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Basic Study

Effect and mechanism of Qingre Huashi decoction on drug-resistant *Helicobacter pylori*

Miao-Miao Lin, Shan-Shan Yang, Qiu-Yue Huang, Guang-Hui Cui, Xiao-Fen Jia, Yao Yang, Zong-Ming Shi, Hui Ye, Xue-Zhi Zhang

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**Abstract**

**BACKGROUND**

*Helicobacter pylori* (HP), the most common pathogenic microorganism in the stomach, can induce inflammatory reactions in the gastric mucosa, causing chronic gastritis and even gastric cancer. HP infection affects over 4.4 billion people globally, with a worldwide infection rate of up to 50%. The multidrug resistance of HP poses a serious challenge to eradication. It has been demonstrated that compared to bismuth quadruple therapy, Qingre Huashi decoction (QHD) combined with triple therapy exhibits comparable eradication rates but with a lower incidence of adverse reactions; in addition, QHD can directly inhibit and kill HP.

**AIM**

To explore the effect and mechanism of QHD on clinically multidrug-resistant and strong biofilm-forming HP.

**METHODS**

In this study, 12 HP strains were isolated *in vitro* after biopsy during gastroscopy of HP-infected patients. *In vitro*, the minimum inhibitory concentration (MIC) values for clinical HP strains and biofilm quantification were determined through the E-test method and crystal violet staining, respectively. The most robust biofilm-forming strain of HP was selected, and QHD was evaluated for its inhibitory and bactericidal effects on the strain with strong biofilm formation.
assessment was performed using agar dilution, E-test, killing dynamics, and transmission electron microscopy (TEM). The study also explored the impact of QHD on antibiotic resistance in these HP strains with strong biofilm formation. Crystalline violet method, scanning electron microscopy, laser confocal scanning microscopy, and (p)ppGpp chromatographic identification were employed to evaluate the effect of QHD on biofilm in strong biofilm-forming HP strains. The effect of QHD on biofilm and efflux pump-related gene expression was evaluated by quantitative polymerase chain reaction. Non-targeted metabolomics with UHPLC-MS/MS was used to identify potential metabolic pathways and biomarkers which were different between the NC and QHD groups.

RESULTS
HP could form biofilms of different degrees in vitro, and the intensity of formation was associated with the drug resistance of the strain. QHD had strong bacteriostatic and bactericidal effects on HP, with MICs of 32-64 mg/mL. QHD could inhibit the biofilm formation of the strong biofilm-forming HP strains, disrupt the biofilm structure, lower the accumulation of (p)ppGpp, decrease the expression of biofilm-related genes including LuxS, Spot, glup (HP1174), NapA, and CagE, and reduce the expression of efflux pump-related genes such as HP0605, HP0971, HP1327, and HP1489. Based on metabolomic analysis, QHD induced oxidative stress in HP, enhanced metabolism, and potentially inhibited relevant signaling pathways by upregulating adenosine monophosphate (AMP), thereby affecting HP growth, metabolism, and protein synthesis.

CONCLUSION
QHD exerts bacteriostatic and bactericidal effects on HP, and reduces HP drug resistance by inhibiting HP biofilm formation, destroying its biofilm structure, inhibiting the expression of biofilm-related genes and efflux pump-related genes, enhancing HP metabolism, and activating AMP in HP.

Key Words: Qingre Huashi decoction; Helicobacter pylori; Drug resistance; Biofilm; Metabolomics

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Core Tip: Helicobacter pylori (HP) infection affects over 4.4 billion people worldwide, with its multidrug resistance posing a serious challenge. Twelve strains of HP were isolated in vitro following biopsy during gastroscopy of HP-infected patients, with the most robust biofilm-forming strain of HP being selected to investigate the effect and mechanism of Qingre Huashi decoction (QHD) on drug-resistant HP. QHD has bacteriostatic and bactericidal effects on HP, reduces HP drug-resistance by inhibiting biofilm formation, destroying biofilm structure, inhibiting biofilm-related and efflux pump-related gene expression, enhancing HP metabolism, and activating adenosine monophosphate in HP.

INTRODUCTION
Helicobacter pylori (HP), as the most common pathogenic microorganism in the stomach, can induce inflammatory reactions in the gastric mucosa, causing sustained damage to the gastric mucosa and resulting in chronic gastritis and even gastric cancer[1]. The estimated worldwide occurrence of HP infection declined from 58.2% during the 1980-90 era to 43.1% during the 2011-22 period[2]. However, HP infection is still a focus of public health concern. The multidrug resistance of HP poses a serious challenge to eradication therapy, contributing to a decrease in eradication rates and making eradication treatment difficult[3-5].

The mechanisms of HP resistance are highly complex[6], with biofilm formation[7] and efflux pumps[8] being the major factors causing nonspecific resistance. The formation of biofilm provides bacteria with a protective environment, leading to a nonspecific reduction in HP sensitivity to antibiotics[9,10]. Biofilm formation is a vital factor in HP resistance to various antibiotics and is a result of HP quorum sensing[11] and the stringent response. Quorum sensing is a communication mechanism among bacteria, sensing changes in bacterial population density through the release of signaling molecules. This sensing system plays a role in establishing communication among HP within the biofilm. The LuxS gene is the only known quorum-sensing gene present in the sequenced HP genome[12]. The stringent response is a stress signaling system responding to nutrient deprivation, mediated by (p)ppGpp. When HP confronts environmental pressures including nutrient limitation, the SpoT enzyme senses the stress signal and catalyzes the synthesis of (p)ppGpp[13-15]. As reported in the literature, the SpoT-HP1174/NapA-biofilm pathway is a crucial pathway mediating HP resistance[16]. In addition, efflux pumps have been reported as one of the mechanisms responsible for the antimicrobial resistance in biofilm structures[8]. Microorganisms manage their internal environment by eliminating harmful chemicals
including antimicrobials, metabolites, and quorum sensing signal molecules via efflux pumps[8,17]. The efflux pump system contains numerous transport proteins[18,19]. In biofilm-forming strains, the expression levels of HP0605-HP0607 (hefABC), HP0971-HP0969 (hefDEF), HP1327-HP1329 (hefGHI), and HP1489-HP1487 are all higher than those in planktonic bacteria[20]. Metabolites, resulting from microbial genome-environment interactions, are integral to cellular regulatory systems[21]. Investigating metabolites also aids in understanding the biofilm developmental process and crucial bacterial molecular components for biofilm formation[22,23]. The study performed by Wong et al[24] indicates significant differences in metabolites between low and high biofilm formers, including major categories of lipids and metabolites involved in prostaglandin and folate metabolism.

From the perspective of traditional Chinese medicine, HP is considered a specific pathogenic factor characterized by damp-heat. Individuals currently infected with HP usually exhibit characteristics of damp-heat[25,26]. Qingre Huashi decoction (QHD) is an internally formulated prescription at Peking University First Hospital consisting of 11 traditional Chinese medicinal ingredients (Table 1), and it exhibits the efficacy of clearing heat and dampness. Clinical studies have demonstrated the safety and efficacy of QHD combined with triple therapy in the treatment of HP infection. The eradication rate was comparable to that of quadruple therapy with bismuth compounds, and adverse reactions were less pronounced[27]. In basic experiments, it was found that QHD directly inhibited and killed HP in vitro, suppressed HP adhesion, effectively reduced HP colonization density, exerted antioxidant and anti-inflammatory effects, and alleviated gastric mucosal inflammation[28,29]. However, whether QHD exerts the impact of reducing the resistance of HP and its potential mechanism remain unclear. Therefore, in this study, we selected clinically isolated HP strains with strong biofilm-forming ability for in vitro cultivation to investigate the mechanisms of QHD in disrupting HP biofilm and regulating HP efflux pump resistance (Figure 1). The current research aimed to provide a theoretical foundation for the clinical treatment of refractory HP infections with QHD.

**MATERIALS AND METHODS**

**Preparation of QHD**

QHD was prepared using standardized boil-free herbal granules from Beijing Kangrentang Pharmaceutical Company, subjected to quality control using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). In vitro, QHD was dissolved in DD water. The solution was exposed to ultrasound for 3 h until the granular substance was completely dissolved. Then, the solution was centrifuged at 3500 rpm for 10 min, and the supernatant was collected. After being centrifuged at 5000 rpm for 10 min, the supernatant was collected again. Subsequently, centrifugation at 10000 rpm for 10 min was performed, and the supernatant was collected. Finally, the drug concentration was adjusted to 2560 mg/mL, filtered through a 0.22 μm filter for sterilization, and then stored at -80 °C.

**Acquisition, cultivation, and cryopreservation of HP strains**

The 12 strains of HP used in this study were all obtained from the Department of Gastroenterology, Peking University First Hospital. The study was conducted in Beijing, China from March 1, 2021 to November 30, 2021. During gastroscopy examinations of individuals with HP infection, gastric mucosal tissue samples were collected from the antrum and corpus of the stomach. Then, HP was isolated and cultured in vitro (Figure 2). Information for the 12 patients is presented in Table 2. In line with experimental requirements, HP cultivation was performed employing two methods, solid culture or liquid culture. In the solid culture method, HP was inoculated on Columbia blood agar (OXOID, Basingstoke, United Kingdom) plates with 8% sheep blood (Lablead, Beijing, China) and incubated upside-down at 37 °C in a microaerophilic (85% N₂, 10% CO₂, 5% O₂) environment for 48-72 h. In the liquid culture method, HP was cultured in a liquid medium containing Brucella broth (BD, Franklin Lakes, NJ, United States), supplemented with 10% fetal bovine serum (FBS; BI, Kibbutz Beit Haemek, Israel). During incubation, the bacteria were agitated at 37 °C and 120 rpm. HP strains were cryopreserved in a freezer set to -80 °C. The cryopreservation solution was prepared using brain heart infusion (OXOID, Basingstoke, United Kingdom) and glycerol (Solarbio, Beijing, China). The study was approved by the ethics committee of Peking University First Hospital (No. 2022Yan130-002).

**Drug susceptibility test**

**E-test method:** After 48-72 h of culture, HP was pulverised into a cryopreservation solution and diluted to 3 × 10⁸ CFU/mL. The E-test drug susceptibility test strip (Liofilchem, Roseto degli Abruzzi, Italy) was employed to test the bacterial solution, which was pipetted onto the microtiter plate, smeared with L sticks, and incubated at 37 °C for 72 h in a microaerophilic atmosphere. HP antibiotics have a minimum inhibitory concentration (MIC) in the ring area where the bacterium stops growing. EUCAST Clinical Breakpoint standard 2022 identifies drug-resistant HP bacteria as those surviving in amoxicillin (MIC > 0.125 μg/mL), levofloxacin (MIC > 1.000 μg/mL), clarithromycin (MIC > 0.500 μg/mL), and metronidazole (MIC > 8.000 μg/mL). The experiment was performed three times.

**Agar plate dilution method:** Media with QHD concentrations of 256, 128, 64, 32, 16, 8, and 4 μg/mL were produced. Negative controls were drug-free. The bacterial solution was diluted to 3 × 10⁸ CFU/mL and pulverised into the cryopreservation solution. Bacterial solution (1 μL) was inoculated into the drug-containing medium and cultured in a microaerobic environment at 37 °C for 72 h. In addition, the MIC of QHD was determined on a colony-free medium. The experiment was conducted three times.
Table 1 Composition of Qingre Huashi decoction

<table>
<thead>
<tr>
<th>Authorities name</th>
<th>Scientific name</th>
<th>Plant part</th>
<th>Weight ratio (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huanglian</td>
<td>Coptis chinensis Franch.</td>
<td>Rhizome</td>
<td>6</td>
</tr>
<tr>
<td>Zhizi</td>
<td>Gardenia jasminoides Ellis</td>
<td>Fruit</td>
<td>6</td>
</tr>
<tr>
<td>Pugongying</td>
<td>Taraxacum mongolicum Hand-Mazz.</td>
<td>Whole plant</td>
<td>15</td>
</tr>
<tr>
<td>Kuxingren</td>
<td>Prunus armeniaca L. var. ansu Maxim.</td>
<td>Seed</td>
<td>10</td>
</tr>
<tr>
<td>Doukouren</td>
<td>Anomum krawuah Pierre ex Gagnep.</td>
<td>Fruit</td>
<td>6</td>
</tr>
<tr>
<td>Yiyiren</td>
<td>Coix lacryma-jobi L. var. ma-yuen (Roman.) Stapf</td>
<td>Seed</td>
<td>15</td>
</tr>
<tr>
<td>Banxia</td>
<td>Pinellia ternata (Thunb) Breit.</td>
<td>Tuber</td>
<td>10</td>
</tr>
<tr>
<td>Cangzhu</td>
<td>Atractylodes lancea (Thunb.) DC.</td>
<td>Rhizome</td>
<td>10</td>
</tr>
<tr>
<td>Houpo</td>
<td>Magnolia Officinalis Rehd Et Wils.</td>
<td>Bark</td>
<td>10</td>
</tr>
<tr>
<td>Fuling</td>
<td>Poria Cocos (Schw.) Wolf.</td>
<td>Sclerotium</td>
<td>15</td>
</tr>
<tr>
<td>Cheqiancao</td>
<td>Plantago asiatica L.</td>
<td>Whole plant</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 2 Information of 12 Helicobacter pylori-infected individuals

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Age</th>
<th>Gastroscopic diagnosis</th>
<th>Pathological diagnosis</th>
<th>°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>47</td>
<td>Superficial gastritis; duodenal bulb inflammation (mild)</td>
<td>Mild chronic gastritis in the gastric body, activity grade II, lymphoid follicle formation, and mucosal erosion</td>
<td>32.4</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>39</td>
<td>Superficial gastritis</td>
<td>Moderate chronic gastritis in the gastric antrum, activity grade II, and mucosal erosion</td>
<td>26.7</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>54</td>
<td>Chronic atrophic gastritis</td>
<td>Mild chronic gastritis in the gastric antrum, activity grade I, mild intestinal metaplasia. Mild chronic gastritis in the gastric fundus, and severe intestinal metaplasia</td>
<td>19.8</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>64</td>
<td>Reflux esophagitis (LA-A); superficial gastritis; duodenal bulb ulcer (H1 stage)</td>
<td>Moderate chronic gastritis in the gastric antrum, activity grade II, mucosal hyperplasia, lymphoid follicle formation, and fundic gland polyp in the gastric fundus</td>
<td>40.5</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>35</td>
<td>Chronic non-atrophic gastritis; bile reflux</td>
<td>Mild chronic inflammation in the gastric body, and activity grade II. Moderate chronic gastritis in the gastric antrum, activity grade II, and lymphoid follicle formation</td>
<td>42.2</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>42</td>
<td>Chronic non-atrophic gastritis; Helicobacter pylori infection</td>
<td>Moderate chronic inflammation in the gastric antrum, and activity grade II</td>
<td>27.5</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>32</td>
<td>Reflux esophagitis (LA-B); hiatal hernia; chronic atrophic gastritis C2</td>
<td>Moderate chronic gastritis in the gastric antrum, activity grade II</td>
<td>28.9</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>34</td>
<td>Chronic atrophic gastritis with intestinal metaplasia and gastric ulcer</td>
<td>Moderate chronic gastritis in the gastric antrum, activity grade II, mild intestinal metaplasia, mild atrophy, mucosal hyperplasia, lymphoid follicle formation. Mild chronic inflammation in the cardia, and activity grade I</td>
<td>24.3</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>35</td>
<td>Chronic superficial gastritis; bile reflux; duodenal bulb ulcer</td>
<td>Severe chronic inflammation in the gastric antrum, activity grade II, and lymphoid follicle formation</td>
<td>25.4</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>40</td>
<td>Hiatal hernia; chronic gastritis with intestinal metaplasia</td>
<td>Moderate chronic gastritis in the gastric antrum (site 1), activity grade II, mild intestinal metaplasia, and moderate glandular atrophy</td>
<td>17.3</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>46</td>
<td>Duodenal ulcer; chronic gastritis; bile reflux</td>
<td>NA</td>
<td>18.3</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>42</td>
<td>Superficial gastritis</td>
<td>Mild chronic gastritis in the gastric antrum, and focal lymphocyte aggregation</td>
<td>16.4</td>
</tr>
</tbody>
</table>

Inhibiting kinetics assay

The inhibition assay was performed following the method reported previously[30]. For the inhibition kinetics assay, HP cultured for 48-72 h was collected at 0 (control), 0.25, 0.50, and 1.00 times the MIC concentration of QHD in Brucella broth (BD, Franklin Lakes, NJ, United States) added with 10% FBS (BI, Kibbutz Beit Haemek, Israel) and agitated (100-120 rpm) at 37 °C. Subsequently, at 0, 12, 24, 36, 46, 48, 60, and 72 h, 100 μL from each sample was assessed for absorbance at 600 nm. Besides, three replicates were set for each sample, and the experiment was iterated three times.

Biofilm culture

The cultivation of HP biofilm was performed according to the method outlined previously[31]. A sterilised and desiccated nitrocellulose (NC) membrane (Millipore, United States; 1 cm × 1 cm) was positioned atop a new solid medium to promote consistent development of HP at the solid-gas interface. Upon reaching the logarithmic phase, the
bacteria were suspended in Brinell’s liquid medium and the starting concentration was adjusted to obtain an optical density (OD) of 0.2 per unit at 600 nm (OD600). After inoculating 25 μL of the bacterial solution on the NC membrane, it was incubated at 37 °C in a microaerobic atmosphere (85% N₂, 10% CO₂, and 5% O₂) for 3 d.

**Antibiofilm assay**

**Crystal violet staining:** Quantitative analysis of HP biofilm was conducted using the crystal violet staining method[32]. HP biofilm was cultivated in 96-well plates. HP cultivated for 48-72 h was treated in a microaerobic condition at 37 °C for 72 h with various medication doses in Brucella broth. Nine wells were given to each group. Following the incubation, the upper bacterial solution was discarded, the plate was rinsed three times with phosphate buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, United States), 200 μL of anhydrous methanol (Beijing Tongguang Fine Chemical, Beijing, China) was added to each well and incubated for 15 min, the methanol was discarded, and the plate was air-dried. Next, each well was stained with 200 μL of 1% ammonium oxalate crystal violet reagent (Solarbio, Beijing, China) for 5 min before washing with running water. Besides, 200 μL of 95% ethanol (Beijing Tongguang Fine Chemical, Beijing, China) was diluted in each well and shaken at 80 rpm at 37 °C for 30 min after natural drying. The OD was determined at 590 nm using a microplate reader (TECAN, Männedorf, Switzerland). The interpretation of biofilm production was conducted according to the criteria of Stepanović et al[32] (Table 3).

**Confocal laser scanning microscopy**

The LIVE/DEAD™ BacLight™ Bacterial Cell Activity Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States) was used for fluorescence staining. Dye A (SYTO™ 9 dye) binds to DNA and stains bacteria green after it enters the cell membrane. Dye B (propidium iodide) only penetrates partial bacterial cell membranes; as bacteria die, the membrane permeability changes, it stains the dead bacteria red. The biofilm, which was prepared as described earlier, was aseptically removed from the incubated NC membrane, transferred to a 24-well plate, and then washed three times with PBS. Next, 3 μL of dye A was combined and dye B in a 1:1 ratio, supplemented with 1 mL of normal saline, and 100 μL was dispensed into each well, followed by incubation in darkness for 15 min. Subsequently, the NC membrane was transferred onto a glass slide, and glycerol was applied for microscopic observation. Samples were observed within 1 h to mitigate the potential impact of prolonged exposure-induced bacterial death. Confocal laser scanning microscope (CLSM; Leica, Wetzlar, Germany) utilized a 488 nm-excited argon laser, where the blue channel detected the green signal and the 560 nm green channel obtained the red signal. Scanning occurred layer-by-layer at 1 μm intervals, which moved from the free side of the HP biofilm to the attached side of the slide. This method was primarily based on the previous work[33].

---

Table 3 Interpretation of biofilm production

<table>
<thead>
<tr>
<th>Average optical density value</th>
<th>Biofilm production</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ ODc</td>
<td>No</td>
</tr>
<tr>
<td>ODc &lt; OD ≤ 2 × ODc</td>
<td>Weak</td>
</tr>
<tr>
<td>2 × ODc &lt; OD ≤ 4 × ODc</td>
<td>Moderate</td>
</tr>
<tr>
<td>&gt; 4 × ODc</td>
<td>Strong</td>
</tr>
</tbody>
</table>

Optical density cut-off value (ODc) = average optical density of negative control + 3 × SD of negative control. OD: Optical density; ODc: Optical density cut-off value.

**Clinical HP strain acquisition**

1. Gastroscopy in patients with HP infection
2. Gastric mucosal biopsy
3. Multi-sample tissue grinder
4. In-vitro isolation of HP
5. Helicobacter pylori, HP

**Scanning electron microscopy**

The formation of HP biofilms was observed using a scanning electron microscope (SEM, JSM-7900F, JEOL, Japan). The NC membrane (GE, Boston, Mass, United States) was cut into 1 cm × 1 cm pieces to prepare a solid medium comprising NC membranes and varying drug concentrations. After 48-72 h of growth, HP was pulverized into cryopreservation solution and diluted to 3 × 10⁸ CFU/mL. In addition, 10 μL of bacterial solution was added to the NC membrane, distributed uniformly, and incubated at 37 °C in a microaerophilic atmosphere for 72 h. After the removal of the NC membrane, it was placed with glutaraldehyde (Regen Biotechnology Co., LTD, Beijing, China) in a 6-well plate at 4 °C for 2 h. Subsequently, the samples were air-dried, sputter-coated with gold (10 nm), and observed by SEM.

**Detection of (p)ppGpp accumulation patterns**

The method for the detection of (p)ppGpp accumulation patterns was referenced from a previous report with slight modifications[34]. The biofilm bacteria were washed with PBS, and diluted to OD600 = 0.2. Each sample, consisting of 5 mL, was centrifuged at 6000 rpm for 5 min, and subsequently suspended in 250 μL of liquid culture medium. Next, 100 μCi/mL of ³²P (Amersham)-labeled KH₂PO₄ was added and allowed to act for 2 h at 37 °C. During this process, varying concentrations of QHD (128, 64, 32, and 16 mg/mL) were added for 1 h of intervention in a shaking culture environment at 37 °C and 120 rpm. After labeling, a 50 μL sample was taken, and centrifuged at 8000 rpm for 5 min for two washes. Then, the 50 μL sample was resuspended in an equal volume of 2 M formic acid. Subsequently, the sample was incubated on ice for 15 min, undergoing 5 cycles of freeze-thaw and then being centrifuged at 16000 g for 5 min. Approximately 3 μL of the supernatant was taken, and applied to polyethyleneimine-cellulose plates (Sigma-Aldrich), dried, and chromatographed for 2.5 h with 1.5 M KH₂PO₄ as the eluent. The data were obtained using phosphor screen scanning (Bio-Rad).
TEM
TEM was used to evaluate the impact of QHD on HP cell structure. Sample processing followed a stated technique[30]. In brief, HP overnight cultures were treated with QHD (16, 32, 64, and 128 mg/mL) for 2 h. PBS was used as a negative control. For agarose pre-embedding, the fixed bacteria were centrifuged, washed in 0.1 M PBS (pH 7.4) for 3 min, and then suspended in a 1% agarose solution before solidification. Afterwards, the agarose blocks were post-fixed with 1% OsO4 (Ted Pella Inc, America), rinsed, and dehydrated at room temperature. Following dehydration, the samples underwent resin penetration and embedding, polymerization, and ultrathin sectioning. The following steps included staining with uranium acetate and lead citrate, drying the cuprum grids, as well as overnight observation and image capture under a TEM (HITACHI, Japan).

Quantitative real-time polymerase chain reaction
HP cultivated for 48-72 h was shaken (100-120 rpm) at 37 °C for 2 h in Brucella broth with varying medication doses. TRIzol (Thermo Fisher Scientific, Waltham, MA, United States) was used to extract HP total RNA. Then, 1 μg RNA was reverse transcribed with PrimeScript™ RT Master Mix (TAKARA, Tokyo, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) detection was conducted on the ABI 7500 system (Applied Biosystems, Waltham, MA, United States) using PowerUp™ SYBR™Green Master Mix (Thermo Fisher Scientific, Waltham, MA, United States). Relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Table 4 lists the primers used in this experiment.

PCR-agarose gel electrophoresis
PCR-agarose gel electrophoresis was routinely performed to detect whether the target gene is expressed in HP strains. Table 5 lists the primers used in this experiment.

Metabolomics
The HP in the NC group was cultured for 72 h in Brucella broth with 10% FBS to form biofilm. In the QHD group, a 2 h intervention with QHD at a 1/2 MIC concentration was performed to disrupt biofilm structure, based on the NC group. Then, the bacterial cultures were harvested and washed three times with BHI broth at 10000 g for 10 min at 4 °C. In Brucella broth, the bacterial suspensions were adjusted to OD600 = 1.0 and subsequently used for metabolite extraction. The NC group and QHD group were analyzed using untargeted metabolomics. Further experimental procedures are provided in the Supplementary material.

Statistical analysis
At least three independent experiments were performed to calculate the mean and SE. Statistical analyses were conducted with GraphPad Prism 9. As indicated, Student’s t test, Mann-Whitney U test, analysis of variance, or Spearman correlation tests were used. A P-value ≤ 0.05 was considered significant ($^aP < 0.05; ^bP < 0.01; ^cP < 0.001; ^dP < 0.0001; NS, not significant; P > 0.05$).

RESULTS

Gastroscopy and pathological information from 12 patients
All 12 patients revealed varying degrees of gastritis during gastroscopy, and the pathological diagnosis confirmed chronic gastritis with signs of activity. Some patients exhibited lymphoid follicle formation, mucosal erosion, and intestinal metaplasia. $^{13}$C breath test results indicated current HP infection in all 12 patients (Table 2), with infection by type I HP strains, which are highly virulent (Table 6).

MICs for clinical HP strains
Following EUCAST Clinical Breakpoint standard 2022, HP strains with AML MIC > 0.125 μg/mL, CLR MIC > 0.500 μg/mL, LEV MIC > 1.000 μg/mL, and MTZ MIC > 8.000 μg/mL were antibiotic-resistant. The 12 clinical isolates of HP included 4 strains with multidrug resistance, 3 with dual drug resistance, 2 with single drug resistance, and the remaining 3 that were fully sensitive (Table 7).

Different strains of HP exhibit varying abilities to form biofilms in vitro
Among the 12 clinical strains of HP, 4 were classified as strong biofilm-forming HP, 5 as moderate biofilm-forming HP, and 3 as weak biofilm-forming HP (Figure 3). The strains with multidrug resistance and dual drug resistance exhibited stronger biofilm-forming abilities. The strain with the strongest biofilm-forming capability, Strain 1, was selected for in vitro intervention with QHD. The aim was to find the mechanism by which QHD intervenes in HP resistance using nonspecific pathways.

In vitro antibacterial activities of QHD on HP
The MICs of QHD against the drug-resistant strains were determined using the agar dilution method. The results showed that QHD had considerable antibacterial activity against drug-resistant HP strains, with MIC values ranging from 32 mg/mL to 64 mg/mL (Table 8). This result suggested that there existed differences in the antibacterial activity against different strains.
Table 4 Primer sequences for quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
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<tr>
<td>16S rRNA-F</td>
<td>GCTAAGAGATCAGCCTATGGCC</td>
<td>Spot-F</td>
<td>TGGCACAACCTGCGCTAAT</td>
</tr>
<tr>
<td>16S rRNA-R</td>
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</tr>
<tr>
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<td>HP1327-F</td>
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<td>HP1327-R</td>
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<td>HP0971-R</td>
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Table 5 Primers sequences for polymerase chain reaction-agarose gel electrophoresis

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<th>Primer name</th>
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Table 6 Typing of *Helicobacter pylori* strains

<table>
<thead>
<tr>
<th>No.</th>
<th>CagA</th>
<th>VacA</th>
<th>UreA</th>
<th>UreB</th>
<th><em>Helicobacter pylori</em> strain type</th>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>I</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>I</td>
</tr>
</tbody>
</table>

*: Positive, -: Negative; Type I *Helicobacter pylori* strains: CagA and/or VacA (+); Type II *Helicobacter pylori* strains: CagA and VacA (-).

QHD inhibits growth of HP and rapidly permeabilizes HP membranes, causing cell lysis

Inhibitory kinetics indicated that QHD inhibited the growth of HP in a time- and dose-dependent manner (Figure 4A). At a concentration of 16 mg/mL (1/4 MIC), QHD significantly inhibited the growth of HP. After intervention with QHD at concentrations of 32 mg/mL (1/2 MIC) and 64 mg/mL (MIC), there was no significant increase in the concentration of HP in the liquid culture medium, implying that HP did not grow under these conditions (Figure 4A).
Table 7 Minimum inhibitory concentration of antibiotics against 12 clinical strains of *Helicobacter pylori*

<table>
<thead>
<tr>
<th><em>Helicobacter pylori</em> strain</th>
<th>AML (μg/mL)</th>
<th>CLR (μg/mL)</th>
<th>LEV (μg/mL)</th>
<th>MTZ (μg/mL)</th>
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<tr>
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<td>32.000</td>
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<td>2</td>
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<td>32.000</td>
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<td>3.000</td>
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<td>0.016</td>
<td>0.032</td>
<td>1.500</td>
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</table>

1Antibiotic resistance.

MIC: Minimum inhibitory concentration; AML: Amoxicillin; CLR: Clarithromycin; LEV: Levofloxacin; MTZ: Metronidazole.

Table 8 Minimum inhibitory concentration of Qingre Huashi decoction against *Helicobacter pylori*

<table>
<thead>
<tr>
<th><em>Helicobacter pylori</em> strain</th>
<th>Concentration of QHD (mg/mL)</th>
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<td></td>
<td>NC (0)</td>
</tr>
<tr>
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</table>

MIC: Minimum inhibitory concentration; QHD: Qingre Huashi decoction; +: Existing colonies; −: No colony growth.

The bactericidal mechanism of QHD was investigated through bacterial morphology observation and vesicle leakage assessment. After intervention with varying concentrations of QHD (16, 32, 64, and 128 mg/mL) for 2 h, the cell membrane morphology was observed by TEM. As shown in Figure 4B, certain differences in damage were found among the five groups of HP. The group with a concentration of 128 mg/mL exhibited the most severe overall damage, with extensive structural necrosis and disappearance, significant dissolution of plasmids and ribosomes, and obvious separation of the cell wall. The group with a concentration of 64 mg/mL had a slightly lower level of damage, with some structures presenting swelling and necrosis, and some structures exhibiting significant damage changes, with local sparse dissolution of plasmids and ribosomes and partial separation of the cell wall. The overall damage to the groups with concentrations of 16 and 32 mg/mL was shown to be slightly milder, with a small amount of viable structures, abundant plasmids and ribosomes, and mild local separation of the cell wall. The group without QHD (NC group) showed the least damage, with abundant bacteria, some viable structures, and rich, uniform plasmids and ribosomes, without obvious separation of the cell wall. This also indicated that QHD exerted a disruptive effect on the HP membrane.

Figure 3 Biofilm formation of 12 clinical strains in vitro. Strains 1-4 were classified as strong biofilm-forming *Helicobacter pylori* (HP), strains 5-9 as moderate biofilm-forming HP, and strains 10-12 as weak biofilm-forming HP. HP: *Helicobacter pylori*, OD: Optical density, ODc: Optical density cut-off value.

QHD inhibits and disrupts clinical HP biofilm formation in vitro

Crystal violet staining: When assessing the in vitro biofilm formation of HP using the crystal violet method, strains with OD ≤ OD cut-off value (ODc) are classified as HP strains with no biofilm formation; strains with ODc < OD ≤ 2 × ODc are identified as strains with weak biofilm formation; strains with 2 × ODc < OD ≤ 4 × ODc are categorized as strains with moderate biofilm formation; strains with OD > 4 × ODc are considered as strains with strong biofilm-forming ability. Strain 1 was classified as a strain with strong biofilm-forming ability, and the results indicated that QHD inhibited the in vitro biofilm formation of HP (16-128 mg/mL) in a concentration-dependent manner, with significant inhibition observed at a concentration of 16 mg/mL (1/4 MIC) (Figure 5A).

CLSM: By CLSM, cell viability assays revealed the impact of QHD on HP biofilms and the viability of the bacteria (Figure 5B). Green represents viable bacteria, while red stands for dead bacteria. Strain 1 exhibited robust survival and high bacterial activity in vitro, forming a dense biofilm. Intervention with QHD at different concentrations (16-128 mg/mL) resulted in a significant reduction in HP viability, decreased activity, and the formation of thinner and more loosely structured biofilms. With the drug concentration reaching 64 mg/mL (MIC), bacteria were nearly inactive, and biofilm formation was virtually absent.

SEM: By SEM, we observed the effect of QHD on the HP biofilm (Figure 5B). The NC group presented the normal biofilm structure of HP in vitro, where most of the bacteria appeared to be rod-shaped, densely distributed, and closely connected, with an intact biofilm matrix. Following QHD intervention, bacterial binding in the biofilm became less tight, and the biofilm matrix was incomplete, exhibiting numerous voids. At a drug concentration of 64 mg/mL (MIC) and higher concentrations, SEM observations revealed the absence of clearly formed rod-shaped structures and a lack of an intact biofilm matrix.

qRT-PCR

QHD-treated clinical strain’s biofilm gene mRNA expression levels were measured by qRT-PCR (Figure 5C-G). QHD dose-dependently inhibited LuxS and NapA expression. At 64 mg/mL, QHD inhibited Spot, HP1174, and CagE expression, with significant differences. Thus, QHD could affect biofilm formation by suppressing biofilm-related gene expression.

Detection of (p)ppGpp accumulation patterns

(p)ppGpp is a key molecule regulating bacterial biofilm formation and multidrug resistance. HP produces a significant amount of (p)ppGpp after the formation of a biofilm. The 32P-labeled nucleotides in HP were detected by thin-layer chromatography after drug intervention. QHD significantly inhibited HP (p)ppGpp molecule expression (Figure 5H and I).

QHD decreases HP efflux pump-related gene expression in vitro

The efflux pump gene mRNA expression levels in the clinical strain treated with QHD were measured using qRT-PCR. QHD significantly inhibited HP1489 and HP1327 expression. It also suppressed HP0971 and HP0605 expression at 32 mg/mL (1/2 MIC) and 64 mg/mL (MIC), respectively, with all differences being of statistical significance (Figure 6).

Metabolic differences analysis and metabolomic pathway enrichment analysis for QHD vs NC

In metabolomics, totally 6023 metabolites were detected in this study. The classification results are illustrated in Figure 7A using class-based pie charts. OPLS-DA analysis revealed that the samples from the NC group and the QHD group exhibited distinct clustering patterns, demonstrating substantial metabolic differences between the two groups (Figure 7B).
Figure 4 Bacteriostatic and bactericidal effects of Qingre Huashi decoction on Helicobacter pylori. A: Inhibitory kinetics show that Qingre Huashi decoction (QHD) inhibited the growth of Helicobacter pylori (HP) in a time- and dose-dependent manner. At a concentration of 16 mg/mL (1/4 MIC), QHD significantly inhibited the growth of HP. After intervention with QHD at concentrations of 32 mg/mL (1/2 MIC) and 64 mg/mL (MIC), no significant increase was found in the concentration of HP in the liquid culture medium, indicating that HP did not grow under these conditions; B: Transmission electron microscopy showing that the cell membranes of the five groups of HP exhibited varying degrees of differences, which indicated different levels of damage. OD: Optical density.

Metabolic differences analysis for QHD vs NC: Compared to the NC group, there were 103 upregulated differential metabolites and 24 downregulated differential metabolites in the QHD group (Figure 7C and D). To visually reveal the relationship between samples and the expression differences of metabolites between the two groups, a hierarchical clustering heatmap (Figure 7E) was generated, showing the expression levels of the top 50 significant differential metabolites based on variable importance in projection (VIP) scores. Previous literature reported that strains with low biofilm-forming ability produced more metabolites than those with high biofilm-forming ability [24]. In this study, the upregulated metabolites after QHD intervention were significantly more than the downregulated metabolites, which could further validate the inhibitory and disruptive effects of QHD on HP biofilms.

Metabolic pathway enrichment analysis for QHD vs NC: To investigate the metabolic mechanisms underlying QHD intervention on HP biofilms, pathway enrichment analysis was performed using metabolites significantly altered in the QHD group compared to the NC group (Figure 7F-H). Figure 7F shows the enriched pathways for significantly altered metabolites between the two groups, while Figure 7G illustrates the upregulated pathways in QHD vs NC, and Figure 7H presents the downregulated pathways in QHD vs NC. Among the upregulated pathways, ten were enriched in the upregulation of the metabolite adenosine monophosphate (AMP).

DISCUSSION

HP antibiotic resistance is a problem globally [5]. Its adaptation to harsh environments, resistance to external pressures,
and long-term survival on the host gastric mucosa depend on biofilm and efflux pump mechanisms\cite{6,35}. We examined how QHD influences HP, its biofilm, and efflux pumps in clinically isolated and cultured HP strains with high antibiotic resistance and strong biofilm-forming ability. It was shown in this study that QHD interacted with the SpoT-HP1174/NapA-biofilm pathway, mediated HP quorum sensing, and controlled HP biofilm formation tightly. In addition, it downregulated efflux pump-related genes and inhibited efflux pump transport.

It is reported that the components of QHD, including *Coptis chinensis*\cite{36}, *Gardenia jasminoides*\cite{37}, *Taraxacum mongolicum*\cite{38}, *Amomum kravanh*\cite{39}, and *Magnolia officinalis*\cite{40}, exhibit inhibitory effects on HP growth both in *vivo* and *in vitro*. They also possess antioxidant properties and can improve inflammation caused by HP in the stomach. *Plantago asiatica*\cite{41} is reported to have antioxidant, anti-inflammatory, and antibacterial effects. *In vitro*, *Atractylodes lancea* extracts inhibit HP NCTC11637 strain growth and biofilm formation\cite{42}. The antibacterial effects of herbal medicine on HP are mostly caused by single herbs or monomer extracts in domestic and international reports. There are hardly any traditional Chinese medicine formulations concentrating on the study of drug resistance mechanisms in HP. Traditional Chinese medicine formulations, with multiple components, targets, and diversity, may delay absorption and excretion, which can therefore prolong therapeutic effects\cite{43,44}. Moreover, they also possess complex regulatory effects that restore and balance the body’s physiological environment.

Through gastroscopy, 12 HP strains were obtained from patients who were clinically infected with HP. Then, these strains were cultured *in vitro* to conduct drug susceptibility testing and biofilm culture. Out of the 12 strains, 9 were found to be resistant to one or more drugs, and all exhibited high expression of virulence genes [CagA and/or VacA (+)]. Our study revealed variations in the *in vitro* biofilm formation capability among different strains of HP. Multi-resistant strains demonstrated strong biofilm-forming ability, while mono-resistant and dual-resistant strains formed medium biofilms. In addition, non-resistant strains formed weak biofilms. However, owing to the limited sample size, the overall characteristics could not be fully represented. Besides, further studies are required to clarify this conclusion.

To investigate the impact of QHD on HP biofilm and understand its mechanism in regulating HP biofilm and efflux pump resistance, the HP strain with the strongest biofilm-forming ability was selected and treated with QHD. QHD exhibited a bactericidal effect against HP. Initially, the agar dilution method was used to assess the MIC of QHD against 12 clinical strains of HP. The MIC for 11 HP strains was found to be 64 mg/mL. A strain representative was chosen to determine the inhibition curve of QHD. Meanwhile, the effect of QHD on HP structure was observed using TEM. Our findings demonstrated that QHD displayed bacteriostatic and bactericidal properties by infiltrating and disrupting the external and internal membranes of HP. This resulted in the dissolution of plasmids and ribosomes, separation of the membranes, and ultimately the demise of HP. The development of HP biofilm structure is strongly associated with HP outer membrane proteins\cite{45}. Therefore, the interference of QHD with the structure of the HP membrane could potentially cause instability in membrane proteins, resulting in the destruction of biofilms.

Biofilms contain polysaccharides, proteins, and other molecules\cite{35}. Crystal violet dye selectively binds biofilm polysaccharides and proteins\cite{46}. HP biofilm formation *in vitro* is measured by crystal violet staining. With SEM and
Figure 6 Qingre Huashi decoction inhibits expression of *Helicobacter pylori* efflux pump-related genes in vitro. The expression levels of efflux pump-related genes were examined by quantitative real-time polymerase chain reaction before and after Qingre Huashi decoction (QHD) intervention. The results suggested that QHD could inhibit the expression of these efflux pump-related genes. A: HP1489; B: HP1327; C: HP0971; D: HP0605. *P < 0.05; †P < 0.01; ‡P < 0.001; ††P < 0.0001.

CLSM, it provides a more complete HP biofilm picture. Crystal violet staining showed that QHD reduced biofilm formation in HP strains, especially strong biofilm-forming strains. The biofilm formation of the strain was significantly reduced by the decoction at 16 mg/mL (1/4 MIC). SEM and CLSM showed that QHD significantly inhibited HP biofilm formation and prevented biofilm matrix formation. In addition to damaging the outer membrane of HP, QHD may have other mechanisms for reducing HP biofilm formation and disrupting its structure. (p)ppGpp is a key molecule in regulating bacterial biofilm formation and multidrug resistance[47]. QHD can inhibit the expression of (p)ppGpp molecules in HP, therefore influencing biofilm formation. LuxS is the only known quorum-sensing gene[48], and the SpoT-HP1174/NapA-biofilm pathway is a vital pathway mediating HP resistance[16,34]. CagE is involved in the interaction and signal transduction between HP and host cells. According to this mechanism, it was found that QHD at low concentrations effectively reduced the expression of LuxS, and significantly reduced the expression of SpoT, HP1174, NapA, and CagE at MIC concentrations. This indicated that QHD regulated biofilm formation by affecting the HP quorum-sensing system and the SpoT-HP1174/NapA-biofilm pathway. The efflux pump system is a mechanism contributing to antibiotic resistance in HP biofilm structures[49]. In the current study, it was found that QHD effectively suppressed the expression of efflux pump genes HP1489 and HP1327. At a concentration of 32 mg/mL (1/2 MIC), it also inhibited the expression of HP0971, and it inhibited the expression of HP0605 at a concentration of 64 mg/mL (MIC).

Metabolic difference analysis and metabolomic pathway enrichment analysis for QHD vs NC were conducted. Compared to the NC group, significantly upregulated metabolites in the QHD group are involved in inducing HP oxidative stress, including N-(N-L-gamma-glutamyl-/cysteinyl) glycine monoethyl ester, S-hydroxymethylglutathione[50], and 3-hydroxyocta-2,4-dieneoylcarnitine. These metabolites can induce oxidative stress in HP, which damages its biofilm and intracellular structures, ultimately leading to bacterial death. In lipid metabolism, phospholipids (PLs) like PE [19:1(9Z)/0:0], PC (0:0/18:0), PC (O-16:0/2:0), PC (O-1:0/16:0), PC (18:1(11Z)/0:0), and Pc (16:0/0:0) (Rac) were significantly upregulated. These PLs are crucial constituents of cell membranes, and are involved in membrane construction, energy metabolism, and signal transduction (Figure 7E). A previous study indicated increased PL levels in
Lin MM et al. Impact of QHD on drug-resistant H. pylori
Figure 7 Metabolic difference analysis and metabolomic pathway enrichment analysis for Qingre Huashi decoction vs NC. A: Classification of metabolites; B: OPLS-DA analysis revealed that the samples from the NC group and Qingre Huashi decoction (QHD) group exhibited distinct clustering patterns; C: Number of differential metabolites between the QHD group and NC group; D: QHD vs NC. Red dots represent significantly upregulated differential metabolites ($P < 0.05$, variable importance in projection (VIP) > 1 and FC > 1), blue dots refer to significantly downregulated differential metabolites ($P < 0.05$, VIP > 1 and FC < 1), and gray dots stand for metabolites with no significant difference. Each dot in the plot represents a metabolite, with the x-axis showing the log2 (FC) values of the comparison between the two groups, and the y-axis representing the -log10 ($P$ value) values; E: Hierarchical clustering of all significantly differentially expressed metabolites and top 50 differentially expressed metabolites ranked by VIP. The horizontal axis represents sample names, while the vertical axis indicates differential metabolites. The color gradient from blue to red indicates the abundance of metabolite expression, with red showing higher expression levels of differential metabolites; F-H: KEGG pathway enrichment analysis on differentially expressed metabolites. The x-axis represents the -log10 $P$-value for each pathway, the y-axis stands for different pathway names, and the numbers on the bars indicate the number of differentially expressed metabolites annotated to that pathway.
strains with low biofilm formation[24]. After QHD intervention, PL metabolites were elevated in strains exhibiting high biofilm-forming ability, suggesting an impact on their biofilm architecture. AMP acts as a crucial signaling molecule within bacteria, which is capable of activating AMP-activated protein kinase (AMPK). In the QHD group, AMP exhibited significant upregulation, potentially further stimulating AMPK. Activation of AMPK in relevant pathways may cause the inhibition of specific signaling pathways, thereby potentially influencing biological processes including growth, metabolism, and protein synthesis in HP.

**CONCLUSION**

To conclude, the formation of HP biofilm structures and the efflux pump mechanism significantly contribute to clinical multidrug resistance, leading to a decrease in the eradication rate of HP. Inhibiting the formation of HP biofilms and the efflux pump mechanism is an effective strategy to reduce HP resistance. This study clarifies that QHD has bacteriostatic and bactericidal effects on HP. In addition, it can achieve a reduction in HP resistance by inhibiting the formation of HP biofilms and the efflux pump mechanism. Based on metabolomic analysis, QHD induces oxidative stress in HP, enhances metabolism, and potentially inhibits relevant signaling pathways by upregulating AMP, thereby affecting HP growth, metabolism, and protein synthesis. As a traditional Chinese medicine formula, QHD, compared to single-component decoctions, is more comprehensive and diverse, which can provide a better approach to address the complex etiology and pathogenesis of HP resistance. This holds significant clinical importance and presents an urgent need for developing new-generation drugs to treat HP. However, the complexity of the formula also poses challenges in research and application.

**ACKNOWLEDGEMENTS**

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**FOOTNOTES**

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