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# *Helicobacter pylori* infection: Pathogenesis, antibiotic resistance, advances and therapy, new treatment strategies

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# *Helicobacter pylori* infection: Pathogenesis, antibiotic resistance, advances and therapy, new treatment strategies

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# Table of contents

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# Editorial: *Helicobacter pylori* infection: pathogenesis, antibiotic resistance, advances and therapy, new treatment strategies

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***Helicobacter pylori* infection, molecular diagnostics, antibiotics resistance, non-traditional therapies, updated treatment strategies**

Editorial on the Research Topic
*Helicobacter pylori* infection: pathogenesis, antibiotic resistance, advances and therapy, new treatment strategies

*Helicobacter pylori* (*Hp*) is a microorganism discovered only 40 years ago but since then its importance has grown in many pathologies as chronic gastritis, peptic ulcer, gastric carcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma, as well as other endothelial dysfunctions leading to vascular diseases (Ando et al., 2006). Gastric cancer mainly represents the fifth cancer for incidence and the third cause of death in the developed countries (Rawla and Barsouk, 2019). Epidemiology, transmission, and pathogenesis of *Hp* have been deeply examined as well as the resistance to antibiotics (Eusebi et al., 2014; Kayali et al., 2018; Mascellino et al., 2020). The development of new drugs capable of eradicating this pathogen is highly recommended such as combination therapy or research into other alternatives and new strategies (Makipour and Friedenberg, 2011; Yuan et al., 2021).

The influence of previous therapies in *Hp* eradication rate is reported in the paper by Choe et al. in which the past use of metronidazole (MZ) is taken into consideration in a group of subjects hospitalized in Korea. It was noted that in patients who underwent the BQT (Bismuth Quadruple Therapy) for 14 days and in those with no MZ previous treatment, the eradication rate was higher than in subjects who underwent the BQT for less than 14 days or in patients with MZ previous use.

The susceptibility of *Hp* to antibiotics in the different niches of the stomach (antrum and corpus) is another issue based on the microorganism heteroresistance. The authors Goni et al. take into account the severity of atrophic gastritis from the antrum and corpus

biopsies, where *H. pylori* strains were isolated and tested for antibiotics susceptibility. The severity of atrophy seemed to be correlated with the increase in MZ and clarithromycin (CLA) resistance. Thus, the severity of atrophy was a crucial element in order to establish a correct treatment. The high CLA and MZ resistance in atrophic gastritis should be carefully considered.

As far as antibiotic resistance is concerned, interesting is the article by Guo et al. in which the genetic methods (polymerase chain reaction, whole genome sequencing) and the phenotypic method (broth micro-dilution) were compared in *H. pylori*-infected patients with treatment failure for at least twice in order to assess the efficacy of different antimicrobial resistance–guided quadruple therapies in refractory *H. pylori* infection. As such, the genotypic resistance determined using gastric biopsy specimens correlated well with the phenotypic resistance both achieving high eradication rates.

The question of whether susceptibility testing is essential in guiding therapeutic strategies has been debated in many studies (Gomollon et al., 2000; Zullo et al., 2003; Graham, 2015).

In this Research Topic, two articles play an important role for this purpose. The first one by Nyssen et al. deals with the comparison of empirical vs. susceptibility-guided *Hp* treatment. The authors infer that the benefit of susceptibility-guided treatment over empirical therapy could not be demonstrated, even in first-line therapy if the most updated quadruple regimen (BQT) is prescribed.

In the same way in the article by Li P. et al., both susceptibility-guided therapy based on the resistance of CLA or minocycline and empiric quadruple therapy containing furazolidone may achieve the same level of eradication. The empirical quadruple therapy containing furazolidone, bismuth, and esomeprazole might be selected as a correct first-line regimen.

A series of articles in this Research Topic is based on the research for alternative products different from antibiotics and able to treat *Hp* infection (Ayala et al., 2014; Gopal et al., 2014; Ruggiero, 2014).

For example, Sosa et al. found that new therapies based on plant extracts such as extra virgin olive oil show an effect *in vitro* on *Hp* strains and *in vivo* on the gastric mucosa of mice infected orally with an *H. pylori* suspension, greatly preventing the formation of the stomach erosions after the treatment.

The same situation is found in the paper of Ibáñez et al. that examines the *Hp* antimicrobial activity of *Asclepias curassavica* L. a derivative by a plant from South America and Tropical areas, which was considered a therapeutic adjuvant and a safe nutraceutical product. Asclepain showed an interesting activity towards *Hp* even against the drug-resistant isolates. The MIC resulted as being 1–2 μg/ml and the MBC between 2 and 4 μg/ml without toxic effects. Its activity was based on the reduction of *Hp* virulence genes such as *ureA*, ompA, and *flaA*.

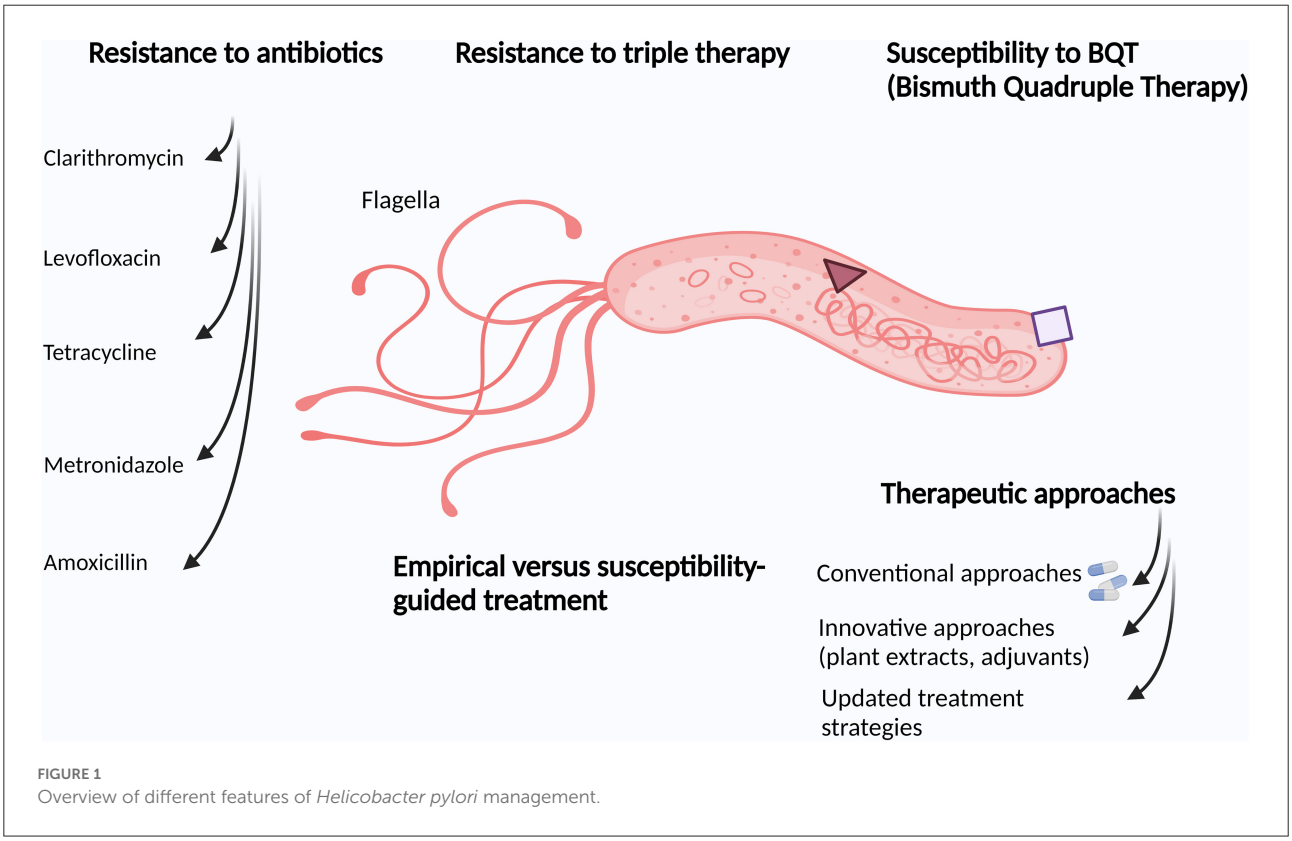


FIGURE 1
Overview of different features of *Helicobacter pylori* management.

The following three articles of this Research Topic concern other compounds included in the group of non-antibiotic substances showing an antimicrobial activity. The first study (Jia et al.) takes into consideration the Jinghua Weikang Capsule (JWC) that is the first patent medicine approved in China for the treatment of gastritis and peptic ulcers caused by *Hp*. Its major component *Chenopodium ambrosioides* L. inhibits biofilm formation even though the exact mechanism of its efficacy against drug-resistant *Hp* is still uncertain. It also seems to be able to induce the reversal of MZ resistance.

The second study concerns the use of 1,4-dihydropyridine (DHP)-based antihypertensive drug, which seems to exhibit a strong bactericidal activity against *H. pylori*. The results presented in this study by González et al. strongly support the use of 1,4-DHP as a tool for novel antimicrobials against *H. pylori*. The MIC values are reported to be comparable with those achieved by first-line antibiotics.

In the third study, Li R.-J. et al. selected from the natural product forsythia, the Phillygenin, an effective antibacterial component against *Hp* even if the values of MICs and MBCs are shown to be quite high (16 μg/ml). It was found to be non-toxic to gastric epithelial cells and its mechanism of action was mainly associated with the inhibition of biofilm formation. Phillygenin could also cause ATP leakage in a concentration and time-dependent way. This mechanism seemed to be multiple targets.

In the context of products that can be suitable for helping to combat *Hp* infection either alone or associated with antibiotics, probiotics play a crucial role. They might interact with the gastric microbiota bringing benefits in the clinical *Hp* management. These concepts are discussed in the last article by Marinelli et al. who studied *Lactococcus rhamnosum (LG)* supplementation in combination with BQT (Bismuth Quadruple Therapy) to determine a possible improvement in eradication rate, tolerability, and compliance. The authors found that the influence of LG to BQT in the management of *Hp*-related infection was very useful in terms of efficacy and tolerability but mainly in the persistence decrease of post-treatment dyspepsia.

In conclusion, *Hp* antibiotic resistance has been increasing all over the world in recent years, and this phenomenon constitutes an important challenge for the treatment of this fastidious bacterium (Figure 1). This has led to an obstinate search for new solutions such as treatments based on the use of natural resources such as plants, probiotics, nutraceuticals, and bacteriophages. As such, some interesting non-traditional therapies have been indicated in this Research Topic as a mean to target this important gastric pathogen. Notably, it was also shown in this study that successful *Hp* eradication might be achieved in almost all patients even without susceptibility tests that are expensive and time-consuming.

## Author contributions

MM organized the editorial and wrote the paper. SP revised the manuscript. AV checked the references. PM gave a complete overview of the whole article. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

Ando, T., Minami, M., Ishiguro, K., Maeda, O., Watanabe, O., Mizuno, T., et al. (2006). Changes in biochemical parameters related to atherosclerosis after *Helicobacter pylori* eradication. *Aliment. Pharmacol. Ther.* 24, 58–64. doi: 10.1111/j.1365-2036.2006.00026.x

Ayala, G., Escobedo-Hinojosa, W. I., de la Cruz-Herrera, C. F., Herrera, C., and Romer, I. (2014). Exploring alternative treatment for *Helicobacter pylori* infection. *World J. Gastroenterol.* 20, 1450–1469. doi: 10.3748/wjg.v20.i6.1450

Eusebi, L. H., Zagari, R. M., and Bazzoli, F. (2014). Epidemiology of *Helicobacter pylori* infection. *Helicobacter* 19, 1–5. doi: 10.1111/hel.12165

Gomollon, F., Sicilia, B., Ducóns, J. A., Sierra, E., and Revillo, M. J. (2000). Third line treatment for *Helicobacter pylori:* a prospective, cultured-guided study in peptic ulcer patients. *Aliment. Pharmacol. Ther.* 14, 1335–1338. doi: 10.1046/j.1365-2036.2000.00833.x

Gopal, R., Jeong, E., Seo, C. H., and Park, Y. (2014). Role of antimicrobical peptides expressed by host cells upon infection by *Helicobacter pylori*. *Protein Pept. Lett.* 21, 1057–1064. doi: 10.2174/0929866521666140708092032

Graham, D. Y. (2015). Editorial: avoiding unethical *Helicobacter pylori* clinical trials: susceptibility-based studies and probiotics as adjuvants. *Helicobacter* 20, 321–325. doi: 10.1111/hel.12244

Kayali, S., Manfredi, M., Gaiani, F., Bianchi, L., Bizzarri, B., Leandro, G., et al. (2018). *Helicobacter pylori*, transmission routes and recurrence of infection: state of the art. *Acta Biomed*. 89, 72–76. doi: 10.23750/abm.v89i8-S.7947

Makipour, K., and Friedenberg, F. K. (2011). The potential role of N-acetylcysteine for the treatment of *Helicobacter pylori*. *J. Clin. Gastroenterol*. 45, 841–843. doi: 10.1097/MCG.0b013e31822be4d6

Mascellino, M. T., Oliva, A., Miele, M. C., De Angelis, M., Bruno, G., and Severi, C. (2020). Secondary antibiotic resistance, correlation between genotypic and phenotypic methods and treatment in *Helicobacter pylori* infected patients: a retrospective study. *Antibiotics* 9, 549. doi: 10.3390/antibiotics9090549

Rawla, P., and Barsouk, A. (2019). Epidemiology of gastric cancer: global trends, risk factors and prevention. *Prz. Gastroenterol*. 14, 26–38. doi: 10.5114/pg.2018.80001

Ruggiero, P. (2014). Use of probiotics in the fight against *Helicobacter pylori*. *World J. Gastrointest. Pathophysiol*. 5, 384–391. doi: 10.4291/wjgp.v5.i4.384

Yuan, Z., Xiao, S., Li, S., Suo, B., Wang, Y., Meng, L., et al. (2021). The impact OF *Helicobacter pylor* infection, eradication therapy, and probiotics intervention on gastric microbiota in young adults. *Helicobacter* 26, e12848. doi: 10.1111/hel.12848

Zullo, A., Hassan, C., Lorenzetti, R., Winn, S., and Morini, S. (2003). A clinical practice viewpoint: to culture or not to culture *Helicobacter pylori*? *Dig. Liver Dis*. 35, 357–361. doi: 10.1016/S1590-8658(03)00081-1

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# Correlation Analysis Among Genotype Resistance, Phenotype Resistance, and Eradication Effect After Resistance-Guided Quadruple Therapies in Refractory *Helicobacter pylori* Infections

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**Objectives:** The antimicrobial resistance of *Helicobacter pylori* (*H. pylori*) in most countries and regions has increased significantly. It has not been fully confirmed whether the detection of *H. pylori* resistance gene mutation can replace antibiotic drug sensitivity test to guide the clinical personalized treatment. The objective of this study was to assess and compare the efficacy of different antimicrobial resistance-guided quadruple therapies in refractory *H. pylori*-infected individuals who had undergone unsuccessful prior eradication treatments.

**Methods:** From January 2019 to February 2020, genotypic and phenotypic resistances were determined by polymerase chain reaction (PCR), whole genome sequencing (WGS) and broth microdilution test, respectively, in 39 *H. pylori*-infected patients who have failed eradication for at least twice. The patients were retreated with bismuth quadruple therapy for 14 days according to individual antibiotic resistance results. Eradication status was determined by the $^{13}$C-urea breath test.

**Results:** The overall eradication rate was 79.5% (31/39, 95% CI 64.2–89.5%) in the intention-to-treat (ITT) analysis and 88.6% (31/35, 95% CI 73.5–96.1%) in the per-protocol analysis (PP) analysis. The presence of amoxicillin resistance (OR, 15.60; 95% CI, 1.34–182.09; $p = 0.028$), female sex (OR, 12.50; 95% CI, 1.10–142.31; $p = 0.042$) and no less than 3 prior eradication treatments (OR, 20.25; 95% CI, 1.67–245.44; $p = 0.018$), but not the methods for guiding therapy ($p > 0.05$) were associated with treatment failure. Resistance-guided therapy achieved eradication rates of more than 80% in these patients. The eradication rate of *H. pylori* in the phenotypic resistance-guided group was correlated well with genotype resistance-guided groups, including PCR and WGS.

**Conclusion:** Culture or molecular method guiding therapy can enable personalized, promise salvage treatments, and achieve comparably high eradication rates in patients

with refractory *H. pylori* infection. The detection of *H. pylori* resistance mutations has a good clinical application prospect.

**Protocol Study Register:** [clinicaltrials.gov], identifier [ChiCTR1800020009].

**Keywords: *Helicobacter pylori*, antibiotic resistance, mutations, whole-genome sequencing, eradication**

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a Gram-negative microaerophilic bacterium that persistently colonizes the stomach of approximately 50% of the world's population, equivalent to approximately 4.4 billion people (O'Connor et al., 2017). This infection establishes lifelong chronic progressive gastric inflammation, leading to a stepwise progression through gastric atrophy, intestinal metaplasia, and dysplasia, to the development of carcinoma (McColl, 2010). In the absence of an effective vaccine, treatment of chronic *H. pylori* infection has emerged as the main strategy for preventing subsequent gastric cancer development (Ford et al., 2014; Choi et al., 2018; Doorakkers et al., 2018).

Bismuth quadruple therapy, containing two antibiotics, plus bismuth and proton pump inhibitor (PPI) for 14 days, is recommended as a first-line empirical treatment for *H. pylori* infections by the Masstricht V Consensus (Malfertheiner et al., 2017). However, curing *H. pylori* infection has been proved remarkably difficult as the cure rates of empirical treatments are often <70% due to the increasing antimicrobial resistance (Malfertheiner et al., 2012; López-Góngora et al., 2015). And personalized treatment could be the main direction in the future, as it can significantly enhance the eradication rate, diminish the abuse of antibiotics, and avoid secondary antibiotic resistance (Fallone et al., 2016; Chey et al., 2017). Consequently, evaluating the resistance of *H. pylori* to drugs is of major clinical importance for guiding decisions about appropriate therapies in individuals and treatment policies in populations.

Susceptibility of *H. pylori* to antibiotics can be assessed by culture-based or molecular-based drug susceptibility testing (DST) techniques. Culture-based techniques are the standard DST for *H. pylori* and provide *in vitro* phenotypic susceptibly information (Miura and Hokari, 2012). However, this method is hampered by a relatively high rate of false negative, ranging from 45 to 90% (Mégraud and Lehours, 2007). Growth of *H. pylori* can be affected by many environmental factors and determination of the minimum inhibitory concentration (MIC) for *H. pylori* is cumbersome with a long turn-around-time, which limits their clinical application (Kjøller et al., 1991; Giorgio et al., 2016). Molecular-based methods rely mainly on the detection of specific *H. pylori* mutations encoding resistance. Globally, polymerase chain reaction (PCR)-based techniques have been developed to shorten the turn-around time and provide rapid detection of genotypic resistance in *H. pylori* (Cattoir et al., 2007). Besides, next-generation sequencing (NGS) refers to technologies that enable massively parallel sequencing (of DNA or RNA) to provide high-throughput genetic data at relatively low cost. Consequently, NGS would be applied in combination with culture for whole-genome sequencing (WGS), which is a powerful tool for antibiotic resistance prediction in *H. pylori* (Nezami et al., 2019).

However, in view of the correlation between genotypic resistance and phenotypic resistance and clinical treatment effect have not yet been fully elucidated, especially in China. Hence, we tried to further verify their consistency and the clinical application prospect of molecular techniques (PCR and WGS) guided eradication therapy. We conducted this trial by testing the resistance of antibiotics *in vitro*, detecting the mutation in the antibiotic resistance site by PCR and WGS, following up the efficacy of resistance-guided clinical eradication therapy, and analyzing the general data (demography, clinical diagnosis, and medical history, etc.) of the patients with refractory *H. pylori* infection.

## MATERIALS AND METHODS

### Study Design and Population

This prospective, open-label trial was conducted in Wuhan Union Hospital and Pathogen Microbiology Laboratory, Tongji Medical College, Huazhong University of Science and Technology (clinicaltrials.gov identifier: ChiCTR1800020009). Between January 2019 and February 2020, a total of 39 *H. pylori*-infected patients who failed at least twice were prospectively enrolled in this study. The study protocol was approved by the Institutional Review Board of Tongji Medical College, Huazhong University of Science and Technology. Written informed consent was obtained from all patients prior to enrollment. We searched published works from PubMed, MEDLINE and the Cochrane Library for the terms "*H. pylori*," "quadruple therapy," "third-line," "phenotypic resistance," "genotypic resistance," "polymerase chain reaction," and "next generation sequencing" with no language or date limits. No publication of clinical trials that assessed and compared the efficacy of phenotypic resistance-guided and genotypic resistance-guided quadruple therapy in the at least third-line treatment of *H. pylori* infection was identified.

### Eligibility Criteria and Randomization

Patients were excluded from the study if any of the following criteria was present: (i) children and teenagers aged <18 years old; (ii) history of gastrectomy or non-curable malignancy; (iii) contraindication or previous allergic reaction to proton

**Abbreviations:** *H. pylori*, *Helicobacter pylori*; PPI, proton pump inhibitor; DST, drug susceptibility testing; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; NGS, next-generation sequencing; WGS, whole-genome sequencing; $^{13}$C-UBT, $^{13}$C-urea breath test; $^{14}$C-UBT, $^{14}$C-urea breath test; ORs, odds ratios; CIs, confidence intervals; CLR, clarithromycin; AMX, amoxicillin; LVX, levofloxacin; TET, tetracycline; FZD, furazolidone.

pump inhibitors (PPI), bismuth, or antibiotics (clarithromycin, amoxicillin, metronidazole, levofloxacin, furazolidone, and tetracycline); (iv) pregnant or lactating women or severe concurrent diseases; (v) any of the three methods for detecting antibiotic resistance failed. Before *H. pylori* eradication, patients were randomly assigned to three groups using a random-number chart: (i) treated according to culture-based antibiotic susceptibility testing; (ii) treated according to PCR-based testing; (iii) treated according to WGS.

## Determination of *Helicobacter pylori* Status

Before enrollment, the status of *H. pylori* infection was determined by the $^{13}$C-urea breath test ($^{13}$C-UBT) or $^{14}$C-urea breath test ($^{14}$C-UBT). Patients with either positive $^{13}$C-UBT or positive $^{14}$C-UBT at least twice were defined as refractory to previous treatment and were eligible for enrollment. Oesophago-gastro-duodenoscopy with biopsies from gastric antrum (two pieces for *H. pylori* culture, one pieces for PCR test and necessary number of pieces for histology) were performed for all patients. After treatment, *H. pylori* status was determined by $^{13}$C-UBT or $^{14}$C-UBT $\geq$4 weeks after the completion of eradication therapy. Successful eradication of *H. pylori* was defined as a negative $^{13}$C-UBT or $^{14}$C-UBT result. All patients were asked to stop proton pump inhibitors (PPI) for $\geq$2 weeks and antibiotics or bismuth for $\geq$4 weeks before endoscopy examination. Positive and negative results of $^{13}$C-UBT were defined according to the results of previous study as a cut-off value of $\geq$5 and <2.5‰, respectively (Chen et al., 2003). Patients with uncertain results received another $^{13}$C-UBT until the result was conclusive.

## Determination of Phenotypic and Genotypic Resistance

### Phenotypic Resistance: Culture-Based Drug Susceptibility Testing

The biopsy specimens were cultured on chocolate agar plates containing 10% sheep blood and incubated under microaerobic conditions (5% $O_2$, 10% $CO_2$, and 85% $N_2$) for 5–7 days (**Figure 1**). Phenotypic resistance was determined by the broth microdilution test if strains were available. *H. pylori* was inoculated onto antibiotic-containing broth medium supplemented with 5% defibrinated sheep blood. *H. pylori* ATCC 26695 was used as the quality control strain. The MIC of each antibiotic was determined after 72 h of incubation. The breakpoints for amoxicillin, clarithromycin, levofloxacin, tetracycline, metronidazole, and furazolidone resistance were defined as $\geq$0.5, $\geq$1, $\geq$1, $\geq$1, $\geq$8, and $\geq$2 μg/mL, respectively (Midolo et al., 1997; Liou et al., 2010; Chung et al., 2017). Each experiment was performed in triplicate, and experiments were repeated at least three times per strain.

### Genotypic Resistance: Polymerase Chain Reaction-Based Assays

The DNA of *H. pylori* was extracted from gastric biopsy tissues using DNA extraction kit (Gentra DNA Purification Kit, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The gene fragment of *H. pylori* correlated with different antibiotics was amplified by PCR with specific primers and then sequenced (**Table 1**). As established by previous studies, the presence of the specific variants is listed (**Supplementary Table 1**). All PCR mixtures were prepared in a final volume of 25 μL containing 50 ng DNA from the samples served as the template for PCR performed in a thermal cycler (Master cycler gradient, Eppendorf, Germany), 10 μM of each primer, 8.5 μL dd$H_2O$, 2 μL template DNA, 12.5 μL Mix of Taq DNA polymerase. Thermal amplification of PCR products was performed at 94°C for 4 min and then for 35 cycles of 94°C for 1 min, 55°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 7 min, with a final hold at 10°C in a PCR thermal cycler (Master cycler gradient). The PCR amplified products (10 μL) were subjected to electrophoresis in 1.5% agarose gel in 1 × TBE buffer at 80 V for 30 min stained with a solution of ethidium bromide (EMD Millipore, Billerica, MA, United States). And examined under ultraviolet illumination (Cleaver Scientific Ltd., Rugby, United Kingdom). The genotypic information of *H. pylori* ATCC 26695 was used as reference.

### Genotypic Resistance: Library Preparation and Whole Genome Sequencing of *Helicobacter pylori* Strains

Library preparation was performed using the Qiagen® QIAseq FX DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Furthermore, next-generation sequencing (MiSeq next-generation sequencer; Illumina, San Diego, CA, United States) was used to analyze all strains for mutation status of 23S rRNA, PBP-1A, gyrA, 16S rRNA, oorD, and porD genotypes. The BLAST algorithm implemented in the CLC Genomics Workbench software (ver. 11; Qiagen NV, Venlo, Netherlands) was used for the analysis. The sequences of hp0425, hp0597, hp0701, hp0374, hp0954, oorD, and porD of the strain 26695 (GenBank accession number AE000511.1) were used as queries to obtain the 23S rRNA, PBP-1A, gyrA, 16S rRNA, rdxA, oorD, and porD sequences, respectively, from the next-generation sequencing data. Subsequently, each codon of the strains was compared to the reference sequence hp 26699 using our original PERL script and confirmed by visual inspection. Discrepancies found between the strains and reference sequence, which was included in the variants of **Supplementary Table 1**, was considered as variants related to antibiotic resistance.

## Susceptibility-Guided Intervention and Assessment of Adverse Effects

Treatment recommendations based on susceptibility testing were given on request in line with guideline from the fifth national consensus report on the treatment of *H. pylori* infection (Liu et al., 2018; **Table 2**). Six antibiotics are available for use in combined therapies in *H. pylori* eradication regimes: clarithromycin, amoxicillin, levofloxacin, metronidazole, tetracycline, and furazolidone. Patients were treated with quadruple therapy containing 30 mg of lansoprazole, 200 mg of pectin bismuth and 2 combined antibiotics for 14 days, according to the phenotypic or genotypic resistance determined using biopsy specimens or isolated strains. If the determined antibiotics for *H. pylori* sensitivity were less than 2 or not included in the regimens (**Table 2**), the patients would be treated empirically according to their previous medication history

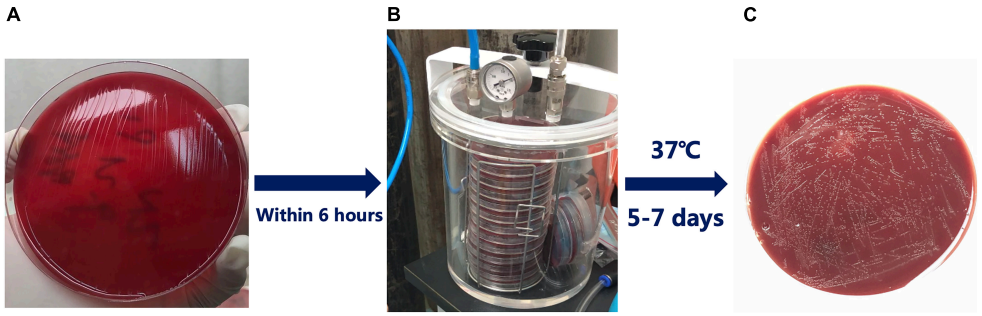


**FIGURE 1 |** Isolation of *Helicobacter pylori* (*H. pylori*) from clinical biopsy specimens. **(A)** The biopsy specimens were applied to chocolate agar plates containing 10% sheep blood; **(B)** Sealing device for culturing *H. pylori*; **(C)** The transparent color colonies of *H. pylori* after successful culture.

to avoid the reuse of unnecessary antibiotics. Patients were informed of the common adverse effects and were asked to record their symptoms during treatment.

**TABLE 1 |** Polymerase chain reaction primers used for detecting mutations of *H. pylori* strains in this study.

| Antibiotics | Genes | Primer sequence (5′–3′) |
|---|---|---|
| clarithromycin | 23S rRNA | F: ACAGCACTTTGCCAACTCGTAA<br>R: GCTTGTGCCATTACACTCAACTTG |
| amoxicillin | PBP-1A | F: GCCATTCTTATCGCTCAAGTTTGG<br>R: ATCGCTAAAATGTTACGCATGAAATACG |
| levofloxacin | gyrA | F: TGGGGATTGATTCTTCTATTGAAGA<br>R: TGCACTAAAGCGTCTATGATTTCA |
| Tetracycline | 16S rRNA | F: TCCGTAGAGATCAAGAGGAATACTCATTG<br>R: TCACCGCAACATGGCTGATTTG |
| furazolidone | oorD | F: GGCTTGCCTGGAAATCCTGTAG<br>R: AACCGATTTGCTCCACTTTCAATGA |
| | porD | F: GCAAGAAGTCATTGACGC<br>R: GGGGTGATAGGATAGGCT |
| metronidazole | rdxA | F: GCAGGAGCATCAGATAGT<br>R: GGGTGATTTCTTGGTTGC |

**TABLE 2 |** Recommended combined regimens of *H. pylori* in China.†

| Regimen‡ | Drug 1 | Drug 2 |
|---|---|---|
| 1 | AMX, 1000 mg, bid | CLR, 500 mg, bid |
| 2 | AMX, 1000 mg, bid | LVX, 500 mg, qd/200 mg, bid |
| 3 | AMX, 1000 mg, bid | FZD, 100 mg, bid |
| 4 | TET, 500 mg, tid/qid | MTZ,400 mg, tid/qid |
| 5 | TET, 500 mg, tid/qid | FZD, 100 mg, bid |
| 6 | AMX, 1000 mg, bid | MTZ,400 mg, tid/qid |
| 7 | AMX, 1000 mg, bid | TET, 500 mg, tid/qid |

*CLR, clarithromycin; AMX, amoxicillin; LVX, levofloxacin; MTZ, metronidazole; TET, tetracycline; FZD, furazolidone. Qd, once daily; bid, twice daily; tid, three-times daily; qid, four-times daily.*

†*Table adapted from the fifth national consensus report on the treatment of Helicobacter pylori infection, Chin J. Gastroenterol., 2017, Vol. 22, No. 6.*

‡*Standard dose (PPI + bismuth) (bid, orally half an hour before a meal) + 2 antibacterial drugs (orally after a meal). The standard dose of PPI is lansoprazole 30 mg; the standard dose of bismuth is 200 mg of pectin bismuth.*

## Statistical Analysis

The statistical analyses were performed using the SPSS 26.0 statistical software for Windows. Categorical data were compared using the Chi-square test or Fisher's exact test, as appropriate. Continuous data were compared with Student's *t*-test and expressed as the mean (SD). The kappa coefficient was used to assess the agreement between genotypic resistance and phenotypic resistance. Logistic regression analysis was performed to analyze factors affecting the eradication rates. The statistical significance level was set at 0.05. And odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

# RESULTS

## Baseline Characteristics of Patients

Thirty-six *H. pylori* strains were isolated from 39 patients, and eventually 35 patients were analyzed for *H. pylori* eradication as a result of 1 follow-up loss (**Figure 2**). The culture-based DST was successfully determined in all of them with broth microdilution testing. The genotypic resistance was successfully determined using PCR tests as well as WGS in the same thirty-six patients above. There were 17 male patients and 19 female patients. The average age of the patients was 47 years old, ranging from 27 to 62 years old. Of the 36 patients with adequate information regarding their medication history, 100% (36/36) received clarithromycin and amoxicillin in their previous eradication regimens, compared with 11.1% (4/36), 8.3% (3/36), 2.8% (1/36), and 16.7% (6/36) for levofloxacin, metronidazole, tetracycline, and furazolidone, respectively. The baseline characteristics of patients included in the study are summarized (**Table 3**). Among three groups, all of baseline characteristics are of no significance.

## Prevalence of Antibiotic Resistance

As all patients had undergone prior unsuccessful eradication treatments, the proportions of *H. pylori* antimicrobial resistant to clarithromycin, levofloxacin and metronidazole were markedly high and associated with the number of treatment failures (**Table 4**). However, none of the strains tested was resistant to furazolidone or tetracycline. And the prevalence of resistance

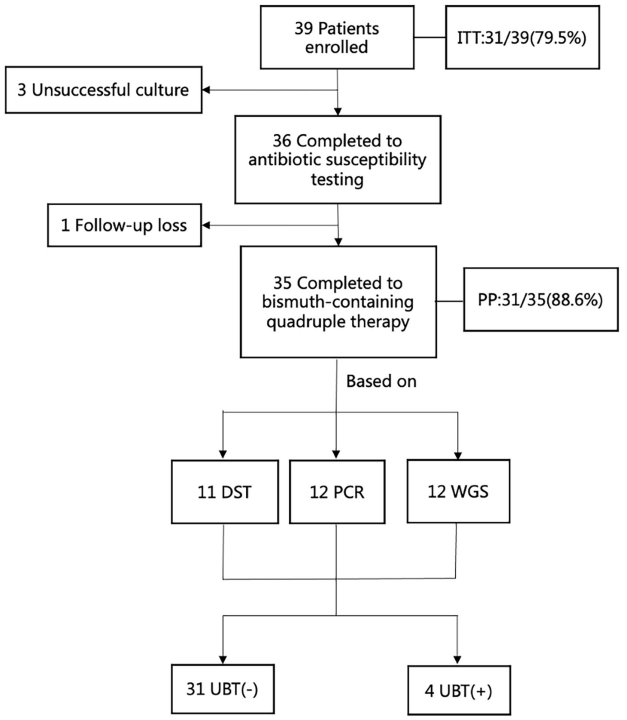


**FIGURE 2 |** The flow chart of enrollment in this study. ITT, intention -to-treat; PP, per-protocol; UBT, urea breath test; DST, drug susceptibility testing; PCR, polymerase chain reaction; WGS, whole genome sequencing.

**TABLE 3 |** Baseline characteristics of patients included in the study ($n$ = 36).

| Characteristic | Basis for treatment | | | *P* value* |
|---|---|---|---|---|
| | DST ($n$ = 12), $n$ (%) | PCR ($n$ = 12), $n$ (%) | WGS ($n$ = 12), $n$ (%) | |
| Age (years), mean (range) | 42 (27–62) | 43 (26–60) | 45 (29–63) | 0.137 |
| Gender, male | 5 (41.67) | 7 (58.33) | 5 (41.67) | 0.640 |
| Current smoker | 3 (25.00) | 5 (41.67) | 2 (16.67) | 0.381 |
| Endoscopic findings | | | | 0.449 |
| peptic ulcer | 2 (16.67) | 3 (25.00) | 0 (0.00) | |
| intestinal metaplasia | 4 (33.33) | 3 (25.00) | 4 (33.33) | |
| atrophic gastritis | 3 (25.00) | 4 (33.33) | 6 (50.00) | |
| others | 3 (25.00) | 2 (16.67) | 2 (16.67) | |
| Number of prior eradication treatments | | | | 0.074 |
| 2 | 10 (83.33) | 9 (75.00) | 5 (41.67) | |
| ≥3 | 2 (16.67) | 3 (25.00) | 7 (58.33) | |
| Prior treatment | | | | |
| clarithromycin | 12 (100.0) | 12 (100.0) | 12 (100.0) | NA |
| amoxicillin | 12 (100.0) | 12 (100.0) | 12 (100.0) | NA |
| levofloxacin | 1 (8.33) | 2 (16.67) | 1 (8.33) | 0.766 |
| metronidazole | 1 (8.33) | 0 (0.00) | 2 (16.67) | 0.336 |
| tetracycline | 1 (8.33) | 0 (0.00) | 0 (0.00) | 0.324 |
| furazolidone | 1 (8.33) | 4 (33.33) | 1 (8.33) | 0.183 |
| Virulence factors | | | | |
| cagA positive | 12 (100.0) | 11 (91.67) | 11 (91.67) | 0.432 |
| ureA/ureB positive | 12 (100.0) | 12 (100.0) | 12 (100.0) | NA |

*DST, drug susceptibility testing; PCR, polymerase chain reaction; WGS, whole genome sequencing; cagA, cytotoxin-associated gene A; vacA, gene encoding vacuolating cytotoxin A.*

**All P values were for testing the difference between DST, PCR, and WGS subgroups and were calculated by Student's t test or Fisher's exact test.*

to clarithromycin, levofloxacin, amoxicillin, and metronidazole was 91.7% (33/36), 50.0% (18/36), 25.8 (8/36), 41.7 (15/36), respectively. Markedly, more double resistant *H. pylori* isolates were observed in these patients (91.7%) (**Table 4**).

The mutant genes of *H. pylori* isolated from 36 patients with unsuccessful prior eradication treatments according to PCR or WGS were shown (**Table 5**). The proportions of mutant 23S rRNA, PBP-1A, gyrA, and rdxA using PCR were 88.9% (32/36), 22.2% (8/36), 47.2% (17/36), and 44.4% (16/36), respectively. While the proportions of mutant 23S rRNA, PBP-1A, gyrA, and rdxA using whole genome sequencing were 81.7% (33/36), 22.2% (8/36), 50.0% (18/36), and 50.0% (18/36), respectively. None of mutations were found either in 16S rRNA gene or oorD/porD gene (**Table 5**).

The agreement between culture-based DST results and identified point mutations by PCR or WGS was shown (**Table 6**), conferring resistance to CLR, LVX, AMX, and MTZ. The genotypic resistance determined using PCR in biopsy specimens correlated well with the phenotypic DST determined using strains for both clarithromycin and levofloxacin, amoxicillin, as well as metronidazole ($k > 0.80$) (**Table 6**). Overall, there was a comparatively high congruence of >90% between phenotypic DST results for clarithromycin, levofloxacin, amoxicillin, and metronidazole and SNPs identified by WGS in the 23S rRNA, gyrA, PBP-1A, and rdxA genes.

## Eradication Rates and Factors Affecting the Efficacy

In the present study, four antibiotic combination regimens were applied in the quadruple therapy, TET + FZD, TET + MTZ, AMX + FZD, and AMX + LVX, respectively. Moreover, AMX + FZD was the main regimen used in the quadruple therapy for patients in the DST and WGS groups, while AMX + LVX was the main regimen used in the quadruple therapy for patients in the PCR group (**Table 7**). The overall eradication rate was 79.5% (31/39, 95% CI 64.2–89.5%) in the ITT analysis and 88.6% (31/35, 95% CI 73.5–96.1%) in the PP analysis. Factors possibly related to eradication failure are summarized (**Table 8**). Furthermore, the four patients with failed eradication consisted of one from DST group, two from PCR group and one from WGS group with treatment regimens of TET + FZD, TET + MTZ, AMX + LVX, and TET + MTZ, respectively. In univariate analysis, there were statistically significant clinical factors associated with eradication failure, including male: female ratio ($p = 0.042$), number of prior eradication treatments

**TABLE 4 |** Antimicrobial resistance of *H. pylori* isolated from 36 patients with unsuccessful prior eradication treatments.

| Resistant to | Number of prior eradication treatments | | | | Overall (*n* = 36) | |
|---|---|---|---|---|---|---|
| | 2 (*n* = 24) | | ≥3 (*n* = 12) | | | |
| | *n* | % | *n* | % | *n* | % |
| CLR* | 21 | 87.5 | 12 | 100.0 | 33 | 91.7 |
| LVX** | 11 | 45.8 | 7 | 58.3 | 18 | 50.0 |
| MTZ*** | 9 | 37.5 | 6 | 50.0 | 15 | 41.7 |
| AMX** | 5 | 20.8 | 3 | 25.0 | 8 | 25.8 |
| CLR/LVX† | 10 | 41.7 | 6 | 50.0 | 16 | 44.4 |
| CLR/MTZ† | 7 | 29.2 | 4 | 33.3 | 11 | 30.6 |
| CLR/AMX† | 1 | 4.2 | 2 | 16.7 | 3 | 8.3 |
| AMX/MTZ† | 2 | 8.3 | 1 | 8.3 | 3 | 8.3 |
| CLR/LVX/AMX†† | 0 | 0.0 | 1 | 100.0 | 1 | 2.8 |
| CLR/LVX/MTZ/AMX††† | 0 | 0.0 | 1 | 100.0 | 1 | 2.8 |

*CLR, clarithromycin; AMX, amoxicillin; LVX, levofloxacin; MTZ, metronidazole.*
**Resistance included monoresistance, double resistance, triple resistance, and quadruple resistance.*
***Resistance included double resistance, triple resistance, and quadruple resistance.*
****Resistance included double resistance and quadruple resistance.*
†*Double resistance.*
††*Triple resistance.*
†††*Quadruple resistance.*

($p$ = 0.018) and AMX resistance ($p$ = 0.028). Besides, we also found no statistical difference in eradication failure rates related to age, cigarette smoking, alcohol intake, diabetes, hypertension, high salt diet, family history of GC, method for guiding therapy, endoscopic findings, CLR resistance, LVX resistance, MTZ resistance, CagA, and VacA ($p > 0.05$). It is note-worthy that the eradication rate based on DST, PCR and WGS was 90.9% (10/11), 83.3% (10/12), and 91.7% (11/12), respectively, which indicated that genotypic resistance-guided therapy might achieve satisfactory results as same as phenotypic resistance-guided therapy. Furthermore, logistic regression analysis revealed that female sex (OR, 12.50; 95% CI, 1.10–142.31; $p$ = 0.042), not less than 3 prior eradication treatments (OR, 20.25; 95% CI, 1.67–245.44; $p$ = 0.018) and AMX resistance (OR, 15.60; 95% CI, 1.34–182.09; $p$ = 0.028) were significantly associated with eradication failure (**Table 9**).

## The Side Effects of *Helicobacter pylori* Eradication

Of the 35 participants, 15 participants (42.9%) complained of adverse events after 14 days of quadruple therapy. The side effects were not significantly different between the success group and failure group (**Supplementary Table 2**). In addition, the combination of TET and FZD was significantly associated with side effects of *H. pylori* eradication ($p$ = 0.026), but the presence of TET and FZD alone was not associated with the development of adverse reactions (TET + MTZ, $p$ = 0.581; AMX + FZD, $p$ = 0.557) (**Supplementary Table 3**).

# DISCUSSION

The work presented above lead to several novel findings relevant to the optimization of refractory *H. pylori* eradication. This was the first study to compare the prevalence of refractory antibiotic resistance of six antibiotics in *H. pylori* by using phenotypic DST and PCR-based assays as well as WGS-based assays at the same time. We further showed that resistance-guided modified quadruple therapy was effective (>80%) in the treatment of refractory *H. pylori* infection. More importantly, the eradication rates appeared to be same in patients treated with genotypic resistance-guided therapy as compared with those treated with phenotypic resistance-guided therapy. Besides, we found that not less than 3 prior eradication treatments, the presence of amoxicillin resistance and female sex were associated with treatment failure in patients treated with resistance-guided quadruple therapy.

In the present study, we have demonstrated that the genotypic resistance determined using gastric biopsy specimens as well as WGS correlated well with the phenotypic resistance determined in *H. pylori* strains. We could show a clear correlation between the occurrence of mutations in the 23S rRNA, gyrA, PBP-1A, and rdxA genes of *H. pylori* and clarithromycin, fluoroquinolone,

**TABLE 5 |** Mutant genes of *H. pylori* isolated from 36 patients with unsuccessful prior eradication treatments.

| Antibiotic | Mutant genes | Genotypic resistance testing | |
|---|---|---|---|
| | | PCR, *n* (%) | WGS, *n* (%) |
| Clarithromycin | 23S rRNA | 32 (88.9) | 33 (91.7) |
| Amoxicillin | PBP-1A | 8 (22.2) | 8 (22.2) |
| Levofloxacin | gyrA | 17 (47.2) | 18 (50.0) |
| Metronidazole | rdxA | 16 (44.4) | 18 (50.0) |
| Tetracycline | 16S rRNA | 0 (NA) | 0 (NA) |
| Furazolidone | oorD/porD | 0 (NA) | 0 (NA) |

*PCR, polymerase chain reaction; WGS, whole genome sequencing.*

**TABLE 6 |** Correlations between phenotypic resistance by DST and genotypic resistance determined by PCR and WGS.

| Phenotypic | | | Genotypic | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 23S rRNA (PCR) | | *P** | *k* coefficient | 23S rRNA (WGS) | | *P** | *k* coefficient |
| **CLR MIC** | **W** | **M** | | | **W** | **M** | | |
| S | 3 | 0 | <0.001 | 0.842 | 3 | 0 | <0.001 | 1.000 |
| R | 1 | 32 | | | 0 | 33 | | |
| **LVX MIC** | **gyrA (PCR)** | | ***P**** | ***k* coefficient** | **gyrA (WGS)** | | ***P**** | ***k* coefficient** |
| | **W** | **M** | | | **W** | **M** | | |
| S | 17 | 1 | <0.001 | 0.833 | 18 | 0 | <0.001 | 0.944 |
| R | 2 | 16 | | | 1 | 17 | | |
| **AMX MIC** | **PBP-1A (PCR)** | | ***P**** | ***k* coefficient** | **PBP-1A (WGS)** | | ***P**** | ***k* coefficient** |
| | **S** | **R** | | | **W** | **M** | | |
| S | 27 | 1 | <0.001 | 0.839 | 28 | 0 | <0.001 | 0.916 |
| R | 1 | 7 | | | 1 | 7 | | |
| **MTZ MIC** | **rdxA (PCR)** | | ***P**** | ***k* coefficient** | **rdxA (WGS)** | | ***P**** | ***k* coefficient** |
| | **S** | **R** | | | **S** | **R** | | |
| S | 20 | 1 | <0.001 | 0.943 | 18 | 3 | <0.001 | 0.833 |
| R | 0 | 15 | | | 0 | 15 | | |

*DST, drug susceptibility testing; PCR, polymerase chain reaction; WGS, whole genome sequencing; MIC, minimum inhibitory concentration; CLR, clarithromycin; AMX, amoxicillin; LVX, levofloxacin; MTZ, metronidazole; S, susceptible; R, resistant; W, wild type; M, mutant.*
**All P values were calculated by Fisher's exact test.*

**TABLE 7 |** Antibiotic combination regimens in three groups of patients in the quadruple therapy.

| Regimen | Basis for treatment | | | Overall (*n* = 36), *n* (%) |
|---|---|---|---|---|
| | DST (*n* = 12), *n* (%) | PCR (*n* = 12), *n* (%) | WGS (*n* = 12), *n* (%) | |
| TET + FZD | 1 (8.3) | 2 (16.7) | 1 (8.3) | 4 (11.1) |
| TET + MTZ | 1 (8.3) | 1 (8.3) | 2 (16.7) | 4 (11.1) |
| AMX + FZD | 7 (58.3) | 3 (25.0) | 6 (50.0) | 16 (44.4) |
| AMX + LVX | 3 (25.0) | 6 (50.0) | 3 (25.0) | 12 (33.3) |

*AMX, amoxicillin; LVX, levofloxacin; MTZ, metronidazole; TET, tetracycline; FZD, furazolidone.*
*DST, drug susceptibility testing; PCR, polymerase chain reaction; WGS, whole genome sequencing.*

amoxicillin, and metronidazole resistance, respectively. In a prospective study, Konrad also found that clarithromycin and levofloxacin gene resistance is consistent with the phenotypic resistance of *H. pylori*, which was consistent with our results (Egli et al., 2020). Few molecular diagnostic tests were generally performed for detecting the tetracycline or furazolidone resistance on gastric tissue samples because this may be of a little significance for initial antibiotic selection at the beginning of therapy, owing to the well documented low resistance rate to tetracycline/furazolidone (Fiorini et al., 2018; Palmitessa et al., 2020). And in this study, interestingly, neither phenotypic resistance nor genotypic resistance appeared in tetracycline and furazolidone, which demonstrated that it might be reasonable to use tetracycline and furazolidone empirically even in patients who have failed multiple treatment.

During this study, we realized that the efficacies appeared to be similar in patients treated with therapies guided by three types of testing as *p* value was of no significance among them. The successful cultivation from gastric biopsy specimens and DST are the gold standards for the phenotypic sensitivity test of *H. pylori*, which provide reliable information for the development of personalized clinical programs (Gerrits et al., 2006). However, this method is challenging due to the pathogen's fastidious growth requirements for laboratory environment, taking up to at least 10 days to obtain results (Gerrits et al., 2006). Besides, culture-based methods could be hampered by a few technical factors, such as quality of the clinical specimen, occurrence of microbial commensal flora and inappropriate transport conditions (Cuadrado-Lavín et al., 2012). And most clinical laboratories, especially small and medium-sized hospitals

**TABLE 8 |** Clinical characteristics in eradication success and failure groups ($n$ = 35).

| Factor | Success group ($n$ = 31), $n$ (%) | Failure group ($n$ = 4), $n$ (%) | $P$ value* |
|---|---|---|---|
| Age, yr. | 42.6 ± 11.0 | 42.5 ± 9.7 | 0.993 |
| Male: Female ratio | 25:6 (80.6:19.4) | 1:3 (25.0:75.0) | 0.044 |
| Cigarette smoking | 9 (29.0) | 1 (25.0) | 0.681 |
| Alcohol intake | 19 (38.7) | 1 (25.0) | 0.522 |
| Diabetes | 3 (9.7) | 0 (0.0) | 0.687 |
| Hypertension | 8 (25.8) | 1 (25.0) | 0.732 |
| High salt diet | 19 (61.3) | 2 (50.0) | 0.530 |
| Family history of GC | 2 (6.5) | 0 (0.0) | 0.782 |
| Method for guiding therapy | | | 0.788 |
| DST | 10 (32.3) | 1 (25.0) | |
| PCR | 10 (32.3) | 2 (50.0) | |
| WGS | 11 (31.4) | 1 (25.0) | |
| Number of prior eradication treatments | | | 0.019 |
| 2 | 27 (87.1) | 1 (25.0) | |
| ≥3 | 4 (12.9) | 3 (75.0) | |
| Endoscopic findings | | | 0.478 |
| Non-atrophic | 11 (35.5) | 2 (50.0) | |
| Atrophic | 20 (64.5) | 2 (50.0) | |
| CLR resistance† | 29 (93.5) | 3 (75.0) | 0.313 |
| AMX resistance† | 5 (16.1) | 3 (75.0) | 0.030 |
| LVX resistance† | 10 (55.6) | 2 (50.0) | 0.632 |
| MTZ resistance† | 9 (60.0) | 3 (75.0) | 0.525 |
| CagA | | | 0.218 |
| Negative | 1 (3.2) | 1 (25.0) | |
| Positive | 30 (96.8) | 3 (75.0) | |
| VacA | | | 0.732 |
| m1 | 8 (25.8) | 1 (25.0) | |
| m2/m1 + m2 | 23 (74.2) | 3 (75.0) | |

*GC, gastric cancer; DST, drug susceptibility testing; PCR, Polymerase chain reaction; WGS, whole genome sequencing; CLR, clarithromycin; AMX, amoxicillin; LVX, levofloxacin; MTZ, metronidazole; cagA, cytotoxin-associated gene A; vacA, gene encoding vacuolating cytotoxin A.*
**All P values were for testing the difference between Success group and failure group and were calculated by Student's t test or Fisher's exact test.*
†*The resistance determined by antibiotic susceptibility testing.*

**TABLE 9 |** Logistic regression analysis for *Helicobacter pylori* eradication failure.

| Variable | Unadjusted OR | 95% CI | $P$ value* |
|---|---|---|---|
| Female sex | 12.50 | 1.10–142.31 | 0.042 |
| Not less than 3 prior eradication treatments | 20.25 | 1.67–245.44 | 0.018 |
| AMX resistance† | 15.60 | 1.34–182.09 | 0.028 |

*OR, odds ratio; CI, confidence interval; AMX, amoxicillin.*
**All P values were calculated by logistic regression analysis.*
†*The resistance determined by antibiotic susceptibility testing.*

in China, could not carry out this clinical project on a large scale, and most patients are not willing to wait for 2 weeks to be treated. Therefore, more rapid, and convenient molecular detection technology is ready. The determination of genotypic resistance using biopsy specimens is more expedient (culture not needed) and speedier, and it is easier to transfer the specimen (Cui et al., 2021) and has a higher success rate compared with traditional susceptibility tests (100.0 versus 92.3% in the present study).

Moreover, WGS delivers a more complete picture of resistance determinants present in a clinical isolate than PCR that can only examine a limited number of nucleotide positions (Binh et al., 2015). And the relevance of new polymorphisms can easily be assessed by later retrospective analysis of WGS data (Binh et al., 2014). However, WGS is obviously costlier than qPCRs. Anyway, genotypic resistance-guided quadruple therapies might be practical strategies in the treatment of refractory *H. pylori* infection in future clinical practice.

We demonstrated that female sex, not less than 3 prior eradication treatments and the presence of AMX resistance were associated with treatment failure in patients treated with resistance-guided quadruple therapy. However, the use of AMX under resistance guidelines remains acceptable in the third-line treatment of AMX-sensitive patients, as the rate of resistance to AMX remained relatively low in patients who have failed at least two eradication therapies. Besides, patients with AMX resistant can use a combination regimen containing TET with a lower resistance rate under resistance guidance. Recommended antimicrobial combinations for bismuth quadruple regimens include: (i) TET + MTZ; (ii) TET + FZD; (iii) TET + LVX. In addition, Vonoprazan, a new potassium competitive acid blocker, can be used to replace PPI. Vonoprazan is stable, highly efficacious, and less affected by CYP2C19 gene polymorphism, which is beneficial to improve *H. pylori* eradication rate (Akazawa et al., 2016). There are several reports that female gender can influence *H. pylori* eradication (Binh et al., 2014, 2015). One study suggested that there might be a difference in gastric physiology between males and females (Akazawa et al., 2016). Our study showed that the female gender is an unfavorable factor affecting eradication. However, the cause of gender differences in the eradication rate needs further research. Besides, data has shown that after just one unsuccessful therapy, resistance to clarithromycin rose to 60%, and further vain treatment attempts resulted in resistance as high as 80% (Wüppenhorst et al., 2014; Yahaghi et al., 2014; Thung et al., 2016). Although we found the efficacy of eradication was affected by these three factors, the results should be validated in further studies because of the wide CIs, which indicated small case numbers for these three variables.

However, this study had some limitations. First, it was a single-arm prospective research which did not include the relatively large sample size in third-line therapy. Second, the prevalence of CLR and AMX resistance is higher than average status because of nearly all of patients enrolled had prior treatments in Wuhan Union Hospital, where the empirically first-line regimen included antibiotic combination of CLR and AMX. Third, we should focus on predicating new point mutations of drug resistance in *H. pylori* based on the bacterium's genome using next generation sequencing (NGS) technology in the future.

In conclusion, the results from this study show that genotypic resistance-guided quadruple therapy can achieve comparably high eradication rates as compared with phenotypic

resistance-guided therapy in the treatment of refractory *H. pylori* infection. The detection of *H. pylori* resistance genes could be a good clinical application in the eradication of *H. pylori*.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Tongji Medical College, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

RL and ZG designed the project. ST and WW supplied samples. ZG performed all experiments, collected, and analyzed data. YZ and JL followed the participants. ZG, ST, and WW drafted and revised the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

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## REFERENCES

Akazawa, Y., Fukuda, D., and Fukuda, Y. (2016). Vonoprazan-based therapy for *Helicobacter pylori* eradication: experience and clinical evidence. *Therap Adv. Gastroenterol.* 9, 845–852.

Binh, T. T., Shiota, S., Suzuki, R., Matsuda, M., Trang, T. T., Kwon, D. H., et al. (2014). Discovery of novel mutations for clarithromycin resistance in *Helicobacter pylori* by using next-generation sequencing. *J. Antimicrob Chemother.* 69, 1796–1803. doi: 10.1093/jac/dku050

Binh, T. T., Suzuki, R., Trang, T. T., Kwon, D. H., and Yamaoka, Y. (2015). Search for novel candidate mutations for metronidazole resistance in *Helicobacter pylori* using next-generation sequencing. *Antimicrob Agents Chemother.* 59, 2343–2348. doi: 10.1128/AAC.04852-14

Cattoir, V., Nectoux, J., Lascols, C., Deforges, L., Delchier, J. C., Megraud, F., et al. (2007). Update on fluoroquinolone resistance in *Helicobacter pylori*: new mutations leading to resistance and first description of a gyrA polymorphism associated with hypersusceptibility. *Int. J. Antimicrob Agents* 29, 389–396. doi: 10.1016/j.ijantimicag.2006.11.007

Chen, T. S., Chang, F. Y., Chen, P. C., Huang, T. W., Ou, J. T., Tsai, M. H., et al. (2003). Simplified 13C-urea breath test with a new infrared spectrometer for diagnosis of *Helicobacter pylori* infection. *J. Gastroenterol. Hepatol.* 18, 1237–1243. doi: 10.1046/j.1440-1746.2003.03139.x

Chey, W. D., Leontiadis, G. I., Howden, C. W., and Moss, S. F. (2017). ACG clinical guideline: treatment of *Helicobacter pylori* infection. *Am. J. Gastroenterol.* 112, 212–239. doi: 10.1038/ajg.2016.563

Choi, I. J., Kook, M. C., Kim, Y. I., Cho, S. J., Lee, J. Y., Kim, C. G., et al. (2018). *Helicobacter pylori* therapy for the prevention of metachronous gastric Cancer. *N. Engl. J. Med.* 378, 1085–1095. doi: 10.1056/NEJMoa1708423

Chung, J. W., Kim, S. Y., Park, H. J., Chung, C. S., Lee, H. W., Lee, S. M., et al. (2017). *In vitro* activity of diphenyleneiodonium toward multidrug-resistant *Helicobacter pylori* strains. *Gut Liver* 11, 648–654.

Cuadrado-Lavín, A., Salcines-Caviedes, J. R., Carrascosa, M. F., Mellado, P., Monteagudo, I., Llorca, J., et al. (2012). Antimicrobial susceptibility of *Helicobacter pylori* to six antibiotics currently used in Spain. *J. Antimicrob Chemother.* 67, 170–173. doi: 10.1093/jac/dkr410

Cui, R., Song, Z., Suo, B., Tian, X., Xue, Y., Meng, L., et al. (2021). Correlation analysis among genotype resistance, phenotype resistance and eradication effect of *Helicobacter pylori*. *Infect Drug Resist.* 14, 1747–1756. doi: 10.2147/IDR.S305996

Doorakkers, E., Lagergren, J., Engstrand, L., and Brusselaers, N. (2018). *Helicobacter pylori* eradication treatment and the risk of gastric adenocarcinoma in a Western population. *Gut* 67, 2092–2096.

Egli, K., Wagner, K., Keller, P. M., Risch, L., Risch, M., and Bodmer, T. (2020). Comparison of the diagnostic performance of qPCR, sanger sequencing, and whole-genome sequencing in determining clarithromycin and levofloxacin resistance in *Helicobacter pylori*. *Front. Cell Infect Microbiol.* 10:596371. doi: 10.3389/fcimb.2020.596371

Fallone, C. A., Chiba, N., van Zanten, S. V., Fischbach, L., Gisbert, J. P., Hunt, R. H., et al. (2016). The toronto consensus for the treatment of *Helicobacter pylori* infection in adults. *Gastroenterology* 151, 51–69.e14.

Fiorini, G., Zullo, A., Saracino, I. M., Pavoni, M., and Vaira, D. (2018). Antibiotic resistance pattern of *Helicobacter pylori* strains isolated in Italy during 2010-2016. *Scand. J. Gastroenterol.* 53, 661–664.

Ford, A. C., Forman, D., Hunt, R. H., Yuan, Y., and Moayyedi, P. (2014). *Helicobacter pylori* eradication therapy to prevent gastric cancer in healthy asymptomatic infected individuals: systematic review and meta-analysis of randomised controlled trials. *BMJ* 348:g3174. doi: 10.1136/bmj.g3174

Gerrits, M. M., van Vliet, A. H., Kuipers, E. J., and Kusters, J. G. (2006). *Helicobacter pylori* and antimicrobial resistance: molecular mechanisms and clinical implications. *Lancet Infect Dis.* 6, 699–709. doi: 10.1016/S1473-3099(06)70627-2

Giorgio, F., Ierardi, E., Sorrentino, C., Principi, M., Barone, M., Losurdo, G., et al. (2016). *Helicobacter pylori* DNA isolation in the stool: an essential pre-requisite for bacterial noninvasive molecular analysis. *Scand. J. Gastroenterol.* 51, 1429–1432. doi: 10.1080/00365521.2016.1216592

Kjøller, M., Fischer, A., and Justesen, T. (1991). Transport conditions and number of biopsies necessary for culture of *Helicobacter pylori*. *Eur. J. Clin. Microbiol. Infect. Dis.* 10, 166–167. doi: 10.1007/BF01964450

Liou, J. M., Lin, J. T., Chang, C. Y., Chen, M. J., Cheng, T. Y., Lee, Y. C., et al. (2010). Levofloxacin-based and clarithromycin-based triple therapies as first-line and second-line treatments for *Helicobacter pylori* infection: a randomised comparative trial with crossover design. *Gut* 59, 572–578. doi: 10.1136/gut.2009.198309

Liu, W. Z., Xie, Y., Lu, H., Cheng, H., Zeng, Z. R., Zhou, L. Y., et al. (2018). Fifth chinese national consensus report on the management of *Helicobacter pylori* infection. *Helicobacter* 23:e12475. doi: 10.1111/hel.12475

López-Góngora, S., Puig, I., Calvet, X., Villoria, A., Baylina, M., Muñoz, N., et al. (2015). Systematic review and meta-analysis: susceptibility-guided versus empirical antibiotic treatment for *Helicobacter pylori* infection. *J. Antimicrob Chemother.* 70, 2447–2455. doi: 10.1093/jac/dkv155

Malfertheiner, P., Megraud, F., O'Morain, C. A., Atherton, J., Axon, A. T., Bazzoli, F., et al. (2012). Management of *Helicobacter pylori* infection–the Maastricht IV/ florence consensus report. *Gut* 61, 646–664. doi: 10.1136/gutjnl-2012-302084

Malfertheiner, P., Megraud, F., O'Morain, C. A., Gisbert, J. P., Kuipers, E. J., Axon, A. T., et al. (2017). Management of *Helicobacter pylori* infection-the maastricht V/Florence consensus report. *Gut* 66, 6–30. doi: 10.1136/gutjnl-2016-312288

McColl, K. E. (2010). Clinical practice. *Helicobacter pylori* infection. *N. Engl. J. Med.* 362, 1597–1604.

Mégraud, F., and Lehours, P. (2007). *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin. Microbiol. Rev.* 20, 280–322.

Midolo, P. D., Bell, J. M., Lambert, J. R., Turnidge, J. D., and Grayson, M. L. (1997). Antimicrobial resistance testing of *Helicobacter pylori*: a comparison of Etest and disk diffusion methods. *Pathology* 29, 411–414. doi: 10.1080/00313029700169415

Miura, S., and Hokari, R. (2012). Seeking an optimal eradication therapy for *Helicobacter pylori* infection. *J. Gastroenterol. Hepatol.* 27, 7–9. doi: 10.1111/j.1440-1746.2011.06953.x

Nezami, B. G., Jani, M., Alouani, D., Rhoads, D. D., and Sadri, N. (2019). *Helicobacter pylori* mutations detected by next-generation sequencing in formalin-fixed, paraffin-embedded gastric biopsy specimens are associated with treatment failure. *J. Clin. Microbiol.* 57:e01834-18. doi: 10.1128/JCM.01834-18

O'Connor, A., O'Morain, C. A., and Ford, A. C. (2017). Population screening and treatment of *Helicobacter pylori* infection. *Nat. Rev. Gastroenterol. Hepatol.* 14, 230–240.

Palmitessa, V., Monno, R., Panarese, A., Cuppone, R., Burattini, O., Marangi, S., et al. (2020). Evaluation of antibiotic resistance of *Helicobacter pylori* strains isolated in bari, southern italy, in 2017-2018 by phenotypic and genotyping methods. *Microb Drug Resist.* 26, 909–917. doi: 10.1089/mdr.2019.0262

Thung, I., Aramin, H., Vavinskaya, V., Gupta, S., Park, J. Y., Crowe, S. E., et al. (2016). Review article: the global emergence of *Helicobacter pylori* antibiotic resistance. *Aliment Pharmacol. Ther.* 43, 514–533. doi: 10.1111/apt.13497

Wüppenhorst, N., Draeger, S., Stüger, H. P., Hobmaier, B., Vorreiter, J., Kist, M., et al. (2014). Prospective multicentre study on antimicrobial resistance of *Helicobacter pylori* in Germany. *J. Antimicrob Chemother.* 69, 3127–3133. doi: 10.1093/jac/dku243

Yahaghi, E., Khamesipour, F., Mashayekhi, F., Safarpoor Dehkordi, F., Sakhaei, M. H., Masoudimanesh, M., et al. (2014). *Helicobacter pylori* in vegetables and salads: genotyping and antimicrobial resistance properties. *Biomed. Res. Int.* 2014:757941. doi: 10.1155/2014/757941

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# The Influence of Past Metronidazole Exposure on the Outcome of *Helicobacter pylori* Eradication

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**Background:** Bismuth quadruple therapy (BQT) is recommended as empirical first-line therapy because it is not affected by antibiotic resistance. We examined whether past exposure to metronidazole affected BQT outcomes.

**Methods:** The records of seven hospitals were searched for patients who received BQT for *Helicobacter pylori* eradication between 2009 and 2020. The association between past metronidazole exposure and the eradication rate was evaluated.

**Results:** This study was a multicenter retrospective study. Around 37,602 people tested for *H. pylori* infection were identified, and 7,233 received BQT. About 2,802 (38.7%) underwent a 13C-urea breath test to confirm eradication. The BQT efficacy was 86.4% among patients without metronidazole exposure and 72.8% among patients with exposure ($p < 0.001$). The eradication rate of BQT 14 days in patients with past exposure was higher than that of BQT <14 days (85.5 vs. 66.0%, $p = 0.009$). Multivariate analysis revealed that past metronidazole exposure [odds ratio (OR) 2.6, 95% CI 1.8–3.7; $p < 0.001$] and BQT <14 days (OR 1.5, 95% CI 1.2–2.0; $p = 0.002$) were independent risk factors for eradication failure.

**Conclusion:** Past metronidazole exposure significantly lowered the BQT eradication rate. BQT 14 days should be recommended for patients with suspected metronidazole exposure.

**Keywords: anti-bacterial agents, bismuth quadruple therapy, microbial drug resistance, duration of therapy, eradication rate, *Helicobacter* infections, metronidazole**

## INTRODUCTION

*Helicobacter pylori* is the primary etiologic cause of gastric adenocarcinoma (Lee et al., 2016; Hooi et al., 2017; Chiang et al., 2021). A recent systematic review reported that eradication resulted in a 46% reduced incidence of and 39% reduced mortality from gastric cancer (de Martel et al., 2020; Ford et al., 2020). The Kyoto global consensus and Houston consensus conferences recommend treating all individuals with proven *H. pylori* infection (Sugano et al., 2015; El-Serag et al., 2018). Traditionally, eradication for *H. pylori* was based on a standard triple regimen consisting of a proton pump inhibitor (PPI), amoxicillin, and clarithromycin. However, this regimen is no longer recommended without susceptibility testing due to the high rates of clarithromycin resistance in many countries (Malfertheiner et al., 2011; Yeo et al., 2018).

Many current guidelines recommend bismuth quadruple therapy (BQT) as first-line therapy in areas with high clarithromycin resistance because BQT is less affected by antibiotic resistance (Fallone et al., 2016; Chey et al., 2017; Malfertheiner et al., 2017; Chen et al., 2019; Jung et al., 2020; Shah et al., 2021). However, BQT is difficult to use due to its frequent dosing, side effects, and availability (Howden and Graham, 2021). According to the recent European Registry, BQT users reported higher rates of side effects than those on other regimens (Nyssen et al., 2021a). BQT is generally recommended for 14 days rather than 7 or 10 days (Fallone et al., 2019; Kim et al., 2020; Shah et al., 2021). This is because prolonging the duration of metronidazole use to 14 days is expected to overcome resistance. However, few studies have confirmed this in clinical trials.

Several studies reported that clarithromycin-based triple therapy was affected by past macrolides exposure (Boltin et al., 2019; Kwon et al., 2019). There have been studies examining the relationship between past metronidazole exposure and BQT eradication rates. However, no meaningful additional analysis was performed other than the eradication rate due to the small number of patients (McNulty et al., 2012; Boltin et al., 2020; Lee et al., 2020). Our purpose was to investigate whether past metronidazole exposure influenced the eradication rates of the BQT regimen. We also examined the eradication rates according to the BQT duration in patients with or without metronidazole exposure.

## MATERIALS AND METHODS

### Study Design and Data Extraction

This was a multicenter retrospective study of patients from seven university hospitals from 2009 to 2020. We identified patients aged 20–79 years who underwent an endoscopy and were diagnosed with *H. pylori* by histology or a rapid urease test. Patients who were prescribed BQT for eradication were included. Age, sex, body mass index, smoking history, endoscopic findings, and pathologic results were identified. We also investigated the BQT duration and the type of PPI used for the BQT regimen. Medical records were reviewed to identify patients who had been prescribed metronidazole before their BQT prescription.

This study protocol was approved by the Institutional Review Board (IRB) of Catholic Medical Center (IRB approval number, XC20WIDI0119). The requirement for written informed consent was waived because anonymous data were used. This study followed the ethical principles of the Declaration of Helsinki.

### Past Metronidazole Exposure

Patients' medical records were examined to determine whether they had received metronidazole before the prescription of BQT. All inpatient or outpatient antibiotic prescription records were searched, and metronidazole prescription records were extracted. The patient's diagnosis, drug administration method, dosage, the number of doses per day, and total administration duration were examined together with all metronidazole prescription records. However, only the intravenous or oral administration cases were extracted, and topical preparations were excluded. We also investigated past metronidazole exposure intervals up to the BQT therapy regimen.

### Confirmation of *Helicobacter pylori* Infection and Eradication Regimen

*Helicobacter pylori* infection was confirmed by rapid urease test, histopathology, or urea breath test. Dual priming oligonucleotide (DPO)-based multiplex PCR (Seeplex® ClaR-*H. pylori* ACE detection kit, Seegene Inc., Seoul, Korea) was performed in some patients confirmed with *H. pylori* infection to examine the presence of clarithromycin resistance. The precise methods of DPO-PCR have been described elsewhere (Posteraro et al., 2006; Woo et al., 2009; Lehours et al., 2011). BQT was prescribed as second-line therapy after the failure of the first-line triple therapy or first-line therapy in patients confirmed with clarithromycin resistance by DPO-PCR methods.

Bismuth quadruple therapy consisted of a PPI (standard dose) twice daily, bismuthate tripotassium dicitrate (300 mg) and tetracycline (500 mg) four times daily, and metronidazole (500 mg) three times daily. The PPIs used were lansoprazole (30 mg/T), pantoprazole (40 mg/T), rabeprazole (20 mg/T), esomeprazole (40 mg/C), and ilaprazole (10 mg/T). The BQT duration was 7, 10, or 14 days at the physician's discretion. All patients underwent a urea breath test after 4–12 weeks to confirm the success of eradication. For the urea breath test, 13C-urea 100 mg tablets (UBiTkit™, Otsuka Electronics, Co., Ltd., Osaka, Japan) were orally taken with water after fasting for at least 4 h. Exhaled breath samples obtained after that were analyzed using the 13C-urea breath test (UBiT-IR300®), and the cut-off value for judging *H. pylori*-positive and negative was set to 2.5‰ (Graham et al., 1987; Graham and Klein, 1991; Atherton and Spiller, 1994).

**Abbreviations:** BQT, Bismuth quadruple therapy; DPO, Dual-priming oligonucleotide; *H. pylori*, *Helicobacter pylori*; PCR, Polymerase chain reaction; PPI, Proton pump inhibitor.

## Endpoints and Statistical Analysis

The primary endpoint was to compare the BQT eradication rates according to patients' past metronidazole exposure. The secondary endpoint was to identify risk factors associated with eradication failure. We examined whether past metronidazole exposure influenced the BQT eradication rate and whether prolonging the BQT duration was associated with treatment outcomes. We also examined whether the duration of past metronidazole exposure affected the BQT outcome and whether the interval between the past exposure and the prescription of BQT affected *H. pylori* eradication rates.

The baseline patient characteristics are summarized using descriptive statistics. Continuous data are presented as the mean (SD) or median (interquartile range), and categorical data are given quantities and proportions. The student's *t*-test was used to compare continuous variables. Categorical variables were compared using the $\chi^2$ test or Fisher's exact test. The duration of the past treatment was analyzed by linear-by-linear association because the independent variables were classified to be three or more. Logistic regression analysis was used to identify independent risk factors for eradication failure, and multiple regression analysis was used to analyze the correlation between these factors and eradication failure (i.e., factors that showed significant differences in univariate analysis).

The SPSS statistical program, version 22 (SPSS, Chicago, Illinois), was used for all analyses, and the significance level was set at a value of $p < 0.05$.

# RESULTS

## Baseline Characteristics

Around 37,602 patients were tested for *H. pylori* infection in the seven participating hospitals, and 7,238 of them received metronidazole containing eradication therapy. In total, 7,233 patients were prescribed BQT. Of the 7,233 patients prescribed BQT, 2,802 were followed up after eradication and included in this study (**Figure 1**). The mean age was 57.8 ± 11.4 years, and 53.2% of patients were male. About 158 (5.6%) patients had previously received metronidazole intravenously or orally. The baseline characteristics of the groups with and without past metronidazole exposure are shown in **Table 1**.

Bismuth quadruple therapy regimen was prescribed as second-line therapy in 2,561 (91.4%) patients and as first-line therapy in 241 (8.6%) patients. BQT was prescribed more frequently as a first-line regimen in patients without metronidazole exposure ($p < 0.001$). The BQT duration was 7 days in 64.1%, 10 days in 8.3%, and 14 days in 27.6%. There was no significant difference in the duration of BQT in patients with or without metronidazole exposure ($p = 0.115$).

## Past Metronidazole Exposure and *Helicobacter pylori* Eradication Rate

The BQT eradication rates for *H. pylori* were 86.4% in patients without metronidazole exposure and 72.8% in patients with exposure ($p < 0.001$; **Table 2**). There was no significant difference in the eradication rates of BQT <14 days and 14 days in

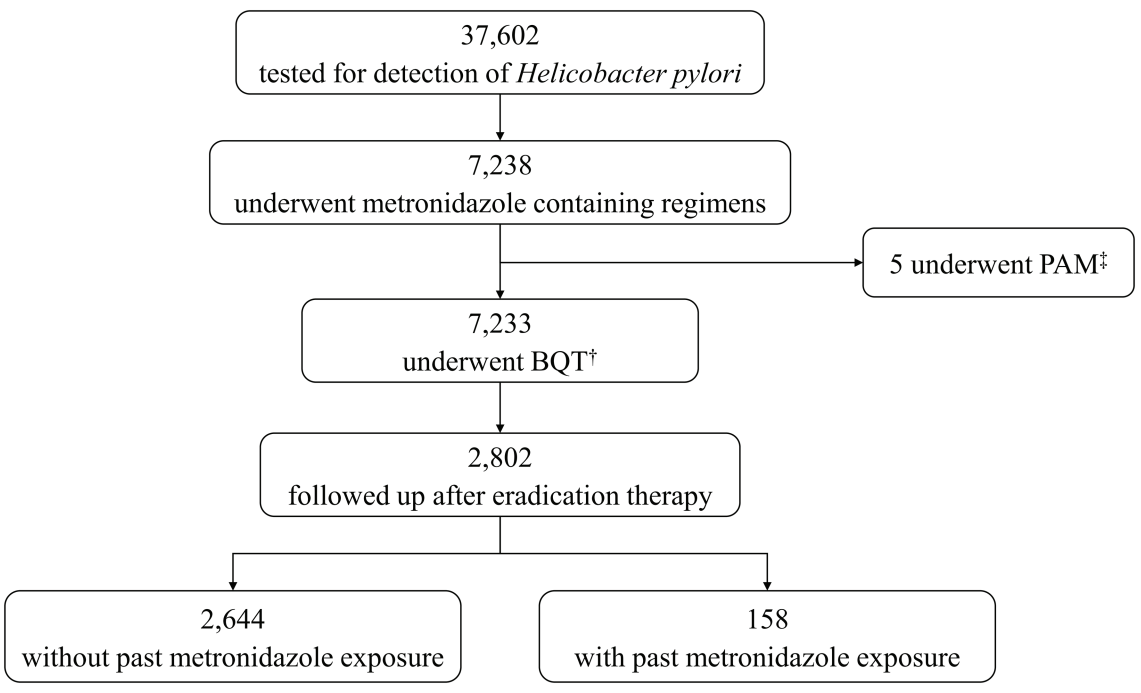


**FIGURE 1 |** Flow chart. †BQT, bismuth quadruple therapy, ‡PAM, proton pump inhibitor, amoxicillin, and metronidazole.

**TABLE 1** | Baseline characteristics.

| | Total (*N* = 2,802) | Without metronidazole exposure (*N* = 2,644) | With metronidazole exposure (*N* = 158) | *p*-value |
|---|---|---|---|---|
| Age, years | 57.8 ± 11.4 | 57.9 ± 11.4 | 56.3 ± 11.0 | 0.085 |
| Male sex | 1,492/2,802 (53.2) | 1,415/2,644 (53.5) | 77/158 (48.7) | 0.242 |
| BMI | 24.4 ± 3.4 | 24.4 ± 3.4 | 24.0 ± 3.2 | 0.210 |
| Current smoker | 325/1,787 (11.6) | 307/1,669 (18.4) | 18/118 (15.3) | 0.498 |
| Order of BQT | | | | |
| First-line† | 241/2,802 (8.6) | 239/2,644 (9.0) | 2/158 (1.3) | <0.001 |
| Second-line | 2,561/2,802 (91.4) | 2,405/2,644 (91.0) | 156/158 (98.7) | |
| Type of PPI | | | | |
| Lansoprazole | 963/2,802 (34.4) | 900/2,644 (34.0) | 63/158 (39.9) | 0.657 |
| Pantoprazole | 844/2,802 (30.1) | 818/2,644 (30.9) | 26/158 (16.5) | |
| Rabeprazole | 801/2,802 (28.6) | 736/2,644 (27.8) | 65/158 (41.1) | |
| Esomeprazole | 128/2,802 (4.6) | 125/2,644 (4.7) | 3/158 (1.9) | |
| Ilaprazole | 66/2,802 (2.4) | 65/2,644 (2.5) | 1/158 (0.6) | |
| BQT duration | | | | |
| 7 days | 1,796/2,802 (64.1) | 1,705/2,644 (64.5) | 91/158 (57.6) | 0.115 |
| 10 days | 233/2,802 (8.3) | 221/2,644 (8.4) | 12/158 (7.6) | |
| 14 days | 773/2,802 (27.6) | 718/2,644 (27.2) | 55/158 (34.8) | |

*Data represent the number of patients (%) or mean ± SD. †Clarithromycin resistance. BMI, body mass index; BQT, bismuth quadruple therapy; and PPI, proton pump inhibitor.*

**TABLE 2** | Eradication rates of bismuth quadruple therapy for *Helicobacter pylori* with and without past metronidazole exposure.

| | Total | Without metronidazole exposure (*N* = 2,644) | With metronidazole exposure (*N* = 158) | *p*-value |
|---|---|---|---|---|
| Eradication rates | 2,399/2,802 (85.6) | 2,284/2,644 (86.4) | 115/158 (72.8) | <0.001 |
| BQT <14 days | 1,711/2,029 (84.3) | 1,643/1,926 (85.3) | 68/103 (66.0) | <0.001 |
| BQT 14 days | 688/773 (89.0) | 641/718 (89.3) | 47/55 (85.5) | 0.383 |

*Data represent the number of patients (%). BQT, bismuth quadruple therapy.*

patients without metronidazole exposure (87.5 vs. 89.1%, $p = 0.289$). The eradication rates of BQT 14 days were higher than BQT <14 days in patients with exposure (85.5 vs. 67.0%, $p = 0.014$).

## Eradication Rates According to the Duration of Past Metronidazole Exposure and Interval to BQT

We examined whether the duration of past metronidazole exposure and interval to BQT affected eradication. The eradication rates for less than 5 days, 5–9 days, and more than 10 days of past exposure were 81.8, 72.0, and 61.1% (**Table 3**). **Figure 2A** showed a tendency for the eradication rate to increase as the metronidazole exposure duration in the past increases ($p < 0.001$ by linear-by-linear association).

**TABLE 3** | Past metronidazole exposure duration in people receiving bismuth quadruple therapy.

| | Total number of patients | Number of patients successfully eradicated | Ratio |
|---|---|---|---|
| Metronidazole exposure duration, days | | | |
| Median (range) | 5 (1–19) | | |
| 0 | 2,644 | 2,284 | 86.4% |
| 1–4 | 33 | 27 | 81.8% |
| 5–9 | 108 | 77 | 71.3% |
| ≥10 | 17 | 11 | 64.7% |

*Value of $p < 0.001$ analyzed according to linear-by-linear association according to exposure duration.*

We also examined if the interval from past exposure to BQT prescription affected its outcomes. The intervals between past metronidazole exposure were 73, 70, and 75% for patients less than 12 months, 12–36 months, and ≥36 months, respectively. There was no significant difference between the exposure interval and the current BQT eradication rate (**Figure 2B**).

## Univariate and Multivariate Analysis of Factors Related to *Helicobacter pylori* Eradication Failure

We performed a logistic regression analysis to identify *H. pylori* eradication failure (**Table 4**). Univariate analysis revealed BQT prescribed as first-line, past metronidazole exposure and BQT <14 days to be significantly associated with eradication failure. Multivariate analysis confirmed that BQT prescribed as first-line (odds ratio 1.79; 95% CI, 1.28–2.50; $p = 0.001$), past metronidazole exposure (odds ratio 2.57; 95% CI, 1.77–3.73; $p < 0.001$), and BQT <14 days (odds ratio 1.51; 95% CI, 1.16–1.96; $p = 0.002$) were independent risk factors for *H. pylori* eradication failure.

# DISCUSSION

Current guidelines recommend BQT as empirical therapy because it has proven to be effective in areas with high antibiotic resistance (Fallone et al., 2016; Chey et al., 2017; Malfertheiner et al., 2017; Chen et al., 2019; Shah et al., 2021). Metronidazole resistance is high in many countries, and a recent study reported global resistance rates to be 40–70% (Savoldi et al., 2018; Schubert et al., 2020; Megraud et al., 2021). BQT 14 days is generally recommended in areas with high metronidazole resistance as extending the duration to 14 days is expected to overcome resistance. In our clinical practice, patients often complain of difficulty taking BQT for 14 days due to the complexity of the regimen and adverse events. According to a European Registry survey from 2013 to 2018, patients prescribed BQT had the highest risk of adverse events compared with other eradication regimens (Nyssen et al., 2021b). A systematic review reported that adverse events increased as the BQT

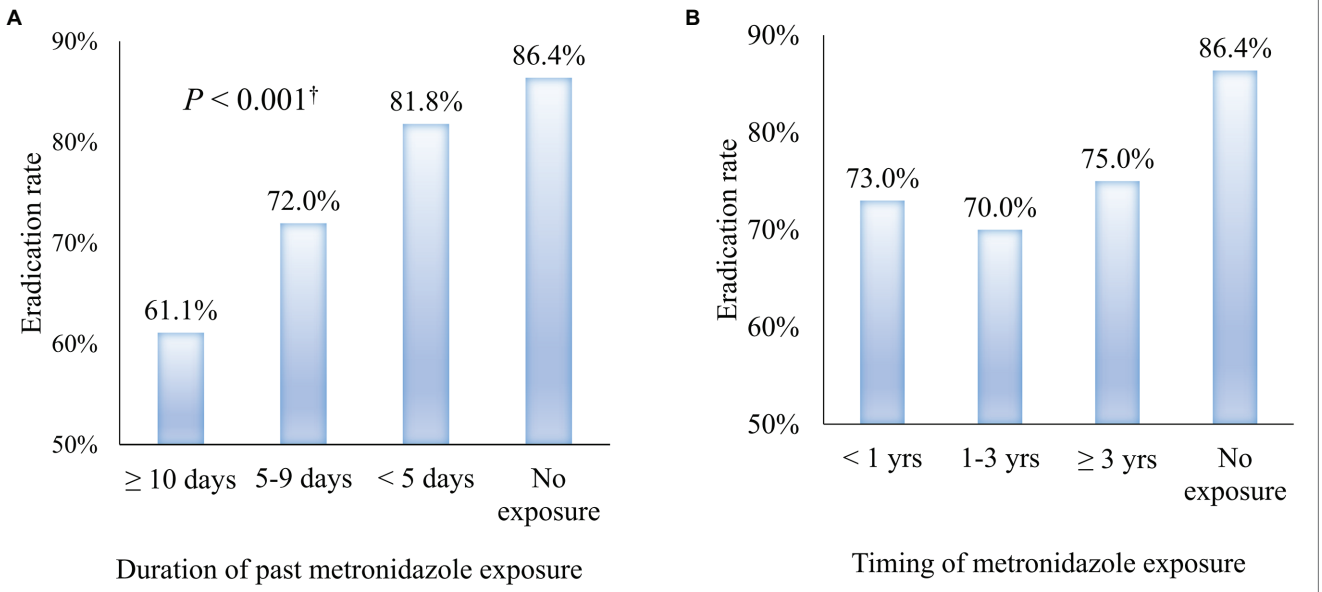


**FIGURE 2 |** The trend of *Helicobacter pylori* eradication rate according to past metronidazole exposure. **(A)** Eradication rate of *H. pylori* by the duration of previous metronidazole exposure **(B)** Eradication rate of *H. pylori* by the interval from past metronidazole exposure to the prescription of bismuth quadruple therapy.

**TABLE 4 |** Univariate and multivariate analysis of factors related to *Helicobacter pylori* eradication failure.

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | **Odds ratio (95% CI)** | ***p*-value** | **Odds ratio (95% CI)** | ***p*-value** |
| Age ≥ 60 years | 0.88 (0.71–1.09) | 0.248 | | |
| Male | 0.99 (0.80–1.23) | 0.949 | | |
| BMI ≥ 25 kg/m$^2$ | 1.04 (0.79–1.37) | 0.790 | | |
| Current smoker | 1.01 (0.87–1.17) | 0.942 | | |
| Past history of gastric cancer | 1.14 (0.39–3.32) | 0.817 | | |
| Prescribed BQT for the 1st line | 1.88 (1.36–2.59) | <0.001 | 1.79 (1.28–2.50) | 0.001 |
| Past metronidazole exposure | 2.37 (1.64–3.43) | <0.001 | 2.57 (1.77–3.73) | <0.001 |
| BQT <14 days | 1.50 (1.17–1.94) | 0.002 | 1.51 (1.16–1.96) | 0.002 |

*BMI, body mass index; BQT, bismuth quadruple therapy; and CI, confidence interval.*

duration prolonged from 7 to 14 days (Yuan et al., 2013). These studies suggest that the dosage frequency and adverse events of BQT can affect patient compliance. There is also little evidence that increasing BQT duration to 14 days is more effective than 7 or 10 days in patients with metronidazole resistance.

Metronidazole resistance is not easy to demonstrate. Culture tests can confirm antibiotic resistance. However, *H. pylori* are intricate to incubate, and the long time deters its use in clinical practice. The E-test, widely used to measure antibiotic resistance, is known to overestimate metronidazole resistance (Osato et al., 2001). An *in vitro* test for metronidazole resistance is not an absolute preclusion criterion for use because *in vitro* resistance does not reliably correlate with *H. pylori* eradication failure. A recent study reported no association between metronidazole resistance determined by molecular-based experiments and eradication success (Argueta et al., 2021). Phenotypic methods for metronidazole resistance testing are not well standardized, and results can vary depending on the method used, which can result in lower predictive value.

In our study, we found that past metronidazole exposure lowered the BQT eradication rate. Prolonging the BQT duration to 14 days significantly increased eradication rates in patients with past exposure. The eradication rates of BQT <14 days in patients without exposure were similar to BQT 14 days. This suggest that BQT regimens of shorter duration may be effective in patients without metronidazole exposure history or metronidazole resistance. For patients suspected with a past metronidazole exposure, BQT should be prescribed for 14 days or levofloxacin-based regimens may be considered in areas with low quinolone resistance.

A previous study investigated the relationship between the dose of past macrolides exposure and the eradication rate of standard triple therapy (Boltin et al., 2019). They reported that increased macrolides exposure was associated with lower eradication rates. To our knowledge, no study examined if the duration of past metronidazole exposure and the interval from exposure to eradication affected the eradication rate. In our study, the eradication rate decreased as the duration of past metronidazole exposure increased. However, the interval from metronidazole exposure to BQT prescription was not related to the eradication rate. Based on our results, BQT 14 days may be recommended for patients with suspected past metronidazole exposure, regardless of the interval from exposure. BQT 7 days can achieve high eradication rates with fewer side

effects in areas with low metronidazole resistance or in patients without metronidazole exposure.

A three-in-one formulation (Pylera®) of three drugs, bismuth subcitrate, metronidazole, and tetracycline, is frequently prescribed in the United States and Europe. This formulation is preferred to BQT because of its small number of capsules but consists of 10 days. Few studies have compared the BQT eradication rates for 10 and 14 days (Dore et al., 2011). According to our study, 10-day BQT may not be sufficient in patients with past metronidazole exposure, suggesting that 10-day regimens should be avoided in areas with high metronidazole resistance.

Our study has several limitations. First, our study is a retrospective study which inevitably includes inherent limitations due to its study design. Most importantly, the baseline characteristics of patients with and without exposure were different. Specifically, BQT was prescribed as first-line regimens more often in patients without past metronidazole exposure. Second, patients classified as unexposed in this study might have been prescribed metronidazole at other hospitals. However, it is essential to note that metronidazole is not commonly prescribed in primary clinics in Korea (Park et al., 2017; Health Insurance Review and Assessment Service, 2021). Also, reclassification of these patients into the exposed group is expected to strengthen our findings further. Third, the follow-up loss rate was high in patients who were prescribed BQT. However, our study was retrospective, and the high follow-up loss rate reflects the reality of actual clinics. Another study reported that only 34.9% of those prescribed *H. pylori* eradication regimens visited the hospital to check their eradication results (Kumar et al., 2021). Finally, this study was conducted in a region with high metronidazole resistance. Our results may not be applicable in areas with different metronidazole.

In conclusion, past metronidazole exposure lowered the *H. pylori* eradication rate of BQT regimens, and longer exposure duration was associated with lower eradication rates. Prolonging the BQT duration to 14 days should be considered in patients with past metronidazole exposure.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of Catholic Medical Center (IRB approval number, XC20WIDI0119). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

YC designed the study, reviewed the literature, acquired and analyzed data, administrated the project, performed statistical analyses, wrote the original draft, and reviewed and edited the manuscript. JK supervised the study and was responsible for paper conception and manuscript review, and editing. JP acquired data and reviewed and edited the manuscript. HC, DK, JO, TK, and DC acquired data and edited the manuscript. WC, B-WK, and SK analyzed data and edited the manuscript. All authors contributed to the article and approved the submitted version.

## REFERENCES


Argueta, E. A., Alsamman, M. A., Moss, S. F., and D'Agata, E. M. C. (2021). Impact of antimicrobial resistance rates on eradication of *Helicobacter pylori* in a US population. *Gastroenterology* 160, 2181.e1–2183.e1. doi: 10.1053/j.gastro.2021.02.014

Atherton, J. C., and Spiller, R. C. (1994). The urea breath test for *Helicobacter pylori*. *Gut* 35, 723–725. doi: 10.1136/gut.35.6.723

Boltin, D., Levi, Z., Gingold-Belfer, R., Gabay, H., Shochat, T., Niv, Y., et al. (2019). Impact of previous exposure to macrolide antibiotics on *Helicobacter pylori* infection treatment outcomes. *Am. J. Gastroenterol.* 114, 900–906. doi: 10.14309/ajg.0000000000000223

Boltin, D., Levi, Z., Gingold-Belfer, R., Gabay, H., Shochat, T., Schmilovitz-Weiss, H., et al. (2020). Effect of previous nitroimidazole treatment on *Helicobacter pylori* eradication success. *J. Clin. Gastroenterol.* 54, 333–337. doi: 10.1097/mcg.0000000000001237

Chen, Q., Long, X., Ji, Y., Liang, X., Li, D., Gao, H., et al. (2019). Randomised controlled trial: susceptibility-guided therapy versus empiric bismuth quadruple therapy for first-line *Helicobacter pylori* treatment. *Aliment. Pharmacol. Ther.* 49, 1385–1394. doi: 10.1111/apt.15273

Chey, W. D., Leontiadis, G. I., Howden, C. W., and Moss, S. F. (2017). ACG clinical guideline: treatment of *Helicobacter pylori* infection. *Am. J. Gastroenterol.* 112, 212–239. doi: 10.1038/ajg.2016.563

Chiang, T. H. C., Tseng, C. C., Liou, P. H., Wu, J. M., Shun, M. S., Lee, C. T., et al. (2021). Bismuth salts with versus without acid suppression for *Helicobacter pylori* infection: a transmission electron microscope study. *Helicobacter* 26:e12801. doi: 10.1111/hel.12801

de Martel, C., Georges, D., Bray, F., Ferlay, J., and Clifford, G. M. (2020). Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis. *Lancet Glob. Health* 8, e180–e190. doi: 10.1016/s2214-109x(19)30488-7

Dore, M. P., Farina, V., Cuccu, M., Mameli, L., Massarelli, G., and Graham, D. Y. (2011). Twice-a-day bismuth-containing quadruple therapy for *Helicobacter pylori* eradication: a randomized trial of 10 and 14 days. *Helicobacter* 16, 295–300. doi: 10.1111/j.1523-5378.2011.00857.x

El-Serag, H. B., Kao, J. Y., Kanwal, F., Gilger, M., LoVecchio, F., Moss, S. F., et al. (2018). Houston consensus conference on testing for *Helicobacter pylori* infection in the United States. *Clin. Gastroenterol. Hepatol.* 16, 992.e6–1002.e6. doi: 10.1016/j.cgh.2018.03.013

Fallone, C. A., Chiba, N., van Zanten, S. V., Fischbach, L., Gisbert, J. P., Hunt, R. H., et al. (2016). The Toronto consensus for the treatment of *Helicobacter pylori* infection in adults. *Gastroenterology* 151, 51.e14–69.e14. doi: 10.1053/j.gastro.2016.04.006

Fallone, C. A., Moss, S. F., and Malfertheiner, P. (2019). Reconciliation of recent *Helicobacter pylori* treatment guidelines in a time of increasing resistance to antibiotics. *Gastroenterology* 157, 44–53. doi: 10.1053/j.gastro.2019.04.011

Ford, A. C., Yuan, Y., and Moayyedi, P. (2020). *Helicobacter pylori* eradication therapy to prevent gastric cancer: systematic review and meta-analysis. *Gut* 69, 2113–2121. doi: 10.1136/gutjnl-2020-320839

Graham, D. Y., and Klein, P. D. (1991). What you should know about the methods, problems, interpretations, and uses of urea breath tests. *Am. J. Gastroenterol.* 86, 1118–1122

Graham, D. Y., Klein, P. D., Evans, D. J. Jr., Evans, D. G., Alpert, L. C., Opekun, A. R., et al. (1987). Campylobacter pylori detected noninvasively by the 13C-urea breath test. *Lancet* 1, 1174–1177. doi: 10.1016/s0140-6736(87)92145-3

Health Insurance Review and Assessment Service (2021). "[Assessment Report of the Adequacy of Drug Use, 2020]." July 2021 edition. Available at: http://www.hira.or.kr/bbsDummy.do?pgmid=HIRAA020002000100 (Accessed January 29, 2022).

Hooi, J. K. Y., Lai, W. Y., Ng, W. K., Suen, M. M. Y., Underwood, F. E., Tanyingoh, D., et al. (2017). Global prevalence of *Helicobacter pylori* infection: systematic review and meta-analysis. *Gastroenterology* 153, 420–429. doi: 10.1053/j.gastro.2017.04.022

Howden, C. W., and Graham, D. Y. (2021). Recent developments pertaining to *H. pylori* infection. *Am. J. Gastroenterol.* 116, 1–3. doi: 10.14309/ajg.0000000000001031

Jung, H. K., Kang, S. J., Lee, Y. C., Yang, H.-J., Park, S.-Y., Shin, C. M., et al. (2020). Evidence-based guidelines for the treatment of *Helicobacter pylori* infection in Korea: 2020 revised edition. *Korean J. Helicobacter Up. Gastrointest. Res.* 20, 261–287. doi: 10.7704/kjhugr.2020.0045

Kim, T. H., Park, J. M., Cheung, D. Y., and Oh, J. H. (2020). Comparison of 7- and 14-day eradication therapy for *Helicobacter pylori* with first- and second-line regimen: randomized clinical trial. *J. Korean Med. Sci.* 35:e33. doi: 10.3346/jkms.2020.35.e33

Kumar, S., Metz, D. C., Kaplan, D. E., and Goldberg, D. S. (2021). Low rates of retesting for eradication of *Helicobacter pylori* infection after treatment in the veterans health administration. *Clin. Gastroenterol. Hepatol.* 19, 305.e1–313.e1. doi: 10.1016/j.cgh.2020.03.059

Kwon, Y. H., Jeon, S. W., Nam, S. Y., Lee, H. S., and Park, J. H. (2019). Efficacy of tailored therapy for *Helicobacter pylori* eradication based on clarithromycin resistance and survey of previous antibiotic exposure: A single-center prospective pilot study. *Helicobacter* 24:e12585. doi: 10.1111/hel.12585

Lee, Y. C., Chiang, T. H., Chou, C. K., Tu, Y. K., Liao, W. C., Wu, M. S., et al. (2016). Association between *Helicobacter pylori* eradication and gastric cancer incidence: a systematic review and meta-analysis. *Gastroenterology* 150, 1113.e5–1124.e5. doi: 10.1053/j.gastro.2016.01.028

Lee, G. H., Lee, K. M., Shin, S. J., Kang, J. K., Noh, C. K., Kim, J. H., et al. (2020). Impact of previous metronidazole exposure on metronidazole-based second-line quadruple therapy for *Helicobacter pylori* infection. *Korean J. Intern. Med.* 35, 1094–1103. doi: 10.3904/kjim.2020.174

Lehours, P., Siffré, E., and Mégraud, F. (2011). DPO multiplex PCR as an alternative to culture and susceptibility testing to detect *Helicobacter pylori* and its resistance to clarithromycin. *BMC Gastroenterol.* 11:112. doi: 10.1186/1471-230x-11-112

Malfertheiner, P., Bazzoli, F., Delchier, J. C., Celiński, K., Giguère, M., Rivière, M., et al. (2011). *Helicobacter pylori* eradication with a capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: a randomised, open-label, non-inferiority, phase 3 trial. *Lancet* 377, 905–913. doi: 10.1016/s0140-6736(11)60020-2

Malfertheiner, P., Megraud, F., O'Morain, C. A., Gisbert, J. P., Kuipers, E. J., Axon, A. T., et al. (2017). Management of *Helicobacter pylori* infection-the Maastricht V/florence consensus report. *Gut* 66, 6–30. doi: 10.1136/gutjnl-2016-312288

McNulty, C. A., Lasseter, G., Shaw, I., Nichols, T., D'Arcy, S., Lawson, A. J., et al. (2012). Is *Helicobacter pylori* antibiotic resistance surveillance needed and how can it be delivered? *Aliment. Pharmacol. Ther.* 35, 1221–1230. doi: 10.1111/j.1365-2036.2012.05083.x

Megraud, F., Bruyndonckx, R., Coenen, S., Wittkop, L., Huang, T. D., Hoebeke, M., et al. (2021). *Helicobacter pylori* resistance to antibiotics in Europe in 2018 and its relationship to antibiotic consumption in the community. *Gut* 70, 1815–1822. doi: 10.1136/gutjnl-2021-324032

Nyssen, O. P., Bordin, D., Tepes, B., Perez-Aisa, A., Vaira, D., Caldas, M., et al. (2021a). European registry on *Helicobacter pylori* management (Hp-EuReg): patterns and trends in first-line empirical eradication prescription and outcomes of 5 years and 21 533 patients. *Gut* 70, 40–54. doi: 10.1136/gutjnl-2020-321372

Nyssen, O. P., Perez-Aisa, A., Tepes, B., Castro-Fernandez, M., Kupcinskas, J., Jonaitis, L., et al. (2021b). Adverse event profile during the treatment of *Helicobacter pylori*: a real-world experience of 22,000 patients from the European Registry on *H. pylori* management (Hp-EuReg). *Am. J. Gastroenterol.* 116, 1220–1229. doi: 10.14309/ajg.0000000000001246

Osato, M. S., Reddy, R., Reddy, S. G., Penland, R. L., and Graham, D. Y. (2001). Comparison of the Etest and the NCCLS-approved agar dilution method to detect metronidazole and clarithromycin resistant *Helicobacter pylori*. *Int. J. Antimicrob. Agents* 17, 39–44. doi: 10.1016/s0924-8579(00)00320-4

Park, J., Han, E., Lee, S. O., and Kim, D. S. (2017). Antibiotic use in South Korea from 2007 to 2014: a health insurance database-generated time series analysis. *PLoS One* 12:e0177435. doi: 10.1371/journal.pone.0177435

Posteraro, P., Branca, G., Sanguinetti, M., Ranno, S., Cammarota, G., Rahimi, S., et al. (2006). Rapid detection of clarithromycin resistance in *Helicobacter pylori* using a PCR-based denaturing HPLC assay. *J. Antimicrob. Chemother.* 57, 71–78. doi: 10.1093/jac/dki406

Savoldi, A., Carrara, E., Graham, D. Y., Conti, M., and Tacconelli, E. (2018). Prevalence of antibiotic resistance in *Helicobacter pylori*: a systematic review and meta-analysis in World Health Organization regions. *Gastroenterology* 155, 1372.e17–1382.e17. doi: 10.1053/j.gastro.2018.07.007

Schubert, J. P., Gehlert, J., Rayner, C. K., Roberts-Thomson, I. C., Costello, S., Mangoni, A. A., et al. (2020). Antibiotic resistance of *Helicobacter pylori* in Australia and New Zealand: a systematic review and meta-analysis. *J. Gastroenterol. Hepatol.* 36, 1450–1456. doi: 10.1111/jgh.15352

Shah, S. C., Iyer, P. G., and Moss, S. F. (2021). AGA clinical practice update on the management of refractory *Helicobacter pylori* infection: expert review. *Gastroenterology* 160, 1831–1841. doi: 10.1053/j.gastro.2020.11.059

Sugano, K., Tack, J., Kuipers, E. J., Graham, D. Y., El-Omar, E. M., Miura, S., et al. (2015). Kyoto global consensus report on *Helicobacter pylori* gastritis. *Gut* 64, 1353–1367. doi: 10.1136/gutjnl-2015-309252

Woo, H. Y., Park, D. I., Park, H., Kim, M. K., Kim, D. H., Kim, I. S., et al. (2009). Dual-priming oligonucleotide-based multiplex PCR for the detection of *Helicobacter pylori* and determination of clarithromycin resistance with gastric biopsy specimens. *Helicobacter* 14, 22–28. doi: 10.1111/j.1523-5378.2009.00654.x

Yeo, Y. H., Shiu, S. I., Ho, H. J., Zou, B., Lin, J. T., Wu, M. S., et al. (2018). First-line *Helicobacter pylori* eradication therapies in countries with high and low clarithromycin resistance: a systematic review and network meta-analysis. *Gut* 67, 20–27. doi: 10.1136/gutjnl-2016-311868

Yuan, Y., Ford, A. C., Khan, K. J., Gisbert, J. P., Forman, D., Leontiadis, G. I., et al. (2013). Optimum duration of regimens for *Helicobacter pylori* eradication. *Cochrane Database Syst. Rev.* 12:Cd008337. doi: 10.1002/14651858.CD008337.pub2

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# Phillygenin Inhibits *Helicobacter pylori* by Preventing Biofilm Formation and Inducing ATP Leakage

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With the widespread use and abuse of antibiotics, *Helicobacter pylori* (*H. pylori*) has become seriously drug resistant. The development of new antibiotics is an important way to solve *H. pylori*'s drug resistance. Screening antibacterial ingredients from natural products is a convenient way to develop new antibiotics. Phillygenin, an effective antibacterial component, was selected from the natural product, forsythia, in this study. Its minimal inhibitory concentration (MIC) for 18 *H. pylori* strains was 16–32 μg/ml. The minimum bactericidal concentration (MBC) of *H. pylori* G27 was 128 μg/ml; the higher the drug concentration and the longer the time, the better the sterilization effect. It was non-toxic to gastric epithelial cell (GES)-1 and BGC823 cells at the concentration of 100 μg/ml. It presented a better antibacterial effect on *H. pylori* in an acidic environment, and after 24 days of induction on *H. pylori* with 1/4 MIC of phillygenin, no change was found in the MIC of *H. pylori*. In the mechanism of action, phillygenin could cause ATP leakage and inhibit the biofilm formation; the latter was associated with the regulation of *spoT* and *Hp1174* genes. In addition, phillygenin could regulate the genes of *Nhac, caggamma, MATE, MdoB, flagellinA*, and *lptB*, leading to the weakening of *H. pylori*'s acid resistance and virulence, the diminishing of *H. pylori*'s capacity for drug efflux, *H. pylori*'s DNA methylation, the initiation of human immune response, and the ATP leakage of *H. pylori*, thus accelerating the death of *H. pylori*. In conclusion, phillygenin was a main ingredient inhibiting *H. pylori* in *Forsythia suspensa*, with a good antibacterial activity, high safety, strong specificity, better antibacterial effect under acidic conditions, and low risk of resistance development by *H. pylori*. Its mechanism of action was mainly associated with inhibiting the biofilm formation and resulting in ATP leakage. In addition, phillygenin was shown to be able to reduce the acid resistance and virulence of *H. pylori*.

**Keywords: *Helicobacter pylori*, *Forsythia suspensa*, phillygenin, bacteriostasis, mechanism**

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## INTRODUCTION

*Helicobacter pylori (H. pylori)* is an important cause of multiple extra gastrointestinal diseases, chronic gastritis, peptic ulcer, and gastric cancer (Amnon et al., 2010; Plummer et al., 2016; Sultan et al., 2016). At present, the treatment of Western medicine to eradicate *H. pylori* mainly includes the standard triple therapy, the non-tantalum four-link schemes, the bismuth-based quadruple therapy, etc. (Nagy et al., 2016), the increasingly higher resistance rate, and increasingly lower eradication rate of *H. pylori*, which are

attributed to the long-term use, and abuse of antibiotics poses a serious threat to the health and safety of the public (Hooi et al., 2017). However, the search for new therapeutic drugs is the fundamental solution to the current problem, and screening effective ingredients from the bacteriostatic natural products serves as a convenient way to develop new drugs. *Forsythia suspensa* (*F. suspensa*), a kind of natural plant from the *Forsythia* of *Oleaceae*, is a precious traditional Chinese medicinal material (Yang et al., 2019) that contains active ingredients such as the coumarin, phillyrin, and rutin, with antibacterial, antitumor, and anti-inflammatory properties and the ability to enhance immunity (Seung et al., 2009; Yuan et al., 2015; Bae et al., 2020). *Forsythia suspensa* has various components with complex mechanisms of action; it is difficult to determine on which microorganism it has a better inhibitory effect and to identify an effective active ingredient from it and explore this ingredient's mechanism of action. Dong et al. (2016) reported that *F. suspensa* had a certain inhibitory effects on *H. pylori* with no discussing its functions, ingredients, and mechanism. In this study, phillygenin was screened from *F. suspensa* as the main antibacterial ingredient for *H. pylori* and evaluated as having a good druggability in terms of its toxicity, antibacterial spectrum, acid resistance, and drug resistance, besides the exploration of phillygenin's mechanism through experiments on inhibiting biofilm formation, ATP leakage detection, the transcriptome detection and verification, etc.

# MATERIALS AND METHODS

## Recovery and Cultivation of Bacterial Strains

*Helicobacter pylori* strains containing the preserving liquid were removed from the refrigerator where they were stored at −80°C (standard strains 26695, NSH57, MSD132, and G27 were presented by professor Hongkai Bi from Nanjing Medical University; the clinical strains HPBS001-HPBS016 were isolated by our laboratory), Information on other strains are provided in **Supplementary Table 1**. *Helicobacter pylori* strains were cultured on the Columbia blood agar plate (OXOID, UK) or in the brain heart infusion (BHI, OXOID, UK) broth medium containing 10% serum (Pingrui, China). Bacterial species from non-*H. pylori* group were used as an experimental control and were cultured on nutrient agar (NA) plates or Luria-Bertani (LB) plates.

## Detection of MIC

The active ingredients of *F. suspensa* purchased from Chengdu Herbpurify Co., Ltd. (CAS: 487-39-8, etc.) were dissolved into 4 mg/ml using absolute ethanol. The first well of a 96-well plate with bacteria was added with 173.6 μl of culture medium and then with 6.4 μl of antibacterial drugs, and every well from the first to the eighth was all diluted in proportion (the drug concentrations of the first to eighth wells were 128, 64, 32, 16, 8, 4, 2, and 1 μg/ml) with negative wells (sterile, only with medium, and drugs) and positive wells (no drugs, only with medium, and bacteria) as controls. Bacteria at the logarithmic growth phase were taken from the solid plate and made into bacterial suspensions with the corresponding mediums: the concentration of *H. pylori* was adjusted to 0.3 [$1.0 \times 10^8$ colony forming unit (CFU)/ml] and diluted 10 times to $1.0 \times 10^7$ CFU/ml. The concentration of other bacteria at OD600 was adjusted to 0.3 ($1 \times 10^8$ CFU/ml) and diluted 100 times to $1 \times 10^6$ CFU/ml. The concentration of fungi was adjusted to 0.5 ($5 \times 10^6$ CFU/ml) and diluted 1,000 times to $5 \times 10^3$ CFU/ml. Notably, 10 μl was taken and added from the first to eighth well (the concentration of bacterium per well was about $1.0 \times 10^6$ CFU/ml) and the wells with bacteria were cultivated for 24–72 h to judge the results (Huang et al., 2019).

## Detection of MBC

Phillygenin was dissolved to 4 mg/ml by absolute ethanol. The first well of the 96-well plate with bacteria was added with 173.6 μl culture medium (pH 3.0, pH 4.5, pH 6.0, and pH 7.0) with 90 μl culture medium added to the other wells. Thereafter, 6.4 ml of phillygenin was added to the first well, with every well from the first to the fifth well diluted in proportion (the drug concentrations were 128, 64, 32, and 16, 8 μg/ml) and phosphate-buffered saline (PBS) (sangon, China) used as a positive control. The *H. pylori* G27 strains at the logarithmic growth phase on the solid medium were employed to make a bacterial suspension with a BHI medium; the concentration of the bacteria liquid was adjusted to $1 \times 10^8$ CFU/ml (OD600 was 0.3), diluted by ten times, and kept in reserve. The first to fifth wells were added with 10 μl of the bacteria liquid (the concentration of bacteria liquid per well was about $1 \times 10^6$ CFU/ml) and cultured in a three-gas incubator. The bacteria liquid after the drug action for a certain period of time (such as 2 h) was diluted (100 times, 1,000 times, etc.), coated on a Columbia agar plate, and cultured in a three-gas incubator for 4–5 days. The number of bacteria growing on the agar plate was calculated, with the drug concentration at which the number of bacteria was suppressed by 99.9% as the MBC.

## Drug Resistance Detection of Phillygenin

*Helicobacter pylori* G27 strain was used to detect the drug resistance of phillygenin. First, the MICs for *metronidazole* and phillygenin were 2 and 16 μg/ml, respectively. The induction on the strains was performed with one-fourth MIC concentration of *metronidazole* and phillygenin, with the detection performed every 3 days during a total of 24 days of induction. The induced concentrations were adjusted with the change of MICs. For example, the induced concentration was adjusted to 4 μg/ml when the MIC of metronidazole was 16 μg/ml.

## Cytotoxicity Test of Phillygenin

The cell suspensions of gastric epithelial cell (GES)-1 and BGC823 (KeyGEN BioTECH, Nanjing, China) were prepared with their concentrations adjusted to $1 \times 10^5$. The suspensions were then inoculated into a 96-well plate, 100 μl per well. Three repeats of the same sample were performed and cultured in the incubator at 37°C for 24 h. Moreover, 10 μl of phillygenin (the working concentrations were 300, 200, 100, 50, and 0 μg/ml) was added to each well and inoculated at 37°C for 24 h. Furthermore, 10 μl of CCK8 (Beyotime, China) was added per well, tapped, and mixed well and then incubated for 4 h. The absorbance at 450 nm was measured, and the survival rate was calculated according to the formula: cell survival rate = [(As-Ab)]/[(Ac-Ab)] × 100%. As refers to the wells that contain the culture medium of cells,

drugs, and CCK-8. Ac refers to the wells that contain the culture medium of cells and CCK-8 with no drug. Ab refers to the wells that contain the culture medium of cells and CCK-8, with no cell and drug. A survival curve based on the survival rate was established.

## Animal Toxicity Test of Phillygenin

Specific pathogen-free (SPF) C57BL/6 mice aged 6–8 weeks were purchased from Changsha Tianqin Biological Co., Ltd., the number of SPF animal license: SYXK Gui 2017-0004, and animal experiment Ethics Number: No. 2019112501. The evaluation of drug efficacy for animal *in vivo* as follows was performed in accordance with the same experiment ethics. The mice were raised in an SPF environment and randomly divided into the medicine administration group and negative control group with 10 in each group. Ten times therapeutic dose of phillygenin was administered to the treatment group for 3 consecutive days, once a day; the negative control group was given the PBS buffer solution with the same times of administration and dosage as the administration group. The mice were weighed a day before the administration and weighed after the administration for 7 days. On the third day after the drug withdrawal, the mice in the infection group were weighed, and their average weight was calculated. The blood from their eyeballs was collected. Thereafter, the mice were sacrificed by cervical dislocation, their stomachs, kidneys, livers, and spleens were made into pathological sections, stained with H & E.

## Detection of the Phillygenin Inhibitory Effect *in vivo*

Phillygenin, omeprazole (Sigma-Aldrich, Germany), amoxicillin (Sigma-Aldrich, Germany), and clarithromycin (Sigma-Aldrich, Germany) were dissolved and diluted to 10 mg/ml. The models of SPF C57BL/6 mice aged 6 weeks were established (HPBS001). The mice were divided into four groups, namely, the omeprazole + amoxicillin + clarithromycin group (the triple-therapy group), the omeprazole + phillygenin group (28 mg/kg), the omeprazole + phillygenin group (7 mg/kg), and the PBS group, each with 10 mice. In addition, 10 mice that were not infected with *H. pylori* were treated as the negative control. The treatment group was given an intragastric administration. The groups that contained omeprazole were administered omeprazole first and then other drugs 30 min later. After the administration, the mice were made to fast and deprived of water for 4 h. The dosage was 138.2 mg/kg of omeprazole, 28.5 mg/kg of amoxicillin, and 14.3 mg/kg of clarithromycin, once a day for 3 consecutive days; the control group was given the PBS buffer solution with the same volume and times of administration as above. Two days after the drug withdrawal, the blood was collected from the eyeballs of the mice, which were then sacrificed by cervical dislocation with their stomach tissues taken and broken to acquire *H. pylori* that was then isolated, cultured, and identified with the amount of colonization calculated. A part of the stomach tissues was made into paraffin sections with H&E staining, TUNEL immunohistochemistry, and fluorescence immunoassay performed thereon.

## Inhibition Experiment of Phillygenin on *H. pylori's* Biofilms

The OD of the *H. pylori* G27 bacterial suspension was adjusted to 0.1 and inoculated into a 96-well plate under microaerobic conditions at 37°C for 3 days to form *H. pylori* biofilms (Hathroubi et al., 2020), which were then added with phillygenin (concentrations were 128, 64, 32, and 16 μg/ml). The antibiofilm effect of phillygenin was evaluated through crystal violet (Macklin, China) staining and detection using the Alamar blue assay (Solarbio, China). Biofilms were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher, America) in the dark for 15 min and then visualized using the confocal laser scanning microscopy. The wavelength of the SYTO 9-stained live cells that had been activated was 411 nm, while that of the PI-stained dead cells was 568 nm. The protein content of these biofilms was determined by the BCA Protein Quantification Kit (Beyotime, China).

## Inhibition Experiment of Phillygenin on ATP

*Helicobacter pylori* G27 was cultured to the logarithmic phase, and the concentration of the bacteria solution was adjusted to $1 \times 10^7$ CFU/ml. Meanwhile, three gradients of the working concentration of phillygenin were set, which were 32, 64, and 128 μg/ml, respectively. Polymyxin B (Macklin, China) was used for the positive control and PBS for the negative control. They were cultured for 2 h and centrifuged to acquire the supernatant and bacteria. Adenosine triphosphate (ATP) was detected by ATP Assay Kit (Beyotime, China) through the multifunctional enzyme microplate reader (BioTek, USA).

## Transcriptomics Detection

In the exploration of the semi-inhibition curve of phillygenin, the concentration of the bacterial suspension was adjusted to $1 \times 10^8$ CFU/ml, with drug action lasting (16, 32, 64, and 128 μg/ml) for 0, 2, and 8 h. Thereafter, the OD value at 600 nm (HITACHI, Japan) was measured with the semi-inhibitory drug concentration confirmed when the OD value remained unchanged. The bacteria were extracted for a transcriptome sequencing, which was completed by Nanjing Fengzi Biopharm Technology using the second-generation Illumina high-throughput sequencing platform and PE150 sequencing strategy. As a result, a total of 80,448,750 raw read pairs were measured with 79,746,845 clean read pairs obtained after quality control. Bowtie2 and Rockhopper were employed to carry out the comparison and sliced transcript analysis. Thereafter, all genes were quantitatively analyzed, and a total of 1,214 differential genes were identified. A functional enrichment analysis was performed on these differential genes to explore their features.

## Reverse Transcription and RT-qPCR

*Helicobacter pylori* G27 was cultured to the logarithmic phase with the concentration of the bacterial solution adjusted to $1 \times 10^8$ CFU/ml. The bacterial solution was added with phillygenin for a certain time based on the semi-inhibitory growth curve and centrifuged to obtain the precipitate. Zero hour was labeled as F_1 group, 2 h as F_2 group, and 8 h as F_3 group. Three biological repeats were performed in each group, labeled as A,

B, and C. The TRIZOL reagent (Takara, China) was used to extract RNA, the transcription of which was reversed into cDNA through a reverse transcription kit (MonPure, China). RT-qPCR was performed applying the LightCycler according to the RT-PCR kit (MonPure, China); the sample was subjected to 95° predenaturation for 30 s, 95° denaturation for 10 s, and 60° annealing and extension for 30 s, about 40 cycles. The primers are displayed in **Supplementary Table 2**. The 16S rRNA amplicon (Thermo Fisher, America) was used as an internal control for data standardization, and the remaining primers were purchased from Sangon Biotech (Shanghai). The change at the transcriptional level was determined by applying the relative quantification method ($2^{-\Delta\Delta CT}$). The dissociation curve was analyzed to verify the homogeneity of the products.

## Statistical Methods

All data were expressed as mean ± standard deviation (SD). Data analyzed by one-way analysis of variance were performed using SPSS 25.0 and $p < 0.05$ considered to be statistically significant.

# RESULTS

## Antibacterial Effect of Phillygenin *in vitro*

A total of 12 components of *F. suspensa* were screened, among which phillygenin had the best antibacterial effect. The liquid chromatogram of phillygenin is displayed in **Figure 1**. The MIC of phillygenin against *H. pylori* was 16–32 μg/ml, and the MICs of other components were all >128 μg/ml, as illustrated in **Table 1**. The inhibition effects of phillygenin on 18 *H. pylori* strains were tested, in which it was found to have a good inhibitory effect on sensitive, drug-resistant, and multiple-resistant strains with the MIC being 16–32 μg/ml, as displayed in **Table 2**. The MBC of phillygenin against *H. pylori* was 16 times the MIC in the normal medium (PH 7.0), with the antibacterial rate reaching 99.9% after 6 h and 99.999% after 8 h. The antibacterial rate was 90, 99, and

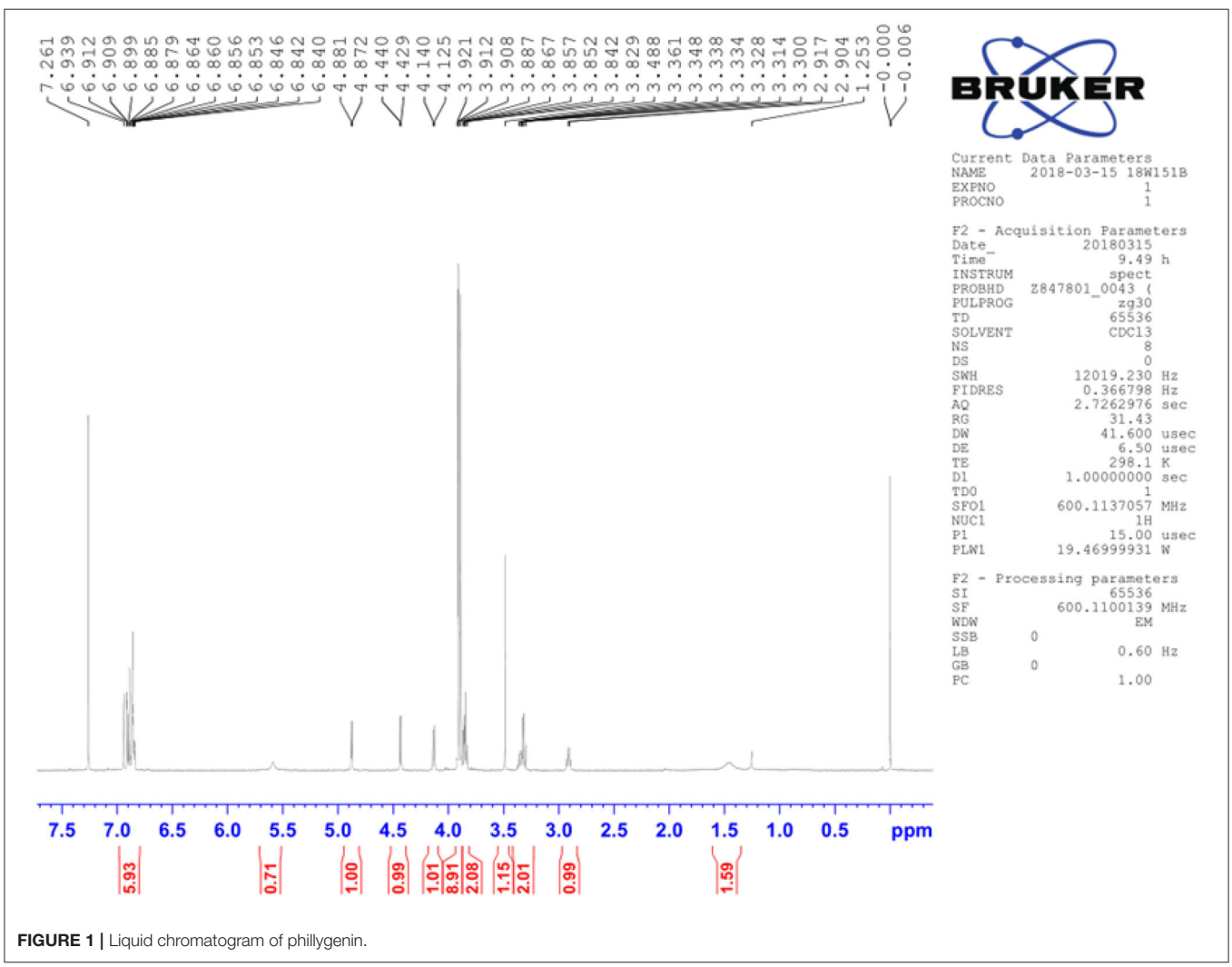


**FIGURE 1 |** Liquid chromatogram of phillygenin.

**TABLE 1 |** Minimum inhibitory concentrations (MICs) of 12 monomer components of *Forsythia suspensa* against *Helicobacter pylori* (μg/ml).

| Ingredient | 26,695 | HPBS001 |
|---|---|---|
| Phillygenin | 32 | 16 |
| Phillyrin | >128 | >128 |
| Phillygenin A | >128 | >128 |
| Esculin | >128 | >128 |
| Arctigenin | >128 | >128 |
| Wedelolactone | >128 | >128 |
| Demethylwedelolactone | >128 | >128 |
| Mycoporphyrin | >128 | >128 |
| Phillygenin E | >128 | >128 |
| Phillygenin F | >128 | >128 |
| Isoforsythiaside | >128 | >128 |
| (+)-Pinoresin-β-D-glucopyranoside | >128 | >128 |

*26,695 is a sensitive strain; HPBS001 is a resistant strain (resistant to levofloxacin, clarithromycin, and metronidazole).*

**TABLE 2 |** The MICs of phillygenin against *H. pylori* (μg/ml).

| Strain | Drug resistance | Phillygenin |
|---|---|---|
| 26695 | Sensitive | 32 |
| G27 | Sensitive | 16 |
| MSD132 | Sensitive | 16 |
| NSH57 | Sensitive | 32 |
| HPBS001 | Resistant to LEV, CLA, and MET | 16 |
| HPBS002 | Resistant to MET | 16 |
| HPBS003 | Resistant to CLA | 16 |
| HPBS004 | Resistant to LEV | 32 |
| HPBS005 | Resistant to LEV and LEV | 32 |
| HPBS006 | Resistant to CLA and MET | 16 |
| HPBS007 | Resistant to CLA | 32 |
| HPBS010 | Resistant to MET, CLA, and LEV | 16 |
| HPBS011 | Resistant to MET and CLA | 16 |
| HPBS012 | Sensitive | 32 |
| HPBS013 | Resistant to MET, CLA, and LEV | 16 |
| HPBS014 | Resistant to MET, CLA, AMO, and LEV | 16 |
| HPBS015 | Sensitive | 16 |
| HPBS016 | Sensitive | 16 |

*The abbreviations for antibiotics are as follows, LEV, levofloxacin; CLA, clarithromycin; MET, metronidazole; AMO, amoxicillin.*

99.9% with phillygenin at a concentration of 8 times the MIC for 4, 6, and 8 h in a dose-and time-dependent manner, as illustrated in **Figure 2**.

## Antibacterial Effect of Phillygenin *in vivo*

The efficacy of phillygenin on *H. pylori in vivo* was evaluated by the acute gastritis mice models, which had been confirmed to be infected with *H. pylori* (HPBS001). According to the counted amount of colonization, the antibacterial effect of phillygenin was better than that of the triple therapy with no significant difference

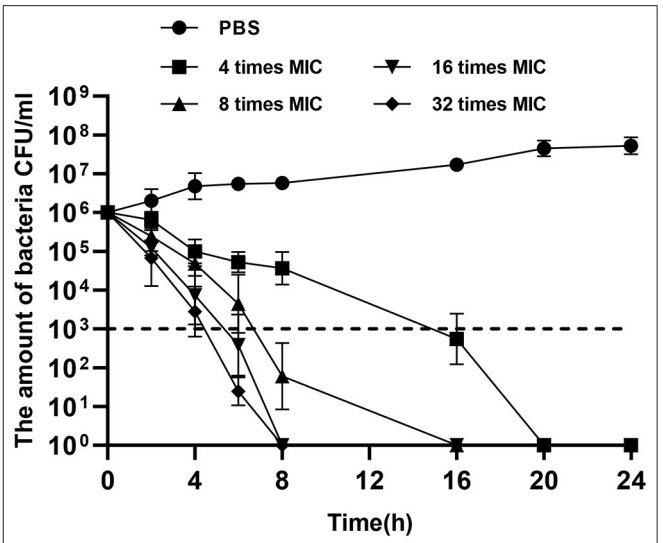


**FIGURE 2 |** Minimum bactericidal concentrations (MBCs) of phillygenin against *H. pylori*.

between high and low concentrations, as displayed in **Figure 3A**. The H&E staining and immunohistochemical images of the phillygenin group showed that the number of the apoptotic cells in the gastric mucosae of the mice and that of the inflammatory factors were significantly decreased (**Figure 3B**). In addition, the expressions of several inflammatory factors in the samples of gastric tissues were detected, with results revealing that the expression levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1β were the lowest in the phillygenin group, as shown in **Figures 3B,C**, which suggested that phillygenin had a good antibacterial effect *in vivo*.

## Safety Evaluation of Phillygenin

The toxicity test of phillygenin was carried out. The results showed that phillygenin at 100 μg/ml had no obvious cytotoxic effect on GES-1 and gastric cancer cells BGC823, with a survival rate above 90% (**Figures 4A,B**). After the intragastric administration of 10 times the therapeutic dose of phillygenin, there was no significant change in the weights of mice within 7 days, as shown in **Figure 4C**. Besides, no obvious pathological damage was found in the stomachs, livers, spleens, and kidneys of mice, as shown in **Figure 4D**. Phillygenin that was found to have a low toxicity *in vitro* and *in vivo* and high safety could be used as the first-line therapy for *H. pylori*.

## Advantages of Phillygenin

To check the selectivity of phillygenin against *H. pylori*, a total of 20 non-*H. pylori* strains were used and showed MICs all >128 μg/ml. Phillygenin that was found to have a single effect on *H. pylori* (**Supplementary Table 3**) was a narrow-spectrum antibiotic with a specificity and not much impact on other microflora. The inhibitory effects of phillygenin under different pH conditions (i.e., 3.0, 4.5, 6.0, and 7.0) were explored for it exerted effects after entering the stomach under low pH

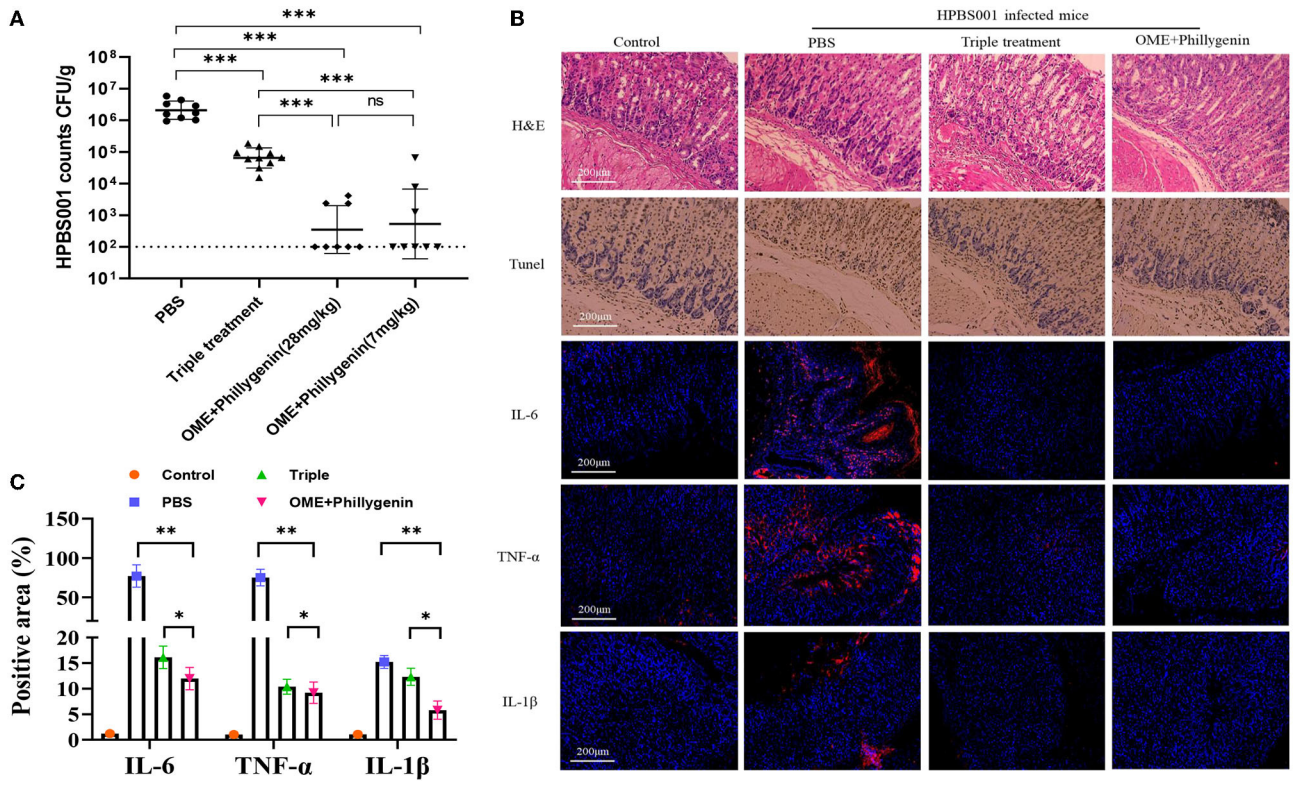


**FIGURE 3 |** Evaluation of the antibacterial effect of phillygenin in mice. **(A)** Presents the amount of HPBS001 colonization of mice with acute gastritis; **(B)** displays the repair of gastric mucosal tissues of mice with acute gastritis; and **(C)** shows the quantitative graph of inflammatory factors. ns $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

conditions, as shown in **Figure 5A**. At pH 3.0 and 4.5, the antibacterial rate reached 99.9% 2 h after the administration of 8 times and 16 times MICs of phillygenin, which could not be found at pH 6.0 and 7.0. These results indicated that phillygenin were more effective in an acidic environment with acid resistance. The drug resistance induction of phillygenin on *H. pylori* G27 strains was detected (**Figure 5B**). The results showed that during the 24 days drug resistance induction, the MIC of phillygenin only doubled on the 12th day with no change at other times, while the MIC of metronidazole increased by 64 times. Phillygenin that was shown to have difficulty in making *H. pylori* develop resistance could be used for clinical treatment.

## Inhibition of Phillygenin on Biofilms and ATP

The formation of biofilms is an important cause for the drug resistance of *H. pylori* (Krzyzek et al., 2021). Phillygenin was shown to inhibit biofilms of *H. pylori* at 100–150 μg/ml (about 6–8 times MIC) through a detection employing the Alamar blue assay (**Figure 6A**). Besides, it was found through the crystal violet staining assay that a drug concentration eight times of the MIC could inhibit 50% of the biofilm growth, with an effect better than that of clarithromycin (**Figure 6B**). Since the main component of biofilm is protein, the detection of the protein content of the biofilms of *H. pylori* was carried out. The results showed that a drug concentration 8 times of the MIC could inhibit 50% of the protein in biofilms, which was consistent with the results of the crystal violet staining and Alamar blue assay, as shown in **Figure 6C**. Phillygenin at a concentration 8 times of the MIC could destroy biofilms of *H. pylori* as shown in **Figure 6D** photographed by the confocal microscope at a 400 magnification.

ATP serves as the direct energy source for life activities and changes in levels of ATP will affect functions of cell. After the effect of phillygenin on *H. pylori* for 2 h, the intracellular ATP gradually leaked to the outside of the cells with most thereof leaking at a drug concentration of two times of the MIC. However, almost all the ATP leaked at both the drug concentrations of four and eight times of the MIC. The degree of leakage was equivalent to that of polymyxin B in a dose- and time-dependent manner (**Figure 6E**).

The detection of the expression of biofilm-related genes was mainly conducted by detecting the differential expressions of phillygenin on suspended bacteria and bacteria biofilms at 0 h and 4 h. The effect of a drug concentration two times of the MIC on suspended bacteria was shown in **Figure 6F**; the effect of a drug concentration four times of the MIC on suspended bacteria was displayed in **Figure 6G**. After drug action, the *Hp1174* genes that formed through biofilm regulation were significantly

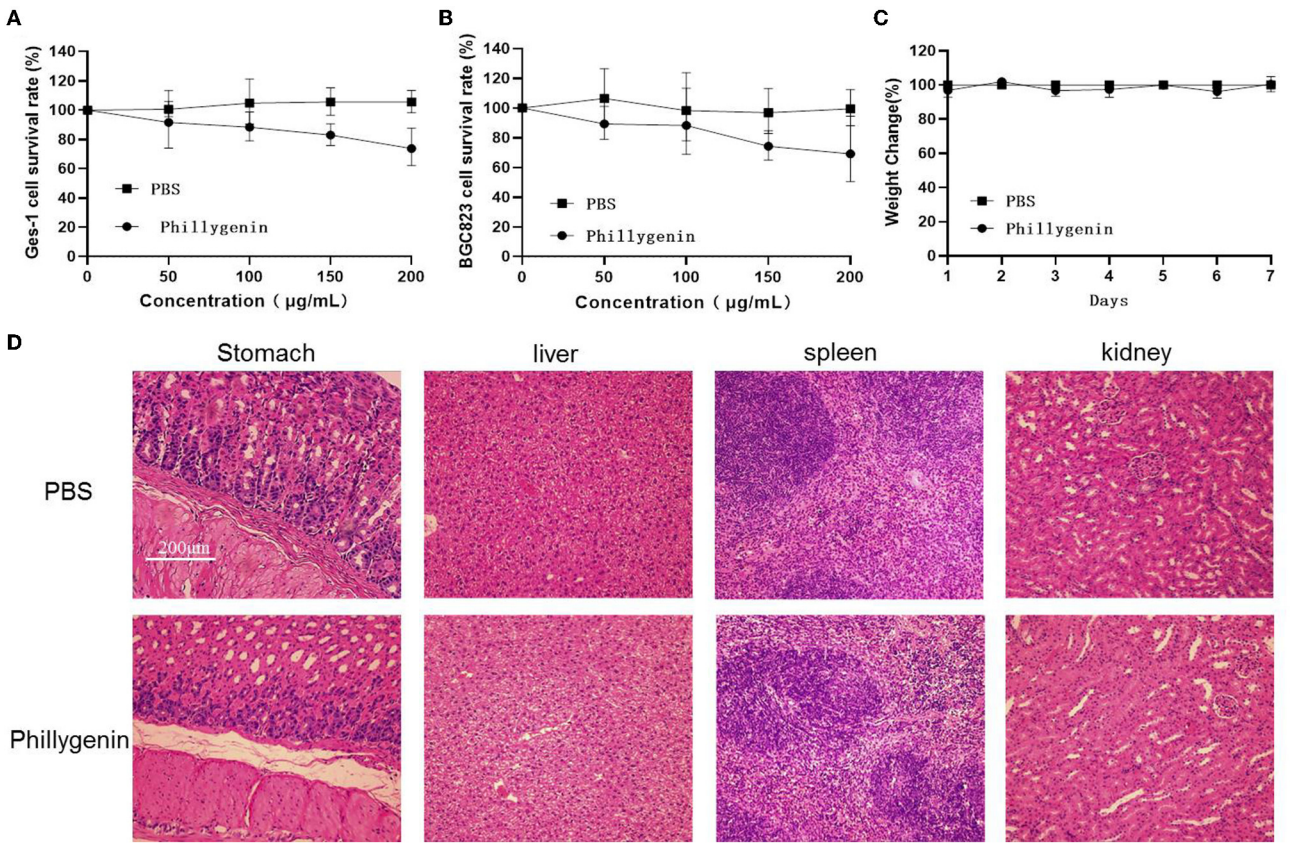


**FIGURE 4 |** Phillygenin toxicity test. **(A)** The phillygenin toxicity test for gastric epithelial cell (GES)-1 cells; **(B)** the phillygenin cytotoxicity test for BGC823; **(C)** the effect of phillygenin on the weights of mice; and **(D)** the detection of the damage of phillygenin to stomachs, livers, spleens, and kidneys in mice.

downregulated, with the remaining ones upregulated. The effect of a drug concentration two times of the MIC on the bacteria in biofilms was as shown in **Figure 6H** and that of a drug concentration four times of the MIC on the bacteria in biofilms was displayed in **Figure 6I**. After the drug action, the *spoT* genes and *Hp1174* genes that regulated the formation of biofilms were significantly downregulated; after the effects of phillygenin at the drug concentrations two times and four times of the MIC, there was no significant change in *hefA, HP0939, HP0497,* and *HP0471*. The *Hp1174* genes involved in the biofilm formation, whether in the suspension or bacteria in biofilms, were significantly downregulated after drug actions at different concentrations. Therefore, it could be inferred that phillygenin might inhibit biofilm formation mainly through the downregulation of *Hp1174* genes. It is noteworthy that in planktonic bacteria, *spoT* genes were upregulated, while *spoT* genes in biofilm bacteria were downregulated, which might be attributed to the characteristics of the bifunctional hydrolase in *spoT* (Bahareh et al., 2017): guanosine tetraphosphate ((p)ppGpp) that was the key to bacterial biofilm formation could be both hydrolyzed and promoted; therefore, it could be inferred that in suspended bacteria, *spoT* might hydrolyze (p)ppGpp and promote the generation thereof after biofilm formation (Cai et al., 2020).

## Transcriptome Sequencing

In the proposed transcriptome sequencing, the drug action of phillygenin at different times was first detected, and the curves of half inhibitory concentrations were drawn as shown in **Figure 7A**, whereas, there was no change in the OD value at a drug concentration of 2 times of the MIC. The RNA-seq correlation analysis of the results showed a good biological repeatability (**Figure 7B**). Using the PCA, differences were shown between groups (**Figure 7C**). Through a pairwise comparison, 929 differential genes were detected between F_1 group and F_2 group, of which 445 were upregulated and 484 were downregulated; 1,015 differential genes were detected between F_1 group and F_3 group, of which 467 were upregulated and 548 were downregulated; 596 differential genes were detected between F_2 group and F_3 group, of which 315 were upregulated and 281 were downregulated. In the Venn diagram of differentially expressed genes, it was shown that there were 299 repetitive differentially expressed genes among three groups (**Figure 7D**). The differentially expressed genes

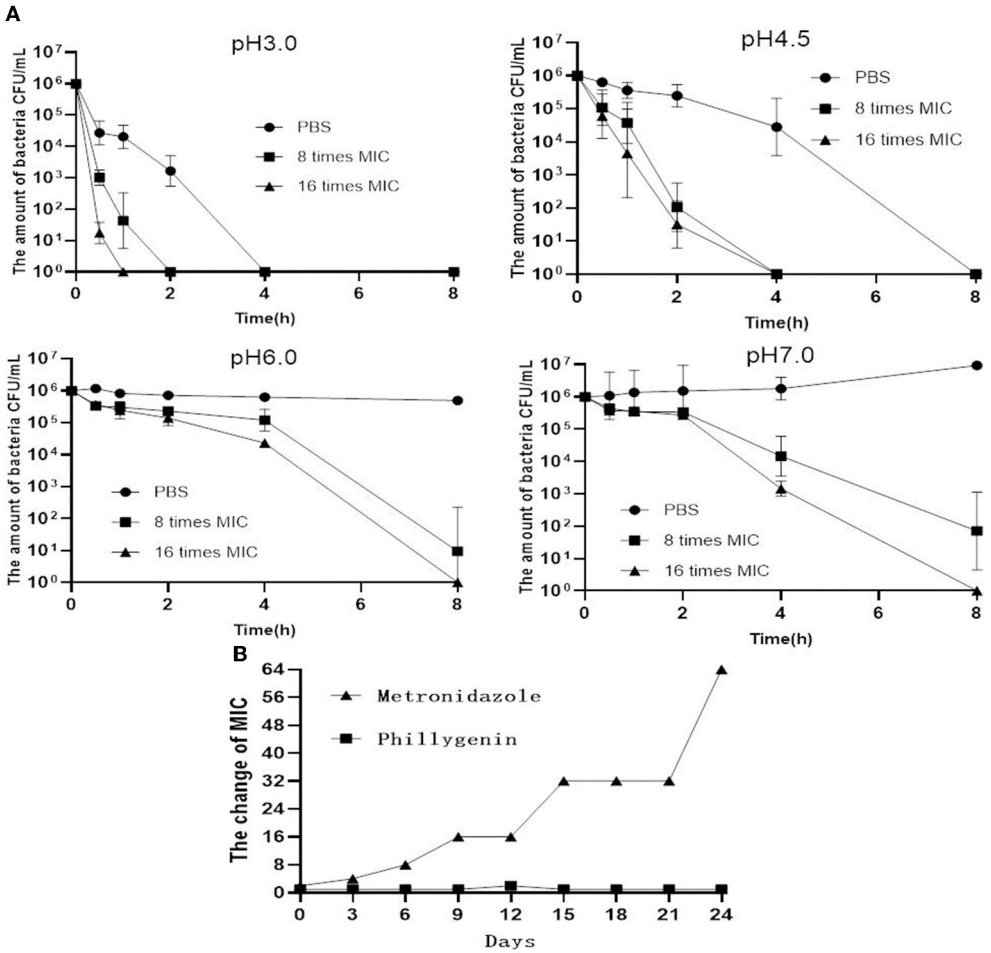


**FIGURE 5 |** Acid response and drug resistance induction of phillygenin. **(A)** The detection of MBCs of phillygenin (μg/ml) against *H. pylori* at different pHs and **(B)** the detection of inducing drug-resistant *H. pylori* to phillygenin.

were drawn into a gene ontology (GO) enrichment analysis histogram and divided into three categories, namely, biological processes, cellular components, and molecular functions. The differential genes between each group were found to mostly concentrate on pathways such as obsolete electron transport, transporter activity, ion binding, etc. (**Figure 7E**). According to the correlation between groups (log 2-fold change), and the comparison of repeated genes between groups to the exclusion of unknown proteins, six repeated differentially expressed genes that were most relevant were screened (including two upregulated and four downregulated ones), as shown in **Table 3**. According to the results of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment and GO analysis, the differential genes mainly concentrated on the pathway for efflux transport, which was further verified by RT-qPCR to compare the differential expression levels of the effect of phillygenin on suspended bacteria with 0 h and 8 h, as shown in **Figure 7F**. *Nhac, caggamma, MATE,* and *MdoB* genes were downregulated, and *flagellin A* and *lptB* genes were upregulated, which were consistent with the results of the transcriptome sequencing.

## DISCUSSION

*Helicobacter pylori* is a cause for the occurrence of various intestinal and extra-intestinal diseases and severe drug resistance (Sugano et al., 2015; Okuda et al., 2016). The development of new antibiotics is an important way to solve *H. pylori*'s drug resistance, and screening of effective ingredients from natural products is a convenient method for new drug development. In this study, phillygenin, which is an effective antibacterial component of *F. suspensa* belonging to the lignans, was screened out (Liu et al., 2018). Studies have reported that phillygenin had the effects of regulating the intestinal microbiota, reducing fibrosis herein (Sun et al., 2021), inhibiting the release of inflammatory cytokines (Wang et al., 2021), inhibiting adhesion and migration, etc. (Quan et al., 2021), while, less has been studied about its antibacterial effects.

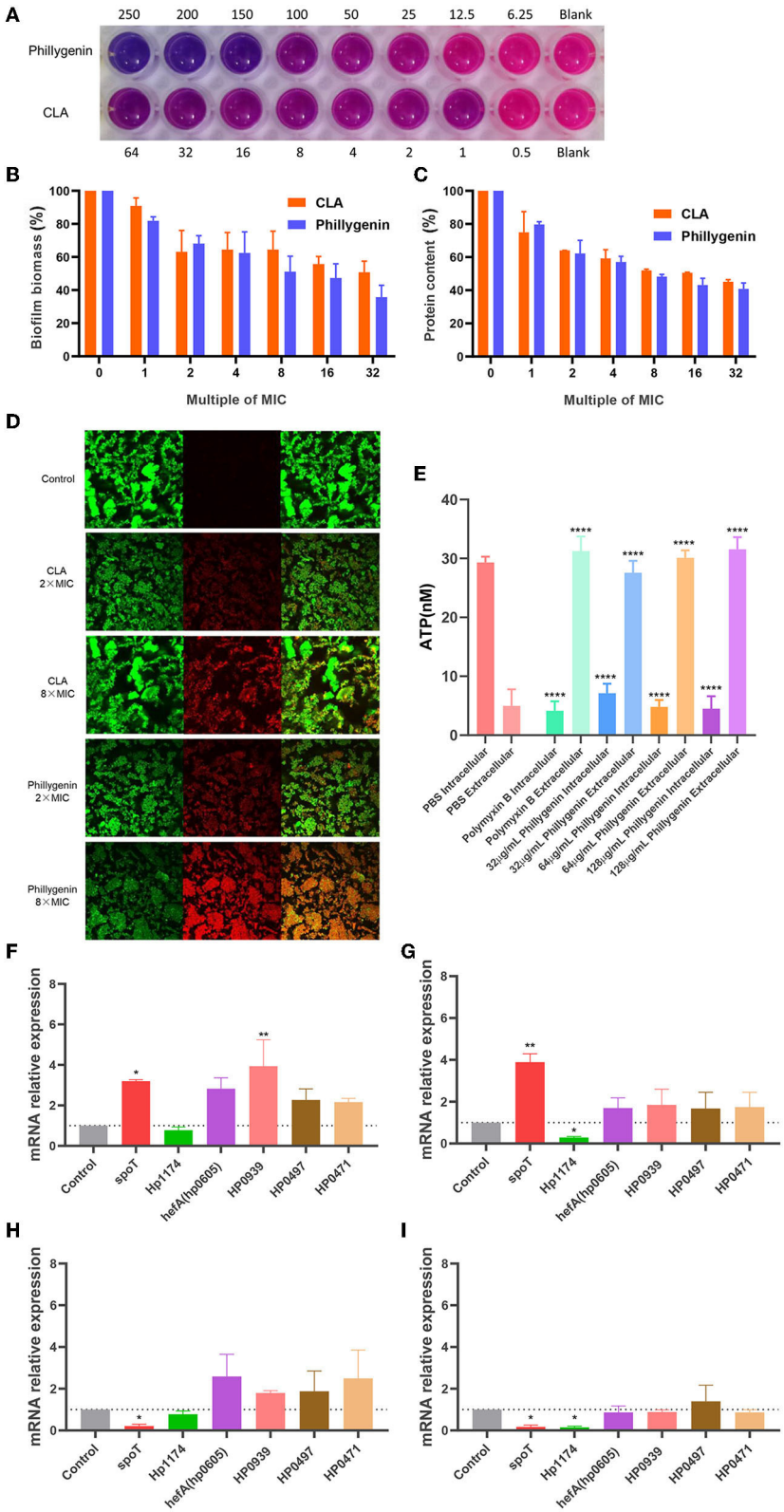


**FIGURE 6 |** The inhibitory effect of phillygenin on biofilms and adenosine triphosphate (ATP). **(A)** The detection of the inhibition of biofilms using the Alamar blue assay; **(B)** the detection of biofilm expression levels through the crystal violet staining; **(C)** the expression levels of biofilm protein; **(D)** the detection of biofilm through the confocal laser scanning microscopy at a 400 magnification; **(E)** ATP detection; **(F)** the changes in the relative expression quantity of the mRNA of biofilm-related genes in suspended bacteria treated with phillygenin at a concentration of two times of the MIC; **(G)** the changes in the relative expression quantity of the mRNA of genes in

*(Continued)*

**FIGURE 6 |** planktonic bacteria treated with phillygenin at a concentration of four times of the MIC; **(H)** the changes in the relative expression quantity of the mRNA of biofilm-related genes in bacteria of biofilms treated with phillygenin at a concentration of two times of the MIC; **(I)** the changes in the relative expression quantity of the mRNA of biofilm-related genes in bacteria of biofilms treated with phillygenin at a concentration of four times of the MIC. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

It was observed that phillygenin has the same effect on both sensitive and drug-resistant *H. pylori* strains. The effect was a concentration- and time-dependent. In the efficacy evaluation *in vitro*, the MICs of phillygenin against 20 non-*H. pylori* strains were detected, and phillygenin was found to have a specific inhibitory effect on *H. pylori*. In the CCK-8 cytotoxicity test, the survival rates of GES-1 and BGC823 cells were above 90% with phillygenin at a concentration of 100 μg/ml (6.25 times MIC); in addition, after the intragastric administration of 10 times of the therapeutic dose to mice, no damage was found in the organs; therefore, phillygenin was found to have a high safety through the *in vivo* and *in vitro*. In the efficacy evaluation *in vivo*, the treatment of mice infected with HPBS001 (resistant to levofloxacin, clarithromycin, and metronidazole) using phillygenin, its antibacterial effect was found to be better than that of the triple therapy; therefore, it could be suggested that phillygenin which exerted a good therapeutic effect on drug-resistant strains *in vivo* and on refractory gastritis caused by clinically drug-resistant *H. pylori* infection, can serve as a lead drug or candidate drug for treating *H. pylori.*

The long-term use of antibiotics in general is more prone to rendering *H. pylori* drug-tolerant, but the degrees of drug resistance vary among different antibiotics (Lu et al., 2020). In the comparison of phillygenin and MET, no obvious drug resistance to phillygenin was found after 24 days of drug resistance induction with the MIC of phillygenin increasing by two times and that of metronidazole by 64 times. It could be suggested that phillygenin had difficulty in rendering *H. pylori* drug resistant, which might be attributed to its plant origin and multiple targets. The discovery of this strength, whereas, may lay the foundation for increasing the dosage or usage of phillygenin in the treatment of *H. pylori* in the future and help this compound become a lead drug. On the contrary, oral drugs were found to fail to achieve the desired effect due to the low pH environment in gastric juice (Li et al., 2021), while the antibacterial rates of phillygenin at the concentrations of eight times and 16 times of the MIC at pH 3.0 and 4.5 reached 99.9% after 2 h of administration, an antibacterial rate that was not observed at pH 6.0 and 7.0. It could be suggested that phillygenin exerted a better antibacterial effect under low pH conditions and resisted the acidic environment in the stomach, which was its another advantage. The *Nhac* genes mainly regulated the transport of $Na^+/H^+$, which helped *H. pylori* better colonize in the acidic environment (Ge et al., 2018). However, phillygenin could downregulate the *Nhac* genes and exert better effect in the acidic environment.

At present, the international competition of drug research is mainly on the research of drug targets. Generally speaking, a new target of drug action once discovered will tend to become a breakthrough in the discovery of a series of new drugs (Wu et al., 2021). The bacterial biofilm is a bacterial community called extracellular polymer (EPS) in a self-assembled matrix, and this produced by *H. pylori* is mainly composed of proteins (Hathroubi et al., 2018). The bacterial biofilm once formed becomes a refuge for bacteria to resist the antibiotic treatment and immune defense, which is also referred to as the development of drug resistance (Rizzato et al., 2019). Guanosine tetraphosphate (p-ppGpp) can affect the formation of bacterial biofilms. *SpoT* is a bifunctional enzyme with the properties of p-ppGpp synthase and hydrolase (Hathroubi et al., 2017). It is noteworthy that in planktonic bacteria, spoT genes were upregulated, while *spoT* genes in biofilm bacteria were downregulated, which might be attributed to the characteristics of the bifunctional hydrolase in *spoT* (Bury-Moné et al., 2004): guanosine tetraphosphate ((p)ppGpp) that was the key to bacterial biofilm formation could be both hydrolyzed and promoted; therefore, it could be inferred that in suspended bacteria, *SpoT* might hydrolyze (p)ppGpp and promote the generation thereof after biofilm formation (Wang et al., 2011). The efflux pump is also one of the mechanisms leading to the antimicrobial properties of biofilms (Atkinson et al., 2011). *Hp1174* is a gene of the major facilitator superfamily (MFS) efflux pump family. Studies have found that *spoT* and *Hp1174* genes were involved in the formation of biofilms (De-Kievit et al., 2001). The phillygenin alluded to in this research was found to inhibit the formation of biofilms and downregulate the *spoT* and *Hp1174* genes that regulated biofilm formation. *SpoT, Hp1174, lptB, Nhac,* and *MATE* genes are all transmembrane proteins, indicating that the mechanism of phillygenin might also inhibit the formation of biofilms and change the permeability of the membranes. The *Caggamma (Cag4)* gene, which is a lytic transglycosylase encoded by the Cag pathogenicity island, can hydrolyze peptidoglycan layer of bacteria, release intracellular proteins into the periplasmic space, promote the assembly and formation of the IV secretion system, help the host evade immune detection, and contribute to the long-term colonization of bacteria (Lai et al., 2017). Phillygenin was shown to downregulate the Caggamma genes and prevent bacterial colonization. The spatial organization of the population, such as biofilm, was found to increase the production of some virulence factors (Ge et al., 2018); therefore, phillygenin that downregulated the *Caggamma* genes of the virulence factors could partly confirm the inhibitory effect of phillygenin on biofilms.

ATP was found to provide energy in many key cellular processes and reactions (Quinn et al., 2021). The decrease of ATP level suggested that the functions of mitochondria were impaired, and the cells would therefore undergo apoptosis and necrosis (Arya et al., 2019). The Lpt protein family was found to be required in the export of lipopolysaccharide (LPS) to the cell surface (Turkina et al., 2015). The results of this study suggested that the drug action of phillygenin on *H. pylori* would cause the leakage of intracellular ATP, the degree of which was positively correlated with the concentration of phillygenin. This might be associated with the upregulation of the lptB genes (Sperandeo et al., 2007). The *LptB* gene was

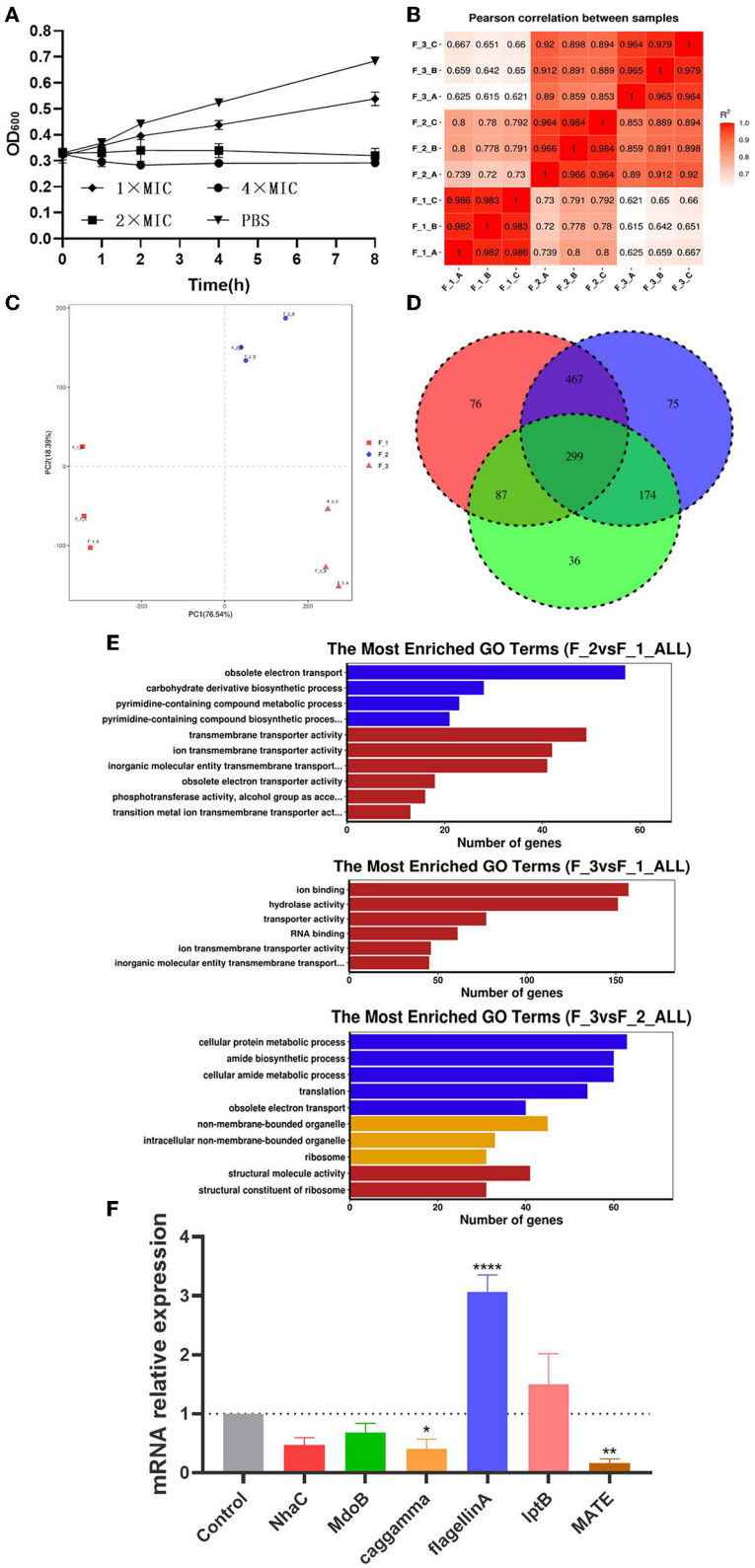


**FIGURE 7 |** Phillygenin inhibiting *Helicobacter pylori* by Transcriptome analysis. **(A)** The half inhibitory concentration curve; **(B)** the RNA-Seq correlation analysis; **(C)** the principal component analysis (PCA); **(D)** volcano (red represents the upregulation, green represents the downregulation, and blue represents no change); **(E)** the gene ontology (GO) enrichment analysis histogram of differentially expressed genes (blue represents biological processes, yellow represents the cellular component, and red represents the molecular function); **(F)** the changes in the relative expression quantity of the mRNA of differentially expressed genes detected by the transcriptome sequencing in suspended bacteria treated with phillygenin at a concentration of two times of the MIC. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

**TABLE 3 |** Differentially expressed genes.

| Gene | Name | Log2 fold change | Description | Enrichment pathway |
|---|---|---|---|---|
| *Nhac* | HPG27_RS04635 | −7.1404 | Sodium:protonantiporter | The inorganic molecular entity transmembrane transporter activity |
| *Caggamma* | HPG27_RS02515 | −5.0855 | Sodium:calciumantiporter | The epithelial cell signaling in Helicobacter pylori infection |
| *MATE* | HPG27_RS03695 | −5.9103 | MATEfamilyeffluxtransporter | The transmembrane transporter activity |
| *MdoB* | HPG27_RS02805 | −7.4155 | LTAsynthasefamilyprotein | The sulfuric ester hydrolase activity |
| *flagellinA* | HPG27_RS02925 | 4.0196 | FlagellinA | The two-component system/Flagellar assembly |
| *lptB* | HPG27_RS03465 | 5.3738 | LPSexportABCtransporterATP-bindingprotein | The ABC transporters |

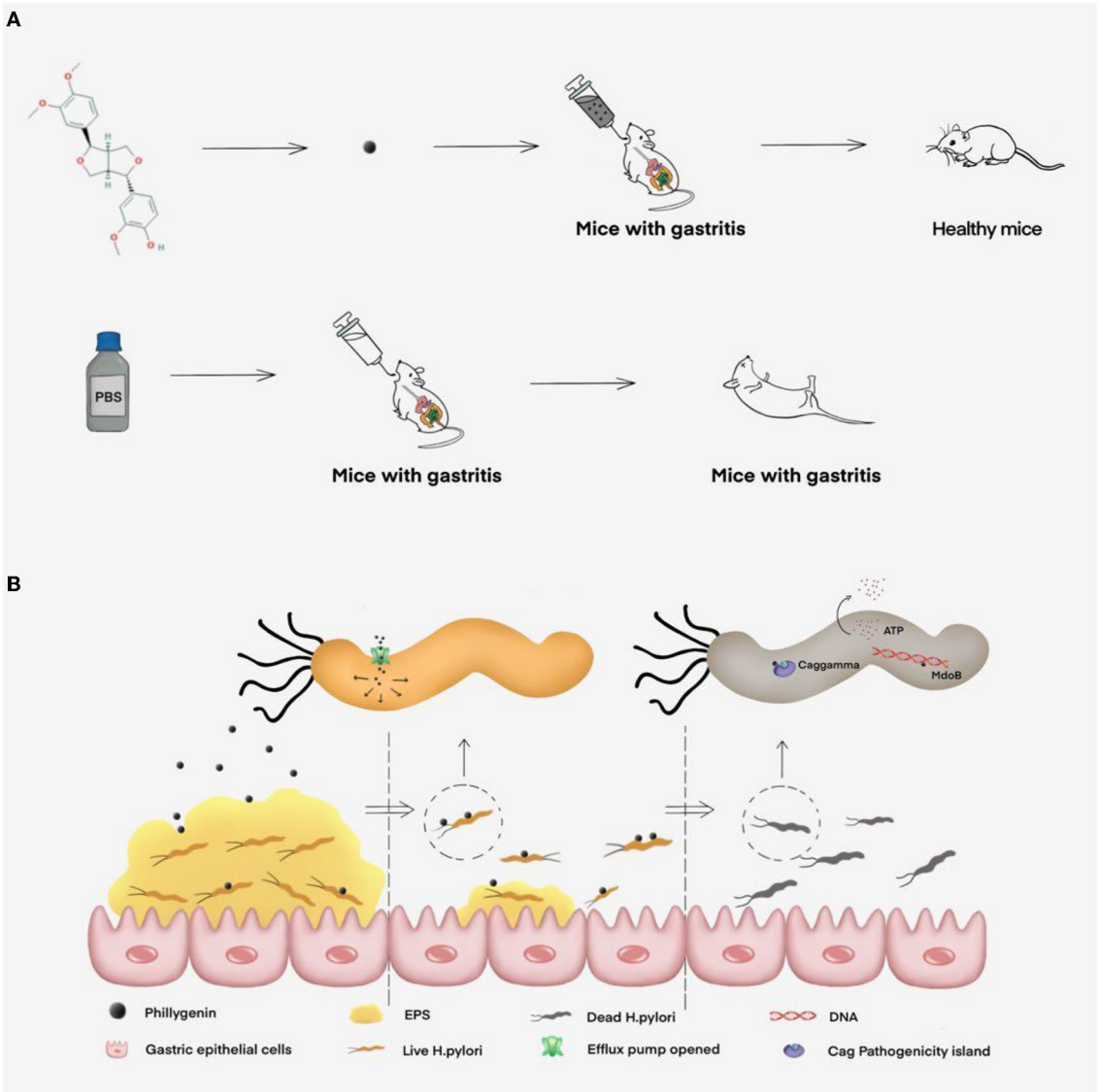


**FIGURE 8 |** The inhibitory effect of phillygenin on *H. pylori* and its mechanism map. **(A)** The therapeutic effect of phillygenin on *H. pylori*-infected acute gastritis in mice and **(B)** the mechanism of phillygenin, mainly including inhibiting biofilm formation, causing ATP leakage, weakening *H. pylori*'s virulence, etc.

found to bind to the transmembrane *LptFG* complex on the cytoplasmic side to hydrolyze ATP, thereby providing energy to accelerate the transport of ATP (Martorana et al., 2016). The leakage of ATP alluded to in this research, which might be associated with the upregulation of the *LptB* genes by phillygenin, was consistent in the phenotype with those of the ATP metabolism, acid resistance, and inhibition of biofilm formation in the above.

In the transcriptome sequencing, it was found that the drug action of phillygenin on *H. pylori*, *Nhac, MATE,* and *MdoB* genes was downregulated and *flagellin A* genes upregulated. The *MATE* genes, which were found to mainly regulate the efflux of drugs, could be significantly downregulated by phillygenin to decrease the efflux of drugs. The *MdoB* gene, which is a kind of DNA methyltransferase, is involved in DNA methylation (Tan et al., 2016). The downregulation of this gene, however, indicates the damage of DNA and cell death. The *Flagellin A (FlaA)* gene not only regulates the composition of mastigoneme but is also one of the main antigens that produce the serum IgG and gastrointestinal IgA (Zarei et al., 2017). The upregulation of *FlaA* genes, however, may be associated with the immune response produced by the body. In addition, the antiadhesion effects and *in vivo* oxidation (ROS) were studied, both of which did not work effectively at low concentrations but worked effectively under high concentrations, as show in **Supplementary Figures 1, 2**. Although biofilm-related genes were not preferentially revealed in transcriptome sequencing, this may be related to the drug action time because the strains extracted by transcriptome sequencing were treated with drugs for 8 h, while the strains extracted during biofilm-related gene detection were treated with drugs for 4 h.

## CONCLUSION AND OUTLOOK

Phillygenin has good antibacterial effects *in vivo* and *in vitro* by causing ATP leakage and inhibit the biofilm formation (**Figure 8**), but its mechanism of action is multiple targets and pathways, which require further experimental exploration. In addition, phillygenin has the advantage of low toxicity, a difficulty in making *H. pylori* form a drug resistance, and a specific drug action on *H. pylori* and is a drug with great application potential. This study can provide experimental basis for phillygenin-inhibiting *H. pylori* and ideas for the clinical treatment of *H. pylori* and development of new drugs.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI; PRJNA802695.

## ETHICS STATEMENT

The animal study was reviewed and approved by SYXK Gui 2017-0004.

## AUTHOR CONTRIBUTIONS

R-JL was responsible for the experimental research. CQ, G-RH, and L-JL performed to consult literature and write the first draft. X-QM and Y-QH designed, checked, modified, and finalized the manuscript. All authors proofed the revised manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.863624/full#supplementary-material

## REFERENCES

Amnon, S., Richard, H. L., and Robert, M. G. (2010). A national study of *Helicobacter pylori* infection in gastric biopsy specimens. *Gastroenterology* 139, 1894–1901.e4. doi: 10.1053/j.gastro.2010.10.033

Arya, T., Oudouhou, F., Casu, B., Bessette, B., Sygusch, J., and Baron, C. (2019). Fragment-based screening identifies inhibitors of ATPase activity and of hexamer formation of Cagα from the *Helicobacter pylori* type IV secretion system. *Sci Rep*. (2019) 9:6474. doi: 10.1038/s41598-019-42876-6

Atkinson, G. C., Tenson, T., and Hauryliuk, V. (2011). The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. *PLoS ONE*. 6, e23479. doi: 10.1371/journal.pone.0023479

Bae, W. Y., Kim, H. Y., Yu, H. S., Chang, K. H., Hong, Y. H., Lee, N. K., et al. (2020). Antimicrobial effects of three herbs (*Brassica juncea*, *Forsythia suspensa*, and *Inula britannica*) on membrane permeability and apoptosis in Salmonella. *J. Appl. Microbiol.* 130, 394–404. doi: 10.1111/jam.14800

Bahareh, A., Tahereh, F., and Nassim, G. (2017). Effect of biofilm formation by clinical isolates of *Helicobacter pylori* on the efflux-mediated resistance to commonly used antibiotics. *World J. Gastroenterol.* 23, 1163–1170. doi: 10.3748/wjg.v23.i7.1163

Bury-Moné, S., Thiberge, J. M., Contreras, M., Maitournam, A., Labigne, A., and De Reuse, H. (2004). Responsiveness to acidity *via* metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. *Mol. Microbiol.* 53, 623–638. doi: 10.1111/j.1365-2958.2004.04137.x

Cai, Y., Wang, C., Chen, Z., Xu, Z., Li, H., Li, W., et al. (2020). Transporters HP0939, HP0497, and HP0471 participate in intrinsic multidrug resistance and biofilm formation in Helicobacter pylori by enhancing drug efflux. *Helicobacter* 25, 12715. doi: 10.1111/hel.12715

De-Kievit, T. R., Parkins, M. D., Gillis, R. J., Srikumar, R., Ceri, H., and Poole, K. (2001). Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in Pseudomonas aeruginosa biofilms. *Antimicrob Agents Chemother*. 45, 1761–70. doi: 10.1128/AAC.45.6.1761-1770.2001

Dong, X., Chen, Z. L., and Li, X. (2016). The effect of Shengdi Lianqiao Recipe in controlling dental plaque on the clearance of *Helicobacter pylori* in patients with gastritis. *Sichuan Tradit. Chin. Med.* 34, 80–82.

Ge, X., Cai, Y., Chen, Z., Gao, S., Geng, X., Li, Y., et al. (2018). Bifunctional enzyme SpoT is involved in biofilm formation of *Helicobacter pylori* with multidrug resistance by upregulating efflux pump Hp1174 (gluP). *Antimicrob. Agents Chemother.* 62, e00957-18. doi: 10.1128/AAC.00957-18

Hathroubi, S., Hu, S., and Ottemann, K. M. (2020). Genetic requirements and transcriptomics of Helicobacter pylori biofilm formation on abiotic

and biotic surfaces. *NPJ Biofilms Microb.* 6, 56–56. doi: 10.1038/s41522-020-00167-3

Hathroubi, S., Mekni, M. A., Domenico, P., Nguyen, D., and Jacques, M. (2017). Biofilms: microbial shelters against antibiotics. *Microb Drug Resist.* 23, 147–156. doi: 10.1089/mdr.2016.0087

Hathroubi, S., Servetas, S. L., Windham, I., Merrell, D. S., and Ottemann, K. M. (2018). *Helicobacter pylori* biofilm formation and its potential role in pathogenesis. *Microbiol. Mol. Biol. Rev.* 82, e00001–18. doi: 10.1128/MMBR.00001-18

Hooi, J. K. Y., Lai, W. Y., Ng, W. K., Suen, M. M. Y., Underwood, F. E., Tanyingoh, D., et al. (2017). Global prevalence of *Helicobacter pylori* infection: systematic review and meta-analysis. *Gastroenterology*. 153:420–429. doi: 10.1053/j.gastro.2017.04.022

Huang, Y., Hang, X., Jiang, X., Zeng, L., Jia, J., Xie, Y., et al. (2019). *In vitro* and *In vivo* activities of zinc linolenate, a selective antibacterial agent against *Helicobacter pylori*. *Antimicrob Agents Chemother*. 63, e00004–19. doi: 10.1128/AAC.00004-19

Krzyzek, P., Junka, A., Słupski, W., Dołowacka-Józwiak, A., Płachno, B. J., Sobiecka, A., et al. (2021). Antibiofilm and antimicrobial-enhancing activity of Chelidonium majus and Corydalis cheilanthifolia extracts against multidrug-resistant *Helicobacter pylori*. *Pathogens* 10, 1033. doi: 10.3390/pathogens10081033

Krzyzek, P., Grande, R., Migdał, P., Paluc, E, and Gościniak, G. (2020). Biofilm formation as a complex result of virulence and adaptive responses of *Helicobacter pylori*. *Pathogens* 9, 1062. doi: 10.3390/pathogens9121062

Lai, S. J., Tu, I. F., Wu, W. L., Yang, J. T., Luk, L. Y. P., and Lai, M. C. (2017). Site-specific His/Asp phosphoproteomic analysis of prokaryotes reveals putative targets for drug resistance. *BMC Microbiology* 17(1). doi: 10.1186/s12866-017-1034-2

Li, Y., Lin, R., Jin, Y., Jin, S., Chen, B., and Wu, X. (2021). Genotyping *Helicobacter pylori* antibiotic resistance and virulence-associated genes in patients with gastric cancer in Wenzhou, China. *Arab. J. Gastroenterol.* 22, 267–271. doi: 10.1016/j.ajg.2021.05.017

Liu, W. Z., Xie, Y., Lu, H., Cheng, H., Zeng, Z. R., Zhou, L. Y., et al. (2018). Chinese society of gastroenterology, Chinese study group on *Helicobacter pylori* and peptic ulcer. Fifth Chinese National Consensus Report on the management of Helicobacter pylori infection. *Helicobacter.* 23, e12475. doi: 10.1111/hel.12475

Lu, Z. B., Liu, S. H., Ou, J. Y., Cao, H. H., Shi, L. Z., Liu, D. Y., et al. (2020). Corrigendum to "Forsythoside A inhibits adhesion and migration of monocytes to type II alveolar epithelial cells in lipopolysaccharide-induced acute lung injury through upregulating miR-124" [TOXICOL APPL PHARM 407 (2020) 115252]. *Toxicol. Appl. Pharmacol.* 409, 115328–115328. doi: 10.1016/j.taap.2020.115328

Martorana, A. M., Benedet, M., Maccagni, E. A., Sperandeo, P., Villa, R., Dehò, G., et al. (2016). Functional interaction between the cytoplasmic ABC protein LptB and the inner membrane LptC protein, components of the lipopolysaccharide transport machinery in *Escherichia coli*. *J. Bacteriol.* 198, 2192–2203. doi: 10.1128/JB.00329-16

Nagy, P., Johansson, S., and Molloy-Bland, M. (2016). Systematic review of time trends in the prevalence of *Helicobacter pylori* infection in China and the USA. *Gut. Pathog.* 8, 8. doi: 10.1186/s13099-016-0091-7

Okuda, S., Sherman, D. J., Silhavy, T. J., Ruiz, N., and Kahne, D. (2016). Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model. *Nat. Rev. Microbiol.* 14, 337–345. doi: 10.1038/nrmicro.2016.25

Plummer, M., de Martel, C., Vignat, J., Ferlay, J., Bray, F., and Franceschi, S. (2016). Global burden of cancers attributable to infections in 2012:a synthetic analysis. *Lancet Global Health* 4, e609–616. doi: 10.1016/S2214-109X(16)30143-7

Quan, X. H., Liu, H. H., Ye, D. M., Ding, X. L., and Su, X. L. (2021). Forsythoside A alleviates high glucose-induced oxidative stress and inflammation in podocytes by inactivating MAPK signaling *via* MMP12 inhibition. *Diabetes Metab. Synd. Obesity Targets Ther.* 14, 1885–1895. doi: 10.2147/DMSO.S305092

Quinn, R. J., Barraza, I., García-Diéguez, L., Pajon, C., Krausfeldt, L. E., Ibrahim, K., et al. (2021). Periodically disturbing the spatial structure of biofilms can affect the production of an essential virulence factor in *Pseudomonas aeruginosa*. *mSystems*. 6, e0096121. doi: 10.1128/mSystems.00961-21

Rizzato, C., Torres, J., Kasamatsu, E, Camorlinga, P. M., Bravo, M. M., Canzian, F., et al. (2019). Potential role of biofilm formation in the development of digestive tract cancer with special reference to *Helicobacter pylori* infection. *Front. Microbiol.* 10, 846. doi: 10.3389/fmicb.2019.00846

Seung, J. S., Chan, E. P., Nam, I. B., In, S. C., and Chang, H. P. (2009). Betulinic and oleanolic acids isolated from Forsythia suspensa Vahl inhibit urease activity of *Helicobacter pylori*. *Biotechnol. Bioprocess Eng.* 14, 140–145. doi: 10.1007/s12257-008-0272-4

Sperandeo, P., Cescutti, R., Villa, R., Di Benedetto, C., Candia, D., Dehò, G., et al. (2007). Characterization of lptA and lptB, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. *J. Bacteriol.* 189, 244–253. doi: 10.1128/JB.01126-06

Sugano, K., Tack, J., Kuipers, E. J., Graham, D. Y., El-Omar, E. M., Miura, S., et al. (2015). Kyoto global consensus report on *Helicobacter pylori* gastritis. *Gut.* 64, 1353–1367. doi: 10.1136/gutjnl-2015-309252

Sultan, S., Ahmed, S. I., Murad, S., and Irfan, S. M. (2016). Primary versus secondary immune thrombocytopenia in adults; a comparative analysis of clinical and laboratory attributes in newly diagnosed patients in Southern Pakistan. *Med. J. Malaysia.* 71, 269–274.

Sun, C. Z., Ma, N., Wang, Y. J., Li, C., and Pei, Y. H. (2021). Isolation and identification of the chemical components of Forsythia suspense. *Chinese J. Med. Chem.* 31, 286–291.

Tan, A., Hill, D. M., Harrison, O. B., Srikhanta, Y. N., Jennings, M. P., Maiden, M. C., et al. (2016). Distribution of the type III DNA methyltransferases modA, modB and modD among Neisseria meningitidis genotypes: implications for gene regulation and virulence. *Sci. Rep.* 6, B64–B70. doi: 10.1038/srep21015

Turkina, M. V., Olofsson, A., Magnusson, K. E., Arnqvist, A., and Vikström, E. (2015). *Helicobacter pylori* vesicles carrying CagA localize in the vicinity of cell-cell contacts and induce histone H1 binding to ATP in epithelial cells. *FEMS Microbiol. Lett.* 362, fnv076. doi: 10.1093/femsle/fnv076

Wang, C., Ma, C., Fu, K., Gong, L. H., Zhang, Y. F., Zhou, H. L., et al. (2021). Phillygenin attenuates carbon tetrachloride-induced liver fibrosis *via* modulating inflammation and gut microbiota. *Front. Pharmacol.* 12, 756924–756924. doi: 10.3389/fphar.2021.756924

Wang, W., Zhong, Q., Xie, L., and Shao, S. (2011). Heterogenous expression and characterization of cag4 in *Helicobacter pylori*. *Wei sheng wu xue bao = Acta Microbiol. Sinica*. 51, 630–6.

Wu, Y., Song, Z., Deng, G., Jiang, K., Wang, H., Zhang, X., et al. (2021). Gastric acid powered nanomotors release antibiotics for *in vivo* treatment of *Helicobacter pylori* infection. *Small* 17, 877. doi: 10.1002/smll.202006877

Yang, Q., Shang, Q., Wei, G. Q., Guo, Q. H., Ka, S. Q., Zhang, Z. Y., et al. (2019). Jinghuaweikang capsules combined with Quadruple therapy in the treatment of *Helicobacter pylori* infection:a multicenter, randomized, controlled, clinical study. *Zhonghua yi xue za zhi.* 99, 295–300. doi: 10.3760/cma.j.issn.0376-2491.2019.04.012

Yuan, J. F., Zhao, J. F., Sun, J. J., He, L. M., Zhang, Z. Q., and Liu, H. (2015). Determination of flavonoids and triterpene acids in forsythia suspensa leaves from Henan and Shanxi Province. *Food Sci.* 36, 164–167.

Zarei, M., Mosayebi, G., Khansarinejad, B., and Abtahi, H. (2017). Antigenic and immunogenic evaluation of Helicobacter pylori FlaA epitopes. *Iran. J. Basic Med. Sci.* 20, 920–926. doi: 10.22038/IJBMS.2017.9115

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# 1,4-Dihydropyridine as a Promising Scaffold for Novel Antimicrobials Against *Helicobacter pylori*

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The increasing occurrence of multidrug-resistant strains of the gastric carcinogenic bacterium *Helicobacter pylori* threatens the efficacy of current eradication therapies. In a previous work, we found that several 1,4-dihydropyridine (DHP)-based antihypertensive drugs exhibited strong bactericidal activities against *H. pylori* by targeting the essential response regulator HsrA. To further evaluate the potential of 1,4-DHP as a scaffold for novel antimicrobials against *H. pylori*, we determined the antibacterial effects of 12 novel DHP derivatives that have previously failed to effectively block L- and T-type calcium channels. Six of these molecules exhibited potent antimicrobial activities (MIC ≤ 8 mg/L) against three different antibiotic-resistant strains of *H. pylori*, while at least one compound resulted as effective as metronidazole. Such antimicrobial actions appeared to be specific against *Epsilonproteobacteria*, since no deleterious effects were appreciated on *Escherichia coli* and *Staphylococcus epidermidis*. The new bactericidal DHP derivatives targeted the *H. pylori* regulator HsrA and inhibited its DNA binding activity according to both *in vitro* and *in vivo* analyses. Molecular docking predicted a potential druggable binding pocket in HsrA, which could open the door to structure-based design of novel anti-*H. pylori* drugs.

**Keywords: *Helicobacter pylori*, HsrA, hexahydroquinoline, novel antimicrobial drugs, antibiotic resistance, dihydropyridine**

## INTRODUCTION

Chronic infection of the human gastric mucosa with the microaerophilic Gram-negative bacterium *Helicobacter pylori* can cause a variety of upper gastrointestinal diseases, including chronic gastritis, peptic ulcer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer (GC; Kusters et al., 2006). Notably, more than a half of the world's population is

estimated to be infected with this microbial type I carcinogen (World Health Organization, 1994; Hooi et al., 2017). Unless properly treated, about 1% of the *H. pylori* infected people is estimated to develop GC along their lives (Kuipers, 1999). This calculation means that more than 4 million persons worldwide are currently at high risk or actually suffering GC due to untreated or mistreated *H. pylori* infections.

Eradication of *H. pylori* infection significantly reduces the risk of GC (Leung et al., 2018; Nam et al., 2019), even in persons with a family history of this malignancy in first-degree relatives (Choi et al., 2020). However, the increasing occurrence of multidrug-resistant strains of this clinically relevant pathogen worldwide begins to limit the efficacy of current eradication therapies (Boyanova et al., 2016, 2019; Savoldi et al., 2018). In 2017, the WHO included *H. pylori* in its first ever list of antibiotic-resistant "priority pathogens," a catalogue of 12 families of bacteria that pose at present the greatest threat to human health, for which novel classes of antibiotics are urgently needed (Tacconelli et al., 2018). As consequence, multiple efforts are being made to discover and develop new therapeutic options, including both drug repurposing and *de novo* identification of bioactive compounds directed against novel and validated therapeutic targets in *H. pylori* (Salillas et al., 2019; González et al., 2019a,b, 2020, 2021; Roszczenko-Jasinska et al., 2020; Salillas and Sancho, 2020).

The *H. pylori* OmpR-like "orphan" response regulator HsrA (also known as HP1043) constitutes a promising therapeutic target (González et al., 2019a,b). This protein is unique and highly conserved among *Epsilonproteobacteria* (Muller et al., 2007), and its expression appears essential for cell viability (Beier and Frank, 2000; McDaniel et al., 2001). HsrA modulates the transcription of a plethora of genes and operons involved in relevant physiological processes including translation, transcription, chemiotaxis, energy metabolism, nitrogen metabolism and redox homeostasis (Delany et al., 2002; Olekhnovich et al., 2014; Pelliciari et al., 2017). Hence, the protein acts as a global homeostatic regulator, synchronizing metabolic functions and virulence with the availability of nutrients and cell division. In a previous work, we found that several 1,4-dihydropyridine (DHP)-class antihypertensive and highly prescribed drugs, such as nifedipine, nicardipine, nisoldipine, nimodipine, nitrendipine, and lercanidipine acted as low-molecular-weight ligands of HsrA and noticeably inhibited its *in vitro* DNA binding activity (González et al., 2019a). Some of these HsrA inhibitors exhibited potent bactericidal actions against different strains of *H. pylori*, including both clarithromycin- and metronidazole-resistant strains, and showed additive interactions with first-line antibiotics in checkerboard assays. Experimental therapies with 100 mg/kg/day of marketed formulations of nimodipine or nitrendipine, in combination with omeprazole (140 mg/kg/day) daily during 7 days, led to significant reductions in the *H. pylori* (strain PMSS1) gastric colonization in mice (González et al., 2019a). These results strongly supported the use of 1,4-DHPs as novel repurposable antimicrobial drugs against *H. pylori*; however, the repositioning of these antihypertensive drugs as antimicrobials could be linked to undesirable side effects associated with their intrinsic vasodilatation action and therefore the occurrence of potential hypotensive effects in both hypertensive and non-hypertensive patients. In this context, the use of 1,4-DHP as a scaffold for novel derivatives with similar or enhanced antimicrobial actions and mitigated side effects appears as a promising strategy for the development of novel antibiotics.

A new class of DHP derivatives, in which substituted cyclohexane rings are fused to 1,4-DHP forming a hexahydroquinoline (HHQ) group, has been previously achieved *via* modified Hantzsch reactions and tested for their L- and T-type calcium channel blocking activities by whole-cell patch clamp technique (Schaller et al., 2018; Aygün Cevher et al., 2019). Notably, some of these DHP-based HHQ derivatives failed to effectively block L- and T-type calcium channels. In the present study, we determined the anti-*H. pylori* activities of 12 condensed DHPs with no significant effect on calcium channels, and analyzed how different substituents on the molecule backbone affected the antimicrobial activity. At least six of these DHP derivatives demonstrated potent bactericidal activities against three antibiotic-resistant strains of *H. pylori*. As previously observed with commercial 1,4-DHP class antihypertensive drugs, the new DHPs appeared to target the essential response regulator HsrA according to both *in vitro* and *in vivo* evidences. The results further support the use of 1,4-DHP as a promising scaffold for novel antimicrobial drugs against *H. pylori*.

# MATERIALS AND METHODS

## Chemicals

DHP-based HHQ derivatives were synthesized by the reaction of dimethyl-1,3-cyclohexanedione, substituted benzaldehyde, appropriate alkyl acetoacetate, and ammonium acetate as previously described (Schaller et al., 2018; Aygün Cevher et al., 2019), and stored as neat solid compounds at −20°C in amber tubes until use. DHP-class antihypertensive drugs were purchased from Sigma-Aldrich (Saint Louis, MO, United States), and properly stored according to the manufacturer's instructions. Stock solutions of each 1,4-DHP derivative were freshly prepared at 20 mM in 100% dimethyl sulfoxide (DMSO) for electrophoretic mobility shift assays and isothermal titration calorimetry analyses, and at 10.24 g/L in 100% DMSO for determination of minimal inhibitory/bactericidal concentrations. Since DHPs are light-sensitive compounds, all stock solutions were protected from light. Metronidazole, clarithromycin, levofloxacin, and ampicillin were obtained from Sigma-Aldrich. Stock solutions of these antibiotics in 100% DMSO were prepared at 10.24 g/L and stored at −20°C for up to 30 days.

## Bacterial Strains and Culture Conditions

*Helicobacter pylori* reference strains ATCC 43504 (metronidazole-resistant) and ATCC 700684 (clarithromycin-resistant) were purchased from the American Type Culture Collection (Rockville, MD, United States). The *H. pylori* clinical isolate Donostia 2, resistant to levofloxacin, was isolated from gastroduodenal biopsies at Donostia University Hospital (San Sebastian, Spain). The *H. pylori* strain 26695 (ATCC 700392) was used in some

experiments. All *H. pylori* strains were routinely grown in Blood Agar Base No. 2 (OXOID, Basingstoke, United Kingdom) supplemented with 8% defibrinated horse blood (OXOID) in a humidified microaerobic incubator (85% $N_2$, 10% $CO_2$, and 5% $O_2$) at 37°C for 48–72 h. For certain experiments, bacteria were grown for 48–72 h at 37°C in brain heart infusion broth (OXOID) supplemented with 4% fetal bovine serum (Gibco, Carlsbad, CA, United States).

*Escherichia coli* ATCC 25922 and *Staphylococcus epidermidis* ATCC 12228 were kindly provided by Jose Antonio Aínsa (University of Zaragoza), and belong to the microbial culture collection of the Department of Microbiology, from this university. Both strains were routinely cultured overnight in Mueller-Hilton agar/broth (PanReac AppliChem, Barcelona, Spain) at 37°C.

## Minimal Inhibitory and Bactericidal Concentrations

Minimal inhibitory concentrations (MICs) were determined by the microdilution method using sterile 96-well flat-bottom microtiter plates as previously described (González et al., 2020), with slight modifications. Briefly, inoculum suspensions of *H. pylori* strains ATCC 43504 (metronidazole-resistant), ATCC 700684 (clarithromycin-resistant), and Donostia 2 (levofloxacin-resistant) were freshly prepared from cultures grown during 48 h at 37°C on Blood Agar Base No. 2 supplemented with 8% defibrinated horse blood under microaerobic conditions (85% $N_2$, 10% $CO_2$, and 5% $O_2$). Bacterial growth from two blood agar plates was aseptically resuspended in 10 ml of brain heart infusion (BHI) broth supplemented with 4% fetal bovine serum (BHI + FBS) and next diluted to $OD_{600} = 0.01$ [~$10^6$ colony forming units (CFU) per ml] in the same medium. A range of concentrations from 64 to 0.031 mg/L was tested for each 1,4-DHP derivative against each *H. pylori* strain. DMSO (vehicle) and conventional antibiotics were included as controls in all assays. Microtiter plates were incubated under microaerobic conditions at 37°C for 48 h; then, inhibition of microbial growth was colorimetrically revealed after the addition of filter-sterilized resazurin (Sigma-Aldrich, Saint Louis, MO, United States) to a final concentration of 0.01 mg/ml, and further incubation of 6 h. To determine the minimal bactericidal concentration (MBC), 10 µl aliquots of several dilutions around the MIC value were aseptically seeded on blood agar plates and incubated at 37°C for 72 h under microaerobic conditions. Each experiment was performed twice in triplicate.

Antimicrobial activities of selected compounds against the *E. coli* reference strain ATCC 25922 and the *S. epidermidis* reference strain ATCC 12228 were determined according to the EUCAST Guidelines [European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society for Clinical Microbiology and Infectious Diseases (ESCMID), 2003]. Briefly, standardized inoculums equivalent to a 0.5 McFarland standard turbidity (~$1.5 \times 10^8$ CFU per ml) were freshly prepared from overnight colonies on Mueller–Hinton agar plates of both microorganisms. Final bacterial suspensions at $5 \times 10^5$ CFU per ml in Mueller–Hinton broth were faced to a range of concentrations from 64 to 0.031 mg/L of selected DHP derivatives. DMSO and ampicillin were included as controls in all assays. Plates were incubated at 37°C overnight, and MIC values were colorimetrically defined by the addition of 0.01 mg/ml resazurin. Aliquots were seeded on Mueller–Hinton agar for MBC determinations. Experiments were performed twice in triplicate.

## Time-Kill Kinetics Assays

Time-kill kinetics of selected DHP-based HHQ derivatives were conducted as previously described (González et al., 2019a). DHPs at concentrations of twice (2×) their MIC values were individually mixed with a freshly prepared suspension of *H. pylori* strain ATCC 700684 at ~$10^6$ CFU/ml in BHI + FBS. DMSO instead of DHP was used as negative control in all assays. Mixtures of bacteria and DHPs were incubated under microaerobic conditions (85% $N_2$, 10% $CO_2$, and 5% $O_2$) at 37°C with mild shaking. Aliquots of 10 µl were aseptically taken at time intervals of 0, 2, 4, 8, and 24 h after exposure, and seeded on Blood Agar Base No. 2 supplemented with 8% defibrinated horse blood. Plates were incubated at 37°C for 72 h under microaerobic conditions and CFU were determined. Experiments were performed twice in triplicate, and the results were presented as log10 CFU/ml versus incubation time. Statistical significances were considered if $p < 0.05$, according to the Mann–Whitney U test.

## Checkerboard Assays

Potential antimicrobial synergies between selected DHP-based HHQ derivatives and conventional antibiotics including metronidazole, clarithromycin and levofloxacin were evaluated by the checkerboard assay (González et al., 2019b). As a first step, the pairs of compounds to be evaluated were twofold serially diluted in BHI + FBS using two microtiter plates; one compound was diluted along all the rows of a first plate, while the other compound was diluted along all the columns of a second plate. Next, equal volumes of both gradients were mixed in a third microtiter plate, which were immediately inoculated with a freshly prepared bacterial suspension of *H. pylori* adjusted at $2 \times 10^6$ CFU/ml in BHI + FBS. Plates were incubated at 37°C for 48 h under microaerobic conditions, and then, the microbial growth was colorimetrically revealed by the addition of 0.01 mg/ml resazurin. Fractional inhibitory concentration index (FICI) was calculated as $FIC_A$ ($MIC_A$ in the presence of $B/MIC_A$ alone) + $FIC_B$ ($MIC_B$ in the presence of $A/MIC_B$ alone), where A and B represent two different antimicrobial agents. Values of $FICI \leq 0.5$, $0.5 < FICI \leq 1$, $1 < FICI \leq 4$, and $FICI > 4$ indicate synergistic, additive, neutral or antagonist interactions, respectively (González et al., 2019a,b).

## HsrA Expression and Purification

The HsrA response regulator from *H. pylori* strain 26695 (ATCC 700392) was overexpressed in *E. coli* BL21(DE3), and purified by immobilized metal-affinity chromatography (IMAC) according to previously described procedures (González et al., 2019b). The recombinant protein was finally dialyzed against the store buffer [50 mM Tris–HCl (pH 8), 300 mM NaCl, 10% glycerol] and conserved at −20°C until use. Protein concentration was determined using the BCA™ Protein Assay kit (Thermo Fisher Scientific, Bothell, WA, United States).

## Electrophoretic Mobility Shift Assays

The inhibitory action of DHP-based HHQ derivatives on the *in vitro* DNA binding activity of HsrA was assessed by electrophoretic mobility shift assay (EMSA), as previously described (González et al., 2019b). Briefly, recombinant HsrA protein (5 μM) was mixed with 120 ng of its target promoter (P*porGDAB*) in a 20 μl reaction volume containing 10 mM bis-Tris (pH 7.5), 40 mM KCl, 100 mg/L BSA, 1 mM DTT and 5% glycerol, in the presence of 3, 2, and 1 mM of each 1,4-DHP derivative. DMSO instead of DHP was included as vehicle control, while an internal sequence of the gene *pkn22* (*alr2502*) from *Anabaena* sp. PCC 7120 was used as non-specific competitor DNA in all assays. Mixtures were incubated at room temperature for 20 min and subsequently separated in a 6% native polyacrylamide gel electrophoresis. Gels were stained with SYBR Safe® (Thermo Fisher Scientific) and analyzed by using a Bio-Rad Gel Doc 2000 Imaging System (Bio-Rad Laboratories, Hercules, CA, United States).

## Isothermal Titration Calorimetry Assays

Isothermal titration calorimetry (ITC) experiments were carried out in an Auto-iTC200 calorimeter (MicroCal, Malvern-Panalytical, Malvern, United Kingdom) in order to determine several thermodynamic parameters of the molecular interaction between HsrA and selected DHP derivatives. The protein, located in the calorimetric cell at 20 μM, was titrated with each ligand, located in the injection syringe at 200 μM, by programming a series of 19 injections of 2 μl, with 150 s time spacing, 10 μcal/s reference power, and 750 rpm stirring speed (Velázquez-Campoy et al., 2015). Experiments were performed in buffer 50 mM Tris–HCl (pH 8), 150 mM NaCl, 10% glycerol, 1% DMSO at two different temperatures (15°C and 25°C) in order to obtain the best signal. The heat effect per injection was normalized by the amount of ligand injected, and the interaction isotherm was analyzed by nonlinear least-squares regression data analysis considering an interaction model with a single ligand binding site in the protein, using user-defined fitting routines in Origin 7.0 (OriginLab, Northampton, MA, United States).

## Molecular Docking Analysis

The tridimensional structure of the *H. pylori* HsrA response regulator (2HQR, model 1, chain A) was retrieved from the Protein Data Bank.[1] The 3D structures of selected DHPs were built in Corina Classic[2] and energy minimized using the AMMOS software (Pencheva et al., 2008). If compounds contain an unspecified stereocenter, both enantiomers were built for each ligand and used in docking studies. The protein and the ligands were prepared using the AutoDockTools 1.5.6 program. Molecular docking analyses were performed using AutoDock Vina (Trott and Olson, 2010). Rotatable bonds were defined as free for the ligands and rigid for the protein. The AutoGrid4 algorithm was used to estimate the interaction energy of each ligand pose. The pose that exhibited the lowest free energy of interaction ($\Delta G$) for each ligand was considered as its predicted model of binding to the target protein. HsrA-ligand complex structures were visualized by PyMOL.[3]

## *In vivo* Inhibition of HsrA and RNA Isolation

*Helicobacter pylori* strain 26695 (ATCC 700392) was grown during 48 h at 37°C on Blood Agar Base No. 2 supplemented with 8% defibrinated horse blood under microaerobic conditions (85% $N_2$, 10% $CO_2$, and 5% $O_2$). Cells from four blood agar plates were aseptically resuspended in BHI + FBS and diluted to 80 ml at $OD_{600} = 0.1$ (~$10^7$ CFU/ml) in the same medium. BHI broth cultures were incubated at 37°C for 24 h under microaerobic conditions in T75 culture flasks. Next, cultures were pooled and subsequently divided into equal samples of 12 ml, which were exposed either to 4 × MIC (16 mg/L) of the DHP-based HHQ derivative MD7, or to the same volume of DMSO (vehicle). After 2 h of exposure under microaerobic conditions, 1.5 ml of ice-cold RNA stop solution (95% EtOH, 5% acid-buffered phenol) was added to each culture in order to preserve RNA integrity. Bacterial cells were immediately harvested by centrifugation (10,000 rpm for 5 min) at 4°C, washed once with ice-cold 50 mM Tris–HCl pH 7.4, 100 mM EDTA, and then lysed to extract total RNA as previously described (Sarasa-Buisan et al., 2021). Genomic DNA was removed with the TURBO DNA-free™ Kit (Thermo Fisher Scientific). The absence of residual DNA in RNA preparations was assessed by qPCR. Quality of RNA samples was checked using a NanoDrop spectrophotometer (Thermo Scientific) and agarose gel electrophoresis.

## Quantitative Real-Time PCR

Reverse transcription of 2 μg of total RNA from each sample was carried out using SuperScript retrotranscriptase (Invitrogen) in 40 μl of reaction volume containing 150 ng of random primers (Invitrogen), 1 mM dNTP mix (GE Healthcare) and 10 mM DTT. Real-time PCR (qPCR) was performed using the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). Each reaction was set up in a final volume of 30 μl containing 10 ng of cDNA template, 12.5 μl of SYBR Green PCR Master Mix and 160 nM of each primer. Negative controls with no cDNA were included. The sequences of primers used for transcript quantification of selected genes are defined in **Supplementary Table S1**. Relative quantification was performed according to the ΔΔCt method (Livak and Schmittgen, 2001). Expression levels were normalized using 16S rRNA as housekeeping gene.

## Cytotoxicity and Therapeutic Index

The *in vitro* toxicity of selected DHP-based HHQ derivatives toward HeLa cells was determined by the PrestoBlue™ assay (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, cells were cultured in Dulbecco's modified

[1]https://www.rcsb.org/
[2]https://mn-am.com/
[3]https://pymol.org/

Eagle's medium containing 10% fetal bovine serum, 1% L-Glutamax solution and 1% penicillin/streptomycin solution using a humid incubator at 37°C with 5% $CO_2$ until 80% confluence was achieved. Next, cells were detached with 0.25% trypsin, counted with a Neubauer chamber, and seeded in 96-well microplates at a density of 10,000 cells per well. Then, cells were allowed to adhere for 24 h and subsequently exposed to either DMSO (1.2% final concentration) or selected DHP derivatives at a range of concentrations from 128 to 0.125 mg/L. After 24 h of exposure, the PrestoBlue cell viability reagent was added to each well at 10% v/v and plates were incubated for another 2 h under the same conditions. The absorbance of each well was measured using a Synergy HT microplate reader (Excitation, 530 nm; Emission, 590 nm, BioTek Instruments, Winooski, United States). Experiments were performed twice in triplicate. The 50% cytotoxic concentration ($CC_{50}$) was defined as the compound concentration required for the reduction of cell viability of DMSO (vehicle)-treated cell cultures by 50%, and it was calculated by regression analysis using Microsoft Excel. Therapeutic index (TI) values for each compound of interest were calculated as the ratio of the $CC_{50}$ (cytotoxic activity) to the MIC (antibacterial activity) (Pitucha et al., 2016).

## RESULTS

### DHP-Based HHQ Derivatives Exhibited Strong Bactericidal Activities Against Antibiotic-Resistant Strains of *Helicobacter pylori*

Twelve DHP derivatives in which substituted cyclohexane rings were fused to the 1,4-DHP ring leading to a condensed ring system (HHQ) were analyzed regarding their potential as novel antimicrobial candidates against *H. pylori* (**Figure 1**). All the DHP-based HHQ derivatives evaluated in this work had previously shown significant detriments in their abilities to block L- and T-type calcium channels (Schaller et al., 2018; Aygün Cevher et al., 2019). Although all of these compounds carry an HHQ core, they differ from each other by their substitution patterns at the C-4 phenyl ring, the type of the alkyl group at the C-3 ester functionality, and the position of two additional methyl substituents at C6/C7 of the HHQ ring (**Figure 1**; **Supplementary Table S2**).

MIC and MBC values of all DHP derivatives were determined against three different antibiotic-resistant strains of *H. pylori*, showing resistance to clarithromycin (ATCC 700684), metronidazole (ATCC 43504), and levofloxacin (Donostia 2). As shown in **Table 1**, at least six condensed DHP derivatives (MD1, MD2, MD6, MD7, HM4, and HM6) exhibited strong bactericidal activities against all the *H. pylori* resistant-strains tested, with MIC values in the range of 1–8 mg/L. While compounds MD10 and MD19 exhibited moderate anti-*H. pylori* activities, other DHPs like MD3, MD12, and MD13 appeared poorly effective as antimicrobials. No relevant differences were observed in the antimicrobial activities of these molecules with respect to the antibiotic-resistance pattern of the *H. pylori* strains used in the assays.

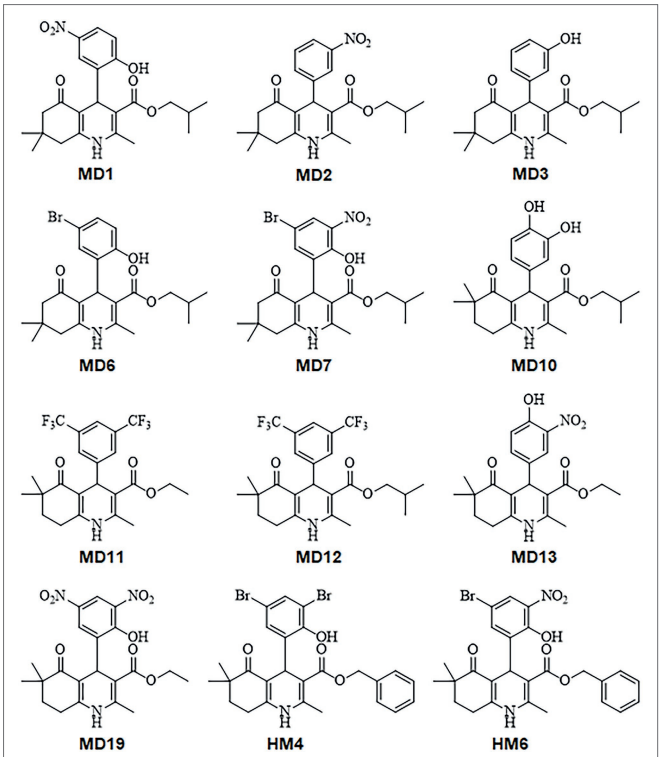



**FIGURE 1 |** Chemical structures of the 1,4-dihydropyridine (DHP)-based hexahydroquinoline (HHQ) derivatives evaluated in this work.

**TABLE 1 |** Minimal inhibitory and bactericidal concentrations of 12 DHP-based HHQ derivatives against different antibiotic-resistant strains of *H. pylori.*

| DHP | MIC (MBC), mg/L | | |
|---|---|---|---|
| | ATCC 700684 (CLR-R) | ATCC 43504 (MTZ-R) | Donostia 2 (LVX-R) |
| MD1 | 8 (8) | 8 (8) | 8 (8) |
| MD2 | 8 (8) | 8 (8) | 4 (8) |
| MD3 | >64 (>64) | 64 (64) | 64 (64) |
| MD6 | 4 (4) | 4 (4) | 4 (4) |
| MD7 | 4 (4) | 4 (8) | 4 (8) |
| MD10 | 32 (64) | 32 (32) | 32 (64) |
| MD11 | 64 (64) | 64 (64) | 32 (64) |
| MD12 | >64 (>64) | >64 (>64) | >64 (>64) |
| MD13 | >64 (>64) | 64 (>64) | >64 (>64) |
| MD19 | 32 (32) | 64 (64) | 32 (64) |
| HM4 | 2 (2) | 2 (4) | 4 (4) |
| HM6 | 1 (1) | 2 (2) | 2 (2) |
| Clarithromycin | 16 (32) | <0.03 (<0.03) | <0.03 (<0.03) |
| Metronidazole | 1 (2) | 64 (128) | 8 (8) |
| Levofloxacin | 0.125 (0.125) | 0.5 (0.5) | 16 (32) |

*MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; CLR-R, clarithromycin-resistant strain; MTZ-R, metronidazole-resistant strain; and LVX-R, levofloxacin-resistant strain.*

To further characterize the bactericidal activities of most effective DHP-based HHQ derivatives, time-kill kinetic assays were carried out by exposing the *H. pylori* strain ATCC 700684 to 2 × MIC of compounds MD1, MD2, MD6, MD7, HM4, and HM6 (**Figure 2**). Despite the fact that no CFUs

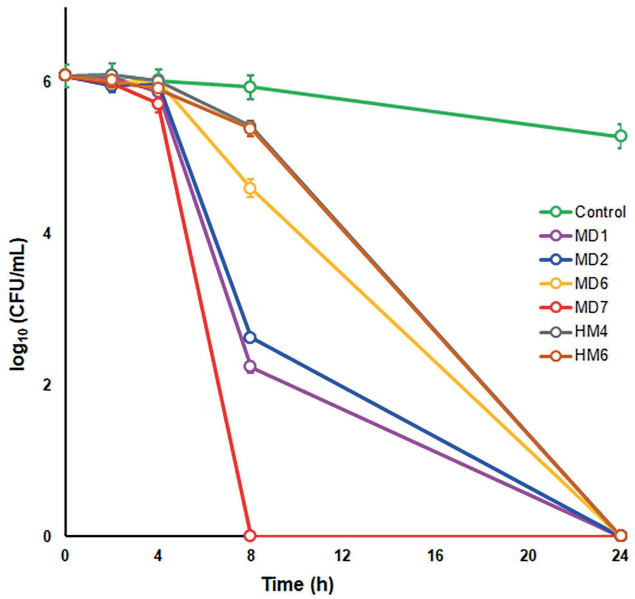



**FIGURE 2 |** Time–kill kinetics of selected DHP-based HHQ derivatives against *Helicobacter pylori* strain ATCC 700684. Bacterial counts were determined at time zero and after 2, 4, 8, and 24 h of exposure to two times the MIC of each compound. Mixtures of bacteria with dimethyl sulfoxide (DMSO; vehicle) instead of DHP were used as controls. Values are the averages of six independent determinations; vertical bars represent SDs. Please note that in some instances, the error bar is smaller than the symbols used.

could be detected after 24 h of exposure to this concentration of any of the DHP evaluated, significant differences were appreciated in the rate of killing produced by each compound from 8 h of exposure. Thus, the decline in bacterial counts occurred significantly faster ($p < 0.05$) after treatment with MD7, MD1, and MD2. Notably, MD7 was completely lethal at 8 h.

In order to preliminarily estimate undesirable side effects of these novel DHP-class antimicrobials, we determine the MIC/MBC values of MD1, MD2, MD6, MD7, HM4, and HM6 against both a Gram-negative and a Gram-positive representative species of the human normal microbiota. As shown in **Table 2**, none of the six most bactericidal DHPs against *H. pylori* exhibited relevant antimicrobial effects against *E. coli* or *S. epidermidis*, which could suggest a specific mechanism of action of these compounds against a molecular target expressed by *H. pylori* or *Epsilonproteobacteria*, not shared with other bacterial families.

The FICI values calculated after the exposure of *H. pylori* to MD1, MD2, MD6, MD7, HM4, and HM6 in combination with either clarithromycin, metronidazole or levofloxacin resulted in the range between >1 and ≤4 in all cases, according to checkerboard assays. Hence, no synergistic or additive effects appeared to occur with the use of these novel anti-*H. pylori* compounds in combination with conventional first-line antibiotics.

**TABLE 2 |** Antimicrobial activities of selected DHP-based HHQ derivatives against two representative species of the human normal microbiota.

| DHP | MIC (MBC), mg/L | |
|---|---|---|
| | ***E. coli* ATCC 25922** | ***S. epidermidis* ATCC 12228** |
| MD1 | >64 (>64) | >64 (>64) |
| MD2 | >64 (>64) | >64 (>64) |
| MD6 | >64 (>64) | >64 (>64) |
| MD7 | >64 (>64) | >64 (>64) |
| HM4 | >64 (>64) | >64 (>64) |
| HM6 | >64 (>64) | >64 (>64) |
| Ampicillin | 4 (4) | 4 (4) |

*MIC, minimal inhibitory concentration and MBC, minimal bactericidal concentration.*

## Bactericidal DHP-Based HHQ Derivatives Inhibited the Biological Activity of the *Helicobacter pylori* Essential Response Regulator HsrA

Previous studies have demonstrated that several commercially available DHP-class antihypertensive drugs act as low-molecular-weight ligands of the *H. pylori* essential response regulator HsrA and sensibly inhibited the *in vitro* DNA binding activity of this protein (González et al., 2019a). In order to evaluate *in vitro* the inhibitory action of the most effective anti-*H. pylori* DHP-based HHQ derivatives, we carried out EMSA experiments in the presence of increasing concentrations of MD1, MD2, MD6, MD7, HM4, and HM6 (**Figure 3**).

All the DHPs tested affected the *in vitro* affinity of HsrA by its target promoter P*porGDAB*. However, due to the poor solubility of DHP-based HHQ derivatives in aqueous solutions like the EMSA reaction buffer, the differences observed in the magnitude of binding inhibition could not be strictly associated with corresponding differences in the affinity of such ligands by HsrA. This thermodynamic parameter of DHP-HsrA interactions was subsequently evaluated by ITC.

In addition to the *in vitro* EMSA experiments, the inhibitory action of DHPs on the regulatory activity of HsrA *in vivo* was assessed by quantitative real-time PCR (qPCR). For this purpose, a cell suspension of *H. pylori* strain 26695 at $10^7$ CFU per ml in BHI broth +4% FBS was exposed during 2 h to 4 × MIC (16 mg/L) of the DHP-based HHQ derivative MD7. At this time, cells were treated with RNA stop solution (95% EtOH, 5% acid-buffered phenol) and total RNA was extracted. qPCR analyses were carried out in order to evaluate changes in the transcript abundance of genes *porA* (*hp1110*) and *tlpB* (*hp0103*), which have been previously recognized as targets of HsrA transcriptional activation (Olekhnovich et al., 2014). The 16S rRNA gene (*hprrnA16S*) was used as housekeeping gene, while *nixA* (*hp1077*) was included as negative control (Pelliciari et al., 2017).

Exposure of *H. pylori* cells to lethal concentrations of the HsrA inhibitor MD7 during 2 h induced a 1.7-fold decrease in the abundance of *porA* transcripts and 1.8-fold decrease in the level of *tlpB* mRNA with respect to DMSO (vehicle)-treated cells. However, the treatment with this HsrA inhibitor did not lead to an appreciable change in the

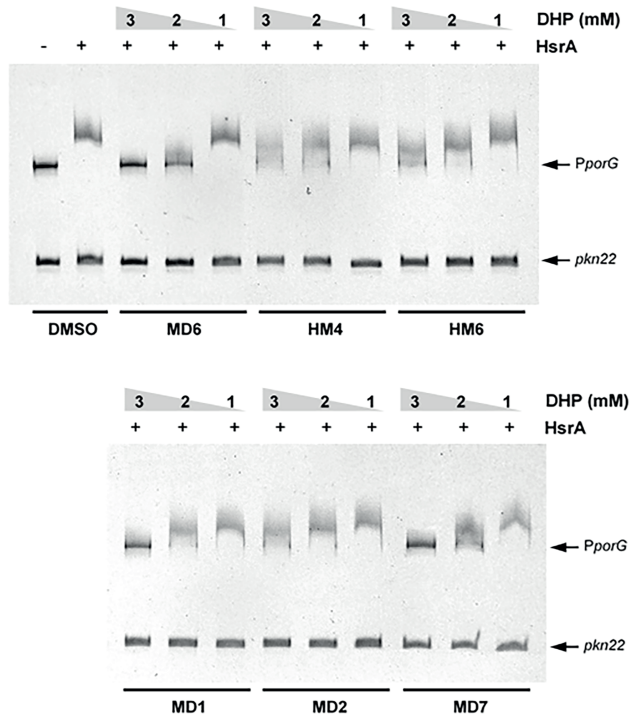



**FIGURE 3 |** Electrophoretic mobility shift assays (EMSAs) showing the ability of selected DHP-based HHQ derivatives to specifically inhibit the *in vitro* DNA binding activity of the *H. pylori* response regulator HsrA. The recombinant protein (5 μM) was mixed with 120 ng of target promoter (*porGDAB* operon) in the presence of 3, 2, and 1 mM of DHP. The *Anabaena* gene *pkn22* was included as non-specific competitor DNA in all assays. Mixture of protein and DNA with DMSO (vehicle) instead of DHP was used as control. Protein-DNA interactions were analyzed by 6% PAGE using SYBR Safe® staining. Representative gel images are shown in a black-white inverted mode.

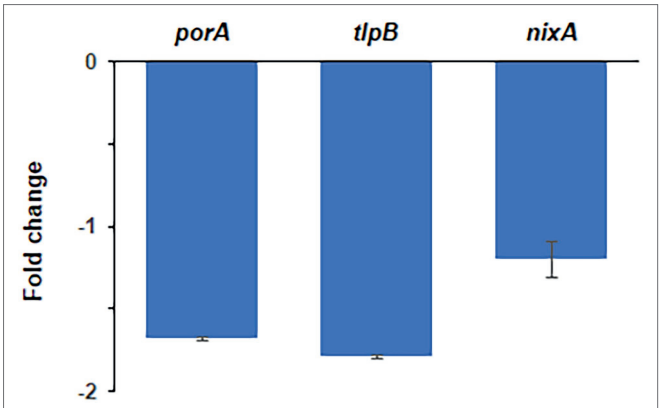



**FIGURE 4 |** Quantitative real-time PCR (qPCR) analysis of transcript abundance changes of selected genes in response to the exposure to lethal concentrations of the DHP-based HHQ derivative MD7. Total RNA was extracted from *H. pylori* strain 26695 after 2 h exposure to 4 × MIC (16 mg/L) of MD7. Relative transcription of genes *porA*, *tlpB* and *nixA* in MD7-treated cells with respect to DMSO (vehicle) treated cells are indicated as fold changes. Values correspond to the average of two independent biological samples, each analyzed in three technical replicates. Error bars indicate the SDs.

transcript level of *nixA*, a gene without a direct control of HsrA (**Figure 4**).

## DHP-Based HHQ Derivatives Interact With the DNA-Binding Domain of HsrA at 1:1 Stoichiometry in the Micromolar Range

Thermodynamic parameters of the molecular interactions between HsrA and its bactericidal inhibitors MD1, MD2, MD6, MD7, HM4, and HM6 were analyzed by isothermal titration calorimetry (ITC). As previously observed with other HsrA inhibitors, DHP-based HHQ derivatives interacted with this response regulator following a 1:1 stoichiometry, that is, each HsrA monomer binds one molecule of DHP (**Supplementary Figure S1**). Despite all HsrA-DHP complexes showed dissociation constants in the micromolar range, some little differences in the binding affinities of these ligands could be intuited according to their $K_d$ values, which appear to suggest that MD1, HM4, and HM6 interact with more affinity with the protein than the rest of the ligands tested (**Table 3**).

Notably, the molecular docking analyses predicted that all the six highly bactericidal DHP-based HHQ derivatives interact with HsrA in the same binding site, consisting in a pocket on the surface of the C-terminal DNA binding domain which involved several amino acid residues of the helix-turn-helix (HTH) DNA binding motif (**Table 3**; **Figure 5**). This ligand-binding pocked is predominantly shaped by nonpolar residues including I135, V142, V144, G146, P148, F149, L152, M195, P198, and L199, but also comprises few polar amino acids such as Y137, K145, and K194.

Analysis of the best-ranked pose of each DHP ligand inside this common binding pocket revealed similar interaction patterns, with slight changes in the spatial arrangement of the DHP molecule promoted by differences in the chemical properties, sizes and positions of the substituent groups on the DHP scaffold. Several non-covalent interactions between these DHP ligands and neighboring HsrA amino acids appeared to define little differences in the affinity of each inhibitor by their target regulator. While some intermolecular interactions appear inherent to the DHP-based HHQ structure, other contacts are established or reinforced with dependence on the type of substituent. Thus, the phenyl ring present in all DHPs establishes a CH/π interaction with P198, while additional hydrophobic interactions occur between the HHQ condensed ring system and V144. In addition, the carbonyl group of the condensed ring interacts by hydrogen bonds with the $NH_3^+$ group of the K194 side chain (**Figure 5**).

The hydroxyl substituent in the *ortho* position of the phenyl ring in MD1, MD6, MD7, HM4, and HM7 forms a hydrogen bond with the $NH_3^+$ group of the K194 side chain, but this interaction is absent in MD2. The bromine substituent in the *meta* position of the phenyl ring in MD6, MD7, HM4, and HM6 could perform halogen bonding with the hydroxyl oxygen of Y137 side as well as hydrophobic interactions with I135, V142 and V144 (**Figures 5E,G**); however, the nitro group at this position does not appear to form any favorable interaction with the protein. Similarly, the dimethyl group at the condensed

**TABLE 3** | Thermodynamic parameters and interacting amino acid residues of the protein-ligand complexes formed between HsrA and selected DHP-based HHQ derivatives, according to ITC and molecular docking analyses.

| DHP | ITC[1] | | | Molecular docking[2] |
|---|---|---|---|---|
| | $K_d$ (μM) | Δ*H* (kcal/mol) | Δ*G* (kcal/mol) | Interacting residues |
| MD1 | 3.5 | −1.5 | −7.4 | I135, V144, F149, L152, **K194**, **M195**, **P198**, **L199** |
| MD2 | 16 | −3.1 | −6.5 | I135, Y137, V144, K145, G146, **K194**, **P198** |
| MD6 | 25 | −2.0 | −6.3 | I135, Y137, V142, V144, P148, F149, L152, **K194**, **P198**, **L199** |
| MD7 | 23 | −7.8 | −6.3 | I135, Y137, V142, V144, F149, L152, **K194**, **M195**, **P198**, **L199** |
| HM4 | 4.0 | −0.7 | −7.4 | I135, Y137, V142, V144, G146, P148, F149, **K194**, **P198** |
| HM6 | 5.4 | −2.7 | −7.2 | I135, Y137, V142, V144, G146, P148, **K194**, **P198** |

[1]*Relative error in $K_d$ is 15%, absolute error in ΔH is 0.4 kcal/mol, absolute error in ΔG is 0.1 kcal/mol.*

[2]*Amino acid residues directly involved in forming the helix-turn-helix (HTH) DNA binding motif of HsrA are highlighted in bold fonts.*

ring system in MD1, MD2, MD6, and MD7 is not involved in any favorable interactions, but the change in the position of this substituent in HM4 and HM6 allowed additional hydrophobic contacts with G146 and P148 (**Figure 5G**). The isobutyl ester moiety of MD1, MD2, MD6, and MD7 establishes hydrophobic interactions with adjacent I135, V142, Y137, L199; however, the conformation of ligand-protein complexes could be further stabilized in HM4 and HM6 by π-π stacking interactions of their benzyl ester moiety with Y137 and CH/π interactions with P198 (**Figure 5G**).

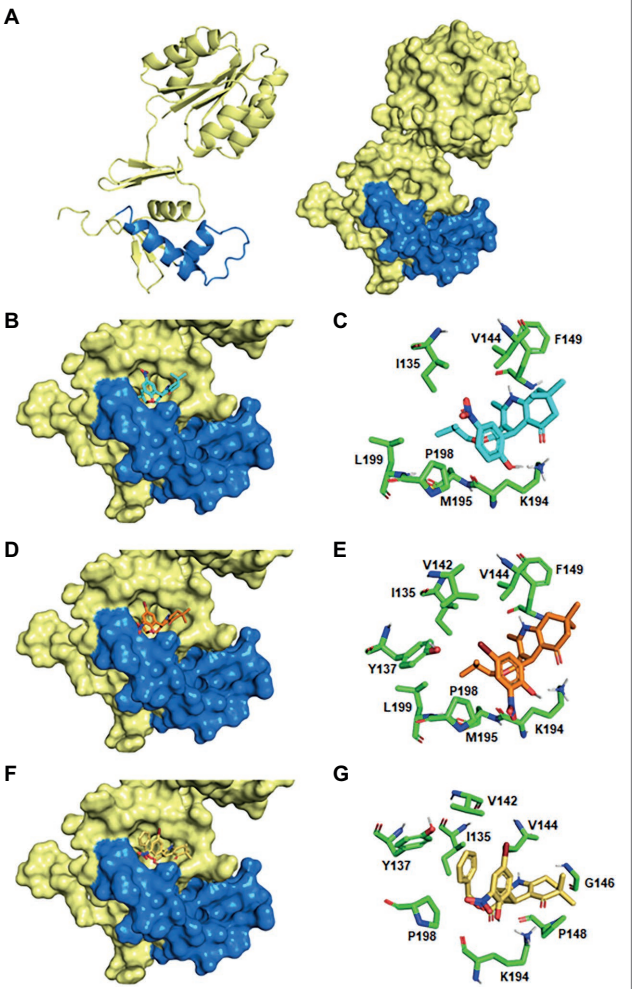



**FIGURE 5** | Predicted models of HsrA-DHP interactions. **(A)** Ribbon diagram and molecular surface of an HsrA monomer. The helix-turn-helix (HTH) DNA binding motif in the C-terminal domain has been highlighted in blue. **(B,D,F)** 3D views of the best-ranked docking poses of MD1, MD7, and HM6. **(C,E,G)** Detailed views of the HsrA amino acid residues involved in the interaction with the respective DHP.

## Most of the Highly Bactericidal DHP-Based HHQ Derivatives Exhibited Cytotoxicity Levels Comparable With Those of Commercial DHP Drugs in HeLa Cells

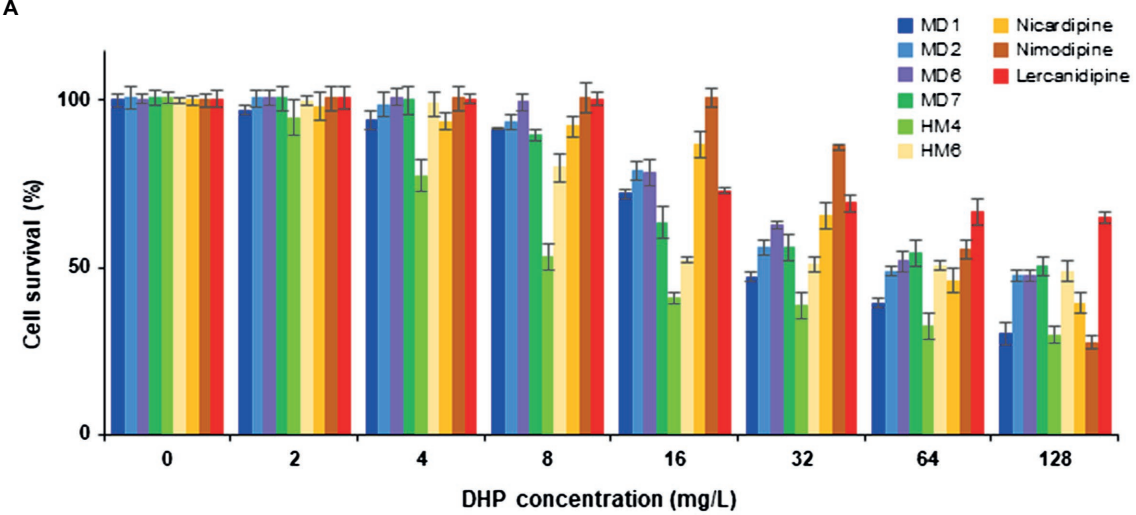
Due to the absence of previous reports related to the cytotoxic potential of DHP-based HHQ derivatives, cytotoxicity studies were conducted in HeLa cells by the method of PrestoBlue (Lall et al., 2013). Cell cultures were exposed during 24 h to selected DHP-based HHQ derivatives at concentrations ranging from the lowest MIC value previously determined for *H. pylori* (1 mg/L) to more than 120 times this value. Three commercially available DHP-class antihypertensive drugs (nicardipine, nimodipine, and lercanidipine) were included as control in the assays.

As shown in **Figure 6A**, no relevant cytotoxicity was observed at 4 mg/L with most of the DHP derivatives tested, with the exception of HM4, which reduced HeLa cell viability to 77% at this concentration. The 50% cytotoxic concentration ($CC_{50}$) of MD2, MD6, MD7, and HM6 was comparable to those exhibited by commercial DHP drugs (**Figure 6B**). However, the therapeutic index (TI) values, which were calculated as the ratio between $CC_{50}$ and MIC, resulted higher than 3 in all cases. This fact supposes a wide therapeutic window for all of these DHP derivatives as anti-*H. pylori* antimicrobial candidates.

## DISCUSSION

Antimicrobial resistance is nowadays a major global challenge for public health. The constant development of new resistance mechanisms, the unstoppable spread of antibiotic resistance genes among veterinary and clinical relevant pathogens but also in commensal bacteria, and the rapid dissemination of multidrug-resistant strains in a globalized world already threaten our capacities to face some infectious diseases (Chen et al., 2017; Ofori-Asenso, 2017; Nkansa-Gyamfi et al., 2019; Karakonstantis et al., 2020). In recent years, the efficacies of

A




B

| DHP | $CC_{50}$ (mg/L) | TI ($CC_{50}$ / MIC) |
|---|---|---|
| MD1 | 30 | 3.8 |
| MD2 | 59.5 | 7.4 |
| MD6 | 90.6 | 22.6 |
| MD7 | 128 | 32 |
| HM4 | 10.2 | 5.1 |
| HM6 | 83.1 | 83.1 |
| Nicardipine | 57.7 | 7.2 |
| Nimodipine | 74.6 | 18.6 |
| Lercanidipine | >128 | >16 |

**FIGURE 6 |** Cytotoxicity and therapeutic index of several DHP-class anti-*H. pylori* compounds. **(A)** Cytotoxicity of selected DHP-based HHQ derivatives and some commercial DHP drugs toward HeLa cells was assessed at 24 h of exposure through the PrestoBlue method. Experiments were performed twice in triplicate, vertical bars represent SDs. **(B)** The 50% cytotoxic concentration ($CC_{50}$) was defined as the compound concentration that reduced the viability of DMSO (vehicle)-treated cell cultures by 50%. The indicated therapeutic index (TI) values were calculated as the ratio between $CC_{50}$ and the MIC value for the *H. pylori* strain ATCC 700684.

several antimicrobial combinatory therapies commonly used to eradicate infection by the carcinogenic bacterium *H. pylori* have drastically decreased worldwide because of an increasing emergence of resistance to first-line antibiotics (Boyanova et al., 2019; Kuo et al., 2021). Efforts are underway to discover new antimicrobial candidates directed against novel therapeutic targets in this pathogen that allow for overcoming the current resistome (Krzyzek et al., 2019, 2020; González et al., 2019b, 2020; Roszczenko-Jasinska et al., 2020; Salillas and Sancho, 2020).

In a previous work, we found that several antihypertensive drugs of the DHP class targeted the *H. pylori* response regulator HsrA and inhibited its essential function *in vitro*. Such DHP derivatives exhibited strong bactericidal activities against antibiotic-resistant strains of *H. pylori* and significantly reduced gastric colonization by this pathogen in the mouse model (González et al., 2019a); however, the hypotensive activity of these highly prescribed drugs could slow down their repurposing as novel antimicrobials.

1,4-DHP is one of the most privileged heterocyclic scaffolds in medicinal chemistry covering a broad spectrum of biological activities and therapeutic effects including antihypertensive, antianginal, neuroprotective, antioxidant, anti-inflammatory, anticancer, and antimicrobial (Ioan et al., 2011; Carosati et al., 2012; Mishra et al., 2019; Ling et al., 2021). Most 1,4-DHP derivatives share some structural features, such as the unsaturated 1,4-DHP core ring with unsubstituted N1 atom, small alkyl groups (usually methyl) at the C2 and C6 positions, ester groups at the C3 and C5 positions, and a phenyl ring with different substituent types and patterns at the C4 position (Olejnikova et al., 2014; Ling et al., 2021).

To further evaluate the potential of 1,4-DHP as a scaffold for novel antimicrobial drugs against *H. pylori*, we determined the antibacterial effect of 12 DHP-based hexahydroquinoline derivatives which have previously shown no significant blocking effects on calcium channels. As previously described with other biological activities exerted by this class of compounds, the observed antimicrobial effects against *H. pylori* of these DHP-based HHQ derivatives depended on the substitution pattern of the DHP scaffold. Thus, the most potent bactericidal activities against *H. pylori* were observed in the presence of the DHP derivatives HM4 and HM6. These two compounds are distinguished by possessing a benzyl moiety in the ester

functionality at the C3 position, and dimethyl substituents at the C6 position in the HHQ condensed system, one or two bromine in the *meta* positions of the phenyl ring, and a hydroxyl group in the *ortho* position.

Notably, the change of the alkyl group of the ester moiety from benzyl (HM6) to isobutyl (MD7), and the modification of the position of the dimethyl group from C6 (HM6) to C7 (MD7) on the HHQ ring system, resulted in up to 4-fold reduction in the antimicrobial activity against *H. pylori*. These structure-associated differences in the anti-*H. pylori* activities between HM6 and MD7 were partially supported by differences in the binding affinities of these DHPs for their target HsrA, according to ITC and molecular docking analyses. However, MD7 led to a significantly faster decline in bacterial counts when compared to HM6 in time-kill kinetic assays, maybe associated to a faster translocation across the cell membrane because of its smaller molecular size. Reduction of the hydrophobicity of the ester moiety by changing the alkyl group from benzyl or isobutyl to ethyl led to a severe detriment of the antimicrobial potential of these DHPs against *H. pylori*.

The addition of one or two bromine atoms in the *meta* position of the phenyl ring favored the antimicrobial activity as the result of additional noncovalent interactions between the DHPs and the target HsrA protein. This effect was less evident with the nitro group at the same positions, while the inclusion of trifluoromethyl substituents appreciably reduced the anti-*H. pylori* activity. Likewise, the presence of only hydroxyl substituents in the phenyl ring resulted in a low antimicrobial potential.

The use of 1,4-DHP as a scaffold for novel antimicrobials has been the focus of several investigations since decades ago. Fiszer-Maliszewska and co-workers in 1985 observed that some DHPs inhibited the *in vitro* growth of antibiotic-resistant *Mycobacterium tuberculosis* strains at 3.1 mg/L (Fiszer-Maliszewska et al., 1985). Since then, the antitubercular properties of other DHP derivatives have been reported by many other researchers (Trivedi et al., 2011; Desai et al., 2015; Zandhaghighi et al., 2017; Venugopala et al., 2021). DHPs have been also evaluated as antimicrobials against other pathogenic bacteria including *S. aureus* (Ceviz et al., 1997; Olejnikova et al., 2014; Mahmoodi et al., 2015; Nkosi et al., 2016; Nosrati et al., 2021), *E. coli* (Murthy et al., 2012; Olejnikova et al., 2014; Mahmoodi et al., 2015; Nkosi et al., 2016; Ahamed et al., 2018), *Pseudomonas aeruginosa* (Mahmoodi et al., 2015; Nkosi et al., 2016; Ahamed et al., 2018), *Vibrio cholerae* (Lavanya et al., 2021) and *Klebsiella pneumoniae* (Murthy et al., 2012); but also against parasites (Núñez-Vergara et al., 1997; Palit and Ali, 2008; Reimao et al., 2010; Pollo et al., 2017; Jeddi et al., 2021) and fungi (Chhillar et al., 2006; Jezikova et al., 2017; Ahamed et al., 2018). This class of molecules have been also studied as inhibitors of bacterial transmembrane efflux pumps, acting thereby as enhancers of the action of conventional antibiotics (Lentz et al., 2016, 2018, 2019).

Several DHP-class antihypertensive drugs including nifedipine, nicardipine, nisoldipine, nimodipine, nitrendipine, and lercanidipine have previously shown MIC values in the range of 4–32 mg/L against different strains of *H. pylori* (González et al., 2019a). In the present study, the chemical modifications carried out on the 1,4-DHP scaffold led to a noticeable increase in the anti-*H. pylori* activity, with MIC values ranging from 1 to 4 mg/L in the case of compounds HM4 and HM6. This strong antimicrobial effect against *H. pylori* is comparable with those achieved by some first-line conventional antibiotics like metronidazole, and up to 4-fold greater than those previously observed with commercial 1,4-DHP drugs.

The MIC values of those DHP-based HHQ derivatives that exhibited the most potent bactericidal effects against *H. pylori* were in all cases >3 times higher than the concentration necessary to induce damage in HeLa cells (TI > 3), suggesting wide therapeutic windows of these molecules as potential new antimicrobial drugs. On the other hand, these novel anti-*H. pylori* compounds resulted poorly deleterious for *E. coli* and *S. epidermidis*, a Gram-positive and Gram-negative representative species of the human normal microbiota. These results might indicate a narrow-spectrum in the antimicrobial action of these novel compounds, and consequently, a reduced risk of associated dysbiosis (Francino, 2015; Konstantinidis et al., 2020). Our findings suggest that the mechanistic base of such specific antimicrobial action is based on the inhibitory effect of DHPs on the essential transcriptional regulatory activity of HsrA, an orphan response regulator unique in *Epsilonproteobacteria* (Muller et al., 2007; Olekhnovich et al., 2013; Pelliciari et al., 2017). Both *in vitro* and *in vivo* experiments showed an inhibition of the DNA binding activity of this regulatory protein in the presence of selected bactericidal DHPs, while ITC studies revealed HsrA-DHP interactions with 1:1 stoichiometry and dissociation constants in the micromolar range. These thermodynamic parameters of the molecular interactions between HsrA and the novel DHP derivatives are similar to those exhibited by all the low-molecular-weight inhibitors described so far for this protein (González et al., 2019a,b).

Notably, despite differences in the chemical structure, molecular size and physicochemical properties of six selected highly bactericidal DHP-based HHQ derivatives, all of these molecules appear to interact with HsrA in a common binding site located in a pocket on the protein surface at the C-terminal DNA binding domain (Hong et al., 2007), according to molecular docking predictions. This binding site, partially shaped by several amino acid residues directly involved in the HTH DNA binding motif, seemed to be occupied also by other previously recognized HsrA inhibitors including the natural flavonoids apigenin and hesperetin (González et al., 2019b), and the DHP-class antihypertensive drug nimodipine (González et al., 2019a). Hence, this predicted binding pocket could be a potential druggable binding site on HsrA, a validated therapeutic target in *H. pylori*.

Overall, the results presented here strongly support the use of 1,4-DHP as a scaffold for novel antimicrobials against *H. pylori*. New highly bactericidal 1,4-DHP derivatives showing MIC values against *H. pylori* comparable with those achieved by first-line antibiotics have been obtained. Molecular docking analysis of several HsrA-DHP interactions predicted a potential druggable binding pocket in the C-terminal DNA binding domain of this essential regulatory protein. Further studies should be carried out to experimentally validate and best

characterize this binding pocket, which could open the door to structure-based design of improved HsrA inhibitors and lead to the definition of new strategies for drug discovery against *H. pylori* infection.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

AG and MG designed the study and wrote the manuscript. AG, JC, MG, BS, AV-C, CS-B, and MM performed the experiments. AG, JC, MG, AV-C, CS-B, and MM analyzed and validated the data. MF, EP, and ÁL gave important technical advices and supervised some experiments. MG, MF, and ÁL contributed to the funding acquisition. All authors have checked, read, and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.874709/full#supplementary-material

## REFERENCES

Ahamed, A., Arif, I. A., Mateen, M., Surendra Kumar, R., and Idhayadhulla, A. (2018). Antimicrobial, anticoagulant, and cytotoxic evaluation of multidrug resistance of new 1,4-dihydropyridine derivatives. *Saudi J. Biol. Sci.* 25, 1227–1235. doi: 10.1016/j.sjbs.2018.03.001

Aygün Cevher, H., Schaller, D., Gandini, M. A., Kaplan, O., Gambeta, E., Zhang, F. X., et al. (2019). Discovery of Michael acceptor containing 1,4-dihydropyridines as first covalent inhibitors of L-/T-type calcium channels. *Bioorg. Chem.* 91:103187. doi: 10.1016/j.bioorg.2019.103187

Beier, D., and Frank, R. (2000). Molecular characterization of two-component systems of *Helicobacter pylori*. *J. Bacteriol.* 182, 2068–2076. doi: 10.1128/JB.182.8.2068-2076.2000

Boyanova, L., Evstatiev, I., Yordanov, D., Markovska, R., and Mitov, I. (2016). Three unsuccessful treatments of *Helicobacter pylori* infection by a highly virulent strain with quadruple antibiotic resistance. *Folia Microbiol.* 61, 307–310. doi: 10.1007/s12223-015-0439-2

Boyanova, L., Hadzhiyski, P., Kandilarov, N., Markovska, R., and Mitov, I. (2019). Multidrug resistance in *Helicobacter pylori*: current state and future directions. *Expert. Rev. Clin. Pharmacol.* 12, 909–915. doi: 10.1080/17512433.2019.1654858

Carosati, E., Ioan, P., Micucci, M., Broccatelli, F., Cruciani, G., Zhorov, B. S., et al. (2012). 1,4-Dihydropyridine scaffold in medicinal chemistry, the story so far and perspectives (part 2): action in other targets and antitargets. *Curr. Med. Chem.* 19, 4306–4323. doi: 10.2174/092986712802884204

Ceviz, A., Inaloz, S. S., Cicek, R., Sari, I., Gul, K., and Sanli, A. (1997). Effects of nimodipine and ofloxacin on staphylococcal brain abscesses in rats. *Arzneimittelforschung* 47, 1402–1405. PMID: 9450172

Chen, L., Todd, R., Kiehlbauch, J., Walters, M., and Kallen, A. (2017). Notes from the field: pan-resistant New Delhi metallo-beta-lactamase-producing *Klebsiella pneumoniae* - Washoe County, Nevada, 2016. *MMWR Morb. Mortal. Wkly Rep.* 66, 33. doi: 10.15585/mmwr.mm6601a7

Chhillar, A. K., Arya, P., Mukherjee, C., Kumar, P., Yadav, Y., Sharma, A. K., et al. (2006). Microwave-assisted synthesis of antimicrobial dihydropyridines and tetrahydropyrimidin-2-ones: novel compounds against aspergillosis. *Bioorg. Med. Chem.* 14, 973–981. doi: 10.1016/j.bmc.2005.09.014

Choi, I. J., Kim, C. G., Lee, J. Y., Kim, Y. I., Kook, M. C., Park, B., et al. (2020). Family history of gastric cancer and *Helicobacter pylori* treatment. *N. Engl. J. Med.* 382, 427–436. doi: 10.1056/NEJMoa1909666

Delany, I., Spohn, G., Rappuoli, R., and Scarlato, V. (2002). Growth phase-dependent regulation of target gene promoters for binding of the essential orphan response regulator HP1043 of *Helicobacter pylori*. *J. Bacteriol.* 184, 4800–4810. doi: 10.1128/JB.184.17.4800-4810.2002

Desai, N. C., Trivedi, A. R., Somani, H. C., and Bhatt, K. A. (2015). Design, synthesis, and biological evaluation of 1,4-dihydropyridine derivatives as potent antitubercular agents. *Chem. Biol. Drug Des.* 86, 370–377. doi: 10.1111/cbdd.12502

European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) (2003). EUCAST discussion document E. dis 5.1: determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin. Microbiol. Infect.* 6, 509–515.

Fiszer-Maliszewska, L., Wieczorek, J., Mordarski, M., Balicki, R., Kaczmarek, L., and Nantka-Namirski, P. (1985). Biological activity of 1,4-dihydropyridine derivatives. *Arch. Immunol. Ther. Exp.* 33, 345–352. PMID: 4084011

Francino, M. P. (2015). Antibiotics and the human gut microbiome: dysbioses and accumulation of resistances. *Front. Microbiol.* 6:1543. doi: 10.3389/fmicb.2015.01543

González, A., Casado, J., Chueca, E., Salillas, S., Velázquez-Campoy, A., Espinosa Angarica, V., et al. (2019a). Repurposing dihydropyridines for treatment of *Helicobacter pylori* infection. *Pharmaceutics* 11:681. doi: 10.3390/pharmaceutics11120681

González, A., Casado, J., Chueca, E., Salillas, S., Velázquez-Campoy, A., Sancho, J., et al. (2020). Small molecule inhibitors of the response regulator ArsR exhibit bactericidal activity against *Helicobacter pylori*. *Microorganisms* 8:503. doi: 10.3390/microorganisms8040503

González, A., Casado, J., and Lanas, A. (2021). Fighting the antibiotic crisis: flavonoids as promising antibacterial drugs against *Helicobacter pylori* infection. *Front. Cell. Infect. Microbiol.* 11:709749. doi: 10.3389/fcimb.2021.709749

González, A., Salillas, S., Velázquez-Campoy, A., Espinosa Angarica, V., Fillat, M. F., Sancho, J., et al. (2019b). Identifying potential novel drugs against *Helicobacter pylori* by targeting the essential response regulator HsrA. *Sci. Rep.* 9:11294. doi: 10.1038/s41598-019-47746-9

Hong, E., Lee, H. M., Ko, H., Kim, D. U., Jeon, B. Y., Jung, J., et al. (2007). Structure of an atypical orphan response regulator protein supports a new phosphorylation-independent regulatory mechanism. *J. Biol. Chem.* 282, 20667–20675. doi: 10.1074/jbc.M609104200

Hooi, J. K. Y., Lai, W. Y., Ng, W. K., Suen, M. M. Y., Underwood, F. E., Tanyingoh, D., et al. (2017). Global prevalence of *Helicobacter pylori* infection: systematic review and meta-analysis. *Gastroenterology* 153, 420–429. doi: 10.1053/j.gastro.2017.04.022

Ioan, P., Carosati, E., Micucci, M., Cruciani, G., Broccatelli, F., Zhorov, B. S., et al. (2011). 1,4-Dihydropyridine scaffold in medicinal chemistry, the story

so far and perspectives (part 1): action in ion channels and GPCRs. *Curr. Med. Chem.* 18, 4901–4922. doi: 10.2174/092986711797535173

Jeddi, B., Saberi, S., Menendez, J. C., and Sepehri, S. (2021). Synthesis and biological evaluation of tetrahydropyrimidine and dihydropyridine derivatives against *Leishmania major*. *Acta Parasitol.* 67, 255–266. doi: 10.1007/s11686-021-00457-6

Jezikova, Z., Pagac, T., Pfeiferova, B., Bujdakova, H., Dizova, S., Jancikova, I., et al. (2017). Synergy between azoles and 1,4-dihydropyridine derivative as an option to control fungal infections. *Antonie Van Leeuwenhoek* 110, 1219–1226. doi: 10.1007/s10482-017-0895-6

Karakonstantis, S., Kritsotakis, E. I., and Gikas, A. (2020). Pandrug-resistant gram-negative bacteria: a systematic review of current epidemiology, prognosis and treatment options. *J. Antimicrob. Chemother.* 75, 271–282. doi: 10.1093/jac/dkz401

Konstantinidis, T., Tsigalou, C., Karvelas, A., Stavropoulou, E., Voidarou, C., and Bezirtzoglou, E. (2020). Effects of antibiotics upon the gut microbiome: a review of the literature. *Biomedicine* 8:502. doi: 10.3390/biomedicines8110502

Krzyzek, P., Franiczek, R., Krzyzanowska, B., Laczmanski, L., Migdal, P., and Gosciniak, G. (2019). In vitro activity of sertraline, an antidepressant, against antibiotic-susceptible and antibiotic-resistant *Helicobacter pylori* strains. *Pathogens* 8:228. doi: 10.3390/pathogens8040228

Krzyzek, P., Paluch, E., and Gosciniak, G. (2020). Synergistic therapies as a promising option for the treatment of antibiotic-resistant *Helicobacter pylori*. *Antibiotics* 9:658. doi: 10.3390/antibiotics9100658

Kuipers, E. J. (1999). Review article: exploring the link between *Helicobacter pylori* and gastric cancer. *Aliment. Pharmacol. Ther.* 13(Suppl 1), 3–11. doi: 10.1046/j.1365-2036.1999.00002.x

Kuo, C. J., Lee, C. H., Chang, M. L., Lin, C. Y., Lin, W. R., Su, M. Y., et al. (2021). Multidrug resistance: the clinical dilemma of refractory *Helicobacter pylori* infection. *J. Microbiol. Immunol. Infect.* 54, 1184–1187. doi: 10.1016/j.jmii.2021.03.006

Kusters, J. G., van Vliet, A. H., and Kuipers, E. J. (2006). Pathogenesis of *Helicobacter pylori* infection. *Clin. Microbiol. Rev.* 19, 449–490. doi: 10.1128/CMR.00054-05

Lall, N., Henley-Smith, C. J., De Canha, M. N., Oosthuizen, C. B., and Berrington, D. (2013). Viability reagent, PrestoBlue, in comparison with other available reagents, utilized in cytotoxicity and antimicrobial assays. *Int. J. Microbiol.* 2013:420601. doi: 10.1155/2013/420601

Lavanya, G., Magesh, C. J., Venkatapathy, K., Perumal, P. T., and Prema, S. (2021). Design, synthesis, spectral characterization and molecular docking studies of novel pyranoquinolinyl dihydropyridine carboxylates as potential antibacterial agents including *Vibrio cholerae* with minimal cytotoxity towards fibroblast cell line (L-929). *Bioorg. Chem.* 107:104582. doi: 10.1016/j.bioorg.2020.104582

Lentz, F., Hemmer, M., Reiling, N., and Hilgeroth, A. (2016). Discovery of novel N-phenyl 1,4-dihydropyridines with a dual mode of antimycobacterial activity. *Bioorg. Med. Chem. Lett.* 26, 5896–5898. doi: 10.1016/j.bmcl.2016.11.010

Lentz, F., Reiling, N., Martins, A., Molnar, J., and Hilgeroth, A. (2018). Discovery of novel enhancers of isoniazid toxicity in *Mycobacterium tuberculosis*. *Molecules* 23:825. doi: 10.3390/molecules23040825

Lentz, F., Reiling, N., Spengler, G., Kincses, A., Csonka, A., Molnar, J., et al. (2019). Dually acting nonclassical 1,4-dihydropyridines promote the anti-tuberculosis (Tb) activities of clofazimine. *Molecules* 24:2873. doi: 10.3390/molecules24162873

Leung, W. K., Wong, I. O. L., Cheung, K. S., Yeung, K. F., Chan, E. W., Wong, A. Y. S., et al. (2018). Effects of *Helicobacter pylori* treatment on incidence of gastric cancer in older individuals. *Gastroenterology* 155, 67–75. doi: 10.1053/j.gastro.2018.03.028

Ling, Y., Hao, Z. Y., Liang, D., Zhang, C. L., Liu, Y. F., and Wang, Y. (2021). The expanding role of pyridine and dihydropyridine scaffolds in drug design. *Drug Des. Devel. Ther.* 15, 4289–4338. doi: 10.2147/DDDT.S329547

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Mahmoodi, N. O., Ramzanpour, S., and Ghanbari Pirbasti, F. (2015). One-pot multi-component synthesis of 1,4-dihydropyridines using Zn(2+) @KSF and evaluating their antibacterial and antioxidant activities. *Arch. Pharm.* 348, 275–282. doi: 10.1002/ardp.201400414

McDaniel, T. K., Dewalt, K. C., Salama, N. R., and Falkow, S. (2001). New approaches for validation of lethal phenotypes and genetic reversion in *Helicobacter pylori*. *Helicobacter* 6, 15–23. doi: 10.1046/j.1523-5378.2001.00001.x

Mishra, A. P., Bajpai, A., and Rai, A. K. (2019). 1,4-Dihydropyridine: a dependable heterocyclic ring with the promising and the most anticipable therapeutic effects. *Mini-Rev. Med. Chem.* 19, 1219–1254. doi: 10.2174/1389557519666190425184749

Muller, S., Pflock, M., Schar, J., Kennard, S., and Beier, D. (2007). Regulation of expression of atypical orphan response regulators of *Helicobacter pylori*. *Microbiol. Res.* 162, 1–14. doi: 10.1016/j.micres.2006.01.003

Murthy, Y. L., Rajack, A., Moturu, T. R., Jeson Babu, J., Praveen, C., and Aruna Lakshmi, K. (2012). Design, solvent free synthesis, and antimicrobial evaluation of 1,4 dihydropyridines. *Bioorg. Med. Chem. Lett.* 22, 6016–6023. doi: 10.1016/j.bmcl.2012.05.003

Nam, S. Y., Park, B. J., Nam, J. H., and Kook, M. C. (2019). Effect of *Helicobacter pylori* eradication and high-density lipoprotein on the risk of de novo gastric cancer development. *Gastrointest. Endosc.* 90, 448.e1–456.e1. doi: 10.1016/j.gie.2019.04.232

Nkansa-Gyamfi, N. A., Kazibwe, J., Traore, D. A. K., and Nji, E. (2019). Prevalence of multidrug-, extensive drug-, and pandrug-resistant commensal *Escherichia coli* isolated from healthy humans in community settings in low- and middle-income countries: a systematic review and meta-analysis. *Glob. Health Action* 12(Sup1):1815272. doi: 10.1080/16549716.2020.1815272

Nkosi, S. M., Anand, K., Anandakumar, S., Singh, S., Chuturgoon, A. A., and Gengan, R. M. (2016). Design, synthesis, anticancer, antimicrobial activities and molecular docking studies of novel quinoline bearing dihydropyridines. *J. Photochem. Photobiol. B* 165, 266–276. doi: 10.1016/j.jphotobiol.2016.10.009

Nosrati, A., Amirnejat, S., and Javanshir, S. (2021). Preparation, antibacterial activity, and catalytic application of magnetic graphene oxide-fucoidan in the synthesis of 1,4-dihydropyridines and polyhydroquinolines. *ChemistryOpen* 10, 1186–1196. doi: 10.1002/open.202100221

Núñez-Vergara, L. J., Squella, J. A., Bollo-Dragnic, S., Morello, A., Repetto, Y., Aldunate, J., et al. (1997). Nitro aryl 1,4-dihydropyridine derivatives: effects on *Trypanosoma cruzi*. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 118, 105–111. doi: 10.1016/s0742-8413(97)00078-9

Ofori-Asenso, R. (2017). "When the bug cannot be killed"-The rising challenge of antimicrobial resistance. *Medicines.* 4:40. doi: 10.3390/medicines4020040

Olejnikova, P., Svorc, L., Olsovska, D., Panakova, A., Vihonska, Z., Kovaryova, K., et al. (2014). Antimicrobial activity of novel c2-substituted 1,4-dihydropyridine analogues. *Sci. Pharm.* 82, 221–232. doi: 10.3797/scipharm.1311-04

Olekhnovich, I. N., Vitko, S., Chertihin, O., Hontecillas, R., Viladomiu, M., Bassaganya-Riera, J., et al. (2013). Mutations to essential orphan response regulator HP1043 of *Helicobacter pylori* result in growth-stage regulatory defects. *Infect. Immun.* 81, 1439–1449. doi: 10.1128/IAI.01193-12

Olekhnovich, I. N., Vitko, S., Valliere, M., and Hoffman, P. S. (2014). Response to metronidazole and oxidative stress is mediated through homeostatic regulator HsrA (HP1043) in *Helicobacter pylori*. *J. Bacteriol.* 196, 729–739. doi: 10.1128/JB.01047-13

Palit, P., and Ali, N. (2008). Oral therapy with amlodipine and lacidipine, 1,4-dihydropyridine derivatives showing activity against experimental visceral leishmaniasis. *Antimicrob. Agents Chemother.* 52, 374–377. doi: 10.1128/AAC.00522-07

Pelliciari, S., Pinatel, E., Vannini, A., Peano, C., Puccio, S., De Bellis, G., et al. (2017). Insight into the essential role of the *Helicobacter pylori* HP1043 orphan response regulator: genome-wide identification and characterization of the DNA-binding sites. *Sci. Rep.* 7:41063. doi: 10.1038/srep41063

Pencheva, T., Lagorce, D., Pajeva, I., Villoutreix, B. O., and Miteva, M. A. (2008). AMMOS: automated molecular mechanics optimization tool for in silico screening. *BMC Bioinform.* 9:438. doi: 10.1186/1471-2105-9-438

Pitucha, M., Wos, M., Miazga-Karska, M., Klimek, K., Miroslaw, B., Pachuta-Stec, A., et al. (2016). Synthesis, antibacterial and antiproliferative potential of some new 1-pyridinecarbonyl-4-substituted thiosemicarbazide derivatives. *Med. Chem. Res.* 25, 1666–1677. doi: 10.1007/s00044-016-1599-6

Pollo, L. A. E., de Moraes, M. H., Cisilotto, J., Creczynski-Pasa, T. B., Biavatti, M. W., Steindel, M., et al. (2017). Synthesis and *in vitro* evaluation of Ca(2+) channel blockers 1,4-dihydropyridines analogues against *Trypanosoma cruzi* and *Leishmania amazonensis*: SAR analysis. *Parasitol. Int.* 66, 789–797. doi: 10.1016/j.parint.2017.08.005

Reimao, J. Q., Scotti, M. T., and Tempone, A. G. (2010). Anti-leishmanial and anti-trypanosomal activities of 1,4-dihydropyridines: *in vitro* evaluation and structure-activity relationship study. *Bioorg. Med. Chem.* 18, 8044–8053. doi: 10.1016/j.bmc.2010.09.015

Roszczenko-Jasinska, P., Wojtys, M. I., and Jagusztyn-Krynicka, E. K. (2020). *Helicobacter pylori* treatment in the post-antibiotics era-searching for new drug targets. *Appl. Microbiol. Biotechnol.* 104, 9891–9905. doi: 10.1007/s00253-020-10945-w

Salillas, S., Alias, M., Michel, V., Mahia, A., Lucia, A., Rodrigues, L., et al. (2019). Design, synthesis, and efficacy testing of nitroethylene- and 7-nitrobenzoxadiazol-based flavodoxin inhibitors against *Helicobacter pylori* drug-resistant clinical strains and in *Helicobacter pylori*-infected mice. *J. Med. Chem.* 62, 6102–6115. doi: 10.1021/acs.jmedchem.9b00355

Salillas, S., and Sancho, J. (2020). Flavodoxins as novel therapeutic targets against *Helicobacter pylori* and other gastric pathogens. *Int. J. Mol. Sci.* 21:1881. doi: 10.3390/ijms21051881

Sarasa-Buisan, C., Guio, J., Broset, E., Peleato, M. L., Fillat, M. F., and Sevilla, E. (2021). FurC (PerR) from *Anabaena* sp. PCC7120: a versatile transcriptional regulator engaged in the regulatory network of heterocyst development and nitrogen fixation. *Environ. Microbiol.* 24, 566–582. doi: 10.1111/1462-2920.15552

Savoldi, A., Carrara, E., Graham, D. Y., Conti, M., and Tacconelli, E. (2018). Prevalence of antibiotic resistance in *Helicobacter pylori*: a systematic review and meta-analysis in World Health Organization regions. *Gastroenterology* 155, 1372.e17–1382.e17. doi: 10.1053/j.gastro.2018.07.007

Schaller, D., Gündüz, M. G., Zhang, F. X., Zamponi, G. W., and Wolber, G. (2018). Binding mechanism investigations guiding the synthesis of novel condensed 1,4-dihydropyridine derivatives with L-/T-type calcium channel blocking activity. *Eur. J. Med. Chem.* 155, 1–12. doi: 10.1016/j.ejmech.2018.05.032

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., et al. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* 18, 318–327. doi: 10.1016/S1473-3099(17)30753-3

Trivedi, A., Dodiya, D., Dholariya, B., Kataria, V., Bhuva, V., and Shah, V. (2011). Synthesis and biological evaluation of some novel 1,4-dihydropyridines as potential antitubercular agents. *Chem. Biol. Drug Des.* 78, 881–886. doi: 10.1111/j.1747-0285.2011.01233.x

Trott, O., and Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461. doi: 10.1002/jcc.21334

Velázquez-Campoy, A., Leavitt, S. A., and Freire, E. (2015). Characterization of protein-protein interactions by isothermal titration calorimetry. *Methods Mol. Biol.* 1278, 183–204. doi: 10.1007/978-1-4939-2425-7_11

Venugopala, K. N., Deb, P. K., Pillay, M., Chopra, D., Chandrashekharappa, S., Morsy, M. A., et al. (2021). 4-Aryl-1,4-dihydropyridines as potential enoyl-acyl carrier protein reductase inhibitors: antitubercular activity and molecular docking study. *Curr. Top. Med. Chem.* 21, 295–306. doi: 10.2174/1568026620666201102121606

World Health Organization (1994). "IARC monographs on the evaluation of carcinogenic risks to humans," in *Schistosomes, Liver Flukes and Helicobacter pylori. Vol. 61*. ed. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (Lyon: World Health Organization).

Zandhaghighi, M., Hadizadeh, F., Soleimanpour, S., Meshkat, Z., Rezaee, S. A., Derakhshan, M., et al. (2017). *In vitro* bactericidal activities of two novel dihydropyridine derivatives against *Mycobacterium tuberculosis*. *J. Infect. Dev. Ctries.* 11, 453–458. doi: 10.3855/jidc.7966

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# Empirical vs. Susceptibility-Guided Treatment of *Helicobacter pylori* Infection: A Systematic Review and Meta-Analysis

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**Background:** Treating *Helicobacter pylori* infection according to antibiotic resistance has been frequently recommended. However, information on its real effectiveness is scarce.

**Aim:** The aim of this study is to perform a meta-analysis comparing empirical vs. susceptibility-guided treatment of *H. pylori*.

**Methods:** *Selection of studies*: Studies comparing empirical versus susceptibility-guided treatment were selected. *Search strategy*: electronic and manual up to August 2021. *Data synthesis*: by intention-to-treat (random-effects model).

**Results:** Overall, 54 studies were included (6,705 patients in the susceptibility-guided group and 7,895 in the empirical group). *H. pylori* eradication rate was 86 vs. 76%, respectively (RR: 1.12; 95% CI: 1.08–1.17; $I^2$: 83%). Similar results were found when only RCTs were evaluated (24 studies; RR: 1.16; 95% CI: 1.11–1.22; $I^2$: 71%) and when susceptibility testing was assessed by culture (RR: 1.12; 95% CI: 1.06–1.18) or PCR (RR: 1.14; 95% CI: 1.05–1.23). For first-line treatments (naïve patients; 30 studies), better efficacy results were obtained with the susceptibility-guided strategy (RR: 1.15; 95% CI: 1.11–1.20; $I^2$: 79%). However, for empirical first-line quadruple regimens, in particular (both with and without bismuth, excluding the suboptimal triple therapies), not based on CYP2C19 gene polymorphism, no differences in efficacy were found compared with the susceptibility-guided group (RR: 1.04; 95% CI: 0.99–1.09); this lack of difference was confirmed in RCTs (RR: 1.05; 95% CI: 0.99–1.12). For rescue therapies (13 studies, most 2$^{nd}$-line), similar results were demonstrated for both strategies, including all studies (RR: 1.09; 95% CI: 0.97–1.22; $I^2$: 82%) and when only RCTs were considered (RR: 1.15; 95% CI: 0.97–1.36).

**Conclusion:** The benefit of susceptibility-guided treatment over empirical treatment of *H. pylori* infection could not be demonstrated, either in first-line (if the most updated quadruple regimens are prescribed) or in rescue therapies.

**Keywords:** ***Helicobacter pylori*, culture, tailored, susceptibility, empirical**

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# INTRODUCTION

*Helicobacter pylori* (*H. pylori*) infection affects billions of people worldwide, which is the main cause of gastritis, peptic ulcer disease, and gastric cancer (Hooi et al., 2017). However, after more than 30 years of experience in the management of this infection, the ideal treatment regimen remains undefined.

Antibiotic resistance has been identified as the major factor affecting our ability to cure *H. pylori* infection, and the rate of resistance to several antibiotics—mainly clarithromycin—is steadily increasing in many geographic areas (Dore et al., 2000; Megraud et al., 2013; Camargo et al., 2014; Thung et al., 2016). A recent systematic review and meta-analysis assessed the distribution of *H. pylori* resistance to commonly used antibiotics in 65 countries and found that primary resistance rates to clarithromycin, metronidazole, and levofloxacin were ≥15% in most regions. Furthermore, increasing antibiotic resistance was observed in most countries (Savoldi et al., 2018). Accordingly, the World Health Organization (WHO) has designated clarithromycin-resistant *H. pylori* a high priority for antibiotic research and development.

Since antibiotic resistance is an evolving process, it seems mandatory to carry out point prevalence surveys on a regular basis to guide clinicians in their therapeutic choice (Megraud et al., 2013). A strategy that has been suggested to increase the eradication rate is individualized treatment according to antibiotic susceptibility testing (personalized treatment). However, the true utility of culture—with consequent antibiotic susceptibility testing—and the moment when it must be performed (before the first treatment or only after eradication failure) are both controversial. Of note, *H. pylori* culture is time-consuming, not always available on a routine basis, offers quite low sensitivity, and implies the performance of an endoscopic exploration (Zullo et al., 2003; Gisbert, 2011). Furthermore, culture is relatively expensive, not because of the cost of the procedure *per se*, but mainly because of the costs of the associated endoscopy required to obtain biopsy specimens.

Although susceptibility-guided therapy is recommended by many *H. pylori* consensus reports, the number of studies evaluating this strategy is, however, quite limited, and the evidence available to date regarding when and in whom culture should be performed is surprisingly scant. Currently, most physicians treat *H. pylori* infection without relying on antimicrobial susceptibility testing to choose the best regimen (Gisbert, 2020).

Therefore, the present study aimed to perform a meta-analysis comparing empirical vs. susceptibility-guided treatment of *H. pylori* including both first-line and rescue regimens.

# METHODS

## General Criteria for Considering Studies for This Review

Randomized, quasi-randomized, and non-randomized controlled trials were eligible for inclusion in this review, whereas case reports, letters, editorials, comments, and reviews were excluded. Full-text forms and abstracts of the articles selected (in each of the searches) were reviewed, and those dealing with the susceptibility-guided treatment of *H. pylori* infection were recorded and were eligible for inclusion. No restrictions by date of publication or by language were considered.

The studied population included adults or children diagnosed as positive for *H. pylori*. Patients could be treated with any of the available eradication treatments for *H. pylori* infection in any line of treatment. Trials had to compare the efficacy of an *H. pylori* eradication treatment based on a previous susceptibility-guided diagnostic test with that of empirical treatment. Pre-treatment diagnostic methods for *H. pylori* detection should comprise one or more of the most commonly validated tests: urea breath test, histology, rapid urease test, and stool antigen test; for susceptibility-guided treatments, studies should include methods to test antimicrobial susceptibility on gastric biopsies such as PCR or culture.

Eligible studies should include accessible data on successful eradication rates in both tailored and empirical groups.

## Outcome Measures

The primary endpoint was intention-to-treat efficacy (*H. pylori* eradication rate). Reported efficacy was considered as the rate (proportion) of patients cured among the total of treated patients. Trials were included if they reported the number of patients with *H. pylori* eradication in each treatment arm; otherwise, the numerator was calculated from the percentage of eradication reported and the intention-to-treat (ITT) sample size.

Trials were eligible if *H. pylori* eradication was confirmed using a rapid urease test, histology or culture of an endoscopic biopsy sample, or by a urea breath test or a monoclonal stool antigen test, at least 4 weeks after completion of treatment. Trials, in which only serology test was performed, were excluded.

## Search Methods for Identification of Studies

### Search Strategy

Bibliographical searches were performed in the MEDLINE, EMBASE, and the Cochrane Library electronic databases up to August 2021 based on the following words (all fields): pylori AND [(culture OR culture-based OR culture-guided OR tailored OR susceptibility OR susceptibility-guided OR "antimicrobial susceptibility" OR "susceptibility testing") OR (empiric OR empirical)].

Reference lists of the articles selected by electronic searching were examined in detail to further identify relevant studies. In addition, references of articles retrieved, significant reviews, and the personal databases of the authors were also checked for eligible publications.

## Data Collection and Analysis

### Selection of Studies

The Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) approach (www.prismastatement.org) was used to develop a diagram to schematize the different steps of study selection (Liberati et al., 2009; Page et al., 2021).

**Abbreviations:** *H. pylori*, *Helicobacter pylori*; MIC, minimal inhibitory concentration; PPI, proton pump inhibitor; RCT, randomized controlled trial.

Before the selection of studies, duplicates were removed in the citation manager. The selection of studies was conducted in two phases: a first screening of titles and abstracts to identify potentially relevant citations; and a second phase, where full texts of the previously selected studies were retrieved. Selection criteria were applied to full texts for definite inclusion. Two reviewers (OPN and ME) performed the screenings independently; disagreements were resolved by consensus with a third reviewer (JPG). The reason for the exclusion of a given study was reported in the second phase only as appropriate.

## Data Extraction

A pre-tested data extraction form was used in a pilot test before the final collection of data to test its reliability. The following information was extracted from each study: first author; year of publication of the study; country; population (adult or children); study design (RCT or non-RCT); treatment line; susceptibility test; clarithromycin resistance rate (%); metronidazole resistance rate (%); levofloxacin resistance rate (%); type of empirical regimen; eradication rate with the empirical regimen; and eradication rate with the susceptibility-guided regimen. Two reviewers (OPN and ME) performed the data extraction independently; disagreements were resolved by consensus with a third reviewer (JPG).

## Assessment of the Risk of Bias in Included Studies

The risk of bias was assessed independently by two reviewers (OPN and ME); disagreements were resolved by consensus with a third reviewer (JPG) in accordance with the Cochrane Collaboration's current recommendations (Higgins et al., 2009).

For RCTs, the Cochrane Risk of Bias (RoB) tool was used and the six quality items were evaluated: random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), and selective reporting (reporting bias). A study was considered to be an RCT if it was explicitly described as "randomized." This should include the use of words such as "random," "randomly," or "randomization." We then rated the potential randomized trial as truly random, pseudo-random (randomization was mentioned but the method used was not reported), or non-random, based on the definitions by the Cochrane Handbook (Higgins et al., 2009).

For quasi-randomized trials (that is, non-random but controlled studies) and non-RCTs, the RoB criteria for EPOC Reviews (Guide for review authors on assessing study quality) advocated by the Cochrane was used. The same quality domains (as for RCTs) were assessed, but that related to the evaluation of randomization was reported as "high risk of bias" as no allocation of the sequence was generated as per the study design.

## Assessment of Heterogeneity

The possible sources of diversity in the trial's characteristics were evaluated. We performed the Chi² test for heterogeneity for each combined analysis, where $P < 0.10$ indicated significant heterogeneity between studies (Higgins and Thompson, 2002). Graphical methods (forest plots) were also used to complete the Chi² test assessment.

The $I^2$ statistic was used to assess the heterogeneity of the studies, following the recommendation of the Cochrane Collaboration's Handbook for Systematic Reviews of Interventions (Higgins et al., 2009), as follows: 0 to 40%, unimportant heterogeneity; 40 to 75%, moderate heterogeneity; 75 to 100% considerable heterogeneity.

## Assessment of Reporting Biases

To assess publication bias, funnel plot asymmetry was inspected visually by examining the relationship between the treatment effects and the standard error of the estimate.

## Data Synthesis

To collate, combine, and summarize the information obtained, a quantitative approach was undertaken. The evidence collected in the included studies was synthesized by summarizing the information related to the effect size of all studies, for each comparison and for each subgroup analysis. A meta-analysis was therefore performed combining the calculated risk ratios (RRs) of the individual studies with their corresponding 95% confidence intervals (CIs), using a random effects model (Mantel–Haenszel). Additional sensitivity analyses were performed to check the robustness of the results (DerSimonian and Laird, 1986; Egger et al., 1997).

The subgroup analyses were pre-planned to explore the possible sources of heterogeneity according to the study design (RCT vs. non-RCT), treatment line (naïve vs. rescue), susceptibility testing (culture vs. PCR), RCT by treatment line, and RCT by susceptibility test. The last group evaluating the empirical first-line quadruple treatments only was also included, to perform the most equitable comparison according to the most updated recommended first empirical quadruple treatments (i.e., non-bismuth and bismuth quadruple therapy) (Malfertheiner et al., 2017).

Analyses were performed using the freeware program Review Manager (RevMan) version 5.4.1 (2020).

# RESULTS

## Description of Studies

In total, 17,383 citations were retrieved from the following electronic databases: PubMed and EMBASE, up to August 2021. After removing duplicates, a total of 13,941 citations were screened. After reviewing the abstracts and full texts, 47 studies (Romano et al., 2000, 2003; Toracchio et al., 2000; Avidan et al., 2001; Street et al., 2001; Lamouliatte et al., 2003; Miwa et al., 2003; Neri et al., 2003; Marzio et al., 2006; Yahav et al., 2006; Furuta et al., 2007; Kawai et al., 2008; Wang et al., 2008; Zhou et al., 2010, 2016; Bontems et al., 2011; Molina-Infante et al., 2012; Cosme et al., 2013, 2016; Lee et al., 2013; Martos et al., 2014; Park et al., 2014; Dong et al., 2015; Zhuo et al., 2015; Kwon et al., 2016; Miyaki et al., 2016; Ferenc et al., 2017; Gweon et al., 2018; Huang et al., 2018; Liou et al., 2018; Mascellino et al., 2018; Tanabe et al., 2018; Byambajav et al., 2019; Chen et al., 2019; Cho et al., 2019; Choi et al., 2019, 2021; Delchier et al., 2019; Ong et al., 2019;

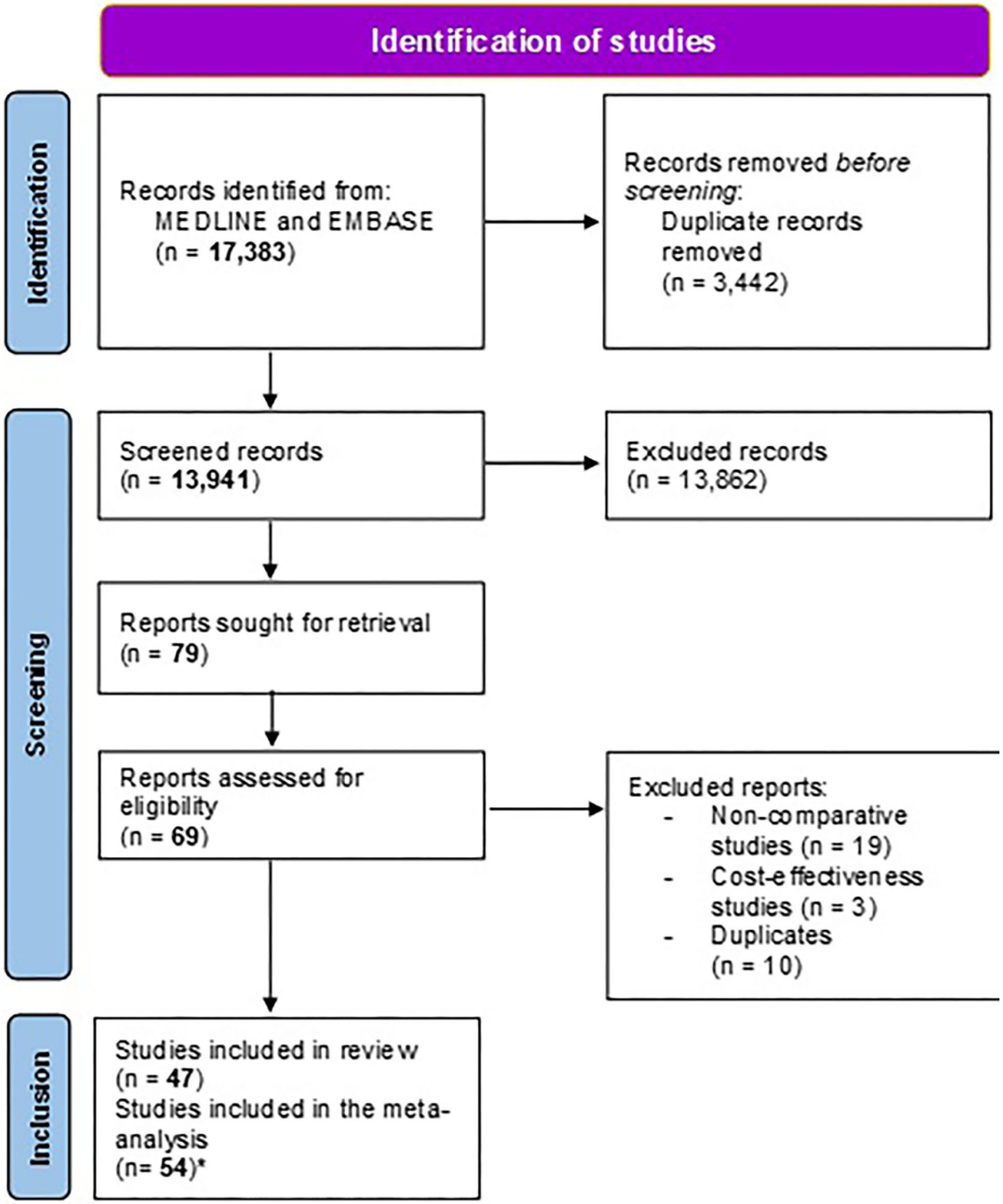


**FIGURE 1 |** Study flow chart. *The number of studies included in the meta-analysis is higher than the number of studies included in the systematic review because certain studies included different treatment groups that were meta-analyzed separately (i.e., we considered as many study groups/comparisons as treatment arms).

Pan et al., 2020; Saracino et al., 2020; Zhang et al., 2020; Bonoso Criado et al., 2021; Cha et al., 2021; Chang et al., 2021; Choe et al., 2021) were finally included in the present systematic review. A total of 54 different treatment comparisons were evaluated both in the quantitative and qualitative synthesis since some studies assessed more than one treatment comparison.

The different steps in the selection of studies and the reasons for the exclusion of studies [non-comparative (Gasbarrini et al., 2000; Gomollon et al., 2000; Chi et al., 2003; Cammarota et al., 2004; Fiorini et al., 2013; Liou et al., 2013; Choi et al., 2014; Sugimoto et al., 2014; Draeger et al., 2015; Stamboliyska et al., 2015; Han et al., 2016; Cosme et al., 2017, 2019; Costa et al.,

2017; Králícek et al., 2017; Blümel et al., 2018; Kwon et al., 2019; Lee et al., 2019; Yu et al., 2019; Zhu and Wu, 2019) and cost-effectiveness (Breuer and Graham, 1999; Qasim et al., 2004; Faber et al., 2005; Cammarota et al., 2014)] are reported in the PRISMA flow chart (**Figure 1**).

The search time span was from the year 2000 to 2021, and published studies were mainly from Asian and European countries. All studies were on adults except for two, which evaluated children. One of them was an RCT (Bontems et al., 2011) and the other one was a prospective observational cohort (Zhang et al., 2020), and both used culture for the diagnosis of the infection and tailoring of the treatment.

We found 31 RCTs and 23 non-RCTs. In 30 studies, patients were naïve to treatment and the majority assessed patients treated with a second-line rescue therapy, except for three studies (Bontems et al., 2011; Huang et al., 2018; Liou et al., 2018), in which patients were treated with a third-line therapy. Baseline characteristics, diagnostic methods, and prescribed treatments are reported in **Table 1**. Most studies are tested for clarithromycin resistance. In four studies, (Miwa et al., 2003; Furuta et al., 2007; Zhou et al., 2016; Zhang et al., 2020) patients received a tailored therapy based on CYP2C19 polymorphism.

## Overall Results

From all studies, 14,600 patients were analyzed (6,705 patients in the susceptibility-guided group and 7,895 in the empirical group). Overall, *H. pylori* eradication was significantly better for the susceptibility-guided treatment than for the empirical treatment, 86 vs. 76%, respectively (RR: 1.12; 95% CI: 1.08–1.17; $I^2$: 83%; **Supplementary Figure 1**).

Results of meta-analyses comparing the effectiveness of the empirical and the susceptibility-guided treatments between different groups (by treatment line, study design, tailored therapy, or recommended empirical quadruple therapy) are detailed below.

## Treatment Line

### First-Line Therapy

A total of 35 studies were included in this analysis, with 10,894 patients treated with first-line treatment. Statistically significant differences were found in cure rates in favor of susceptibility-guided therapy (87%) vs. empirical treatment (78%); however, results were highly heterogeneous (RR: 1.13; 95%CI: 1.08, 1.17; $I^2$: 83%; **Supplementary Figure 2**).

Sensitivity analyses confirmed that susceptibility-guided therapy was also superior to first-line clarithromycin-based triple therapy, in areas with high (i.e., over 20%) clarithromycin resistance (RR: 1.13; 95% CI: 1.03, 1.25; $I^2$: 90%) and also in those with low clarithromycin resistance (RR: 1.24; 95% CI: 1.15, 1.32; $I^2$: 45%).

### Rescue Therapy

A total of 16 studies were on rescue (more than one treatment failure) therapy. When patients receiving a second- (1,131) or third-line (152) treatment were evaluated separately, no differences were found between groups. Likewise, when all rescue lines were grouped (from 2$^{nd}$ to 3$^{rd}$) and analyzed together (1,356 participants), no differences were reported (RR: 1.07; 95% CI: 0.97–1.18; $I^2$: 78%, **Supplementary Figure 2**).

## Susceptibility Test

Similar results were reported when culture (36 studies; RR: 1.11; 95% CI: 1.05–1.16; $I^2$: 83%) or PCR (16 studies; RR: 1.08; 95% CI: 1,01–1,16; $I^2$: 84%; **Supplementary Figure 3**) was used as a method to test antibiotic susceptibility; in both cases, the efficacy of the susceptibility-guided treatment was higher than that of the empirical treatment (85 vs. 77% and 86 vs. 78%, respectively). Heterogeneity between groups was high ($I^2$: 83%); however, no significant variation in the mean effects was found between the different subgroups ($P = 0.64$).

## Randomized Controlled Trials vs. Non-randomized Controlled Trials

In total, 27 RCTs (encompassing 31 comparisons) were included in the meta-analysis; that is, 7,325 patients (3,502 in the susceptibility-guided and 3,823 in the empirical treatment group) were evaluated. *H. pylori* eradication was achieved in 85% of patients in the susceptibility-guided group vs. 76% in the empirical group (RR: 1.13; 95% CI: 1.07–1.18; $I^2$: 74%; **Supplementary Figure 4**). In this subgroup, one study (Bontems et al., 2011) was on children, nevertheless, excluding this study from the group did not vary the result of the sensitivity analysis. Heterogeneity was considerable in the RCT group, but lower than that of the overall assessment including all study designs (74 vs. 82%; respectively).

In non-RCTs, 8,000 patients (3,698 in the susceptibility-guided group and 4,302 in the empirical treatment group) were analyzed. In this sub-group, eradication was also higher in the susceptibility-guided group than in the empirical group (RR: 1.07; 95% CI: 1.01–1.14; $I^2$: 86%). Likewise, the exclusion of one study (Zhang et al., 2020) on children did not vary the overall result of the sensitivity analysis. In addition, heterogeneity was significantly higher in the non-RCT group than when only RCTs were evaluated (86 vs. 74%, respectively; $p < 0.001$).

### Randomized-Controlled Trials by Treatment Line

All the RCTs included could be meta-analyzed by treatment line except for the one by Bontems et al. (2011), in which eradication data could not be extracted separately for the first- and second-line treatment arms.

In total, 21 comparisons were evaluated, where 5,819 naïve patients had been randomized to receive either a first-line empirical therapy or a susceptibility-guided treatment. Statistically significant differences were reported in eradication rates between groups (78 vs. 87%, respectively), with moderate heterogeneity between arms (RR: 1.14; 95% CI: 1.08–1.20; $I^2$: 75%; **Figure 2**).

No statistical differences were observed in second- (RR: 1.10; 95% CI: 0.85–1.42; $I^2$: 84%) or subsequent rescue treatment lines; that is, when participants received more than one eradication treatment (RR: 1.10; 95% CI: 0.97–1.25; $I^2$: 69%). Two RCTs (Liou et al., 2018; Bonoso Criado et al., 2021) reported data on patients treated with a third-line treatment, with no differences between treatment arms.

**TABLE 1 |** Characteristics of studies comparing empirical vs. susceptibility-guided treatment for *H. pylori* infection.

| Author | Year | Country | Population | Design | Treatment line | Susceptibility test | C res (%) | M res (%) | L res (%) | Empirical regimen | Eradication rate with empiric regimen | Eradication rate with susceptibility-guided regimen |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Toracchio et al. (2000) | 2000 | Italy | Adults | R | 1st | Agar dilution | 13 | 33 | - | PPI-C-M, 10 d | 42/56 (75%) | 48/53 (91%) |
| Romano et al. (2000) | 2000 | Italy | Adults | R | 1st | E-test | 12 | 22 | - | PPI-C-M, 7 d | 31/40 (77%) | 38/40 (95%) |
| Street et al. (2001) | 2001 | Italy | Adults | NR | 1st | E-test | 16 | 56 | - | PPI/Ra-A-C, 8 d | 61/75 (81%) | 62/63 (98%) |
| Avidan et al. (2001) | 2001 | Israel | Adults | R | 2nd | E-test | 100 | 0 | - | PPI-A-C, 10 d | 5/5 (100%) | 5/5 (100%) |
| Miwa et al. (2003) | 2003 | Japan | Adults | R | 2nd | Dry plate | 71 | - | - | PPI-A-M, 10 d | 36/39 (92%) | 31/38 (82%)* |
| Neri et al. (2003) | 2003 | Italy | Adults | R | 1st | E-test | 7 | 12 | - | PPI-A-C, 7 d<br>RBC-C-M, 7 d | 78/116 (67%) | 88/116 (76%) |
| Romano et al. (2003) | 2003 | Italy | Adults | R | 1st | E-test | 12 | 22 | - | PPI-C-M, 7 d | 58/75 (77%) | 71/75 (95%) |
| Lamouliatte et al. (2003) | 2003 | France | Adults | R | 2nd | E-test | 64 | 53 | - | PPI-A-C, 7 d<br>PPI-A-C, 14 d<br>PPI-A-M, 14 d | 83/172 (48%) | 84/113 (74%) |
| Marzio et al. (2006) | 2006 | Italy | Adults | R | 1st | Agar dilution | 22 | 32 | 10 | PPI-A-L, 10 d | 36/39 (92%) | 39/41 (95%) |
| Marzio et al. (2006) | 2006 | Italy | Adults | R | 2nd | Agar dilution | 43 | 70 | 12 | PPI-A-L, 10 d | 26/32 (81%) | 50/51 (98%) |
| Yahav et al. (2006) | 2006 | Israel | Adults | NR | 2nd | E-test | 59 | 47 | - | PPI-A-C, 7 d<br>PPI-A-M, 7 d<br>PPI-B-T-M, 7 d | 31/49 (63%) | 42/49 (86%) |
| Furuta et al. (2007) | 2007 | Japan | Adults | R | 1st | PCR | 30 | - | - | PPI-A-C, 7 d | 105/150 (70%) | 144/150 (96%)* |
| Kawai et al. (2008) | 2018 | Japan | Adults | R | 1st | PCR (stools) | 48 | - | - | PPI-A-C, 7 d | 25/35 (71%) | 33/35 (94%) |
| Wang et al. (2008) | 2008 | China | Adults | R | 1st | Culture | 10 | - | - | PPI-A-C, 7 d<br>PPI-C-M, 7 d | 57/80 (71%) | 36/40 (90%) |
| Zhou et al. (2010) | 2010 | China | Adults | R | 1st | Agar dilution | 15 | - | - | PPI-C-M, 10 d | 107/135 (79%) | 117/125 (94%) |
| Bontems et al. (2011) | 2011 | Brussels, Italy, France | Children | R | 1st and ≥2nd | E-test | 16 | 20 | - | PPI-A, 5 d + PPI-C-M, 5 d | 68/83 (82%) | 59/82 (72%) |
| Molina-Infante et al. (2012) | 2012 | Spain | Adults | NR | 1st | E-test | 20 | 34 | - | PPI-A-C-M, 10 d | 182/209 (87%) | 70/87 (80%) |
| Lee et al. (2013) | 2013 | Korea | Adults | NR | 1st | PCR | 22 | - | - | PPI-A-C, 7 d<br>PPI-C-M, 7 d | 433/616 (70%) | 176/218 (81%) |
| Cosme et al. (2013) | 2013 | Spain | Adults | NR | 1st | E-test | 13 | - | - | PPI-A-C, 10 d | 51/104 (49%) | 113/134 (84%) |
| Park et al. (2014) | 2014 | Korea | Adults | R | 1st | Agar dilution | 25 | 46 | 37 | PPI-A-C, 7 d | 41/57 (72%) | 54/57 (95% |
| Martos et al. (2014) | 2014 | Spain | Adults | R | 1st | E-test | 9 | - | - | PPI+A+C, 10 d | 36/54 (67%) | 52/55 (94%) |
| Zhuo et al. (2015) | 2015 | China | Adults | R | 1st | Agar dilution | 17 | 95 | 28 | PPI-A-C-B, 14 d | 405/500 (81%) | 281/313 (90%) |
| Dong et al. (2015) | 2015 | China | Adults | R | 1st | E-test, PCR | 40 | 53 | 56 | PPI-A-C-B, 14 d | 33/45 (73%) | 41/45 (91%) |
| Zhou et al. (2016) | 2016 | China | Adults | R | 1st | E-test | 49 | 66 | - | PPI-A-C-B, 10 d<br>PPI-A-C-M, 10 d | 545/700 (78%) | 282/318 (89%)* |
| Kwon et al. (2016) | 2016 | Korea | Adults | NR | 2nd | Agar dilution | 85 | 52 | - | PPI-B-T-M, 14 d<br>PPI-A-Mo, 14 d | 130/178 (73%) | 36/41 (88%) |
| Cosme et al. (2016) | 2016 | Spain | Adults | NR | 1st | E-test | 16 | - | - | PPI-A-C-M, 10 d | 103/118 (87%) | 98/104 (94%) |
| Miyaki et al. (2016) | 2016 | Japan | Adults | NR | 1st | Agar dilution | 30 | - | - | PPI-A-C, 7 d | 101/132 (76%) | 119/128 (93%) |
| Ferenc et al. (2017) | 2017 | Poland | Adults | NR | 1st | E-test | 55 | 57 | 6 | PPI-A, 5 d + PPI-C-M, 5 d<br>PPI-A-L, 14 d | 26/30 (87%) | 43/45 (95%) |

*(Continued)*

**TABLE 1** | Continued

| Author | Year | Country | Population | Design | Treatment line | Susceptibility test | C res (%) | M res (%) | L res (%) | Empirical regimen | Eradication rate with empiric regimen | Eradication rate with susceptibility-guided regimen |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Liou et al. (2018) | 2018 | Taiwan | Adults | R | ≥3rd | PCR | 92 | 69 | 70 | PPI-A, 7 d + PPI-M-L/C/T, 7 d | 12/20 (60%) | 17/21 (81%) |
| Liou et al. (2018) | 2018 | Taiwan | Adults | R | ≥3rd | PCR | 93 | 66 | 60 | PPI-A, 7 d + PPI-M-L/C/T, 7 d | 148/205 (72%) | 160/205 (78%) |
| Gweon et al. (2018) | 2018 | Korea | Adults | NR | 1st | PCR | 37 | - | - | PPI-A-C, 7 d | 230/319 (72%) | 191/208 (92%) |
| Gweon et al. (2018) | 2018 | Korea | Adults | NR | 2nd | PCR | 37 | - | - | PPI-B-T-M, 7 d | 66/75 (88%) | 8/9 (89%) |
| Huang et al. (2018) | 2018 | Taiwan | Adults | NR | 3rd | E-test | 75 | 67 | 95 | PPI-A-T-M, 14 d | 14/27 (52%) | 35/43 (81%) |
| Mascellino et al. (2018) | 2018 | Italy | Adults | NR | ≥2nd | E-test | 50 | 68 | - | PPI-B-T-M, 14 d PPI-A-L, PPI-A-R Other regimens | 8/10 (80%) | 20/30 (67%) |
| Tanabe et al. (2018) | 2018 | Japan | Adults | NR | 1st | Agar dilution | 23 | 4 | - | PPI-A-C, 7 d | 619/780 /79%) | 198/212 (93%) |
| Ong et al. (2019) | 2019 | Korea | Adults | R | 1st | PCR | 26 | - | - | PPI-A-C-M, 14 d | 169/196 (86%) | 164/201 (82%) |
| Chen et al. (2019) | 2019 | China | Adults | R | 1st | Agar dilution | 35 | 83 | 47 | PPI-B-A-M, 14 d | 82/96 (85%) | 262/286 (92%) |
| Cho et al. (2019) | 2019 | Korea | Adults | NR | 1st | PCR | 23 | - | - | PPI-A-M, 7 d | 186/327 (57%) | 115/150 (77%) |
| Choi et al. (2019) | 2019 | Korea | Adults | NR | 1st | PCR | 24 | - | - | PPI-B-T-M, 14 d | 98/104 (94%) | 48/50 (96%) |
| Byambajav et al. (2019) | 2019 | Mongolia | Adults | NR | 1st | Agar dilution | 37 | 74 | - | PPI-A-C, 10 d PPI-A-C-B, 10 d PPI-A, 5 d + PPI-C-M, 5 d | 204/270 (75%) | 41/46 (89%) |
| Delchier et al. (2019) | 2019 | France | Adults | R | 1st | PCR | 23 | - | 13 | PPI-A-C, 7 d | 152/208 (73%) | 177/207 (85%) |
| Zhang et al. (2020) | 2020 | China | Children | NR | 2nd | Culture | 96 | 4 | 7 | PPI-A-M-B | 74/75 (99%) | 46/64 (72%)* |
| Saracino et al. (2020) | 2020 | Italy | Adults | NR | ≥2nd | E-test | 83 | 67 | 47 | PPI-Pylera®, 10 d | 161/186 (87%) | 875/1037 (84%) |
| Pan et al. (2020) | 2020 | China | Adults | R | 1st | Agar dilution | 67 | 86 | 64 | PPI-A-C-B, 14 d | 100/157 (64%) | 238/310 (77%) |
| Ji et al. (2020) | 2020 | China | Adults | R | ≥2nd | Agar dilution | 67 | 98 | 51 | PPI-A-L-B, 14 d PPI-A-F-B, 14 d | 156/210 (74%) | 164/210 (78%) |
| Bonoso Criado et al. (2021) | 2021 | Spain | Adults | R | 1st | Culture | 23 | 25 | 19 | PPI-B-T-M, 10 d | 43/45 (96%) | 39/43 (91%) |
| Bonoso Criado et al. (2021) | 2021 | Spain | Adults | R | 2nd | Culture | 23 | 25 | 19 | PPI-B-T-M, 10 d | 6/6 (100%) | 8/9 (89%) |
| Bonoso Criado et al. (2021) | 2021 | Spain | Adults | R | 3rd | Culture | 23 | 25 | 19 | PPI-B-T-M, 10 d | 2/4 (50%) | 1/1 (100%) |
| Chang et al. (2021) | 2021 | Korea | Adults | NR | 1st | PCR | 32 | - | - | PPI-A-C, 7d | 183/198 (92%) | 256/292 (88%) |
| Choe et al. (2021) | 2021 | Korea | Adults | NR | 1st | PCR | - | - | - | PPI-A-C, 14d | 22/27 (82%) | 124/139 (89%) |
| Choe et al. (2021) | 2021 | Korea | Adults | NR | 1st | PCR | - | - | - | PPI-A, 5 d + PPI-C-M, 5 d | 91/111 (82%) | 8/10 (80%) |
| Choe et al. (2021) | 2021 | Korea | Adults | NR | 1st | PCR | - | - | - | PPI-B-T-M, 14 d | 15/17 (88%) | 55/60 (92%) |
| Choi et al. (2021) | 2021 | Korea | Adults | R | 1st | PCR | 26 | - | - | PPI-A-C-M, 10 d | 88/107 (82%) | 91/110 (83%) |
| Cha et al. (2021) | 2021 | Korea | Adults | R | 1st | PCR | 22 | - | - | PPI-B-T-M, 7 d | 142/161 (88%) | 118/147 (80%) |

*R, randomized; NR, non-randomized; C res, resistance to clarithromycin; M res, resistance to metronidazole; L res, resistance to levofloxacin; PPI, proton pump inhibitor; A, amoxicillin; C, clarithromycin; M, metronidazole (or tinidazole); B, bismuth; T, tetracycline; L, levofloxacin; Mo, moxifloxacin; R, rifabutine; Ra, ranitidine; RBC, ranitidine bismuth citrate; Pylera®, single capsule containing bismuth, tetracycline, and metronidazole; -, information was not reported/available. Eradication rate was calculated by intention-to-treat analysis. *The dose of PPI in the susceptibility-guided regimen was also based on CYP2C19 gene polymorphism.*

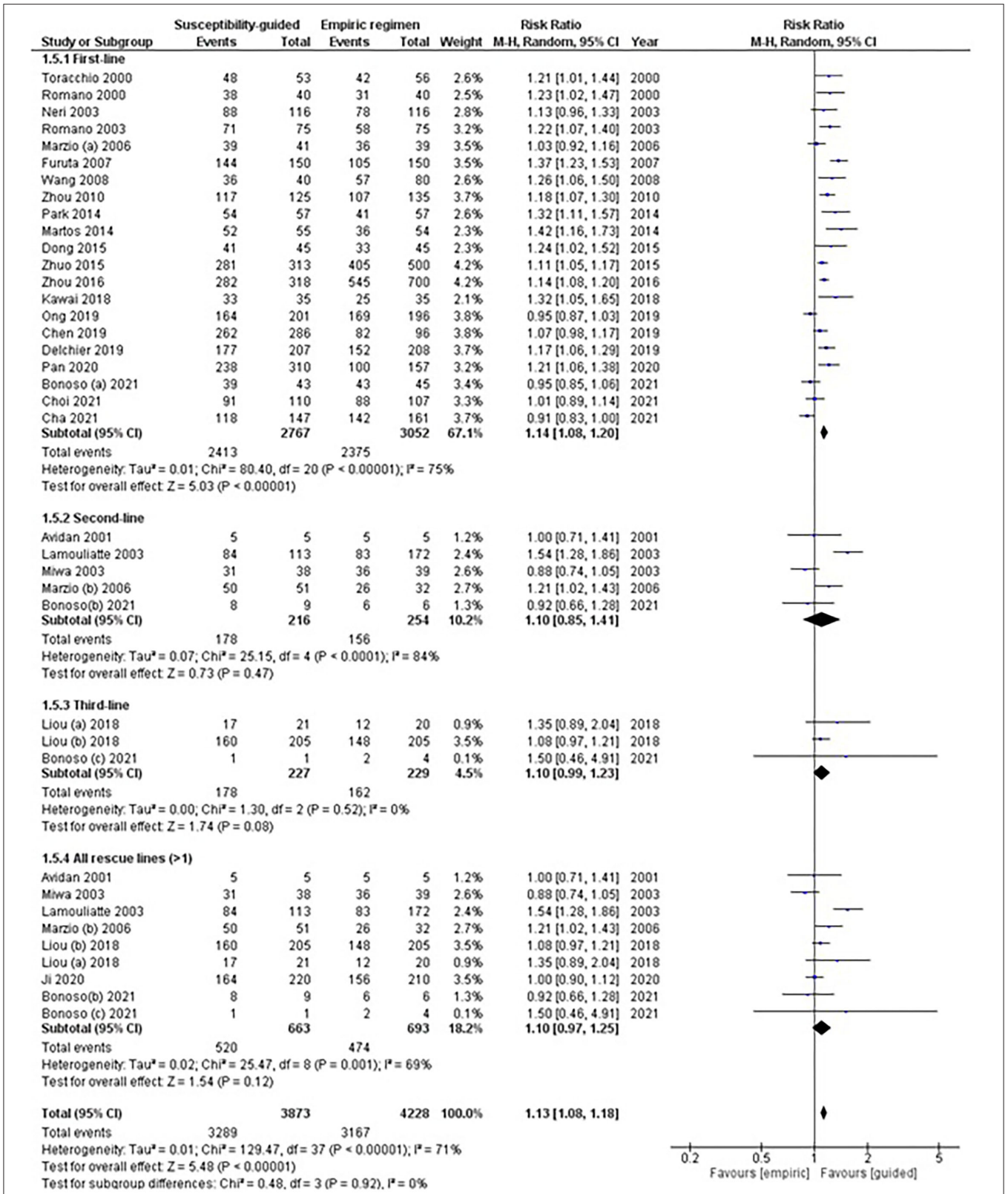


**FIGURE 2 |** Forest plot of randomized controlled studies comparing the effectiveness of susceptibility-guided and empirical treatment according to treatment line. M-H, Mantel–Haenszel; CI, confidence interval.

### Randomized-Controlled Trials by Susceptibility Test

A total of 24 RCTs used culture and 8 PCR-based methods to determine (on gastric biopsies only) any bacterial antibiotic resistance (**Table 1**). Overall data were reported with moderate to high heterogeneity for each of the subgroup analyses and the test for subgroup differences was reported not significant.

Among studies with culture testing, results were moderately heterogeneous (RR: 1.13, 95% CI: 1.08–1.19, $I^2$: 65%; **Figure 3**), and eradication rates were statistically higher in the guided-treatment arm than in empirically treated participants (86 vs. 76%; respectively). In this same subgroup, sensitivity analyses performed among naïve patients showed similar results (RR: 1.13, 95% CI: 1.07–1.20, $I^2$: 81%). However, when rescue treatments (more than one treatment failure) were considered, no statistically significant differences were found between treatment arms (RR: 1.05, 95% CI: 0.95–1.17, $I^2$: 85%).

Among studies using PCR, no statistically significant differences were found between treatment groups (RR: 1.10, 95% CI: 0.99–1.24, $I^2$: 85%). In this subgroup, sensitivity analyses performed among naïve patients showed similar results (RR: 1.10, 95% CI: 0.95–1.26, $I^2$: 89%). One study (Liou et al., 2018) assessing third-line treatment, where two comparisons were available, showed no significant differences between treatments when data from both comparisons were pooled (RR: 1.10, 95% CI: 0.98–1.24, $I^2$: 3%).

Two other studies (Kawai et al., 2008; Dong et al., 2015) were not included in this subgroup meta-analysis as they used PCR on stool, and both PCR and E-test, respectively, and results were not reported separately.

## Empirical First-Line Quadruple Treatment

The recommendations of the Consensus guidelines on *H. pylori* first-line therapy were used to select studies for this subgroup meta-analysis. Only those studies evaluating naïve patients treated with an empirical first-line quadruple therapy—either with or without bismuth—and excluding unaccepted and suboptimal triple therapies and tailored treatment based on the CYP2C19 polymorphism were included.

In total, 12 studies (RCTs and non-RCTs) including 2,762 naïve patients (1,455 in the susceptibility-guided and 1,307 in the empirical group) were evaluated. No statistically significant differences were found in cure rates between the guided therapy (87%) and the empirical treatment (78%), with low to moderate heterogeneity between treatment arms (RR: 1.04; 95% CI: 0.99–1.09; $I^2$: 72%; **Figure 4**). A *post hoc* sensitivity analysis including only RCTs (eight studies) in this same subgroup meta-analysis also confirmed the lack of difference in effectiveness between both groups (RR: 1.05; 95% CI: 0.99–1.12; $I^2$: 77%). Moreover, excluding the RCT by Zhou et al. (2016), because the susceptibility-guided treatment was based on the CYP2C19 polymorphism, the effectiveness results remained similar in both treatment arms, with no significant differences (RR: 1.04; 95% CI: 0.97–1.12; $I^2$: 75%).

## Quality Assessment

A summary of the quality of included studies is shown in **Supplementary Figures 5**, **6**.

Quality assessment of all studies included in the meta-analysis is presented in the summary table, following Cochrane instructions for evaluation of comparative studies (both RCTs and non-RCTs) with the RoB tool.

The RoB for both the randomization and allocation items was either unclear or high-risk in 50 to 75% of the studies (**Supplementary Figure 5**). The quality items related to the blinding of participants and personnel and to the outcome assessment are unlikely to affect the eradication outcome because *H. pylori* is an objective measurable endpoint. Therefore, these items were considered as introducing a low risk of bias, even for open-labeled studies.

All studies reported complete outcome data with no imbalance between arms in the patient's participation flow; therefore, no attrition bias was identified. Likewise, no selective reporting bias was detected [except for one study (Bontems et al., 2011)], as results of the primary endpoint were always correctly reported and data could be extracted (**Supplementary Figure 6**).

The funnel plot comparing the susceptibility-guided vs. empirical regimen groups of all included studies are shown in **Supplementary Figure 7**. This plot shows asymmetry suggesting a possible publication bias.

# DISCUSSION

Susceptibility testing has been proposed for antibiotic stewardship, aiming to reduce unnecessary antibiotic prescriptions; theoretically, treatment of *H. pylori* infections should not be an exception (Dang and Graham, 2017). Furthermore, through the application of susceptibility testing before treatment, the development of antimicrobial resistance could be minimized (Arslan et al., 2017), as antibiotic resistance in the outpatient community is positively correlated with antibiotic use (Megraud et al., 2013). However, in the present study (meta-analysis), the benefit of susceptibility-guided treatment over empirical treatment of *H. pylori* infection could not be demonstrated.

Several meta-analyses have previously compared cure rates of susceptibility-guided vs. empirical therapy for *H. pylori* first-line treatment, but all of them had limitations. The first meta-analysis was published by Wenzhen et al. (2010) and was focused specifically on first-line treatment. It only included five RCTs and concluded that culture-guided triple therapy was more effective than standard triple therapy (which was the regimen prescribed in most studies at that time) for first-line treatment. The second meta-analysis was published by Lopez-Gongora et al. (2015) and concluded that, in first-line treatment (nine studies only), susceptibility-guided therapy was more efficacious than empirical 7- to 10-day triple therapy (which, again, was the generally prescribed treatment at that time). The third meta-analysis was published by Chen et al. (2016) (including also nine studies only), and, again, showed that first-line tailored therapy achieved higher eradication rates than empirical regimens. The fourth meta-analysis was published by Gingold-Belfer et al. (2021) (including 16 studies only), also focusing mainly on first-line treatment (as only three RCTs were on rescue treatment), and concluded

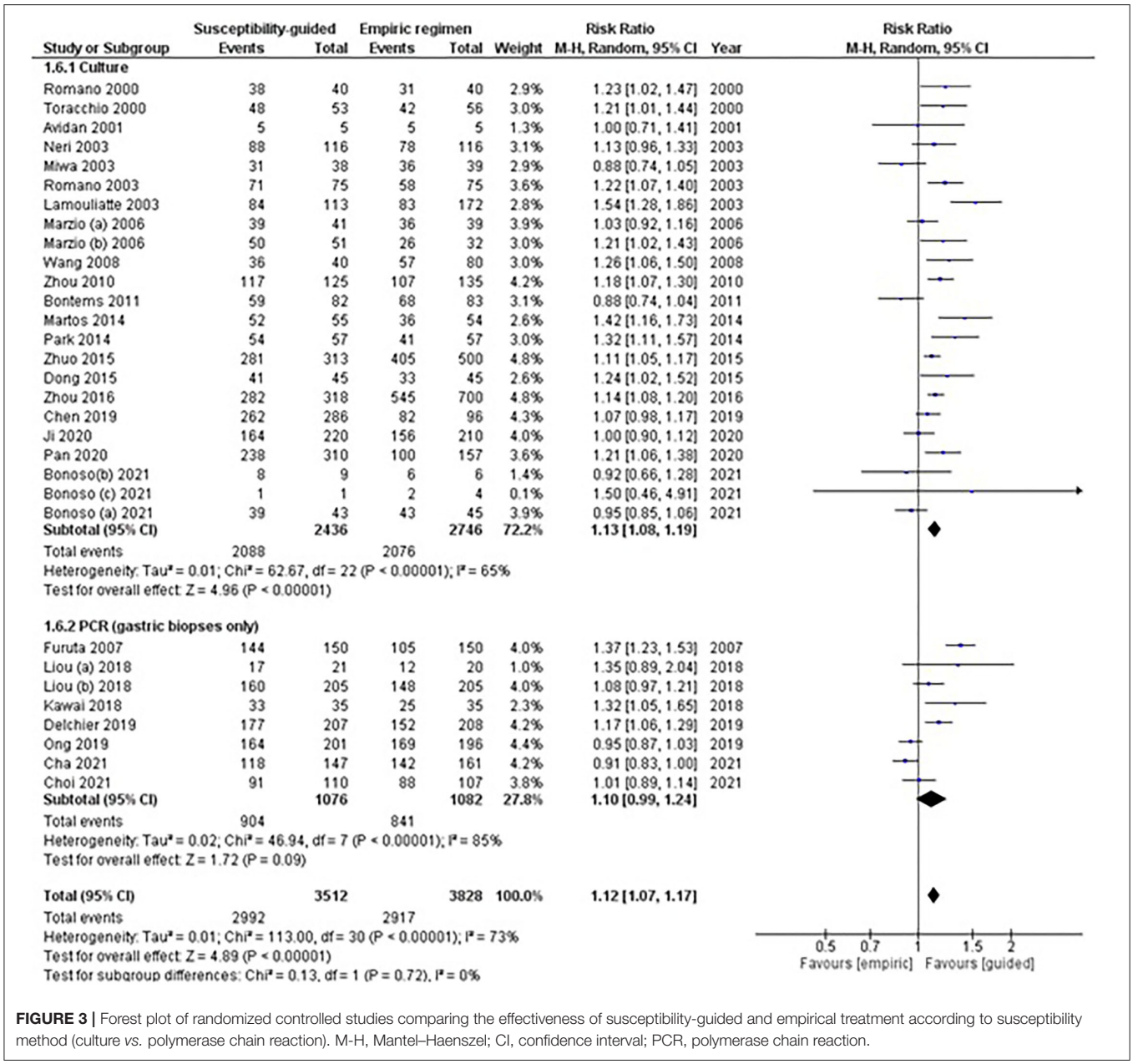


**FIGURE 3 |** Forest plot of randomized controlled studies comparing the effectiveness of susceptibility-guided and empirical treatment according to susceptibility method (culture *vs.* polymerase chain reaction). M-H, Mantel–Haenszel; CI, confidence interval; PCR, polymerase chain reaction.

that susceptibility-guided therapy was superior to first-line clarithromycin-based triple therapy only when clarithromycin resistance exceeded 20%.

In our meta-analysis, the most updated in the literature, we have included 47 comparative studies (involving 6,705 patients in the susceptibility-guided group and 7,895 in the empirical group and including both RCTs and non-RCTs). Therefore, this study presents the highest number of studies in each subgroup published so far. Furthermore, the subgroup analyses performed in the present meta-analysis were more comprehensive than those of previous systematic reviews, and our protocol established no language restrictions.

Overall better efficacy results were obtained with the susceptibility-guided strategy for first-line treatments (naïve patients, 35 studies), although the results were borderline statistically significant (RR: 1.13; 95% CI: 1.08, 1.17). However, when prescribing only empirical quadruple regimens—that is, excluding the suboptimal triple therapies—no differences in efficacy were found vs. the susceptibility-guided group; this lack of difference was confirmed when only RCTs were considered. Therefore, we may conclude that susceptibility-guided treatment of *H. pylori* infection is not better than empirical treatment in first-line if the most updated bismuth or non-bismuth quadruple regimens are empirically prescribed, in agreement with a previous study (Gingold-Belfer et al., 2021).

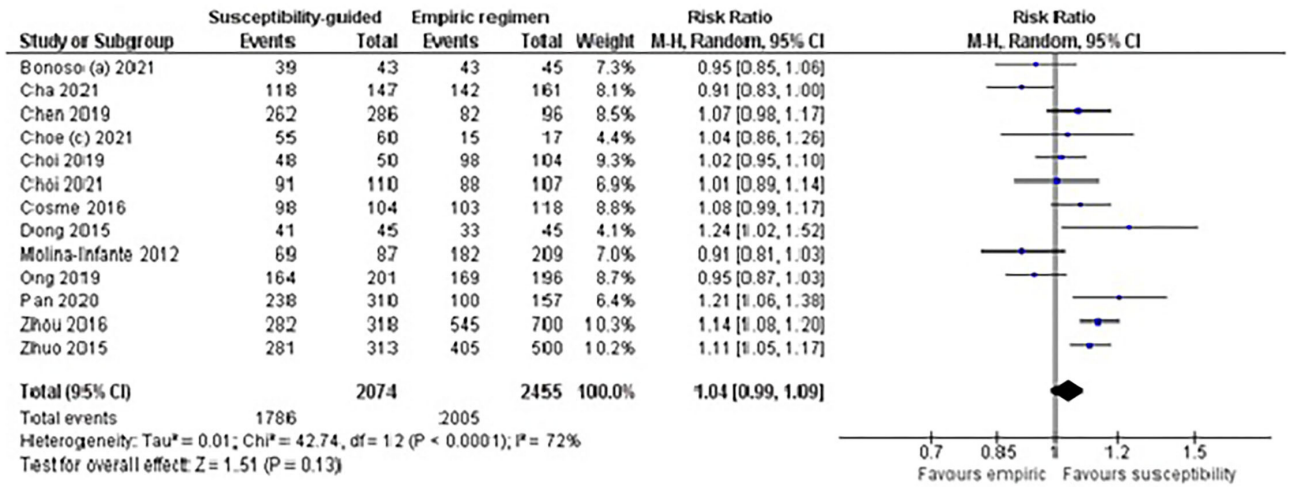


**FIGURE 4 |** Forest plot of studies comparing the effectiveness of susceptibility-guided and empirical treatment in naïve patients treated with a (bismuth or non-bismuth) quadruple therapy. M-H, Mantel–Haenszel; CI, confidence interval.

The results of our meta-analysis are in agreement with the well-known high effectiveness of bismuth quadruple therapy, even in patients with clarithromycin or metronidazole resistance. In particular, when a bismuth quadruple therapy [either with tetracycline (Choi et al., 2019) or with amoxicillin (Chen et al., 2019)] was empirically prescribed, the efficacy was similar to that obtained with the susceptibility-based strategy. As an example, in the study by Choi et al. (2019), the eradication rate with the empirical bismuth quadruple and the susceptibility-based therapy was 94 and 96%, respectively. An advantage of prescribing a bismuth-based quadruple therapy is that we do not need to worry about previous antibiotic use or antimicrobial resistance as the risk of having a tetracycline or amoxicillin-resistant strain is extremely low and metronidazole resistance has limited impact on the effectiveness of this regimen (Gisbert and Pajares, 2002; Gisbert, 2020). In addition, the results of our meta-analysis are also in agreement with the encouraging results that are generally obtained with the empirical use of non-bismuth quadruple concomitant therapy, even when single clarithromycin or metronidazole resistance is present (only dual clarithromycin and metronidazole resistance seems to jeopardize effectiveness with this regimen) (Gisbert and Calvet, 2011).

Some meta-analyses have compared *H. pylori* cure rates of susceptibility-guided therapies with those of empirical therapy specifically for second-line treatment. In the meta-analysis by Lopez-Gongora et al., only four RCTs assessing *H. pylori* second-line rescue therapies were included (Lopez-Gongora et al., 2015). Results were highly heterogeneous and no significant differences were found between susceptibility-guided and empirical strategies in terms of efficacy. The other meta-analysis, performed by Chen et al., also found no differences between tailored and empirical rescue regimens, although only three studies were included (Chen et al., 2016). Finally, in our updated meta-analysis, for rescue therapies (16 studies, mostly as second-line), similar efficacy results were demonstrated with the two strategies—tailored and empirical—both when all the comparative studies were included and when only RCTs were considered.

It has been frequently recommended that performing culture at first-line treatment or after a first eradication failure may not be necessary and therefore assessing *H. pylori* sensitivity to antibiotics in clinical practice may be suggested only after failure of the second treatment (O'Connor et al., 2000). However, previous meta-analyses could not find any RCT comparing cure rates of susceptibility-guided therapies vs. those of empirical third-line therapy (Lopez-Gongora et al., 2015). Another systematic review aimed to evaluate the effectiveness of susceptibility-guided therapy as third-line therapy (without comparing it with empirical treatment) (Puig et al., 2016): four observational studies were included (no comparative studies were found), and the pooled mean eradication rate with susceptibility-guided therapy was only 72%. Therefore, the authors concluded that cure rates with susceptibility-guided therapy were, at best, moderate (Puig et al., 2016). Similarly, a more recent meta-analysis identified up to three studies and one sub-study showing a third-line therapy success of 79.9% in the susceptibility-guided therapy group vs. 65.2% in the empirical one (Gingold-Belfer et al., 2021). In our meta-analysis, four studies (of which two were RCTs) evaluated this comparison in the scenario of third-line treatment (Huang et al., 2018; Liou et al., 2018; Bonoso Criado et al., 2021; Choe et al., 2021), reporting no differences between the empirical and the susceptibility-guided arms. Therefore, the evidence is in favor of susceptibility-guided therapy as rescue therapy is currently insufficient to recommend its use.

In routine clinical laboratories, the detection of *H. pylori* antimicrobial resistance is mainly based on phenotypic methods performed after culture, including gradient diffusion susceptibility testing (E-test) and the agar dilution method (Arslan et al., 2017). In the last years, different PCR-based

approaches have been developed as alternative tools to bacterium culture (Ierardi et al., 2017). In our meta-analysis, similar results were observed when susceptibility testing was assessed by culture or by PCR. Molecular tests are accurate in finding even minimal genotypic traces of certain resistant strains and are faster than conventional culture-based assays. Furthermore, PCR is technically feasible for clinical application in small- and medium-sized hospitals in developing countries (Xuan et al., 2016). However, the correlation between both methods is not perfect, probably due to the relatively low sensitivity of phenotypic assessment, the possibility that the E-test may identify resistant strains with point mutations different from those tested by PCR, or its inability to detect hetero-resistance (Ierardi et al., 2017; Jung et al., 2018).

Finally, some limitations of the strategy of performing culture systematically in all patients should be recognized: (1) culture implies the performance of endoscopic exploration, which is uncomfortable, expensive, and not free of risk. In addition, as endoscopy centers have been facing increasing demands, the technique frequently involves prolonged waiting times. As a consequence of the aforementioned problems, several diagnostic policies have been proposed for selecting patients with symptoms of dyspepsia, the most outstanding being the so-called "test-and-treat" strategy. Several prospective studies and decision analyses support the use of the test-and-treat strategy for dyspeptic patients (Gisbert and Calvet, 2013; Beresniak et al., 2020). Accordingly, this strategy has been recommended by all international consensus conferences (Fallone et al., 2016; Chey et al., 2017; Malfertheiner et al., 2017). (2) Culture is not always available on a routine basis. (3) The sensitivity of bacterial culture is not 100% (Megraud et al., 1997); indeed, even in the optimal conditions usually encountered in therapeutic trials, culture sensitivity is <90% (Zullo et al., 2003; Cammarota et al., 2014; Baylina et al., 2019). (4) Antibiotic susceptibility testing in clinical practice yields useful information only for a few antibiotics: clarithromycin, quinolones, and, less clearly, metronidazole (the relevance of *in vitro* metronidazole resistance for the *in vivo* treatment is quite limited) (Gisbert and Pajares, 2002); on the other hand, resistance to amoxicillin and tetracycline is extremely low. (5) Even knowing the susceptibility of *H. pylori*, eradication rates do not achieve 100%, as the results observed *in vivo* by following *in vitro* susceptibility to antibiotics are often disappointing (Guslandi, 2001; Gisbert and Pajares, 2002; Zullo et al., 2003; Baylina et al., 2019). The reverse situation is also possible, as *H. pylori* eradication may, nonetheless, be achieved in the presence of *H. pylori* metronidazole- or clarithromycin-resistant strains even with a drug combination including these antibiotics (Zullo et al., 2003; Bujanda et al., 2020, 2021). Furthermore, probably due to the synergistic effect of bismuth, the addition of this drug to triple therapy with clarithromycin may allow achieving a cure rate of approximately 90% even in patients with resistance against this antibiotic (Gisbert and McNicholl, 2017; Gisbert and Nyssen, 2021). (6) As previously mentioned, high eradication rates (≥90%) have been obtained with current up-to-date empirical first-line treatments, such as the bismuth or non-bismuth quadruple regimens. (7) Some studies have evaluated different empirical regimens after the failure of one, two, or more eradication treatments and have achieved a final (overall) eradication rate of almost 100% (Bock et al., 2000; Chan et al., 2000; Gasbarrini et al., 2000; Gomollon et al., 2000; Perri et al., 2000; Seppala et al., 2000; Beales, 2001; Canducci et al., 2001; Zullo et al., 2001, 2003; Treiber et al., 2002; Dore et al., 2003; Gisbert et al., 2003, 2004, 2008; Rokkas et al., 2009; Burgos-Santamaria et al., 2019). Thus, the empirical strategy should be based on the avoidance of repeating similar eradicating schemes, mainly clarithromycin- and quinolone-containing regimens, in the same patients during different eradicating regimens (Gisbert and Pajares, 2002; Roccarina et al., 2012; Calvet, 2018; Baylina et al., 2019; Nyssen et al., 2022). (8) Finally, different cost-effectiveness studies of the susceptibility-guided treatment of *H. pylori* infection have achieved contradictory results (Breuer and Graham, 1999; Romano et al., 2003; Qasim et al., 2004; Faber et al., 2005; Furuta et al., 2007; Cosme et al., 2013; Cammarota et al., 2014; Gweon et al., 2018; Liou et al., 2018; Cho et al., 2019).

Some relevant limitations affect studies comparing empirical vs. susceptibility-guided strategies, and consequently also the reliability of our meta-analysis. A major limitation of the current evidence regarding susceptibility-guided therapy is that comparative studies of susceptibility-guided therapy randomized patients after diagnostic endoscopy or even after successful culture (Lopez-Gongora et al., 2015). Therefore, the comparative effectiveness of susceptibility-guided therapy vs. the current non-invasive diagnosis and empirical treatment policy in patients with *suspected H. pylori* infection has not been evaluated in RCTs (Lopez-Gongora et al., 2015). Thus, a study adequately evaluating the effectiveness of susceptibility-guided therapy as a first-line treatment should randomize patients with non-investigated dyspepsia into non-invasive testing and endoscopy plus culture groups. In this same line, most of the studies evaluate the effectiveness of susceptibility-guided therapy as rescue therapy included the patients when the culture had been already obtained. Therefore, the effectiveness of susceptibility-guided therapy and empirical rescue therapy has never been properly compared (Puig et al., 2016). On the other hand, most studies using susceptibility-guided therapy only include patients with a positive culture. Therefore, the number of susceptibility-guided therapy failures due to patients' refusal of endoscopy has not been estimated or included (Baylina et al., 2019). When the applicability and effectiveness of this strategy were reviewed (Baylina et al., 2019), the rate of acceptance of endoscopy for biopsy and culture was described only in one article with only 60 patients and was reported to be as low as 60% (Matsumoto et al., 2005). In addition, given the diversity of studies included, our meta-analysis showed considerable heterogeneity (with asymmetric funnel plots) of the different *a priori* subgroup analyses performed comparing both therapeutic strategies; although such variability was investigated, it only could be partially explained. However, it is important to highlight that overall methodological quality was frequently high, and most studies were likely to avoid performance or detection biases (as per the therapeutic context) as well as attrition or reporting biases (as per the robustness of the outcome).

In summary, we think that susceptibility tests (culture or PCR) should be routinely performed, even before prescribing first-line treatment, in specialized centers with an interest in *H. pylori* management, to evaluate the prevalence of antibiotic resistance in the treatment of naïve patients and the influence of such resistances on the efficacy of up-to-date first-line eradication treatments. However, the present meta-analysis shows that the evidence is too limited to support the generalized use of susceptibility-guided therapy for *H. pylori* treatment in routine clinical practice, either as first-line or as rescue treatment. Undoubtedly, the most effective first-line *H. pylori* eradication treatment—that is, those regimens that have demonstrated to achieve cure rates $\geq$90% in our setting—must always be prescribed and the rescue treatment should be carefully chosen depending on which treatment was used initially. The results (*H. pylori* cure rates) of our clinical practice should be continuously audited to confirm that we always maintain a high success rate.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

OPN and JPG interpreted the data. OPN performed the analysis and prepared the first draft of the manuscript. ME, JPG, and OPN contributed to the literature search and data extraction. ME reviewed and approved the final draft of the manuscript. JPG analyzed and reviewed the draft manuscript. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.913436/full#supplementary-material

## REFERENCES


Arslan, N., Yilmaz, O., and Demiray-Gurbuz, E. (2017). Importance of antimicrobial susceptibility testing for the management of eradication in Helicobacter pylori infection. *World J. Gastroenterol.* 23, 2854–2869. doi: 10.3748/wjg.v23.i16.2854

Avidan, B., Melzer, E., Keller, N., and Bar-Meir, S. (2001). The effect of culture results for Helicobacter pylori on the choice of treatment following failure of initial eradication. *Isr. Med. Assoc. J.* 3, 163–165.

Baylina, M., Munoz, N., Sanchez-Delgado, J., Lopez-Gongora, S., Calvet, X., and Puig, I. (2019). Systematic review: Would susceptibility-guided treatment achieve acceptable cure rates for second-line Helicobacter pylori therapy as currently practiced? *Helicobacter.* 24, e12584. doi: 10.1111/hel.12584

Beales, I. L. (2001). Efficacy of Helicobacter pylori eradication therapies: a single centre observational study. *BMC Gastroenterol.* 1, 7. doi: 10.1186/1471-230X-1-7

Beresniak, A., Malfertheiner, P., Franceschi, F., Liebaert, F., Salhi, H., and Gisbert, J. P. (2020). Helicobacter pylori "Test-and-Treat" strategy with urea breath test: A cost-effective strategy for the management of dyspepsia and the prevention of ulcer and gastric cancer in Spain-Results of the Hp-Breath initiative. *Helicobacter*. 13, e12693. doi: 10.1111/hel.12693

Blümel, B., Goelz, H., Kist, M., and Glocker, E. O. (2018). Retrospective study on outcome of salvage Helicobacter pylori eradication therapies based on molecular genetic susceptibility testing. *Helicobacter.* 23, e12494. doi: 10.1111/hel.12494

Bock, H., Koop, H., Lehn, N., and Heep, M. (2000). Rifabutin-based triple therapy after failure of Helicobacter pylori eradication treatment: preliminary experience. *J. Clin. Gastroenterol.* 31, 222–225. doi: 10.1097/00004836-200010000-00007

Bonoso Criado, R., Pérez Citores, L., Pérez Millán, G., Montero Moretón, A., González de Castro, E., Cabezudo Molleda, L., et al. (2021). Estudio comparativo prospectivo del tratamiento de Helicobacter pylori con triple terapia guiada por antibiograma frente a cuádruple terapia con subcitrato de bismuto-metronidazol-tetraciclina. *Rev. Esp. Enferm. Dig.* 113, 597–601.

Bontems, P., Kalach, N., Oderda, G., Salame, A., Muyshont, L., and Miendje, D. Y. (2011). Sequential therapy versus tailored triple therapies for Helicobacter pylori infection in children. *J. Pediatr. Gastroenterol. Nutr.* 53, 646–650. doi: 10.1097/MPG.0b013e318229c769

Breuer, T., and Graham, D. Y. (1999). Costs of diagnosis and treatment of Helicobacter pylori infection: when does choosing the treatment regimen based on susceptibility testing become cost effective? *Am. J. Gastroenterol.* 94, 725–729. doi: 10.1111/j.1572-0241.1999.00943.x

Bujanda, L., Nyssen, O. P., Cosme, A., Bordin, D. S., Tepes, B., and Perez-Aisa, A. (2020). Impact of Helicobacter pylori clarithromycin resistance on the treatment effectiveness: data of the European Registry on H. pylori Management (Hp-EuReg). *United Eur. Gastroenterol. J.* 8, 256–257.

Bujanda, L., Nyssen, O. P., Vaira, D., Saracino, I. M., Fiorini, G., and Lerang, F. (2021). Antibiotic Resistance Prevalence and Trends in Patients Infected with Helicobacter pylori in the Period 2013-2020: Results of the European Registry on H. pylori Management (Hp-EuReg). *Antibiotics.* 10, 1058. doi: 10.3390/antibiotics10091058

Burgos-Santamaria, D., McNicholl, A. G., and Gisbert, J. P. (2019). Empirical Helicobacter pylori rescue therapy: an 18-year singlecentre study of 1200 patients. *GastroHep*. 1, 311–324. doi: 10.1002/ygh2.372

Byambajav, T. O., Bira, N., Choijamts, G., Davaadorj, D., Gantuya, B., and Sarantuya, T. (2019). Initial trials with susceptibility-based and empiric anti-h. pylori therapies in mongolia. *Front. Pharmacol.* 10, 394. doi: 10.3389/fphar.2019.00394

Calvet, X. (2018). Dealing with uncertainty in the treatment of Helicobacter pylori. *Ther. Adv. Chronic. Dis.* 9, 93–102. doi: 10.1177/2040622318758240

Camargo, M. C., Garcia, A., Riquelme, A., Otero, W., Camargo, C. A., and Hernandez-Garcia, T. (2014). The problem of Helicobacter pylori resistance to antibiotics: a systematic review in Latin America. *Am. J. Gastroenterol.* 109, 485–495. doi: 10.1038/ajg.2014.24

Cammarota, G., Ianiro, G., Bibb,ò, S., Di Rienzo, T. A., Masucci, L., Sanguinetti, M., et al. (2014). Culture-guided treatment approach for Helicobacter pylori infection: review of the literature. *World J. Gastroenterol.* 20, 5205–5211. doi: 10.3748/wjg.v20.i18.5205

Cammarota, G., Martino, A., Pirozzi, G., Cianci, R., Branca, G., and Nista, E. C. (2004). High efficacy of 1-week doxycycline- and amoxicillin-based quadruple regimen in a culture-guided, third-line treatment approach for Helicobacter pylori infection. *Aliment. Pharmacol. Therap.* 19, 789–795. doi: 10.1111/j.1365-2036.2004.01910.x

Canducci, F., Ojetti, V., Pola, P., Gasbarrini, G., and Gasbarrini, A. (2001). Rifabutin-based Helicobacter pylori eradication 'rescue therapy'. *Aliment. Pharmacol. Therap.* 15, 143. doi: 10.1046/j.1365-2036.2001.00909.x

Cha, B., Bang, B. W., Shin, J. B., Ko, E. J., Ko, W., and Kwon, K. S. (2021). Bismuth containing quadruple therapy versus tailored therapy as first-line treatments for Helicobacter pylori infection in a high clarithromycin resistance area. *Scandin. J. Gastroenterol.* 56, 1017–1022. doi: 10.1080/00365521.2021.1948606

Chan, F. K., Sung, J. J., Suen, R., Wu, J. C., Ling, T. K., and Chung, S. C. (2000). Salvage therapies after failure of Helicobacter pylori eradication with ranitidine bismuth citrate-based therapies. *Aliment. Pharmacol. Therap.* 14, 91–95. doi: 10.1046/j.1365-2036.2000.00674.x

Chang, Y. W., Shin, G. Y., Kim, J. W., Moon, J. C., Chang, E. J., and Oh, C. H. (2021). Cost-effectiveness of empirical bismuth-based quadruple therapy and tailored therapy after clarithromycin resistance tests for helicobacter pylori eradication. *Dig. Dis. Sci.* 23, 1–9. doi: 10.1007/s10620-021-06938-y

Chen, H., Dang, Y., Zhou, X., Liu, B., Liu, S., and Zhang, G. (2016). Tailored therapy versus empiric chosen treatment for helicobacter pylori eradication: a meta-analysis. *Medicine.* 95, e2750. doi: 10.1097/MD.0000000000002750

Chen, Q., Long, X., Ji, Y., Liang, X., Li, D., Gao, H., et al. (2019). Randomised controlled trial: susceptibility-guided therapy versus empiric bismuth quadruple therapy for first-line Helicobacter pylori treatment. *Aliment. Pharmacol. Therap.* 49, 1385–1394. doi: 10.1111/apt.15273

Chey, W. D., Leontiadis, G. I., Howden, C. W., and Moss, S. F. A. C. G. C. (2017). Treatment of Helicobacter pylori Infection. *Am. J. Gastroenterol.* 112, 212–239. doi: 10.1038/ajg.2016.563

Chi, C. H., Lin, C. Y., Sheu, B. S., Yang, H. B., Huang, A. H., and Wu, J. J. (2003). Quadruple therapy containing amoxicillin and tetracycline is an effective regimen to rescue failed triple therapy by overcoming the antimicrobial resistance of Helicobacter pylori. *Aliment. Pharmacol. Therap.* 18:347–353. doi: 10.1046/j.1365-2036.2003.01653.x

Cho, J. H., Jeon, S. R., Kim, H. G., Jin, S. Y., and Park, S. (2019). Cost-effectiveness of a tailored Helicobacter pylori eradication strategy based on the presence of a 23S ribosomal RNA point mutation that causes clarithromycin resistance in Korean patients. *J. Gastroenterol. Hepatol.* 34, 700–706. doi: 10.1111/jgh.14383

Choe, A. R., Shim, K. N., Park, Y., Song, E. M., Tae, C. H., and Jung, S. A. (2021). Cost-effectiveness, efficacy, and safety analysis of tailored therapy in patients with helicobacter pylori infection. *J. Clin. Med.* 10, 2619. doi: 10.3390/jcm10122619

Choi, M. G., Lee, J. Y., Lim, C. H., Kim, J. S., Park, J. M., and Lee, I. S. (2014). Helicobacter pylori eradication according to23s ribosomal RNA point mutations. *Gastroenterology.* 146, S-557. doi: 10.1016/S0016-5085(14)62017-5

Choi, Y. I., Chung, J. W., Kim, K. O., Kwon, K. A., Kim, Y. J., and Kim, J. H. (2021). Tailored eradication strategy vs concomitant therapy for Helicobacter pylori eradication treatment in Korean patients. *World J. Gastroenterol.* 27, 5247–5258. doi: 10.3748/wjg.v27.i31.5247

Choi, Y. I., Chung, J. W., Park, D. K., Kim, K. O., Kwon, K. A., and Kim, Y. J. (2019). Tailored eradication vs empirical bismuth-containing quadruple therapy for first-line Helicobacter pylori eradication: A comparative, open trial. *World J. Gastroenterol.* 25, 6743–6751. doi: 10.3748/wjg.v25.i46.6743

Cosme, A., Lizasoan, J., Montes, M., Tamayo, E., Alonso, H., and Mendarte, U. (2016). Antimicrobial susceptibility-guided therapy versus empirical concomitant therapy for eradication of helicobacter pylori in a region with high rate of clarithromycin resistance. *Helicobacter.* 21, 29–34. doi: 10.1111/hel.12231

Cosme, A., Montes, M., Ibarra, B., Tamayo, E., Alonso, H., and Mendarte, U. (2017). Antimicrobial susceptibility testing before first-line treatment for Helicobacter pylori infection in patients with dual or triple antibiotic resistance. *World J. Gastroenterol.* 23, 3367–3373. doi: 10.3748/wjg.v23.i18.3367

Cosme, A., Montes, M., Martos, M., Gil, I., Mendarte, U., and Salicio, Y. (2013). Usefulness of antimicrobial susceptibility in the eradication of Helicobacter pylori. *Clin. Microbiol. Infec.* 19, 379–383. doi: 10.1111/j.1469-0691.2012.03844.x

Cosme, A., Torrente Iranzo, S., Montes Ros, M., Fernandez-Reyes Silvestre, M., Alonso Galan, H., and Lizasoain, J. (2019). Helicobacter pylori antimicrobial resistance during a 5-year period (2013-2017) in northern Spain and its relationship with the eradication therapies. *Helicobacter.* 24, e12557. doi: 10.1111/hel.12557

Costa, S., Soares, J. B., and Goncalves, R. (2017). Efficacy and tolerability of culture-guided treatment for Helicobacter pylori infection. *Eur. J. Gastroenterol. Hepatol.* 29, 1258–1263. doi: 10.1097/MEG.0000000000000960

Dang, B. N., and Graham, D. Y. (2017). Helicobacter pylori infection and antibiotic resistance: a WHO high priority? *Nat. Rev. Gastroenterol. Hepatol.* 14, 383–384. doi: 10.1038/nrgastro.2017.57

Delchier, J. C., Bastuji-Garin, S., Raymond, J., Megraud, F., Amiot, A., and Cambau, E. (2019). Efficacy of a tailored PCR-guided triple therapy in the treatment of Helicobacter pylori infection. *Med. Malad. Infect.* 50, 492–9. doi: 10.1016/j.medmal.2019.06.001

DerSimonian, R., and Laird, N. (1986). Meta-analysis in clinical trials. *Control Clin. Trials.* 7, 177–188. doi: 10.1016/0197-2456(86)90046-2

Dong, F., Ji, D., Huang, R., Zhang, F., Huang, Y., Xiang, P., et al. (2015). Multiple genetic analysis system-based antibiotic susceptibility testing in helicobacter pylori and high eradication rate with phenotypic resistance-guided quadruple therapy. *Medicine.* 94, e2056. doi: 10.1097/MD.0000000000002056

Dore, M. P., Leandro, G., Realdi, G., Sepulveda, A. R., and Graham, D. Y. (2000). Effect of pretreatment antibiotic resistance to metronidazole and clarithromycin on outcome of Helicobacter pylori therapy: a meta-analytical approach. *Dig. Dis. Sci.* 45, 68–76. doi: 10.1023/A:1005457226341

Dore, M. P., Marras, L., Maragkoudakis, E., Nieddu, S., Manca, A., and Graham, D. Y. (2003). Salvage therapy after two or more prior Helicobacter pylori treatment failures: the super salvage regimen. *Helicobacter.* 8, 307–309. doi: 10.1046/j.1523-5378.2003.00150.x

Draeger, S., Wuppenhorst, N., Kist, M., and Glocker, E. O. (2015). Outcome of second- and third-line Helicobacter pylori eradication therapies based on antimicrobial susceptibility testing. *J. Antimicrob. Chemother.* 70, 3141–3145. doi: 10.1093/jac/dkv223

Egger, M., Davey Smith, G., Schneider, M., and Minder, C. (1997). Bias in meta-analysis detected by a simple, graphical test. *BMJ.* 315, 629–634. doi: 10.1136/bmj.315.7109.629

Faber, J., Bar-Meir, M., Rudensky, B., Schlesinger, Y., Rachman, E., and Benenson, S. (2005). Treatment regimens for Helicobacter pylori infection in children: is in vitro susceptibility testing helpful? *J. Pediatr. Gastroenterol. Nutr.* 40, 571–574. doi: 10.1097/01.MPG.0000155567.71902.75

Fallone, C. A., Chiba, N., van Zanten, S. V., Fischbach, L., Gisbert, J. P., and Hunt, R. H. (2016). The Toronto Consensus for the Treatment of Helicobacter pylori Infection in Adults. *Gastroenterology.* 151, 51–69. e14. doi: 10.1053/j.gastro.2016.04.006

Ferenc, S., Gnus, J., Koscielna, M., Kinda, M., Yarka, A., and Stewart, L. (2017). High antibiotic resistance of Helicobacter pylori and its effect on tailored and empiric eradication of the organism in Lower Silesia, Poland. *Helicobacter.* 22, e12365. doi: 10.1111/hel.12365

Fiorini, G., Vakil, N., Zullo, A., Saracino, I. M., Castelli, V., and Ricci, C. (2013). Culture-based selection therapy for patients who did not respond to previous treatment for Helicobacter pylori infection. *Clin. Gastroenterol. Hepatol.* 11, 507–510. doi: 10.1016/j.cgh.2012.12.007

Furuta, T., Shirai, N., Kodaira, M., Sugimoto, M., Nogaki, A., and Kuriyama, S. (2007). Pharmacogenomics-based tailored versus standard therapeutic regimen for eradication of H. pylori. *Clin. Pharmacol. Ther.* 81, 521–528. doi: 10.1038/sj.clpt.6100043

Gasbarrini, A., Ojetti, V., Armuzzi, A., Branca, G., Canducci, F., and Torre, E. S. (2000). Efficacy of a multistep strategy for Helicobacter pylori eradication. *Aliment. Pharmacol. Therap.* 14, 79–83. doi: 10.1046/j.1365-2036.2000.00685.x

Gingold-Belfer, R., Niv, Y., Schmilovitz-Weiss, H., Levi, Z., and Boltin, D. (2021). Susceptibility-guided versus empirical treatment for Helicobacter pylori infection: A systematic review and meta-analysis. *J. Gastroenterol. Hepatol.* 36, 2649–2658. doi: 10.1111/jgh.15575

Gisbert, J. P. (2011). Is culture necessary before first-line treatment for Helicobacter pylori infection? *Intern Med.* 50, 2717. doi: 10.2169/internalmedicine.50.5135

Gisbert, J. P. (2020). Empirical or susceptibility-guided treatment for Helicobacter pylori infection? A comprehensive review. *Therap. Adv. Gastroenterol.* 13, 1756284820968736. doi: 10.1177/1756284820968736

Gisbert, J. P., and Calvet, X. (2011). Review article: non-bismuth quadruple (concomitant) therapy for eradication of Helicobater pylori. *Aliment. Pharmacol. Therap.* 34, 604–617. doi: 10.1111/j.1365-2036.2011.04770.x

Gisbert, J. P., and Calvet, X. (2013). Helicobacter Pylori "Test-and-Treat" strategy for management of dyspepsia: a comprehensive review. *Clin. Transl. Gastroenterol.* 4, e32. doi: 10.1038/ctg.2013.3

Gisbert, J. P., Calvet, X., Bujanda, L., Marcos, S., Gisbert, J. L., and Pajares, J. M. (2003). 'Rescue' therapy with rifabutin after multiple Helicobacter pylori treatment failures. *Helicobacter*. 8, 90–94. doi: 10.1046/j.1523-5378.2003.00128.x

Gisbert, J. P., Gisbert, J. L., Marcos, S., Jimenez-Alonso, I., Moreno-Otero, R., and Pajares, J. M. (2008). Empirical rescue therapy after Helicobacter pylori treatment failure: a 10-year single-centre study of 500 patients. *Aliment. Pharmacol. Therap*. 27, 346–354. doi: 10.1111/j.1365-2036.2007.03573.x

Gisbert, J. P., Gisbert, J. L., Marcos, S., and Pajares, J. M. (2004). Empirical Helicobacter pylori "rescue" therapy after failure of two eradication treatments. *Dig. Liver Dis*. 36, 7–12. doi: 10.1016/j.dld.2003.09.018

Gisbert, J. P., and McNicholl, A. G. (2017). Optimization strategies aimed to increase the efficacy of H. pylori eradication therapies. *Helicobacter*. 22, e12392. doi: 10.1111/hel.12392

Gisbert, J. P., and Nyssen, O. P. (2021). "Ten common errors in the treatment of Helicobacter pylori infection", in *Recent Advances in Gastroenterology*. New Delhi: C Probert Jaypee Brothers Medical Publishers Ltd. p. 106–150. ISBN13 9781909836242.

Gisbert, J. P., and Pajares, J. M. (2002). Review article: Helicobacter pylori "rescue" regimen when proton pump inhibitor-based triple therapies fail. *Aliment. Pharmacol. Therap*. 16, 1047–1057. doi: 10.1046/j.1365-2036.2002.01276.x

Gomollon, F., Sicilia, B., Ducons, J. A., Sierra, E., Revillo, M. J., and Ferrero, M. (2000). Third line treatment for Helicobacter pylori: a prospective, culture-guided study in peptic ulcer patients. *Aliment. Pharmacol. Therap*. 14, 1335–1338. doi: 10.1046/j.1365-2036.2000.00833.x

Guslandi, M. (2001). Review article: alternative antibacterial agents for Helicobacter pylori eradication. *Aliment. Pharmacol. Therap*. 15, 1543–1547. doi: 10.1046/j.1365-2036.2001.01064.x

Gweon, T. G., Kim, J. S., and Kim, B. W. (2018). An economic modeling study of helicobacter pylori eradication: comparison of dual priming oligonucleotide-based multiplex polymerase chain reaction and empirical treatment. *Gut Liver*. 12, 648–654. doi: 10.5009/gnl18079

Han, F., Ji, Z. Z., Jin, X., Wan, L., Cai, C. X., and Chen, Y. P., et al. (2016). Antimicrobial susceptibility testing for helicobacterf pylori treatment: A follow-up study on clinical effects. *World Chin. J. Digestol*. 24, 808–814. doi: 10.11569/wcjd.v24.i5.808

Higgins, J. P., and Thompson, S. G. (2002). Quantifying heterogeneity in a meta-analysis. *Stat. Med*. 21, 1539–1558. doi: 10.1002/sim.1186

Higgins, JPT., Thomas, J., Chandler, J., Cumpston, M., Li, T., Page, MJ., Welcg VA (editors) (2009). *Cochrane Handbook for Systematic Reviews of Interventions version 6, 2*. (updated February 2022). Cochrane, 2022. Available online at: www.training.cochrane.org/handbook

Hooi, J. K. Y., Lai, W. Y., Ng, W. K., Suen, M. M. Y., Underwood, F. E., and Tanyingoh, D. (2017). Global prevalence of helicobacter pylori infection: systematic review and meta-analysis. *Gastroenterology*. 153, 420–429. doi: 10.1053/j.gastro.2017.04.022

Huang, H. T., Wang, H. M., Yang, S. C., Tai, W. C., Liang, C. M., and Wu, K. L. (2018). Efficacy of a 14-day quadruple-therapy regimen for third-line Helicobacter pylori eradication. *Infect. Drug Resist*. 11, 2073–2080. doi: 10.2147/IDR.S185511

Ierardi, E., Giorgio, F., Iannone, A., Losurdo, G., Principi, M., and Barone, M. (2017). Noninvasive molecular analysis of Helicobacter pylori: Is it time for tailored first-line therapy? *World J. Gastroenterol*. 23, 2453–2458. doi: 10.3748/wjg.v23.i14.2453

Ji, C. R., Liu, J., Li, Y. Y., Qiao, C., Qu, J. Y., Hu, J. N., et al. (2020). Susceptibility-guided quadruple therapy is not superior to medication history-guided therapy for the rescue treatment of Helicobacter pylori infection: a randomized controlled trial. *J. Dig. Dis*. 21, 549–557. doi: 10.1111/1751-2980.12934

Jung, D. H., Kim, J. H., Jeong, S. J., Park, S. Y., Kang, I. M., and Lee, K. H. (2018). Peptide Nucleic Acid Probe-Based Analysis as a New Detection Method for Clarithromycin Resistance in Helicobacter pylori. *Gut. liver*. 12, 641–647. doi: 10.5009/gnl18111

Kawai, T., Yamagishi, T., Yagi, K., Kataoka, M., Kawakami, K., and Sofuni, A. (2008). Tailored eradication therapy based on fecal Helicobacter pylori clarithromycin sensitivities. *J. Gastroenterol. Hepatol*. 23, S171–S174. doi: 10.1111/j.1440-1746.2008.05408.x

Králícek, L., Kroupa, R., Ševcíková, A., Otrubová, M., and Kulhánek, V. (2017). Outcome of treatment of Helicobacter pylori infection based on microbiological susceptibility test ing fol low ing the unsucces sful second-line eradication treatment. *Gastroenterol. Hepatol*. 71, 304–309. doi: 10.14735/amgh2017csgh.info02

Kwon, Y. H., Jeon, S. W., Nam, S. Y., Lee, H. S., and Park, J. H. (2019). Efficacy of tailored therapy for Helicobacter pylori eradication based on clarithromycin resistance and survey of previous antibiotic exposure: A single-center prospective pilot study. *Helicobacter*. 24, e12585. doi: 10.1111/hel.12585

Kwon, Y. H., Kim, N., Lee, J. Y., Choi, Y. J., Yoon, K., and Nam, R. H. (2016). Comparison of the efficacy of culture-based tailored therapy for Helicobacter pylori eradication with that of the traditional second-line rescue therapy in Korean patients: a prospective single tertiary center study. *Scandin. J. Gastroenterol*. 51, 270–276. doi: 10.3109/00365521.2015.1095352

Lamouliatte, H., Megraud, F., Delchier, J. C., Bretagne, J. F., Courillon-Mallet, A., and De Korwin, J. D. (2003). Second-line treatment for failure to eradicate Helicobacter pylori: a randomized trial comparing four treatment strategies. *Aliment. Pharmacol. Therap*. 18, 791–797. doi: 10.1046/j.1365-2036.2003.01759.x

Lee, H. J., Kim, J. I., Cheung, D. Y., Kim, T. H., Jun, E. J., and Oh, J. H. (2013). Eradication of Helicobacter pylori according to 23S ribosomal RNA point mutations associated with clarithromycin resistance. *J. Infect. Dis*. 208, 1123–1130. doi: 10.1093/infdis/jit287

Lee, J. W., Kim, N., Nam, R. H., Lee, S. M., Kwon, Y. H., and Sohn, S. D. (2019). Favorable outcomes of culture-based Helicobacter pylori eradication therapy in a region with high antimicrobial resistance. *Helicobacter*. 24, e12561. doi: 10.1111/hel.12561

Liberati, A., Altman, D. G., Tetzlaff, J., Mulrow, C., Gotzsche, P. C., and Ioannidis, J. P. (2009). The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *BMJ*. 339, b2700. doi: 10.1136/bmj.b2700

Liou, J. M., Chen, C. C., Chang, C. Y., Chen, M. J., Fang, Y. J., and Lee, J. Y. (2013). Efficacy of genotypic resistance-guided sequential therapy in the third-line treatment of refractory Helicobacter pylori infection: a multicentre clinical trial. *J. Antimicrob. Chemother*. 68, 450–456. doi: 10.1093/jac/dks407

Liou, J. M., Chen, P. Y., Luo, J. C., Lee, J. Y., Chen, C. C., and Fang, Y. J. (2018). Efficacies of genotypic resistance-guided vs empirical therapy for refractory helicobacter pylori infection. *Gastroenterology*. 155, 1109–1119. doi: 10.1053/j.gastro.2018.06.047

Lopez-Gongora, S., Puig, I., Calvet, X., Villoria, A., Baylina, M., and Munoz, N. (2015). Systematic review and meta-analysis: susceptibility-guided versus empirical antibiotic treatment for Helicobacter pylori infection. *J. Antimicrob. Chemother*. 70, 2447–2455. doi: 10.1093/jac/dkv155

Malfertheiner, P., Megraud, F., O'Morain, C. A., Gisbert, J. P., Kuipers, E. J., and Axon, A. T. (2017). Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut*. 66, 6–30. doi: 10.1136/gutjnl-2016-312288

Martos, M., Bujanda, L., Salicio, Y., Sarasqueta, C., Ibarra, B., and Mendarte, U. (2014). Clarithromycin for first-line treatment of Helicobacter pylori infection after culture in high-resistance regions. *Eur. J. Gastroenterol. Hepatol*. 26, 1380–1384. doi: 10.1097/MEG.0000000000000197

Marzio, L., Coraggio, D., Capodicasa, S., Grossi, L., and Cappello, G. (2006). Role of the preliminary susceptibility testing for initial and after failed therapy of Helicobacter pylori infection with levofloxacin, amoxicillin, and esomeprazole. *Helicobacter*. 11, 237–242. doi: 10.1111/j.1523-5378.2006.00407.x

Mascellino, M. T., Oliva, A., De Angelis, M., Pontone, S., and Porowska, B. (2018). Helicobacter pylori infection: antibiotic resistance and eradication rate in patients with gastritis showing previous treatment failures. *New Microbiol*. 41, 306–309.

Matsumoto, Y., Miki, I., Aoyama, N., Shirasaka, D., Watanabe, Y., and Morita, Y. (2005). Levofloxacin- versus metronidazole-based rescue therapy for H. pylori infection in Japan. *Dig. Liver. Dis*. 37, 821–825. doi: 10.1016/j.dld.2005.06.002

Megraud, F., Coenen, S., Versporten, A., Kist, M., Lopez-Brea, M., and Hirschl, A. M. (2013). Helicobacter pylori resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut*. 62, 34–42. doi: 10.1136/gutjnl-2012-302254

Megraud, F., O'MORAIN, C., and Malfertheiner, P. (1997). Technical annex: tests used to assess Helicobacter pylori infection. Working Party of the European Helicobacter pylori Study Group. *Gut*. 41, S10–S18.

Miwa, H., Nagahara, A., Kurosawa, A., Ohkusa, T., Ohkura, R., and Hojo, M. (2003). Is antimicrobial susceptibility testing necessary before second-line treatment for Helicobacter pylori infection? *Aliment. Pharmacol. Therap*. 17, 1545–1551. doi: 10.1046/j.1365-2036.2003.01541.x

Miyaki, A., Yamaguchi, K., Ida, A., and Miyauchi, T. (2016). An assessment of the efficacy of first-line Helicobacter pylori-eradication therapy based on clarithromycin susceptibility. *Minerva Gastroenterol. Dietol*. 62, 234–239.

Molina-Infante, J., Pazos-Pacheco, C., Vinagre-Rodriguez, G., Perez-Gallardo, B., Duenas-Sadornil, C., and Hernandez-Alonso, M. (2012). Nonbismuth quadruple (concomitant) therapy: empirical and tailored efficacy versus standard triple therapy for clarithromycin-susceptible Helicobacter pylori and versus sequential therapy for clarithromycin-resistant strains. *Helicobacter*. 17, 269–276. doi: 10.1111/j.1523-5378.2012.00947.x

Neri, M., Milano, A., Laterza, F., Di Bonaventura, G., Piccolomini, R., Caldarella, M. P., et al. (2003). Role of antibiotic sensitivity testing before first-line Helicobacter pylori eradication treatments. *Aliment. Pharmacol. Therap*. 18, 821–827. doi: 10.1046/j.1365-2036.2003.01757.x

Nyssen, O. P., Vaira, D., Tepes, B., Kupcinskas, L., Bordin, D., and Perez-Aisa, A. (2022). Room for Improvement in the Treatment of Helicobacter pylori Infection: Lessons from the European Registry on H. pylori Management (Hp-EuReg). *J. Clin. Gastroenterol*. 56, e98–e108. doi: 10.1097/MCG.0000000000001482

O'Connor, A., Furuta, T., Gisbert, J. P., and O'Morain, C. (2000). Regular review: treatment of Helicobacter pylori infection. *BMJ*. 320, 31–34. doi: 10.1136/bmj.320.7226.31

Ong, S., Kim, S. E., Kim, J. H., Yi, N. H., Kim, T. Y., Jung, K., et al. (2019). Helicobacter pylori eradication rates with concomitant and tailored therapy based on 23S rRNA point mutation: A multicenter randomized controlled trial. *Helicobacter*. 24, e12654. doi: 10.1111/hel.12654

Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., et al. (2021). The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ*. 372, n71. doi: 10.1136/bmj.n71

Pan, J., Shi, Z., Lin, D., Yang, N., Meng, F., and Lin, L. (2020). Is tailored therapy based on antibiotic susceptibility effective? a multicenter, open-label, randomized trial. *Front. Med*. 14, 43–50. doi: 10.1007/s11684-019-0706-8

Park, C. S., Lee, S. M., Park, C. H., Koh, H. R., Jun, C. H., and Park, S. Y. (2014). Pretreatment antimicrobial susceptibility-guided vs. clarithromycin-based triple therapy for Helicobacter pylori eradication in a region with high rates of multiple drug resistance. *Am. J. Gastroenterol*. 109, 1595–1602. doi: 10.1038/ajg.2014.222

Perri, F., Festa, V., Clemente, R., Quitadamo, M., and Andriulli, A. (2000). Rifabutin-based 'rescue therapy' for Helicobacter pylori infected patients after failure of standard regimens. *Aliment. Pharmacol. Therap*. 14, 311–316. doi: 10.1046/j.1365-2036.2000.00719.x

Puig, I., Lopez-Gongora, S., Calvet, X., Villoria, A., Baylina, M., and Sanchez-Delgado, J. (2016). Systematic review: third-line susceptibility-guided treatment for Helicobacter pylori infection. *Therap. Adv. Gastroenterol*. 9, 437–448. doi: 10.1177/1756283X15621229

Qasim, A., Sebastian, S., Buckley, M., O'Connor, H., and O'Morain, C. (2004). Pretreatment antimicrobial susceptibility testing is not cost saving in the standard eradication of Helicobacter pylori. *Clin. Gastroenterol. Hepatol*. 2, 85. discussion doi: 10.1016/S1542-3565(03)00296-9

Roccarina, D., Franceschi, F., Zocco, M. A., Garcovich, M., Gasbarrini, G., and Gasbarrini, A. (2012). Different Antibiotic No Culture Eradicating (DANCE) strategy: an easy way to manage H. pylori eradication. *Dig. Liver Dis*. 44, 889–892. doi: 10.1016/j.dld.2012.05.010

Rokkas, T., Sechopoulos, P., Robotis, I., Margantinis, G., and Pistiolas, D. C. (2009). Pylori eradication rates in clinical practice by adopting first and second-line regimens proposed by the Maastricht III consensus and a third-line empirical regimen. *Am. J. Gastroenterol*. 104, 21–25. doi: 10.1038/ajg.2008.87

Romano, M., Iovene, M. R., Montella, F., Vitale, L. M., De Simone, T., and Blanco, C. D. V. (2000). Pretreatment antimicrobial-susceptibility testing in the eradication of H. pylori infection. *Am. J. Gastroenterol*. 95, 3317–3318. doi: 10.1111/j.1572-0241.2000.03317.x

Romano, M., Marmo, R., Cuomo, A., De Simone, T., Mucherino, C., and Iovene, M. R. (2003). Pretreatment antimicrobial susceptibility testing is cost saving in the eradication of Helicobacter pylori. *Clin Gastroenterol Hepatol*. 1, 273–278. doi: 10.1016/S1542-3565(03)00131-9

Saracino, I. M., Pavoni, M., Zullo, A., Fiorini, G., Saccomanno, L., and Lazzarotto, T. (2020). Antibiotic resistance and therapy outcome in h. pylori eradication failure patients. *Antibiotics*. 9, 121. doi: 10.3390/jcm9051299

Savoldi, A., Carrara, E., Graham, D. Y., Conti, M., and Tacconelli, E. (2018). Prevalence of antibiotic resistance in helicobacter pylori: a systematic review and meta-analysis in world health organization regions. *Gastroenterology*. 155, 1372–82.e17. doi: 10.1053/j.gastro.2018.07.007

Seppala, K., Kosunen, T. U., Nuutinen, H., Sipponen, P., Rautelin, H., and Sarna, S. (2000). Cure of Helicobacter pylori infection after failed primary treatment: one-center results from 120 patients. *Scandin. J. Gastroenterol*. 35, 929–934. doi: 10.1080/003655200750022977

Stamboliyska, M., Kaludova, V., Mirchev, M., Kotzev, I., Madjov, R., and Metodiev, K. (2015). Role and importance of Microbiology testing in the Diagnosis and Treatment of Helicobacter pylori infection. *J. IMAB – Ann. Proc*. 21, 969–973. doi: 10.5272/jimab.2015214.969

Street, M. E., Caruana, P., Caffarelli, C., Magliani, W., Manfredi, M., and Fornaroli, F. (2001). Antibiotic resistance and antibiotic sensitivity based treatment in Helicobacter pylori infection: advantages and outcome. *Arch. Dis. Child*. 84, 419–422. doi: 10.1136/adc.84.5.419

Sugimoto, M., Uotani, T., Sahara, S., Ichikawa, H., Yamade, M., and Sugimoto, K. (2014). Efficacy of tailored Helicobacter pylori eradication treatment based on clarithromycin susceptibility and maintenance of acid secretion. *Helicobacter*. 19, 312–318. doi: 10.1111/hel.12128

Tanabe, H., Yoshino, K., Ando, K., Nomura, Y., Ohta, K., and Satoh, K. (2018). Vonoprazan-based triple therapy is non-inferior to susceptibility-guided proton pump inhibitor-based triple therapy for Helicobacter pylori eradication. *Ann. Clin. Microbiol. Antimicrob*. 17, 29. doi: 10.1186/s12941-018-0281-x

Thung, I., Aramin, H., Vavinskaya, V., Gupta, S., Park, J. Y., and Crowe, S. E. (2016). Review article: the global emergence of Helicobacter pylori antibiotic resistance. *Aliment. Pharmacol Therap*. 43, 514–533. doi: 10.1111/apt.13497

Toracchio, S., Cellini, L., Di Campli, E., Cappello, G., Malatesta, M. G., Ferri, A., et al. (2000). Role of antimicrobial susceptibility testing on efficacy of triple therapy in Helicobacter pylori eradication. *Aliment. Pharmacol Therap*. 14, 1639–1643. doi: 10.1046/j.1365-2036.2000.00870.x

Treiber, G., Ammon, S., Malfertheiner, P., and Klotz, U. (2002). Impact of furazolidone-based quadruple therapy for eradication of Helicobacter pylori after previous treatment failures. *Helicobacter*. 7, 225–231. doi: 10.1046/j.1523-5378.2002.00087.x

Wang, G., Zhao, Q., and Li, S. (2008). Study of drug sensitivity test in Helicobacter pylori eradication therapy. *J. Clin. Intern. Med*. 25, 474–477.

Wenzhen, Y., Yumin, L., Quanlin, G., Kehu, Y., Lei, J., and Donghai, W. (2010). Is antimicrobial susceptibility testing necessary before first-line treatment for Helicobacter pylori infection? Meta-analysis of randomized controlled trials. *Intern. Med*. 49, 1103–1109. doi: 10.2169/internalmedicine.49.3031

Xuan, S. H., Wu, L. P., Zhou, Y. G., and Xiao, M. B. (2016). Detection of clarithromycin-resistant Helicobacter pylori in clinical specimens by molecular methods: A review. *J. Glob. Antimicrob. Resist*. 4, 35–41. doi: 10.1016/j.jgar.2016.01.002

Yahav, J., Samra, Z., Niv, Y., Evans, C. T., Passaro, D. J., and Dinari, G. (2006). Susceptibility-guided vs. empiric retreatment of Helicobacter pylori infection after treatment failure. *Dig. Dis. Sci*. 51, 2316–2321. doi: 10.1007/s10620-006-9302-2

Yu, L., Luo, L., Long, X., Liang, X., Ji, Y., Chen, Q., et al. (2019). Susceptibility-guided therapy for Helicobacter pylori infection treatment failures. *Therap. Adv. Gastroenterol*. 12, 1756284819874922. doi: 10.1177/1756284819874922

Zhang, Y. D., Dong, Q. W., Zhang, S. H., Gu, F., Zhang, Y., and Song, H. B. (2020). Effectiveness of eradication regimen based on the bacterial susceptibility and CYP2C19 genotype in children with refractory Helicobacter pylori infection. *Zhonghua er ke za zhi = Chinese J. Pediat*. 58, 41–45. doi: 10.3760/cma.j.issn.0578-1310.2020.01.010

Zhou, J., Wu, M., and Jiang, X. (2010). Role of drug sensitivity test in the triple therapy for eradication of Helicobacter pylori. *Chin. J. Gastroenterol*. 15, 358–360.

Zhou, L., Zhang, J., Song, Z., He, L., and Qian, L., i. Y. (2016). Tailored versus triple plus bismuth or concomitant therapy as initial helicobacter pylori treatment: a randomized trial. *Helicobacter*. 21, 91–99. doi: 10.1111/hel.12242

Zhu, D. B., and Wu, J. M. (2019). Risk factors for treatment ineffectiveness in patients with H. Pylori infection undergoing eradication treatment according

to drug susceptibility testing results. *World Chin. J. Digestol.* 27, 509–514. doi: 10.11569/wcjd.v27.i8.509

Zhuo, R. P., Chen, X. P., Wu, S. Z., Xie, J. L., and Hu, S. K. (2015). Clinical effects of quadruple therapy based on antimicrobial susceptibility testing in treatment of Helicobacter pylori associated upper digestive tract diseases. *World Chin. J. Digestol.* 23, 196–201. doi: 10.11569/wcjd.v23.i2.196

Zullo, A., Hassan, C., Campo, S. M., Lorenzetti, R., Febbraro, I., Matthaeis, D., et al. (2001). A triple therapy regimen after failed Helicobacter pylori treatments. *Aliment. Pharmacol. Therap.* 15, 1193–1197. doi: 10.1046/j.1365-2036.2001.01028.x

Zullo, A., Hassan, C., Lorenzetti, R., Winn, S., and Morini, S. (2003). A clinical practice viewpoint: to culture or not to culture Helicobacter pylori? *Dig. Liver Dis.* 35, 357–361. doi: 10.1016/S1590-8658(03)00081-1

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# Extra virgin olive oil inhibits *Helicobacter pylori* growth *in vitro* and the development of mice gastric mucosa lesions *in vivo*

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*Helicobacter pylori* infection is widespread worldwide, with more than a half of the world population infected. *H. pylori* antibiotic-resistant strains and non-compliance to therapy are the major causes of *H. pylori* eradication failure. The search for new therapies based on plant extracts is a scientific interest field. The present study was conducted to evaluate the effect *in vitro* of extra virgin olive oil (EVOO), hydroxytyrosol (HT), and oleuropein (Olp) against two *H. pylori* strains and the effect *in vivo* of the oral administration of EVOO on the gastric mucosa of BALB/c mice infected with this microorganism. The broth microdilution method assayed the antibacterial *in vitro* activity of EVOO, HT, and Olp against *H. pylori* strains. For *in vivo* studies, male BALB/c mice were infected orally with an *H. pylori* suspension every 72 h. Four groups were used: (1) Control, (2) *H. pylori*-infected (HP), (3) EVOO, and (4) HP + EVOO. Mice were sacrificed at 7, 15, and 30 days. The stomachs were removed and observed under a microscope. Scoring of the degree of erosion was determined. Samples were processed by histological techniques for light microscopy. Macroscopic analysis showed that the presence of small erosions increased, both in number and size, in the infected group. Animals infected and treated with EVOO exhibited the presence of fewer erosions, which decreased in number as the treatment progressed. The mucosa of the control and EVOO groups showed normal histological characteristics at the three times studied. The mucosa of animals infected with *H. pylori* showed disruptions of the lining epithelium, damage to gastric glands, and vasodilation. The mucosa of animals infected with *H. pylori* and treated with EVOO showed morphological characteristics similar to those of normal and EVOO mucosa. For the first time, the current study showed the effect *in vitro* and *in vivo* of EVOO and combined administration of HT and Olp against

*H. pylori* using an animal model. Future studies are needed to establish the mechanism of EVOO's action at the gastric mucosa level to propose this product as a natural antimicrobial agent for the treatment of gastric *H. pylori* infections.

KEYWORDS

olive oil, EVOO, hydroxytyrosol, oleuropein, *Helicobacter pylori*, gastric mucosa

## Introduction

*Helicobacter pylori* is a Gram-negative bacterium that infects about half of the world's population and about 80% of the population in developing countries (Ranjbar et al., 2017; Alipour, 2021; Siwe et al., 2021). It colonizes the gastric mucosa and triggers pathologic conditions such as peptic ulcer, chronic gastritis, gastric mucosa-associated lymphoid tissue lymphoma (Matongo and Nwodo, 2014; Huang et al., 2017; Park et al., 2018). Besides, in 1994, *H. pylori* was classified as a type I carcinogen by the World Health Organization, since infection with this microorganism is considered the main factor for the development of gastric cancer (Mladenova, 2021). In addition to this, in recent years it has also been associated with various extra-gastric pathologies such as autoimmune, cardiovascular, colonic, respiratory, skin, neurological, and hematological diseases (Ranjbar et al., 2017; Gravina et al., 2020). The infection with this microorganism leads to infiltration of chronic inflammatory cells and accumulation of neutrophil leukocytes in the gastric mucosa, thus leading to continuing inflammation (Palacios-Espinosa et al., 2014). Bacterial eradication therapies involve using bismuth, or a proton pump inhibitor, and two antibiotics, such as amoxicillin, tetracycline, clarithromycin, or metronidazole, in triple or quadruple therapies. However, eradication is not always successful because some patients fail to respond to the treatment or due to antibiotic resistance, where in some regions an increase in resistance to clarithromycin and metronidazole greater than 80% has been reported (Dudley et al., 2017; Malfertheiner et al., 2017; Ozturk et al., 2017; Ranjbar et al., 2017). Because of this, there is a strong demand in the search for new antimicrobial agents. Natural medicine contains active principles such as antimicrobial, anti-inflammatory, antioxidant, and anticancer properties (Baker, 2020). Extra virgin olive oil (EVOO) is obtained by the mechanical pressing of the fruits of the olive tree (*Olea europaea* L.) (Nazzaro et al., 2019) without other treatments, preserving in this way high amounts of phenolic constituents that have beneficial effects on the human health when is part of the diet (Thielmann et al., 2017; Marcelino et al., 2019). EVOO has been associated with reduced incidence of degenerative diseases, such as coronary heart disease and several types of cancer. More specifically, it has been shown that EVOO exerts health benefits mainly, but not only, *via* phenolic constituents such as hydroxytyrosol (HT) and oleuropein (Olp) (Waterman and Lockwood, 2007; Jimenez-Lopez et al., 2020; Bilal et al., 2021). Hydroxytyrosol is one of the main polyphenols of EVOO. It has anti-inflammatory and antioxidant properties, reducing oxidative stress and the activation of inflammatory cells (Marcelino et al., 2019). Oleuropein is a glycosylated seco-iridoid, a phenolic bitter compound with antioxidant and anti-inflammatory activities (Ahamad et al., 2019).

Based on this background, the present study was conducted to evaluate the effect *in vitro* of EVOO, hydroxytyrosol, and oleuropein against two *H. pylori* strains and the effect *in vivo* of the oral administration of EVOO on the gastric mucosa of BALB/c mice infected with this microorganism. This work will allow us to understand the effectiveness of EVOO, and its main phenolic constituents, in eradicating *H. pylori* infection.

## Materials and methods

### Chemicals, reagents, and oil samples

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich Chemical Inc. (St. Louis, MO, United States). Hydroxytyrosol (HT) and Oleuropein (Olp) were supplied by Extrasynthèse (Lyon, France). These polyphenols were dissolved in a solution containing 6.7 mM $Na_2HPO_4$, 6.7 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, 0.8 mM $CaCl_2$, 0.5 g/l albumin, and 1 g/l glucose, adjusted to pH 7.2, and stored at -20°C until required. The stock solutions were then diluted to the desired final concentration with the same solution. An extra virgin olive oil commercially available at the international level was selected for the study (EVOO) due to its high total antioxidant power. A sunflower oil (SFO) commercially available in Argentina was used for comparative studies due to significantly lower total antioxidant power compared to EVOO.

The EVOO used in this work had the following composition:

*Fatty acid profile*

Myristic: 0.00%

Myristoleic acid: 0.00%

Palmitoleic: 13.91%

Palmitoleic: 1.31%
Margaric: 0.11%
Heptadecenic: 0.23%
Stearic: 2.05%
Oleic: 69.59%
Linoleic: 11.17%
Arachidic: 0.38%
Eicosaenoic: 0.38%
Behenic: 0.11%
Erucic: 0.00%
Lignoseric: 0.00%
Nervonic: 0.00%
Pentadecanoic: 0.00%
Arachidonic: 0.00%
Eicosapentanoic: 0.00%
Docosapentanic: 0.00%
Docosahexaenoic: 0.00%

*Sterols profile*

Cholesterol: 0.15%
Brassicasterol: <0.05%
Cholesterol: 4.49%
Campesterol: 4.49%
Stigmasterol: 0.82%
Beta-Sitosterol: 93.66%
Delta 7 Stigmasterol: 0.11%

*Phenolic compounds/secoiridoids profile*

Oleocanthal: 123 mg/kg EVOO
Oleacein: 216 mg/kg EVOO
Oleuropein aglycone: 215 mg/kg EVOO
Ligstroside aglycone: 30 mg/kg EVOO
Oleokoronal: 113 mg/kg EVOO
Oleomissional: 88 mg/kg EVOO
S-(*E*)-elenolide: 1054 mg/kg EVOO
Hydroxytyrosol: 5.95 mg/kg EVOO
Tyrosol: 3.07 mg/kg EVOO
Cinnamic acid: 0.90 mg/kg EVOO
Pinoresinol: 13.3 mg/kg EVOO
Apigenin: 2.53 mg/kg EVOO

## Total antioxidant activity of oil samples

The antioxidant activity of EVOO and SFO was determined spectrophotometrically as a measure of radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) (La et al., 2021). A control sample containing a volume of solvent (methanol) equivalent to oil was used to measure the maximum DPPH absorbance. Aliquots of 0.1 ml of 100 μM solution of 5% DPPH in methanol were mixed with 0.1 ml of each sample. Samples in triplicate were mixed and incubated at room temperature in the dark for 30 min. The absorbance at 517 nm was recorded to determine the concentration of residual DPPH. The percentage of inhibition of the maximal absorbance was calculated according to the following equation:

$$\%\text{inhibition} = [(A_{\text{DPPH}} - -A_{\text{OIL}})/A_{\text{DPPH}}] \times 100$$

in which $A$ is the absorbance of DPPH and oil, respectively. EC50 values correspond to the concentration of the sample, which scavenge 50% of DPPH free radicals.

## Strains and culture conditions

Strain NCTC 11638 (kindly provided by Dra. Teresa Alarcón Cavero, Microbiology Service of Hospital Universitario de la Princesa, Madrid, Spain) and strain HP661 (clinical strain, isolated from the gastric mucosa of a human patient) were used. Both *H. pylori* strains were grown in Mueller- Hinton agar (MHA) supplemented with 7% horse blood (MHA-HB) at 37°C under microaerophilic conditions and identified by microscopy, urease, catalase, and oxidase tests. A bacterial suspension of 1–1 × $10^8$ colony forming units for milliliter (CFU/ml) was prepared for *in vitro* assays.

## Animals and experiment protocol

Male adult BALB/c wild-type (WT) mice were used ($n = 60$). Three independent experiments were carried out with 5 mice *per* group ($n = 20$ mice *per* experiment). Animals were kept under a 12-h dark/light cycle in a temperature-controlled room (24–25°C) with free access to drinking water and laboratory food. All animal experiments were evaluated and approved by the Institutional Committee for Care and Use of Laboratory Animals (IACUC), Universidad Nacional de San Luis (Protocol No. B-328/19). Regulations of this Committee are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH, United States) to comply with established international regulations and guidelines.

## *In vitro* antimicrobial activity of oil samples, hydroxytyrosol, and oleuropein

The anti-bacterial activity of EVOO, SFO, HT, and Olp against *H. pylori* strains was assayed by broth microdilution method using Mueller Hinton Broth (MHB) according to CLSI (2007) guidelines. Serial dilutions of amoxicillin (AMX) (Sigma-Aldrich Co., St Louis, MO, United States) were used to control the susceptibility test. Broth microdilution methods were carried out in 96-well microtiter plates. Aliquots (100 μl) of each extract dilution and each bacterial suspension adjusted

to a scale of 0.5 in MacFarland's standard [1 × $10^8$ colony forming units (CFU)/mL] were dispensed into each well. Two hundred microliters of extract, bacterial suspensions, MHB, and 0.9% saline were also included. Plates were incubated in microaerophilic conditions at 37°C for 3 days. The results were evaluated by colorimetric evaluation using 2,3,5-Triphenyltetrazolium chloride (TTC) as an indicator. Minimal inhibitory concentration (MIC) was measured by determining the smallest amount of extract or antibiotic needed to inhibit the visible growth of the microorganism. The Checkerboard microdilution test was applied to measure the probable synergistic effect of HT and Olp (Martinez-Irujo et al., 1996). The checkerboard assay was performed by mixing the two chemicals in different concentrations and determining the MIC for HT and Olp. A positive control with AMX was performed. All tests were performed in duplicate (**Figures 1**, **2**).

## Experimental protocol and *Helicobacter pylori* infection for *in vivo* studies

Mice were distributed into four groups: (1) Control (C), (2) *H. pylori*-infected (HP), (3) EVOO (E), and (4) HP + EVOO (HPE). The control group received the ordinary diet (without EVOO) and a sterile phosphosaline buffer solution (PBS × pH 7.4) instead of the microorganisms. EVOO was administered with food (150 ml EVOO/kg pellet), being mixed well with the ordinary diet, to EVOO and HP + EVOO groups. Animals in the infected groups were administered intragastrically with 300 μl of the microorganism suspension (1–5 × $10^8$ CFU/ml) every 3 days for 3, 15, or 30 days (Robinson et al., 2017). Mice were sacrificed by cervical dislocation 3, 15, and 30 days after the first administration of saline solution or *Helicobacter pylori* suspensions. Their stomachs were removed aseptically, opened along the greater curvature, and washed gently with ice-cold saline solution. Immediately after the collection, the degree of erosion was assessed with a scoring system and the observation of histological preparations for light microscopy (**Figure 3**).

## Scoring system

The degree of erosion was assessed from a scoring system designed by Marazzi-Uberti and Turba (Ohta et al., 2005) as follows: 0, no erosions; 1, 1–3 small erosions (4 mm diameter or smaller); 2, more than 3 small erosions or one large erosion; 3, one large erosion and more than 3 small erosions; 4, 3–4 large erosions; 5, any very large erosion or ulcer perforation. The results were expressed in terms of an ulcer factor, which is the average severity of erosions *per* mouse for each group on a scale from 0 to 5. The sum of these values was divided by the number of animals. This procedure was performed using a Nikon binocular stereomicroscope (×40 magnification).

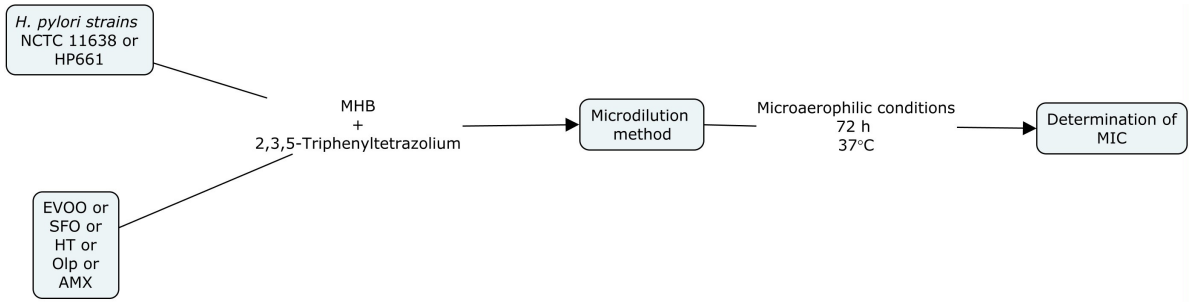


FIGURE 1
Flow diagram of the *in vitro* study of extra virgin olive oil (EVOO), sunflower oil (SFO), hydroxytyrosol (HT), oleuropein (Olp), and amoxicillin (AMX) against *Helicobacter pylori* strains NCTC 11638 and HP661 (Behzadi and Gajdács, 2021).

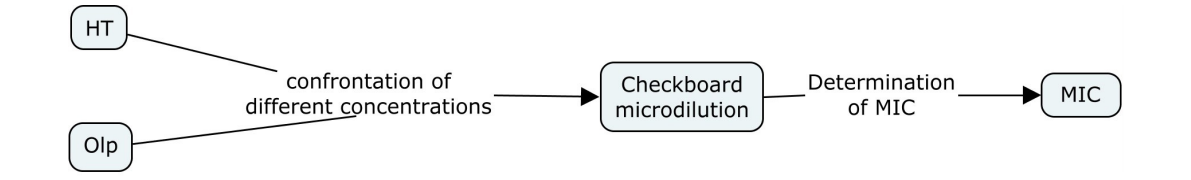


FIGURE 2
Flow diagram of the *in vitro* study of different concentrations of hydroxytyrosol (HT) together with oleuropein (Olp) against *Helicobacter pylori* strains NCTC 11638 and HP661 (Behzadi and Gajdács, 2021).

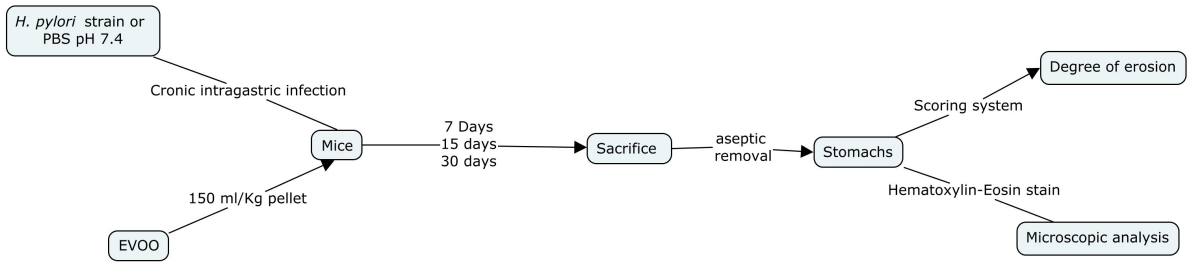


FIGURE 3
Flow diagram of the *in vivo* study of extra virgin olive oil (EVOO) against the infection of *Helicobacter pylori* strains NCTC 11638 in the gastric mucosa of mice (Behzadi and Gajdács, 2021).

## Light microscopy

The samples for light microscopy were fixed for 24 h in a 10% formaldehyde solution (prepared with saline solution, pH = 7), dehydrated in graded ethanol and xylol, and embedded in paraffin. Serial sections (6 μm) were mounted on glass slides and deparaffinized. Sections were stained with hematoxylin–eosin in order to evaluate the general histoarchitecture and the degree of gastric lesions.

## Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows and GraphPad In Stat version 3.00 for Windows (GraphPad Software,[1] San Diego, CA, United States). All data are expressed as the mean ± standard error of mean (S.E.M). A probability of $p < 0.05$ was considered statistically significant.

# Results

## *In vitro* evaluation of the antibacterial effect of extra virgin olive oil, sunflower oil, hydroxytyrosol, and oleuropein against *Helicobacter pylori*

The results of this analysis are summarized in Table 1. Briefly, the antimicrobial effect of EVOO against both strains showed a MIC of 229.5 μg/ml. Separately, HT and Olp showed no effect at concentration assayed range 25–4000 μg/ml. Interestingly, the combination of both compounds assayed by the checkerboard method showed an inhibitory effect at values of 19.27 μg/ml (HT) + 2.1 μg/ml (Olp) against NCTC strain, while the values were 154.16 μg/ml (HT) + 8.44 μg/ml (Olp) against 661 strain. On the other hand, no inhibitory effect was observed with SFO (Table 1). Total antioxidant power values of oil samples can be seen in Table 2.

## Macroscopic evaluation after the *in vivo* action of chronic administration of extra virgin olive oil on the gastric mucosa in animals infected with *Helicobacter pylori*

Figure 4 shows representative images of the gastric mucosa surface after 7, 15, and 30 days of treatment from four experimental groups: (1) Control, (2) EVOO, (3) *H. pylori*, and (4) EVOO + *H. pylori*. The gastric mucosa surface from the control and EVOO groups shows a healthy macroscopic appearance with score values of the ulcerogenic index of 1 and 2 (control) and 2 (EVOO). The ulcerogenic index of the *H. pylori* group's stomachs is significantly higher than that of

TABLE 1 Minimal inhibitory concentration (MIC) of the extra virgin olive oil (EVOO) and the combined effect of hydroxytyrosol (HT) and oleuropein (Olp).

| *H. pylori* strain | MIC EVOO (μ g/mL) | MIC H + O (μ g/mL) | MIC AMX (μ g/mL) |
|---|---|---|---|
| NCTC 11638 | 229.5 | 19.27 + 2.1 | 0.5 |
| HP661 | 229.5 | 154.16 + 8.44 | 0.25 |

This table shows the concentration in μg/ml of EVOO and the combination of HT and Olp that had an inhibitory effect against *Helicobacter pylori* strains.

TABLE 2 The values of EC50 represent the average of total antioxidant power of each sample oil, with their standard deviation.

| Oil sample | EC50 | MIC AMX (μ g/mL) |
|---|---|---|
| EVOO | 1.7 ± 0.01 | 0.5 |
| SFO | 3 ± 0.02 | 0.25 |

The higher the EC50, the lower the total antioxidant power. Results are expressed as mean ± SEM.

1 www.graphpad.com

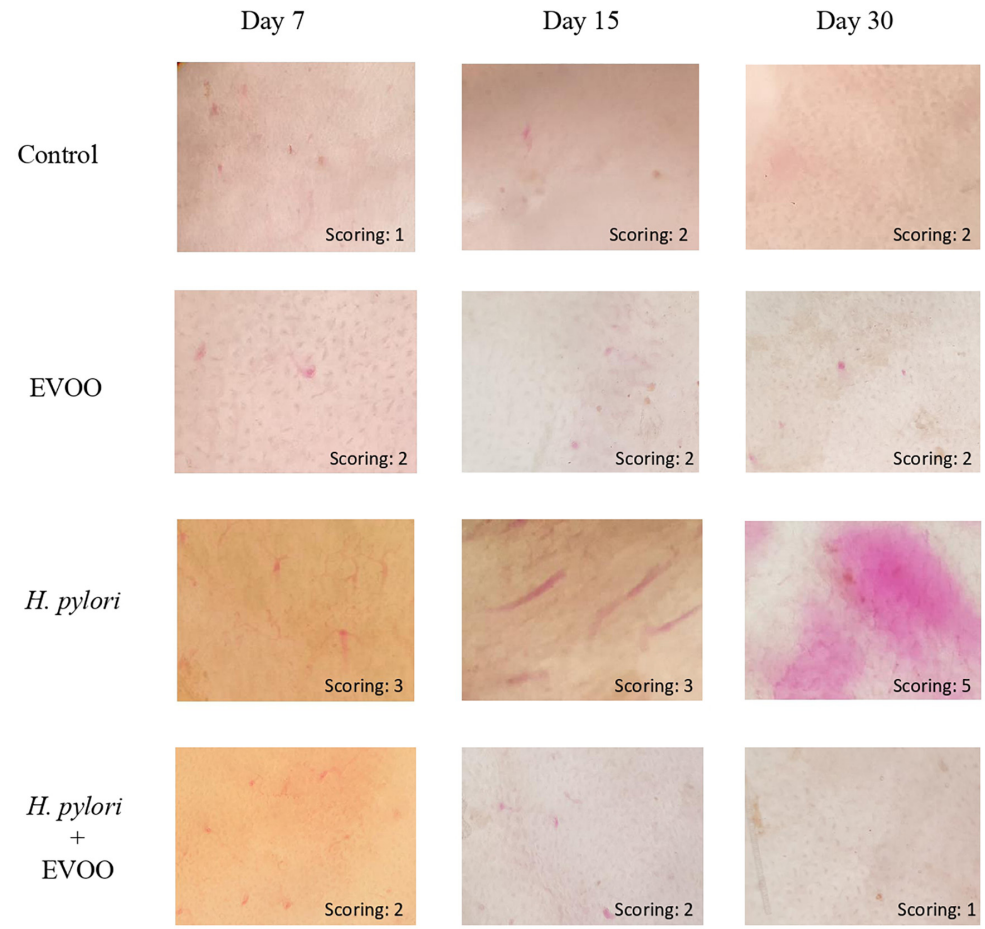


FIGURE 4
Macroscopic evaluation of the gastric mucosa surface of mice after 7, 15, or 30 days of treatment from four experimental groups: (1) Control, (2) Extra virgin olive oil (EVOO), (3) *Helicobacter pylori*, and (4) EVOO + *H. pylori*. Gastric mucosa from the control and EVOO groups shows a healthy appearance, with a score of 1 and 2 (control) and 2 (EVOO) at different infection times. *Helicobacter pylori* group had an increasing scoring with the time, reaching the highest value at 30 days, while in the group infected and treated with EVOO, the gastric mucosa structure is similar to that of the control and EVOO groups.

the control and EVOO groups at 7 and 15 days ($p < 0.005$ and $p < 0.0001$) (Figure 5). Gastric mucosa from infected animals and without EVOO show the presence of small and medium-size erosions that were increasing, both in number and size, reaching the highest level (5) after 30 days from the first infection ($p < 0.0001$). Regions with mild, intense hyperemia are observed. In contrast, the group infected and treated with EVOO exhibited the presence of few erosions, which decreased in number as the treatment progressed. Hyperemia was not observed at any of the times analyzed in this group ($p < 0.0001$) (Figure 5).

## Histological analysis

Figures 6–8 show micrographs, at different magnifications, of histological sections of hematoxylin–eosin-stained mouse stomachs on days 7, 15, and 30 after infection, respectively. In these images, it is possible to observe the histoarchitecture of the gastric mucosa representative of the different experimental groups. The mucosa of the control and EVOO groups shows normal histological characteristics at the three times studied. It is possible to observe the lining epithelium, the glandular epithelium, the lamina propria, and the *muscularis mucosae* without structural alterations. The mucosa of animals infected with *H. pylori* at 7 days shows apparent injuries, mainly disruptions of the lining epithelium and damage in the isthmus and the neck of gastric glands. A decrease in the size of the gland epithelial cells can be observed. Blood, remnants of injured tissue, and some mucus filaments are observed in the lumen. At 15 days post-infection, an increase in the stomach lumen with vasodilation and increased blood supply is observed. Infiltration of inflammatory cells can also be seen. At 30 days, significantly damaged tissue is observed with the presence of a

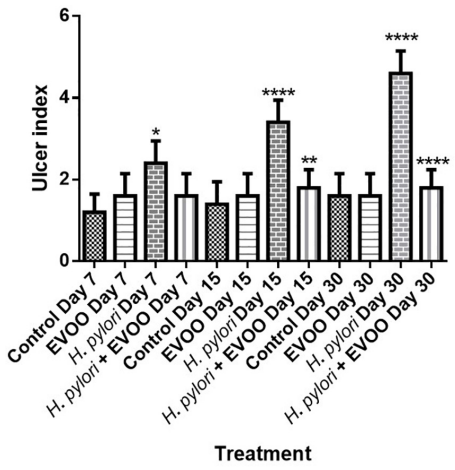


FIGURE 5
Effects of extra virgin olive oil (EVOO) on gastric lesions induced by the infection of *Helicobacter pylori* in mice. Asterisks denote significant differences between groups (*$p < 0.05$, **$p < 0.01$, and ****$p < 0.001$). All values are expressed as mean ± S.E.M.

large number of blood vessels. The mucosa of animals infected with *H. pylori* and treated with EVOO shows morphological characteristics similar to those of normal and EVOO mucosa, even though slight damage is observed at 7 days, which increases at 15 days. However, the cells are in better condition compared to the HP group.

## Discussion

*Helicobacter pylori* is a microorganism that produces chronic gastritis, peptic ulcers, and gastric neoplasms in the human stomach mucosa (Matongo and Nwodo, 2014; Huang et al., 2017; Park et al., 2018). Due the localization of *H. pylori,* drugs must penetrate the gastric mucosa layer and it is due to this, that in the treatments two to three antibiotics (metronidazole, amoxicillin, or tetracycline) are used, among a proton-pump inhibitor is used, but most of the time, the elimination is not successful due to the antibiotic resistance, patient compromise, and age (Castro et al., 2012; Ranjbar et al., 2017).

Today, natural products and derivates are being studied due to their different properties. Extra virgin olive oil is a vegetable oil obtained from olive fruit by a mechanical process without any solvents and at a temperature that does not degrade it. It contains monounsaturated fatty acids, primarily oleic acid, carotenoids, sterols, lycopene, and hydrophilic phenolics such as hydroxytyrosol and oleuropein (Osman et al., 2017; Negm et al., 2020). Different properties have been described for EVOO, like antioxidant, anti-inflammatory, anti-cancer, anti-atherogenic, hypoglucemic, hepatic-, cardiac-, and neuro-protective, anti-viral, and anti-bacterial (Barbaro et al., 2014).

This work aimed to study the effect of EVOO and its main phenolic compounds (hydroxytyrosol and oleuropein) *in vitro* against *H. pylori* strains and evaluate the action of the administration of EVOO *in vivo* using an animal model on the gastric mucosa infected with *H. pylori*.

Castro et al. (2012) studied the effect *in vitro* in three *H. pylori* strains of olive oil extracts in PBS. Starting from an initial inoculum of $10^6$ CFU/ml, where after 5 min of contact, all the strains were killed, and no growth was observed. These extracts contained high levels of dialdehyde form of decarboxmethyl elenolic acid linked to tyrosol and dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol, in a concentration of 30–60 μg/ml, but also, the last wash show

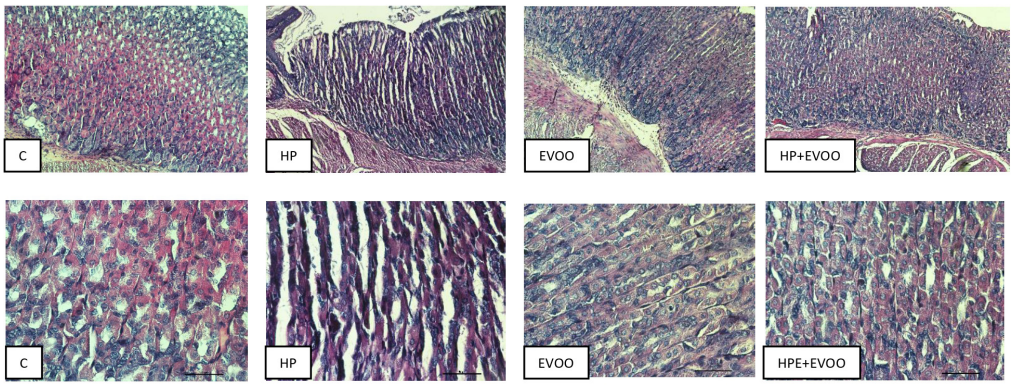


FIGURE 6
Hematoxylin–Eosin stain at 7 days after infection. The mucosa of the control and extra virgin olive oil (EVOO) groups shows normal histological characteristics. The mucosa of animals infected with *Helicobacter pylori* shows apparent injuries, mainly disruptions of the lining epithelium and damage in the isthmus and the neck of gastric glands. A decrease in the size of the gland epithelial cells can be observed. Blood, remnants of injured tissue, and some mucus filaments are observed in the lumen. The mucosa of animals infected with *H. pylori* and treated with EVOO shows morphological characteristics similar to those of normal and EVOO mucosa, even though slight damage is observed. Scale bar: 40 μm.

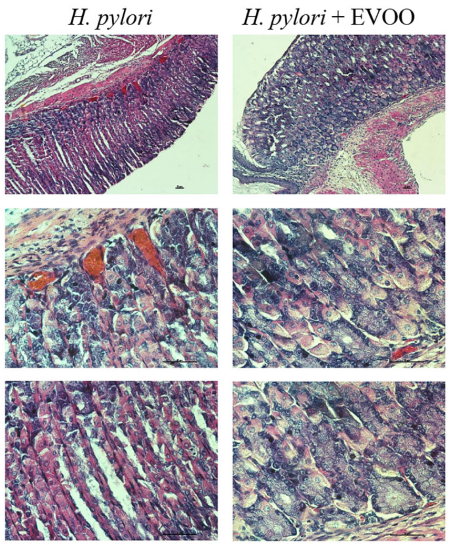


FIGURE 7
Hematoxylin–Eosin stain of *Helicobacter pylori* group and *H. pylori* and treatment group at 15 days. The mucosa of animals infected with *H. pylori* shows structural alterations, such as an increase of the gland gastric, vasodilation, and increased blood supply. The mucosa of animals infected with *H. pylori* and treated simultaneously with EVOO show morphological characteristics similar to those of normal and EVOO mucosa, even though slight vasodilation is observed. Scale bar: 40 μm.

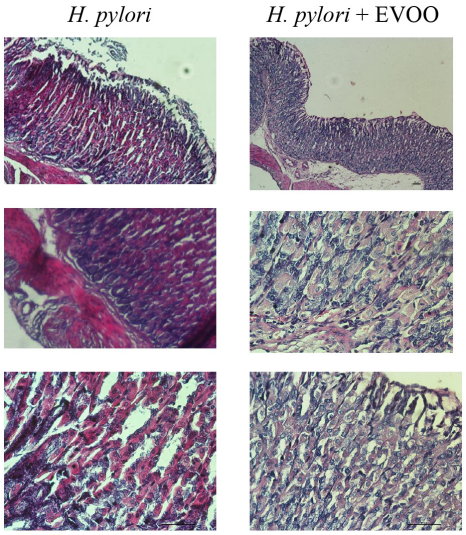


FIGURE 8
Hematoxylin–Eosin stain of *Helicobacter pylori* group and *H. pylori* and treatment group at 30 days. Significantly damaged tissue is observed with many blood vessels in gastric mucosa from the *H. pylori* group. At 30 days, significantly damaged tissue is observed with the presence of a large number of blood vessels. The mucosa of animals infected with *H. pylori* and treated with EVOO shows morphological characteristics similar to those of normal and EVOO mucosa. HP + EVOO group shows a cytoprotected gastric mucosa. Scale bar: 40 μm.

effect, where the concentration was 5–10 μg/ml. In our study, the initial concentration was $10^8$ UFC/ml, and the strains were put in contact with different concentrations of EVOO using the board technique, where no growth was observed at 229 μg/ml. The differences with respect to our results may be due to the fact that the extracts were rich in certain compounds, while in this study, pure extra virgin olive oil was used.

Romero et al. (2007) studied the *in vitro* effects of phenolic compounds of EVOO against *H. pylori*, where the extract that had nine polyphenols, including hydroxytyrosol and dialdehydic form of decarboxymethyl oleuropein aglycon (Hy-EDA), showed bactericidal effect time-dependent in three of the eight strains tested. But when isolated compounds were tested against the most resistant strain of *H. pylori*, none of them showed significant bactericidal effects except Hy-EDA. This accompanies our results, where no effect was observed on the part of the compounds separately, but synergy was seen when hydroxytyrosol and oleouropein were put in contact at the concentrations of H = 19.27 μg/ml + O = 2.1 μg/ml against NCTC strain, while in 661 strain, the values were H = 154.16 μg/ml + O = 8.44 μ g/ml.

Castro et al. (2012) also studied the effect of the administration of washed olive oil for 14 days in patients, using the $^{13}$C-urea breath test to confirm the presence or absence of *H. pylori*. In two different trials, several patients were abandoned because of the taste or nausea. In both cases, only the 26 and 10% showed eradication of *H. pylori*, and several patients who had been negative for *H. pylori* were positive after one month of treatment. In our study, using an animal model of mice, the infection of *H. pylori* in the stomach mucosal was demonstrated by histology, and the group of mice who were infected and subsequently administered EVOO continuously not only showed eradication of the microorganism but also an improvement in the damage to the gastric mucosa, with a resolution of the ulcers.

Gastric ulcers are lesions in the gastric mucosa that extend along the *muscularis mucosae*, which are characterized by different stages of necrosis, neutrophil infiltration, reduced blood flow, increased oxidative stress, and inflammation (Siwe et al., 2021). Bhattamisra et al. (2018) had similar results in the treatment of ulcers with geraniol, with a reduction of ulcers in comparison with infected animals with *H. pylo*ri. Our results show a protective effect in the formation of ulcers in the first days of infection, having and keeping a scoring of 2 ($p < 0.005$), while the animal infected show an increase in the number and size of ulcers ($p < 0.0001$) (**Figure 5**).

Extra virgin olive oil is rich in polyphenols, an important group of polar components that have numerous biochemist activities like preventing and inhibiting radical reactions in the human body. Free radicals cause oxidative damage to biomolecules like lipids and DNA, increasing the risk of chronic diseases (Cicerale et al., 2012; Nazzaro et al., 2019), and it

has been shown that the ingestion of food rich in polyphenols radically decreases the generation of hydroxyperoxides. It has been observed that reactive oxygen species (ROS) and lipid peroxidation are involved in the gastric ulceration (Palacios-Espinosa et al., 2014) and that *H. pylori* produce urease, an enzyme that hydrolyzes urea to ammonia that provides, and leads to pH elevation, favoring gastric colonization and providing protection from the stomach hydrochloric acid, creating an alkaline microenvironment favoring the Michael reaction, where some free radicals could interact with cysteine, lysine, and histidine within proteins which could have consequences in the cells of the stomach (Matongo and Nwodo, 2014; Cherkas and Zarkovic, 2018). This could explain the effect observed in this work of the administration *in vivo* of EVOO in mice infected with *H. pylori*, where the inhibition of ROS and lipid peroxidation or the stimulation of antioxidants reduce ulcer and help the healing process. Over 30 days, in the beginning, was observed a decrease in the size of the epithelial cells of the stomach, followed in the following days by vasodilation with increased blood supply, observing in the last days great damage to the tissue and the presence of ulcers, but in the group infected and have the administration of EVOO in the diet, show a decrease in damage over 30 days.

In addition to all this, inflammation is a defense of the organism that involves the local changes, with vasodilatation and migration of inflammatory cells. It is documented that the phenolic compounds of EVOO have anti-inflammatory effects. Osman et al. (2017) studied the anti-inflammatory effects of EVOO compared to ibuprofen in the treatment paw of male mice, and a decrease in the volume of the inflammation was seen compared with controls but was lower than the mixture of EVOO with ibuprofen. In our results, the administration of EVOO over time shows an improvement in the tissue compared to the infected group, where the epithelial cells are in better condition, and there is not as much tissue disruption.

In conclusion, the current study shows, for the first time, the effect *in vitro* and *in vivo* of extra virgin olive oil against *H. pylori* using an animal model. Hydroxytyrosol and oleuropien had no effect on their own against *H. pylori*, but they did show effects in a combination of both. At the same time, EVOO showed *in vitro* effect against both strains. Also, in mice chronically infected with *H. pylori*, the administration of EVOO protects the gastric mucosa avoiding the formation of small erosions and ulcers. Future studies are needed to establish the mechanism of EVOO's action at the gastric mucosa level in order to propose this product as a natural antimicrobial agent for the treatment of gastric *H. pylori* infections.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was reviewed and approved by Institutional Committee for Care and Use of Laboratory Animals (IACUC), Universidad Nacional de San Luis (Protocol No. B-328/19).

## Author contributions

AA, MM, AV, and AP: study ideation, design, and manuscript–critical revision. AA, AV, and AP: sample collection, preparation, and manuscript–initial draft. AA: sample processing, experimentation, and statistical analysis. AP, MM, and AA: data acquisition and measurements. AV and AP: funding acquisition. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# References


Ahamad, J., Toufeeq, I., Khan, M. A., Ameen, M. S. M., Anwer, E. T., Uthirapathy, S., et al. (2019). Oleuropein: a natural antioxidant molecule in the treatment of metabolic syndrome. *Phytother. Res.* 33, 3112–3128. doi: 10.1002/ptr.6511

Alipour, M. (2021). Molecular mechanism of *Helicobacter pylori*-induced gastric cancer. *J. Gastrointest. Cancer* 52, 23–30. doi: 10.1007/s12029-020-00518-5

Baker, D. A. (2020). Plants against *Helicobacter pylori* to combat resistance: an ethnopharmacological review. *Biotechnol. Rep.* 26:e00470. doi: 10.1016/j.btre.2020.e00470

Barbaro, B., Toietta, G., Maggio, R., Arciello, M., Tarocchi, M., Galli, A., et al. (2014). Effects of the olive-derived polyphenol oleuropein on human health. *Int. J. Mol. Sci.* 15, 18508–18524. doi: 10.3390/ijms151018508

Behzadi, P., and Gajdács, M. (2021). Writing a strong scientific paper in medicine and the biomedical sciences: a checklist and recommendations for early career researchers. *Biol. Futur.* 72, 395–407. doi: 10.1007/s42977-021-00095-z

Bhattamisra, S. K., Yean Yan, V. L., Koh Lee, C., Hui Kuean, C., Candasamy, M., Liew, Y. K., et al. (2018). Protective activity of geraniol against acetic acid and *Helicobacter pylori*- induced gastric ulcers in rats. *J. Tradit. Complement. Med.* 9, 206–214. doi: 10.1016/j.jtcme.2018.05.001

Bilal, R. M., Liu, C., Zhao, H., Wang, Y., Farag, M. R., Alagawany, M., et al. (2021). Olive Oil: nutritional applications, beneficial health aspects and its prospective application in poultry production. *Front. Pharmacol.* 12:723040. doi: 10.3389/fphar.2021.723040

Castro, M., Romero, C., de Castro, A., Vargas, J., Medina, E., Millán, R., et al. (2012). Assessment of *Helicobacter pylori* eradication by virgin olive oil. *Helicobacter* 17, 305–311. doi: 10.1111/j.1523-5378.2012.00949.x

Cherkas, A., and Zarkovic, N. (2018). 4-Hydroxynonenal in redox homeostasis of gastrointestinal mucosa: implications for the stomach in health and diseases. *Antioxidants* 7:118. doi: 10.3390/antiox7090118

Cicerale, S., Lucas, L. J., and Keast, R. S. (2012). Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. *Curr. Opin. Biotechnol.* 23, 129–135.

CLSI (2007). *Performance standards for antimicrobial susceptibility testing; seventeenth information supplement. CLSI document M100-S17 (M2-A7 and M7-A7)*, Vol. 27. Wayne, PA: Clinical and Laboratory Standards institute.

Dudley, J., Wieczorek, T., Selig, M., Cheung, H., Shen, J., Odze, R., et al. (2017). Clinicopathological characteristics of invasive gastric *Helicobacter pylori*. *Hum. Pathol.* 61, 19–25. doi: 10.1016/j.humpath.2016.09.029

Gravina, A. G., Priadko, K., Ciamarra, P., Granata, L., Facchiano, A., Miranda, A., et al. (2020). Extra-gastric manifestations of *Helicobacter pylori* infection. *J. Clin. Med.* 9:3887. doi: 10.3390/jcm9123887

Huang, C. C., Tsai, K. W., Tsai, T. J., and Hsu, P. I. (2017). Update on the first-line treatment for *Helicobacter pylori* infection - a continuing challenge from an old enemy. *Biomark. Res.* 11, 5–23. doi: 10.1186/s40364-017-0103-x

Jimenez-Lopez, C., Carpena, M., Lourenço-Lopes, C., Gallardo-Gomez, M., Lorenzo, J. M., Barba, F. J., et al. (2020). Bioactive compounds and quality of extra virgin olive oil. *Foods* 9:1014. doi: 10.3390/foods9081014

La, J., Kim, M. J., and Lee, J. (2021). Evaluation of solvent effects on the DPPH reactivity for determining the antioxidant activity in oil matrix. *Food Sci. Biotechnol.* 30, 367–375. doi: 10.1007/s10068-020-00874-9

Malfertheiner, P., Megraud, F., O'Morain, C. A., Gisbert, J. P., Kuipers, E. J., Axon, A. T., et al. (2017). Management of *Helicobacter pylori* infection-the Maastricht v/Florence consensus report European *Helicobacter* and microbiota study group and consensus panel. *Gut* 66, 6–30.

Marcelino, G., Hiane, P. A., Freitas, K. C., Santana, L. F., Pott, A., Donadon, J. R., et al. (2019). Effects of olive oil and its minor components on cardiovascular diseases, inflammation, and gut microbiota. *Nutrients* 11:1826. doi: 10.3390/nu11081826

Martinez-Irujo, J. J., Villahermosa, M. L., Alberdi, E., and Santiago, E. (1996). A checkerboard method to evaluate interactions between drugs. *Biochem. Pharmacol.* 51, 635–644.

Matongo, F., and Nwodo, U. U. (2014). *In vitro* assessment of *Helicobacter pylori* ureases inhibition by honey fractions. *Arch. Med. Res.* 45, 540–546.

Mladenova, I. (2021). Clinical relevance of *Helicobacter pylori* infection. *J. Clin. Med.* 10:3473. doi: 10.3390/jcm10163473

Nazzaro, F., Fratianni, F., Cozzolino, R., Martignetti, A., Malorni, L., De Feo, V., et al. (2019). Antibacterial activity of three extra virgin olive oils of the Campania region, Southern Italy, related to their polyphenol content and composition. *Microorganisms* 7:321. doi: 10.3390/microorganisms7090321

Negm, S., Moustafa, M., Sayed, M., Alamri, S., Alghamdii, H., Shati, A., et al. (2020). Antimicrobial activities of silver nanoparticles of extra virgin olive oil and sunflower oil against human pathogenic microbes. *Pak. J. Pharm. Sci.* 33, 2285–2291.

Ohta, Y., Kamiya, Y., Imai, Y., Arisawa, T., and Nakano, H. (2005). Plaunotol prevents the progression of acute gastric mucosal lesions induced by compound 48/80, a mast cell degranulator, in rats. *Pharmacology* 74, 182–192. doi: 10.1159/000085388

Osman, W. A., Labib, D. A., Abdelhalim, M. O., and Elrokh, E. M. (2017). Synergistic analgesic, anti-pyretic and anti-inflammatory effects of extra virgin olive oil and ibuprofen in different experimental models of albino mice. *Int. J. Rheum. Dis.* 20, 1326–1336. doi: 10.1111/1756-185X.13105

Ozturk, O., Doganaya, L., Colak, Y., Yilmaz, Enc F, Ulasoglu, C., Ozdil, K., et al. (2017). Therapeutic success with bismuth-containing sequential and quadruple regimens in *Helicobacter pylori* eradication. *Arab J. Gastroenterol.* 18, 62–67. doi: 10.1016/j.ajg.2017.05.002

Palacios-Espinosa, J. F., Arroyo-García, O., García-Valencia, G., Linares, E., Bye, R., and Romero, I. (2014). Evidence of the anti-*Helicobacter pylori*, gastroprotective and anti-inflammatory activities of *Cuphea aequipetala* infusion. *J. Ethnopharmacol.* 151, 990–998. doi: 10.1016/j.jep.2013.12.012

Park, H. S., Wijerathne, C. U. B., Jeong, H. Y., Seo, C. S., Ha, H., and Kwun, H. J. (2018). Gastroprotective effects of Hwanglyeonhaedok-tang against *Helicobacter pylori*-induced gastric cell injury. *J. Ethnopharmacol.* 216, 239–250. doi: 10.1016/j.jep.2018.01.025

Ranjbar, R., Behzadi, P., and Farshad, S. (2017). Advances in diagnosis and treatment of *Helicobacter pylori* infection. *Acta Microbiol. Immunol. Hung.* 64, 273–292. doi: 10.1556/030.64.2017.008

Robinson, K., Letley, D. P., and Kaneko, K. (2017). The human stomach in health and disease: infection strategies by *Helicobacter pylori*. *Curr. Top. Microbiol. Immunol.* 400, 1–26. doi: 10.1007/978-3-319-50520-6_1

Romero, C., Medina, E., Vargas, J., Brenes, M., and De Castro, A. (2007). In vitro activity of olive oil polyphenols against *Helicobacter pylori*. *J. Agric. Food Chem.* 55, 680–686.

Siwe, G. T., Maharjan, R., Amang, A. P., Mezui, C., Zondegoumba, N. E., Enow-Orock, G. E., et al. (2021). *Eremomastax speciosa* (Hochst.) Cufod. counteracts the delaying effect of indomethacin on *Helicobacter pylori*-associated chronic gastric ulcers healing. *J. Ethnopharmacol.* 279:114374. doi: 10.1016/j.jep.2021.114374

Thielmann, J., Kohnen, S., and Hauser, C. (2017). Antimicrobial activity of *Olea europaea* Linné extracts and their applicability as natural food preservative agents. *Int. J. Food Microbiol.* 251, 48–66. doi: 10.1016/j.ijfoodmicro.2017.03.019

Waterman, E., and Lockwood, B. (2007). Active components and clinical applications of olive oil. *Altern. Med. Rev.* 12, 331–342.

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# Asclepain cI, a proteolytic enzyme from *Asclepias curassavica* L., a south American plant, against *Helicobacter pylori*

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*Helicobacter pylori* is a Gram negative bacterium most frequently associated with human gastrointestinal infections worldwide. The increasing occurrence of antibiotic-resistant isolates of *H. pylori* constitutes a challenge. The eradication of the microorganism is currently being considered a "high priority" by the World Health Organization (WHO). In this context, bioactive compounds found in natural products seem to be an effective therapeutic option to develop new antibiotics against the pathogen. In this study, we investigated the effect of asclepain cI, the main purified proteolytic enzyme of the latex of petioles and stems from *Asclepia curassavica* L. (Asclepiadaceae), a South American native plant, against *H. pylori*; in order to obtain a natural therapeutic adjuvant and a safe nutraceutical product. Asclepain cI showed antibacterial activity against reference strains and drug-resistant clinical isolates of *H. pylori in vitro*. A range of minimal inhibitory concentration (MIC) from 1 to 2 μg/ml and minimal bactericidal concentration (MBC) from 2 to 4 μg/ml was obtained, respectively. The action of asclepain cI on the transcription of *omp*18, *ure*A, *fla*A genes showed a significantly decreased expression of the selected pathogenic factors. Furthermore, asclepain cI did not induce toxic effects at the concentrations assayed. Asclepain cI could be considered a highly feasible option to be used as a natural therapeutic adjuvant and a safe nutraceutical product against *H. pylori*.

KEYWORDS

**asclepain cI, *Asclepia curassavica* L. (Asclepiadaceae), safe nutraceutical product, antimicrobial proteolytic enzyme, *helicobacter pylori*, natural therapeutic adjuvant**

## Introduction

*Asclepias curassavica* L. (Asclepiadaceae), which is also locally known as flor de sangre (blood flower), bandera española (Spanish flag), algodoncillo (scarlet milkweed), platanillo or hierba Maria (Marie herb), is an erect evergreen perennial subshrub with a woody base, that grows up to 1.6 m tall (Asclepiadaceae, 2022). The plant is native to the South American biographical region although it has become a naturalized weed in tropical and subtropical areas around the world (Floridata, 2022). The dull green/reddish stem is smooth and round. The 8–12 cm long and 5–24 cm wide leaves are simple, opposite, and lanceolate, with short petioles. The umbel-shaped inflorescence has 6–15 flowers that head on short axillary and terminal peduncles. Flowers are bright red or orange around a yellow center (Bendre and Kumar, 2014).

The roots, leaves, and stems of *A. curassavica* contain several biologically active molecules, such as flavonoids, triterpenes, polyphenols, proteins, carbohydrates, fixed oils, saponin, and steroids (Bate and Smith, 1962; Oliver-Bever, 1986; Hembing, 2000). After drying and decoction, the entire plant is used in traditional medicine for its anti-inflammatory, antimicrobial, anticancer, antithrombotic, antioxidant, and hemostatic properties (Moulin-Traffort et al., 1990; Shivaprasad et al., 2009a; Baskar et al., 2012; Lee et al., 2012; Yuan et al., 2016; Qamar et al., 2019; Zheng et al., 2019; Nakano et al., 2020; Alonso-Castro et al., 2021).

Two cysteine-type phytoproteases, asclepain cI and asclepain cII, were isolated, biochemically characterized, and then purified from petioles and stem latex. Both proteases were inhibited using cysteine proteases inhibitors like E-64, maximal proteolytic activity was exhibited at pH 8.0–8.5, which remained stable within that pH range. The proteases showed significant thermal stability at temperatures between 40 and 60°C but were completely inactivated at 70°C. The isoelectric points of both proteases were greater than 9.3. The highest endosterolytic activity on the synthetic substrates N-α-carbobenzoxy-p-nitrophenyl esters of amino acids was expressed with the glutamine derivative. Although asclepain cII is the minor proteolytic component of the enzymatic extract, it showed higher specific activity than asclepain cI (Liggieri et al., 2004, 2009).

Cysteine proteases from the Asclepiadaceae plants latex have exhibited thrombin and plasmin like activities (Shivaprasad et al., 2009b).

The primary scientific novelty of this work lies in the fact that there are no existing reports within the literature on the antibacterial activity of asclepain cI and cII or on the *Helicobacter pylori* activity of asclepain cI and cII.

*Helicobacter pylori* is a Gram-negative bacterium, which has been defined as a group I carcinogen since 1994. It is currently clinically associated with the most frequent human infections worldwide, such as gastritis, peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (Ansari and Yamaoka, 2017; Asgari et al., 2018; Akeel et al., 2019; Nagata et al., 2020; Holmes et al., 2021).

The bacterium colonizes an exclusive ecological niche, the human stomach, through several pathogenic factors, like outer membrane proteins, urease, motility, chemotaxis, and its helical shape (Ansari and Yamaoka, 2019).

These factors allow *H. pylori* to move within the viscous gastric mucosa, facilitate its attachment to epithelial cells and counteract the acidic environment of the human stomach. Also, the production of carbon dioxide ($CO_2$) and ammonia ($NH_3$) by urea hydrolysis, provides a nitrogen source for the bacterium (Cheok et al., 2021; Woo et al., 2021).

The management of *H. pylori* infection includes triple or quadruple antibiotic therapy with clarithromycin (CLA), metronidazole (MTZ), or amoxicillin (AML) plus a proton pump inhibitor (Ogasawara et al., 2020). However, treatment failure is a global concern due to the increase in resistant strains (Kim et al., 2015; Marcus et al., 2016; Dang and Graham, 2017). In this sense, the WHO encourages the urgent search for safe and efficient compounds for the priority treatment of *H. pylori* and other multiresistant bacteria (Roszczenko-Jasińska et al., 2020).

Natural plant extracts provide a feasible alternative to either attack *H. pylori* targets or modulate the host's immune system (Salinas Ibáñez et al., 2017; Korona-Glowniak et al., 2020).

Studies carried out with polyphenols from wine, apple peel, or olive oil have shown that they can cause the rupture of the outer membrane of some bacteria and the decrease in the release of urease and adhesion factors such as SabA or Vac (Parreira et al., 2016). On the other hand, Brown et al. (2016) demonstrated that adverse environmental conditions can increase the transcription of the gene for the survival and virulence of the bacteria (Brown et al., 2016).

The group of proteolytic enzymes shows different structures, an affinity for substrate, and a reaction mechanism (Vallés and Cantera, 2018; Barcia et al., 2020). Thus, subtilisin proves to be effective against *Pseudomonas* sp, *Bacillus* sp, and *Listeria monocytogenes* (Longhi et al., 2008; Molobela et al., 2010). Lysostaphin is capable of breaking the pentaglycine bond between the peptidoglycan chains and lysing cell walls (Boksha et al., 2016); while pronase did not show an additive effect in eradicating *H. pylori* infection when combined with AML and CLA (Bang et al., 2015).

Adaro et al. observed that the partially purified proteolytic extract (≥50 μg of protein/ml) of the fruits from *Solanum granuloso-leprosum* (Dunal) decreased ($p \leq 0.05$) the growth of *Escherichia coli* ATCC 25922. Nevertheless, no effect was observed against *Staphylococcus aureus* ATCC 25923 (Adaro et al., 2019). There is no further information in the literature regarding the use of native plant proteases with antibacterial

activity against *H. pylori* (Bruno et al., 2003, 2006; Liggieri et al., 2004; Pardo et al., 2010; Cimino et al., 2015; Bersi et al., 2019).

The aim of the present study was to investigate the action of asclepain cI, the main purified protease of the latex of petioles and stems from *A. curassavica* L. (Asclepiadaceae) against *H. pylori,* and thus obtain a natural therapeutic adjuvant and a safe nutraceutical product.

# Materials and methods

## Plant material

Petioles and stems of *A. curassavica* L. (Asclepiadaceae), which grow in the city of La Plata, Province of Buenos Aires, Argentina, were collected. The latex was obtained by making incisions on the surface of both parts of the plant. The crude proteolytic extract was obtained by receiving the latex in cold 0.1 M citric phosphate buffer (pH 6.5) with 5 mM EDTA and 2 mM cysteine. This suspension was centrifuged at 16,000 *g* for 30 min at 4°C to remove gums and cell debris. Then, the supernatant was ultra-centrifuged at 100,000 *g* for 1 h at 4°C and kept at −20°C until purified (Ansari and Yamaoka, 2017).

## Purification of asclepain cI

The crude proteolytic extract was purified by cation exchange chromatography (FPLC). Two active fractions were isolated. The major purified protease (asclepain cI) showed a molecular mass of 23.2 kDa by mass spectrometry and a pI higher than 9.3 (Ansari and Yamaoka, 2017).

## Electrophoresis (SDS-PAGE)

Samples containing proteases were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5 % (w/v) polyacrylamide gels. The current was kept constant at 40 mA during stacking and then increased to 60 mA and kept constant for 40 min. Gels were stained with Coomassie Brilliant Blue R-250. Finally, protein purity was verified using the silver staining procedure (Ansari and Yamaoka, 2017). The electrophoretic profiles were analyzed by densitography using the latest version of ImageJ 1.31, Wayne Rasband of the Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA.

## Protein concentration and specific proteolytic activity

The Bradford method was used for measuring the protein concentration of asclepain cI (Bradford, 1976). Proteolytic assays were performed during purification steps using 340 µl of 1 % w/v azocasein solution as substrate in a reaction mixture containing 340 µl of asclepain cI and 340 µl of 100 mM TRIS hydrochloride buffer (TCB) with pH 7.5 containing 15 mM cysteine. This buffer was also used for preparing substrate and enzyme solutions. The reaction was carried out for 10 min at 37°C, then stopped by the addition of 340 µl of 10 % w/v TCA. The mixture was centrifuged for 20 min at 15,400 × *g*, and the supernatant enzymatic activity was spectrophotometrically measured and defined in terms of the Azocaseinolytic Unit (Uazo). One Uazo is the amount of enzyme that produces an increase of one absorbance unit measured at λ: 337 nm after 1 min, under the test conditions. Negative control was performed by replacing the enzyme with buffer.

## Physicochemical properties of asclepain cI

### Stability upon storage

Asclepain cI was stored for 24 months at −20°C. Its residual proteolytic activity was measured every 24 h using 0.5 ml of 10 mM N-alpha-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BANI) (Sigma-Aldrich, USA) as substrate in a reaction mixture containing 0.5 ml of enzyme in 100 mM TCB pH 8 and 0.5 ml of 20 mM L-cysteine in 100 mM TCB pH 8. The reaction was carried out for 5 min at 37°C under 200 rpm of agitation. The absorbance was measured within the linearity range at λ: 410. Proteolytic enzyme activity was expressed in terms of international unit (IU). One IU was defined as the amount of enzyme that cleaves 1 µmol of BANI per min under previously defined conditions. A control was carried out by replacing the enzyme with buffer.

### Solubility

Enzyme solubility is defined as the amount of soluble nitrogen in an aqueous solution or dispersion that is not sedimented by moderate centrifugal forces. The protein concentration (%) was determined by the Kjeldahl method (Kjeldahl, 1883). The performance of asclepain cI in emulsions, foams, and gels can be predicted by protein solubility, and it depends on pH, ionic strength, and temperature (Morr, 1985). Solubility is expressed as protein solubility index (PSI) (American Oil Chemists' Society, 1998), as follows (Equation 1):

$$\text{PSI} = \frac{\text{Protein content in the supernatant (mg/ml)} \times \text{Volume of supernatant (ml)}}{\text{Sample weight (mg)} \times \text{Protein content in the sample (mg/100 mg of sample)}} \times 100 \quad (1)$$

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## Emulsifying properties

The emulsifying property of an enzyme is related to the amount needed to coat an interfacial area. Emulsifying activity index (EAI, $m^2/g$) and emulsion stability index (ESI, min) were determined by using the turbidimetric technique as described by Pearce and Kinsella (1978) and modified by Tang et al. (2006). These indexes were calculated by means of Equations (2) and (3):

$$EAI = \frac{2 \times 2{,}303 \times Ao \times DF}{c \times \phi \times (1-\theta)} \quad (2)$$

$$ESI = \frac{A_0}{(A_0 - A_{20})} \times 20 \quad (3)$$

Where:

c: is the protein concentration (mg/ml).

$\phi$: is the optical path (0.01 m).

θ: is the fraction of oil used to form the emulsion.

DF: is the dilution factor.

## Viscosity

A rheometer (Model DV-III, AMETEK Brookfield, MA, USA) was used for measuring the viscosity of asclepain cI (10 mg of protein/mL), from 100 to 140 rpm at 24°C. Mean viscosity was calculated (Miroslaw and Surówka, 2004).

## Hydration properties

The amount of water that a protein can retain influences the formulation, processing, and storing of goods. Held water (HW, %) and water retention capacity (WHC, g of water/g of dry residue) of asclepain cI were determined under centrifugation at 3000 × *g* for 30 min at 20°C (Piva et al., 1981). HW and WHC were calculated by means of Equations (4) and (5).

$$HW = \frac{\text{weight of H2O in pellet}}{\text{weight of H2O in pellet} + \text{weight of H2O in supernatant}} \quad (4)$$

$$WHC = \frac{\text{weight of H2O in pellet}}{\text{weight of dry pellet}} \times 100 \quad (5)$$

## Bacterial strains

In this study, we used *H. pylori* NCTC 11638 as a reference strain, which was supplied by Dr. Teresa Alarcon Cavero (Hospital Universitario de la Princesa; Madrid, Spain), and also 12 clinical strains isolated from patients of San Luis (Argentina). The characterization of the strains is shown in Table 1.

The strains were confirmed by gram staining, and positive biochemical reaction for urease, catalase, and oxidase. They were stored at −80°C in trypticase soy broth (TSB, Britania) supplemented with 20% glycerol (Biopack, Buenos Aires, Argentina). The antimicrobial sensitivity of the strains was determined by the MIC breakpoint values, according to the Clinical and Laboratory Standards Institute [Clinical and Laboratory Standards Institute (CLSI), 2010].

TABLE 1 Bacterial strains.

| *H. pylori* strains | Antimicrobial susceptibility (mm) | | | |
|---|---|---|---|---|
| | AML | CLA | MTZ | LEV |
| HP109 | S | S | R | S |
| HP137 | S | R | S | S |
| HP145 | S | S | R | S |
| HP148 | S | R | S | S |
| HP152 | S | R | R | S |
| HP155 | S | S | S | S |
| HP166 | S | S | S | S |
| HP179 | S | R | S | S |
| HP294 | S | R | R | S |
| HP659 | S | S | S | S |
| HP661 | S | S | S | R |
| HP662 | S | S | R | S |
| NCTC 11638 | S | S | S | S |

R, resistant strains; S, sensible strains. Amoxicillin (AML, 10 μg), clarithromycin (CLA, 15 μg), metronidazole (MTZ, 5 μg) (Oxoid™, Argentina), and levofloxacin (LEV, 5 μg) (Britania Laboratories, Argentina). The sensitivity of the strains was evaluated by the MIC breakpoint values, with ≤25 mm for AML (10 μg/ml), ≤28 mm for CLA (15 μg/ml), ≤18 mm for MTZ (5 μg/ml), and ≤18 mm for LEV (5μg/ml).

## Animals

BALB/c mice of 18 to 20 g of body weight were provided by the UNSL Animal Facility. The handling and care were carried out according to the norms of the Institutional Committee for the Care and Use of Animals (CICUA-UNSL) and the Guide for the Care and Experimental Use of Animals (DHEW publication NIH 80-23).

## Antibacterial activity of asclepain cI

The antibacterial activity of asclepain cI against *H. pylori* strains was evaluated using the broth microdilution method as previously described by Bang et al. (2015), Salinas Ibáñez et al. (2017). Asclepain cI concentration ranging from 32 to 0.125 μg of protein/ml was used and 2,3,5-triphenyl tetrazolium chloride (TTC, Merck KGaA, Darmstadt, Germany) solution was added as a viability indicator. Both positive and negative controls were included in all assays. Minimal inhibitory concentrations (MICs) were determined after 72 h of incubation at 37°C,

as the lowest concentration of asclepain cI that inhibited microbial growth. Minimum bactericidal concentration (MBC) was defined as the least concentration of asclepain cI that prevented the growth of microorganisms on antibiotic-free blood agar media [Clinical and Laboratory Standards Institute (CLSI), 2015].

## Effects of asclepain cI subinhibitory concentrations (subMICs) on cultures

Effects of asclepain cI subMICs on viability and on the morphology of 13 *H. pylori* strains were determined by viable cell counts and microscopic studies. Aliquots of 14 ml of cultures of each strain were added with 1 ml of the 1 μg/ml subinhibitory concentration (subMIC) of asclepain cI and incubated at 37°C for 24 h. Then, serial dilution ($10^{-2}$ to $10^{-6}$) viable cell counts were plated in duplicate onto Mueller Hinton Agar (MHA, Britania, Buenos Aires, Argentina) supplemented with 7% horse blood (MHA-HB) and incubated at 37°C for 24 h. Viable cell counts were expressed as colony forming units per ml (CFU/ml). The effect on cell morphology of *H. pylori* in culture treated and untreated with subMICs of asclepain cI was analyzed. Smears were made from the planktonic cultures. Gram stain was subsequently performed. Then, it was observed and photographed under an optical microscope with an immersion objective. In addition, 100 μl of culture was taken, placed on a coverslip, and allowed to dry. Coverslips were gold coated and processed on a standard sputter. Observations were made by scanning electron microscopy (SEM) using a Zeiss LEO 1450VP microscope.

## Gastroprotective effect of asclepain cI

The gastroprotective effect of asclepain cI on the damage induced by intragastrical administration of *H. pylori* was examined. Four groups of teen animals were used. Group 1 was treated with 250 μl of a suspension of *H. pylori* at 1–2 × $10^8$ CFU/mL. Group 2 was treated with 250 μl of asclepain (2 μg/ml), 60 min prior to infection with *H. pylori*. Group 3 was treated with 250 μl of asclepain (2 μg/ml), and Group 4 was treated with 250 μl of phosphate buffered saline (PBS). This procedure was performed for a week every 48 h. The animals were sacrificed by cervical dislocation 4 days after the last inoculation. Lesions in the stomach were observed under an illuminated magnifying microscope, and the number and size of long lesions were both measured. Petechial lesions were counted; five such petechial lesions were taken as 1 mm of ulcer (Awaad et al., 2017).

TABLE 2 Primers used for RT-PCR targeting *Helicobacter pylori* genes.

| Primer | Primer sequence (5′-3′) | Size amplicon (bp) |
|---|---|---|
| 16S *rRNA* -F | GGAGGATGAAGGTTTTAGGATTG | 390 |
| 16S *rRNA* -R | TCGTTTAGGGCGTGGACT | |
| *omp*18-F | TGCTTTTGGAAGGCAATACC | 165 |
| *omp*18-R | CATTTGGGTTTGGTTTCACC | |
| *ure*A-F | GCCAATGGTAAATTAGTT | 411 |
| *ure*A-R | CTCCTTAATTGTTTTTAC | |
| *fla*A -F | GTGGCGCAAAAAGTGGCTAA | 237 |
| *fla*A-R | GTAATCGGCCGGTTTCAAGC | |

## Effects of sub-inhibitory concentrations (subMICs) of asclepain cI on the transcription (expression) of *H. pylori* genes encoding pathogenic factors

Gene expression assays were evaluated with SubMICs (½ MIC) of asclepain cI. Total RNA was isolated from cultures of 13 strains with or without enzyme treatment, using TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Buenos Aires, Argentina), being stored at −20°C. The cDNA was obtained as previously described by Salinas Ibáñez et al. (2017).

Reverse transcription of *omp18, ureA, flaA* genes was carried out using 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen, Buenos Aires, Argentina). PCR amplification was performed using the primer pairs shown in Table 2 and the protocols described in Table 3.

RT-PCR products were identified in agarose gel electrophoresis (1.8%), stained with GelRed Nucleic Acid Gel Stain (Biotium Inc. Hayward, CA, USA), visualized with UV light, and finally photographed. Molecular mass reference (PBL, Quilmes, Buenos Aires, Argentina) was included. Java image processing and analysis software (ImageJ, Maryland, USA) were used for the semi-quantification of the DNA amplicons.

## Hepatotoxicity and nephrotoxicity assays

The animals used in cytotoxicity tests were those described in point 2.7 [National Institutes of Health (NIH), 1980]. The potential hepatotoxicity and nephrotoxicity of asclepain cl were evaluated using two groups of animals. The first group was treated with an intragastric administration of 1 × PBS. The second group was treated with three doses of 250 μl of asclepain cI (2 μg/ml) at 48 h intervals for 3 days. Then, the animals were sacrificed and the mice serums (before and upon treatment) were collected to determine aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine activity, using

TABLE 3 Protocols of PCR amplification for *Helicobacter pylori* genes.

| Steps | Temperature (°C) | Time (min) |
|---|---|---|
| **16S*rRNA* and *omp*18 genes** | | |
| Initial denaturalization | 94 | 3 |
| 30 cycles | 94 | 1 |
| | 58 | 1 |
| | 72 | 1 |
| Final extension | 72 | 10 |
| ***ureA* and *flaA* genes** | | |
| Initial denaturalization | 95 | 5 |
| | 94 | 1 |
| 35 cycles | 45 | 1 |
| | 72 | 1 |
| Final extension | 72 | 7 |

Transaminases 200 and Creatinine commercial kits (Wiener Lab, Rosario, Santa Fe, Argentina). The results were expressed as IU/L and mg/L, for aminotransferase and creatinine activity, respectively (American Gastroenterological Association, 2002). Hepatotoxicity was evaluated using AST and ALT assay. The color change produced by the reaction of pyruvate and 2,4-dinitrophenylhydrazine was measured at λ: 505 nm. Nephrotoxicity assays were carried out with serum (100 μl) and 41.4 mmol/L picric acid (500 μl) in a hemolysis tube. The mixture was allowed to stand for 10 min and centrifuged at 3,000 rpm for 5 min. Next, 63 μl of glycine buffer/1.0 M NaOH was added to supernatant (375 μl) and incubated for 20 min at room temperature. The reaction of creatinine with alkaline picrate in a buffered medium produced a chromogenic compound which was measured at λ: 510 nm. A Cintra 2020 UV-Vis Spectrometer (GBS Scientific Equipment Pty Ltd., Braeside, Victoria 3195 Australia) was used for measuring the absorbance.

## Hemolytic activity

The hemolytic activity of asclepain cI was tested against human erythrocytes. Blood was collected with heparin and the erythrocytes were washed and suspended with 35 mM phosphate buffer and 0.15 mM NaCl (pH 7). In total, 500 μl of 0.5% (v/v) of erythrocyte suspension was added to an equal volume of enzyme solutions (2 μg/ml). The mixture was incubated for 1 h at 37°C and centrifuged at 2,800 rpm for 5 min. Hemolysis of the supernatant was determined at λ: 414 nm. Phosphate buffered saline was used as the negative control while Triton X-100 of 0.1% v/v as the positive control (Gonzalez et al., 2010).

$$\text{Hemolysis percentage} = \frac{\text{Aa - Ab}}{\text{Ac - Ab}} \times 100 \quad (6)$$

Where:

Aa: Absorbance of asclepain cI.

Ab: Absorbance of the negative control.

Ac: Absorbance of the positive control.

## Statistical analysis

All different determinations, specific enzyme activity, protein content, enzyme physicochemical properties, determination of MIC and MBC, gene expression, viable counts, microscopic techniques, and cytotoxicity test were performed in duplicate as three separate assays. The results were expressed as mean ± standard deviation (SD) using InfoStat/L Statistical Software for Windows (Universidad Nacional de Córdoba, Córdoba, Argentina). A value of $p < 0.05$ was considered significant according to Student's t-test. The linear region of the reaction progress of enzyme activity was also determined.

# Results and discussion

## Protein concentration and specific proteolytic activity

Figure 1 shows the electrophoretic profiles analyzed by densitography using the latest version of ImageJ 1.31. The molecular weights of the two purified fractions from the petioles and stem latex of *A. curassavica*, named asclepain cI and asclepain cII, were 23.422 and 24.653 kDa, respectively. These values were similar to those obtained by MALDI-MS/TOF (Ansari and Yamaoka, 2017; Holmes et al., 2021).

The purified fraction of the asclepain cI showed a total protein content of 135 mg/ml and specific proteolytic activity of 7.74 IU/mg of protein.

## Physicochemical properties of asclepain cI

Asclepain cI was shown to be stable at −20°C for at least 24 months. The enzyme showed a low solubility value (PSI: 0.11 %) and behavior of the Newtonian fluid, with a viscosity of 1.3 cP. Besides, the enzyme showed high water retention (HW: 47 % or WHC: 36.2 g of $H_2O$/g of dry residue) and good emulsifying properties (EAI: 3,644 $m^2$/g, ESI: 167.6 min). The properties of asclepain cI were similar to those of other foods (Martínez and Carballo, 2021).

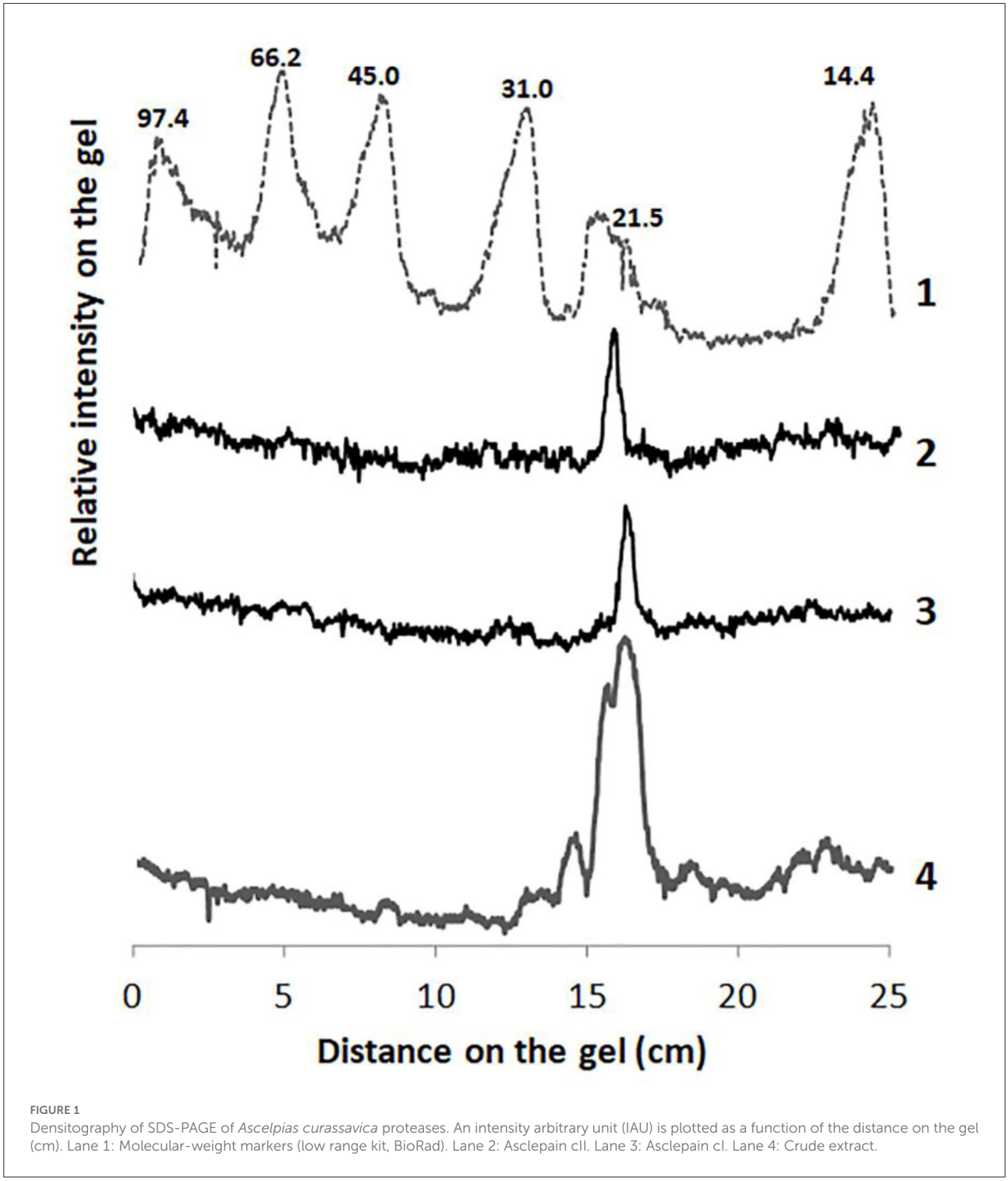


FIGURE 1
Densitography of SDS-PAGE of *Ascelpias curassavica* proteases. An intensity arbitrary unit (IAU) is plotted as a function of the distance on the gel (cm). Lane 1: Molecular-weight markers (low range kit, BioRad). Lane 2: Asclepain cII. Lane 3: Asclepain cI. Lane 4: Crude extract.

## Antibacterial activity of asclepain cI

The antibacterial activity of asclepain cI against *H. pylori* NCTC 11638 (reference strain) and 12 clinical isolates, and the pathologies associated with them, are shown in Table 1 (section Bacterial strains).

All *H. pylori* strains were susceptible to asclepain cI, and MIC values of 1–2 μg/ml and MBC values of 2–4 μg/ml were obtained (Table 4).

TABLE 4 Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of asclepain cI against *H. pylori* strains by means of the broth micro dilution method.

| *H pylori* strains | Pathology | Asclepain cI | |
|---|---|---|---|
| | | MIC (μg/mL) | MBC (μg/mL) |
| **Sensitive to AML, MTZ, LEV and CLA** | | | |
| NCTC 11638 | Reference | 2 | 2 |
| HP155 | Chronic gastritis | 1 | 2 |
| HP166 | Chronic gastritis | 1 | 2 |
| HP179 | Chronic gastritis | 1 | 2 |
| HP659 | Chronic gastritis | 2 | 2 |
| **Single-drug resistant** | | | |
| HP109 | Chronic gastritis R MTZ | 1 | 2 |
| HP137 | Chronic gastritis R CLA | 1 | 2 |
| HP145 | Duodenal ulcer R MTZ | 2 | 4 |
| HP148 | Gastric ulcer R CLA | 1 | 2 |
| HP661 | Gastric ulcer R LEV | 2 | 2 |
| HP662 | Chronic gastritis R MTZ | 2 | 2 |
| **Multidrug-resistant** | | | |
| HP152 | Gastric ulcer R MTZ R CLA | 2 | 4 |
| HP294 | Duodenal ulcer R MTZ R CLA | 2 | 4 |

Inhibition zone diameters to or below 25 mm for AML (10 μg/ml), 28 mm for CLA (15 μg/ml), 18 mm for MTZ (5 μg/ml), and 18 mm for LEV (5 μg/ml) are considered sensitive strains.
The values were expressed as mean of the experiments in triplicate (n = 3). No visual color difference was observed between triplicate tests performed under the same conditions.

The MBC of asclepain cI is 2 μg/ml for all *H. pylori* strains studied, regardless of their sensitivity to antibiotics (CLA, LEV, MTZ, and AML). On the other hand, *H. pylori* HP 145 (resistant to MTZ) and HP 152 (resistant to MTZ and CLA) showed an MBC value as high as 4 μg/ml. Similar behavior was not observed for other *H. pylori* resistant strains, such as HP 109, HP 662 (resistant to MTZ), and HP 294 (resistant to MTZ and CLA).

Several plant extracts used in traditional medicine for gastrointestinal disorders have demonstrated antibacterial activity against *H. pylori* activity. Ethyl acetate extract of leaves and flowers from *Hibiscus rosa-sinensis* L. (Malvaceae) elicited antibacterial and anti-ulcer genic activity, showing MIC of 0.2–0.25 mg/ml and MBC of 1.25–1.5 mg/ml against resistant and sensible *H. pylori* strains (Ngan et al., 2021). Those values were at least 100 times higher than the MIC and MBC values obtained using asclepain cI.

Lien et al. (2019) have reported that ovatodiolide, isolated from *Anisomeles indica*, inhibited the growth of both reference strain and clinical multidrug-resistant isolates of *H. pylori*. The MIC and MBC values ranged from 10 to 20 μM and from 100 to 200 μM, respectively (Lien et al., 2019).

Concentrated ethanol extracts of the entire plant of *A. curassavica* (100 mg/ml) were assayed against 18 bacterial strains by using the agar-diffusion method. Those extracts showed antibacterial activity against *Clostridium histolyticum* ATCC 6282, an anaerobic gram-positive bacterium that can cause gas gangrene in humans and animals, and against *Escherichia coli* ATCC 8739, a gram-negative, facultative anaerobe, no sporulation coliform bacterium. However, the same extracts did neither inhibit the growth of *Bacteroides fragilis* ATCC 23745 (an anaerobic gram-negative bacillus) nor in several gram-positive bacteria, such as *Staphylococcus aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *S. capitis* ATCC 35661, *S. cohnii* ATCC 35662, *Streptococcus pyogenes* ATCC 19615, *S. bovis* ATCC 49133, *S. agalac tiae* ATCC 13813, *S. pneumoniae* ATCC 6303, *S. lactis* ATCC 7962, *Streptococcus sp.* ATCC 12388, *Bacillus subtilis* ATCC 6633, *B. megaterium* ATCC 89, *Corynebacterium diphtheriae* ATCC 13812, and *C. pseudodiphtheriticum* ATCC 10700 (Neto et al., 2002).

In addition, other authors reported that among all the tested species, *E. coli* and *Klebsiella pneumoniae* showed the greatest sensitivity against methanol and petroleum spirit root extracts of *A. curassavica* (Hemadri Reddy et al., 2012). The chloroform extract of *A. curassavica* obtained by the Soxhlet method also showed good activity against the gram-negative bacteria *K. pneumoniae* and *Pseudomonas aeruginosa* but did not show any antifungal activity. The water extract of *A. curassavica* was moderately active against the bacterial strain *P. aeruginosa* and the fungal strain *C. albicans* (Kurdekar et al., 2012). However, those *A. curassavica* extracts have not yet been investigated against *H. pylori*.

In this study, the antibacterial activity of asclepain cI was assayed against *H. pylori* strains that were resistant to one or more drugs. The obtained results demonstrated that asclepain cI exerted significant antibacterial activity against all *H. pylori* strains, including multidrug-resistant strains.

## Effects of subinhibitory concentrations (subMICs) of asclepain cI on cultures

The effect of asclepain cI subMIC (1 and 0.5 μg/ml) on cultures of 13 *H. pylori* strains was evaluated by means of viable cell counts.

A significant decrease in viable cell counts of treated cultures (TP) was regarded as untreated cultures (UTP) ($p < 0.05$). Resistant *H. pylori* strains showed near 2 log units while sensible *H. pylori* strains exhibited 3 log units lesser compared to the control group.

According to literature, 7-O-butylnaringenin (flavonoid) decreased the viable cell count of *H pylori* strains in 2 log units (Moon et al., 2013). Besides, armeniaspirol A, a product isolated from *Streptomyces armeniacus* led to a three-log decrease in viable cell count of *H. pylori* strains (Jia et al., 2022).

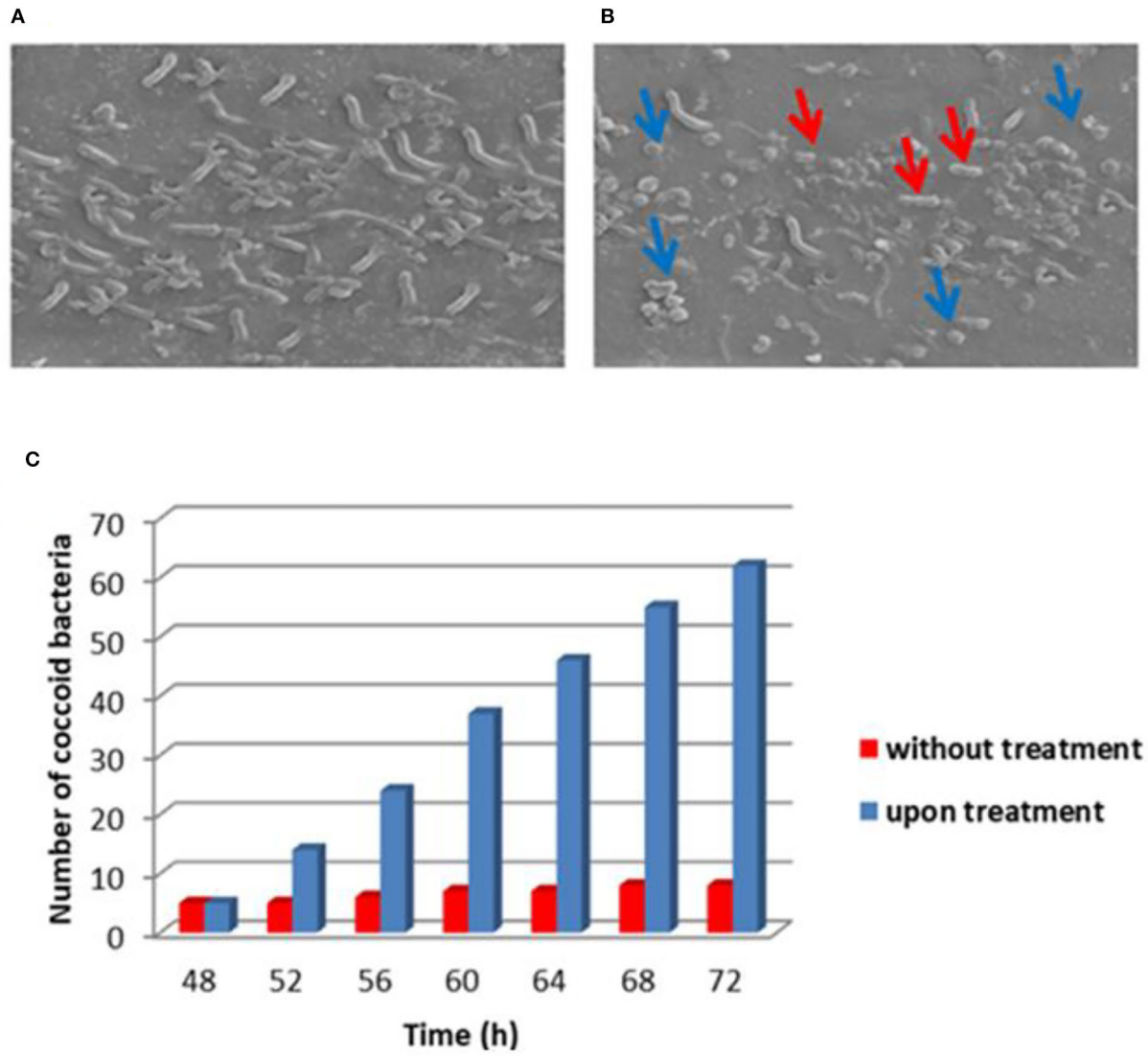


FIGURE 2
Electron microscopy image of *Helicobacter pylori* NCTC 11638 strain untreated **(A)** and treated **(B)** with asclepain cl. Histogram with statistical analysis of the number of coccoid cells **(C)**.

On the other hand, we performed both optical and electron microscopy of treated and untreated *H. pylori* cells with asclepain cI.

Figure 2 shows the electron microscopy image of *H. pylori* NCTC 11638 strain untreated and treated with asclepain cI. No evidence of morphological changes or cell damage was observed in the control cultures (without treatment) and the helical shape was maintained. By contrast, the asclepain cI-treated cells showed coccoid (blue arrows) or coccobacilli (red arrows) forms.

The conversion of the helical shape to the coccoid form of the microbial strain hinders the survival and colonization of *H. pylori* in the gastric mucosa.

Other authors have reported that several organic compounds such as methyl gallate, paeonol, 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose from the root extract of *Paeonia lactiflora*, and the ethyl acetate fraction of the flower from *H. rosa-sinensis* elicited the conversion of helical to coccoid form (Neto et al., 2002; Ngan et al., 2021). The ellagic acid generally found in walnuts, pomegranates, strawberries, blackberries, cloudberries, and raspberries have promoted coccoid morphology in *H pylori* strains (De et al., 2018). In our laboratory, the proteolytic extract of fruits from *Solanum granuloso-Leprosum* and its main purified fraction (granulosain I) both showed similar results (Salinas Ibáñez et al., 2021).

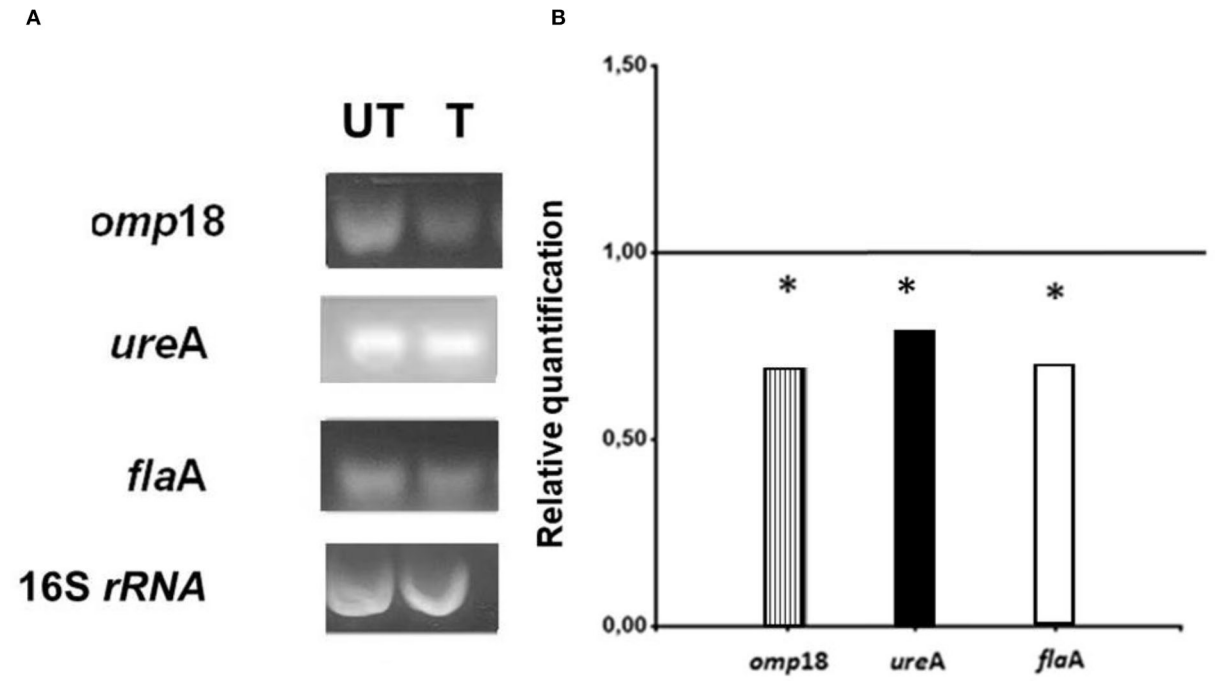


FIGURE 3
**(A)** Electrophoretic gels: *Helicobacter pylori* amplicons resulting from RT-PCR in 1.8 % agarose gels stained with Gel Red®. (T) Treated cultures and (UT) Untreated cultures of *H. pylori* with asclepain cI. **(B)** Relative quantification of the *H. pylori* virulence gene expression, before and after treating *H. pylori* cultures with asclepain cI. The relative quantification represents the mean ± SD of three independent experiments. * $p \leq 0.05$ according to the Student's *t*-test.

## Effects of subinhibitory concentrations (subMICs) of asclepain cI on the transcription (expression) of *H. pylori* genes encoding pathogenic factors

The effect of subinhibitory concentrations (sub-MICs) of asclepain cI (1 μg/ml) on the expression of the *H. pylori* genes encoding pathogenic factors, such as *omp*18, *ure*A, and *fla*A genes, was determined by using RT-PCR.

Amplicons of *H. pylori* cultures, which were grown in the presence (T, treated cultures) and in the absence (UT, untreated cultures) of asclepain cI, are shown in Figure 3A.

The expression levels of the *omp*18, *ure*A, and *fla*A pathogenic factors obtained from T and UT were normalized, using the expression level of the 16S rRNA gene (value 1). A comparison was made of the normalized gene expression between treated and untreated cultures and the resulting values were graphed. The mRNA expression levels in *omp*18, *ure*A, and *fla*A significantly decreased in treated cultures ($p < 0.05$) (Figure 3B).

The ability of *H. pylori* to establish a persistent infection depends on the coordinated expression of genes encoding virulence factors that allow the pathogen to adapt to adverse stomach environmental conditions.

The secretion of urease and spiral structure of *H. pylori* are relevant pathogenic factors to establish initial colonization (Matsuo et al., 2017; Ansari and Yamaoka, 2019). Consequently, urease started to be considered an important therapeutic target to explore (Olivera-Severo et al., 2017).

*Helicobacter pylori* has ∼4% of the bacterial genome that encodes for a diverse family of outer membrane proteins such as BabA, SabA, OipA, and Omp18. Those proteins facilitate the attachment of bacteria to host cells and help establish persistent infections. In addition, they have an important role in osmotic and structural stability, metabolism, ion transport, and antibiotic resistance (Matsuo et al., 2017; Ansari and Yamaoka, 2019; Šterbenc et al., 2019; Liu et al., 2022).

In *H. pylori*, the *fla*A-encoded flagellin protein is part of the motility organ complex (Kao et al., 2016; Zhao et al., 2019). Evidence-based studies report that the colonization capacity of *H. pylori* is due to the bacillary morphology and the presence of 4–6 unipolar flagella. Additionally, the flagellated strains are correlated with the degree of infectivity and the ability to form bacterial biofilm.

The results obtained suggest that the mechanism of antimicrobial action of asclepain cI is based on the inhibitory effect of the transcription of *H. pylori* genes encoding pathogenic factors.

The literature reports that aqueous extracts of *Lithraea molleoides* (Vell.) Engl. (Anacardiaceae), a regional plant of San Luis Province, Argentina, caused a decrease in the expression of the *ure*A gene of *H. pylori* (Salinas Ibáñez et al., 2017). Besides, the partially purified proteolytic extract of the fruits from *Solanum granuloso-leprosum* and granulosain I (the main purified fraction) significantly decreased the expression of pathogenic factors: *omp*18, *ure*A, *and fla*A (Salinas Ibáñez et al., 2021). Disulfiram, an irreversible inhibitor of aldehyde dehydrogenase (ALDH), also decreased the expression levels of urease from *H. pylori* (Kobatake et al., 2021).

## Gastroprotective effects of asclepain cI

The design of this experiment included four groups of five mice each. The first group consisted of five mice infected with a $1 \times 10^6$ suspension of *H. pylori* (Group 1). The second group was of five mice treated with 2 μg/ml of asclepain cI 1 h before being infected with the microorganism (Group 2). The third group included five mice treated with asclepain cI alone (Group 3), and the fourth group consisted of five mice inoculated with PBS (Group 4).

Figure 4A shows the number of injuries in the gastric mucosa by direct microscopy (10×) for Group 1 (X: 69), which was significantly higher than the injuries found in the other groups. The differences between groups were significant. Figure 4B shows an image of mucosa after going through different treatments. Asclepain cI showed a noticeable gastroprotective effect.

The mouse infection model has been widely used in the exploration of host responses and eradication of *H. pylori*. A similar gastroprotective effect that ascleplain cI was shown for a tricyclic sesquiterpene extracted from *Pogostemon cablin* (Blanco) Benth (Lian et al., 2018). On the other hand, the gastric mucosal damage caused by *H. pylori* infection was repaired by an extract of *Sanguisorba officinalis* (Shen et al., 2021).

## Cytotoxicity assays

The toxicological effect of asclepain cI was evaluated through the determination of the activities of transaminases and creatinine, enzymes involved in liver and kidney function. The activities of these enzymes did not show significant differences ($p < 0.05$) compared to the control group (Figures 5A,B). Consequently, asclepain cI did not show toxicological effects at the concentrations studied. Similar results have been reported with both the partially purified proteolytic extract of the fruits from *Solanum granuloso-leprosum* (Dunal) and the purified fraction named granulosain I, against *H. pylori* (Salinas Ibáñez et al., 2021).

## Hemolytic activity

A hemolytic activity assay is a versatile tool for evaluating the rapid initial toxicity.

The anti-hemolytic activity at the MIC concentration of asclepain cI was 70%. This value shows that asclepain cI has about 1.75–3.5 times better capacity to protect the human erythrocytes than *Chinese keemun black* tea grades (*Camellia sinensis*) (Zhang et al., 2019). It is highly likely that this behavior is based on the ability of the enzyme to form hydrogen bonds with the erythrocyte cell membrane. According to other authors, this interaction can increase the stiffness of the membrane, making it less susceptible to hemolysis (Sato et al., 1993).

# Conclusion

The current persistence and rise of antibiotic resistant bacteria have become a serious concern for global public health, this is all due to the lack of new antimicrobials. Diverse initiatives worldwide yearn to develop novel and more effective antimicrobial compounds and novel strategies. Meanwhile, the potential uses of the compounds found in natural sources for the treatment of *H. pylori* strains are ultimately becoming a safe alternative.

The aim of this paper was to study the effect of asclepain cI, the main purified proteolytic enzyme of the latex of petioles and stems from *Asclepia curassavica* L. (Apocynaceae), a South American native plant, against *H. pylori*, for the purpose of obtaining a natural therapeutic adjuvant and a safe nutraceutical product.

Asclepain cI showed a very good antibacterial activity against 30 sensitive and resistant *H. pylori* strains, with MIC of 1–2 μg/ml and MBC of 2–4 μg/ml.

Besides, asclepain cI significantly decreased the expression of pathogenic factors: *omp*18, *ure*A, and *fla*A. The obtained results allow us to conclude that asclepain cI act on several pathogenic facts which are involved in the structure of the outer membrane protein, in the urea cleavage which allows the bacterium survival, and in the motility of *H. pylori*.

The enzyme showed, on the one hand, a relevant gastroprotective effect in an animal model, and on the other hand, no toxicological effects at the concentrations studied.

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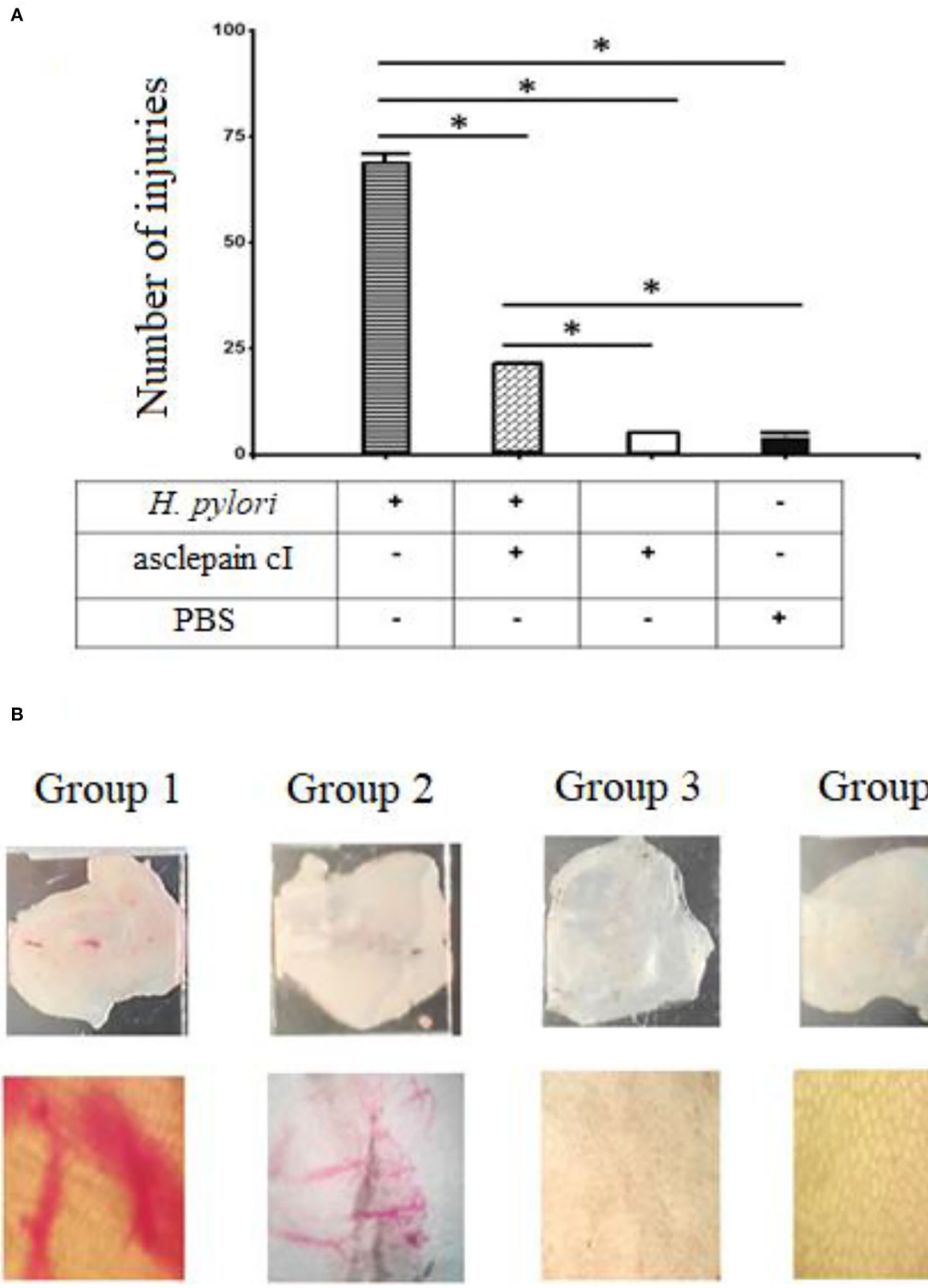


FIGURE 4
**(A,B)** Gastroprotective effects of asclepain cl. Group 1: Stomach infected with *H. pylori.* Group 2: Stomach treated with asclepain cl and infected with *H. pylori.* Group 3: Stomach treated with asclepain cl. Group 4: Stomach treated with PBS (Control). The * symbol indicates the significant differences between groups (*$p < 0.05$). All values are expressed as mean ± S.E.M.

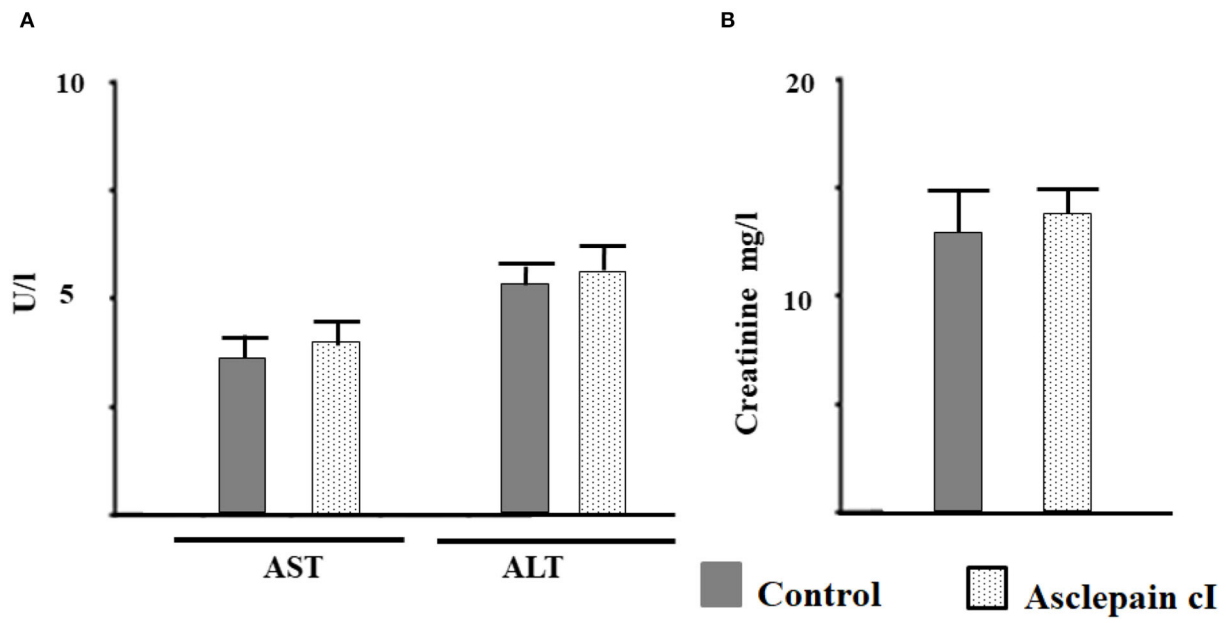


FIGURE 5
Toxicological effect of asclepain cI evaluated as: **(A)** The activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT); **(B)** creatinine in serum.

Asclepain cI could be successfully used as a natural therapeutic adjuvant against *H. pylori* and also as a safe nutraceutical product.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was reviewed and approved by Comité Institucional de Cuidado y Uso de Animales, CICUA—UNSL (Institutional Committee for the Care and Use of Animals—National University of San Luis, San Luis, Argentina). Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

Adaro, M. O., Vallés, D., Cantera, A. M., Talia, J. M., and Barberis, S. E. (2019). Antibacterial activity of the proteolytic extract from fruits of *Solanum granuloso*-leprosum (Solanaceae). *Lat. Am. J. Pharm*. 38, 2032–2035.

Akeel, M., Shehata, A., Elhafey, A., Elmakki, E., Aboshouk, T., Ageely, H., et al. (2019). *Helicobacter pylori* vacA, cagA and iceA genotypes in dyspeptic patients from southwestern region, Saudi Arabia: distribution and association with clinical outcomes and histopathological changes. *BMC Gastroenterol*. 19, 16. doi: 10.1186/s12876-019-0934-z

Alonso-Castro, A. J., Arana-Argáez, V., Yáñez-Barrientos, E., Torres-Romero, J. C., Chable-Cetz, R. J., Worbel, K., et al. (2021). Pharmacological activities of *Asclepias curassavica* L. (Apocynaceae) aerial parts. *J. Ethnopharmacol*. 281, 114554. doi: 10.1016/j.jep.2021.114554

American Gastroenterological Association (2002). American Gastroenterological Association medical position statement: evaluation of liver chemistry tests. *Gastroenterology* 123, 1364–1366. doi: 10.1053/gast.2002.36060

American Oil Chemists' Society (1998). "Analytical methods," in: *Official Methods and Recommended Practices of the AOCS. 5th ed*., ed Firestone D. Champaign, IL: American Oil Chemists' Society Editorial. p. 1200.

Ansari, S., and Yamaoka, Y. (2017). Survival of *Helicobacter pylori* in gastric acidic territory. *Helicobacteria* 22, 19. doi: 10.1111/hel.12386

Ansari, S., and Yamaoka, Y. (2019). *Helicobacter pylori* virulence factors exploiting gastric colonization and its pathogenicity. *Toxins (Basel)*. 11, 677. doi: 10.3390/toxins11110677

Asclepiadaceae (2022). *Asclepias curassavica L*. Available online at: http://www.conabio.gob.mx/malezasdemexico/asclepiadaceae/asclepias-curassavica/fichas/ficha.htm (accessed August 8, 2022).

Asgari, B., Kermanian, F., Derakhshan, N., Asna-Ashari, M., Sadat, Z. R. N., Yaslianifard, S., et al. (2018). Honey-derived *Lactobacillus rhamnosus* alleviates *Helicobacter pylori*-induced gastro-intestinal infection and gastric inflammation in C57BL/6 mice: an immuno-histologic study. *Arq Gastroenterol*. 55, 279–282. doi: 10.1590/s0004-2803.201800000-70

Awaad, A. S., Alafeefy, A. M., Alasmary, F. A. S., El-Meligy, R. M., Zain, M. E., Alqasoumi, S. I., et al. (2017). Novel essential amino acid-sulfanilamide hybrid as safe anti-ulcerogenic agent with anti-*Helicobacter pylori* activity. *Saudi Pharm. J*. 25, 967–971. doi: 10.1016/j.jsps.2017.02.012

Bang, C. S., Kim, Y. S., Park, S. H., Kim, J. B., Baik, G. H., Suk, K. T., et al. (2015). Additive effect of pronase on the eradication rate of first-line therapy for *Helicobacter pylori* infection. *Gut Liver*. 9, 340–345. doi: 10.5009/gnl13399

Barcia, C., Coelho, A. S., Barberis, S., and Veríssimo, P. (2020). Acaciain peptidase: the first South American pollen peptidase potentially involved in respiratory allergy. *Biotechnol. Appl. Biochem*. 67, 224–233. doi: 10.1002/bab.1837

Baskar, A. A., Al Numair, K. S., Alsaif, M. A., and Ignacimuthu, S. (2012). In vitro antioxidant and antiproliferative potential of medicinal plants used in traditional Indian medicine to treat cancer. *Redox Rep*. 17, 145–156. doi: 10.1179/1351000212Y.0000000017

Bate, E. C., and Smith, F. L. S. (1962). The phenolic constituents of plants and their taxonomic significance. I Dicotyledons. *J. Linn. Soc. Lond. Bot*. 58, 95–173. doi: 10.1111/j.1095-8339.1962.tb00890.x

Bendre, A. M., and Kumar, A. A. (2014). *Text Book of Practical Botany*. New Delhi: Rastogi Publications (2014). p. 464.

Bersi, G., Vallés, D., Penna, F., Cantera, A. M., and Barberis, S. (2019). Valorization of fruit by-products of *Bromelia antiacantha* Bertol: protease obtaining and its potential as additive for laundry detergents. *Biocatal. Agric. Biotechnol*. 18, 101099. doi: 10.1016/j.bcab.2019.101099

Boksha, I. S., Lavrova, N. V., Grishin, A. V., Demidenko, A. V., Lyashchuk, A. M., Galushkina, Z. M., et al. (2016). *Staphylococcus simulans* recombinant lysostaphin: Production, purification, and determination of antistaphylococcal activity. *Biochemistry (Mosc)*. 81, 502–510. doi: 10.1134/S0006297916050072

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem*. 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Brown, A., Fernandez, I. S., Gordiyenko, Y., and Ramakrishnan, V. (2016). Ribosome-dependent activation of stringent control. *Nature*. 534, 277–280. doi: 10.1038/nature17675

Bruno, M. A., Pardo, M. F., Caffini, N. O., and López, L. M. I. (2003). Hieronymain I, a new cysteine peptidase isolated from unripe fruits of *Bromelia hieronymi* Mez (Bromeliaceae). *J. Protein Chem*. 22, 127–134. doi: 10.1023/A:1023418812832

Bruno, M. A., Trejo, S. A., Aviles, X. F., Caffini, N. O., and López, L. M. I. (2006). Isolation and characterization of hieronymain II, another peptidase isolated from fruits of *Bromelia hieronymi* Mez. (Bromeliaceae). *Protein J*. 25, 224–231. doi: 10.1007/s10930-006-9005-8

Cheok, Y. Y., Lee, C. Y. Q., Cheong, H. C., Vadivelu, J., Looi, C. Y., Abdullah, S., et al. (2021). An overview of *Helicobacter pylori* survival tactics in the hostile human stomach environment. *Microorganisms*. 9, 2502. doi: 10.3390/microorganisms9122502

Cimino, C. V., Colombo, M. L., Liggieri, C., Bruno, M., and Vairo-Cavalli, S. (2015). Partial molecular characterization of Arctium minus aspartylendopeptidase and preparation of bioactive peptides by whey protein hydrolysis. *J. Med. Food*. 18, 856–864. doi: 10.1089/jmf.2014.0101

Clinical and Laboratory Standards Institute (CLSI) (2010). *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Approved Guideline Second Edition*. CLSI document M45-A2 30, 18. Wayne, PA: Clinical and Laboratory Standard Institute.

Clinical and Laboratory Standards Institute (CLSI) (2015). *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria. Approved Standard-Eighth Edition*. CLSI document M11-A8: Wayne, PA: Clinical and Laboratory Standard Institute, 1–39.

Dang, B. N., and Graham, D. Y. (2017). Helicobacter pylori infection and antibiotic resistance: a WHO high priority? *Nat. Rev. Gastroenterol. Hepatol*. 14, 383–384. doi: 10.1038/nrgastro.2017.57

De, R., Sarkar, A., Ghosh, P., Ganguly, M., Karmakar, B. C., Saha, D. R., et al. (2018). Antimicrobial activity of ellagic acid against *Helicobacter pylori* isolates from India and during infections in mice. *J. Antimicrob. Chemother*. 73, 1595–1603. doi: 10.1093/jac/dky079

Floridata (2022). *A Floridata Plant Profile*. Floridata ID#: 652 *Asclepias curassavica*, Floridata.com - Edgewood, Kentucky/Tallahassee, Florida USA. Available online at: https://Floridata.com/planta/652 (accessed August 8, 2022).

Gonzalez, R., Albericio, F., Cascone, O., and Iannucci, N. B. (2010). Improved antimicrobial activity of h-lysozyme (107–115) by rational Ala substitution. *J. Pept. Sci*. 16, 424–429. doi: 10.1002/psc.1258

Hemadri Reddy, S., Chakravarthi, M., Chandrashekara, K. N., and Naidu, C. V. (2012). Phytochemical screening and antibacterial studies on leaf and root extracts of *Asclepias curassavica* (L). *IOSR J. Pharm. Biol. Sci*. 2, 2278–3008. doi: 10.9790/3008-0213944

Hembing, W. (2000). *Ensiklopedia milenium: Bunga-bungaan*. Jakarta: PRESTASI, 81–86.

Holmes, L., Rios, J., Berice, B., Benson, J., Bafford, N., Parson, K., Halloran, D. (2021). Predictive effect of *Helicobacter pylori* in gastric carcinoma development: systematic review and quantitative evidence synthesis. *Medicines (Basel)*. 8, 1. doi: 10.3390/medicines8010001

Jia, J., Zhang, C., Liu, Y., Huang, Y., Bai, Y., Hang, X., et al. (2022). Armeniaspirol A: a novel anti-*Helicobacter pylori* agent. *Microb. Biotechnol*. 15, 442–454. doi: 10.1111/1751-7915.13807

Kao, C. Y., Sheu, B. S., and Wu, J. J. (2016). *Helicobacter pylori* infection: an overview of bacterial virulence factors and pathogenesis. *Rev. Biomed. J*. 39, 14–23. doi: 10.1016/j.bj.2015.06.002

Kim, S. Y., Choi, D. J., and Chung, J. W. (2015). Antibiotic treatment for *Helicobacter pylori*: Is the end coming? *Rev. World J. Gastrointest. Pharmacol. Ther.* 6, 183–198. doi: 10.4292/wjgpt.v6.i4.183

Kjeldahl, J. (1883). Neue methode zur bestimmung des stickstoffs in organischen körpern. *Fresenius J. Anal. Chem.* 22, 366–382. doi: 10.1007/BF01338151

Kobatake, T., Ogino, K., Sakae, H., Gotoh, K., Watanabe, A., Matsushita, O., et al. (2021). Antibacterial effects of disulfiram in *Helicobacter pylori*. *Infect Drug Resist.* 14, 1757–1764. doi: 10.2147/IDR.S299177

Korona-Glowniak, I., Glowniak-Lipa, A., Ludwiczuk, A., Baj, T., and Malm, A. (2020). The in vitro activity of essential oils against *Helicobacter pylori* growth and urease activity. *Molecules.* 25, 586. doi: 10.3390/molecules25030586

Kurdekar, R. R., Hegde, G. R., and Hebbar, S. S. (2012). Antimicrobial efficacy of *Bridelia retusa* (Linn.) Spreng. and *Asclepias curassavica* Linn. *Indian J. Nat. Prod. Resour.* 3, 589–593.

Lee, C. C., Lin, Y. H., Chan, W. H., Wu, Y. C., and Chang, J. G. (2012). The small molecule calactin induces DNA damage and apoptosis in human leukemia cells. *Eur. J. Cancer Prev.* 5, 467–473. doi: 10.1097/CEJ.0b013e3283498e66

Lian, D., Xu, Y., Ren, W., Fu, L., Chen, F., Tang, L., et al. (2018). Unraveling the novel protective effect of patchouli alcohol against *Helicobacter pylori*-induced gastritis: insights into the molecular mechanism in vitro and in vivo. *Front. Pharmacol.* 9, 1347. doi: 10.3389/fphar.2018.01347

Lien, H. M., Wu, H. Y., Hung, C. L., Chen, C. J., Wu, C. L., Chen, K.-W., et al. (2019). Antibacterial activity of ovatodiolide isolated from *Anisomeles indica* against Helicobacter pylori. *Sci. Rep.* 9, 4205. doi: 10.1038/s41598-019-40735-y

Liggieri, C., Arribére, M. C., Trejo, S. A., Canals, F., and Avilés, F. X., Priolo, N. S., et al. (2004). Purification and biochemical characterization of asclepain cI from the latex of *Asclepias curassavica* L. *Protein J.* 23, 403–411. doi: 10.1023/B:JOPC.0000039554.18157.69

Liggieri, C., Obregon, W., Trejo, S., and Priolo, N. (2009). Biochemical analysis of a papain-like protease isolated from the latex of *Asclepias curassavica* L. *Acta Biochim. Biophys. Sin. (Shanghai).* 41, 154–162. doi: 10.1093/abbs/gmn018

Liu, Y., Yang, F., Wang, S., Chi, W., Ding, L., Liu, T., et al. (2022). HopE and HopD porin-mediated drug influx contributes to intrinsic antimicrobial susceptibility and inhibits streptomycin resistance acquisition by natural transformation in *Helicobacter pylori*. *Microbiol Spectr.* 10, e0198721. doi: 10.1128/spectrum.01987-21

Longhi, C., Scoarughi, G. L., Poggiali, F., Cellini, A., Carpentieri, A., Seganti, L., et al. (2008). Protease treatment affects both invasion ability and biofilm formation in *Listeria monocytogenes*. *Microb. Pathog.* 45, 45–52. doi: 10.1016/j.micpath.2008.01.007

Marcus, E. A., Sachs, G., and Scott, D. R. (2016). Eradication of *Helicobacter pylori* infection. *Curr. Gastroenterol. Rep.* 18, 33. doi: 10.1007/s11894-016-0509-x

Martínez, S., and Carballo, J. (2021). Physicochemical, sensory and nutritional properties of foods affected by processing and storage. *Foods.* 10, 2970. doi: 10.3390/foods10122970

Matsuo, Y., Kido, Y., and Yamaoka, Y. (2017). *Helicobacter pylori* outer membrane protein-related pathogenesis. *Toxins (Basel).* 9, 101. doi: 10.3390/toxins9030101

Miroslaw, F., and Surówka, K. (2004). Autoproteolysis rate of rainbow trout muscle proteins. *Nahrung.* 48, 104–109. doi: 10.1002/food.200300344

Molobela, P., Cloete, T. E., and Beukes, M. (2010). Protease and amylase enzymes for biofilm removal and degradation of extracellular polymeric substances (EPS) produced by Pseudomonas fluorescens bacteria. *Afr. J. Microbiol. Res.* 4, 1515–1524. Available online at: http://www.academicjournals.org/ajmr

Moon, S. H., Lee, J. H., Kim, K. T., Park, Y. S., Nah, S. Y., Ahn, D. U., et al. (2013). Antimicrobial effect of 7-O-butylnaringenin, a novel flavonoid, and various natural flavonoids against *Helicobacter pylori* strains. *Int. J. Environ. Res. Public Health.* 10, 5459–5469. doi: 10.3390/ijerph10115459

Morr, C. V. (1985). Composition, physicochemical and functional properties of reference whey protein concentrates. *J. Food Sci.* 50, 1406–1411. doi: 10.1111/j.1365-2621.1985.tb10488.x

Moulin-Traffort, J., Giordani, R., and Régli, P. (1990). Antifungal action of latex saps from *Lactuca sativa* L. and *Asclepias curassavica* L. *Mycoses.* 33, 383–392. doi: 10.1111/myc.1990.33.7-8.383

Nagata, R., Ohsumi, T., Takenaka, S., and Noiri, Y. (2020). Current prevalence of oral *Helicobacter pylori* among japanese adults determined using a nested polymerase chain reaction assay. *Pathogens.* 10, 10. doi: 10.3390/pathogens10010010

Nakano, D., Ishitsuka, K., Takashima, M., Arima, R., Satou, A., Tsuchihashi, R., et al. (2020). Screening of promising chemotherapeutic candidates from plants against human adult T-cell leukemia/Lymphoma (VI): Cardenolides from *Asclepias curassavica*. *Biol. Pharm. Bull.* 43, 1609–1614. doi: 10.1248/bpb.b20-00465

National Institutes of Health (NIH) (1980). *Guide for the Care and Use of Laboratory Animals*. DHEW Publication (NIH) 80-23. Bethesda, MD: Office of Science and Health Reports, National Institutes of Health.

Neto, C. C., Owens, C. W., Langfield, R. D., Comeau, A. B., St Onge, J., Vaisberg, A. J., et al. (2002). Antibacterial activity of some Peruvian medicinal plants from the Callejón de Huaylas. *J. Ethnopharmacol.* 79, 133–138. doi: 10.1016/S0378-8741(01)00398-1

Ngan, L. T. M., Tan, M. T., Hoang, N. V. M., Thanh, D. T., Linh, N. T. T., Hoa, T. T. H., et al. (2021). Antibacterial activity of *Hibiscus rosa-sinensis* L. red flower against antibiotic-resistant strains of *Helicobacter pylori* and identification of the flower constituents. *Braz J Med Biol Res.* 54, e10889. doi: 10.1590/1414-431x2020e10889

Ogasawara, K., Nakajima, S., Sato, H., and Sasaki, T. (2020). *Helicobacter pylori* eradication using laser endoscope and methylene blue. *Laser Ther.* 29, 19–27. doi: 10.5978/islsm.20-OR-02

Olivera-Severo, D., Uberti, A. F., Marques, M. S., Pinto, M. T., Gomez-Lazaro, M., Figueiredo, C., et al. (2017). New role for *Helicobacter pylori* urease: contributions to angiogenesis. *Front Microbiol.* 8, 1883. doi: 10.3389/fmicb.2017.01883

Oliver-Bever, B. (1986). *Medicinal Plants in Tropical West Africa*. Cambridge, London: Cambridge University Press. 23.

Pardo, M., Bruno, M., Sequeiros, C., Trejo, S., López, L., Caffini, N., et al. (2010). New plant endopeptidases with potential application in cheesemaking. *Acta Aliment.* 39, 211–221. doi: 10.1556/AAlim.39.2010.2.12

Parreira, P., Duarte, M. F., Reis, C. A., and Martins, M. C. L. (2016). *Helicobacter pylori* infection: a brief overview on alternative natural treatments to conventional therapy. *Crit. Rev. Microbiol.* 42, 94–105. doi: 10.3109/1040841X.2014.892055

Pearce, K. N., and Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a turbidimetric technique. *J. Agric. Food Chem.* 26, 716–723. doi: 10.1021/jf60217a041

Piva, M., Pinnavaia, G., and Lerici, C. R. (1981). "Idratazione," in: *Propietá Funzionali delle Protein*, ed Consiglio Nazionale delle Ricerche (Italy: Consiglio Nazionale delle Ricerche Ed.), 34–48.

Qamar, H., Rehman, S., and Chauhan, D. K. (2019). Current status and future perspective for research on medicinal plants with anticancerous activity and minimum cytotoxic value. *Curr. Drug Targets.* 20, 1227–1243. doi: 10.2174/1389450120666190429120314

Roszczenko-Jasińska, P., Wojtyś, M. I., and Jagusztyn-Krynicka, E. K. (2020). Helicobacter pylori treatment in the post-antibiotics era searching for new drug targets. *Appl. Microbiol. Biotechnol.* 104, 9891–9905. doi: 10.1007/s00253-020-10945-w

Salinas Ibáñez, A. G., Arismendi Sosa, A. C., Ferramola, F. F., Paredes, J., Wendel, G., María, A. O., et al. (2017). Inhibition of *Helicobacter pylori* and its associated urease by two regional plants of San Luis Argentina. *Int. J. Curr. Microbiol. App. Sci.* 6, 2097–2106. doi: 10.20546/ijcmas.2017.609.258

Salinas Ibáñez, Á. G., Vallés, D., Adaro, M., Barberis, S., and Vega, A. E. (2021). Antimicrobial effect of a proteolytic enzyme from the fruits of *Solanum granuloso-leprosum* (Dunal) against *Helicobacter pylori*. *Front Nutr.* 8, 699955. doi: 10.3389/fnut.2021.699955

Sato, Y., Yamakose, H., and Suzuki, Y. (1993). Mechanism of hypotonic hemolysis of human erythrocytes. *Biol. Pharm. Bull.* 16, 506–512. doi: 10.1248/bpb.16.506

Shen, X., Zhang, W., Peng, C., Yan, J., Chen, P., Jiang, C., et al. (2021). *In vitro* anti-bacterial activity and network pharmacology analysis of *Sanguisorba officinalis* L. against *Helicobacter pylori* infection. *Chin Med.* 16, 33. doi: 10.1186/s13020-021-00442-1

Shivaprasad, H. V., Rajesh, R., Nanda, B. L., Dharmappa, K. K., and Vishwanath, B. S. (2009a). Thrombin like activity of *Asclepias curassavica* L. latex: action of cysteine proteases. *J. Ethnopharmacol.* 123, 106–109. doi: 10.1016/j.jep.2009.02.016

Shivaprasad, H. V., Riyaz, M., Venkatesh Kumar, R., Dharmappa, K. K., Tarannum, S., Siddesha, J. M., et al. (2009b). Cysteine proteases from the Asclepiadaceae plants latex exhibited thrombin and plasmin like activities. *J. Thromb. Thrombolysis.* 3, 304–308. doi: 10.1007/s11239-008-0290-2

Šterbenc, A., Jarc, E., Poljak, M., and Homan, M. (2019). *Helicobacter pylori* virulence genes. *World J. Gastroenterol.* 25, 4870–4884. doi: 10.3748/wjg.v25.i33.4870

Tang, C.-H., Ten, Z., Wang, X.-Sh., and Yang, X.-Q. (2006). Physicochemical and functional properties of hemp (*Cannabis sativa* L.) protein isolate. *J. Agric. Food Chem.* 54, 8945–8950. doi: 10.1021/jf0619176

Vallés, D., and Cantera, A. M. B. (2018). Antiacanthain A: new proteases isolated from *Bromelia antiacantha* Bertol. (Bromeliaceae). *Int. J. Biol. Macromol.* 113, 916–923. doi: 10.1016/j.ijbiomac.2018.03.025

Woo, H. J., Yang, J. Y., Lee, P., Kim, J.-B., and Kim, S.-H. (2021). Zerumbone inhibits *Helicobacter pylori* urease activity. *Molecules*. 26, 2663. doi: 10.3390/molecules26092663

Yuan, W. Q., Zhang, R. R., Wang, J., Ma, Y., Li, W. X., Jiang, R. W., et al. (2016). Asclepiasterol, a novel C21 steroidal glycoside derived from *Asclepias curassavica*, reverses tumor multidrug resistance by down-regulating P-glycoprotein expression. *Oncotarget*. 7, 31466–31483. doi: 10.18632/oncotarget.8965

Zhang, L., Santos, J. S., Cruz, T. M., Marques, M. B., do Carmo, M. A. V., Azevedo, L., et al. (2019). Multivariate effects of Chinese keemun black tea grades (*Camellia sinensis* var sinensis) on the phenolic composition, antioxidant, antihemolytic and cytotoxic/cytoprotection activities. *Food Res. Int*. 125, 108516. doi: 10.1016/j.foodres.2019.108516

Zhao, H., Wu, Y., Xu, Z., Ma, R., Ding, Y., Bai, X., et al. (2019). Mechanistic insight into the interaction between *Helicobacter pylori* urease subunit α and its molecular chaperone Hsp60. *Front. Microbiol*. 10, 1–11. doi: 10.3389/fmicb.2019.00153

Zheng, X., Xu, Y., Liu, B., Qi, Y., Zhang, Y., Li, X., et al. Wan, C. (2019). Ethyl acetate extract of Asclepias curassavica induced apoptosis in human cancer cells via activating p38 and JNK MAPK signaling pathways. *Evid. Based Complement Alternat. Med.* 2019, 9076269. doi: 10.1155/2019/9076269

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# Revealing the novel effect of Jinghua Weikang capsule against the antibiotic resistance of *Helicobacter pylori*

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**Background:** *Helicobacter pylori* (*H. pylori*) infects half of the human population globally. Eradication rates with triple or quadruple therapy have decreased owing to the increasing rate of antibiotic resistance. Jinghua Weikang capsule (JWC) is the first and most popular Chinese patent medicine approved by the state for the treatment of gastritis and peptic ulcers caused by *H. pylori* infection in China. Previous studies have found that JWC has a certain bactericidal effect on drug-resistant *H. pylori* and its major component, *Chenopodium ambrosioides* L. inhibits biofilm formation, but the mechanism remains unclear. This study focused on drug-resistant *H. pylori* and explored whether JWC could reverse drug resistance and its related mechanisms.

**Method:** The agar plate dilution method, E-test method, and killing kinetics assay were used to evaluate the bactericidal effect of JWC on antibiotic-resistant *H. pylori* and its effect on antibiotic resistance. Sanger sequencing was used to detect mutations in drug resistance genes. The crystal violet method, scanning electron microscopy, and confocal laser scanning microscopy were used to evaluate the effects of JWC on biofilms. qPCR was performed to evaluate the effect of JWC on the expression of efflux pump-related genes. qPCR and immunofluorescence were used to evaluate the effects of JWC on *H. pylori* adhesion.

**Results:** JWC showed considerable antibacterial activity against drug-resistant *H. pylori* strains, with minimum inhibitory concentration (MIC) values ranging from 64 to 1,024μg/ml. The MIC of metronidazole (MTZ) against *H. pylori* 26,695–16R decreased from 64 to 6μg/ml after treatment with 1/2 MIC of JWC. The resistance of *H. pylori* 26,695–16R to MTZ was reversed by JWC, and its effect was better than that of PaβN and CCCP. *H. pylori* 26,695–16R is a moderate biofilm-forming strain, and JWC (16–64μg/ml) can inhibit the formation of biofilms in *H. pylori* 26,695–16R. JWC reduced the expression of HP0605-HP0607 (*hefABC*), HP0971-HP0969 (*hefDEF*), HP1327-HP1329 (*hefGHI*), and HP1489-HP1487. JWC reduced the adhesion of *H. pylori* to GES-1 cells and the expression of adhesives *NapA*, *SabA*, and *BabA*.

**Conclusion:** The reversal of MTZ resistance by JWC may be achieved through the adhesin/efflux pump-biofilm pathway.

KEYWORDS

*Helicobacter pylori*, antibiotic resistance, metronidazole, biofilm, efflux pump, adhesion, Jinghua Weikang capsule

## Introduction

*Helicobacter pylori* (*H. pylori*) infects half of the global population and can cause a variety of gastric diseases, such as peptic ulcers, chronic gastritis, gastric cancer, and extragastric diseases (Zamani et al., 2018; Ren et al., 2022). The eradication of *H. pylori* has significantly reduced the incidence and mortality rates of gastric cancer (Chiang et al., 2021). However, large-scale eradication has led to increasing rates of *H. pylori* resistance to multiple antibiotics, the main cause of eradication failure (Zhong et al., 2022). The eradication rate of triple therapy is currently less than 70% (Liu et al., 2018). Since 2017, *H. pylori* has been listed by the World Health Organization as one of the 20 pathogens that pose the most serious threat to human health owing to its drug resistance (Tacconelli et al., 2018). It is difficult to reduce the resistance rate. The causes and mechanisms of antibiotic resistance are complicated and include specific resistance factors against a particular antibiotic (resistance gene mutation; Tshibangu-Kabamba and Yamaoka, 2021), as well as nonspecific resistance factors, such as biofilms and efflux pumps (Zanotti and Cendron, 2019). Bacterial biofilms are complexes composed of bacteria and extracellular polymers (EPS) such as proteins, polysaccharides, lipids, and DNA secreted by bacteria, which create a protective environment for bacteria (Høiby et al., 2010; Rather et al., 2021). Bacteria that form biofilm structures are highly resistant to harsh external environments such as antibiotic exposure. It has been demonstrated that bacteria are 10–1,000 times more resistant to antibiotics when they form biofilms (Chen and Wen, 2011; Yonezawa et al., 2019; Hou et al., 2022). The formation of *H. pylori* biofilms includes four steps: adhesion, growth, maturation, and diffusion (Hou et al., 2022). Adhesion is the first step and a prerequisite for biofilm formation. The adhesion of *H. pylori* is mediated by dozens of specific adhesin receptors, among which blood group antigen-binding adhesin (*BabA*), sialic acid adhesin (*SabA*), and neutrophil-activating protein A (*NapA*) play major roles (Fu, 2014; Doohan et al., 2021; Matos et al., 2021). The bacterial efflux pump is a transmembrane transporter protein that mediates the pumping of intracellular drugs out of the cell, thereby reducing the intracellular drug concentration and promoting drug resistance. The efflux effect of the active efflux pump system in bacteria is an important mechanism underlying nonspecific drug resistance. The resistance nodulation and cell division (RND) family, major facilitator super (MFS) family, and ATP-binding cassette (ABC) family are the predominant efflux pump families in *H. pylori*. Several studies have shown that the efflux pump expression in biofilm-forming bacteria is higher than that in planktonic cells (Soto, 2013). The expression levels of HP0605–HP0607 (*hefABC*), HP0971-HP0969 (*hefDEF*), HP1327–HP1329 (*hefGHI*), and HP1489–HP1487 in biofilm-forming strains are higher than those in planktonic bacteria (Yonezawa et al., 2019). The expression level of Hp1174 [glucose/galactose transporter (*gluP*)] also follows these rules (Ge et al., 2018). This suggests that efflux pumps and biofilms may interact or act synergistically to increase drug resistance.

As an exogenous pathogenic factor, *H. pylori* is equivalent to "evil *qi*" in traditional Chinese medicine (TCM). According to TCM, *H. pylori* infection mostly presents with basic symptoms of cold and heat in complexity and deficiency in complexity. Jinghua Weikang capsules (JWC) were obtained from *Chenopodium ambrosioides L.* (CAL) and Rubiaceae adina pilulifera (RAP). CAL regulates *qi*, disperses cold, and kills insects, while RAP clears heat and removes blood stasis. The two are used in combination to harmonize the spleen and stomach. It is widely used in digestive diseases related to *H. pylori* infection and has a good basis for application and clinical efficacy (Hui and Xuezhi, 2014). Previous studies have found that JWC and its main component, CAL, can kill and inhibit standard drug-resistant *H. pylori*, and CAL can inhibit the formation of drug-resistant *H. pylori* biofilms (Liu et al., 2013; Ye et al., 2015; Enen et al., 2020). In the remedial treatment of patients with chronic gastritis or peptic ulcer suffering from *H. pylori* infection relapses, the addition of JWC improved the eradication rate of *H. pylori* compared to bismuth quadruple therapy (90.0 *vs.* 82.0%; Hong et al., 2016). Antibiotic resistance is the main reason for *H. pylori* eradication failure. JWC can contribute toward improving the eradication rate of *H. pylori* in remedial treatment, however, its mechanism is still unknown. This study explores whether JWC can reverse the drug resistance and related mechanisms in *H. pylori*.

## Materials and methods

### Drug preparation

The volatile oil of JWC (Tasly Pharmaceutical Group Co. LTD, Tianjin, China) was mixed with dimethyl sulfoxide (DMSO, Thermo Fisher Scientific, Waltham, MA, United States) at a 1:4 volume ratio and dissolved in sterile deionized water. The density of JWC was 937 mg/ml.

### Bacterial culture and identification

The *H. pylori* strains used in this experiment (Table 1) were all obtained from the Department of Gastroenterology, Peking University First Hospital, among which *H. pylori* Strains1–6 were

TABLE 1 The primers used in Sanger sequencing.

| Primer name | Sequence (5′→3′) | References |
|---|---|---|
| *rdxA* fwd | ATGGTAATTGTTTCGTTAGGG | Teh et al. (2014) |
| *rdxA* rev | CTCCTTGAACTTTAATTTAG | Teh et al. (2014) |
| *frxA* fwd | TGGATATGGCAGCCGTTTA | Teh et al. (2014) |
| *frxA* rev | GGTTATCAAAAAGCTAACAGCG | Teh et al. (2014) |
| 23S rRNA fwd | CCACAGCGATGTGGTCTCAG | Teh et al. (2014) |
| 23S rRNA rev | CTCCATAAGA GCCAAAGCCC | Teh et al. (2014) |
| *gyrA* fwd | AGCTTATTCCATGAGCGTGA | Teh et al. (2014) |
| *gyrA* rev | TCAGGCCCTTTGACAA ATTC | Teh et al. (2014) |
| *gyrB* fwd | CCCTAACGAAGCCAAAATCA | Teh et al. (2014) |
| *gyrB* rev | GGGCGCAAATAACG ATAGAA | Teh et al. (2014) |

isolated from clinical patients who had previously failed *H. pylori* eradication therapy and required drug sensitivity test to guide the eradication regimen and *H. pylori* 26,695–16R is an *rdxA* null deletion mutant derivative of *H. pylori* 26,695 (Sisson et al., 2000). *H. pylori* strains were frozen in −80°C refrigerator. The cryopreservation solution was prepared by brain heart infusion (OXOID, Basingstoke, United Kingdom) and glycerol (Solarbio, Beijing, China). *H. pylori* was inoculated on Columbia blood agar (OXOID, Basingstoke, United Kingdom) plates containing 8% sheep blood (Lablead, Beijing, China), placed upside down at 37°C in a microaerophilic (85% $N_2$, 10% $CO_2$, 5% $O_2$) environment for 48–72 h, and the positive ones were sub-cultured, generally no more than seven generations. The strains were identified by colony morphology, Gram staining, and rapid urease tests.

## Drug susceptibility test

### E-test method

*Helicobacter pylori* cultured for 48–72 h was uniformly ground into a cryopreservation solution, and the bacterial solution was diluted to $3 \times 10^8$ CFU/ml. One hundred microliters of bacterial solution was pipetted onto the surface of the medium, smeared evenly with L sticks, placed on an E-test drug susceptibility test strip (Liofilchem, Roseto degli Abruzzi, Italy), and incubated at 37°C for 72 h in a microaerophilic environment to read the results. The value corresponding to the ring region where the bacteria stops growing is the minimum inhibitory concentration (MIC) of an antibiotic for *H. pylori*. According to EUCAST Clinical Breakpoint standard 2022, *H. pylori* strains that could grow in medium containing amoxicillin (MIC > 0.125 μg/ml), levofloxacin (MIC > 1 μg/ml), clarithromycin (MIC > 0.5 μg/ml), and metronidazole (MIC > 8 μg/ml) were identified as drug-resistant strains. Phenylalanine-arginine β-naphthylamide (PaβN, Sigma-Aldrich, St. Louis, MO, United States) and carbonyl cyanide m-chlorophenylhydrazonequinoline (CCCP, Sigma-Aldrich, St. Louis, MO, United States) are the most common inhibitors of efflux pumps and have also been found to inhibit biofilm formation (Zhang et al., 2010; Kinana et al., 2016; Tang et al., 2020; Dawan et al., 2022).

### Agar plate dilution method

Media containing different concentrations of JWC (2048, 1,024, 512, 256, 128, 64, 32, and 16 μg/ml) were prepared. Drug-free and DMSO (Sigma-Aldrich, St. Louis, MO, United States) -containing media were used as controls. *H. pylori* cultured for 48–72 h was uniformly ground into the cryopreservation solution, and the bacterial solution was diluted to $3 \times 10^8$ cfu/ml. A 1 μl sterile inoculating ring was used to inoculate the bacterial solution on the surface of the drug-containing medium, and observed after 72 h of culture in a microaerobic environment at 37°C. The MIC of the lowest drug concentration on a medium without colonies was the MIC for JWC.

## Inhibiting kinetics and killing kinetics assay

The inhibition and killing kinetics assays were performed as previously reported (Shen et al., 2021; Peng et al., 2022). For the inhibition kinetics assay, *H. pylori* 26,695–16R cultured for 48–72 h was collected at 0 (control), 0.25, 0.5, and 1 times the MIC concentration for JWC in Brucella Broth (BD, Franklin Lakes, NJ, United States) containing 10% Foetal Bovine Serum (FBS, BI, Kibbutz Beit Haemek, Israel) and shaken (100–120 rpm) at 37°C. Then, at 0, 12, 24, 36, 46, 48, 60, and 72 h, 100 μl of each sample was tested for absorbance at 600 nm. Three holes were set in each sample, and the experiment was repeated three times. For the killing kinetics assay, *H. pylori* 26,695–16R cultured for 48–72 h was collected at 0 (control), 1, 2, and 4 times the MIC concentration of JWC in Brucella broth containing 10% FBS and treated in a shaker (100–120 rpm) at 37°C. At 0, 4, 8, 12, and 24 h, 30 μl was removed from each sample, and serial 10-fold dilutions were prepared in Brucella broth. One hundred microliters of the diluted solution were plated on Columbia blood agar plates and incubated at 37°C, and colonies were counted and averaged after 3 days. The results are expressed as $Log_{10}$ (CFU/ml). This experiment was repeated twice.

## Sanger sequencing

Bacterial genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), and the samples were stored at −80°C. The *23S rRNA*, *gyrA* (hp0701), *gyrB* (hp0501), *rdxA* (hp0954), and *frxA* (hp0642) fragments were amplified using PCR. Primers used are listed in Table 1 (Teh et al., 2014). PCR amplification products were examined on 1.0% agarose gels and bands were observed. The PCR product was separated and purified using magnetic beads (Ensure Biologicals, Shanghai, China). The PCR product sequencing was performed by Beijing Liuhe Bgi Co. Ltd. (Beijing, China).

## Quantitative real-time PCR

*Helicobacter pylori* cultured for 48–72 h was collected in Brucella broth containing different concentrations of drugs and shaken (100–120 rpm) at 37°C for 2 h. Total RNA of *H. pylori* was extracted using the TRIzol method, mRNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, United States), and PCR amplification was carried out using PowerUp™ SYBR™Green Master Mix (Thermo Fisher Scientific, Waltham, MA, United States). Primers used in this experiment are listed in Table 2 (Yonezawa et al., 2019; Zhong et al., 2021).

TABLE 2 Primers sequences for qPCR.

| Primer name | Sequence (5′→3′) | References |
|---|---|---|
| 16S rRNA fwd | GGGTGAGTAACGCATAGGTCA | Designed for this study |
| 16S rRNA rev | TTTACGCCCAGTGATTCCGA | Designed for this study |
| HP0605 fwd | AGCGCAAGAACTCAGTGTCA | Zhong et al. (2021) |
| HP0605 rev | GCTTGGAGTTGTTGGGTGTT | Zhong et al. (2021) |
| HP0971 fwd | TTACCGGCAAAGGGATACG | Yonezawa et al. (2019) |
| HP0971 rev | AAATTGGATCGCTCGTTGTATG | Yonezawa et al. (2019) |
| HP1327 fwd | GCCAGGCTTGATGAAGAAAA | Yonezawa et al. (2019) |
| HP1327 rev | TTAGCCTGCTTGCCGTAAAT | Yonezawa et al. (2019) |
| HP1489 fwd | TAGGCGCTCAAGTGGCTTAT | Yonezawa et al. (2019) |
| HP1489 rev | TCAGATCGGGCAGATTTTTC | Yonezawa et al. (2019) |
| *BabA* fwd | CCCGCGCTCAAAGAAAACAA | Designed for this study |
| *BabA* rev | GTGGTGGTTACGGTTTTGCC | Designed for this study |
| *SabA* fwd | TCGTCATCAGTGGCGTTTCA | Designed for this study |
| *SabA* rev | TCCCTGTAGCTTGAGCTTGC | Designed for this study |
| *NapA* fwd | TTGGAATGTGAAAGGCACCGATTTT | Designed for this study |
| *NapA* rev | GCCTTCTTTTTCAGCGGTGTTAGA | Designed for this study |

## Crystal violet staining

The biofilm of *H. pylori* was cultured using the 96-well plate method. *H. pylori* cultured for 48–72 h was collected in Brucella broth containing different concentrations of drugs and treated in a microaerobic environment at 37°C for 72 h. Each group was provided with nine holes. After incubation, the upper bacterial solution was gently discarded, the plate was rinsed three times with Phosphate Buffer 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 for 15 min, the methanol was discarded, and the plate was air-dried. Then, 200 μl of 1% ammonium oxalate crystal violet reagent (Solarbio, Beijing, China) was added to each well for staining for 5 min and then washed with running water. After natural drying, 200 μl 95% ethanol (Beijing Tongguang Fine Chemical, Beijing, China) was added to each well and dissolved in a shaker (80 rpm) at 37°C for 30 min. The Optical Density (OD) value was measured using a microplate reader (TECAN, Männedorf, Switzerland; absorbance at 590 nm). Dc is the OD value of the blank wells and D is the mean value of the remaining OD values after removing the outliers. A value of $D > 4 \times Dc$, was determined to be a strong biofilm-forming *H. pylori* strain; $2 \times Dc < D \leq 4 \times Dc$, a moderate biofilm-forming *H. pylori* strain; $Dc < D \leq 2 \times Dc$, a weak biofilm-forming *H. pylori* strain; and $D \leq Dc$, a non-biofilm-forming *H. pylori* strain.

## Scanning electron microscope

The nitrocellulose (NC) membrane (GE, Boston, Mass, United States) was cut into 1 × 1 cm pieces to prepare a solid medium containing NC membranes and different concentrations of drugs. *H. pylori* cultured for 48–72 h was uniformly ground into the cryopreservation solution, and the bacterial solution was diluted to $3 \times 10^8$ cfu/ml. A total of 10 μl of bacterial solution was pipetted onto the NC membrane, spread evenly, and incubated in a microaerophilic environment at 37°C for 72 h. The NC membrane was then removed and placed in a 6-well plate, and an appropriate amount of glutaraldehyde (Regen Biotechnology Co., LTD, Beijing, China) was added to fix it at 4°C for 2 h. After fixation, the membrane was air-dried and the results were observed using a field emission scanning electron microscope (JEOL, Tokyo, Japan). Metronidazole used in this study was obtained from Aladdin, Shanghai, China.

## Confocal laser scanning microscope

A LIVE/DEAD™ *Bac*Light™ Bacterial Cell Activity Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States) was used for fluorescence staining. Dye A (SYTO™ 9 dye) penetrates the bacterial cell membrane and binds to DNA to stain bacteria green. Dye B (propidium iodide) only penetrates incomplete bacterial cell membranes; when the bacteria die, the permeability of the cell membrane changes, and dye B dyes the dead bacteria red. Glycerin was used for microscopic observations.

The biofilm was constructed as described above, and the incubated NC membrane was removed aseptically, placed in a 24-well plate and rinsed 3 times in PBS. Mix 3 μl A and B 1:1, add 1 ml normal saline, add 100 μl to each well, and incubate in the dark for 15 min. The NC membrane was removed and placed on a glass slide, and glycerol was added for observation under a microscope. The samples were observed within 1 h to avoid the effects of bacterial death caused by prolonged exposure. CLSM (Leica, Wetzlar, Germany) was performed using an argon laser at 488 nm excitation, with the blue channel receiving the green signal and the 560 nm green channel receiving the red signal, and scanning from the free side of the *H. pylori* biofilm to the attached side of the slide layer-by-layer at an interval of 1 μm.

## Immunofluorescent staining

GES-1 cells ($1 \times 10^5$) were infected with *H. pylori* (multiplicity of infection [MOI] = 200:1) for 2 h. The drug group was then pre-treated for 2 h. After 2 h, the culture was aspirated, 4% paraformaldehyde (Regen Biotechnology, Beijing, China) was added, incubated at room temperature for 20 min, paraformaldehyde was discarded, and the cells were washed thrice with PBST [PBS + 0.05% Tween 20 (Solarbio, Beijing, China)] and maintained for 5 min each time. After washing, PBST containing 0.1% Triton (Solarbio, Beijing, China) was added for 20 min to permeabilize the cells. Next, blocking solution [TBST containing 5% BSA (Lablead, Beijing, China)] was added for 30 min at room temperature, and the blocking solution was removed and cleaned twice with PBST. Then, the primary anti-Helicobacter pylori antibody (ab20459, Abcam, Cambridge, United Kingdom) was added and incubated in a wet box overnight at 4°C. The cells were washed three times with PBST and Alexa Fluor® 488-labeled goat anti-rabbit IgG secondary antibody (Zhongshan Jinqiao Biotechnology, Beijing, China), incubated at room temperature, and protected from light for 1 h. After 1 h, the secondary antibody was aspirated, washed three times with PBST, and stained with mounting medium containing DAPI (Zhongshan Jinqiao Biotechnology, Beijing, China) for 5 min. The DAPI and fluorescein isothiocyanate (FITC) channels were selected under a fluorescence microscope (Nikon, Tokyo, Japan) and photographed for analysis. The results were expressed as *H. pylori* fluorescence area/DAPI fluorescence area calculated using ImageJ software.

## Statistical analysis

Data are presented as mean ± standard deviation. Differences between groups were assessed using one-way ANOVA. Pairwise comparisons were performed using Dunnett's or Tukey's *post hoc* test. $p$ was set at $p < 0.05$. Statistical analysis was performed using the GraphPad Prism 8.1 software.

# Results

## Screening of drug-resistant *Helicobacter pylori* strains by E-test

According to EUCAST Clinical Breakpoint standard 2022, *H. pylori* strains with AML MIC > 0.125 μg/ml, CLR MIC > 0.5 μg/ml, LEV MIC > 1 μg/ml, and MTZ MIC > 8 μg/ml were antibiotic resistant. Seven drug-resistant *H. pylori* strains were screened, including six multidrug-resistant strains (Nos. 1–6) and one single drug-resistant strain (26695–16R). There were five CLR-resistant strains (Nos. 1, 2, 4, 5, and 6), five LEV-resistant strains (Nos. 1, 2, 3, 4, 6, and 26,695–16R), six MTZ-resistant strains (Nos. 1, 2, 3, 4, 6, and 26,695–16R), and one AML-resistant strain (No. 1). The MICs of the antibiotics against *H. pylori* are listed in Table 3.

## *In vitro* antibacterial activities of JWC on *Helicobacter pylori*

The MICs of JWC against the drug-resistant strains were determined using the agar dilution method. The results showed that JWC had considerable antibacterial activity against drug-resistant *H. pylori* strains, with MIC values ranging from 64 to 1,024 μg/ml (Table 4), suggesting that there were differences in the antibacterial activity against different strains.

## The MICs of antibiotics after JWC intervention

JWC at ½ MIC was used to inhibit drug-resistant *H. pylori* strains. Based on previous studies (Hirata et al., 2010; Tsugawa et al., 2011), the concentration of PaβN used in this study was 20 μg/ml, which had no inhibitory effect on *H. pylori* 26,695–16R growth (Supplementary Table S1). Owing to the toxicity of CCCP, which had an obvious inhibitory effect on *H. pylori* 26,695–16R growth, its MIC against *H. pylori* 26,695–16R was determined to be 5 μg/ml using the agar dilution method (Supplementary Table S2); to exclude the bactericidal effect of CCCP itself, we used a concentration of 1 μg/ml for the test (Supplementary Figure S1). The

TABLE 3 Minimum inhibitory concentration (MIC) of antibiotics against *H. pylori*.

| *H. pylori* strains | MIC of antibiotics against *H. pylori* (μg/ml) | | | |
|---|---|---|---|---|
| | AML | CLR | LEV | MTZ |
| 1 | 0.19* | 32* | 32* | 256* |
| 2 | 0.023 | 24* | 32* | 192* |
| 3 | 0.016 | 0.016 | 32* | 256* |
| 4 | 0.016 | 32* | 32* | 256* |
| 5 | 0.016 | 4* | 32* | 1.5 |
| 6 | 0.016 | 12* | 0.25 | 256* |
| 26,695–16R | 0.016 | 0.016 | 0.25 | 64* |

*Antibiotic resistance. MIC, minimum inhibitory concentration; AML, amoxicillin; CLR, clarithromycin; LEV, levofloxacin; MTZ, metronidazole.

TABLE 4 MIC of JWC against *H. pylori*.

| *H. pylori* strains | Concentration of JWC (μg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | DMSO | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1,024 | 2,048 |
| 1 | + | + | + | + | + | + | + | − | − | − | − | − |
| 2 | + | + | + | + | + | + | + | − | − | − | − | − |
| 3 | + | + | + | + | + | + | + | − | − | − | − | − |
| 4 | + | + | + | + | + | + | − | − | − | − | − | − |
| 5 | + | + | + | + | + | + | + | + | + | + | − | − |
| 6 | + | + | + | + | + | + | − | − | − | − | − | − |
| 26,695–16R | + | + | + | + | + | + | − | − | − | − | − | − |

MIC, minimum inhibitory concentration; JWC, Jinghua Weikang capsule; +, existing colonies; −, no colony growth.

results showed that JWC and efflux pump inhibitors PaβN (20 μg/ml) and CCCP (1 μg/ml) had no effect on the MICs of LEV-resistant strains but had a slight effect on the MICs of CLA-resistant strains. However, the MIC of MTZ against *H. pylori* 26,695–16R decreased from 64 μg/ml to 6 μg/ml after treatment with ½ MIC of JWC. The drug resistance of *H. pylori* 26,695–16R to MTZ was reversed, and its effect was better than that of efflux pump inhibitors PaβN (20 μg/ml) and CCCP (1 μg/ml; Tables 5–7).

In this study, JWC had different MICs for different drug-resistant *H. pylori* strains and showed a unique effect on reducing MTZ resistance in *H. pylori* 26,695–16R, suggesting that its effect on reducing drug resistance may also be affected by bacterial characteristics. To further explore the possible mechanism based on the confirmed effect, *H. pylori* 26,695–16R was selected as the research object in subsequent experiments.

## Inhibiting kinetics and killing kinetics assay

The kinetics of the inhibition and killing of *H. pylori* 26,695–16R by JWC were time-and dose-dependent (Figure 1). JWC inhibited the growth of *H. pylori* 26,695–16R at concentrations as low as 16 μg/ml (1/4 MIC). The $OD_{600}$ of the bacterial solution did not increase significantly after treatment with 32 μg/ml (1/2 MIC) and 64 μg/ml (MIC) concentrations of JWC (Figure 1A). JWC killed *H. pylori* 26,695–16R at 64–256 μg/ml (MIC to 4 MIC), which indicated a 1,000-fold reduction in the number of bacteria compared with the initial inoculation. JWC at 64–256 μg/ml (MIC to 4 MIC) completely killed *H. pylori* 26,695–16R after 8–24 h of intervention (Figure 1B).

## Detection of drug resistance-related genes in *Helicobacter pylori* 26,695–16R

Gene sequencing results showed that only the G210T point mutation occurred in the MTZ resistance-related gene *rdxA* among several genes detected in *H. pylori* 26,695–16R, and intervention with the efflux pump inhibitors PaβN (20 μg/ml), CCCP (1 μg/ml), and JWC did not affect the mutation, as shown by the red ellipse in Figure 2. Primers used in these experiments are listed in Table 1.

TABLE 5 Effects of JWC on MIC (μg/ml) of LEV resistant strains.

| *H. pylori* strains | Con | PaβN | CCCP | JWC |
|---|---|---|---|---|
| 1 | 32 | 32 | 32 | 32 |
| 2 | 32 | 32 | 32 | 32 |
| 3 | 32 | 32 | 32 | 32 |
| 4 | 32 | 32 | 32 | 32 |
| 5 | 32 | 32 | 32 | 32 |

TABLE 6 Effects of JWC on MIC (μg/ml) of CLA resistant strains.

| *H. pylori* strains | Con | PaβN | CCCP | JWC |
|---|---|---|---|---|
| 1 | 32 | 32 | 32 | 32 |
| 2 | 24 | 24 | 24 | 24 |
| 4 | 32 | 32 | 32 | 32 |
| 5 | 4 | 2 | 4 | 4 |
| 6 | 12 | 4 | 8 | 8 |

TABLE 7 Effects of JWC on MIC (μg/ml) of MTZ resistant strains.

| *H. pylori* strains | Con | PaβN | CCCP | JWC |
|---|---|---|---|---|
| 1 | 256 | 256 | 256 | 256 |
| 2 | 192 | 192 | 192 | 192 |
| 3 | 256 | 256 | 256 | 256 |
| 4 | 256 | 256 | 256 | 256 |
| 6 | 256 | 256 | 256 | 256 |
| **26,695–16R** | **64** | **48** | **24** | **6** |

MIC, minimum inhibitory concentration; JWC, Jinghua Weikang Capsule; CLR, clarithromycin; LEV, levofloxacin; MTZ, metronidazole.

## Effect of JWC on the biofilm of *Helicobacter pylori* 26,695–16R by crystal violet method

When D/Dc > 4, it was determined to be a strong biofilm-forming strain; 2 < D/Dc ≤ 4, a moderate biofilm-forming strain;

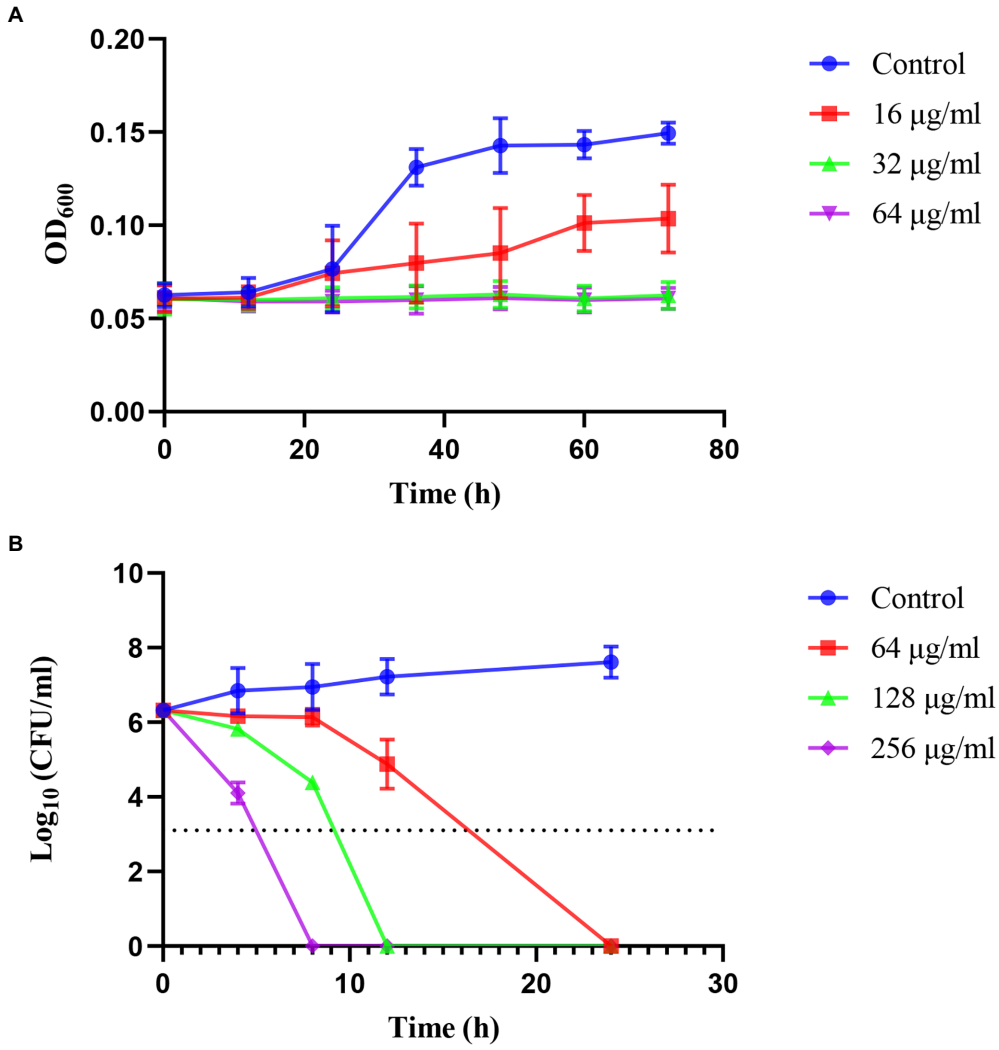


FIGURE 1
Inhibiting kinetic curves and killing kinetic curves. **(A)** Inhibiting kinetics curves of JWC on *Helicobacter pylori* 26,695–16R. **(B)** Killing kinetics curves of JWC on *H. pylori* 26,695–16R. The dotted line represents a 1,000-fold reduction in the number of bacteria compared to the initial inoculation.

and $1 < D/Dc \leq 2$, a weak biofilm-forming strain. $D/Dc \leq 1$ was defined as a strain without biofilm formation. The results showed that *H. pylori* 26,695–16R is a moderate biofilm-forming strain, and CCCP (1 μg/ml) and JWC (16–64 μg/ml) inhibited biofilm formation in *H. pylori* 26,695–16R, and the difference was statistically significant (Figure 3).

## Effect of JWC on the biofilm of *Helicobacter pylori* 26,695–16R by SEM

The normal group showed a biofilm structure formed by *H. pylori* 26,695–16R. Bacteria and extracellular matrix that are closely linked to bacteria can be found in biofilms. The bacteria were mostly rod-shaped with tight connections and fewer voids. After treatment with CCCP (1 μg/ml) and JWC, the biofilm structure of bacteria was disrupted, connections between bacteria became sparse, and the number of voids increased. The degree of destruction of the bacterial biofilm structure increased with increasing JWC concentrations. After treatment with MTZ at a concentration of 64 μg/ml, *H. pylori* 26,695–16R cells converted into a coccoid shape, which is a stress response to MTZ and one of the survival mechanisms of *H. pylori* (Figure 4).

## Effect of JWC on the biofilm of *Helicobacter pylori* 26,695–16R by CLSM

CLSM images provide a rough outline of *H. pylori* biofilms and the viability of the bacteria. Green represents live bacteria and red represents dead bacteria. In the control group, we observed that *H. pylori* 26,695–16R formed a dense biofilm

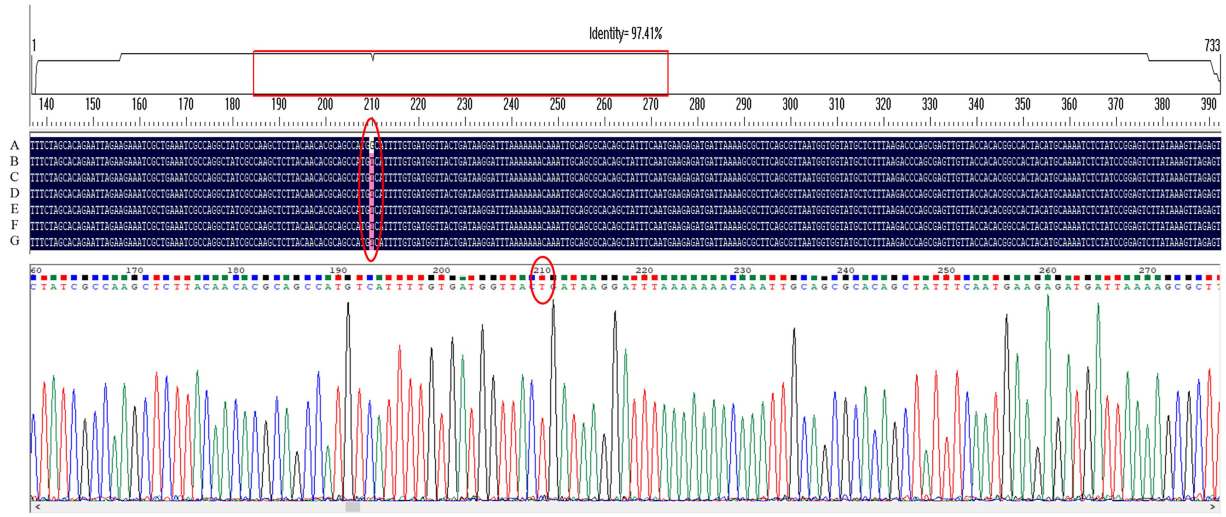


FIGURE 2
G210T point mutation in the MTZ resistance-related gene *rdxA* of *H. pylori* 26,695–16R under different drug interventions. A, *H. pylori* 26,695; B, *H. pylori* 26,695–16R; C, Treated with PaβN (20μg/ml); D, Treated with CCCP (1μg/ml); E, Treated with JWC 16μg/ml; F, Treated with JWC 32μg/ml; G, Treated with JWC 64μg/ml.

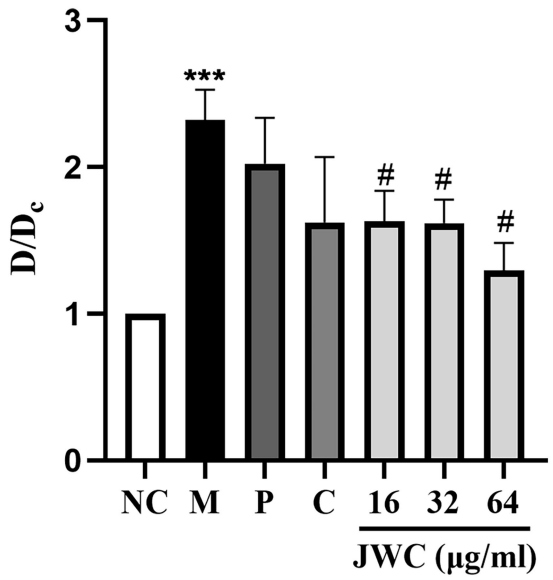


FIGURE 3
Effect of JWC, efflux pump inhibitors and MTZ on the biofilm of *H. pylori* 26,695–16R. Dc is the OD value of the blank control group, and D is the OD value of the other groups. NC: the blank control group, culture medium without *H. pylori* 26,695–16R; M, Model group, culture medium with *H. pylori* 26,695–16R; P, PaβN (20μg/ml); C, CCCP (1μg/ml). ***$p$ <0.001, compared with the results of the control group. $^{\#}p$ <0.05, compared with the results of the model group.

of a certain thickness and good bacterial activity. After treatment with PaβN (20 μg/ml), CCCP (1 μg/ml), and JWC (16–64 μg/ml), the biofilm structure of *H. pylori* 26,695–16R loosened, with a reduced proportion of live bacteria and an increased proportion of dead bacteria, indicating that biofilm formation was inhibited. When the concentration of JWC increased, the ratio of live/dead bacteria decreased and the amount of biofilm formed by bacterial aggregation decreased (Figure 5).

## Influence of JWC on efflux pump gene expression in *Helicobacter pylori* 26,695–16R

qPCR results showed that both CCCP and JWC inhibited the expression of HP0605 and HP0971. CCCP and JWC at 64 μg/ml (MIC) and 128 μg/ml (2 MIC) inhibited the expression of HP1327. CCCP and JWC at 32 μg/ml (1/2 MIC) and 128 μg/ml (2 MIC) inhibited the expression of HP1489, and the difference was statistically significant, as shown in Figure 6. The primers used are listed in Table 2.

## Jinghua Weikang capsule inhibited the adhesion of *Helicobacter pylori* 26,695–16R to GES-1 cells

As shown in Figure 7, blue fluorescence indicates the nucleus of GES-1 cells and green fluorescence indicates *H. pylori*. After observing the pictures in different channels, they were merged using a filter function. The merged results showed that *H. pylori* can adhere to GES-1 cells and that 16, 32, and 64 μg/ml JWC dose-dependently reduced the amount of *H. pylori* adherence (Figure 7A). According to the results of fluorescence intensity analysis using Image J software, the adhesion of *H. pylori* was

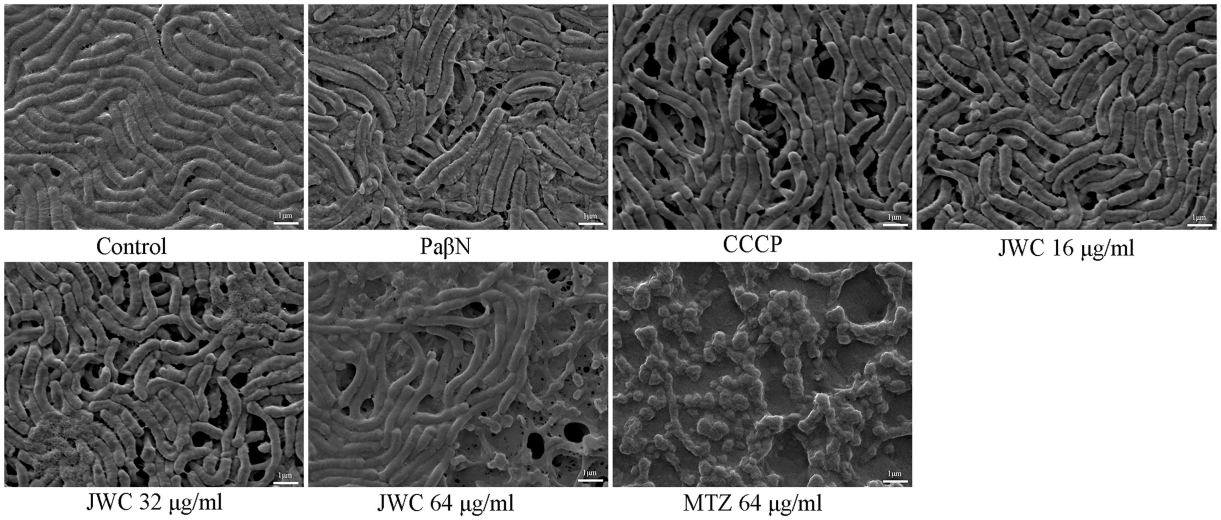


FIGURE 4
Effect of JWC and efflux pump inhibitors on the biofilm of *H. pylori* 26,695–16R.

inhibited by 40–60% when 16–64 μg/ml JWC was used (Figure 7B).

## Effect of JWC on adhesins of *Helicobacter pylori* 26,695–16R

The results showed that 32 μg/ml and 64 μg/ml JWC and CCCP decreased the expression of *SabA*, *BabA* and *NapA*. PaβN only decreased *SabA*. On the whole. JWC decreased the expression of adhesins in a concentration-dependent manner (Figure 8).

# Discussion

Antibiotic resistance of *H. pylori* has been increasing worldwide (Tshibangu-Kabamba and Yamaoka, 2021; Ho et al., 2022; Veenendaal et al., 2022; Zhong et al., 2022). Resistance of *H. pylori* to MTZ is more common than that of other antibiotics (Gerrits et al., 2006; Ho et al., 2022). MTZ resistance rates range from 42 to 96% worldwide, with higher rates in developing countries (Geng et al., 2022; Ho et al., 2022; Liu et al., 2022; Tian et al., 2022; Veenendaal et al., 2022). In triple, quadruple, and sequential therapies, including MTZ, eradication failure is often associated with MTZ resistance (Fischbach and Evans, 2007; Gatta et al., 2009). The nitro moiety of MTZ is reduced to a highly active compound that exerts antibacterial activity against *H. pylori* (Jenks and Edwards, 2002). The inactivation of *rdxA* (which encodes an oxygen-insensitive NADPH nitroreductase), *frxA* (which encodes NADPH flavin oxidoreductase), and *fdxB* (which encodes a ferredoxin-like protein) was closely associated with the failure of enzymatic reduction and MTZ resistance (Jenks and Edwards, 2002). In this study, seven resistant strains were screened, and JWC was found to have a significant anti-drug resistance effect on the MTZ-resistant strain *H. pylori* 26,695–16R, reducing its MIC value against MTZ from 64 μg/ml to 6 μg/ml and reversing resistance. For *H. pylori* 26,695–16R, the effect of JWC on reducing MTZ resistance was better than that of the efflux pump inhibitors PaβN and CCCP. The G210T point mutation in the *rdxA* gene of *H. pylori* 26,695–16R was found by sequencing, but the point mutation did not change after drug treatment, suggesting that the mechanism by which JWC reverses MTZ resistance is not related to the *rdxA* mutation, and that JWC may influence MTZ resistance through other mechanisms.

In addition to gene mutations, biofilm formation *in vivo* is an important mechanism leading to drug resistance. Biofilms are communities of microorganisms attached to a surface, and the surrounding EPS matrix is composed of extracellular polysaccharides, DNA, and proteins (Rabin et al., 2015). Biofilms play an important role in the persistence of bacterial infections, reducing bacterial susceptibility to antibiotics, and counteracting host immune mechanisms, allowing bacteria to survive in hostile environments (Rabin et al., 2015; Del Pozo, 2018). In the initial stage of biofilm formation, *H. pylori* is helical, and after effective adhesion and proliferation on the surface, the morphology changes to helical, rod-shaped, curved, spherical, and filamentous. However, in prolonged culture, all cells in the biofilm eventually transformed into globular cells, indicating that they were involved in survival and had a higher tolerance to adverse environmental factors (Krzyżek et al., 2020). The crystal violet method showed that *H. pylori* 26,695–16R is a moderate biofilm-forming strain, and CCCP and JWC (16–64 μg/ml) could inhibit biofilm formation in *H. pylori* 26,695–16R. After treatment with

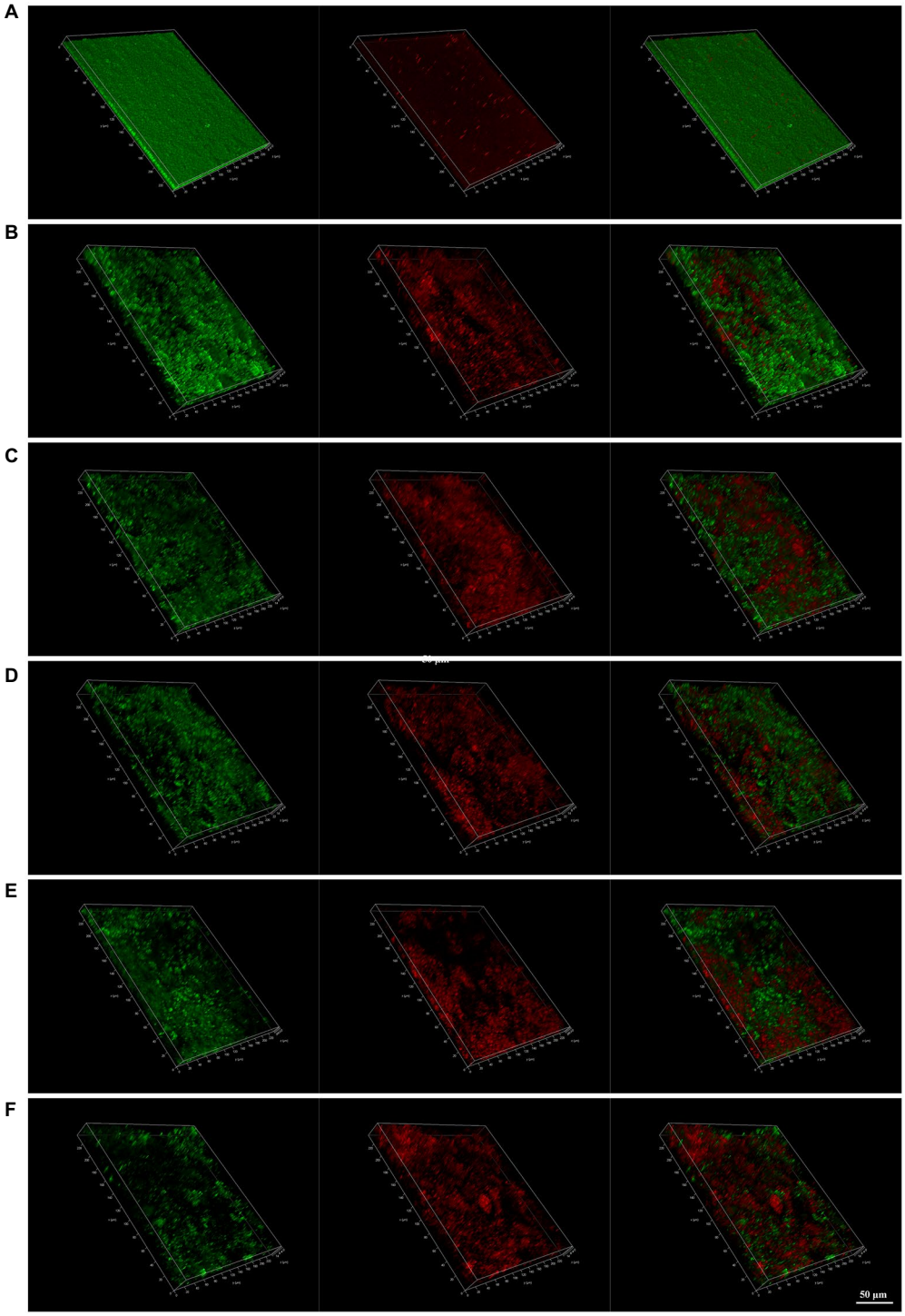


FIGURE 5
Confocal laser scanning microscope (CLSM) images of *H. pylori* 26,695–16R strain biofilms. Cells stained with membrane-permeant SYTO 9 (green) and membrane-impermeant propidium iodide (red) were visualized by CLSM. **(A)** Control group; **(B)** treated with PaβN (20μg/ml); **(C)** treated with CCCP (1μg/ml); **(D)** treated with JWC 16μg/ml; **(E)** treated with JWC 32μg/ml; **(F)** treated with JWC 64μg/ml.

CCCP and JWC, the biofilm structure of bacteria was disrupted, connections between bacteria became sparse, and the number of voids increased. The degree of destruction of the bacterial biofilm structure increased with increasing JWC concentrations. However, after treatment with MTZ at a concentration of 64 μg/ml, the morphology of *H. pylori* 26,695–16R became spherical, indicating that MTZ is a powerful toxic substance to bacteria, and that bacteria obtain

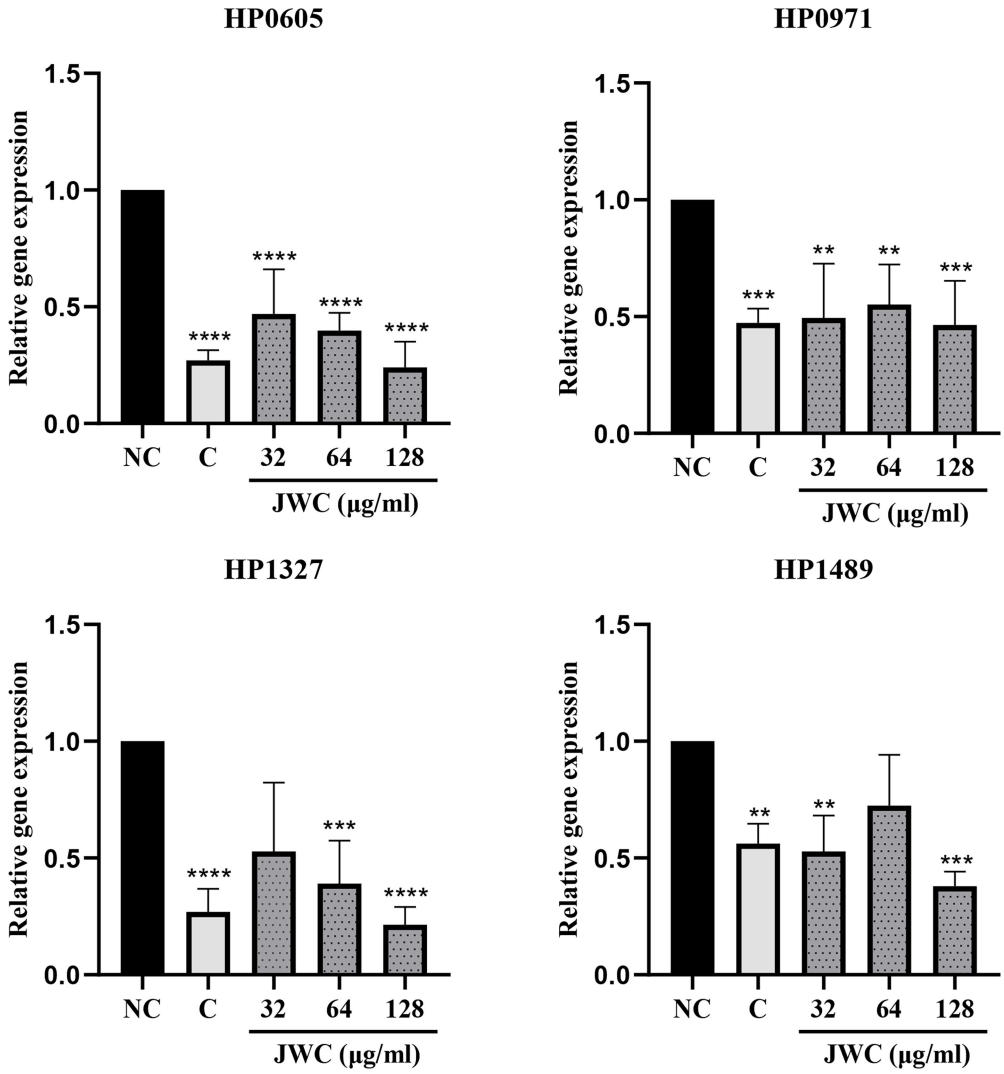


FIGURE 6
Expression of different efflux effect genes of *H. pylori* 26,695–16R after incubation with PaβN, CCCP and different concentrations of JWC. NC, Normal control group; C, CCCP (1μg/ml); ***p* <0.01, ****p* <0.001, *****p* <0.0001, compared with the results of the control group.

stronger self-protection ability through sphericity, which is also the reason for their resistance to MTZ.

The efflux pump system is one of the mechanisms of biofilm formation and is a key nonspecific mechanism of drug resistance in gram-negative bacteria (Hall and Mah, 2017). The efflux pump expels toxic substances such as antibiotics from the bacterial cytosol, thereby reducing the intracellular concentration of antibiotics and conferring antibiotic resistance to the bacteria (Poole, 2007). The RND family, the most studies in regards to their involvement in bacterial biofilm formation, is composed of inner membrane, periplasmic membrane fusion, and outer membrane proteins (Nikaido, 2011). At least one efflux pump, AcrAB-TolC, belongs to the RND family, and four gene clusters encoding RND, namely, HP0605-HP0607 (*hefABC*), HP0971-HP0969 (*hefDEF*), HP1327-HP1329 (*hefGHI*), and HP1489-HP1487, are currently detected in *H. pylori* (Ye et al., 2020). An enhanced efflux system is the first step in the development of MTZ resistance in *H. pylori* (Tsugawa et al., 2011). Several studies have shown that the expression of the efflux pump gene in biofilm-forming cells was significantly higher than that in planktonic cells (Soto, 2013; Attaran et al., 2017). For example, the expression levels of HP0605-HP0607 (*hefABC*), HP0971-HP0969 (*hefDEF*), HP1327-HP1329 (*hefGHI*), and HP1489-HP1487 in biofilm-forming strains are higher than those in planktonic bacteria (Yonezawa et al., 2019). This study found that JWC reduced the expression of HP0605-HP0607 (*hefABC*), HP0971-HP0969 (*hefDEF*), HP1327-HP1329 (*hefGHI*), and HP1489-HP1487, suggesting that JWC may reduce *H. pylori* resistance to MTZ by reducing the expression of efflux pump genes, and may also indirectly affect biofilm formation by reducing the expression of efflux pump genes to reduce *H. pylori* resistance to

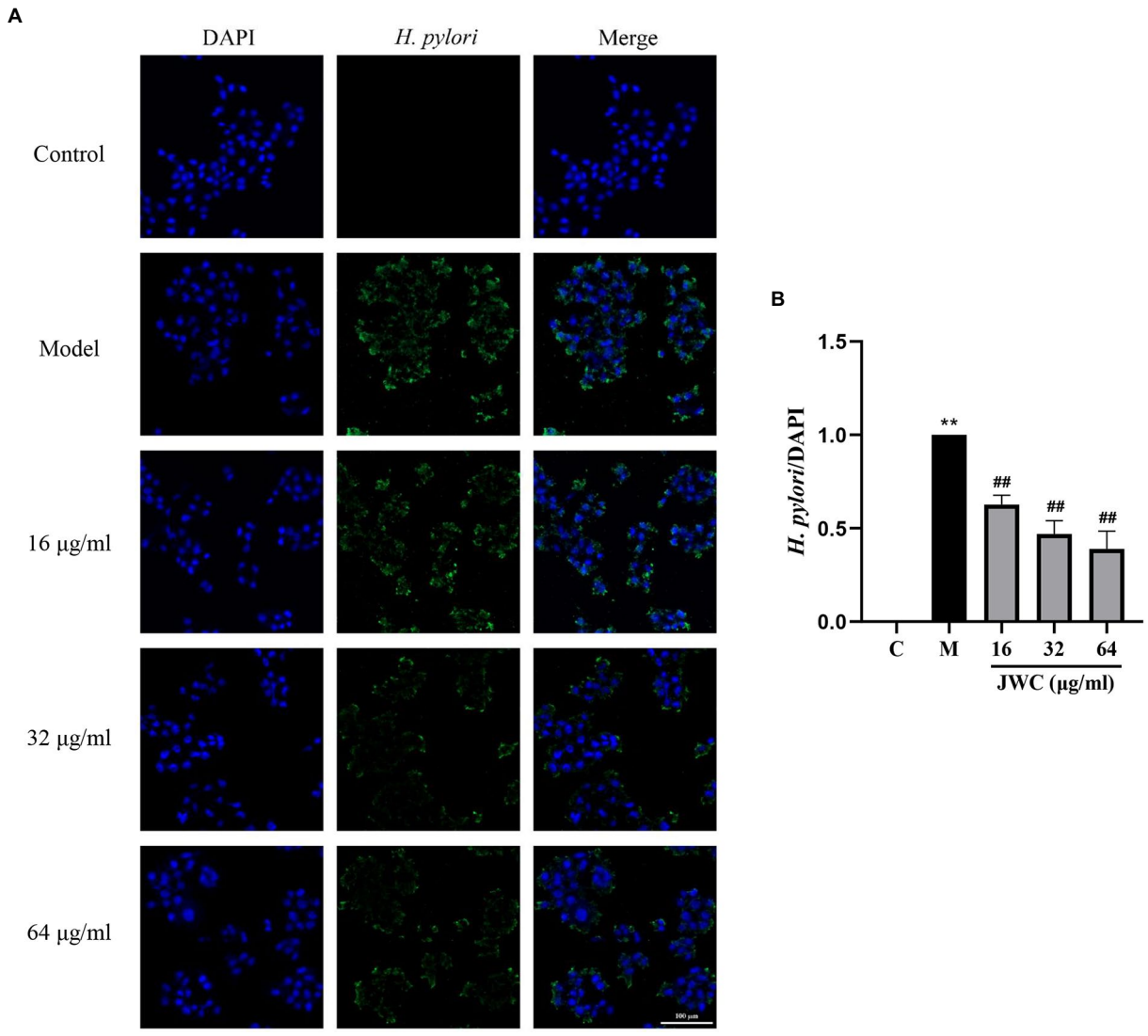


FIGURE 7
Effect of JWC on the adhesion of *H. pylori* 26,695–16R to GES-1 cells. **(A)** Immunofluorescence images of *H. pylori* and GES-1 adhesion after JWC intervention. Control group, only GES-1 cells; Model group, GES-1 and *H. pylori*. **(B)** The *H. pylori* fluorescence area/DAPI fluorescence area calculated by ImageJ software. $^{**}p$ <0.01, compared with the results of the control group. $^{\#\#}p$ <0.01, compared with the results of the model group. Bold values represent a meaningful MIC change of antibiotic and the *H. pylori* strain used in subsequent experiments.

MTZ. However, the specific mechanism by which efflux pumps affect biofilm formation is not clear at present, which may be related to the pumping of substances related to biofilm formation. A previous study found that emodin, baicalin, schizandrin, and berberine significantly decreased the MIC of amoxicillin and tetracycline against some *H. pylori* strains, and the mechanism may be related to the reduction in *hefA* mRNA expression (Huang et al., 2015). Our study is consistent with this view and complements the study of efflux pump genes and related biofilms.

Adhesion is the first step and prerequisite for biofilm formation. The expression of adhesins increases during the transition from the planktonic to the biofilm phase (Krzyżek et al., 2020). Several studies have shown that adhesive proteins, such as *NapA*, *AlpB*, *SabA*, *BabA*, *Homb*, *LabA* and *HopZ* are involved in biofilm formation (Cooksley et al., 2003; Yang et al., 2011; Acio-Pizzarello et al., 2017; Servetas et al., 2018; Zhao et al., 2021). Compared to wild-type strains, ArsRS mutants had high surface attachment and biofilm production, and the expression of genes encoding outer membrane proteins was increased in these mutants, including *AlpB*, *SabA*, *BabA*, *Homb*, *LabA* and *HopZ* (Acio-Pizzarello et al., 2017; Servetas et al., 2018). *NapA* is a surface protein that attracts and activates neutrophils and promotes endothelial adhesion and production of oxygen radicals and chemokines (D'Elios et al., 2007). When Helicobacter pylori is exposed to oxidative stress, *NapA* is highly expressed to resist oxygen stress injury, thus relieving bacterial survival pressure and promoting the formation and aggregation of EPS to promote

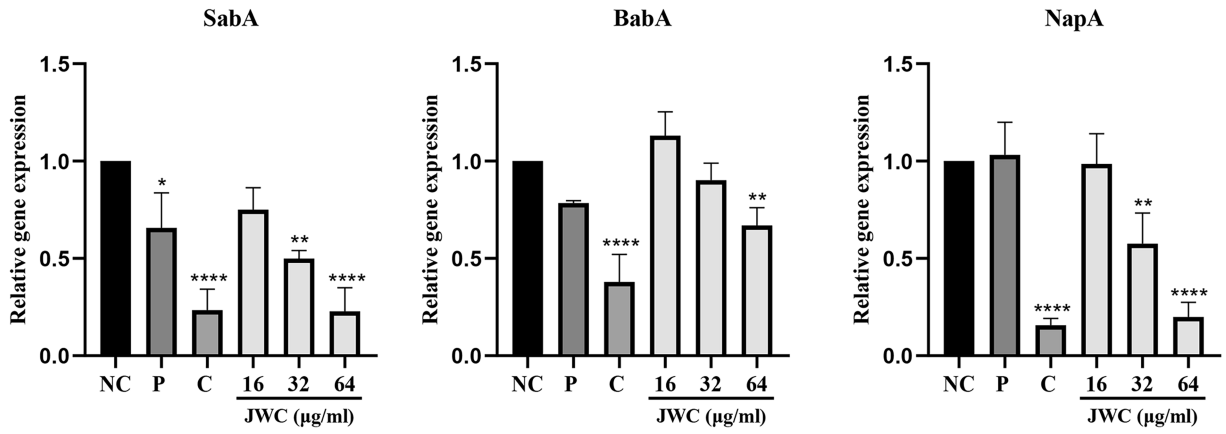


FIGURE 8
Effect of JWC on adhesions of *H. pylori* 26,695–16R. NC, Normal control group, *H. pylori* 26,695–16R; P, PaβN (20μg/ml); C, CCCP (1μg/ml). *$p$<0.05, **$p$<0.01, ****$p$<0.0001, compared with the results of the control group.

biofilm formation (Cooksley et al., 2003; Zhao et al., 2021). Another study also showed that *NapA* plays a role in adhesion to a substratum and *H. pylori* and hence influences biofilm formation (Yang et al., 2011). In this study, we found that JWC dose-dependently reduced the adhesion of *H. pylori* to GES-1 cells and the expression of adhesives *NapA*, *sabA* and *babA*, suggesting that JWC may reduce the adhesion between *H. pylori* and *H. pylori* or GES-1 by decreasing the expression of adhesins, thus affecting the formation of biofilms and inhibiting drug resistance.

Although it was found in this study that JWC considerably reduced the drug resistance of MTZ-resistant strain 26,695–16R, it did not reduce the drug resistance of other strong MTZ-resistant strains, indicating that the ability of JWC to reduce MTZ resistance is not universal and may be related to the strength of MTZ resistance of the strain itself. The reversal of metronidazole resistance by JWC may be achieved through the adhesin/RND efflux pump-biofilm pathway.

## Conclusion and outlook

JWC had good antibacterial effects against drug-resistant *H. pylori* strains and reversed the drug resistance of the MTZ-resistant strain 26,695–16R *in vitro*, the mechanism of which was related to the adhesin/RND efflux pump-biofilm pathway. However, biofilms are difficult to construct in animals because of the low ratophilic nature of *H. pylori*, which requires further experimental exploration *in vivo*. The mechanisms by which efflux pumps and adhesins inhibit biofilm formation require further investigation. In addition to its bactericidal effect, JWC has the advantages of reducing drug resistance and multi-targeting, reflecting the concept of potentiation rather than pure antagonism against drug-resistant *H. pylori*. This study provides an explanation for the mechanism by which JWC inhibits drug-resistant *H. pylori*, experimental support for the clinical application of JWC in combination with triple or quadruple therapy, and ideas for the clinical treatment of *H. pylori* and the development of new drugs.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

XJ performed the experiments, wrote the manuscript, and summarized and analyzed the data. QH and ML participated in performing the experiments. HY and ZS revised the manuscript. YC participated in the data analysis. HY designed the study. XZ and HY finally reviewed and approved the article for publication. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.962354/full#supplementary-material

## References

Acio-Pizzarello, C. R., Acio, A. A., Choi, E. J., Bond, K., Kim, J., Kenan, A. C., et al. (2017). Determinants of the regulation of helicobacter pylori adhesins include repeat sequences in both promoter and coding regions as well as the two-component system ArsRS. *J. Med. Microbiol.* 66, 798–807. doi: 10.1099/jmm.0.000491

Attaran, B., Falsafi, T., and Ghorbanmehr, N. (2017). Effect of biofilm formation by clinical isolates of helicobacter pylori on the efflux-mediated resistance to commonly used antibiotics. *World J. Gastroenterol.* 23, 1163–1170. doi: 10.3748/wjg.v23.i7.1163

Chen, L., and Wen, Y. M. (2011). The role of bacterial biofilm in persistent infections and control strategies. *Int. J. Oral Sci.* 3, 66–73. doi: 10.4248/IJOS11022

Chiang, T. H., Chang, W. J., Chen, S. L., Yen, A. M., Fann, J. C., Chiu, S. Y., et al. (2021). Mass eradication of helicobacter pylori to reduce gastric cancer incidence and mortality: a long-term cohort study on Matsu Islands. *Gut* 70, 243–250. doi: 10.1136/gutjnl-2020-322200

Cooksley, C., Jenks, P. J., Green, A., Cockayne, A., Logan, R. P. H., and Hardie, K. R. (2003). NapA protects helicobacter pylori from oxidative stress damage, and its production is influenced by the ferric uptake regulator. *J. Med. Microbiol.* 52, 461–469. doi: 10.1099/jmm.0.05070-0

Dawan, J., Li, Y., Lu, F., He, X., and Ahn, J. (2022). "Role of efflux pump-mediated antibiotic resistance in quorum sensing-regulated biofilm formation by salmonella Typhimurium," in *Pathogens*. ed. L. S. Young (Switzerland: Basel).

Del Pozo, J. L. (2018). Biofilm-related disease. *Expert Rev. Anti-Infect. Ther.* 16, 51–65. doi: 10.1080/14787210.2018.1417036

D'Elios, M. M., Amedei, A., Cappon, A., Del Prete, G., and de Bernard, M. (2007). The neutrophil-activating protein of helicobacter pylori (HP-NAP) as an immune modulating agent. *FEMS Immunol. Med. Microbiol.* 50, 157–164. doi: 10.1111/j.1574-695X.2007.00258.x

Doohan, D., Rezkitha, Y. A. A., Waskito, L. A., Yamaoka, Y., and Miftahussurur, M. (2021). *Helicobacter pylori* BabA-SabA key roles in the adherence phase: the synergic mechanism for successful colonization and disease development. *Toxins* 13:485. doi: 10.3390/toxins13070485

Enen, Z., Hui, Y., xiaofen, J., Qiuyue, H., Xuezhi, Z., and Yu, L. (2020). Effect of Chenopodium ambrosioides L. on biofilm formation of helicobacter pylori *in vitro*. *Chin. J. Integr. Traditional Western Med. Digest.* 40, 1241–1245. doi: 10.7661/j.cjim.20200904.334

Fischbach, L., and Evans, E. L. (2007). Meta-analysis: the effect of antibiotic resistance status on the efficacy of triple and quadruple first-line therapies for *Helicobacter pylori*. *Aliment. Pharmacol. Ther.* 26, 343–357. doi: 10.1111/j.1365-2036.2007.03386.x

Fu, H. W. (2014). *Helicobacter pylori* neutrophil-activating protein: from molecular pathogenesis to clinical applications. *World J. Gastroenterol.* 20, 5294–5301. doi: 10.3748/wjg.v20.i18.5294

Gatta, L., Vakil, N., Leandro, G., Di Mario, F., and Vaira, D. (2009). Sequential therapy or triple therapy for helicobacter pylori infection: systematic review and meta-analysis of randomized controlled trials in adults and children. *Am. J. Gastroenterol.* 104, 3069–3079. doi: 10.1038/ajg.2009.555

Ge, X., Cai, Y., Chen, Z., Gao, S., Geng, X., Li, Y., et al. (2018). Bifunctional enzyme SpoT is involved in biofilm formation of *Helicobacter pylori* with multidrug resistance by upregulating efflux pump Hp1174 (gluP). *Antimicrob. Agents Chemother.* 62:e00957-18. doi: 10.1128/AAC.00957-18

Geng, T., Yu, Z. S., Zhou, X. X., Liu, B., Zhang, H. H., and Li, Z. Y. (2022). *Antibiotic Resistance of Helicobacter pylori Isolated from children in Chongqing*, China. European Journal of Pediatrics, 2715, 2722.

Gerrits, M. M., van Vliet, A. H., Kuipers, E. J., and Kusters, J. G. (2006). Helicobacter pylori and antimicrobial resistance: molecular mechanisms and clinical implications. *Lancet Infect. Dis.* 6, 699–709. doi: 10.1016/S1473-3099(06)70627-2

Hall, C. W., and Mah, T. F. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiol. Rev.* 41, 276–301. doi: 10.1093/femsre/fux010

Hirata, K., Suzuki, H., Nishizawa, T., Tsugawa, H., Muraoka, H., Saito, Y., et al. (2010). Contribution of efflux pumps to clarithromycin resistance in *Helicobacter pylori*. *J. Gastroenterol. Hepatol.* 25, S75–S79. doi: 10.1111/j.1440-1746.2009.06220.x

Ho, J. J. C., Navarro, M., Sawyer, K., Elfanagely, Y., and Moss, S. F. (2022). *Helicobacter pylori* antibiotic resistance in the United States between 2011-2021: A systematic review and meta-analysis. *Am. J. Gastroenterol.* 117, 1221–1230. doi: 10.14309/ajg.0000000000001828

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* 35, 322–332. doi: 10.1016/j.ijantimicag.2009.12.011

Hong, C., Fulian, H., Jianqiu, S., Hejuan, A., and Le, X. (2016). Jinghuaweikang capsules combined with furazolidone-based triple or quadruple therapy as the rescue treatment for helicobacter pylori infection:a multicenter randomized controlled clinical trial. *Natl. Med. J. China* 96, 3206–3212. doi: 10.3760/cma.j.issn.0376-2491.2016.40.002

Hou, C., Yin, F., Wang, S., Zhao, A., Li, Y., and Liu, Y. (2022). Helicobacter pylori biofilm-related drug resistance and new developments in its anti-biofilm agents. *Infect. Drug Resist.* 15, 1561–1571. doi: 10.2147/IDR.S357473

Huang, Y. Q., Huang, G. R., Wu, M. H., Tang, H. Y., Huang, Z. S., Zhou, X. H., et al. (2015). Inhibitory effects of emodin, baicalin, schizandrin and berberine on hefA gene: treatment of helicobacter pylori-induced multidrug resistance. *World J. Gastroenterol.* 21, 4225–4231. doi: 10.3748/wjg.v21.i14.4225

Hui, Y., and Xuezhi, Z. (2014). Clinical research progress of Jinghua Weikang capsule in the treatment of *Helicobacter pylori* related upper digestive tract diseases. *Chin. J. Integr. Traditional Western Med. Digest.* 22, 694–698. doi: 10.3969/j.issn.1671-038X.2014.11.21

Jenks, P. J., and Edwards, D. I. (2002). Metronidazole resistance in *Helicobacter pylori*. *Int. J. Antimicrob. Agents* 19, 1–7. doi: 10.1016/S0924-8579(01)00468-X

Kinana, A. D., Vargiu, A. V., May, T., and Nikaido, H. (2016). Aminoacyl β-naphthylamides as substrates and modulators of AcrB multidrug efflux pump. *Proc. Natl. Acad. Sci. U. S. A.* 113, 1405–1410. doi: 10.1073/pnas.1525143113

Krzyżek, P., Grande, R., Migdał, P., Paluch, E., and Gościniak, G. (2020). "Biofilm formation as a complex result of virulence and adaptive responses of helicobacter pylori" in *Pathogens* (Switzerland: Basel)

Liu, W., Liu, Y., Zhang, X. Z., Li, N., and Cheng, H. (2013). In vitro bactericidal activity of Jinghua Weikang capsule and its individual herb Chenopodium ambrosioides L. against antibiotic-resistant helicobacter pylori. *Chin. J. Integr. Med.* 19, 54–57. doi: 10.1007/s11655-012-1248-y

Liu, Y., Wang, S., Yang, F., Chi, W., Ding, L., Liu, T., et al. (2022). Antimicrobial resistance patterns and genetic elements associated with the antibiotic resistance of helicobacter pylori strains from Shanghai. *Gut pathogens* 14:14. doi: 10.1186/s13099-022-00488-y

Liu, W. Z., Xie, Y., Lu, H., Cheng, H., Zeng, Z. R., Zhou, L. Y., et al. (2018). Fifth Chinese National Consensus Report on the management of helicobacter pylori infection. *Helicobacter* 23:e12475. doi: 10.1111/hel.12475

Matos, R., Amorim, I., Magalhães, A., Haesebrouck, F., Gärtner, F., and Reis, C. A. (2021). Adhesion of helicobacter species to the human gastric mucosa: a deep look Into Glycans role. *Front. Mol. Biosci.* 8:656439. doi: 10.3389/fmolb.2021.656439

Nikaido, H. (2011). Structure and mechanism of RND-type multidrug efflux pumps. *Adv. Enzymol. Relat. Areas Mol. Biol.* 77, 1–60. doi: 10.1002/9780470920541.ch1

Peng, C., Sang, S., Shen, X., Zhang, W., Yan, J., Chen, P., et al. (2022). In vitro anti-helicobacter pylori activity of *Syzygium aromaticum* and the preliminary mechanism of action. *J. Ethnopharmacol.* 288:114995. doi: 10.1016/j.jep.2022.114995

Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms. *Ann. Med.* 39, 162–176. doi: 10.1080/07853890701195262

Rabin, N., Zheng, Y., Opoku-Temeng, C., Du, Y., Bonsu, E., and Sintim, H. O. (2015). Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future Med. Chem.* 7, 493–512. doi: 10.4155/fmc.15.6

Rather, M. A., Gupta, K., and Mandal, M. (2021). Microbial biofilm: formation, architecture, antibiotic resistance, and control strategies. *Braz. J. Microbiol.* 52, 1701–1718. doi: 10.1007/s42770-021-00624-x

Ren, S., Cai, P., Liu, Y., Wang, T., Zhang, Y., Li, Q., et al. (2022). Prevalence of helicobacter pylori infection in China: a systematic review and meta-analysis. *J. Gastroenterol. Hepatol.* 37, 464–470. doi: 10.1111/jgh.15751

Servetas, S. L., Doster, R. S., Kim, A., Windham, I. H., Cha, J. H., Gaddy, J. A., et al. (2018). ArsRS-dependent regulation of homB contributes to helicobacter pylori biofilm formation. *Front. Microbiol.* 9:1497. doi: 10.3389/fmicb.2018.01497

Shen, X., Zhang, W., Peng, C., Yan, J., Chen, P., Jiang, C., et al. (2021). In vitro anti-bacterial activity and network pharmacology analysis of *Sanguisorba officinalis* L. against helicobacter pylori infection. *Chin. Med.* 16:33. doi: 10.1186/s13020-021-00442-1

Sisson, G., Jeong, J. Y., Goodwin, A., Bryden, L., Rossler, N., Lim-Morrison, S., et al. (2000). Metronidazole activation is mutagenic and causes DNA fragmentation in helicobacter pylori and in Escherichia coli containing a cloned *H. pylori* RdxA(+) (Nitroreductase) gene. *J. Bacteriol.* 182, 5091–5096. doi: 10.1128/JB.182.18.5091-5096.2000

Soto, S. M. (2013). Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence* 4, 223–229. doi: 10.4161/viru.23724

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., et al. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* 18, 318–327. doi: 10.1016/S1473-3099(17)30753-3

Tang, M., Wei, X., Wan, X., Ding, Z., Ding, Y., and Liu, J. (2020). The role and relationship with efflux pump of biofilm formation in *Klebsiella pneumoniae*. *Microb. Pathog.* 147:104244. doi: 10.1016/j.micpath.2020.104244

Teh, X., Khosravi, Y., Lee, W. C., Leow, A. H., Loke, M. F., Vadivelu, J., et al. (2014). Functional and molecular surveillance of *Helicobacter pylori* antibiotic resistance in Kuala Lumpur. *PLoS One* 9:e101481. doi: 10.1371/journal.pone.0101481

Tian, L., Yao, Y., Yin, L., Wang, L., An, Z., Kang, L., et al. (2022). Direct detection of antibiotic resistance in Chinese helicobacter pylori clinical isolates by sequencing-based approach. *J. Healthc. Eng.* 2022, 1–6. doi: 10.1155/2022/6436256

Tshibangu-Kabamba, E., and Yamaoka, Y. (2021). Helicobacter pylori infection and antibiotic resistance—from biology to clinical implications. *Nat. Rev. Gastroenterol. Hepatol.* 18, 613–629. doi: 10.1038/s41575-021-00449-x

Tsugawa, H., Suzuki, H., Muraoka, H., Ikeda, F., Hirata, K., Matsuzaki, J., et al. (2011). Enhanced bacterial efflux system is the first step to the development of metronidazole resistance in *Helicobacter pylori*. *Biochem. Biophys. Res. Commun.* 404, 656–660. doi: 10.1016/j.bbrc.2010.12.034

Veenendaal, R. A., Woudt, S. H. S., Schoffelen, A. F., de Boer, M. G. J., van den Brink, G., Molendijk, I., et al. (2022). Important rise in antibiotic resistance rates in *Helicobacter pylori* in the Netherlands. *Ned. Tijdschr. Geneeskd.* 166:D6434.

Yang, F. L., Hassanbhai, A. M., Chen, H. Y., Huang, Z. Y., Lin, T. L., Wu, S. H., et al. (2011). Proteomannans in biofilm of helicobacter pylori ATCC 43504. *Helicobacter* 16, 89–98. doi: 10.1111/j.1523-5378.2010.00815.x

Ye, H., Liu, Y., Li, N., Yu, J., Cheng, H., Li, J., et al. (2015). Anti-helicobacter pylori activities of *Chenopodium ambrosioides* L. *in vitro* and *in vivo*. *World J. Gastroenterol.* 21, 4178–4183. doi: 10.3748/wjg.v21.i14.4178

Ye, L., Meng, F., Mao, X., Zhang, Y., Wang, J., Liu, Y., et al. (2020). Using next-generation sequencing to analyze helicobacter pylori clones with different levofloxacin resistances from a patient with eradication failure. *Medicine* 99:e20761. doi: 10.1097/MD.0000000000020761

Yonezawa, H., Osaki, T., Hojo, F., and Kamiya, S. (2019). Effect of *Helicobacter pylori* biofilm formation on susceptibility to amoxicillin, metronidazole and clarithromycin. *Microb. Pathog.* 132, 100–108. doi: 10.1016/j.micpath.2019.04.030

Zamani, M., Ebrahimtabar, F., Zamani, V., Miller, W. H., Alizadeh-Navaei, R., Shokri-Shirvani, J., et al. (2018). Systematic review with meta-analysis: the worldwide prevalence of helicobacter pylori infection. *Aliment. Pharmacol. Ther.* 47, 868–876. doi: 10.1111/apt.14561

Zanotti, G., and Cendron, L. (2019). Structural aspects of helicobacter pylori antibiotic resistance. *Adv. Exp. Med. Biol.* 1149, 227–241. doi: 10.1007/5584_2019_368

Zhang, Z., Liu, Z. Q., Zheng, P. Y., Tang, F. A., and Yang, P. C. (2010). Influence of efflux pump inhibitors on the multidrug resistance of helicobacter pylori. *World J. Gastroenterol.* 16, 1279–1284. doi: 10.3748/wjg.v16.i10.1279

Zhao, Y., Cai, Y., Chen, Z., Li, H., Xu, Z., Li, W., et al. (2021). SpoT-mediated NapA upregulation promotes oxidative stress-induced helicobacter pylori biofilm formation and confers multidrug resistance. *Antimicrob. Agents Chemother.* 65. doi: 10.1128/AAC.00152-21

Zhong, Y., Tang, L., Deng, Q., Jing, L., Zhang, J., Zhang, Y., et al. (2021). Unraveling the novel effect of patchouli alcohol against the antibiotic resistance of *Helicobacter pylori*. *Front. Microbiol.* 12:674560. doi: 10.3389/fmicb.2021.674560

Zhong, Z., Zhan, B., Xu, B., and Gao, H. (2022). Emphasizing the importance of successful eradication of helicobacter pylori on initial treatment. *Am. J. Cancer Res.* 12, 1215–1221. PMID: 35411234

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# Susceptibility-guided vs. empirical 10-day quadruple treatment for *Helicobacter pylori*-infected patients: A prospective clinical trial of first-line therapy

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**Background:** The increasing antimicrobial resistance of *Helicobacter pylori (H. pylori)* has resulted in a fall in cure rates. We aimed to assess the effectiveness of first-line susceptibility-guided therapy and furazolidone-based quadruple therapy for *H. pylori*-infected patients.

**Methods:** Subjects with *H. pylori*-infection were randomly assigned to either 10-day susceptibility-guided treatment or empiric treatment in a 2:1 ratio. Susceptibility-guided therapy was based on susceptibility to clarithromycin, and patients with susceptible strains received clarithromycin 500 mg twice daily and otherwise minocycline 100 mg twice a day was administered. Patients in the empiric therapy group was treated with furazolidone 100 mg twice a day. During treatment, all patients were given esomeprazole 20 mg twice daily, colloidal bismuth pectin 200 mg twice daily, and amoxicillin 1 g twice daily.

**Results:** A total of 248 patients were screened and 201 were finally included. Empiric and susceptibility-guided regimens were both successful with per-protocol eradication rates of 90.5% (57/63) vs. 88.5% (108/122) ($p$ = 0.685) and intent-to-treat eradication rates of 85.1% (57/67) vs. 80.6% (108/134) ($p$ = 0.435). No significant difference in eradication rates were observed among the furazolidone group, clarithromycin group and minocycline group.

**Conclusion:** Both susceptibility-guided therapy and quadruple therapy containing furazolidone can achieve good eradication rates. For population with a high rate of resistance, quadruple therapy containing furazolidone and bismuth may be a more practical choice for first-line treatment.

KEYWORDS

***Helicobacter pylori*, eradication, susceptibility-guided therapy, empirical therapy, randomized controlled trial**

# Introduction

Globally, *Helicobacter pylori (H. pylori)* infects approximately 4.4 billion people, making it one of the most prevalent pathogens in humans (Hooi et al., 2017). *H. pylori* is a leading cause of chronic gastritis, peptic ulcer, gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer, and plenty of researchers have emphasized the eradication of *H. pylori* infection to reduce these diseases (Lee et al., 2013; Graham et al., 2017). Moreover, *H. pylori* has also been suggested to be a risk factor for many extra-gastrointestinal diseases, such as cardiovascular diseases (de Korwin et al., 2017; Wang et al., 2017). Thus, *H. pylori* eradication is important for public health. However, the widespread use of antibiotics has led to a rise in antimicrobial resistance, which has decreased the cure rate for *H. pylori* infections (Megraud et al., 2021).

Traditional therapy for infectious disease depends on local, regional, or patient-specific antimicrobial susceptibilities (Graham and Dore, 2016), and susceptibility-guided treatment should be the best strategy if available. The currently recommended first-line *H. pylori* therapy in China is bismuth quadruple therapy (Liu et al., 2018). One study reported that rates of *H. pylori* resistance in China for clarithromycin, metronidazole, and levofloxacin were 35.1, 82.7, and 46.9%, respectively (Chen et al., 2019). Due to China's high antibiotic resistance rates, treatment regimens have become increasingly complex. Personalized treatments based on antibiotic susceptibility represent a novel therapeutic option. However, susceptibility testing of *H. pylori* is difficult to perform and not practical in many clinical setting. As the resistant rate of amoxicillin and furazolidone remains low, the bismuth quadruple therapy containing these two medicines has been proved to be effective in China (Qiao et al., 2021). Besides, randomized controlled trials have shown that a bismuth quadruple therapy containing tetracycline remained highly effective (Chen et al., 2016), while tetracycline is difficult to obtain in many areas including China. Fortunately, several studies have proved the efficacy of minocycline in *H. pylori* eradication, which is a tetracycline derivative (Song et al., 2016).

Here, we conducted a study to assess the resistance of drugs used in *H. pylori* eradication, and to compare the efficacy of susceptibility-guided therapy (containing clarithromycin or minocycline according to susceptibility testing) with empiric therapy containing furazolidone.

# Materials and methods

## Study design

This study was a prospective, interventional, open-label, single-center trial performed between 2019 and 2020 at the Second Affiliated Hospital of Zhejiang University, School of Medicine. Informed consent was obtained from all subjects and the trial was approved by the hospital's Ethics Committee. It was registered in ResMan, a web-based medical research public management platform, and the registration number was ChiCTR2000038308. The study was also conducted in accordance with the Declaration of Helsinki, and the recommendations of the CONSORT statement for reporting randomized controlled trials. *H. pylori* infection was determined by urea breath test ($^{13}$C-UBT or $^{14}$C-UBT) or histology. Subjects would be excluded if they were younger than 18 years of age, previously treated for *H. pylori*, pregnancy or lactation, previous gastric surgery, presence of significant clinical diseases or malignancy, use of antisecretory drugs, antibiotics or bismuth within the past 4 weeks, or allergy to any of the research drugs.

## *Helicobacter pylori* isolation and antimicrobial susceptibility testing

We collected two biopsy specimens (one from gastric antrum, and one from gastric corpus) during gastroscopy (260/290 series, Olympus, Tokyo, Japan) for *H. pylori* isolation. Under microaerophilic conditions (85% $N_2$, 10% $CO_2$, and 5% $O_2$), the specimens were cultured and maintained on brain heart infusion agar medium (Oxoid, Basingstoke, United Kingdom) containing 5% defibrinated sheep blood at 37°C. *H. pylori* isolates were identified by colony morphology, microscopic image of Gram-negative helix-shaped bacterial morphology, and positive for urease, oxidase, and catalase.

The E-test method (AB Biodisk, Solna, Sweden) was applied to determine the minimum inhibitory concentrations (MICs). MIC values were determined after 72 h of incubation and we used *H. pylori* ATCC 43526 for quality control. Resistance to antibiotics was defined as follows: amoxicillin, MIC $\geq$0.5 μg/ml; clarithromycin, MIC >1.0 μg/ml; metronidazole, MIC >8 μg/ml; tetracycline, MIC >4 μg/ml; and levofloxacin, MIC >1 μg/ml.

# Intervention

Patients with *H. pylori* infection were randomly assigned to either 10-day susceptibility-guided treatment or empiric treatment in a 2:1 ratio. Technicians performing culture, antimicrobial susceptibility testing or urea breath test were blinded to treatment allocation. Patients in the empiric therapy was treated with furazolidone 100 mg twice daily for 10 days. Susceptibility-guided therapy was according to susceptibility to clarithromycin, and patients with susceptible strains received clarithromycin 500 mg twice daily and otherwise minocycline 100 mg twice a day was administered. During treatment, all patients were given esomeprazole 20 mg twice daily, colloidal

bismuth pectin 200 mg twice daily, and amoxicillin 1 g twice daily.

At least 4 weeks after therapy completion, $^{13}$C- or $^{14}$C-urea breathe test was performed to assess *H. pylori* eradication, and negative urea breath test result was defined as eradication.

## Statistical analysis

We used intention-to treat (ITT) and per-protocol (PP) analysis to assess eradication rates. For the ITT analysis, all subjects were included, while only subjects who followed the protocol were included in the PP analysis. Patients without follow-up UBT were defined as treatment failures in the ITT analysis. Characteristics of the population and distribution of antibiotic resistance was performed using descriptive statistics. Student's *t*-test was used for continuous data comparation, and chi-square test was applied for categorical data. All *P*-values were two−sided, and $P < 0.05$ was defined as statistically significance. All analyses were conducted using SPSS v.21 Statistics program.

# Results

A total of 248 *H. pylori*-infected patients were evaluated for eligibility, and 47 met exclusion criteria or declined to participate and were excluded. Finally, 201 patients were enrolled and divided into furazolidone group ($n$ = 67) and susceptibility-guided therapy group ($n$ = 134) (as shown in Figure 1). The mean age was 42.5 for furazolidone group

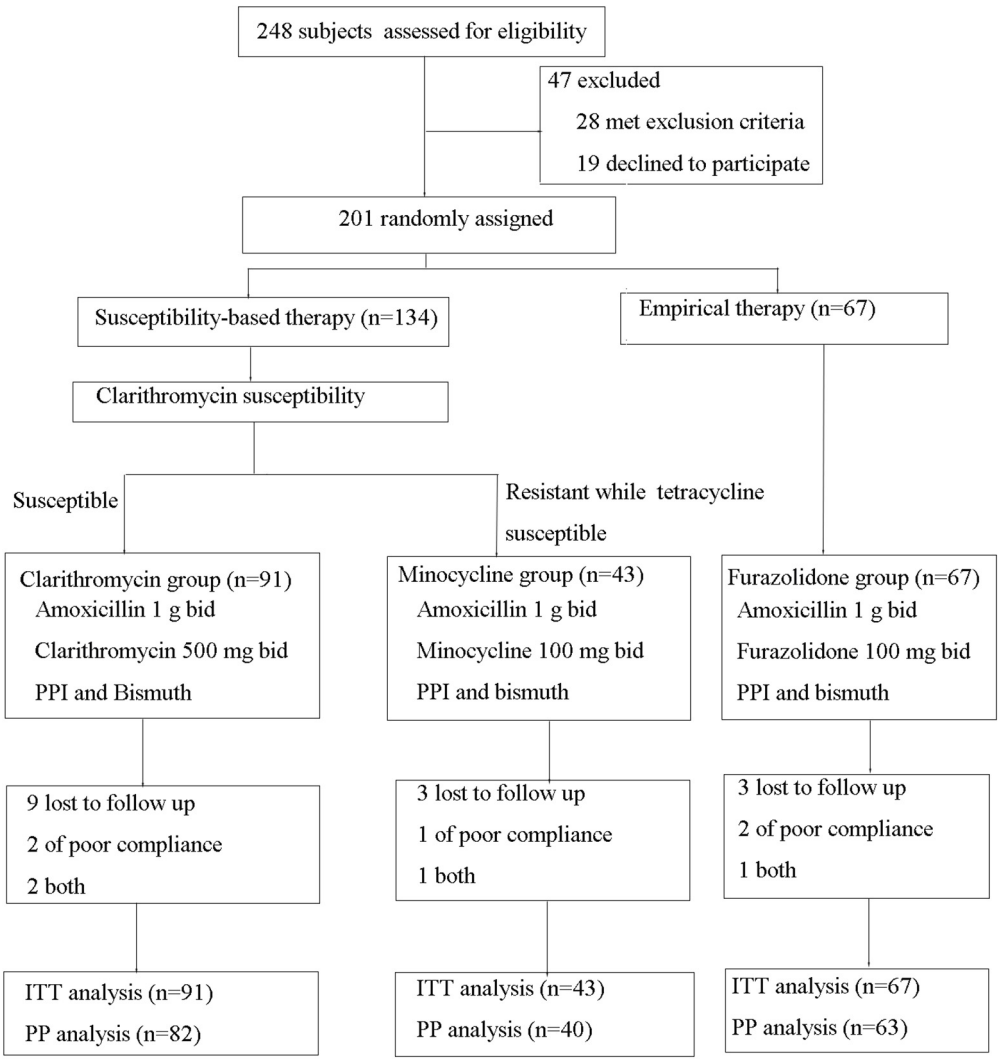


FIGURE 1
Flow diagram of the study.

TABLE 1 Baseline characteristics of the included patients.

| | Susceptibility-guided therapy group ($n$ = 134) | Empiric therapy group ($n$ = 67) | $P$-value |
|---|---|---|---|
| Age (mean) | 45.0 | 42.5 | 0.180 |
| Gender (M/F) | 59/75 | 28/39 | 0.760 |
| **Antibiotic resistance $n$ (%)** | | | |
| Clarithromycin | 44 (32.8%) | 14 (29.2%) | 0.640 |
| Metronidazole | 101 (75.4%) | 27 (56.3%) | 0.013 |
| Levofloxacin | 44 (32.8%) | 10 (20.8%) | 0.118 |
| Tetracycline | 3 (2.2%) | 0 (0%) | 0.296 |
| Furazolidone | 4 (3.0%) | 0 (0%) | 0.226 |
| Amoxicillin | 0 (0%) | 0 (0%) | – |

and 45.0 for susceptibility-guided therapy group ($p$ = 0.180) (Table 1). There were 28 males in furazolidone group (41.8%) and 59 males in susceptibility-guided therapy group (44.0%) ($p$ = 0.760) (Table 1). Overall, 15 patients were lost to follow-up UBT and five patient had poor compliance (four both had poor compliance and lost to follow-up), which were defined as treatment failure in the ITT analysis and were not included in the PP analysis. A total of 185 patients were finally included in the PP analyses (Figure 1).

## Results for antimicrobial susceptibility testing

Among the 201 included subjects, culture was not performed for nine subjects and were failed for another nine patients (in the furazolidone group), and thus 183 subjects were included in the antimicrobial susceptibility test. The rate of metronidazole resistance was highest (70.0%, 128/183), followed by clarithromycin (31.7%, 58/183) and levofloxacin (29.5%, 54/183), furazolidone (2.2%, 4/183) and tetracycline (1.6%, 3/183). There were no cases of amoxicillin resistance. Metronidazole resistance rate was higher in the susceptibility-guided therapy group (75.4%) compared with furazolidone group (56.3%) ($p$ = 0.013), while no significant difference in resistance rate of other antibiotics was observed (Table 1).

In the susceptibility-guided therapy group, 43 were resistant to clarithromycin and were included in the minocycline group and the remaining 91 were included in the clarithromycin group.

TABLE 3 Adverse effects of the included patients.

| | Susceptibility-guided therapy group | Empiric therapy group |
|---|---|---|
| Adverse effects | 9 (6.7%) | 5 (7.5%) |
| Taste alteration | 2 (1.5%) | 1 (1.5%) |
| Skin rash | 1 (0.7%) | 2 (3.0%) |
| Abdominal pain | 3 (2.2%) | 0 (0%) |
| Fever | 0 (0%) | 1 (1.5%) |
| Nausea and vomiting | 2 (1.5%) | 1 (1.5%) |
| Fatigue | 2 (1.5%) | 1 (1.5%) |

All $P > 0.05$.

## *Helicobacter pylori* eradication rates and safety

As shown in Table 2, PP analysis indicated that the eradication rates were 90.5% (57/63) in the furazolidone group and 88.5% (108/122) in the susceptibility-guided therapy group. No significant difference between the two groups was found ($p$ = 0.685). Moreover, there was no significant difference between furazolidone group (90.5%), clarithromycin group (89.0%, 73/82), and the minocycline group (87.5%, 35/40) ($p$ = 0.892).

Intention-to treat analysis also suggested no significant difference in eradication rates between furazolidone group (85.1%, 57/67) and the susceptibility-guided therapy group (80.6%, 108/134) ($p$ = 0.435). There was no significant difference between furazolidone group (85.1%), clarithromycin group (80.2%, 73/91), and the minocycline group (81.4%, 35/43) ($p$ = 0.727) (Table 2). Adverse effects were similar with furazolidone group (7.5%, 5/67) and the susceptibility-guided therapy group (6.7%, 9/134) (Table 3), and no severe adverse effects were observed.

# Discussion

In the current study including 201 patients, the resistant rate of metronidazole was high (70.0%), followed by clarithromycin (31.7%) and levofloxacin (29.5%), while the resistant rate was low for furazolidone (2.2%), tetracycline (1.6%), and amoxicillin (0%). The resistant rate of metronidazole and clarithromycin appeared to be higher than a previous study conducted in Korea, which suggested that resistant rate of metronidazole

TABLE 2 Eradication rate of each group in intention-to treat (ITT) and per-protocol (PP) analysis.

| | Empiric therapy group | Susceptibility-guided therapy group | Clarithromycin group | Minocycline group |
|---|---|---|---|---|
| ITT analysis | 85.1% (57/67) | 80.6% (108/134) | 80.2% (73/91) | 81.4% (35/43) |
| PP analysis | 90.5% (57/63) | 88.5% (108/122) | 89.0% (73/82) | 87.5% (35/40) |

All $P > 0.05$.

and clarithromycin was 29.5 and 17.8%, respectively (Lee et al., 2019). However, the resistant rate was similar with another study performed in China, of which the resistance rates of *H. pylori* for clarithromycin, levofloxacin, metronidazole, amoxicillin, and furazolidone were 26.12, 28.69, 96.79, 0, and 0%, respectively (Pan et al., 2020). The high rates of resistance to antibiotics have significantly reduced eradication rate of *H. pylori* (Sugano et al., 2015; Liu et al., 2018). As reported, traditional therapy eradication rate for *H. pylori* is below 80% in many cities, especially in high-risk areas for antibiotic resistance (Graham et al., 2014; Sebghatollahi et al., 2018).

Theoretically, therapy basing on the results of susceptibility testing for infectious diseases should be recommended, which is associated with higher efficacy, fewer side effects and unnecessary antibiotic use. According to the Maastricht V/Florence Consensus Report, susceptibility-guided therapy has been recommended after the second-line treatment fails (Malfertheiner et al., 2017). In a previous meta-analysis, tailored therapy was found to be more effective than empirically chosen treatment for eradicating *H. pylori* (Gingold-Belfer et al., 2021). The meta-analysis included both first-line and rescue treatments, and the role of susceptibility-guided therapy in first-line *H. pylori* remains unclear. Several studies suggested higher efficacy of susceptibility-guided therapy compared with empirically chosen treatment, while other studies found inconsistent results. Besides, the availability, accuracy, and cost-effectiveness of susceptibility-guided therapy should be considered in the clinical practice of *H. pylori* eradication (Matsumoto et al., 2019).

As a nitrofuran antibiotic, furazolidone damages bacterial DNA and interferes with normal bacterial metabolism. The primary and secondary resistance to furazolidone is low for *H. pylori*, and quadruple therapy containing furazolidone has been widely used in China (Xie et al., 2018). According to guidelines, furazolidone is recommended for eradication of *H. pylori* due to the low resistance (Malfertheiner et al., 2017; Liu et al., 2018). A number of studies have explored the efficacy and safety for rescue therapy and for naïve *H. pylori*-infected patients (Fakheri et al., 2001; Liang et al., 2013; Qiao et al., 2021). As reported by Liang et al. (2013) treatment regimens containing furazolidone were significantly more effective than treatments without furazolidone in rescue therapy of *H. pylori*. Another study compared clarithromycin with furazolidone for naïve *H. pylori*-infected patients, and recommended furazolidone-based quadruple therapy because of the high eradication rate, excellent cost-effectiveness and acceptable safety (de Korwin et al., 2017). In China, bismuth potassium citrate, colloidal bismuth pectin, and colloidal bismuth subcitrate are widely available. Currently, bismuth-containing quadruple therapy is the first-line treatment for *H. pylori* infection because it is effective against both susceptible and resistant strains (Malfertheiner et al., 2017; Liu et al., 2018).

In the current study, we compared the efficacy of susceptibility-guided therapy with empirical quadruple therapy containing furazolidone and bismuth, and both PP and ITT analyses suggested that these two therapies were comparable (90.5% in the furazolidone group and 88.5% in the susceptibility-guided therapy group for PP analysis; 85.1% in the furazolidone group and 80.6% in the susceptibility-guided therapy group for ITT analysis). Moreover, in the susceptibility-guided therapy group, no significant difference was found between clarithromycin group and the minocycline group. We observed a low rate of adverse effects for both furazolidone group (7.5%) and the susceptibility-guided therapy group (6.7%), and no severe adverse effects were found. The results further supported the use of furazolidone and minocycline in *H. pylori* eradication, and supported quadruple therapy containing furazolidone and bismuth when susceptibility test was unavailable.

There were several limitations in the current study. First, this was a single-center randomized controlled trial, which may limit generalizing the results. Second, obtaining tetracycline and minocycline remains difficult in many areas of China. Third, routinely performing *H. pylori* antimicrobial susceptibility test is difficult in most areas, which limits the use of susceptibility-guided therapy.

In conclusion, both susceptibility-guided therapy and empirical quadruple therapy containing furazolidone can achieve good eradication rates. For population with a high rate of resistance, empirical quadruple therapy containing furazolidone and bismuth may be a more practical choice for first-line treatment.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University, School of Medicine. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

YH and PL designed and conceived this study, and prepared for the manuscript. JJ, YC, and JM collected clinical samples and performed the experiments. QD, YH, and PL analyzed

the data. All authors contributed to the article and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

Chen, Q., Long, X., Ji, Y., Liang, X., Li, D., Gao, H., et al. (2019). Randomised controlled trial: susceptibility-guided therapy versus empiric bismuth quadruple therapy for first-line *Helicobacter* pylori treatment. *Aliment Pharmacol. Ther.* 49, 1385–1394. doi: 10.1111/apt.15273

Chen, Q., Zhang, W., Fu, Q., Liang, X., Liu, W., Xiao, S., et al. (2016). Rescue Therapy for *Helicobacter* pylori Eradication: A Randomized Non-Inferiority Trial of Amoxicillin or Tetracycline in Bismuth Quadruple Therapy. *Am. J. Gastroenterol.* 111, 1736–1742.

de Korwin, J. D., Ianiro, G., Gibiino, G., and Gasbarrini, A. (2017). *Helicobacter* pylori infection and extragastric diseases in 2017. *Helicobacter* 1:22. doi: 10.1111/hel.12411

Fakheri, H., Malekzadeh, R., Merat, S., Khatibian, M., Fazel, A., Alizadeh, B. Z., et al. (2001). Clarithromycin vs. furazolidone in quadruple therapy regimens for the treatment of *Helicobacter* pylori in a population with a high metronidazole resistance rate. *Aliment Pharmacol. Ther.* 15, 411–416. doi: 10.1046/j.1365-2036.2001.00931.x

Gingold-Belfer, R., Niv, Y., Schmilovitz-Weiss, H., Levi, Z., and Boltin, D. (2021). Susceptibility-guided versus empirical treatment for *Helicobacter* pylori infection: A systematic review and meta-analysis. *J. Gastroenterol. Hepatol.* 36, 2649–2658.

Graham, D. Y., and Dore, M. P. (2016). *Helicobacter* pylori therapy: a paradigm shift. *Expert Rev. Anti. Infect. Ther.* 14, 577–585. doi: 10.1080/14787210.2016.1178065

Graham, D. Y., Fagoonee, S., and Pellicano, R. (2017). Increasing role for modified bismuth-containing quadruple therapies for *Helicobacter* pylori eradication. *Minerva Gastroenterol. Dietol.* 63, 77–79. doi: 10.23736/S1121-421X.17.02369-8

Graham, D. Y., Lee, Y. C., and Wu, M. S. (2014). Rational *Helicobacter* pylori therapy: evidence-based medicine rather than medicine-based evidence. *Clin Gastroenterol. Hepatol.* 12, 177–186 e173. doi: 10.1016/j.cgh.2013.05.028

Hooi, J. K. Y., Lai, W. Y., Ng, W. K., Suen, M. M. Y., Underwood, F. E., Tanyingoh, D., et al. (2017). Global Prevalence of *Helicobacter* pylori Infection: Systematic Review and Meta-Analysis. *Gastroenterology* 153, 420–429.

Lee, J. H., Ahn, J. Y., Choi, K. D., Jung, H. Y., Kim, J. M., Baik, G. H., et al. (2019). Nationwide antibiotic resistance mapping of *Helicobacter* pylori in Korea: A prospective multicenter study. *Helicobacter* 24:e12592. doi: 10.1111/hel.12592

Lee, Y. C., Chen, T. H., Chiu, H. M., Shun, C. T., Chiang, H., Liu, T. Y., et al. (2013). The benefit of mass eradication of *Helicobacter* pylori infection: a community-based study of gastric cancer prevention. *Gut* 62, 676–682. doi: 10.1136/gutjnl-2012-302240

Liang, X., Xu, X., Zheng, Q., Zhang, W., Sun, Q., Liu, W., et al. (2013). Efficacy of bismuth-containing quadruple therapies for clarithromycin-, metronidazole-, and fluoroquinolone-resistant *Helicobacter* pylori infections in a prospective study. *Clin. Gastroenterol. Hepatol.* 11:e801. doi: 10.1016/j.cgh.2013.01.008

Liu, W. Z., Xie, Y., Lu, H., Cheng, H., Zeng, Z. R., Zhou, L. Y., et al. (2018). Fifth Chinese National Consensus Report on the management of *Helicobacter* pylori infection. *Helicobacter* 23, e12475. doi: 10.1111/hel.12475

Malfertheiner, P., Megraud, F., O'Morain, C. A., Gisbert, J. P., Kuipers, E. J., Axon, A. T., et al. (2017). Management of *Helicobacter* pylori infection-the Maastricht V/Florence Consensus Report. *Gut* 66, 6–30. doi: 10.1136/gutjnl-2016-312288

Matsumoto, H., Shiotani, A., and Graham, D. Y. (2019). Current and Future Treatment of *Helicobacter* pylori Infections. *Adv. Exp. Med. Biol.* 1149, 211–225.

Megraud, F., Bruyndonckx, R., Coenen, S., Wittkop, L., Huang, T. D., Hoebeke, M., et al. (2021). *Helicobacter* pylori resistance to antibiotics in Europe in 2018 and its relationship to antibiotic consumption in the community. *Gut* 70, 1815–1822. doi: 10.1136/gutjnl-2021-324032

Pan, J., Shi, Z., Lin, D., Yang, N., Meng, F., Lin, L., et al. (2020). Is tailored therapy based on antibiotic susceptibility effective ? a multicenter, open-label, randomized trial. *Front. Med.* 14, 43–50. doi: 10.1007/s11684-019-0706-8

Qiao, C., Li, Y., Liu, J., Ji, C., Qu, J., Hu, J., et al. (2021). Clarithromycin versus furazolidone for naive *Helicobacter* pylori infected patients in a high clarithromycin resistance area. *J. Gastroenterol. Hepatol.* 36, 2383–2388. doi: 10.1111/jgh.15468

Sebghatollahi, V., Soheilipour, M., Khodadoostan, M., and Shavakhi, A. (2018). Levofloxacin-containing versus Clarithromycin-containing Therapy for *Helicobacter* pylori Eradication: A Prospective Randomized Controlled Clinical Trial. *Adv. Biomed. Res.* 7:55.

Song, Z., Suo, B., Zhang, L., and Zhou, L. (2016). Rabeprazole, Minocycline, Amoxicillin, and Bismuth as First-Line and Second-Line Regimens for *Helicobacter* pylori Eradication. *Helicobacter* 21, 462–470. doi: 10.1111/hel.12313

Sugano, K., Tack, J., Kuipers, E. J., Graham, D. Y., El-Omar, E. M., Miura, S., et al. (2015). Kyoto global consensus report on *Helicobacter* pylori gastritis. *Gut* 64, 1353–1367.

Wang, Y. C., Lin, T. Y., Shang, S. T., Chen, H. J., Kao, C. H., Wu, C. C., et al. (2017). *Helicobacter* pylori infection increases the risk of adult-onset asthma: a nationwide cohort study. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 1587–1594. doi: 10.1007/s10096-017-2972-1

Xie, Y., Zhang, Z., Hong, J., Liu, W., Lu, H., Du, Y., et al. (2018). Furazolidone-containing triple and quadruple eradication therapy for initial treatment for *Helicobacter* pylori infection: A multicenter randomized controlled trial in China. *Helicobacter* 23:e12496. doi: 10.1111/hel.12496

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# The influence of gastric atrophy on *Helicobacter pylori* antibiotics resistance in therapy-naïve patients

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**Background:** Antibiotic susceptibility of *Helicobacter pylori* to antibiotics may vary among different niches of the stomach. The progression of chronic *H. pylori* gastritis to atrophy changes intragastric physiology that may influence selection of resistant strains.

**Aim:** To study the antibiotic resistance of *H. pylori* taking the severity of atrophic gastritis in antrum and corpus into account.

**Methods:** *Helicobacter pylori*-positive patients ($n$=110, $m$=32, mean age 52.6±13.9years) without prior *H. pylori* eradication undergoing upper gastrointestinal (GI) endoscopy for dyspeptic symptoms were included in a prospective study. Patients were stratified into three groups depending on the grade of atrophy: no atrophy (OLGA Stage 0), mild atrophy (OLGA Stage I–II) and moderate/severe atrophy (OLGA Stage III–IV). Two biopsies each from the antrum and the corpus and one from the angulus were taken and assessed according to the updated Sydney system. *H. pylori* strains were isolated from antrum and corpus biopsies and tested for antibiotic susceptibility (AST) for amoxicillin, clarithromycin, metronidazole, levofloxacin, tetracycline, and rifampicin by the agar dilution methods. A Chi-square test of independence with a 95% confidence interval was used to detect differences in the proportion of patients with susceptible and resistant *H. pylori* strains.

**Results:** Among 110 patients, primary clarithromycin resistance (R) was 30.0%, both in the antrum and corpus; metronidazole resistance accounted for 36.4 and 34.5% in the antrum and corpus; and levofloxacin was 19.1 and 22.7% in the antrum and corpus, respectively. Resistance rates to amoxicillin,

tetracycline, and rifampicin were below 5%. Dual antibiotic resistance rate was 21.8%, and triple resistance rate was 9.1%. There was a significant difference in the resistance rate distribution in antrum ($p$<0.0001) and corpus ($p$<0.0001). With increasing severity of atrophy according to OLGA stages, there was a significant increase in clarithromycin-R and metronidazole-R.

**Conclusion:** In treatment-naïve patients, antibiotic resistance and heteroresistance were related to the severity of atrophy. The high clarithromycin resistance in atrophic gastritis suggests that *H. pylori* antibiotic susceptibility testing should always be performed in this condition before selecting the eradication regimen.

KEYWORDS

**antibiotic resistance rate, antibiotic susceptibility testing, antibiotic stewardship, updated treatment strategies, *Helicobacter pylori* infection, chronic atrophic gastritis, intestinal metaplasia**

## Introduction

*Helicobacter pylori* treatment regimens require the combination of an acid suppressant with two and up to three antibiotics (Sugano et al., 2015; Malfertheiner et al., 2017).

Resistance (R) to commonly used antibiotics (e.g., clarithromycin, metronidazole, and levofloxacin) has dramatically increased and is the main cause of treatment failure in *H. pylori* eradication. The overuse of these antibiotics for other indications (Chey et al., 2017; Fischbach et al., 2017; Malfertheiner et al., 2017) is the most likely explanation for the increasing antibiotic resistance (Austin et al., 1999; Canton and Morosini, 2011; Tacconelli et al., 2018). According to the World Health Organization (WHO), clarithromycin-resistant *H. pylori* is among bacteria with high priority for developing new antibiotics (Tacconelli et al., 2018). Important measures are undertaken by national and international networks for shriveling local, regional and national resistance development (European Study Group on Antibiotic Susceptibility of Helicobacter pylori, 1992; Glupczynski et al., 2001; Megraud et al., 2013; Wuppenhorst et al., 2014). Antibiotic stewardship (ABS) is advocated to better handle the emerging resistance by adopting therapies based on antibiotic susceptibility testing (AST).

In current practice, *H. pylori* eradication regimens in naïve patients are mostly prescribed empirically. A minority of patients undergoes AST before receiving first-line eradication therapy. In a recent European multicentric surveillance study (Hp-EuReg) only 11.1% of 21,533 underwent AST before first-line eradication therapy (Nyssen et al., 2021).

An issue to consider when using a single biopsy for AST is the possibility of synchronous presence of resistant and susceptible strains in the same individual (interniche heteroresistance), a phenomenon reported in 10–20% of cases. Gastric luminal and mucosal factors related to the degree of inflammation, grade of atrophy and gastric pH might interfere with bacterial metabolism and change drug pharmacokinetics. *H. pylori* gastritis is the prototypic environmental, not self-limiting gastritis. *H. pylori* infection leads to a cytotoxic damage of the resident glandular population (i.e., oxyntic glands in the corpus/fundus; mucosecreting glands in the antral mucosa) and it can progress to a loss of native glandular units (atrophy)" (Rugge et al., 2019). These changes in the gastric histology pattern and, consequently, in the gastric physiology (i.e., acid secretion, achlorhydria) influence the bioavailability of antibiotics used in eradication regimens (Mégraud, 2004; Suerbaum and Josenhans, 2007; Wueppenhorst et al., 2009; Mascellino et al., 2017; Ailloud et al., 2019; Kocsmár et al., 2021). Variations in gastric pH can alter the solubility or chemical stability of molecules such as beta-lactams, macrolides, and some azoles; consequently, the bioavailability of these drugs can be reduced if the gastric pH is raised, e.g., by PPI therapy or loss of acid-producing cells. Antibiotics such as tetracycline or fluoroquinolones have reduced bioavailability due to chelation from bi- and tri-valent cations (Kramer et al., 1978; Rugge et al., 2007; Mazzei, 2011). Regarding clarithromycin, it has very poor solubility at neutral intestinal pH, but much better solubility under acidic conditions due to amine protonation. The improved solubility in an acidic environment is based on the poor chemical stability of clarithromycin that is quite labile toward acid-catalyzed degradation (Capelle et al., 2010; Pereira et al., 2013).

The aims of our study are (a) to assess the primary antibiotic resistances in the different stages of severity of chronic atrophic gastritis, and (b) to analyze the relative prevalence of primary resistance in distinct anatomical sites of the stomach. The analysis included AST to amoxicillin, clarithromycin, metronidazole, levofloxacin, tetracycline and rifampicin.

# Materials and methods

## Ethical statement

All investigations were performed at the Department of Gastroenterology, Hepatology, and Infectious Diseases at Otto-von-Guericke University Magdeburg (Germany) from 2011 to 2015. The study was approved by the local Ethics Committee (IRB number 80/11, Otto-von-Guericke University Magdeburg). The study protocol was conducted according to the Declaration of Helsinki and Good Clinical Practice. All study participants provided written informed consent. This work was supported in part by a grant from the BMBF (BMBF-0315905D) within the ERA-Net PathoGenoMics project. Evaluation of data was performed in cooperation with the Medical Department 2 of the Ludwig Maximillian University Munich, Germany.

## Study design

One hundred ten *H. pylori* infected therapy-naïve patients requiring upper gastrointestinal (GI) endoscopy for dyspeptic symptoms were enrolled prospectively from 2011 to 2015. The following exclusion criteria were applied: previous *H. pylori* eradication therapies, operated stomach, upper abdomen irradiation, immunosuppressive therapy, oral anticoagulation, and any antibiotic therapy within the last 2 weeks before entering the study. Ongoing or prior proton pump inhibitor (PPI) therapy was not an exclusion criterion as many patients were already treated with PPI before being appointed for upper endoscopy.

## Methods

Standard video gastroscopes (GIF Q145, GIF 160, and GIF Q180 HD; Olympus Medical, Hamburg, Germany) and standard oval fenestrated cup forceps with a needle (Olympus SwingJaw 2.8 mm FB-240 K_A, Olympus Medical, Hamburg, Germany) were used.

Two biopsies each from the antrum and the corpus and one from the angulus were obtained for the updated Sydney system (lesser and larger curvature of the antrum at 3 cm distance from the pylorus, larger and lesser curvature of the middle corpus). These were immediately fixed in buffered formalin for histopathological assessment. According to OLGA, the degree of atrophic gastritis was staged (Cederbrant et al., 1993; Megraud and Lehours, 2007). One biopsy from the antrum and one biopsy from the corpus were taken for the *H. pylori* culture and stored immediately in Portagerm pylori® tubes (bioMerieux, France). According to the updated Sydney system, a histopathological assessment of the gastric mucosa was performed. Sections were stained with hematoxylin and eosin, PAS staining technique and modified Giemsa to diagnose *H. pylori*.

The culture was performed on a Columbia-agar-based medium that contained 10 vol% washed human erythrocytes and 10 vol% heat-inactivated horse serum (purchased from the NRZ Nationale Referenzzentrum Helicobacter Freiburg, Germany) without and with an antibiotic supplement (vancomycin 10 mg/ml, nystatin 1 mg/ml, and trimethoprim 5 mg/ml) for suppressing the overgrowth of the oral flora. Incubation of the plates was performed under microaerophilic conditions at 37°C with CampyGen™ gasbags (Oxoid, Germany), and examination was done every 2–3 days for up to 10 days. Identification of *H. pylori* was performed by typical morphology on Gram stain and positive urease, oxidase and catalase tests (Glupczynski et al., 1991).

Susceptibility testing to amoxicillin, metronidazole, clarithromycin, tetracycline, levofloxacin and rifampicin was performed with the ETEST method (bioMerieux, France) on Iso-Sensitest agar with 10 vol% defibrinated horse blood (Oxoid, Germany; Selgrad et al., 2014; Bluemel et al., 2020). The ETEST can detect antibiotic-resistant sub-populations. In this study, agar plates were treated with suspensions of *H. pylori* after adjustment to turbidity approximately equal to that of a McFarland standard No. 3. The antibiotics' minimum inhibitory concentrations (MICs) were determined after 3 days of incubation or until the inhibition zone became visible.

The EUCAST criteria were applied for all antibiotic substances tested within this study (European Committee on Antimicrobial Susceptibility Testing). Breakpoint tables for interpretation of MICs and zone diameters for *H. pylori* Version 2.0, 2012[1] were used. A resistant isolate was defined if the MIC was above the following breakpoints (R): amoxicillin 0.125 mg/l, tetracycline and levofloxacin >1 mg/l, clarithromycin >0.5 mg/l, rifampicin >1 mg/l, and metronidazole >8 mg/l (EUCAST, 2018).

## Statistical analysis

The Chi-square test of independence with a 95% confidence interval was used to detect differences in the proportion of patients with susceptible and resistant *H. pylori* strains. *p*-values were considered significant if $p < 0.05$. Subsequently, Cramér's *V* was estimated for the effect size for the Chi-square test of independence. Cramér's *V* determines the degree of associations between two categories. All analyses were performed in R (version R-4.0.4)[2] and R-studio (version 1.3.9.59; R-studio, Boston, MA, United States).

# Results

One hundred ten *H. pylori*-infected patients were included in the analysis ($n = 110$, $m = 32$, mean age 52.6 ± 13.9 years). None of

1 http://www.eucast.org
2 https://www.r-project.org/

TABLE 1 Single, double, triple and quadruple resistance in antrum and corpus, according to the severity of atrophy.

| OLGA Stage | ANTRUM | | | |
|---|---|---|---|---|
| | Single resistance | Double resistance | Triple resistance | Quadruple resistance |
| No atrophy | 12 | 13 | 2 | 0 |
| (OLGA Stage 0) | (26.1%) | (28.3%) | (4.3%) | |
| $n = 46$ | | | | |
| Mild Atrophy | 4 | 8 | 1 | 1 |
| (OLGA Stage I–II) | (10.0%) | (20.0%) | (2.5%) | (2.5%) |
| $n = 40$ | | | | |
| Moderate/severe atrophic gastritis (OLGA Stage III–IV) | 7 | 3 | 2 | 0 |
| $n = 24$ | (29.2%) | (12.5%) | (8.3%) | |
| CORPUS | | | | |
| No atrophy | 11 | 11 | 2 | 0 |
| (OLGA Stage 0) | (24.0%) | (24.0%) | (4.3%) | |
| $n = 46$ | | | | |
| Mild Atrophy | 13 | 8 | 4 | 1 |
| (OLGA Stage I–II) | (32.5%) | (20.0%) | (10.0%) | (2.5%) |
| $n = 40$ | | | | |
| Moderate/severe atrophic gastritis (OLGA Stage III–IV) | 8 | 4 | 3 | 0 |
| $n = 24$ | (33.3%) | (16.7%) | (12.5%) | |

the patients included in the study had been previously treated for *H. pylori* infection.

Overall, 74 patients ($n = 74$, 68.5%) had one or more resistances in the antrum or the corpus; a dual resistance in both antrum and corpus was observed in 64 patients (58%). The single, double, triple and quadruple resistance rates in antrum and in corpus according to OLGA staging are summarized in Table 1. In the antrum, we found resistance to amoxicillin only in one patient (0.9%), to clarithromycin in 33 patients (30.0%), to metronidazole in 40 patients (36.4%), to levofloxacin in 21 patients (19.1%) and to rifampicin in three patients (2.7%). In one patient (0.9%), we observed resistance to tetracycline in the antrum. A similar distribution in the antibiotic resistance was shown in the corpus: resistance to amoxicillin was present only in one patient (0.9%), to clarithromycin in 33 patients (30.0%), to metronidazole in 38 patients (34.5%), to levofloxacin in 25 patients (22.7%) and to rifampicin in four patients (3.6%). One patient had resistance against tetracycline in the corpus (0.9%). In both the antrum and/or corpus, a dual antibiotic resistance was detected in 24 patients (21.8%) and a triple resistance in ten patients (9.1%). Considering the correlation among resistance, age and sex, we found a statistically significant correlation between antibiotic resistance and age of the patients. Resistances were not influenced by sex of patients.

According to the OLGA Staging System, patients were stratified into three groups: no atrophy (OLGA 0, $n = 46$, Group 1), mild atrophic gastritis (OLGA Stage I–II, $n = 40$, Group 2), and moderate/severe atrophic gastritis (OLGA Stage III–IV, $n = 24$, Group 3). The study population characteristics are summarized in Table 2.

TABLE 2 Study population characteristics ($n$=110).

| ***Helicobacter pylori* therapy naïve patients ($n = 110$)<br>Overall resistance rate $n = 74/110$ (67.3%)** | |
|---|---|
| No atrophy (OLGA Stage 0) $n = 46$ | |
| Sex | Males $n = 14$ |
| Age (y.o ± SD) | 47.7 ± 11.8 |
| Resistance rate (overall) | 30 (65.2%) |
| Mild atrophy (OLGA Stage I–II) $n = 40$ | |
| Sex | Males $n = 9$ |
| Age (y.o ± SD) | 48.2 ± 13.8 years |
| Resistance rate (overall) | 28 (70.0%) |
| Moderate/severe atrophic gastritis (OLGA Stage III–IV) $n = 24$ | |
| Sex | Males $n = 9$ |
| Age (y.o ± SD) | 54.9 ± 14.0 years |
| Resistance rate (overall) | 16 (66.7%) |

y.o: years old; SD: standard deviation.

*H. pylori* was detected in the antrum of 96.2% of patients, in the corpus of 92.5% of patients, and both in antrum and corpus in 88.7% of the patients.

Among patients included in Group 1 (no atrophy OLGA 0, $n = 46$, 14 males, mean age 47.7 ± 11.8 years), 30 patients had at least one antibiotic resistance (overall resistance rate 65.2%) and 26 patients (86.7%) were observed having a dual resistance both in the antrum and in the corpus. Resistance against clarithromycin was found in the antrum of 16 patients (34.8%) and the corpus of 15 patients (32.6%). Fourteen patients showed a dual resistance for clarithromycin in both antrum and corpus. Resistance against metronidazole was demonstrated in 21 patients in the antrum (45.6%) and 20 patients in the corpus (43.5%). Resistance against

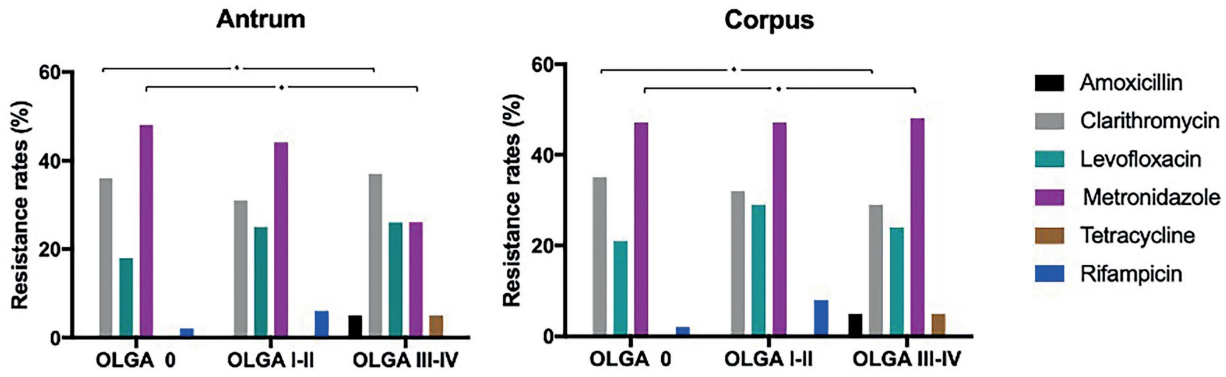


FIGURE 1
Distribution of resistance rate to amoxicillin, clarithromycin, metronidazole, levofloxacin, tetracycline, and rifampicin according to the different grade of atrophic gastritis (OLGA Stage 0, OLGA I–II, OLGA Stage III–IV) in the antrum and corpus. OLGA: Operative Link for Gastritis Assessment. The Chi-square test of independence with a 95% confidence interval was used to detect differences in the proportion of patients with susceptible and resistant *H. pylori* strains. *Clarithromycin (Antrum: $X^2$ Pearson (4)=1.82, $p$=0.03, V Cramer=0.00, Cl 95% 0.00–1.00; corpus: $X^2$ Pearson (4)=3.48, $p$=0.01, V Cramer=0.00, Cl 95% 0.00–1.00) *Metronidazole (Antrum: $X^2$ Pearson (2)=4.95, $p$=0.0098, V Cramer=0.18, Cl 95% 0.00–1.00; Corpus: $X^2$ Pearson (2)=0.62, $p$=0.02, V Cramer=0.00, Cl 95% 0.00–1.00).

levofloxacin was found in eight patients (17.4%) in the antrum, nine patients in the corpus (19.6%) and seven patients in both antrum and corpus. Resistance against rifampicin was observed in one patient in both antrum and corpus (2.2%). Resistance against amoxicillin and tetracycline was not detected in the antrum and the corpus.

The single, double, triple and quadruple resistance rates in Group 1 in antrum and in corpus are summarized in Table 1.

Forty patients were included in Group 2 (mild atrophic gastritis OLGA I-II, n = 40, 9 males, mean age 48.2 ± 13.8 years). Of those, 28 patients had at least one antibiotic resistance (overall resistance rate 70%) and 19 patients (67.8%) were observed a dual resistance both in the antrum and in the corpus. Resistance against clarithromycin was found in the antrum of ten patients (25.0%) and the corpus of 12 patients (30.0%). Nine patients presented with a dual resistance for clarithromycin both in the antrum and corpus. Resistance against metronidazole was demonstrated in 14 patients in the antrum (35.0%) and 17 patients in the corpus (42.5%). Resistance against levofloxacin was found in eight patients (20.0%) in the antrum, in 11 patients (27.5%) in the corpus and in six patients (15.0%) in both antrum and corpus. Resistance against rifampicin was observed in two patients in the antrum (5.0%) and three in the corpus (7.5%). No resistance against amoxicillin and tetracycline was detected both in the antrum and in the corpus in Group 2. The single, double, triple and quadruple resistance rates in Group 2 in antrum and in corpus are reported in Table 1.

Twenty-four patients were diagnosed with moderate-to-severe atrophic gastritis (OLGA Stage III–IV, $n$ = 24, 9 males, mean age 54.9 ± 13 years). *H. pylori* was detected in 96.2% of patients in the antrum, 92.5% in the corpus and 88.7% in both antrum and corpus. Sixteen patients had at least one antibiotic resistance (overall resistance rate 66.7%) and ten patients (62.5%) were observed with a dual resistance both in the antrum and in the corpus.

Resistance against clarithromycin was found in seven patients in the antrum (36.8%), six patients in the corpus (28.6%), and a dual resistance was present in the antrum and the corpus of five patients. Resistance against metronidazole was demonstrated in five patients in the antrum (26.3%), 11 patients in the corpus (52.4%), and five patients (20.8%) in both antrum and corpus. Resistance against levofloxacin was found only as dual resistance—in both antrum and corpus—in five patients (26.3%). One patient had resistance against amoxicillin (5.3%) and one against tetracycline (5.3%). Resistance against rifampicin was not detected in the antrum or the corpus. The single, double, triple and quadruple resistance rates in Group 3 in antrum and in corpus according to OLGA staging are summarized in Table 1.

The overall antibiotic resistance rate among the three groups (OLGA Stage 0 versus OLGA Stage I–II versus OLGA Stage III–IV) is summarized in Figure 1.

Overall, there was a statistically significant difference in the resistance rate distribution to amoxicillin, clarithromycin, metronidazole, levofloxacin, tetracycline, and rifampicin in antrum ($X^2$ Pearson (10) = 112.89, $p$ = 1.39$e$–19, V Cramer = 0.30, Cl 95% 0.23–1.00; Figure 1) and in corpus ($X^2$ Pearson (10) = 131.99, $p$ = 1.83$e$–23, V Cramer = 0.32, Cl 95% 0.25, 1.00; Figure 1).

When comparing OLGA Stage 0 versus OLGA Stage I–II versus OLGA Stage III–IV, our data showed a statistically significant difference in the resistance to clarithromycin ($X^2$ Pearson (4) = 1.82, $p$ = 0.03, V Cramer = 0.00, Cl 95% 0.00–1.00), to metronidazole ($X^2$ Pearson (2) = 4.95, $p$ = 0.0098, V Cramer = 0.18, Cl 95% 0.00–1.00) in antrum, as well as to clarithromycin ($X^2$ Pearson (4) = 3.48, $p$ = 0.01, V Cramer = 0.00, Cl 95% 0.00–1.00) and to metronidazole ($X^2$ Pearson (2) = 0.62, $p$ = 0.02, V Cramer = 0.00, Cl 95% 0.00–1.00) in corpus (Figure 1).

## Discussion

In this study the prevalence of *H. pylori* resistance to clarithromycin and metronidazole increases in parallel to the progression in severity of atrophic gastritis. This is not the case for levofloxacin across all stages of atrophic gastritis. Amoxicillin, tetracycline, and rifampicin resistance were very low, therefore without clinical significance.

The main point of our study is the analysis of susceptibility to key antibiotics used in eradication regimens at different stages of chronic gastritis along with the OLGA staging system. In this therapy-naïve cohort, there is a significant difference in the resistance rate distribution to clarithromycin, metronidazole and levofloxacin, in antrum and corpus, along the cascade of atrophic gastritis.

According to the grade of gastric atrophy, our results showed a significant increase in the prevalence of resistance as well as heteroresistance in general.

When comparing OLGA Stage 0 versus OLGA Stage I–II versus OLGA Stage III–IV, the clarithromycin and metronidazole resistance rates were often different between the antrum and corpus. This was less frequent the case with respect to levofloxacin (18.6–23.8% of resistance). However, in our study, an increased overall resistance rate to levofloxacin compared to earlier studies was observed (Glupczynski et al., 2001; Mégraud, 2004; Megraud et al., 2013; Kocsmár et al., 2021). The same trend was shown to a lesser degree for rifampicin as a reserve antibiotic based on antibiotic susceptibility testing (AST) results. The variations in gastric pH and consequently in the antibiotics bioavailability could explain the statistical difference in the antibiotic resistance rate along the cascade of atrophic gastritis (Kramer et al., 1978; Rugge et al., 2007; Capelle et al., 2010; Mazzei, 2011; Pereira et al., 2013). Furthermore, atrophic gastritis is the long-term expression of *H. pylori* infection and this time-phenotype correlation very likely results in higher exposure to antimicrobial therapies. These factors may all play a crucial role and interact in *H. pylori* resistance development that can occur during infection, resulting in spontaneous mutations (Correa et al., 1975; McGowan Jr., 1983; Harbarth et al., 2001; Albrich et al., 2004; Committee Opinion No. 465, 2010; Ledger and Blaser, 2013; Moss, 2017). According to the current guidelines, due to the increasing number of metronidazole-resistant *H. pylori* strains, metronidazole in first-line treatment is only selectively recommended in Europe (Malfertheiner et al., 2017). Regarding clarithromycin resistance, current guidelines recommend the exclusion of clarithromycin resistance before use or empiric medication depending on local resistance rates (Malfertheiner et al., 2017). However, standardized diagnostic and therapeutic algorithms based on AST in managing *H. pylori* infection could improve the eradication rates and minimize the development of antibiotic resistance worldwide. The WHO recognized the challenge of growing antibiotic resistance rates of *H. pylori* and added this bacterium to 12 pathogens for which new antibiotics are urgently needed (Tacconelli et al., 2018). In several studies, susceptibility-guided therapies showed improved results compared to empirical antibiotic regimes (Wenzhen et al., 2010; Lopez-Gongora et al., 2015; Malfertheiner et al., 2017).

Regarding heteroresistances, in our cohort, patients with mild gastritis (OLGA I–II) had a heteroresistance rate of 45.5%, compared to 81.2% for patients with advanced gastritis (OLGA III–IV). To our knowledge, this is the first study correlating the *H. pylori* antibiotic resistance rate in therapy-naïve patients with the severity of atrophic gastritis; for this reason, limited data on this topic in literature are available. The biological plausibility of this heterogeneous distribution of antibiotic resistance according to the severity of the atrophy remains unclear, but possible explanations are a higher selection pressure on *H. pylori* in severely damaged mucosa and changes in gastric acidity (Kramer et al., 1978; Rugge et al., 2007; Capelle et al., 2010; Mazzei, 2011; Pereira et al., 2013). Furthermore, in our study, more than 60% of antrum samples and more than 70% of corpus samples demonstrated at least one resistance and thus justified the recommendation of early resistance testing, preferably during the first invasive procedure used in the diagnostic workup of patients. Concerning the significant increase and the development of gastric preneoplastic lesions, the subgroup of patients suffering from atrophic gastritis should be highlighted. Several studies demonstrated different antibiotic susceptibilities between antrum and corpus in eradication-naïve and eradication-failed subjects. Various methods are established for *in vitro* susceptibility testing. PCR-based testing opens the AST for additional materials, such as feces and embedded biopsies. This evidence of interniche heteroresistance of *H. pylori* represents a relevant problem in clinical practice and the concomitant presence of *H. pylori* strains with different resistance spectrums in the same patient is likely to cause treatment failure and increase resistant strains' selection; consequently our findings contribute to the ongoing debate regarding the timepoint of AST.

Our study has some limitations. Like most studies in this field, our work was based on the patient history of previous antibiotic exposure, which is not always reliable. The enrollment of subjects in only one tertiary referral center does not allow for the interpretation of quantitative resistance rates, but the relationship between the stratified groups following histopathological results is useful (Kim et al., 2003; Mégraud, 2004; Rimbara et al., 2005; Ayala et al., 2011).

In conclusion, our results report the different distributions of resistance prevalence according to the grade of gastric atrophy. Our findings underline the importance of early AST in the therapeutic algorithm of *H. pylori* infection, especially in patients with moderate/severe atrophy.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving human participants were reviewed and approved by Otto-von-Guericke University Magdeburg. IRB number 80/11. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

CS, EG, KS, and PM: conceptualization and writing—original draft preparation. CS, IT, and DJ: methodology. UM: formal analysis. BA and LM assisted with data analysis. EG, CS, AL, MS, and KS: investigation. EG, RV, KS, AL, and MS: patients' enrollment. CT and AL: biobanking. EG, CS, and KS: data curation. PM and CS: supervision. CS: project administration. PM, KS, and CS: funding acquisition. Each author has approved the submitted version and agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. Each author contributed to the conception, design of the work, acquisition, and analysis and interpretation of data. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

Ailloud, F., Didelot, X., Woltemate, S., Pfaffinger, G., Overmann, J., Bader, R. C., et al. (2019). Within-host evolution of *Helicobacter pylori* shaped by niche-specific adaptation, intragastric migrations and selective sweeps. *Nat. Commun.* 10:2273. doi: 10.1038/s41467-019-10050-1

Albrich, W. C., Monnet, D. L., and Harbarth, S. (2004). Antibiotic selection pressure and resistance in *Streptococcus pneumoniae* and *Streptococcus pyogenes*. *Emerg. Infect. Dis.* 10, 514–517. doi: 10.3201/eid1003.030252

Austin, D. J., Kristinsson, K. G., and Anderson, R. M. (1999). The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc. Natl. Acad. Sci. U S A.* 96, 1152–1156. doi: 10.1073/pnas.96.3.1152

Ayala, G., Galvan-Portillo, M., Chihu, L., Fierros, G., Sanchez, A., Carrillo, B., et al. (2011). Resistance to antibiotics and characterization of *Helicobacter pylori* strains isolated from antrum and body from adults in Mexico. *Microb. Drug Resist.* 17, 149–155. doi: 10.1089/mdr.2010.0154

Bluemel, B., Goelz, H., Goldmann, B., Gruger, J., Hamel, H., Loley, K., et al. (2020). Antimicrobial resistance of *Helicobacter pylori* in Germany, 2015 to 2018. *Clin. Microbiol. Infect.* 26, 235–239. doi: 10.1016/j.cmi.2019.06.007

Canton, R., and Morosini, M. I. (2011). Emergence and spread of antibiotic resistance following exposure to antibiotics. *FEMS Microbiol. Rev.* 35, 977–991. doi: 10.1111/j.1574-6976.2011.00295.x

Capelle, L. G., de Vries, A. C., Haringsma, J., Ter Borg, F., de Vries, R. A., Bruno, M. J., et al. (2010). The staging of gastritis with the OLGA system by using intestinal metaplasia as an accurate alternative for atrophic gastritis. *Gastrointest Endosc.* 71, 1150–1158. doi: 10.1016/j.gie.2009.12.029

Cederbrant, G., Kahlmeter, G., and Ljungh, A. (1993). The E test for antimicrobial susceptibility testing of *Helicobacter pylori*. *J. Antimicrob. Chemother.* 31, 65–71.

Chey, W. D., Leontiadis, G. I., Howden, C. W., and Moss, S. F. (2017). ACG Clinical Guideline: Treatment of *Helicobacter pylori* Infection. *Am. J. Gastroenterol.* 112, 212–239. doi: 10.1038/ajg.2016.563

Committee Opinion No. 465 (2010). Antimicrobial prophylaxis for cesarean delivery: timing of administration. *Obstet Gynecol.* 116, 791–792. doi: 10.1097/AOG.0b013e3181f68086

Correa, P., Haenszel, W., Cuello, C., Tannenbaum, S., and Archer, M. (1975). A model for gastric cancer epidemiology. *Lancet.* 2, 58–60. doi: 10.1016/s0140-6736(75)90498-5

EUCAST (2018). Breakpoint Tables for Interpretation of MICs and Zone Diameters Version 8.1. Available at: http://www.eucast.org/clinical_breakpoints (Accessed February 02, 2021).

European Study Group on Antibiotic Susceptibility of Helicobacter pylori (1992). Results of a multicentre European survey in 1991 of metronidazole resistance in *Helicobacter pylori*. *Eur. J. Clin. Microbiol. Infect. Dis.* 11, 777–781. doi: 10.1007/BF01960875

Fischbach, W., Malfertheiner, P., Jansen, P. L., Bolten, W., Bornschein, J., Buderus, S., et al. (2017). 2S2k-guideline *Helicobacter pylori* and gastroduodenal ulcer disease. *Z. Gastroenterol.* 55, 167–206. doi: 10.1055/s-0042-119653

Glupczynski, Y., Labbe, M., Hansen, W., Crokaert, F., and Yourassowsky, E. (1991). Evaluation of the E test for quantitative antimicrobial susceptibility testing of *Helicobacter pylori*. *J. Clin. Microbiol.* 29, 2072–2075. doi: 10.1128/jcm.29.9.2072-2075.1991

Glupczynski, Y., Megraud, F., Lopez-Brea, M., and Andersen, L. P. (2001). European multicentre survey of *in vitro* antimicrobial resistance in *Helicobacter pylori*. *Eur. J. Clin. Microbiol. Infect. Dis.* 20, 820–823. doi: 10.1007/s100960100611

Harbarth, S., Harris, A. D., Carmeli, Y., and Samore, M. H. (2001). Parallel analysis of individual and aggregated data on antibiotic exposure and resistance in gram-negative bacilli. *Clin. Infect. Dis.* 33, 1462–1468. doi: 10.1086/322677

Kim, J. J., Kim, J. G., and Kwon, D. H. (2003). Mixed-infection of antibiotic susceptible and resistant *Helicobacter pylori* isolates in a single patient and underestimation of antimicrobial susceptibility testing. *Helicobacter* 8, 202–206. doi: 10.1046/j.1523-5378.2003.00145.x

Kocsmár, E., Buzás, G. M., Szirtes, I., Kocsmár, I., Kramer, Z., Szijártó, A., et al. (2021). Primary and secondary clarithromycin resistance in *Helicobacter pylori* and mathematical modeling of the role of macrolides. *Nat. Commun.* 12:2255. doi: 10.1038/s41467-021-22557-7

Kramer, P. A., Chapron, D. J., Benson, J., and Mercik, S. A. (1978). Tetracycline absorption in elderly patients with achlorhydria. *Clin. Pharmacol. Ther.* 23, 467–472. doi: 10.1002/cpt1978234467

Ledger, W. J., and Blaser, M. J. (2013). Are we using too many antibiotics during pregnancy? *BJOG.* 120, 1450–1452. doi: 10.1111/1471-0528.12371

Lopez-Gongora, S., Puig, I., Calvet, X., Villoria, A., Baylina, M., Munoz, N., et al. (2015). Systematic review and meta-analysis: susceptibility-guided versus empirical antibiotic treatment for *Helicobacter pylori* infection. *J. Antimicrob. Chemother.* 70, 2447–2455. doi: 10.1093/jac/dkv155

Malfertheiner, P., Megraud, F., O'Morain, C. A., Gisbert, J. P., Kuipers, E. J., Axon, A. T., et al. (2017). Management of *Helicobacter pylori* infection-the Maastricht V/Florence Consensus Report. *Gut.* 66, 6–30. doi: 10.1136/gutjnl-2016-312288

Mascellino, M. T., Porowska, B., De Angelis, M., and Oliva, A. (2017). Antibiotic susceptibility, heteroresistance, and updated treatment strategies in *Helicobacter pylori* infection. *Drug. Des. Devel. Ther.* 11, 2209–2220. doi: 10.2147/DDDT.S136240

Mazzei, T. (2011). The difficulties of polytherapy: examples from antimicrobial chemotherapy. *Intern. Emerg. Med.* 6, 103–109. doi: 10.1007/s11739-011-0680-x

McGowan, J. E. Jr. (1983). Antimicrobial resistance in hospital organisms and its relation to antibiotic use. *Rev. Infect. Dis.* 5, 1033–1048.

Mégraud, F. (2004). *H pylori* antibiotic resistance: prevalence, importance, and advances in testing. *Gut* 53, 1374–1384. doi: 10.1136/gut.2003.022111

Megraud, F., Coenen, S., Versporten, A., Kist, M., Lopez-Brea, M., Hirschl, A. M., et al. (2013). *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut.* 62, 34–42. doi: 10.1136/gutjnl-2012-302254

Megraud, F., and Lehours, P. (2007). *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin. Microbiol. Rev.* 20, 280–322. doi: 10.1128/CMR.00033-06

Moss, S. F. (2017). The clinical evidence linking *Helicobacter pylori* to gastric cancer. *Cell Mol Gastroenterol Hepatol.* 3, 183–191. doi: 10.1016/j.jcmgh.2016.12.001

Nyssen, O. P., Bordin, D., Tepes, B., Perez-Aisa, A., Vaira, D., Caldas, M., et al. (2021). European Registry on *Helicobacter pylori* management (Hp-EuReg): patterns and trends in first-line empirical eradication prescription and outcomes of 5 years and 21 533 patients. *Gut* 70, 40–54. doi: 10.1136/gutjnl-2020-321372

Pereira, J. M., Mejia-Ariza, R., Ilevbare, G. A., McGettigan, H. E., Sriranganathan, N., Taylor, L. S., et al. (2013). Interplay of degradation, dissolution and stabilization of clarithromycin and its amorphous solid dispersions. *Mol. Pharm.* 10, 4640–4653. doi: 10.1021/mp400441d

Rimbara, E., Noguchi, N., Tanabe, M., Kawai, T., Matsumoto, Y., and Sasatsu, M. (2005). Susceptibilities to clarithromycin, amoxycillin and metronidazole of *Helicobacter pylori* isolates from the antrum and corpus in Tokyo, Japan, 1995-2001. *Clin. Microbiol. Infect.* 11, 307–311. doi: 10.1111/j.1469-0691.2005.01099.x

Rugge, M., Meggio, A., Pennelli, G., Piscioli, F., Giacomelli, L., De Pretis, G., et al. (2007). Gastritis staging in clinical practice: the OLGA staging system. *Gut.* 56, 631–636. doi: 10.1136/gut.2006.106666

Rugge, M., Sugano, K., Scarpignato, C., Sacchi, D., Oblitas, W. J., and Naccarato, A. G. (2019). Gastric cancer prevention targeted on risk assessment: gastritis OLGA staging. *Helicobacter.* 24:e12571. doi: 10.1111/hel.12571

Selgrad, M., Tammer, I., Langner, C., Bornschein, J., Meissle, J., Kandulski, A., et al. (2014). Different antibiotic susceptibility between antrum and corpus of the stomach, a possible reason for treatment failure of *Helicobacter pylori* infection. *World J. Gastroenterol.* 20, 16245–16251. doi: 10.3748/wjg.v20.i43.16245

Suerbaum, S., and Josenhans, C. (2007). *Helicobacter pylori* evolution and phenotypic diversification in a changing host. *Nat. Rev. Microbiol.* 5, 441–452. doi: 10.1038/nrmicro1658

Sugano, K., Tack, J., Kuipers, E. J., Graham, D. Y., El-Omar, E. M., Miura, S., et al. (2015). Kyoto global consensus report on *Helicobacter pylori* gastritis. *Gut.* 64, 1353–1367. doi: 10.1136/gutjnl-2015-309252

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., et al. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis.* 18, 318–327. doi: 10.1016/S1473-3099(17)30753-3

Wenzhen, Y., Yumin, L., Quanlin, G., Kehu, Y., Lei, J., Donghai, W., et al. (2010). Is antimicrobial susceptibility testing necessary before first-line treatment for *Helicobacter pylori* infection? meta-analysis of randomized controlled trials. *Intern Med.* 49, 1103–1109. doi: 10.2169/internalmedicine.49.3031

Wueppenhorst, N., Stueger, H. P., Kist, M., and Glocker, E. (2009). Identification and molecular characterization of triple- and quadruple-resistant *Helicobacter pylori* clinical isolates in Germany. *J Antimicrob Chemother.* 63, 648–653. doi: 10.1093/jac/dkp003

Wuppenhorst, N., Draeger, S., Stuger, H. P., Hobmaier, B., Vorreiter, J., Kist, M., et al. (2014). Prospective multicentre study on antimicrobial resistance of *Helicobacter pylori* in Germany. *J Antimicrob Chemother.* 69, 3127–3133. doi: 10.1093/jac/dku243

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# *Lactobacillus rhamnosus* GG supplementation on eradication rate and dyspepsia in *Helicobacter pylori* infection treated with three-in-one bismuth quadruple therapy

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**Introduction:** *Helicobacter pylori* (Hp)-related dyspepsia has been related to gastroduodenal dysbiosis. The role of probiotic supplementation in the clinical management of Hp infection has been the object of several studies in terms of improvement of efficacy and tolerability of eradication treatments but data on their effects on the outcomes of post-eradication dyspepsia are lacking. The aim of the present study was to evaluate the influence of *Lactobacillus rhamnosus* GG (LGG) supplementation on bismuth quadruple therapy (BQT) in the clinical management of Hp-related infection both in terms of efficacy and tolerability and persistence of post-treatment dyspepsia.

**Methods:** A total of 164 (121 women) Hp-positive adult patients were enrolled in this pilot study and assigned to two different treatment regimens: group A received BQT for 10 days (three capsules qid, IPP bid) and group B received BQT for 10 days in combination with $6 \times 10^9$ CFU LGG (ATCC53103) taken for 24 days (7 days before, 10 days during, and 7 days after therapy). Eradication was assessed after 45 days using the $^{13}$C-urea breath test ($^{13}$C-UBT). Dyspepsia, distinguished into postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS), was assessed at the time of enrollment and 6 months after eradication.

**Results:** Approximately 98 patients were enrolled in group A and 66 patients in group B. At the enrollment, dyspepsia was present in 76.5% of group A and 86.5% of group B. No significant differences were observed in eradication rate between the 2 groups, both in intention-to-treat (ITT) analysis (82.3 vs. 75.0%) and per-protocol (PP) analysis (95 vs. 96%), and in the presence of side effects during the treatment (70.6 vs. 65.4%). At 6 months after eradication of Hp infection, the persistence of dyspepsia was statistically higher in patients of group A than in group B (38.8 vs. 16.1%; $p = 0.032$). The positive influence of LGG supplementation in improving post-eradication dyspepsia resulted in

statistically more effectiveness in PDS dyspepsia, whose remission was 41.7% in group A and 84% in group B patients ($p$ = 0.011).

**Conclusion:** In conclusion, LGG supplementation during Hp eradication therapy, even if not affecting eradication rates and therapy-related side effects, significantly impacts the remission of dyspepsia.

KEYWORDS

***Helicobacter pylori*, dyspepsia, postprandial distress syndrome, epigastric pain syndrome, probiotics, bismuth, gastric dysbiosis**

# Introduction

Recent evidence has shown that *Helicobacter pylori* (Hp) infection is strongly associated with dyspepsia (Kim et al., 2017) which is likely related to Hp-induced gastric and duodenal mucosal inflammation and gastric acid secretion impairment (Hall et al., 2003; Vanheel et al., 2014; Burkitt et al., 2017). Recent consensus on functional dyspepsia indicates that Hp status should be determined in every patient with dyspeptic symptoms and Hp-positive patients should receive eradication therapy (Wauters et al., 2021). Data on improvement in dyspeptic symptoms after Hp eradication are controversial. Although the improvement of dyspeptic symptoms has been reported in a minority of cases (Talley, 2016), a meta-analysis found conflicting results on the improvement of dyspepsia after Hp eradication (Du et al., 2016).

The symptomatic improvement obtained with probiotic treatments highlights the possible influence of gastric dysbiosis in dyspepsia (Cremonini et al., 2001; Nakae et al., 2016; Ohtsu et al., 2017; Tziatzios et al., 2020; Wauters et al., 2020). The efficacy of eradication treatment on the improvement of dyspepsia, which is associated with the type of antibiotic used, also suggests a possible role of dysbiosis in the onset and persistence of dyspepsia after Hp eradication (Kim et al., 2017). Indeed, Hp infection could create a microenvironment that facilitates the proliferation of some bacterial species, despite beneficial ones, that often perpetuates after the infection eradication, partially due to irreversible mucosal alterations caused by Hp infection, and that creates a new gastric microenvironment favored by host-related factors (Gomez-Ramirez et al., 2021).

Considering the evidence that Hp infection determines alterations in the composition of gastric microbiota (Nardone and Compare, 2015; Klymiuk et al., 2017; Bruno et al., 2018), several studies have been conducted to understand if probiotics may interact with the gastric microbiota bringing benefits in the clinical management of Hp infection (Westerik et al., 2018; Zagari et al., 2018; Fang et al., 2019; Yuan et al., 2021). To this aim, various types of probiotics have been used in combination with different antibiotic-based therapeutic regimens to determine a possible improvement in eradication rate, tolerability, and compliance. Results from laboratory studies and clinical trials appear to confirm expectations but there is a lack of clarity regarding standardization on probiotic type, dosage, and time of administration (Westerik et al., 2018; Zagari et al., 2018; Fang et al., 2019; Yuan et al., 2021). Several previous studies have evaluated the benefits of *Lactobacillus rhamnosus* GG (LGG) supplementation in combination with clarithromycin-based treatment regimens, triple therapy, and three-in-one bismuth quadruple therapy (BQT) in terms of eradication rate and tolerability of therapy (Zagari et al., 2018; Fang et al., 2019). LGG is one of the most extensively studied bacteria (Capurso, 2019), with its efficacy both in terms of reducing bacterial load (Do et al., 2021) and improving eradication rate in Hp infection (Zheng et al., 2013; Lü et al., 2016; Chen et al., 2021). In addition, LGG has been successfully used in pediatric functional pathology, demonstrating efficacy also in reducing dyspeptic symptoms (Ding et al., 2019). However, to the best of our knowledge, data on probiotic supplementation to evaluate the improvement of dyspeptic symptoms in eradication therapy are lacking. The aim of the present study was to evaluate the influence of LGG supplementation on BQT in the clinical management of Hp-related infection both in terms of efficacy and tolerability and persistence of post-treatment dyspepsia.

# Materials and methods

## Study design

The observational pilot study was conducted on Hp-positive patients requiring eradication treatment that referred to the Gastritis Outpatient Clinic of the Gastroenterology Unit of the University Hospital, Policlinico Umberto I in Rome, from 2018 to 2020. BQT (three-in-one BQT capsule containing 140 mg bismuth subcitrate potassium, 125 mg tetracycline, and 125 mg metronidazole) was chosen as the first choice treatment given the high level of clarithromycin and metronidazole resistance currently found in Italy (Hu et al., 2016; Thung et al., 2016; Malfertheiner et al., 2017; Fiorini et al., 2018; Savoldi et al., 2018; Fallone et al., 2019; Romano et al., 2022).

Bismuth quadruple therapy eradication schedule was as follows: three capsules four times a day (after main meals and before bedtime), in addition to omeprazole 20 mg two times a day (before breakfast and before dinner) for 10 days. Enrolled patients were assigned to two different treatment regimens, individually chosen at the time of the first visit (**Figure 1**). Group A received the sole BQT eradication treatment plus omeprazole 20 mg bid for 10 days while group B received BQT eradication treatment plus omeprazole 20 mg bid for 10 days with supplementation of six million CFU (colony-forming units) of LGG (strain ATCC 53103) for 24 days, given 7 days before BQT, 10 days during BQT, and 7 days after BQT.

Due to the costs of the probiotic supplementation, this arm of the regimen was a free choice of the patient after appropriate informed consent. The sample size has been assessed on the number of patients who needed to be treated on the basis of 90% (95% CI: 87–92%) BQT eradication rate reported in the literature (Zagari et al., 2018; Nyssen et al., 2021) that resulted in 124 patients. A similar calculation of sample size was not possible for the evaluation of the effects of LGG supplementation on dyspepsia recovery since no data are yet available.

All study participants gave their written informed consent prior to sampling.

## Study subjects

Patients were enrolled based on the following inclusion and exclusion criteria. Inclusion criteria were the age of >18 years, active Hp infection diagnosed by $^{13}$C-urea breath test ($^{13}$C-UBT), or gastric histology. Exclusion criteria were pregnancy status, antibiotic therapy in the month before the enrollment, known allergies to administered drugs, and previous oesophagogastric surgery. At the enrollment time, for each patient, demographic data regarding age, weight, height, and BMI were collected, and a standardized questionnaire, according to the Rome criteria, for the presence of dyspepsia (Drossman, 2016; Drossman and Hasler, 2016) was administered. During the visit, the patient was given the eradication therapy schedule, according to the assigned group, and a questionnaire to complete at home to assess the possible side effects of the therapy.

## Dyspepsia assessment

Dyspepsia was assessed at the enrollment and the follow-up visit, 6 months after the end of treatment. Dyspepsia was defined by the presence of at least one of the following symptoms: (a) postprandial fullness; (b) early satiety; (c) epigastric pain; and (d) epigastric burning. Dyspepsia was distinguished into postprandial distress syndrome PDS and epigastric pain syndrome (EPS) (Stanghellini et al., 2016). The diagnostic criteria defining PDS included one or both of the following, on at least 3 days/week: 1. the postprandial feeling of fullness (e.g., sufficiently severe to have a negative impact on usual activities) and 2. early satiety. The diagnostic criteria defining EPS included at least one of the following symptoms, at least 1 day/week: 1. epigastric pain and 2. epigastric burning.

## Evaluation of side effects

Therapy side effects related to the treatment were evaluated through a standardized questionnaire aimed to qualitatively and quantitatively assess the adverse events encountered during the 10 days of eradication therapy. The completed questionnaire was returned on the day of the 6-month follow-up visit. Side effects were stratified into the following two groups: 1. gastrointestinal (GI) events (diarrhea, constipation, black stools, abdominal bloating and pain, retrosternal burning and pain, nausea, vomiting, and postprandial fullness) and 2. neurovegetative system-related disorders (dysgeusia, headache, and dizziness).

## Evaluation of eradication rate

Eradication treatment outcome was evaluated 45 days after the end of antibiotic therapy by $^{13}$C-UBT. Patients with a delta over baseline (DOB) $\leq$ 3.5% were considered negative.

## Statistical analysis

The MedCalc Statistical Software (MedCalc, Ostend, Belgium) was used for statistical analysis that was conducted by considering separately intention-to-treat (ITT) and per-protocol (PP) groups. For the ITT analysis, all patients to whom eradication therapy was prescribed were considered. For the PP analysis, only those patients who completed eradication therapy, verified eradication by UBT, completed the treatment-related side effect questionnaire, and returned for the 6-month follow-up visit for dyspepsia reassessment were considered. Data are expressed as median (95% CI) and analyzed by Fisher's exact test and Mann–Whitney U test; $p$-value $< 0.05$ is considered statistically significant.

# Results

A total of 164 patients with active Hp infection were enrolled, of whom 73.8% (121/164) were women. The overall median age was 56 years (95% CI: 53.1–60.0). Group A, which received only BQT, consisted of 98 patients; group B, which

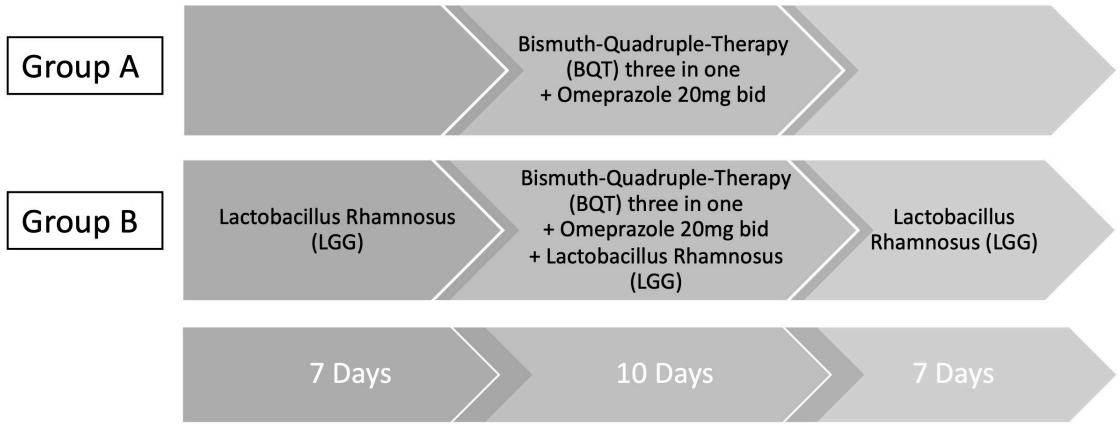


FIGURE 1
Eradication therapy schedule. Three-in-one BQT therapy (140 mg bismuth subcitrate potassium/125 mg metronidazole/125 mg tetracycline hydrochloride) was given as follows: three capsules four times a day (qid) after main meals and before bedtime. Omeprazole was given 20 mg two times a day (bid) (before breakfast and before dinner). Group A received only three-in-one BQT qid plus omeprazole 20 mg bid for 10 days. Group B received three-in-one BQT qid plus omeprazole 20 mg bid for 10 days in combination with six million CFU (colony-forming units) of *Lactobacillus rhamnosus* (LGG), strain ATCC 53103. LGG was given for 24 days (7 days before BQT, 10 days during BQT, and 7 days after BQT).

received BQT with LGG supplementation, consisted of 66 patients. Demographic and anamnestic data of the two groups are shown in Table 1. No statistically significant differences were observed either in median age and BMI between the two study groups.

Overall, a total of 27 patients were lost at follow-up, 13 in group A and 14 in group B, with a total drop-out rate resulting in 16.5% (27/164). Therefore, at PP analysis, 137 patients were considered, 85 patients from group A and 52 patients from group B. After eradication assessment, a 6-month follow-up visit was performed to assess the persistence of dyspepsia, with a further drop-out of four patients (Figure 2).

## Eradication rates

Overall, the Hp eradication rate was 79.9% (131/164) at ITT analysis and 95.6% (131/137) at PP analysis. No significant differences in eradication rates were observed when group A and group B were considered separately, both at ITT and PP analyses (Figure 3).

## Side effects

Overall, no differences were found in the rate of side effects during eradication therapy between the two groups which were present in 70.6% of patients (60/85) in group A and 65.4% (34/52) in group B. Stratifying the side effects into gastrointestinal and neurovegetative disorders, any statistically significant differences were observed both in gastrointestinal and neurovegetative events.

Gastrointestinal side effects occurred in 56.5% of patients (48/85) in group A and 58.5% (31/52) patients in group B, while neurovegetative ones occurred in 44.0% (37/84) in group A and 34.6% (18/52) in group B. Some of the gastrointestinal side effects (black stools, bloating, and postprandial fullness) and neurovegetative ones (headache and fatigue) were more frequent during eradication therapy in the group B that received BQT with LGG supplementation (Table 2).

## Dyspepsia

Overall, at the enrollment, dyspepsia was present in 67.1% (110/164) of patients on ITT analysis, 66.3% in group A, and 68.2% in group B (Table 1). At PP analysis, dyspepsia was present in 64.9% of patients (89/137), 76.5% (65/85) in group A, and 86.5% (45/52) in group B. At 6 months after eradication of Hp infection, the persistence of dyspepsia, in the 84 eradicated dyspeptic patients, resulted significantly higher in group A than in group B [51.1% (26/51) vs. 15.0% (5/33), $p = 0.001$] on ITT analysis. The difference was also present in PP analysis (80 eradicated patients, four drop-outs at 6-months follow-up visit, and two patients for each group) that showed a statistically significant persistence of dyspepsia in 38.8% (19/49) of patients in group A and 16.1% (5/31) in group B ($p = 0.032$) (Figure 4). LGG supplementation appears then to significantly influence the control of post-eradication dyspepsia.

### Subtypes of dyspepsia

At the enrollment, EPS dyspepsia was found in 31% (34/110) and PDS in 55.4% (61/110) of patients with dyspepsia. EPS was present in 38.5% (25/65) of patients in group A and 20.0% (9/45)

TABLE 1 Demographic data of the study population.

| Population characteristics | Group A (BQT) | Group B (BQT + LGG) | *P*-value |
|---|---|---|---|
| Number of patients | 98 | 66 | |
| M/F% (*n*) | 20.4/79.6 (20/78) | 34.8/65.2 (23/43) | 0.047 |
| Age (years) median (95% CI) | 59 (95% CI: 53.9–62.0) | 55(95% CI: 50.0–59.3) | 0.319 |
| BMI (kg/m$^2$) median (95% CI) | 25.0 (95% CI: 24.0–26.0) | 25.0 (95% CI: 23.7–26.8) | 0.829 |
| Number of patients with dyspepsia at enrollment | 65 | 45 | 0.8663 |

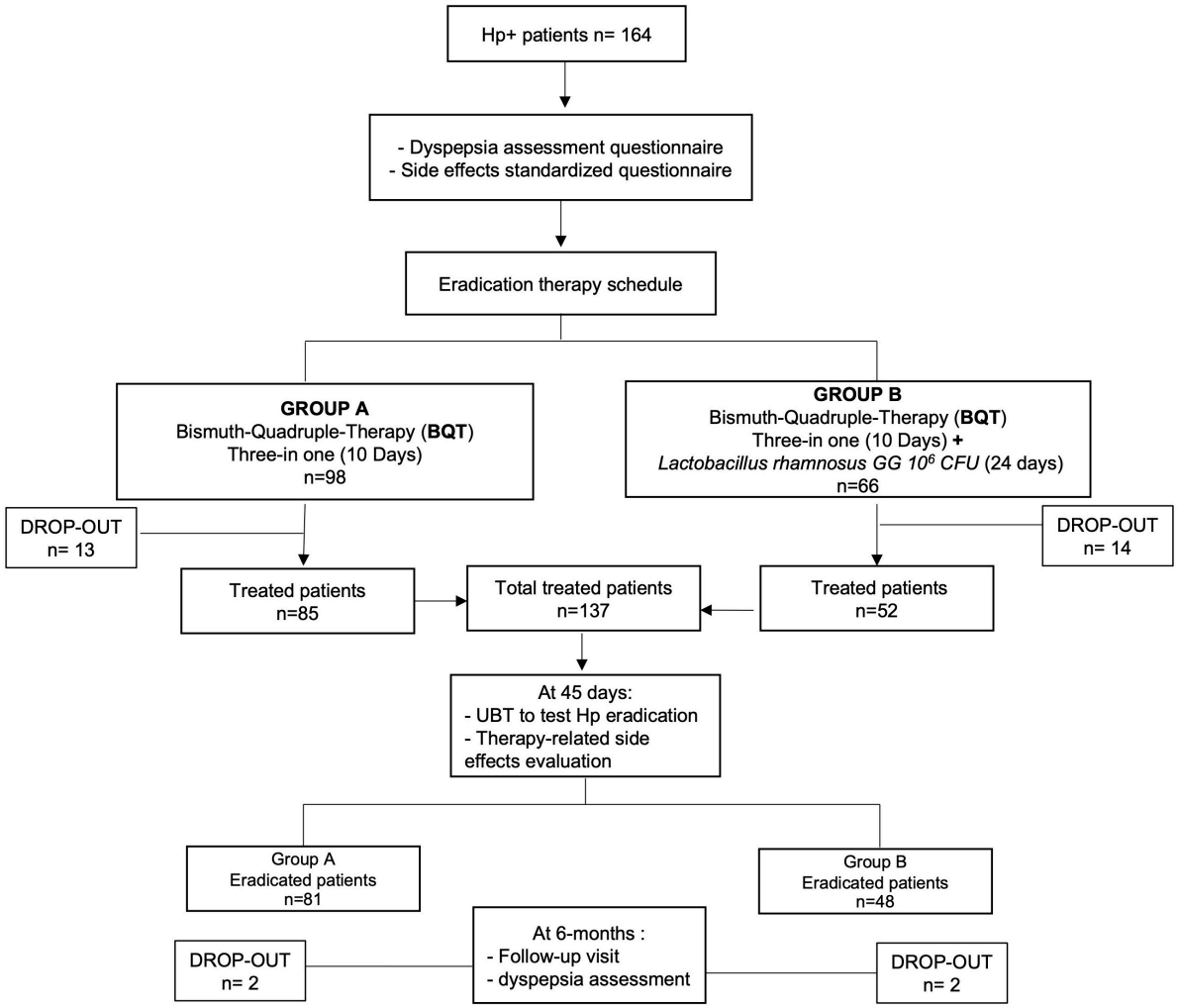


FIGURE 2
Study flowchart.

of patients in group B, while PDS was present in 50.8% (33/65) in group A and 62.2% (28/45) in group B. No effects of LGG were observed on the persistence of EPS-related dyspepsia that was still present in 15.0% (3/20) of patients in group A and 12.5% (1/8) in group B. Instead, LGG supplementation significantly improved the resolution of PDS dyspepsia. The persistence of dyspepsia was present only in 16.0% (3/18) of patients treated with the probiotic supplementation (group B) in respect of 58.3% (14/24) of patients who received the sole BQT (group A) ($p = 0.011$) (Figure 4).

## Discussion

This pilot study focused on the possible beneficial effects of LGG in the clinical-therapeutic management of Hp

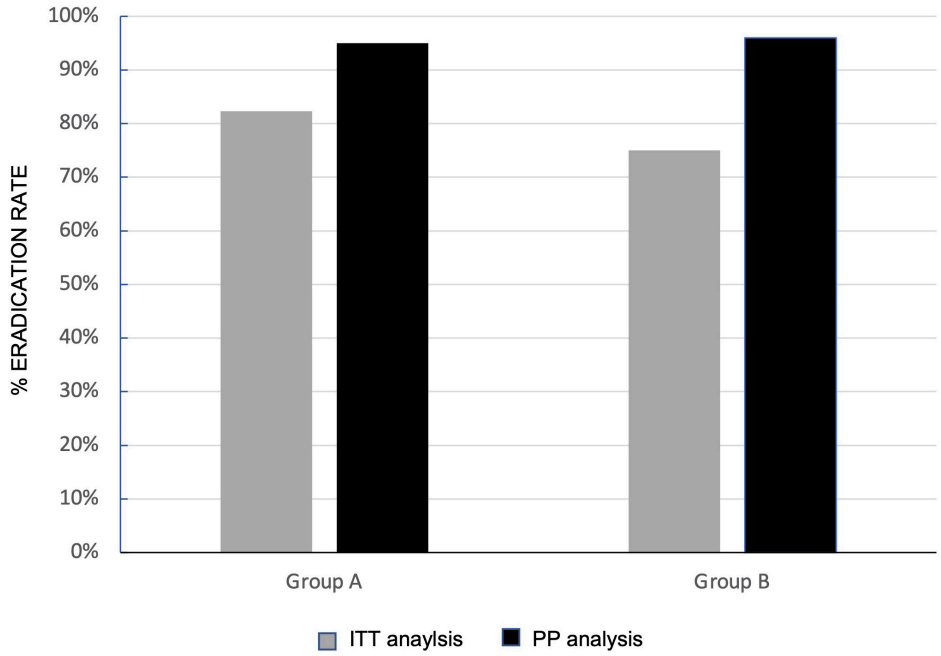


FIGURE 3
Effects of *Lactobacillus rhamnosus* GG (LGG) supplementation in three-in-one bismuth-quadruple therapy (BQT) eradication rates. Eradication rate at intention-to-treat (ITT) (gray column) and per protocol (PP) (black column) in group A (sole BQT treatment) and group B (BQT treatment with LGG supplementation).

TABLE 2 Side effects encountered during the 10 days of three-in-one bismuth-quadruple therapy (BQT) eradication therapy.

| % (Number of patients) | Group A ($n$ = 85) (BQT) | Group B ($n$ = 52) (BQT + LGG) | $P$-value |
|---|---|---|---|
| **Gastrointestinal side effects** | ***n* = 48** | ***n* = 31** | |
| – Diarrhoea | 33.3 (16) | 12.9 (4) | 0.063 |
| – Constipation | 2.2 (1) | 6.4 (2) | 0.557 |
| – Black stools | 60.4 (29) | 87.1 (27) | 0.013 |
| – Bloating | 0 (0) | 32.3 (10) | 0.0001 |
| – Abdominal pain | 18.7 (9) | 32.3 (10) | 0.188 |
| – Heartburn | 4.2 (2) | 6.4 (2) | 0.643 |
| – Back chest pain | 4.2 (2) | 9.7 (3) | 0.375 |
| – Nausea | 22.9 (11) | 38.7 (12) | 0.204 |
| – Vomiting | 6.2 (3) | 0 (0) | 0.275 |
| – Postprandial fullness | 2.2 (1) | 16.1 (5) | 0.032 |
| **Neurovegetative side effects** | ***n* = 37** | ***n* = 18** | |
| – Dysgeusia | 100 (37) | 88.9 (16) | 0.103 |
| – Headache | 0 (0) | 50 (9) | 0.0001 |
| – Dizziness | 0 (0) | 0 (0) | – |
| – Fatigue | 5.4 (2) | 44.4 (8) | 0.001 |

infection and shows the efficacy of LGG supplementation in inducing the remission of dyspepsia after Hp eradication when given in concomitance with three-in-one BQT therapy. Its efficacy in dyspeptic symptoms control after eradication therapy might have important implications in clinical practice since the actual estimated number needed to treat ranges from 8 to 14 (Sugano et al., 2015). Instead, LGG does not improve eradication rates or side effects during therapy.

Considering overall dyspepsia, LGG supplementation in concomitance with eradication treatment significantly improved dyspeptic symptoms with complete remission in

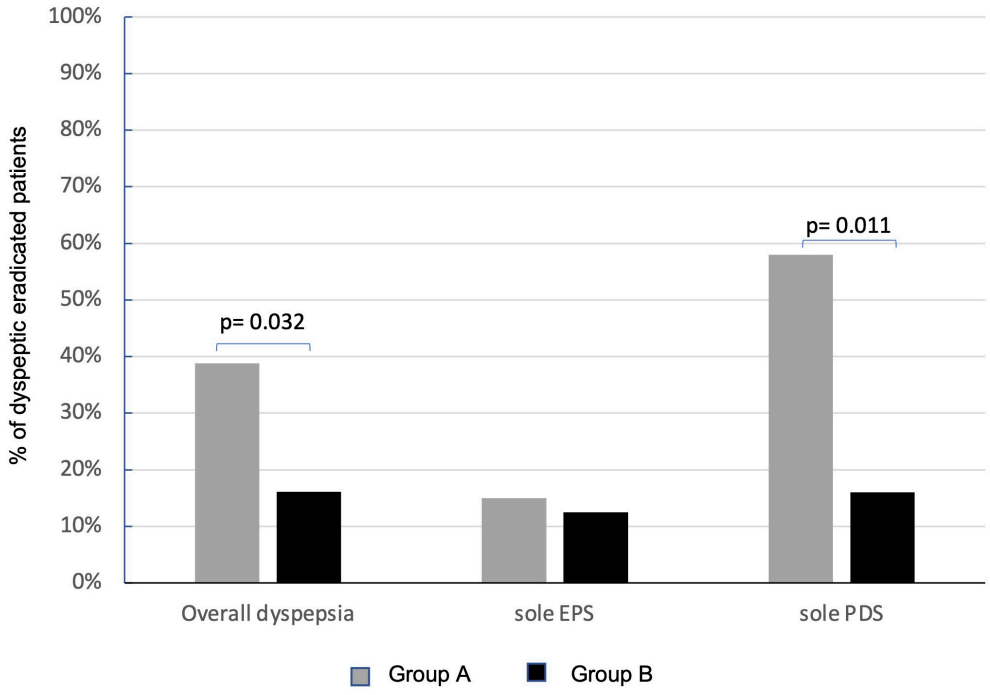


FIGURE 4
Effects of *Lactobacillus rhamnosus* GG (LGG) supplementation in improving overall dyspepsia and relative subtypes. Persistence of dyspepsia at PP analysis in group A (sole BQT treatment and gray columns) and group B (BQT treatment with LGG supplementation and black columns). EPS, epigastric pain syndrome; PDS, postprandial distress syndrome.

84% of patients who received antibiotics and LGG therapy compared with 60% of patients who received eradication therapy alone. Of note, BQT eradication therapy alone presented a higher efficacy on the remission of dyspepsia in respect of other eradication treatments (Moayyedi et al., 2005).

Instead, taking into account dyspeptic subtypes, LGG resulted essentially relevant for the PDS subtype, whose disappearance was significantly higher in patients receiving probiotics than in patients treated with BQT eradication therapy alone. As already reported by Tack and Talley (2013), PDS prevalence was higher than EPS subtypes. The high efficacy of LGG on PDS dyspepsia that was found in the present study could be partly influenced by the time length of the follow-up of 6 months since it has been reported that, after Hp eradication, PDS tends to have a short-term improvement in contrast to EPS whose improvement is long term (Xu et al., 2013). The time length of 6 months to assess dyspepsia outcomes is the time it takes for gastritis to recover but it needs to be taken into account that an increase in the duration of follow-up could end up in different LGG efficacies with respect to dyspeptic subtypes.

The efficacy of LGG in improving the remission of dyspepsia is likely to be ascribed to its influence on Hp-related gastric and duodenal dysbiosis (O'Hara and Shanahan, 2006; Bruno et al., 2018; Filardo et al., 2022) and, likely, the specific LGG administration schedule may also have positively affected the improvement of dyspepsia. The choice of initiating LGG supplementation 7 days prior to the start of BQT therapy was based on the rationale that LGG possesses anti-inflammatory and inhibitory properties on Hp activity that can improve eradication rates. The efficacy of LGG has been reported both in terms of reducing bacterial load (Zhou et al., 2008; Do et al., 2021) and improving the eradication rate of Hp infection (Zheng et al., 2013; Lü et al., 2016; Chen et al., 2021). The use of LGG during and in the next 7 days after eradication therapy was based on the principle that extended use of probiotics promotes eubiosis and restoration of normal intestinal flora for longer in order to result in improved symptomatology.

By itself, patients with functional dyspepsia have alterations in the gastric microbiota and, in some cases, treatment with probiotics resulted in the improvement of functional dyspepsia (Nakae et al., 2016; Ohtsu et al., 2017; Wauters et al., 2020). Furthermore, the presence of Hp in the stomach causes the development of different environments for bacterial growth (Hunt et al., 2015) that results more relevant than the alterations induced by hypochlorhydria alone (Parsons et al., 2017; Gomez-Ramirez et al., 2021). The subversion of the gastric

microbiota due to Hp infection may be related to the intrinsic properties of the bacterium that can create a hostile environment, making it difficult for the other bacteria to survive, thus allowing the establishment of a condition of gastroduodenal dysbiosis (Bruno et al., 2018; Gomez-Ramirez et al., 2021). In Hp-positive subjects, molecular analyses showed a reduction in biodiversity with the absolute prevalence of Hp, followed by *Streptococcus* (Klymiuk et al., 2017). The beneficial effects of probiotics may contribute to the restoration of gastric eubiosis and, specifically, the improvement of dyspeptic symptoms obtained with LGG supplementation agrees with its known anti-inflammatory and anti-apoptotic properties exerted on epithelial cells of the gastrointestinal mucosa (Yan and Polk, 2002).

*Lactobacillus rhamnosus* GG supplementation did not improve the three-in-one BQT eradication rate, as previously reported (Zagari et al., 2018). The positive effects of probiotic supplementation on eradication rates have been reported generally with eradication regimens with lower efficacy rates than BQT (Dang et al., 2014; Fang et al., 2019). Furthermore, LGG supplementation did not reduce gastrointestinal and neurovegetative events occurring during the BQT eradication schedule and the comparative analysis of every single effect indicates that LGG supplementation seems to worsen bismuth-related side effects such as black stools and headaches. Similarly, unfavorable potentiation has already been observed in previous studies using bismuth and LGG in the eradication schedule (Zagari et al., 2018), while LGG supplementation during triple therapy without bismuth reduced the side effects during eradication (Armuzzi et al., 2001).

The main limitation of the present study is that the patient sample is not homogeneously distributed in terms either of the number of patients between the two study groups or in gender distribution. The difference in patient enrollment numbers between the two groups is mainly ascribed to the free choice left to the patients at the time of the first visit to choose between the two therapeutic protocols, in consideration of the increased cost of therapy with probiotic supplementation. Nevertheless, the overall number of enrolled patients with dyspeptic is reliable, considering previous studies (Armuzzi et al., 2001). The female prevalence could be in part due to the higher prevalence of dyspeptic disorders in women, as reported by population studies on functional dyspepsia (Ford et al., 2015). Finally, the efficacy of probiotic administration should have required the presence of a placebo treatment group.

Despite this main limitation of the study, the present results offer promising opportunities to perform a sample size calculation for a future RCT to confirm the present observations.

In conclusion, this study suggests a potential efficacy of LGG supplementation to three-in-one BQT eradication therapy in inducing post-eradication remission of dyspeptic symptoms, suggesting the beneficial effects of probiotics in the restoration of gastric eubiosis. Probiotic supplementation could consequently be proposed as a routine therapeutic line that could have a relevant impact on the clinical management of Hp-related dyspepsia. Anyway, more studies should be performed to obtain a deeper knowledge of probiotic effects on gastric microbiota, with the aim of recommending the association of probiotics with eradication therapy.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The study was approved by the Local Ethical Committee (Code: 6944/2022). The patients/participants provided their written informed consent to participate in this study.

## Author contributions

GBr and CS designed and supervised this study. PM, GS, AC, GBe, and AR contributed to the subject recruitment and questionnaire collection. PM and GS wrote the manuscript under the supervision of GBr and CS. All authors revised the manuscript and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

# References


Armuzzi, A., Cremonini, F., Ojetti, V., Bartolozzi, F., Canducci, F., Candelli, M., et al. (2001). Effect of *Lactobacillus* GG supplementation on antibiotic-associated gastrointestinal side effects during *Helicobacter pylori* eradication therapy: a pilot study. *Digestion* 63, 1–7. doi: 10.1159/00051865

Bruno, G., Rocco, G., Zaccari, P., Porowska, B., Mascellino, M. T., and Severi, C. (2018). *Helicobacter pylori* infection and gastric dysbiosis: Can probiotics administration be useful to treat this condition? *Can. J. Infect. Dis. Med. Microbiol.* 2018:6237239. doi: 10.1155/2018/6237239

Burkitt, M. D., Duckworth, C. A., Williams, J. M., and Pritchard, D. M. (2017). *Helicobacter pylori*-induced gastric pathology: insights from in vivo and ex vivo models. *Dis. Models Mechan.* 10, 89–104. doi: 10.1242/dmm.027649

Capurso, L. (2019). Thirty years of *Lactobacillus rhamnosus* GG: A review. *J. Clin. Gastroenterol.* 53 Suppl 1, S1–S41. doi: 10.1097/MCG.0000000000001170A13

Chen, M. J., Chen, C. C., Huang, Y. C., Tseng, C. C., Hsu, J. T., Lin, Y. F., et al. (2021). The efficacy of Lactobacillus acidophilus and rhamnosus in the reduction of bacterial load of *Helicobacter pylori* and modification of gut microbiota-a double-blind, placebo-controlled, randomized trial. *Helicobacter* 26:e12857. doi: 10.1111/hel.12857

Cremonini, F., Canducci, F., Di Caro, S., Santarelli, L., Armuzzi, A., Khanna, T., et al. (2001). *Helicobacter pylori* treatment: a role for probiotics? *Digestive Dis.* 19, 144–147. doi: 10.1159/000050669

Dang, Y., Reinhardt, J. D., Zhou, X., and Zhang, G. (2014). The effect of probiotics supplementation on *Helicobacter pylori* eradication rates and side effects during eradication therapy: a meta-analysis. *PloS one* 9:e111030. doi: 10.1371/journal.pone.0111030

Ding, F. C. L., Karkhaneh, M., Zorzela, L., Jou, H., and Vohra, S. (2019). Probiotics for paediatric functional abdominal pain disorders: A rapid review. *Paediatr. Child Health* 24, 383–394. doi: 10.1093/pch/pxz036

Do, A. D., Chang, C. C., Su, C. H., and Hsu, Y. M. (2021). Lactobacillus rhamnosus JB3 inhibits *Helicobacter pylori* infection through multiple molecular actions. *Helicobacter* 26:e12806. doi: 10.1111/hel.12806

Drossman, D. A. (2016). Functional gastrointestinal disorders: history, pathophysiology, clinical features, and rome IV. *Gastroenterology* 150, 1262–1279.e2. doi: 10.1053/j.gastro.2016.02.032.

Drossman, D. A., and Hasler, W. L. (2016). Rome IV—functional GI disorders: disorders of gut-brain interaction. *Gastroenterology* 150, 1257–1261. doi: 10.1053/j.gastro.2016.03.035

Du, L. J., Chen, B. R., Kim, J. J., Kim, S., Shen, J. H., and Dai, N. (2016). *Helicobacter pylori* eradication therapy for functional dyspepsia: systematic review and meta-analysis. *World J. Gastroenterol.* 22, 3486–3495. doi: 10.3748/wjg.v22.i12.3486

Fallone, C. A., Moss, S. F., and Malfertheiner, P. (2019). Reconciliation of recent *Helicobacter pylori* treatment guidelines in a time of increasing resistance to antibiotics. *Gastroenterology* 157, 44–53. doi: 10.1053/j.gastro.2019.04.011

Fang, H. R., Zhang, G. Q., Cheng, J. Y., and Li, Z. Y. (2019). Efficacy of *Lactobacillus*-supplemented triple therapy for *Helicobacter pylori* infection in children: a meta-analysis of randomized controlled trials. *Eur. J. Pediatr.* 178, 7–16. doi: 10.1007/s00431-018-3282-z

Filardo, S., Scalese, G., Virili, C., Pontone, S., Di Pietro, M., Covelli, A., et al. (2022). The potential role of hypochlorhydria in the development of duodenal dysbiosis: a preliminary report. *Front. Cell. Infect. Microbiol.* 12:854904. doi: 10.3389/fcimb.2022.854904

Fiorini, G., Zullo, A., Saracino, I. M., Pavoni, M., and Vaira, D. (2018). Antibiotic resistance pattern of *Helicobacter pylori* strains isolated in Italy during 2010-2016. *Scand. J. Gastroenterol.* 53, 661–664. doi: 10.1080/00365521.2018.1464596

Ford, A. C., Marwaha, A., Sood, R., and Moayyedi, P. (2015). Global prevalence of, and risk factors for, uninvestigated dyspepsia: a meta-analysis. *Gut* 64, 1049–1057. doi: 10.1136/gutjnl-2014-307843

Gomez-Ramirez, U., Valencia-Mayoral, P., Mendoza-Elizalde, S., Murillo-Eliosa, J. R., Solórzano Santos, F., Contreras-Rodríguez, A., et al. (2021). Role of *Helicobacter pylori* and other environmental factors in the development of gastric dysbiosis. *Pathogens* 10:1203. doi: 10.3390/pathogens10091203

Hall, W., Buckley, M., Crotty, P., and O'Morain, C. A. (2003). Gastric mucosal mast cells are increased in *Helicobacter pylori*-negative functional dyspepsia. *Clin. Gastroenterol. Hepatol.* 1, 363–369. doi: 10.1053/s1542-3565(03)00184-8

Hu, Y., Zhang, M., Lu, B., and Dai, J. (2016). *Helicobacter pylori* and antibiotic resistance, a continuing and intractable problem. *Helicobacter* 21, 349–363. doi: 10.1111/hel.12299

Hunt, R. H., Camilleri, M., Crowe, S. E., El-Omar, E. M., Fox, J. G., Kuipers, E. J., et al. (2015). The stomach in health and disease. *Gut* 64, 1650–1668. doi: 10.1136/gutjnl-2014-307595

Kim, Y. J., Chung, W. C., Kim, B. W., Kim, S. S., Kim, J. I., Kim, N. J., et al. (2017). Is *Helicobacter pylori* associated functional dyspepsia correlated with dysbiosis? *J. Neurogastroenterol. Motility* 23, 504–516. doi: 10.5056/jnm17066

Klymiuk, I., Bilgilier, C., Stadlmann, A., Thannesberger, J., Kastner, M. T., Högenauer, C., et al. (2017). The human gastric microbiome is predicated upon infection with *Helicobacter pylori*. *Front. Microbiol.* 8:2508. doi: 10.3389/fmicb.2017.02508

Lü, M., Yu, S., Deng, J., Yan, Q., Yang, C., Xia, G., et al. (2016). Efficacy of probiotic supplementation therapy for *Helicobacter pylori* eradication: A meta-analysis of randomized controlled trials. *PLoS One* 11:e0163743. doi: 10.1371/journal.pone.0163743

Malfertheiner, P., Megraud, F., O'Morain, C. A., Gisbert, J. P., Kuipers, E. J., Axon, A. T., et al. (2017). Management of *Helicobacter pylori* infection-the maastricht V/florence consensus report. *Gut* 66, 6–30. doi: 10.1136/gutjnl-2016-312288

Moayyedi, P., Soo, S., Deeks, J., Delaney, B., Harris, A., Innes, M., et al. (2005). Eradication of *Helicobacter pylori* for non-ulcer dyspepsia. *Cochrane Database Systematic Rev.* CD002096. doi: 10.1002/14651858.CD002096.pub2

Nakae, H., Tsuda, A., Matsuoka, T., Mine, T., and Koga, Y. (2016). Gastric microbiota in the functional dyspepsia patients treated with probiotic yogurt. *BMJ Open Gastroenterol.* 3:e000109. doi: 10.1136/bmjgast-2016-000109

Nardone, G., and Compare, D. (2015). The human gastric microbiota: is it time to rethink the pathogenesis of stomach diseases? *U Eur. Gastroenterol. J.* 3, 255–260. doi: 10.1177/2050640614566846

Nyssen, O. P., Perez-Aisa, A., Castro-Fernandez, M., Pellicano, R., Huguet, J. M., Rodrigo, L., et al. (2021). European registry on *Helicobacter* piylori management: Single-capsule bismuth quadruple therapy is effective in real-world clinical practice. *United Eur. Gastroenterol. J.* 9, 38–46. doi: 10.1177/2050640620972615

O'Hara, A. M., and Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Rep.* 7, 688–693. doi: 10.1038/sj.embor.7400731

Ohtsu, T., Takagi, A., Uemura, N., Inoue, K., Sekino, H., Kawashima, A., et al. (2017). The ameliorating effect of *Lactobacillus gasseri* OLL2716 on functional dyspepsia in *Helicobacter pylori*-uninfected individuals: a randomized controlled study. *Digestion* 96, 92–102. doi: 10.1159/000479000

Parsons, B. N., Ijaz, U. Z., D'Amore, R., Burkitt, M. D., Eccles, R., Lenzi, L., et al. (2017). Comparison of the human gastric microbiota in hypochlorhydric states arising as a result of *Helicobacter Pylori*- induced atrophic gastritis, autoimmune atrophic gastritis and proton pump inhibitor use. *PLoS Pathog* 13:e1006653. ppat.1006653

Romano, M., Gravina, A. G., Eusebi, L. H., Pellegrino, R., Palladino, G., Frazzoni, et al. (2022). Management of *Helicobacter pylori* infection: Guidelines of the italian society of gastroenterology (SIGE) and the Italian society of digestive endoscopy (SIED). *Digestive Liver Dis.* 54, 1153–1161. doi: 10.1016/j.dld.2022.06.019

Savoldi, A., Carrara, E., Graham, D. Y., Conti, M., and Tacconelli, E. (2018). Prevalence of antibiotic resistance in *Helicobacter pylori*: a systematic review and meta-analysis in world health organization regions. *Gastroenterology* 155, 1372–1382.e17. doi: 10.1053/j.gastro.2018.07.007.

Stanghellini, V., Chan, F. K., Hasler, W. L., Malagelada, J. R., Suzuki, H., Tack, J., et al. (2016). Gastroduodenal disorders. *Gastroenterology* 150, 1380–1392. doi: 10.1053/j.gastro.2016.02.011

Sugano, K., Tack, J., Kuipers, E. J., Graham, D. Y., El-Omar, E. M., Miura, S., et al. (2015). Kyoto global consensus report on *Helicobacter pylori* gastritis. *Gut* 64, 1353–1367. doi: 10.1136/gutjnl-2015-309252

Tack, J., and Talley, N. J. (2013). Functional dyspepsia–symptoms, definitions and validity of the Rome III criteria. *Nat. Rev. Gastroenterol. Hepatol.* 10, 134–141. doi: 10.1038/nrgastro.2013.14

Talley, N. J. (2016). Functional dyspepsia: new insights into pathogenesis and therapy. *Korean J. Internal Med.* 31, 444–456. doi: 10.3904/kjim.2016.091

Thung, I., Aramin, H., Vavinskaya, V., Gupta, S., Park, J. Y., Crowe, S. E., et al. (2016). Review article: the global emergence of *Helicobacter pylori* antibiotic resistance. *Alimentary Pharmacol. Therapeut.* 43, 514–533. doi: 10.1111/apt.13497

Tziatzios, G., Gkolfakis, P., Papanikolaou, I. S., Mathur, R., Pimentel, M., Giamarellos-Bourboulis, E. J., et al. (2020). Gut microbiota dysbiosis in functional dyspepsia. *Microorganisms* 8:691.

Vanheel, H., Vicario, M., Vanuytsel, T., Van Oudenhove, L., Martinez, C., Keita, et al. (2014). Impaired duodenal mucosal integrity and low-grade inflammation in functional dyspepsia. *Gut* 63, 262–271. doi: 10.1136/gutjnl-2012-303857

Wauters, L., Dickman, R., Drug, V., Mulak, A., Serra, J., Enck, P., et al. (2021). United European Gastroenterology (UEG) and European Society for Neurogastroenterology and Motility (ESNM) consensus on functional dyspepsia. *Neurogastroenterol. Motil.* 33:e14238. doi: 10.1111/nmo.14238

Wauters, L., Talley, N. J., Walker, M. M., Tack, J., and Vanuytsel, T. (2020). Novel concepts in the pathophysiology and treatment of functional dyspepsia. *Gut* 69, 591–600. doi: 10.1136/gutjnl-2019-318536

Westerik, N., Reid, G., Sybesma, W., and Kort, R. (2018). The probiotic *Lactobacillus rhamnosus* for alleviation of *Helicobacter pylori*-associated gastric pathology in East Africa. *Front. Microbiol.* 9:1873. doi: 10.3389/fmicb.2018.01873

Xu, S., Wan, X., Zheng, X., Zhou, Y., Song, Z., Cheng, M., et al. (2013). Symptom improvement after *Helicobacter pylori* eradication in patients with functional dyspepsia-A multicenter, randomized, prospective cohort study. *Int. J. Clin. Exp. Med.* 6, 747–756.

Yan, F., and Polk, D. B. (2002). Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. *J. Biol. Chem.* 277, 50959–50965. doi: 10.1074/jbc.M207050200

Yuan, Z., Xiao, S., Li, S., Suo, B., Wang, Y., Meng, L., et al. (2021). The impact of *Helicobacter Pylori* infection, eradication therapy, and probiotics intervention on gastric microbiota in young adults. *Helicobacter* 26:e12848. doi: 10.1111/hel.12848

Zagari, R. M., Romiti, A., Ierardi, E., Gravina, A. G., Panarese, A., Grande, G., et al. (2018). The "three-in-one" formulation of bismuth quadruple therapy for *Helicobacter pylori* eradication with or without probiotics supplementation: efficacy and safety in daily clinical practice. *Helicobacter* 23:e12502. doi: 10.1111/hel.12502

Zheng, X., Lyu, L., and Mei, Z. (2013). Lactobacillus-containing probiotic supplementation increases *Helicobacter pylori* eradication rate: Evidence from a meta-analysis. *Rev. Esp. Enferm.* 105, 445–453. doi: 10.4321/s1130-01082013000800002

Zhou, C., Ma, F. Z., Deng, X. J., Yuan, H., and Ma, H. S. (2008). Lactobacilli inhibit interleukin-8 production induced by *Helicobacter pylori* lipopolysaccharide-activated Toll-like receptor 4. *World J. Gastroenterol.* 14, 5090–5095. doi: 10.3748/wjg.14.5090

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