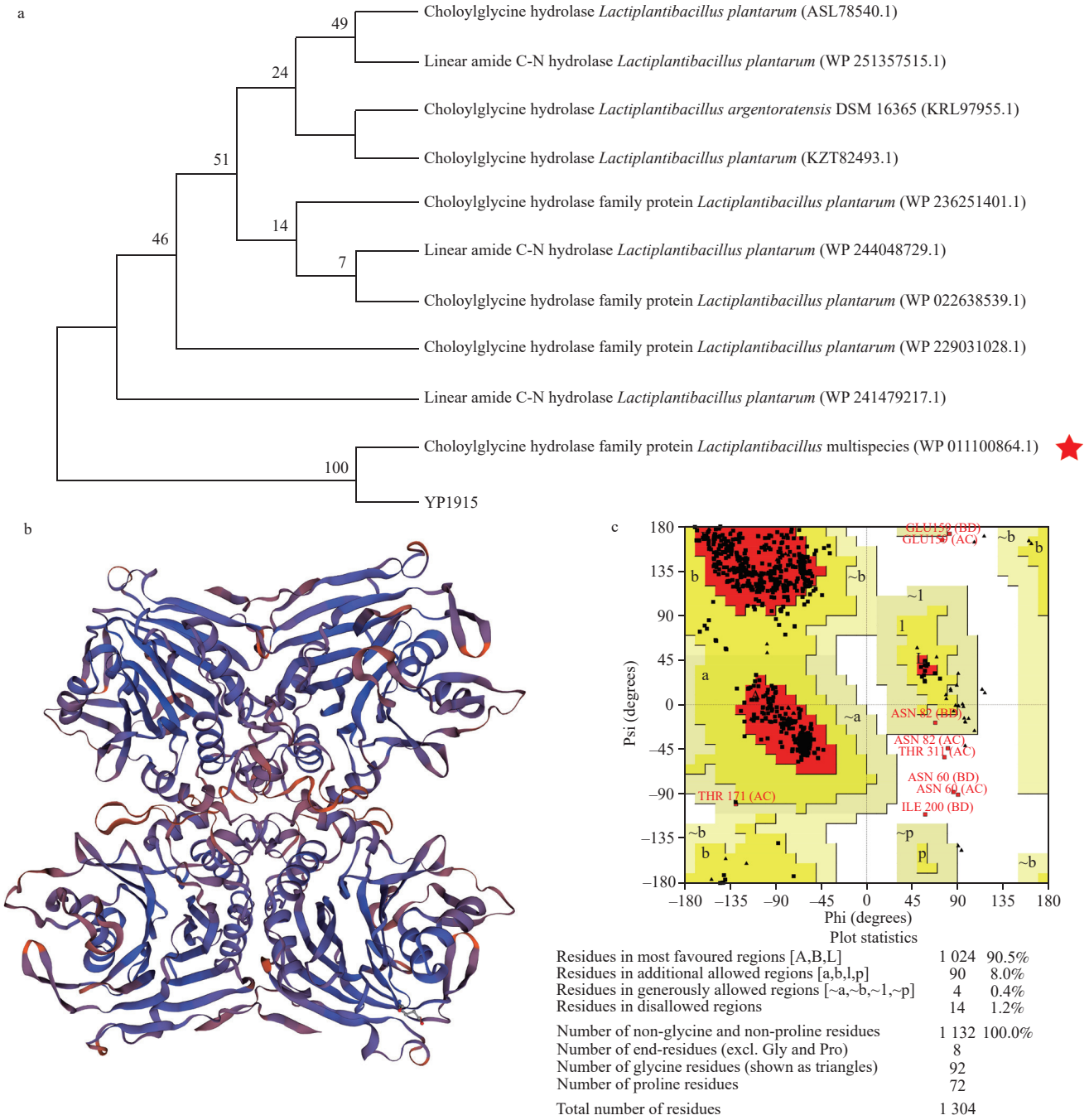


Fig. 5 (a) Phylogenetic tree of the YP1915 sequence. (b) 3D structure of the protein YP1915. (c) Evaluation of the 3D structure of YP1915 using Ramachandran plots.

3.10 Construction and evaluation of the YP1915 protein model

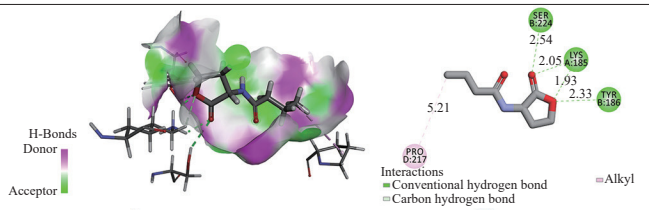
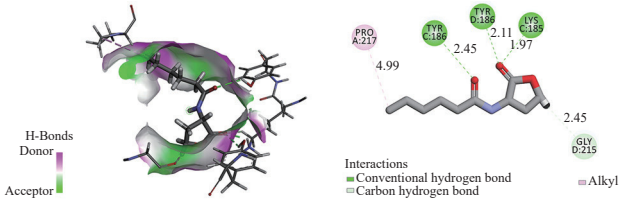
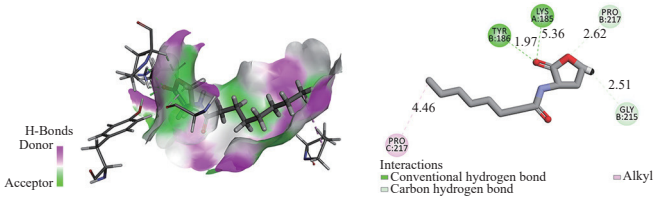
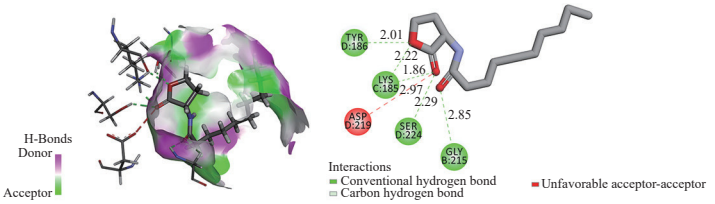
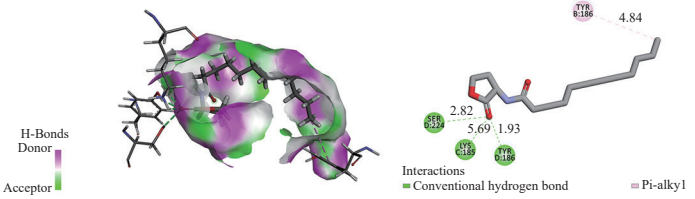
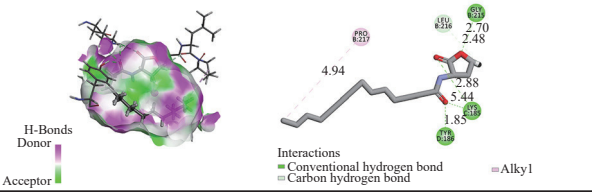
The function of a protein depends on its native three-dimensional structure[46]. To determine the functional characteristics of the YP1915 protein, its tertiary structure was constructed using SWISS model. Based on the comprehensive score, the crystal structure of PVA from *Bacillus subtilis* (Fig. 5b) was selected as a template for YP1915 protein modeling. The similarity of the templates was above 30%, indicating that the build model could predict the target proteins well.

Ramachandran plots were used to evaluate the quality of the three-dimensional structure. These plots can predict whether the amino acids in the protein structure are in a dominant region via energy visualization, reflecting the rationality of conformation. Ramachandran diagrams showed that, in YP1915 models, 90.5% of amino acids were located in the most favoured regions, 8.0% in additionally allowed regions, 0.4% in generously allowed regions, and 1.4% in disallowed regions (Fig. 5c). This demonstrated that the protein model of YP1915 had obtained reasonable conformation.

3.11 Analysis of the active site of LpPVA

Most QQEs have multiple substrates. For example, AiiA lactonase can efficiently inactivate several AHLs (i.e., C_4-, C_6-, C_8-HSL, etc.)[5]. Analysis of these ligands and their interactions can help us understand how a single active site binds to different AHLs. Therefore, in order to clarify the interactions between AHLs and the YP1915 protein from *L. plantarum* YP4-1-2, we used the CDCOCKER docking simulation in Discovery Studio 2017 and predicted the YP1915-AHLs complex. Docking results of the YP1915 proteins with their ligands are listed in Table 1. The results showed that YP1915-AHLs (C_4-, C_6-,

Table 1
Docking results of interaction between YP1915 protein model and AHLs.

AHL	CDOCKER energy (kcal/mol)	Ligand-protein interactions
C_4-HSL	–28.478 8	
C_6-HSL	–32.937 2	
C_8-HSL	–35.464 8	
C_{10}-HSL	–39.549 9	
C_{12}-HSL	–42.765 9	
C_{14}-HSL	–46.443 5	

C_8-, C_{10}-, C_{12}-, and C_{14}-HSL) complexed with CDCOCKER energies of –28.478 8, –32.937 2, –35.464 8, –39.549 9, –42.765 9, and –46.443 5 kcal/mol, respectively. Hence, the docking energy decreased gradually with the growth of the carbon chain. This indicated that the ability of the protein to bind AHLs increased with the length of the carbon chain. This was consistent with the results of previous AHLs quenching experiments. Therefore, we inferred that YP1915 might have a better quenching effect on long-chain AHLs.

The stability of protein structure and specificity of molecular recognition and directionality during interaction are guaranteed by hydrogen bonds. In the YP1915-AHLs docked model, key residues (Ser B:224, Lys A:185, Tyr B:186, Tyr C:186, Tyr D:186, Lys C:185, Gly B: 215, and Ser D: 224) showed hydrogen-bonding interactions with AHLs. In addition, the number of hydrogen bonds can affect the binding affinity, stability, and drug efficacy[47]. These key residues can reveal the molecular interactions involving the binding of substrates to the enzyme, elucidate biological processes, and provide better strategies for subsequent protein engineering. The above analysis demonstrated that the binding ability of *Lp*PVA to long-chain AHLs was better than that to short-chain AHLs. The predict active-site catalytic amino acids were Lys 185, Tyr 186 and Ser B:224.

4. Conclusions

L. plantarum YP4-1-2 has good QQ activity against AHLs. The active substance responsible for quenching is *Lp*PVA and is present in the CCE. *L. plantarum* YP4-1-2 CCE has a strong inhibitory and scavenging activity against *P. fluorescens* biofilms, and also has good inhibitory effects on QS-regulated phenotypes, including extracellular protease and BAs. In our study, qRT-PCR analysis confirmed that *L. plantarum* YP4-1-2 CCE significantly inhibits the expression of the QS signaling molecule receptor (*rhlR*) and the biofilm-related flagellar protein (*flgA*) of *P. fluorescens*. In addition, molecular docking results showed that *Lp*PVA can bind effectively to different AHLs, although it shows stronger binding to long-chain AHLs than to short-chain AHLs.

A large number of studies have shown that the QS system controls the expression of virulence genes, biofilm formation, and drug resistance mechanism of many in bacterial pathogens. Therefore, it is an important target against bacterial infection. Among the QSIs, QQEs are considered to be the most safe and reliable. QQEs weaken the regulation of the QS system in bacteria by quenching QS signals, thus exerting anti-infection effects[3]. The results of this study indicate that *Lp*PVA, produced by *L. plantarum* YP4-1-2, has great potential as a new QSI for controlling *P. fluorescens* contamination and subsequent food spoilage. However, the underlying mechanism of *Lp*PVA inhibition on the QS system of *P. fluorescens* still needs to be thoroughly investigated, and further cell and animal experiments are needed to validate the mechanism. It is worth considering whether the action mode of *Lp*PVA on *P. fluorescens* is *Lp*PVA-specific, and whether *Lp*PVA can inhibit the QS system of other specific spoilage organism to support the protection of food quality in actual production.

Conflict of interest

The authors declare that there is no conflict of interest in the publication of this paper.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250294.

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