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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Lonicera japonica protects *Pelodiscus sinensis* by inhibiting the biofilm formation of *Aeromonas hydrophila*

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Abstract

Aquaculture has suffered significant financial losses as a result of the infection of zoonotic *Aeromonas hydrophila*, which has a high level of resistance to classic antibiotics. In this study, we isolated an *A. hydrophila* strain B3 from diseased soft-shelled turtle (*Pelodiscus sinensis*), which is one of the most commercially significant freshwater farmed reptiles in East Asia, and found that *A. hydrophila* was its dominant pathogen. To better understand the inhibition effect and action mechanism of Chinese herbs on *A. hydrophila*, we conducted Chinese herbs screening and found that *Lonicera japonica* had a significant antibacterial effect on *A. hydrophila* B3. Experimental therapeutics of *L. japonica* on soft-shelled turtle showed that the supplement of 1% *L. japonica* to diet could significantly upregulate the immunity-related gene expression of soft-shelled turtle and protect soft-shelled turtle against *A. hydrophila* infection. Histopathological section results validated the protective effect of *L. japonica*. As the major effective component of *L. japonica*, chlorogenic acid demonstrated significant inhibitory effect on the growth of *A. hydrophila* with MIC at 6.4 mg/mL. The in vitro assay suggested that chlorogenic acid could inhibit the hemolysin/protease production and biofilm formation of *A. hydrophila* and significantly decrease the expression of quorum sensing, biofilm formation, and hemolysin-related genes in *A. hydrophila*. Our results showed that the Chinese herb *L. japonica* would be a promising candidate for the treatment of *A. hydrophila* infections in aquaculture, and it not only improves the immune response of aquatic animals but also inhibits the virulence factor (such as biofilm formation) expression of *A. hydrophila*.

Key points

- *A. hydrophila was the dominant pathogen of the diseased soft-shelled turtle.*
- *L. japonica can protect soft-shelled turtle against A. hydrophila infection.*
- *Chlorogenic acid inhibits the growth and biofilm formation of A. hydrophila.*

Keywords *Aeromonas hydrophila* · Soft-shelled turtle · Chinese herb · Chlorogenic acid · Immune response · Biofilm formation

Introduction

A zoonotic bacteria called *Aeromonas hydrophila* is common in aquatic habitats and can infect fish, amphibians, reptiles, and mammals with various infectious diseases (Rasmussen-Ivey et al. 2016). Pathogenic *A. hydrophila* is the leading cause of bacterial infections in freshwater fish (Akmal et al. 2020). Infections associated with *A. hydrophila* have resulted in enormous economic losses in aquaculture, severely restricting the sustainable and healthy development of fisheries (Mzula et al. 2019).

A. hydrophila's ability to cause disease depends on virulence factors and bacterial mechanisms such as motility,

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bacterial adhesions, toxins, proteases, hemolysins, and biofilm formation (Tanhay Mangoudehi et al. 2020). Sedimentary communities of microorganisms known as biofilms are covered in a slimy extracellular polysaccharide matrix that they self-secrete. The development of biofilms in many bacteria can increase the production of several virulence factors and multidrug resistance (Selvaraj et al. 2020). As a result, reducing the growth of biofilms or preventing their development may be a valuable strategy for reducing the pathogenicity and drug resistance of bacteria. Quorum sensing (QS), the cell-to-cell signaling communication, affects the pathogenicity of various bacterial pathogens and contributes to the development of biofilms (Lee et al. 2018). It has been observed that the QS system positively regulates a number of bacterial virulence factors, including exoprotease production, secretion of T6SS (Type 6 secretion system), bacterial adhesions, motility, and the formation of biofilms (Khajanchi et al. 2012).

Currently, antibiotic misuse is causing bacterial resistance, making it urgently necessary to create new medications to combat the harmful *A. hydrophila* (Chokshi et al. 2019). In drug development, natural products have served as a source of materials with biological activities for many years (Chi et al. 2017). Traditional Chinese medicines, often known as Chinese herbs, have a long history in China and are used frequently to treat infectious diseases in people, aquatic animals, and other creatures on land (Van Hai 2015; Guo et al. 2019; Zhu 2020). Several Chinese herbal formulas have even played a significant role in treating COVID-19 (Fu et al. 2021). Plant extracts containing various compounds exhibit antibacterial, anti-inflammatory, and antioxidant activity (Kim et al. 2014; Huang et al. 2020; Vijayakumar et al. 2021). *Lonicera japonica* is a traditional edible-medicine herb that is extremely widely used. Modern pharmacological studies have confirmed the antiviral, antibacterial, antioxidative, antitumor, liver protective, hypoglycemic activities and anti-inflammatory activities of *L. japonica* (Li et al. 2020; Su et al. 2021). Chlorogenic acid is a naturally occurring substance in many plants, including *L. japonica*. Chlorogenic acid has been discovered to have antibacterial, antiviral, and immunomodulatory properties in vitro and in vivo as a natural plant extract (Naveed et al. 2018; Miao and Xiang 2020). Previous studies have demonstrated that chlorogenic acid can inhibit *Klebsiella pneumoniae*, *Helicobacter pylori*, and *Escherichia coli* by different mechanisms (Lou et al. 2011; Naveed et al. 2018). However, the inhibitory effect of *L. japonica* or chlorogenic acid on *A. hydrophila* virulence has not been clarified.

Soft-shelled turtle (*Pelodiscus sinensis*) is one of the most significant freshwater cultured reptiles in East Asia. Because of their high-protein content, low-fat content, and inclusion of vital elements, including multivitamins and minerals, the soft-shelled turtle is regarded as a high-quality food (Xu et al. 2020; Zhang et al. 2022). Over the past few decades, with the increasing intensive culture, the infectious disease of soft-shelled turtle caused by pathogenic bacteria such as *Citrobacter* sp., *A. hydrophila*, *Pseudomonas aeruginosa*, and *Bacillus cereus* became more and more prevalent (Chen et al. 2013; Liu et al. 2019). In the study, an *A. hydrophila* strain named B3 was isolated from diseased soft-shelled turtles on a farm in Hubei Province, China. By screening the effective Chinese herbs on inhibiting the growth of *A. hydrophila*, we found that the extract of *L. japonica* has significant antibacterial activity on strain B3. Fed with *L. japonica*, the soft-shelled turtle showed more resistance against *A. hydrophila* infection. As the major active compound of *L. japonica*, chlorogenic acid also demonstrated significant antibacterial activity on strain B3 and reduced its virulence via inhibiting the biofilm formation of *A. hydrophila*. Our findings clarify the inhibitory effect of *L. japonica* or chlorogenic acid against *A. hydrophila* virulence and provide a new ecological approach to against *A. hydrophila* infection in aquaculture.

Materials and methods

Soft-shelled turtle samples, isolation, and identification of the pathogenic bacteria

The diseased soft-shelled turtles (*P. sinensis*) from a commercial aquaculture farm in Xiantao, Hubei, China, were used to isolate the suspected pathogen. These diseased soft-shelled turtles were sluggish, often crawling on the shore or the sunbathing platform. Using 0.85% (w/v) saline solution (tissue to saline = 1:5), liver, kidney, and spleen homogenates from the soft-shelled turtle samples were created. They were then disseminated aseptically over LB agar media and incubated at 28 °C for 48 h. Most cultured colonies were characterized by smooth borders, an elevated center, and a light-yellow tint. To test the purity of the isolates, several dominant colonies were subcultured in identical circumstances. One isolate was obtained and named as strain B3.

The bacterial biochemical identification tube (Hangzhou Microbiological Reagent, China) was used for the purpose of characterizing the biochemical characteristics of the isolates. 16S *rRNA* gene sequencing was adopted for the purpose of molecular identification of the isolates. Amplifying the 16S *rRNA* gene from the isolates was performed by PCR using the following primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) as previously described (Park et al. 2015). Tsingke Biotechnology Co., Ltd. sequenced PCR products. The 16S *rRNA* gene was analyzed by comparative alignment as described (Nemec et al. 2011; Park et al. 2015). The nucleotide sequence of the 16S *rRNA* gene of strain B3 has been assigned to GenBank accession number OM906077.1.

Strain B3 (CCTCC AB 2023160) was cultured and publicly deposited in China Center for Type Culture Collection (CCTCC, Wuhan, China).

Artificial infection experiment

Apparently healthy experimental soft-shelled turtles (*P. sinensis*) used in this study weighing about 1000 g were obtained from a farm in Xiantao, Hubei, China, and maintained in fiberglass tanks filled with 26 °C water. Turtles were fed satiation twice daily. No history of illness or abnormality of the turtles were observed, and they were adapted for 2 weeks prior to challenge. The isolated pathogenic bacteria were prepared in a sterile salt solution to a concentration of 1.0×10^6 CFU/mL. The selected soft-shelled turtles were separated into two groups at random: a control group and an experimental group, each with 10 turtles. The experimental group was injected with 200 µL of the bacterial solution intraperitoneally, and the control group was injected with 200 µL of sterile saline, and the injected turtles (two different groups) were cultured in isolated tanks, separately. The activity and mortality of the soft-shelled turtles were observed and recorded every 12 h for 8 days. Survival curves were plotted using GraphPad Prism 8.0.2 software.

Chinese herb screening

As shown in Table 1, 8 different Chinese herbs were used in this study to screen the effective herbs in inhibiting the growth of *A. hydrophila* B3. Briefly, 100 mL distilled water was added with 10 g of each of Chinese herb powder. On a hot plate, the mixture was boiled for 4 h while being constantly stirred. Occasionally, distilled water was added to avoid dryness. After the mixture had finished boiling, distilled water was added to bring the total amount to 100 mL. The mixture was chilled, centrifuged, and filtered. Then, the collected mixture for each of Chinese herb, separately, was used to test their inhibitory effects on *A. hydrophila* B3 using the Oxford cup method (Liang et al. 2021). Following 16 h of incubation at 28 °C, the size of the inhibition circle was determined using vernier calipers, and the inhibition effect of the Chinese herb extracts was assessed using the inhibition circle's diameter.

Table 1 Antibacterial activity of 8 Chinese herbs against *A. hydrophila* B3

Chinese herbs	Diameter of inhibitory plaque (mm)*
Lonicera japonica	26.02 ± 0.91^a
Bupleurum chinense	14.62 ± 0.97^b
Rhei Radix et *Rhizoma*	8.75 ± 0.76^c
Taraxacum mongolicum	6.90 ± 0.46^d
Coptis chinensis	6.86 ± 0.54^d
Viola yedoensis	6.89 ± 0.34^d
Plantaginis Semen	6.87 ± 0.47^d
Scutellaria baicalensis	6.81 ± 0.63^d

*Data are mean ± SD of three replicates. Superscript letters indicate significant difference ($p < 0.05$) among groups

The therapeutic effects of *L. japonica* on soft-shelled turtles infected by *A. hydrophila*

This study tested the protective effects of the screened Chinese herb *L. japonica* on the infection of *A. hydrophila* B3 in soft-shelled turtles. The experimental soft-shelled turtles having a normal weight around 26 g were obtained as described above. The experimental groups were control group, control + B3 (*A. hydrophila* B3) group, 1% Chinese herb (*L. japonica*) + B3 group, 5% Chinese herb (*L. japonica*) + B3 group, and 10% Chinese herb (*L. japonica*) + B3 group, 10 individuals in each group. The control group was soft-shelled turtle group fed without Chinese herb *L. japonica* and injected without *A. hydrophila* B3, and the control + B3 (*A. hydrophila* B3) group was soft-shelled turtle group fed without Chinese herb *L. japonica* but injected with *A. hydrophila* B3. 1% Chinese herb (*L. japonica*) + B3 group was soft-shelled turtle group fed with 1% Chinese herb *L. japonica* for 8 weeks and injected with *A. hydrophila* B3, and so on for 5% Chinese herb (*L. japonica*) + B3 group and 10% Chinese herb (*L. japonica*) + B3 group, respectively. The injected *A. hydrophila* B3 were produced in sterile saline at 1.0×10^6 CFU/mL, and each of the experimental soft-shelled turtles received an intraperitoneal injection of 50 µL of the bacterial solution. The activity and mortality of the soft-shelled turtles were observed and recorded every 12 h for 10 days. Survival curves were plotted using GraphPad Prism 8.0.2 software.

Determination of minimal inhibition concentrations (MICs) and growth curve assay

Chlorogenic acid is the major effective component of Chinese herb *L. japonica* (Shang et al. 2011). To preliminarily elucidate the action mechanism of *L. japonica* on *A. hydrophila*, chlorogenic acid was substituted for *L. japonica* in the following experiments. Different concentrations of chlorogenic acid (purchased from Macklin Biochemical Co. Ltd., Shanghai, China, with a purity concentration of 98%) were co-cultured with *A. hydrophila* B3 to determine the inhibiting effect of chlorogenic acid on the growth of the bacteria. Briefly, *A. hydrophila* B3 was cultured in LB medium at 28 °C overnight; then, the culture was inoculated at 1:1000 (vol/vol) into fresh LB medium. Meanwhile, different concentrations of chlorogenic acid were added, to the final concentrations at 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and

9.6 mg/mL, respectively. The mixtures were subsequently cultured at 28 °C for 16 h while being constantly shaken at 200 rpm, and the culture medium turbidity was observed and recorded.

In addition, we recorded the growth curves of *A. hydrophila* B3 in a culture medium with different concentrations of chlorogenic acid (Macklin, China). Chlorogenic acid at final concentrations of 0, 0.8, 1.6, 3.2, and 6.4 mg/mL, respectively, was added to *A. hydrophila* B3 culture medium. The bacterial growth was observed by reading the OD_{600} values every 2 h during 16 h with constant shaking at 200 rpm.

Hemolysin production assay

Cultures from *A. hydrophila* B3 with different concentrations of chlorogenic acid (Macklin, China) incubated for 18 h in LB medium at 28 °C with shaking (200 rpm) were treated with trypsin (Biosharp, China) at a final concentration of 0.05% at 28 °C for 1 h. The number of hemolytic units per milliliter of cellular filtrate per 1×10^8 CFU was recorded. The culture supernatants were mixed with 2% rabbit red blood cells (1:3) to 200 µL in 96-well plate. After incubation at 37 °C for 1 h, the plate was incubated at 4 °C overnight. Hemolytic activity was evaluated by measuring absorbance of the mixture supernatants at 540 nm. In the experiment, saline and 2% rabbit blood erythrocytes were added in proportion as a negative control (Khajanchi et al. 2012). All experiments were performed in 5 times.

Protease production assay

An aliquot (200 µL) of overnight culture filtrates from *A. hydrophila* B3 with different concentrations of chlorogenic acid (Macklin, China) was added to tubes which contained 800 µL of the DPBS and 5 mg of Hide azure powder (Sigma-Aldrich, USA) substrate. The tubes underwent a 3-h incubation period at 37 °C in a shaker incubator. Blue color was emitted from the substrate as the proteinase in the culture filtrates catalyzed the reaction, and the reaction was measured at OD_{595}. Protease activity was estimated based on 1×10^8 CFU per mL of culture filtrate. A negative control was the substrate treated with just LB medium (Erova et al. 2006). Each experiment was run in 5 times.

Biofilm formation assay

An overnight-grown bacterial culture ($OD_{600} = 1.0$) of *A. hydrophila* B3 with different concentrations of chlorogenic acid (Macklin, China) up to 200 µL were transferred into 96-well plate and statically incubated at 28 °C for 28 h. The medium was carefully removed, and each well was washed three times with deionized water. For the remaining attached bacteria, 200 µL of 99% methanol (Sinopharm Chemical Reagent, China) per well was used for fixation, and after 15 min, the plates were drained and allowed to dry. Plates were then stained using 0.2 mL of 1% Hucker crystal violet (Macklin, China) for Gram staining per well for 5 min. The plate was cleaned of excess stain by being submerged in flowing water. The plates were then air-dried, followed by 100 µL of 33% (v/v) ice acetic acid (Sinopharm Chemical Reagent, China) to extract the dye bound to the structure of the biofilm, and the OD_{595} value was measured for each sample (Stepanović et al. 2000).

Scanning electron microscopy (SEM) analysis

A. hydrophila B3 was grown in 24-well polystyrene microtiter plates containing glass slides ($d = 14$ mm) (Biosharp, China) with and without chlorogenic acid (Macklin, China) at 28 °C for 24 h. Parallels were prepared in triplicate for the control and each concentration group. Planktonic cells and used medium were removed following the incubation, and the glass slides were gently cleaned three times with PBS (pH 7.2). For SEM observation, samples were prepared with the method as described by Zhou et al. (2017). A 2.5% glutaraldehyde solution (Sinopharm Chemical Reagent, China) was used to fix the biofilms to the glass slides, and graded ethanol (Sinopharm Chemical Reagent, China) was used to dehydrate them (50, 70, 80, 90, and 100%). Slides were then lyophilized, gold coated, and observed under SEM (Techcomp, China).

Quantitative real-time PCR analysis

Total RNA was extracted from the bacterial samples using a Bacterial RNA Isolation Kit (Aidlab, Beijing, China). RNA samples from the softshell turtle liver were extracted using 1 mL Trizol reagent (Invitrogen) to identify transcriptional expression of the representative genes in the present study. cDNA synthesis was carried out using an M-MLV Reverse-Transcript Kit (Applied Biological Materials Inc, BC, Canada). Quantitative PCR was carried out using the iQTM SYBR® Green Super mixture (Bio-Rad Laboratories, USA). Full names of the genes, as well as qRT-PCR primer sequences, were listed in Table 2. All experiments were carried out in at least triplicate. Differences were determined using the $2^{-\Delta\Delta Ct}$ comparative quantification method, and data were examined with one-way analysis of variance (ANOVA) and Tukey's post hoc test.

Statistical analysis

Each experiment was carried out at least three times, and the data were presented as mean ± standard deviation (SD). GraphPad Prism 8 was used for the statistical analysis. The statistical significance between groups was assessed using one-way ANOVA plus post hoc Tukey test or two-tail paired *t*-test. To indicate the statistical significance, the terminology used is as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Table 2 Primer pairs used for real-time qPCR

Primer	Sequence	PCR amplicon (bp)
16S-F	GCAACGCGAAGAACCTTACC	111
16S-R	GACACGAGCTGACGACAGCC	
luxS-F	ATGCCGTTATTGGACAGTTTTA	178
luxS-R	ACAGATGCTCAAGGGTGTGGAT	
luxR-F	CTGAAGCAGACCCAGCCCAT	118
luxR-R	AGACACCGTTTCTCACCCCG	
omp-F	GCAGAACAAACCGCTCAAGG	167
omp-R	CGATCAGGGATTCGGTGGAG	
hly-F	TCCGCACTATCTTGGCATCC	189
hly-R	TCTACCTCAACGTCAACCGC	
qseb-F	CCAGTCTTCCCGCATCACCAAC	178
qseb-R	GAGCTCCCTGTCCTCTCCCCGC	
omp48-F	GGCAACGACAAGTGGGAAAC	159
omp48-R	AGCCAGGGTCAGCTTGTAAC	
ompW-F	GATGCCAACAGCAAGGTACG	170
ompW-R	ACGAACCAGCGGTCATTGAT	
MshA-F	GGTTATCCGGAAGCAACCGA	171
MshA-R	GTTCAAAGTTGTCGCTGCGG	
MshB-F	TGACCAATAACGGCCTGGAC	133
MshB-R	GAAGCCGGACGGTAACTGAA	
manB-F	GGCTAGTTTGGCCGGTAAGT	174
manB-R	TGTTGCATCGCAGCATTAGC	
rmlB-F	GGAGTGGTAAAGCGTCTGCT	116
rmlB-R	ACCGTTCAAAGTTGTCGCTG	
ompTS-F	TAACGACGACAAAGCCGTCA	148
ompTS-R	GTAACCTGCGTTCAGACCCA	
β-actin-F	TGAGCTTCGTGTAGCACCTG	137
β-actin-R	AGCAACATACATGGCTGGGG	
il6-F	CTGCCAGATTGCGAGTCCTT	144
il6-R	CTCTGTGATCTTGGGGAGGT	
c3a-F	TTCCCAACTGCAAGAACGAG	123
c3a-R	GAAGGTTGGGATCAGGCTCAA	
dusp7-F	CTCGGCCTTGGAGGACTAAG	135
dusp7-R	GGGTAGGATCTGGACAGGGA	
myd88-F	TAGCCTTTCCCCAGGTGCTC	119
myd88-R	TGCAGGGATTAGTGTAGTCGC	

Results

A. hydrophila was the dominant pathogen of the diseased soft-shelled turtle

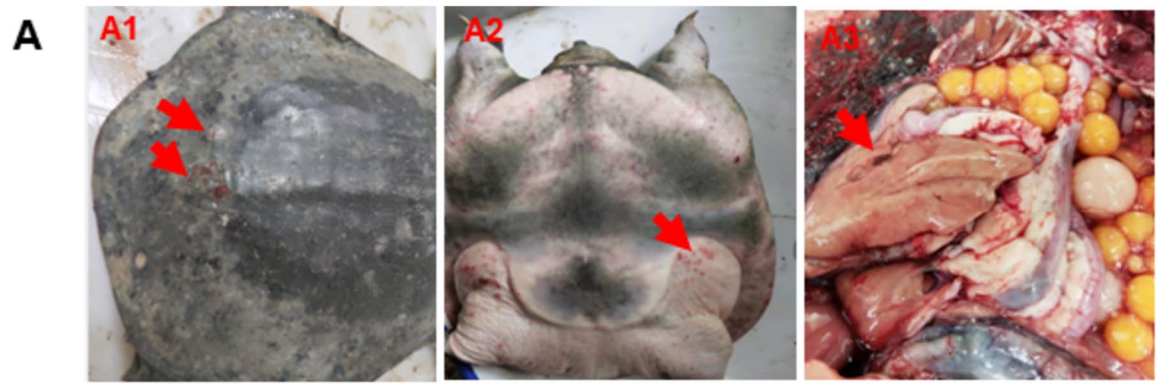
The moribund soft-shelled turtles were adult of 3 years old, weighing about 1600 g. Clinical symptoms such as tiredness and appetite loss were observed in the turtles. Petechial hemorrhages of the body surface (Fig. 1A1), bottom plate with many bleeding spots (Fig. 1A2), and liver hyperemia and hepatomegaly (Fig. 1A3) were also observed.

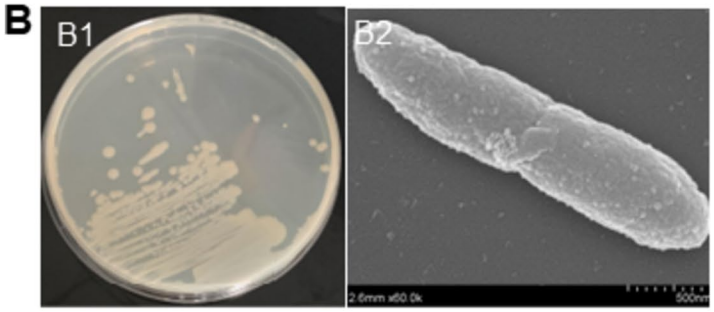
The dominant bacterium with consistent morphological characteristics was isolated from the livers and other samples of the diseased soft-shelled turtles. The colonies were characterized as moist, wet, faint yellow, and smooth with a central raised round shape (Fig. 1B1), and scanning electron microscopy revealed a short rod-shaped bacterium (Fig. 1B2). The dominant strain was purified and named B3. The physiological and biochemical characteristics of strain B3 were consistent with those of *A. hydrophila* strains JAY-1 and JAY-2 isolated from diseased soft-shelled turtles on a farm in Nanchang (Kang et al. 2019) (Fig. 1C). The 16S *rRNA* gene segments from strain B3 had nucleotide sequences that were 99.86% identical to those of *A. hydrophila* ATCC 7966^T (GenBank: X60404.2). Neighbor-joining phylogenetic tree based on the 16S *rRNA* gene of 1492 bp was further constructed, and the result showed that strain B3 was in the same branch where other *A. hydrophila* strains were located (Fig. 1D). These data indicated that the isolate B3 was *A. hydrophila*.

To confirm that strain B3 was the dominating pathogen leading to the septicemia outbreak in the soft-shelled turtle farm, experimental infection with B3 was performed. The experimental group of soft-shelled turtles showed mortality within the first 2 days of infection and all died within 8 days, while none of the control group died within 8 days ($p < 0.001$) (Fig. 1E). The dead individuals exhibited symptoms similar to those of naturally affected individuals. Colonies consistent with the morphology of the attacking strains could be isolated from the livers of the dead soft-shelled turtle in the experimental group, and their identification by the 16S *rRNA* gene showed that they were the corresponding attacking strain. The above data indicated that *A. hydrophila* B3 was the dominant pathogen causing disease and death in the soft-shelled turtles.

Comparison of the antibacterial activity of eight Chinese herbs against *A. hydrophila* B3

As alternatives for treating bacterial diseases in aquaculture, Chinese herbs are gaining global attention because they are inexpensive, simple to prepare, and do not have many negative consequences on the environment or animals (Van Hai 2015). In this study, we screened eight Chinese herbs to find effective Chinese herb against *A. hydrophila* B3. Inhibitory plaque diameters for decocted extracts from eight herbs (100 mg/mL, 200 µL) are presented in Table 1. From these data, it was observed that decocted extracts of *L. japonica* and *Bupleurum chinense* displayed significant antibacterial effects against



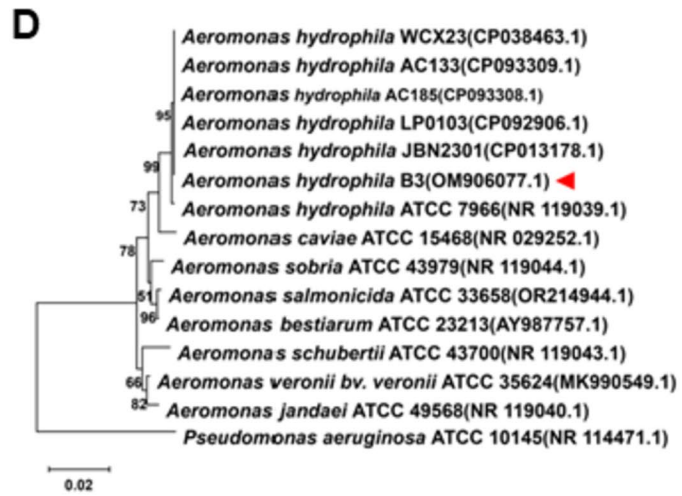


C

Physiological and biochemical characteristics of strain B3

Biochemical pproperties	B3	JAY-1	JAY-2
Glucose	+	+	+
Citrate	+	+	+
Arginine double hydrolysis	+	+	+
Xylose	-	-	-
Maltose	+	+	+
Mannitol	+	*	*
Sorbitol	-	-	-
Lysine	+	+	+
Ornithine	-	-	+
carbamide	-	-	-
Cane sugar	+	+	+

Notes: "+". positive, "-" . negative, "*" means no determination



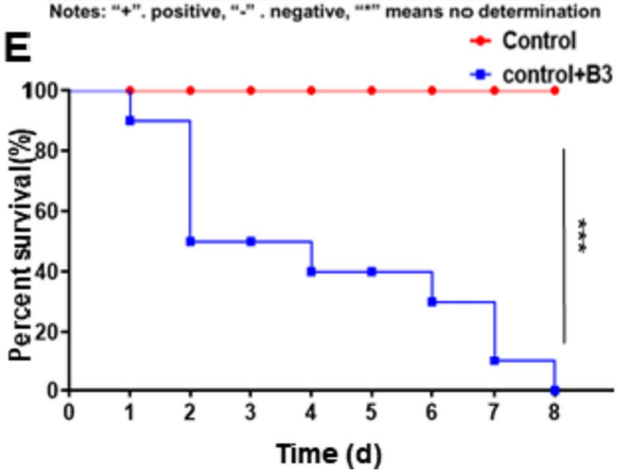


Fig. 1 Clinical signs and identification of the pathogenic bacteria of the diseased soft-shelled turtle. **A** Significant hemorrhage on the back (**A1**), redness and bleeding spots on the baseboard plate (**A2**), and liver with whitening and fibrosis (**A3**) in the diseased turtle. **B** Identification of isolate B3. **B1** Growth of *A. hydrophila* B3 on LB agar plate. **B2** TEM result of *A. hydrophila* B3. **C** Physiological and biochemical properties of isolates B3. **D** Neighbor-joining (N-J) tree based on the comparison of 16S *rRNA* gene sequences of *Aeromonas* spp. including the isolates B3. GenBank accession numbers are given in parentheses. Genetic distances were constructed using the *p*-distance model. Horizontal bar, genetic distance of 0.002. **E** Pathogenicity of the isolates B3 to soft-shelled turtle

A. hydrophila B3, with plaque diameters more than 14 mm in width. The *L. japonica* extract had the most potent antibacterial impact of all of them, and the diameter of the plaque was 26.02. ± 0.91 mm ($p < 0.05$) (Fig. 2A). Chlorogenic acid is the major active compound of *L. japonica* (Shang et al. 2011), and its chemical structure is shown in Fig. 2B. Other Chinese herbs such as *Rhei Radix* et *Rhizoma*, *Taraxacum mongolicum*, and *Coptis chinensis* showed low antibacterial effects due to their small inhibitory plaques (Fig. 2A and Table 1).

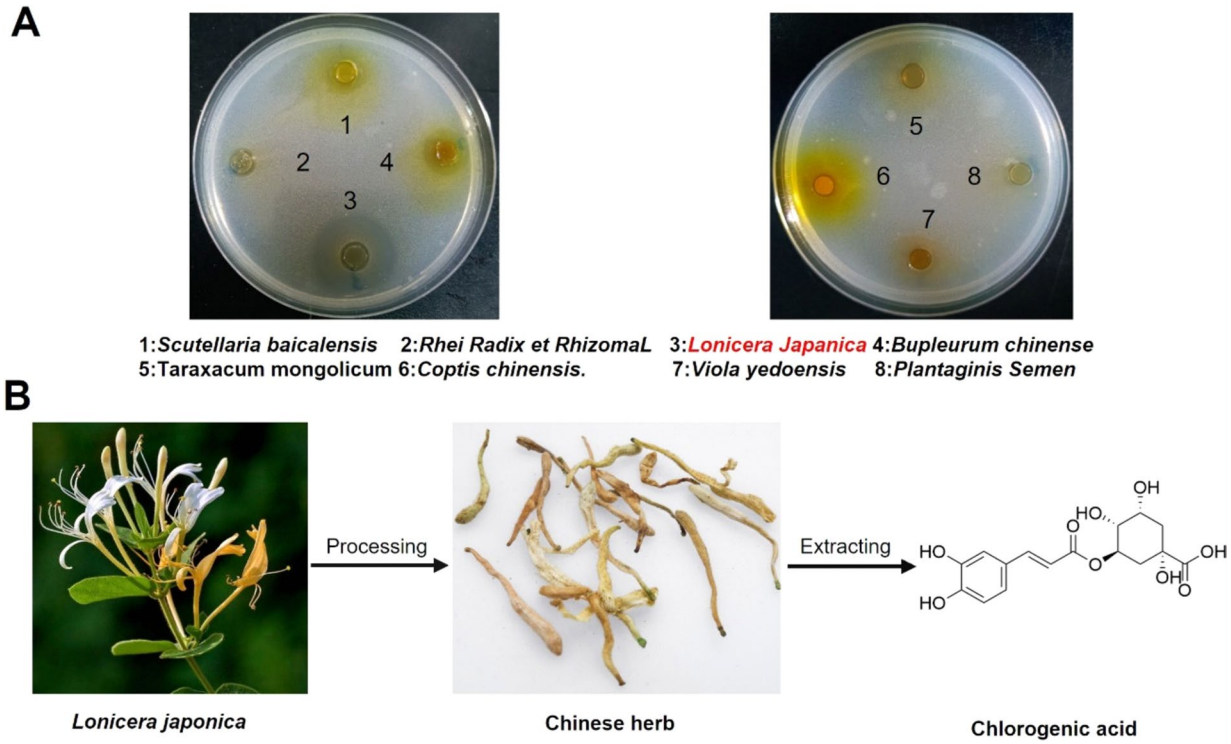


Fig. 2 **A** Antibacterial activities of 8 Chinese herbs against *A. hydrophila* B3. **B** Molecular structure of chlorogenic acid

Chinese herb (*L. japonica*) can protect soft-shelled turtle against *A. hydrophila* infection and enhance its immune response

To study the protective effect of Chinese herb (*L. japonica*) against *A. hydrophila* infestation in the soft-shelled turtles, experimental therapeutics of *L. japonica* on soft-shelled turtles was conduct. In this assay, Chinese herb (*L. japonica*) was included in basal diet with the concentrations of 0% (negative control), 1%, 5%, and 10%. Soft-shelled turtles with an average weight of 26.37 ± 0.79 g were fed the 4 diets separately for 8 weeks. The data showed that the weight gain of the 4 groups were 136.24 ± 5.38%, 182.26 ± 7.85%, 106.6 ± 6.18%, and 88.51 ± 8.03%, respectively. The 1% group had the best growth (body weight) promotion effect ($p < 0.01$) (Fig. 3A).

The impact of the 1% *L. japonica* treatment group on alterations in the immunity-related genes in the soft-shelled turtles was investigated using the qRT-PCR assay. Results showed that *myd88*, *il6*, *dusp7*, and *c3a* significantly increased expression levels by 196.60 ± 34.11%, 117.59 ± 38.32%, 485.97 ± 128.04%, and 358.1 ± 63.67%, respectively, compared to the group without *L. japonica* treatment (Fig. 3B). These data indicated that treating with 1% *L. japonica* had significant ability to activate the immune system of the soft-shelled turtles.

Figure 3C displays the cumulative mortality of soft-shelled turtles over several groups. No soft-shelled turtles perished throughout the 10-day trial in the *L. japonica* safety group or the negative control group. All soft-shelled turtles infected with *A. hydrophila* B3 in the group not receiving Chinese herbs displayed tiredness and a decline in appetite. Six days after infection, 70% of the infected soft-shelled turtles perished throughout the 10-day study period, and on day eight, 100% of mortality was noted. However, in the *L. japonica* therapeutic group, the 5% and 10% groups did not improve the disease resistance of the soft-shelled turtles ($p > 0.05$). In comparison, the 1% group had a significant protective effect on the soft-shelled turtles ($p < 0.001$), with a final survival rate of 40%.

Histopathological section results of the liver and intestine of the soft-shelled turtles from the group of no Chinese herb treating and infecting with *A. hydrophila* B3 (control+B3) and the group of 1% the Chinese herb treating and infecting with *A. hydrophila* B3 (1%+B3), respectively, showed a large number of hemosiderin precipitates, a large amount of red blood cell exudation in the liver, and a large amount of red blood cell exudation in the intestine sections of the control+B3 group. However, there was almost no hemosiderin precipitates or red blood cell exudation in the liver and intestine of the 1%+B3 group (Fig. 3D). These data suggested that treating with 1% *L. japonica* had a significant

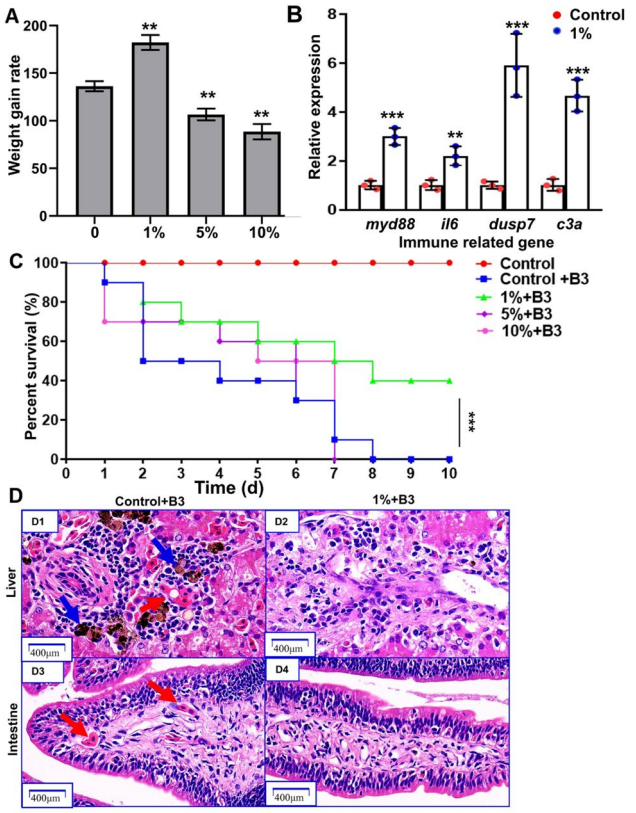


Fig. 3 **A** Weight gain rates of soft-shelled turtles after feeding with different concentrations of Chinese herb *L. japonica*. **B** Expression of immune-related genes in soft-shelled turtle after feeding with 1% Chinese herb *L. japonica*. Data are presented as the expression fold changes of mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by *t*-test. **C** Chinese herb *L. japonica* protects soft-shelled turtle against *A. hydrophila* infection. Survival of *T. sinensis* (n=10) was observed for 10 days. Histopathological sections of soft-shelled turtle after *A. hydrophila* infection. **D1**, **D3** No Chinese herb treating, **D2, D4** 1% treating group, red arrows indicate exudation of red blood cells. Blue arrows indicate deposition of hemosiderin

ability to protect soft-shelled turtles against *A. hydrophila* infection.

Chlorogenic acid has inhibitory effect on the growth of *A. hydrophila*

As shown in Fig. 2A and Table 1, *L. japonica* has significant inhibitory activity against *A. hydrophila*. However, no study on the inhibitory mechanisms of *L. japonica* or chlorogenic acid (the major effective component of *L. japonica*) against *A. hydrophila* has been mentioned. Hereby, we studied the antibacterial effect of chlorogenic acid on *A. hydrophila* B3 in vitro.

The MIC of chlorogenic acid was evaluated by a two-fold dilution method. As shown in Fig. 4A, the MIC of chlorogenic acid against *A. hydrophila* B3 was 6.4 mg/mL. Additionally, it was discovered how chlorogenic acid affected *A. hydrophila* B3 growth. As shown in Fig. 4B, chlorogenic acid did not significantly hinder the growth of *A. hydrophila* B3 in the bacterial cultures when it was added at doses ranging from 0 to 1.6 mg/mL ($p > 0.05$). The growth of B3 with 3.2 mg/mL of chlorogenic acid was significantly slower compared with the control ($p < 0.05$). The growth of B3 with 6.4 mg/mL of chlorogenic acid was almost completely inhibited. Based on these data, chlorogenic acid above 3.2 mg/mL was found to have a significant inhibitory effect on the growth of *A. hydrophila* B3.

Chlorogenic acid inhibits hemolysin and protease production of *A. hydrophila*

The pathogenic nature of *A. hydrophila* has been proven to be in part associated with exoenzyme production, such as hemolysin and protease, which are thought to be the major virulence factor in *A. hydrophila* (Wang et al. 2022). Erythrocytes from rabbit blood were used to determine the hemolytic activity of bacterial supernatants. As shown in Fig. 4C, a dose-dependent decrease in hemolytic activities was observed. At doses greater than 0.8 mg/mL, hemolytic activities were shown to be statistically significant ($p < 0.05$). When treated with 1.6 and 3.2 mg/mL chlorogenic acid, the hemolytic activity was reduced to 79.69 ± 2.52% and 65.59 ± 12.13%, respectively, compared to the control (0 mg/mL). These data suggest that chlorogenic acid significantly inhibited hemolysin production of *A. hydrophila* B3, which proved that the virulence of *A. hydrophila* can be successfully reduced by chlorogenic acid.

Azocasein was used to detect protease activities in bacterial supernatants. As shown in Fig. 4B, the ability of chlorogenic acid to inhibit protease production increases with increasing concentration. Protease production was inhibited at the level of 14.14 ± 2.78%, 34.93 ± 3.27%, and 44.24 ± 1.98%, respectively, in groups supplied with chlorogenic acid at concentrations of 0.8, 1.6, and 3.2 mg/mL, compared to the control (0 mg/mL). These data suggested that chlorogenic acid significantly reduced protease production of *A. hydrophila* B3, and the virulence of *A. hydrophila* will be further diminished by the absence of exotoxins (such hemolysin) that are activated by proteases.

Chlorogenic acid inhibits biofilm formation of *A. hydrophila*

The formation of biofilms represents a characteristic of persistent infection, and many *Aeromonas* infections are associated with this virulence factor (Grim et al. 2013; Ormanci and

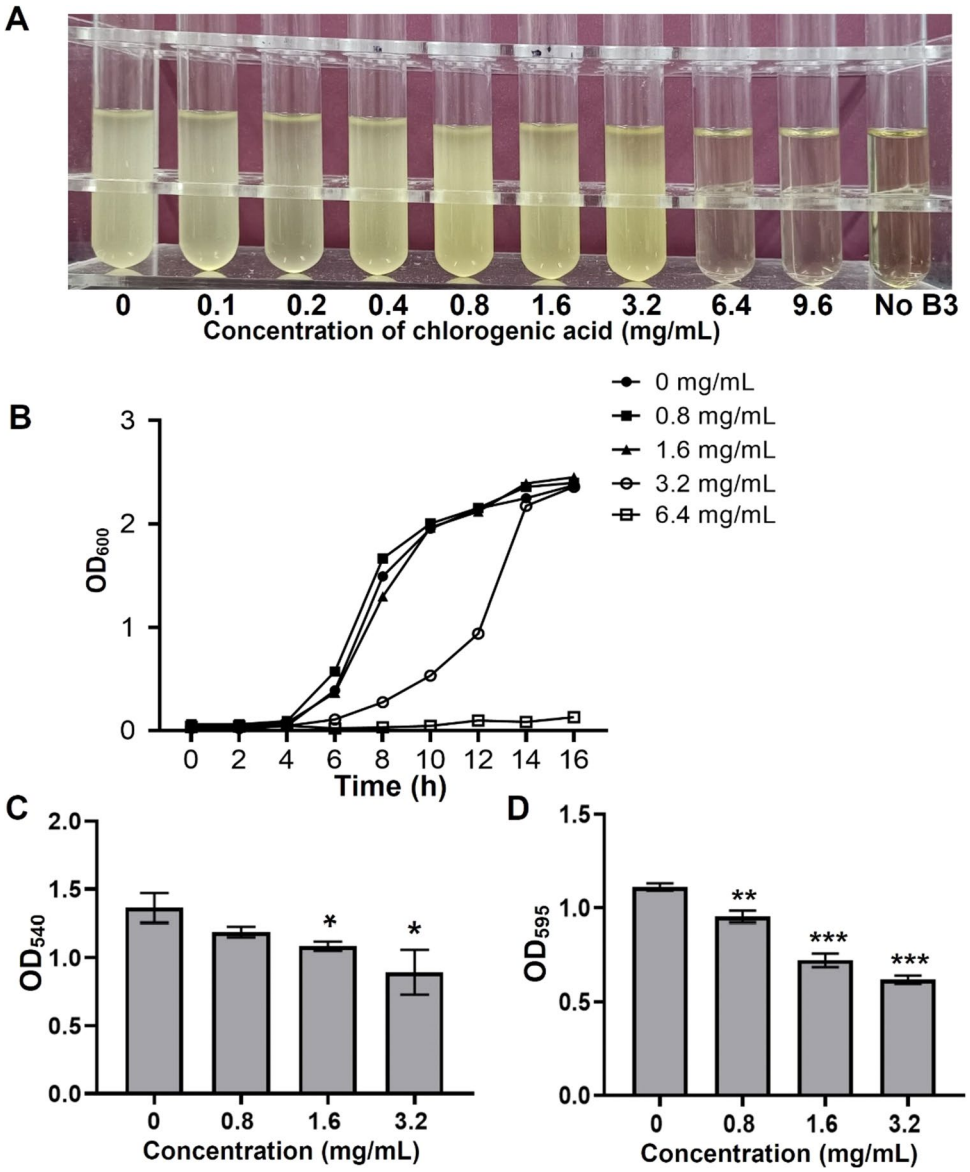


Fig. 4 **A** Growth inhibition of *A. hydrophila* B3 by different concentrations of chlorogenic acid. **B** Curves of different concentrations of chlorogenic acid on *A. hydrophila* B3 growth. **C** Effect of chlorogenic acid at sub-MICs (0.8, 1.6, and 3.2 mg/mL) on hemolysin production of *A. hydrophila* B3. **D** Effect of chlorogenic acid at sub-MICs (0.8, 1.6, and 3.2 mg/mL) on protease activity of *A. hydrophila* B3. Data are presented as the absorbance of mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the 0 mg/mL control group by *t*-test

Yucel 2017). In order to measure biofilm formation associated with the solid surface by *A. hydrophila* B3 with or without chlorogenic acid, we performed a crystal violet (CV) staining assay. As shown in Fig. 5A, the presence of chlorogenic acid significantly inhibited biofilm formation. The inhibitory activity of chlorogenic acid acted in a concentration-dependent manner as the concentration was increased. The biofilm inhibition rate was 24.31 ± 9.29%, 31.97 ± 4.66%, and 41.49 ± 5.04%, respectively, as the bacteria treated with chlorogenic acid at 0.8, 1.6, and 3.2 mg/mL.

Along with quantitatively analyzing the biofilm biomass using the CV staining approach, the SEM examination of the development of the biofilm structure following incubation in the presence and absence of chlorogenic acid was also carried out. SEM scans demonstrated that the biofilm was thick and dense in the control group at 0 mg/mL (Fig. 5B1). In contrast, with chlorogenic acid at the sub-MIC treatment, the biofilm was hindered and eventually became sparse as the concentration was increased (Fig. 5B2–B4). These results suggested that the development of *A. hydrophila* B3's biofilm was considerably suppressed by chlorogenic acid, resulting in a loose and sparse biofilm.

Chlorogenic acid significantly reduces the biofilm formation-associated gene expression of *A. hydrophila*

The qPCR assay was performed to examine the effect of the sub-MIC 1.6 mg/mL of chlorogenic acid on the quorum

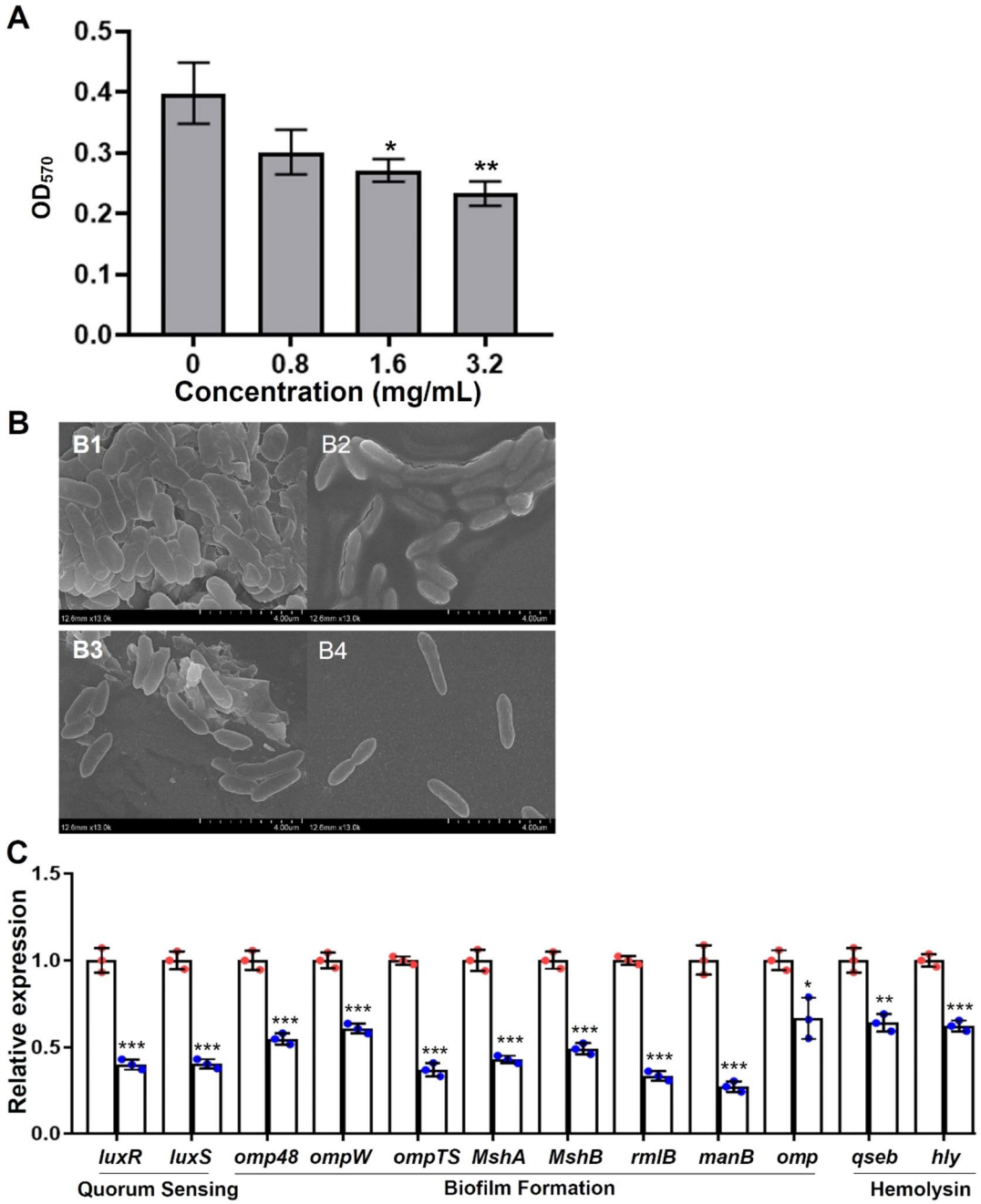


Fig. 5 Effect of chlorogenic acid on biofilm formation, quorum sensing, and hemolysin. **A** Quantitative analysis of biofilm biomass. **B** SEM images of *A. hydrophila* B3 biofilms treated with 0 mg/mL (**B1**), 0.8 mg/mL (**B2**), 1.6 mg/mL (**B3**), and 3.2 mg/mL (**B4**) of chlorogenic acid. **C** Effect of chlorogenic acid at 1.6 mg/mL on the expression of QS, biofilm formation, and hemolysin related genes in *A. hydrophila* B3. Data are presented as the expression fold changes of mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by *t*-test

sensing, biofilm formation, and hemolysin-related gene expression of *A. hydrophila* B3. As shown in Fig. 5C, *luxR*, *luxS* (QS-associated), *omp48*, *ompW*, *ompTS* (outer membrane protein synthesis-associated), *MshA*, *MshB* (flagellum synthesis-associated), *rmlB*, *omp*, *qseb* (extracellular polysaccharide synthesis-associated), and *hly* (hemolysin synthesis-associated) were significantly down regulated ($p < 0.05$, 0.01, or 0.001), and their expression levels were reduced by 59.93 ± 2.94%, 59.56 ± 2.71%, 45.17 ± 3.21%, 39.23 ± 2.88%, 62.92 ± 3.86%, 56.99 ± 2.19%, 50.83 ± 3.20%, 66.45 ± 2.66%, 72.58 ± 3.06%, 33.36 ± 11.83%, 35.96 ± 4.91%, and by 37.62 ± 3.06%, respectively. These

data suggested that chlorogenic acid significantly reduces the expression of QS, biofilm formation, and hemolysin-related genes in *A. hydrophila*.

Discussion

Antibiotic resistance poses a danger to human and animal health, even though the discovery of antibiotics has reduced the mortality rates of humans and other animals suffering from bacterial infections (Helliwell et al. 2020). *A. hydrophila* is regarded as a significant pathogen that causes more severe illnesses including peritonitis and bacteremia in addition to gastroenteritis and skin infections (Chen et al. 2015; Li et al. 2019; Zhang Lishan et al. 2022). Additionally, *A. hydrophila* is a recognized fish pathogen linked to several illnesses, including red sore disease in bass and carp, epizootic ulcer syndrome in catsharks, cod, carp, and gobies, and motile aeromonad septicemia in carp, tilapia, perch, catfish, and salmon (Citterio and Biavasco 2015; Honein et al. 2018; Zhu et al. 2019). The aquaculture industry suffered significant financial losses due to *A. hydrophila* infection, which has garnered widespread attention. With the increasing use of antibiotics in aquaculture, antibiotic resistance has been observed in *A. hydrophila* infections (Bhowmick and Bhattacharjee 2018; Nhinh et al. 2021), suggesting the limitation of the antibiotic application in treating bacterial infections in aquaculture. Meanwhile, abuse of antibiotics in aquaculture also induces environmental and ecological security (Shao et al. 2021). Thus, seeking novel strategies against *A. hydrophila* infections is an urgent problem to be solved in aquaculture.

In a turtle farm in Hubei province, China, from August to October, 2019, many soft-shelled turtles showing clinical signs as petechial hemorrhages in the body surface, bleeding spots in bottom, and with liver hyperemia and hepatomegaly died, and we found that *A. hydrophila* was the predominant pathogenic bacteria responsible for this decease outbreak (Fig. 1). Chung et al. also found that *A. hydrophila* was the main pathogenic bacteria in soft-shelled turtles (Chung et al. 2017), indicating that *A. hydrophila* might be the major bacterial pathogen among the soft-shelled turtle farm industry.

Chinese herbs have been utilized to cure illnesses in both people and animals in China as a medication and immune system booster. Herbs offer several advantages over chemotherapy, including being sourced from natural sources, being relatively inexpensive and easy to prepare, having a known antibacterial impact, and having fewer adverse effects when used as a treatment (Zhu 2020; Wu et al. 2021). It has been reported that Chinese herb sanguinarine can lower channel catfish mortality when exposed to *A. hydrophila* (Lushan et al. 2022). In this study, we performed a small Chinese herb screen and found that *L. japonica* can significantly inhibit *A. hydrophila* growth in vitro, and significantly protect soft-shelled turtles from *A. hydrophila* infection (Figs. 2 and 3, Table 1). The bactericidal activity of ethanol extracts from leaves and floral essential oils was assessed by Rahman et al. in 2009. The extracts have been shown to have a remarkable antibacterial effect against *Listederia monocytogenes*, *Bacillus subtilis*, *Bacillus cereus*, *Salmanella typhimurium*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Enterobacter aerogenes*, and *Escherichia coli* (Rahman and Kang 2009). For the application of *L. japonica*, as reported, feeding tilapia with *L. japonica* remarkably enhanced phagocytosis and increased burst activity of blood phagocytic cells and reduced the mortality rate after *A. hydrophila* infection (Ardó et al. 2008), and the immunomodulation of *L. japonica* ethanol extracts increased the survival rate of crabs challenged by *A. hydrophila* (Zhao et al. 2018), indicating that *L. japonica* is a promising Chinese herb to be utilized in the healthy breeding industry. In this study, we unveil that the significant increase in the expressions of immune and stress-related genes of soft-shelled turtle (Fig. 3B) might be the potential contributors for the increased survival rate in the *L. japonica* feeding group after *A. hydrophila* infection. Meanwhile, *L. japonica* significantly reduced ferric heme precipitation and erythrocyte exudation in the liver and intestine of soft-shelled turtles after *A. hydrophila* infection (Fig. 3D).

As *A. hydrophila* infects animals, bleeding is a frequent occurrence, and both in vivo and in vitro tests can show that the infection is causing hemolysis. The function of hemolysin is highly regarded since it is thought to be the primary virulence factor of *A. hydrophila* (Zhang et al. 2017). The hemolysin, also known as aerolysin, is an exotoxin produced by *A. hydrophila* and is a single polypeptide molecule (Stratev and Odeyemi 2016). Our results show that chlorogenic acid significantly inhibits the hemolysin production and hemolysin gene (*hly*) expression of *A. hydrophila* (Figs. 4C and 5C), which proved that the virulence of the bacteria can be successfully reduced by chlorogenic acid. Extracellular proteases are also considered as the major virulence factors of *A. hydrophila* (Wu et al. 2012; Wang et al. 2019a), and their production could be significantly reduced by chlorogenic acid (Fig. 4D), which also contributes to the virulence attenuation of *A. hydrophila*.

A microbial community known as a biofilm is one in which bacterial cells are immersed inside a self-produced matrix made of lipids, extracellular polysaccharides (EPS), proteins, and nucleic acids that can prevent antimicrobial drugs from penetrating the cells (Flemming and Wingender 2010; Di Martino 2018). Here, we have demonstrated that chlorogenic acid significantly inhibits *A. hydrophila* biofilm formation, rendering it loose and patchy in a concentration-dependent manner (Fig. 5A, B), and significantly reduces the expression of genes associated with biofilm formation,

such as outer membrane protein synthesis genes, flagellum synthesis genes, and EPS genes (Fig. 5C). These results indicated that chlorogenic acid inhibits biofilm formation by affecting the synthesis of the matrix (such as outer membrane protein and EPS) and flagellum of *A. hydrophila*.

QS controls gene expression in response to the density of the cell population and other signals. QS systems have been reported to regulate a variety of bacterial virulence traits, including the secretion of extracellular enzymes, secretion systems of bacteria, bacterial motility, and biofilm formation (Wang et al. 2019b; Huang et al. 2022). QS is therefore thought to be a key factor in bacterial pathogenicity. From this perspective, QS inhibition has received attention for inhibition of bacterial pathogenesis (Haque et al. 2018). In this study, QS, biofilm formation and hemolysin-related genes are significantly down regulated in *A. hydrophila* B3 treated with chlorogenic acid (Fig. 5C), suggesting that chlorogenic acid inhibits *A. hydrophila* B3 *via* destroying its QS, biofilm formation and hemolysin system and may be a good option for a medication to treat bacterial infections in aquaculture.

This study unveils the inhibitory effects of Chinese herb *L. japonica* and chlorogenic acid on the aquatic pathogen *A. hydrophila* and partially explains the effect mode of Chinese herbal medicines.

Author contribution JXL and YL conceived and designed the research. LCH conducted the experiments. LCH, NYL, and CJW analyzed data. LCH wrote the manuscript. JXL and YL revised the manuscript. All authors read and approved the manuscript.

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Data availability Upon reasonable request, the corresponding author will provide the data that back up the study's conclusions.

Declarations

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare no competing interests.

References

Akmal M, Rahimi-Midani A, Hafeez-ur-Rehman M, Hussain A, Choi TJ (2020) Isolation, characterization, and application of a bacteriophage infecting the fish pathogen *Aeromonas hydrophila*. Pathogens 9:215. https://doi.org/10.3390/pathogens9030215

Ardó L, Yin G, Xu P, Váradi L, Szigeti G, Jeney Z, Jeney G (2008) Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila*. Aquaculture 275:26–33. https://doi.org/10.1016/j.aquaculture.2007.12.022

Bhowmick UD, Bhattacharjee S (2018) Bacteriological, clinical and virulence aspects of -associated diseases in humans. Pol J Microbiol 67:137–150. https://doi.org/10.21307/pjm-2018-020

Chen J, Ding X, Zhu N, Kong L, He Z (2015) Prevalence and antimicrobial susceptibility of *Aeromonas* species from diseased Chinese soft-shelled turtles (*Trionyx sinens*). Aquac Res 46:1527–1536. https://doi.org/10.1111/are.12299

Chen J, Zhu N, Kong L, Bei Y, Zheng T, Ding X, He Z (2013) First case of soft shell disease in Chinese soft-shelled turtle (*Trionyx sinens*) associated with *Aeromonas sobria–A. veronii* complex. Aquaculture 406–407:62–67. https://doi.org/10.1016/j.aquaculture.2013.05.006

Chi X, Zhang Z, Xu X, Zhang X, Zhao Z, Liu Y, Wang Q, Wang H, Li Y, Yang G, Guo L, Tang Z, Huang L (2017) Threatened medicinal plants in China: distributions and conservation priorities. Biol Conserv 210:89–95. https://doi.org/10.1016/j.biocon.2017.04.015

Chokshi A, Sifri Z, Cennimo D, Horng H (2019) Global contributors to antibiotic resistance. J Glob Infect Dis 11:36–42. https://doi.org/10.4103/jgid.jgid_110_18

Chung T, Yi S, Kim B, Kim W, Shin G (2017) Identification and antibiotic resistance profiling of bacterial isolates from septicaemic soft-shelled turtles (*Pelodiscus sinensis*). Vet Med (Praha) 62:169–177. https://doi.org/10.17221/65/2016-VETMED

Citterio B, Biavasco F (2015) *Aeromonas hydrophila* virulence. Virulence 6:417–418. https://doi.org/10.1080/21505594.2015.1058479

Di Martino P (2018) Extracellular polymeric substances, a key element in understanding biofilm phenotype. AIMS Microbiol 4:274–288. https://doi.org/10.3934/microbiol.2018.2.274

Erova TE, Pillai L, Fadl AA, Sha J, Wang S, Galindo CL, Chopra AK (2006) DNA adenine methyltransferase influences the virulence of *Aeromonas hydrophila*. Infect Immun 74:410–424. https://doi.org/10.1128/IAI.74.1.410-424.2006

Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633. https://doi.org/10.1038/nrmicro2415

Fu L, Shao S, Feng Y, Ye F, Sun X, Wang Qingling YF, Qisheng W, Huang B, Niu P, Li X, Wong CCL, Qi J, Tan W, Gao GF (2021) Mechanism of microbial metabolite leupeptin in the treatment of COVID-19 by traditional Chinese medicine herbs. mBio 12:e02220–e02221. https://doi.org/10.1128/mBio.02220-21

Grim CJ, Kozlova EV, Sha J, Fitts EC, van Lier CJ, Kirtley ML, Joseph SJ, Read TD, Burd EM, Tall BD, Joseph SW (2013) Characterization of *Aeromonas hydrophila* wound pathotypes by comparative genomic and functional analyses of virulence genes. mBio 4:e00064–e00013. https://doi.org/10.1128/mBio.00064-13

Guo WL, Deng HW, Wang F, Wang SF, Zhong ZH, Sun Y, Chen XF, Wang JH, Zhou YC (2019) *In vitro* and *in vivo* screening of herbal extracts against *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*). Aquaculture 503:412–421. https://doi.org/10.1016/j.aquaculture.2019.01.024

Haque S, Ahmad F, Dar SA, Jawed A, Mandal RK, Wahid M, Lohani M, Khan S, Singh V, Akhter N (2018) Developments in strategies for quorum sensing virulence factor inhibition to combat bacterial drug resistance. Microb Pathog 121:293–302. https://doi.org/10.1016/j.micpath.2018.05.046

Helliwell R, Morris C, Raman S (2020) Antibiotic stewardship and its implications for agricultural animal-human relationships: insights from an intensive dairy farm in England. J Rural Stud 78:447–456. https://doi.org/10.1016/j.jrurstud.2020.07.008

Honein K, Jagoda SSSDS, Arulkanthan A, Ushio H, Asakawa S (2018) Draft genome sequence of *Aeromonas hydrophila* strain Ae25, isolated from a septicemic moribund koi carp (*Cyprinus carpio*) in Sri Lanka. Microbiol Resour Announc 6:e01523–e01517. https://doi.org/10.1128/genomea.01523-17

Huang AG, Tan XP, Cui HB, Qi XZ, Zhu B, Wang GX (2020) Antiviral activity of geniposidic acid against white spot syndrome virus replication in red swamp crayfish *Procambarus clarkii*. Aquaculture 528:735533. https://doi.org/10.1016/j.aquaculture.2020.735533

Huang S, Liu X, Yang W, Ma L, Li H, Liu R, Qiu J, Li Y (2022) Insights into adaptive mechanisms of extreme acidophiles based on quorum sensing/quenching-related proteins. mSystems 7:e01491–e01421. https://doi.org/10.1128/msystems.01491-21

Kang Z, Jia S, Shui Q, Dan J, De F, Chi J, Ding J, Ti Y, Jun Y (2019) Isolation and identification of *Aeromonas hydrophila* from soft-shelled turtle (*Trionyx sinensis*) and in vitro antibacterial test. Anim Breed Feed 5:11–15. https://doi.org/10.13300/j.cnki.cn42-1648/s.2019.05.004

Khajanchi BK, Kozlova EV, Sha J, Popov VL, Chopra AK (2012) The two-component QseBC signalling system regulates *in vitro* and *in vivo* virulence of *Aeromonas hydrophila*. Microbiology 158:259–271. https://doi.org/10.1099/mic.0.051805-0

Kim WS, Choi WJ, Lee S, Kim WJ, Lee DC, Sohn UD, Shin HS, Kim W (2014) Anti-inflammatory, antioxidant and antimicrobial effects of artemisinin extracts from *Artemisia annua* L. Korean J Physiol Pharmacol 19:21. https://doi.org/10.4196/kjpp.2015.19.1.21

Lee KJ, Jung YC, Park SJ, Lee KH (2018) Role of heat shock proteases in quorum-sensing-mediated regulation of biofilm formation by *Vibrio* species. mBio 9:e02086–e02017. https://doi.org/10.1128/mBio.02086-17

Li Y, Li W, Fu C, Song Y, Fu Q (2020) *Lonicerae japonicae* flos and *Lonicerae* flos: a systematic review of ethnopharmacology, phytochemistry and pharmacology. Phytochem Rev 19:1–61. https://doi.org/10.1007/s11101-019-09655-7

Li Z, Wang Y, Li X, Lin Z, Lin Y, Srinivasan R, Lin X (2019) The characteristics of antibiotic resistance and phenotypes in 29 outer-membrane protein mutant strains in *Aeromonas hydrophila*. Environ Microbiol 21:4614–4628. https://doi.org/10.1111/1462-2920.14761

Liang W, Li H, Zhou H, Wang M, Zhao X, Sun X, Li C, Zhang X (2021) Effects of *Taraxacum* and *Astragalus* extracts combined with *probiotic Bacillus* subtilis and *Lactobacillus* on *Escherichia coli*–infected broiler chickens. Poult Sci 100:101007. https://doi.org/10.1016/j.psj.2021.01.030

Liu T, Han Y, Chen S, Zhao H (2019) Genome-wide identification of Toll-like receptors in the Chinese soft-shelled turtle *Pelodiscus sinensis* and expression analysis responding to *Aeromonas hydrophila* infection. Fish Shellfish Immunol 87:478–489. https://doi.org/10.1016/j.fsi.2019.01.052

Lou Z, Wang H, Zhu S, Ma C, Wang Z (2011) Antibacterial activity and mechanism of action of chlorogenic acid. J Food Sci 76:M398–M403. https://doi.org/10.1111/j.1750-3841.2011.02213.x

Lushan Z, Ma L, Yang Q, Liu Y, Ai X, Dong J (2022) Sanguinarine protects channel catfish against *Aeromonas hydrophila* infection by inhibiting aerolysin and biofilm formation. Pathogens 11:323. https://doi.org/10.3390/pathogens11030323

Miao M, Xiang L (2020) Chapter three - Pharmacological action and potential targets of chlorogenic acid. Adv Pharmacol 87:71–88. https://doi.org/10.1016/bs.apha.2019.12.002

Mzula A, Wambura PN, Mdegela RH, Shirima GM (2019) Current state of modern biotechnological-based *Aeromonas hydrophila* vaccines for aquaculture: a systematic review. BioMed Res Int 2019:e3768948. https://doi.org/10.1155/2019/3768948

Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ, Shumzaid M, Ahmad F, Babazadeh D, FangFang X, Modarresi-Ghazani F, WenHua L, XiaoHui Z (2018) Chlorogenic acid (CGA): a pharmacological review and call for further research. Biomed Pharmacothe 97:67–74. https://doi.org/10.1016/j.biopha.2017.10.064

Nemec A, Krizova L, Maixnerova M, Van der Reijden TJK, Deschaght P, Passet V, Vaneechoutte M, Brisse S, Dijkshoorn L (2011) Genotypic and phenotypic characterization of the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex with the proposal of *Acinetobacter pittii sp*. nov (formerly *Acinetobacter* genomic species 3) and *Acinetobacter nosocomialis sp.* nov (formerly *Acinetobacter* genomic species 13TU). Res Microbiol 162:393–404. https://doi.org/10.1016/j.resmic.2011.02.006

Nhinh DT, Le DV, Van KV, Huong Giang NT, Dang LT, Hoai TD (2021) Prevalence, virulence gene distribution and alarming the multidrug resistance of *Aeromonas hydrophila* associated with disease outbreaks in freshwater aquaculture. Antibiotics 10:532. https://doi.org/10.3390/antibiotics10050532

Ormanci S, Yucel N (2017) Biofilm formation on polystyrene and glass surface by *Aeromonas* species isolated from different sources. J Food Process Preserv 41:e13223. https://doi.org/10.1111/jfpp.13223

Park G, Jin WY, Jang SJ, Kook K, Choi JA, Park GC, Lee MJ, Park SN, Li XM, Cho SS, Jang C, Kang SH, Moon DS (2015) Evaluation of four methods of assigning species and genus to medically important bacteria using 16S rRNA gene sequence analysis. J Food Process Preserv 59:285–298. https://doi.org/10.1111/1348-0421.12254

Rahman A, Kang SC (2009) In vitro control of food-borne and food spoilage bacteria by essential oil and ethanol extracts of *Lonicera japonica* Thunb. Food Chem 116:670–675. https://doi.org/10.1016/j.foodchem.2009.03.014

Rasmussen-Ivey CR, Figueras MJ, McGarey D, Liles MR (2016) Virulence factors of *Aeromonas hydrophila*: in the wake of reclassification. Front Microbiol 7. https://doi.org/10.3389/fmicb.2016.01337

Selvaraj A, Valliammai A, Muthuramalingam P, Sethupathy S, Subramenium GA, Ramesh M, Pandian SK (2020) Proteomic and systematic functional profiling unveils citral targeting antibiotic resistance, antioxidant defense, and biofilm-associated two-component systems of *Acinetobacter baumannii* to encumber biofilm and virulence traits. *mSystems* 5:e00986-20. https://doi.org/10.1128/mSystems.00986-20

Shang X, Pan H, Li M, Miao X, Ding H (2011) *Lonicera japonica* Thunb.: ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine. J Ethnopharmacol 138:1–21. https://doi.org/10.1016/j.jep.2011.08.016

Shao Y, Wang Y, Yuan Y, Xie Y (2021) A systematic review on antibiotics misuse in livestock and aquaculture and regulation implications in China. Sci Total Environ 798:149205. https://doi.org/10.1016/j.scitotenv.2021.149205

Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 40:175–179. https://doi.org/10.1016/S0167-7012(00)00122-6

Stratev D, Odeyemi OA (2016) Antimicrobial resistance of *Aeromonas hydrophila* isolated from different food sources: a mini-review. J Infect Public Health 9:535–544. https://doi.org/10.1016/j.jiph.2015.10.006

Su X, Zhu Z, Zhang L, Wang Q, Xu M, Lu C, Zhu Y, Zeng J, Duan JA, Zhao M (2021) Anti-inflammatory property and functional substances of *Lonicerae Japonicae* Caulis. J Ethnopharmacol 267:113502. https://doi.org/10.1016/j.jep.2020.113502

Tanhay Mangoudehi H, Zamani H, Shahangian SS, Mirzanejad L (2020) Effect of curcumin on the expression of *ahyI/R* quorum sensing genes and some associated phenotypes in pathogenic *Aeromonas hydrophila* fish isolates. World J Microbiol Biotechnol 36:1–9. https://doi.org/10.1007/s11274-020-02846-x

Van Hai N (2015) The use of medicinal plants as immunostimulants in aquaculture: a review. Aquaculture 446:88–96. https://doi.org/10.1016/j.aquaculture.2015.03.014

Vijayakumar S, Divya M, Vaseeharan B, Chen J, Biruntha M, Silva LP, Durán-Lara EF, Shreema K, Ranjan S, Dasgupta N (2021)

Biological compound capping of silver nanoparticle with the seed extracts of black cumin (*Nigella sativa*): a potential antibacterial, antidiabetic, anti-inflammatory, and antioxidant. J Inorg Organomet Polym Mater 31:624–635. https://doi.org/10.1007/s10904-020-01713-4

Wang J, Qin T, Chen K, Pan L, Xie J, Xi B (2022) Antimicrobial and antivirulence activities of carvacrol against pathogenic *Aeromonas hydrophila*. Microorganisms 10:2170. https://doi.org/10.3390/microorganisms10112170

Wang Y, Liu B, Grenier D, Yi L (2019b) Regulatory mechanisms of the *LuxS/AI-2* system and bacterial resistance. Antimicrob Agents Chemother 63:e01186–e01119. https://doi.org/10.1128/AAC.01186-19

Wang Y, Wang X, Ali F, Li Z, Fu Y, Yang X, Lin W, Lin X (2019a) Comparative extracellular proteomics of *Aeromonas hydrophila* reveals iron-regulated secreted proteins as potential vaccine candidates. Front Immunol 10:256. https://doi.org/10.3389/fimmu.2019.00256

Wu L, Jiang Y, Tang Q, Lin H, Lu C, Yao H (2012) Development of an *Aeromonas hydrophila* recombinant extracellular protease vaccine. Microb Pathog 53:183–188. https://doi.org/10.1016/j.micpath.2012.07.007

Wu Q, Jiang Y, Chen E, Mu C, Waiho K (2021) Chinese gallnut (*Galla chinensis*) against *Vibrio parahaemolyticus*: in vitro activity and the use of medicated bath method to treat infected mud crab *Scylla paramamosain*. Aquaculture 539:736632. https://doi.org/10.1016/j.aquaculture.2021.736632

Xu C, Xu J, Chen Y, Evensen Ø, Munang'andu HM, Qian G (2020) Immunization with bovine serum albumin (BSA) in oil-adjuvant elicits IgM antibody response in Chinese soft-shelled turtle (*Pelodiscus Sinensis*). Vaccines 8:257. https://doi.org/10.3390/vaccines8020257

Zhang D, Xu DH, Shoemaker CA (2017) Immunization with recombinant aerolysin and haemolysin protected channel catfish against virulent *Aeromonas hydrophila*. Aquac Res 48:875–882. https://doi.org/10.1111/are.12931

Zhang Lishan WY, Yao Z, Wang X, Chen J, Yang W, Yao J, Lin Y, Liu Z, Lin X (2022) A high-throughput screening approach for bacterial quorum sensing inhibitors (QSIs) against *Aeromonas hydrophila* infection. Aquaculture 560:738488. https://doi.org/10.1016/j.aquaculture.2022.738488

Zhang Y, Fang X, Chen X, Wang H, Liu J, Liang X, Gu Y, Fang C, Yang Y (2022) Bacillus cereus causes fatal disease in soft-shelled turtle (*Trionyx sinensis*). Aquaculture 547:737473. https://doi.org/10.1016/j.aquaculture.2021.737473

Zhao Z, Zhang H, Wang M, Zhang C, Kuang P, Zhou Z, Zhang G, Wang Z, Zhang B, Shi X (2018) The ethanol extract of honeysuckle stem modulates the innate immunity of Chinese mitten crab *Eriocheir sinensis* against *Aeromonas hydrophila*. Fish Shellfish Immunol 82:304–311. https://doi.org/10.1016/j.fsi.2018.08.035

Zhou J, Bi S, Chen H, Chen T, Yang R, Li M, Fu Y, Jia AQ (2017) Anti-Biofilm and Antivirulence Activities of Metabolites from Plectosphaerella cucumerina against Pseudomonas aeruginosa. Front Microbiol 8:769. https://doi.org/10.3389/fmicb.2017.00769

Zhu F (2020) A review on the application of herbal medicines in the disease control of aquatic animals. Aquaculture 526:735422. https://doi.org/10.1016/j.aquaculture.2020.735422

Zhu L, Zheng JS, Wang WM, Luo Y (2019) Complete genome sequence of highly virulent *Aeromonas hydrophila* strain D4, isolated from a diseased blunt-snout bream in China. Microbiol Resour Announc 8:e01035–e01018. https://doi.org/10.1128/MRA.01035-18