Antibiotic Eradication of *H. pylori* Infection

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Declaration

Unless otherwise stated, the work presented in this thesis is my own. No part has been submitted at this, or any other institute of learning.

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Dedicated to my parents for their unwavering support in everything I do.

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Abstract

Helicobacter pylori is the major etiological agent of peptic ulcer disease and gastric cancer, infecting nearly 50% of the global population. The rising prevalence of antibiotic resistance threatens *H. pylori* treatment efficacy. While numerous studies have explored the prevalence and impact of resistance on eradication failure, the contribution of the host response in antibiotic eradication success remains relatively underexplored.

This study aimed to analyse 241 clinical isolates of *H. pylori* collected from patients undergoing routine gastroscopy in Nottingham between 2001 and 2018, to assess antibiotic resistance rates. It also aimed to quantify the host innate response to *H. pylori* infection using *ex vivo* gastric biopsies tissue and AGS human gastric adenocarcinoma cell cultures *in vitro.* The anti-inflammatory effects of macrolide antibiotics were also assessed *in vitro*. Additionally, antibiotic resistance was examined, comparing phenotypic data with genome sequence data from clinical isolates.

H. pylori clinical isolates were collected from 162 patients with informed written consent and ethical approval. Their sensitivity to metronidazole, clarithromycin, levofloxacin, tetracycline, and amoxicillin was assessed by culture-based methods using antibiotic discs and E-test strips. mRNA expression levels of cationic antimicrobial peptide defensin genes *DEFB4* (human beta-defensin 4) and *DEFA5* (human alpha-defensin 5) were measured in 56 human gastric antral (lower part of the stomach) biopsies using RT-qPCR. The AGS cell line was infected with several *H. pylori* strains in the presence and absence of the pro-inflammatory cytokine Tumour Necrosis Factor alpha (TNFα), clarithromycin, or azithromycin, and pro-inflammatory chemokine interleukin-8 (IL-8) and human beta-defensin 2 (hβD2) levels were measured using Enzyme-Linked Immunosorbent Assay (ELISA). Illumina MiSeq genome sequence data from 35 *H. pylori* clinical isolates was analysed using SPAdes, QUAST, and Prokka bioinformatics tools on the Galaxy platform. Antibiotic resistance predictions for the genome assemblies were made with the Comprehensive Antibiotic Resistance Database (CARD). Amino acid substitutions in predicted penicillin-binding proteins (PBPs) of 10 amoxicillin-susceptible and 10-resistant *H. pylori* strains were analysed using the *H. pylori* 26695 strain as a reference. The 3D structures of penicillin-binding protein 1A (PBP1A) and penicillin-binding protein 2 (PBP2)

from strain 26695 were predicted using I-TASSER, and protein folding predictions for PBP1A were performed using AlphaFold2 and visualized with UCSF ChimeraX.

The study discovered a high prevalence of resistance to metronidazole (61.8%, 149/241) and clarithromycin (27.8%, 67/241) among *H. pylori* isolates, with resistance rates for these antibiotics rising significantly between 2011 and 2018. Isolates from individuals who had previously undergone *H. pylori* eradication therapy were more likely to be resistant to metronidazole ($p=0.006$) and clarithromycin ($p<0.0001$) compared to those from individuals without prior treatment. Additionally, younger patients (aged 40-60) were more likely to have strains that were resistant to metronidazole ($p=0.0119$) and clarithromycin ($p=0.0454$) than older patients.

DEFB4 mRNA expression levels were 17-fold higher in gastric biopsies from 24 infected patients compared with 21 uninfected patients ($p<0.0001$), whilst the *DEFA5* mRNA levels were reduced by 76-fold ($p=0.0029$). *DEFB4* expression was over 50 times higher in biopsies from patients with atrophic gastritis compared to those without ($p=0.0059$) and similarly, *DEFA5* expression was over 50 times higher in those with atrophy ($p=0.0062$). Higher *DEFB4* expression was also observed in biopsies from patients infected with more virulent cytotoxin-associated gene A positive (*cagA*+) *H. pylori* strains ($p=0.0316$).

IL-8 and hβD2 secretion by AGS cells increased significantly when exposed to *H. pylori* infection at a multiplicity of infection (MOI) of 20 bacteria/cell in the presence of 50 ng/ml TNFα compared to infected cells without TNFα (2-fold higher IL-8 ($p=0.033$) and 2-fold higher HβD2 concentrations ($p=0.0018$)). The addition of macrolides to AGS cell cultures infected with clarithromycin-resistant isolate 873A at an MOI of 20 was found to reduce IL-8 production (clarithromycin: 2.7-fold, $p=0.0051$; azithromycin: 2.7-fold, $p=0.0204$), and a similar result was observed with other isolates. More pronounced effects were observed with *cagA*-positive strains. Macrolides were also observed to decrease the secretion of defensin hβD2 by AGS cells infected with *H. pylori* isolate 873A (clarithromycin: 3-fold, $p=0.0056$; azithromycin: 3-fold, $p=0.0056$), but there were no significant differences between *cagA*-positive and *cagA*-negative strains when additional assays were performed.

Known and novel point mutations and amino acid substitutions were identified in target antimicrobial resistance genes and proteins of *H. pylori* isolates. The A2147G mutation in

the *23S rRNA* was closely associated with resistance to clarithromycin ($p<0.0001$), whilst amino acid changes A85 and R90K in the S-nitrosoglutathione reductase enzyme (FrxA) were significantly linked to metronidazole resistance ($p=0.03$). There was a strong correlation between phenotypic antibiotic susceptibility and genotypic resistance of strains to levofloxacin, with the A subunit of gyrase enzyme (*gyrA*) genotypes accurately indicating strain susceptibility (kappa coefficient of 1). Amoxicillin-resistant strains had higher numbers of amino acid substitutions (13.6 per strain) in PBP1A than amoxicillin-susceptible strains (11.8 per strain). The amino acid substitutions at positions 473, 589, 593, and 595 in *H. pylori* 26695 PBP1A, near the active sites $SAIK_{368-371}$, $SLN_{433-435}$, and $KTGT_{555-558}$ suggest potential structural changes that might reduce the binding affinity or access of amoxicillin.

In conclusion, these studies offer new insights into the prevalence of antibiotic-resistant *H. pylori* in the UK, underscoring the necessity to revise the clinical guidelines for eradication therapy. The guidelines from the National Institute for Health and Care Excellence (NICE) have remained unchanged since 2019 and may no longer be effective considering the increasing prevalence of antibiotic-resistant strains identified in the current research. Ongoing surveillance of resistance patterns is crucial for informing evidence-based updates to treatment protocols, ensuring eradication therapy remains effective in combating *H. pylori* infections.

This study also sheds light on the role of human defensins, particularly *DEFB4* and *DEFA5*, during *H. pylori* infection, highlighting their association with atrophic gastritis and virulence factors. The findings enhance understanding of defensin regulation in gastric pathogenesis especially gastric cancer and its potential as a prognostic tumour biomarker. Assessing defensin levels may aid in identifying pre-malignant lesions in asymptomatic *H. pylori*-infected patients, improving early detection and intervention strategies for gastric cancer. This research contributes valuable insights into host-pathogen interactions and supports the development of diagnostic tools for better management of *H. pylori*-related diseases.

The findings indicate that macrolides can suppress IL-8 and defensin production during *H. pylori* infection, suggesting that they may serve a dual purpose as both antibiotics and immunomodulators. This knowledge could be valuable for clinicians in determining the appropriate treatment for patients where managing inflammation is the primary objective, rather than eradicating the bacteria, such as in cases of refractory or non-eradicable *H.*

pylori infection although inappropriate antibiotic use should be avoided, as this could generate resistance in other organisms of the normal human microbiome. The observation that *cagA*+ strains have a more pronounced effect than *cagA*- strains provides evidence to refine treatment plans, based on the *cagA* status of *H. pylori* isolates, rather than relying solely on empirical therapies, especially for patients who are genetically predisposed to higher-level inflammatory responses.

The identification of point mutations and amino acid substitutions in *H. pylori* antibiotic resistance genes and proteins, obtained from this study helps to inform predictive tools for determining antibiotic susceptibility. Integration of genomic data with phenotypic testing provides a comprehensive view of resistance trends in *H. pylori* populations. In the future, machine learning and artificial intelligence could leverage whole-genome sequencing data from biopsy samples to predict antimicrobial resistance profiles rapidly and recommend optimal antibiotic combinations. These systems could also record local resistance patterns and prior treatment histories to personalize eradication therapies. Improving diagnostic precision and treatment personalization holds the key to overcoming resistance, improving eradication success, and mitigating the global burden of *H. pylori*-associated diseases.

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Suffi Suffian, Elizabeth Garvey, Joanne Rhead, Karen Robinson

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Abbreviations

AGS	Human gastric adenocarcinoma cell line
AI	Artificial intelligence
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
AP-1	Activating protein-1
AST	Antibiotic susceptibility test
BabA	Blood group antigen binding adhesin
BLAST	Basic Local Alignment Search Tool
CagA	Cytotoxin-associated gene A
CagPAI	Cag pathogenicity island
CARD	Comprehensive Antibiotic Resistance Database
cDNA	Complementary DNA
Ct	Cycle threshold
DEFA5	Defensin alpha-5
DEFB4	Defensin beta-4
DMSO	Dimethyl sulfoxide
CD	Crohn's disease
CFU	Colony forming unit
CNV	Copy number variation
DUD	Duodenal ulcer disease
DupA	Duodenal ulcer promoting gene
E	Efficiency (PCR)
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
E-test	Epsilometer test
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GUD	Gastroduodenal ulcer disease
HβD	Human β-defensin
HMM	High molecular mass
HtrA	High-temperature requirement A
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
IM	Intestinal metaplasia
LMM	Low molecular mass
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
ME	Major error

MIC	Minimum inhibitory concentration
ML	Machine learning
MOI	Multiplicity of infection
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa B
NG	Nodular gastropathy
NGS	Next generation sequencing
NOD-1	Nucleotide-binding oligomerization domain 1
NSAID	Nonsteroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Peptidoglycan
PPI	Protein pump inhibitory
PRR	Pattern recognition receptor
PUD	Peptic ulcer disease
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SHP-2	SH2 domain containing tyrosine phosphatase 2
SNP	Single nucleotide polymorphism
TAK1	Transforming growth factor beta-activated kinase 1
T4SS	Type IV secretion system
TLR	Toll-like receptor
TNFα	Tumour necrosis factor A
TNFR	Tumour necrosis factor receptor
TP	Transpeptidase
TRAF	Tumour necrosis factor receptor-associated factor
UC	Ulcerative colitis
VacA	Vacuolating cytotoxin A
VME	Very major error
WGS	Whole genome sequencing

Chapter 1 : Introduction

1.1 *Helicobacter pylori*

Helicobacter pylori is a widespread human pathogen that is accountable for chronic inflammation of the gastric mucosa, gastric and duodenal ulcer disease, and gastric cancer (Qureshi *et al.*, 2019). The Gram-negative genus *Helicobacter* belongs to the subdivision of the *Proteobacteria*, order *Campylobacterales* and family *Helicobacteraceae*. Most of the members of the genus *Helicobacter* are catalase and oxidase-positive, with many of them also urease-positive (Fox, 2002). *H. pylori* is a spiral, flagellated and microaerophilic bacterium (Huang *et al.*, 2016), which measures 2 to 4 µm in length and 0.5 to 1 µm in width (Kusters *et al.*, 1997). However, when *H. pylori* is cultured on solid medium, the bacteria usually appear as rod-shaped, although coccoid forms become visible after prolonged *in-vitro* culture or after antibiotic treatment (Aktaş *et al.*, 2020; Kusters *et al.*, 1997). The organism has 2-6 unipolar, sheathed flagella about 3µm in length and possesses a unique bulb at the end. This adaptation helps with the motility for rapid movement especially in the mucus layer covering the gastric epithelial cells (O'Toole *et al.*, 2000).

The bacterium initially colonizes the stomach's mucosa, and if present, it can be considered the dominant microbial inhabitant of the human stomach (Cover & Blaser, 2009). The bacterium is part of the indigenous biota in the human stomach and persists for decades from early childhood, or lifelong in most people. *H. pylori* survive because of its protected niche in the gastric mucus layer as the pH in the mucus is less extreme than the lumen (the pH at the luminal surface of the stomach ranges from 0.3 to 2.9, with a resting median pH of about 2, while the pH within the mucus layer is approximately 4 when at rest). On ingestion of the bacteria, they rapidly swim into the mucus to survive and establish colonization (Keilberg & Ottemann, 2016).

In general, the adaptation mechanisms of *H. pylori* to the acidic gastric environment are a synergy of several factors including bacterial proteins (e.g. urease), corkscrew morphology (special motility of spiral bacteria which enables them to move rapidly and easily in the viscous environment provided by the mucus that severely impede the movement of more conventional rod-shaped organisms) (Yang *et al.*, 2019), motility via flagella (Keilberg & Ottemann, 2016) , host factors including access to urea and adhesion to protective gastric mucins (Ansari & Yamaoka, 2017). One of the key elements is by *H. pylori* production of large quantities of urease enzyme. The hydrolysis of urea to ammonia by urease elevates the cytoplasmic pH, hence neutralising the acidity of the bacterial microenvironment within the stomach (Ansari & Yamaoka, 2017; Keilberg & Ottemann, 2016).

1.2 *Helicobacter pylori* infection and diseases

H. pylori gastritis is classified as an infectious disease in the 11th revision of the International Classification of Diseases (Malfertheiner *et al.*, 2022). *H. pylori* infection has been prevalent globally, with an overall infection rate reaching 43.2% (95% CI: 40.3–45.9%) in 2011–2022, and interestingly, the prevalence of *H. pylori* infection is distinctly variable among different countries (Li *et al.*, 2023b). The prevalence rates are most commonly high in developing countries (Huang *et al.*, 2024). In developing countries, more than 70% of the population harbours *H. pylori,* and more than half of these infections are acquired before the age of 10 years (Kienesberger *et al.*, 2018). For example, high prevalence is observed in Latin American countries (59%), with lower prevalence rates in North America (36.2%) (Li *et al.*, 2023b; Zamani *et al.*, 2018). Meanwhile, the overall prevalence of *H. pylori* infections among European populations was shown to be 47.5% (95% CI: 43.0–52.1%) (Li *et al.*, 2023b). In 2018, it was reported that the prevalence of *H. pylori* infection in Germany

ranged from 20% and 40% (Fischbach & Malfertheiner, 2018). A recent meta-analysis also found *H. pylori* infection rates of 28.6% in France, 18.8% in Denmark, and 26.3% in the United Kingdom (Chen *et al.*, 2024).

However, the global prevalence of *H. pylori* infection in adults has declined from 58.2% during the 1980-1990 period to 43.1% between 2011 and 2022. While prevalence remained stable from 1991 to 2010, it saw a significant decrease from 2011 to 2022, particularly in the WHO African region (Li *et al.*, 2023b). Overall, a lower prevalence of *H. pylori* infection was mostly attributed to the common use of antibiotics in childhood, advancement in healthcare, improvement of socioeconomic status, living standards, and hygiene conditions (Liou *et al.*, 2020; Park *et al.*, 2021). The widespread use of antibiotics for infections other than *H. pylori* infections may inadvertently eradicate the bacteria, particularly during childhood when infections are often acquired. This has led to younger generations acquiring *H. pylori* at a lower rate compared to older generations, likely because children have lower acid output than adults, making it easier for them to clear the infection. Additionally, it is also uncommon for adults to contract the infection (Cardos *et al.*, 2021; Nguyen *et al.*, 2023).

The current literature also shows that the human transmission of *H. pylori* requires close person-to-person contact, and occurs *via* the oral-oral, faecal-oral, and gastric-oral routes, particularly in settings of poor sanitation and hygiene, and the infection dose for humans is low (Mitchell & Katelaris, 2016). Furthermore, the transmission between family members is thought to be the most prevalent (Kayali *et al.*, 2018).

H. pylori infections are usually first acquired during early childhood and persist for life if untreated, and most infected persons remain asymptomatic (Bashir & Khan, 2023;

Malfertheiner *et al.*, 2023). However, when adults become infected (in very rare cases), this may lead to acute symptoms such as nausea and dyspepsia, along with reduced gastric acid secretion (Robinson & Atherton, 2021). *H. pylori* has been linked to different forms of gastritis, and in some people, the persistent inflammation in the stomach results in chronic atrophic gastritis (Holleczek *et al.*, 2020), which may then lead to further premalignant pathology, such as intestinal metaplasia and adenocarcinoma (Dinis-Ribeiro & Kuipers, 2020).

1.2.1 Peptic ulcer disease

Peptic ulcer disease (PUD) is defined as the presence of an open lesion in the mucosa of the human gastrointestinal tract, and it affects millions of people across the world (Lanas & Chan, 2017). An individual infected with *H. pylori* has approximately a 10% risk for the development of peptic ulcer disease (Oppong *et al.*, 2015), and *H. pylori* infection is the leading cause as it can be diagnosed in 98% of duodenal ulcer patients and 94% of gastric ulcer patients (Chen *et al.*, 2018b).

The pattern of *H. pylori* colonization and the extent of gastritis are the main factors determining whether ulceration occurs in the duodenum or the stomach (Ansari & Yamaoka, 2017). The counteractive effects of acid on bacterial growth and associated mucosal inflammation on acid secretion are crucial in the determination of outcomes of *H. pylori* colonization. *H. pylori* typically colonize the antrum of the stomach, but in conditions of reduced acid secretion, it can also colonize the corpus of the stomach (Figure 1.1). This is often observed in patients who are on long-term acid suppression medication (Lee *et al.*, 1995). In individuals with high gastric acid secretion, *H. pylori* colonize the gastric antrum,

where few acid-secreting parietal cells are present. High local acid concentrations (hyperchlorhydria) increase acid exposure of the duodenal mucosa; hence this colonization pattern is linked to antrum-predominant gastritis, which may lead to duodenal ulcer (Figure 1.2) (Dixon, 2001).

In contrast, if the acid secretion level is stable, the distribution of *H. pylori* colonization in the antrum and corpus are even, which may produce an atrophic pangastritis (Figure 1.2). The colonization and inflammation of the corpus are linked to decreased acid levels (achlorhydria), and this condition can result in the formation of corpus-predominant gastritis and an increased risk of gastric ulcer and gastric cancer (Dixon, 2001). Both types of gastritis can result in the formation of gastric ulcers, typically located on the lesser curvature in the transitional area between the antrum and corpus. Gastric atrophy is regarded as a pre-cancerous condition because it is strongly associated with the development of gastric adenocarcinoma (Atherton, 2006; Atherton & Blaser, 2009).

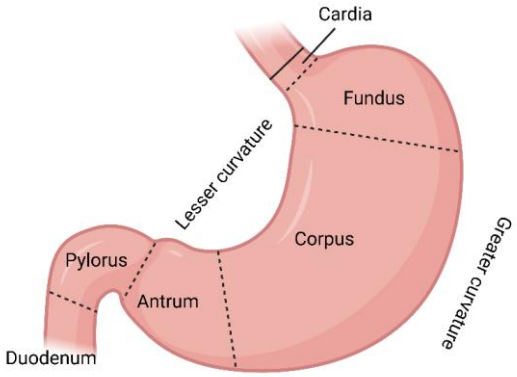


Figure 1.1: Anatomical structure of the human stomach (Created with BioRender.com).

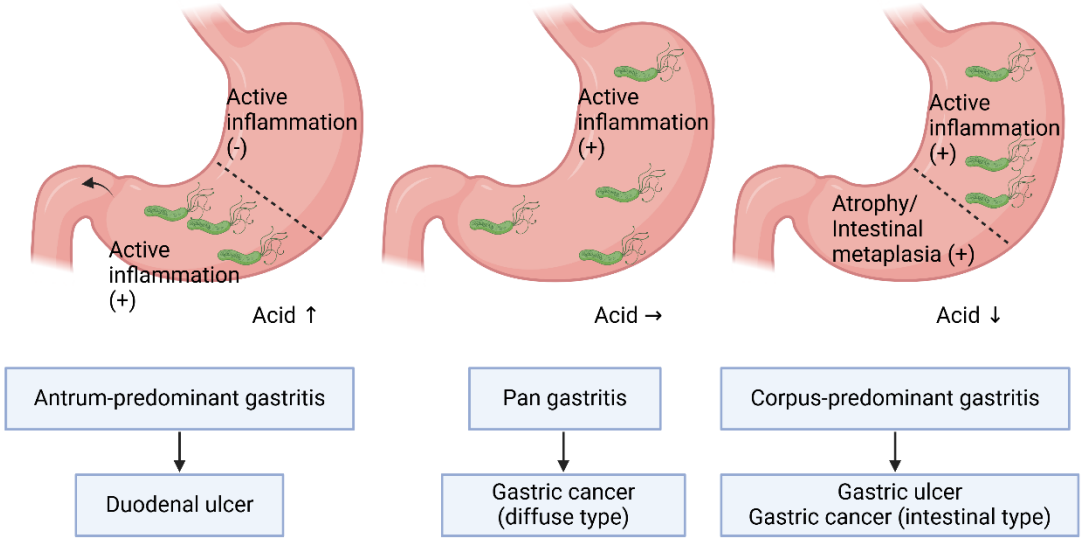


Figure 1.2: The correlation between the pattern of gastritis induced by H. pylori infection, acid secretion, area of active inflammation, and subsequent clinical outcomes (Created with BioRender.com).

1.2.2 Gastric cancer

The bacterium is the first bacterial cancer-causing agent (Wroblewski *et al.*, 2010). In 1994, *H. pylori* was classified as a grade I biological carcinogen by the World Health Organization (WHO) and an International Agency for Research on Cancer consensus group (Humans, 1994). It is the major cause of gastric cancer (Suerbaum & Michetti, 2002). Gastric cancer ranks as the fifth most common malignancy and is the fourth leading cause of cancer-related death globally (Yang *et al.*, 2023). Infection with *H. pylori* is an important risk factor for gastric cancer in humans, however, only about 1-2 % of *H. pylori*-infected individuals globally progress to gastric cancer (Tempera *et al.*, 2022).

It has also been found that the incidence of gastric adenocarcinoma varies profoundly between countries, with some East Asian countries such as China and Vietnam having a more than 10-fold higher prevalence than in Africa, Europe, and the USA (Park *et al.*, 2018b). Even though so much of the world's population is infected with *H. pylori*, gastric cancer is uncommon in some areas, even those with a high prevalence of *H. pylori* infections (Yamaoka, 2010). This implies that different *H. pylori* strains might have different abilities to cause cancer, and these differences could be responsible for the remarkable geographical variations in *H. pylori*-related diseases (Graham, 2014).

Due to the absence of effective treatments and frequent delays in detection, gastric cancer remains a significant global health concern with a poor outlook. Many patients are identified with advanced gastric cancer, leading to noticeably short survival times, due to late diagnosis (Li *et al.*, 2024). Current treatment strategies for gastric cancer focus heavily on consultations with multidisciplinary teams. Despite the abundance of therapeutic options

and insights from clinical trials, most gastric cancer patients benefit from personalized therapies, improving their prognosis. However, some patients continue to face challenges with advanced gastric cancer, including distant metastases and recurrence. A recent study revealed that most gastric cancer patients had diffuse-type gastric cancer (62.2%) and were in advanced stages (89.8%), with 60 patients (61.2%) having metastatic cancer (Bongkotvirawan *et al.*, 2024).

Since *H. pylori* infection is a strong risk factor for gastric cancer, eradicating *H. pylori* before the development of atrophic changes can eliminate the associated cancer risk (Mitchell & Katelaris, 2016). Previous studies have revealed that *H. pylori* eradication therapy was found to reduce the risk of gastric cancer significantly (Correa *et al.*, 2000; Kuipers *et al.*, 2004). The benefits of *H. pylori* eradication therapy that could reduce the risk extend, to those patients older than 60 years who had undergone the therapy at least 10 years earlier (Leung *et al.*, 2018). This strategy is cost-effective in countries with a higher risk of gastric cancer as there is no vaccine; however, population-wide antibiotic eradication would contribute to more antibiotic resistance (Areia *et al.*, 2013).

1.2.3 Gastric MALT lymphoma

The colonization of *H. pylori* also causes various other gastrointestinal diseases in humans including gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Hu *et al.*, 2016a). Gastric MALT lymphoma develops from marginal zone B cells in lymphoid follicles, which proliferate in an uncontrolled manner and frequently become widespread throughout the gastric mucosa (Robinson & Atherton, 2021). Several studies have shown that chronic gastric inflammation causes antigenic stimulation, which then leads to clonal expansion of

B-cell lymphocytes (Banks, 2007; O'Rourke, 2008). Hence, gastric MALT lymphoma is a clear representation of how chronic inflammation by bacterial infection contributes to oncogenesis (Sagaert *et al.*, 2010).

It was firmly established that almost 90% of patients with gastric MALT lymphoma are infected with *H. pylori*. However, only 2% of *H. pylori*-infected individuals worldwide will develop malignant lymphoma (Weber *et al.*, 1994). Gastric MALT lymphoma can range in symptom severity from being long-term asymptomatic, or associated with dyspepsia, abdominal pain, vomiting, diarrhoea, and nausea. Bleeding from the gastrointestinal tract or even perforation may occur occasionally while extensive lesions are present (Medina-Franco *et al.*, 2007). Eradication of *H. pylori* infection by antibiotic treatment causes complete regression of gastric MALT lymphoma in most early-stage cases and has become an established clinical practice (Bacon *et al.*, 2007).

1.3 *H. pylori* virulence factors

The risk of development of these diseases and the severity of gastritis associated with *H. pylori* infection is determined by multiple factors such as bacterial (virulence factors), host, and environmental factors (Kusters *et al.*, 2006b). Studies have revealed that infection with *H. pylori* carrying specific virulence factors such as blood-antigen binding protein A (BabA), *cag* pathogenicity island (*cag*PaI), high-temperature requirement A (HtrA), vacuolating cytotoxin A (VacA) and duodenal-ulcer promoting gene (DupA) may play important pathogenic roles in the development of *H. pylori*-related diseases (Chang *et al.*, 2018).

In general, there are four essential steps for *H. pylori* to establish successful colonization and disease pathogenesis before entering the host stomach: (1) rapid movement from the acidic

stomach lumen into the gastric mucus layer by flagella-mediated motility; (2) buffering against stomach acid by utilizing its urease activity; (3) attachment to host cells via bacterial adhesins interacting with receptors which helps the bacteria avoid displacement from the stomach; (4) accessing nutrients and urea in tissue fluid, for example, using VacA toxin to permeabilise epithelial cell membranes and HtrA directly degrade host cell factors such as occludin and claudin-8 in the tight junctions and E-cadherin in the adherens junctions. The cleavage of junctional proteins leads to disruption of the epithelial barrier paving the way for bacteria to transmigrate across the cell monolayer by a paracellular route (Backert *et al.*, 2018; Kao *et al.*, 2016).

1.3.1 Blood-antigen binding protein A (BabA)

H. pylori adhesion is considered one of the bacterial virulence factors involved in various processes during the early and chronic phases of infection (Kalali *et al.*, 2014). Blood-antigen binding protein A (BabA) is a type of bacterial adhesin that has a molecular mass of nearly 78 kDa. This bacterial cell-surface protein has three identified *bab* alleles including *babA*$_1$, *babA*$_2$, and *babB* (Kao *et al.*, 2016). The BabA adhesin of *H. pylori* recognizes and attaches to the Lewis^b antigen on gastric epithelial cells, a key step in bacterial colonization that drives the onset of gastritis and peptic ulcer disease (Ong & Lin, 2024).

Previous animal and human studies revealed that infection by BabA-expressing strains is associated with higher bacterial density, more severe injury in the gastric mucosa (Fujimoto *et al.*, 2007; Ohno *et al.*, 2011), and were also linked to a higher risk of duodenal ulcers than BabA-negative strains in Western countries (Fujimoto *et al.*, 2007). It also appears that the expression of BabA promotes an increased risk of PUD and gastric cancer in Western

countries, though, the existence of BabA is not correlated to gastric-related diseases in Asians (Chen *et al.*, 2013).

1.3.2 Cytotoxin-associated gene A (CagA)

Following the successful adherence between bacterial adhesins and host cell receptors, *H. pylori* release several effector proteins or toxins, such as cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) causing host tissue damage (Kao *et al.*, 2016). The *cagA* gene is one of more than 30 genes that are present in a 40 kb genomic DNA segment encoding 27-31 proteins known as the *cag* pathogenicity island (*cag*PAI) (Tohidpour, 2016). The type IV secretion system (T4SS) acts as a bacterial "syringe" delivery mechanism used to inject bacterial proteins directly into the cytosol of gastric epithelial cells, and it also plays a crucial role in bacterial adhesion to the epithelium. Strains that either lack the *cag*PAI or possess non-functional mutated alleles show a significantly diminished ability to establish colonization, persist, and exhibit pathogenicity in the host (Kusters *et al.*, 2006a).

The T4SS primarily functions to deliver the CagA protein into gastric epithelial cells, where it is phosphorylated by host Src-dependent kinases. This process involves several T4SS proteins, including CagI, CagL, CagY, and CagA, which interact with the host cell's integrin β1 to facilitate CagA's passage across the cell membrane. The translocation of CagA into host cells requires CagL to bind to the α5β1-integrin on gastric epithelial cells, which is crucial for activating the *cag* T4SS. This interaction serves as an anchor for *H. pylori* on host cells, facilitates the full assembly of the T4SS, and triggers Src-family kinases to phosphorylate CagA (Figure 1.3) (Liu *et al.*, 2023). The binding of CagL is mediated by an RGD motif (Arg-Gly-Asp), which is revealed following acid-induced conformational changes in the stomach.

The CagL-gastric epithelial cells interaction can activate the gastrin promoter, contributing to hypergastrinemia linked with *H. pylori* infection (Cover, 2012).

After CagA is delivered and phosphorylated in the host cell cytosol, it activates Src homology protein 2 (SHP-2), which then dephosphorylates various host proteins, leading to alterations in cellular signalling, activity, and morphology (Backert & Tegtmeyer, 2017). The binding of CagL to integrins on *H. pylori* initiates signalling cascades in gastric epithelial cells, resulting in NFκB activation, IL-8 expression, and the activation of focal adhesion kinase (FAK), epidermal growth factor receptor (EGFR), and MAPK signalling pathways (Naumann *et al.*, 2017).

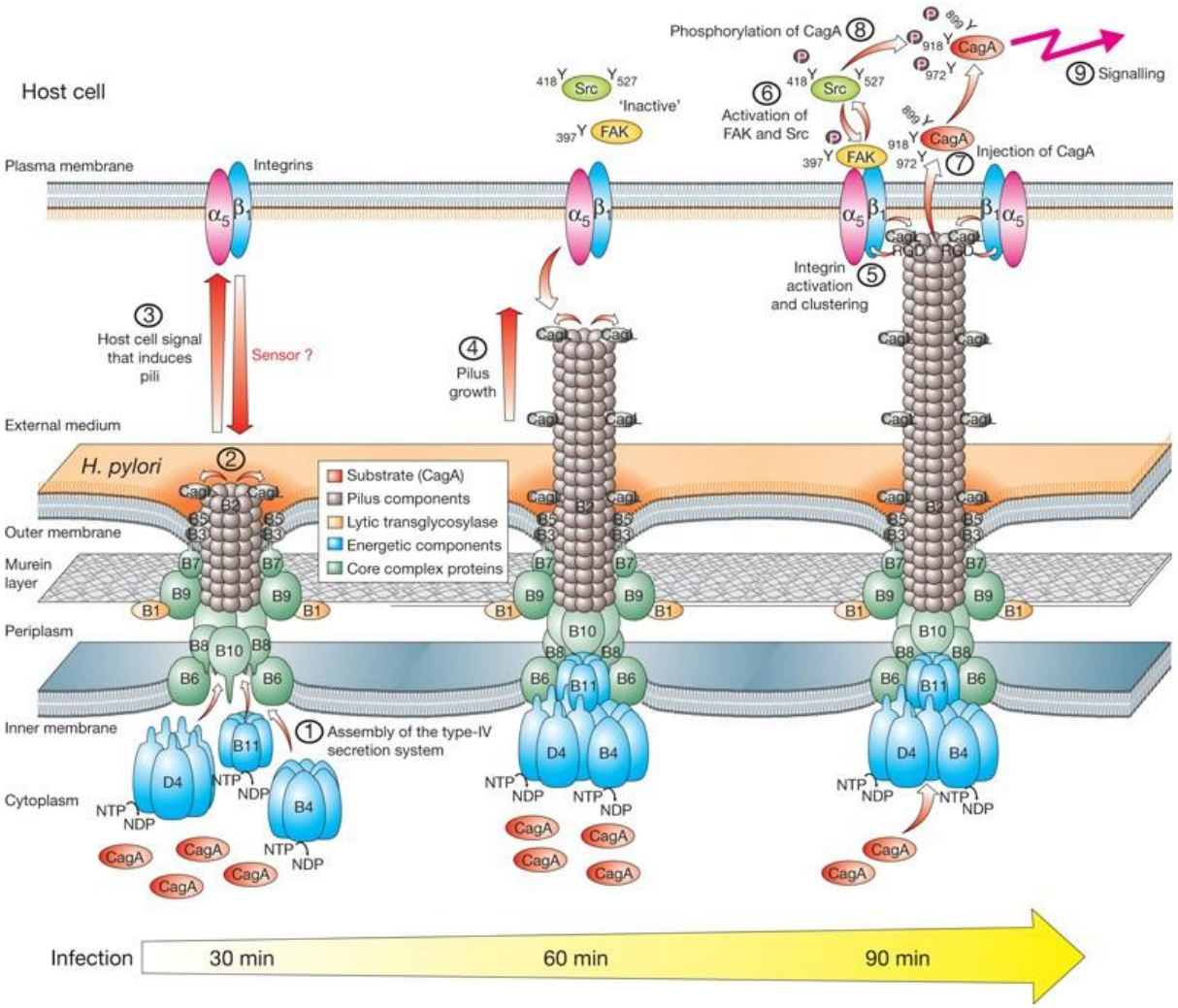


Figure 1.3: Formation of the cag type IV secretion system*. (1) In the early stages of infection, the T4SS subunits are assembled to span the H. pylori membrane. (2, 3, 4) The polymerisation of the pilus causes projection toward host cellular surfaces. (5) CagL is a protein located at the tip of the pilus, CagL binding to α5β1-integrins on the host cell allows full assembly of the T4SS. (6) CagL: integrin association activates host kinases including FAK and Src. (7) CagA can be translocated into the host cytosol upon integrin activation. (8) CagA is phosphorylated by host kinases. (9) CagA phosphorylation leads to altered host signalling which causes cytoskeletal rearrangement, disruption of cell-cell junctions, and inflammatory gene expression, notably IL-8 (Kwok et al., 2007).*

In general, *H. pylori* can be distinguished into two major subpopulations based on the presence or absence of the *cagA* gene that encodes the CagA protein: *cagA*-positive and *cagA*-negative strains (Hatakeyama, 2004). Clinical data show that *cagA*-positive strains are more likely to cause gastric cancer than *cagA*-negative ones (Ji *et al.*, 2024). Although most people infected with *cagA*-positive strains are asymptomatic, but their risk of gastric cancer is about two times greater than with those bearing *cagA*- negative strains (Huang *et al.*, 2003). Furthermore, infections with *H. pylori* strains that express CagA tend to be linked to more severe clinical outcomes than those without the virulence factor CagA, and *cagA*-positive *H. pylori* strains are associated with acute gastritis, peptic ulceration, and gastric cancer (Park *et al.*, 2018b).

Interestingly, *cagA* gene prevalence was about 60% in Western countries, whereas in East Asian countries, it was detected in almost all of the isolates (Matsunari *et al.*, 2016). For instance, in Vietnam, 80% of confirmed *H. pylori* strains were found to have highly virulent East Asian type *cagA* gene which is potent in causing gastric cancer (Nguyen *et al.*, 2021). Extensive molecular epidemiological studies have indicated that individuals infected with East Asian-type CagA ($CagA^{E}$) strains are at a higher risk of developing gastric cancer (Yuan *et al.*, 2017). Transgenic mouse studies have shown that $CagA^{E}$ leads to more severe neoplastic lesions compared to the Western-type A ($CagA^{W}$) (Miura *et al.*, 2009). *In vitro* experiments have also demonstrated that $CagA^{E}$ is more virulent than $CagA^{W}$, causing greater IL-8 secretion and more significant morphological changes (Bridge *et al.*, 2017). These findings suggest that $CagA^{E}$ is more carcinogenic than $CagA^{W}$, although the exact mechanisms remain unclear.

Notably, a study has shown that the East Asian type of CagA binds to SHP2, 100-fold more strongly than the Western CagA (Hayashi *et al.*, 2017). This is because *cagA* is a polymorphic gene that presents different numbers of repeated sequences located in its 3' region, and each repeated region of CagA protein contains Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, including a tyrosine phosphorylation site (Hatakeyama, 2004).

The biological activity of the CagA protein is influenced by the types and numbers of EPIYA motifs in its C-terminal region. These motifs are categorized into four types based on their unique conserved flanking sequences: EPIYA-A, -B, -C, and -D. *H. pylori* isolates from East Asia, where the incidence of gastric cancer is highest, typically feature the EPIYA A-B-D motif (Figure 1.4) (Nishikawa & Hatakeyama, 2017).

In contrast, isolates from Western countries usually exhibit the EPIYA A-B-C motif (Figure 1.4). The A-B-D motif demonstrates a stronger binding affinity for phosphatases (SHP-2) compared to the A-B-C motif and, therefore may causatively account for higher incidence of gastric cancers in East Asian countries than in Western countries (Takahashi-Kanemitsu *et al.*, 2020). This further suggests that CagA sequence polymorphism is also one of the crucial factors that determines the level of oncogenic activity of CagA-positive *H. pylori* strains.

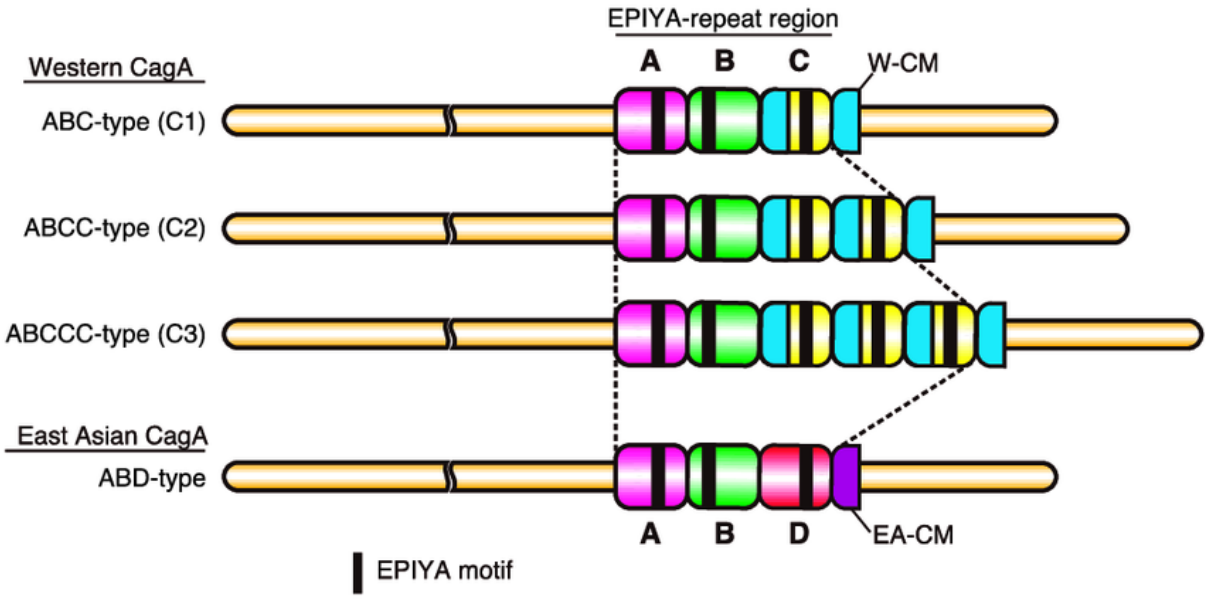


Figure 1.4: Polymorphisms in the C-terminal tail of H. pylori CagA. *The C-terminus of Western CagA is organized into segmental repeats characterized by EPIYA-A, EPIYA-B, and typically 1–3 repeats of EPIYA-C segments, each containing a single EPIYA motif. Note that Western CagA always carries an extra copy of the Western CM motif (W-CM) flanking the distal end of the EPIYA-repeat region. In contrast, the highly oncogenic East Asian CagA usually contains EPIYA-A, EPIYA-B, and a single copy of the EPIYA-D segment. Since the EPIYA-D segment does not contain a CM motif, East Asian CagA typically carries only one East Asian CM motif (EA-CM), which flanks the EPIYA-repeat region at the distal end (Nishikawa & Hatakeyama, 2017).*

1.3.3 High-Temperature Requirement A (HtrA)

High-temperature requirement A (HtrA) in *H. pylori* is a serine protease that plays a key role in the pathogen's ability to colonize and persist in the human stomach. It is a multifunctional protein that significantly contributes to the bacterium's survival, adaptation, and pathogenicity by compromising the integrity and function of the gut epithelial barrier through the disruption of epithelial cell junctions (Taglialegna, 2023). HtrA cleaves E-cadherin (Cdh1), an essential cell adhesion protein and tumour suppressor that plays a pivotal role in maintaining the balance of gastric epithelial cells. Cdh1 creates calcium-dependent homophilic bonds between its extracellular domains within the same cell (cis)

and neighbouring epithelial cells (trans), ensuring strong cell-to-cell connections (Thompson *et al.*, 2021).

The detachment of the Cdh1 ectodomain was linked to infections by *H. pylori*. This bacterium secretes a Cag T4SS-independent factor, the serine protease HtrA, which cuts the extracellular domain of Cdh1 at the cell surface, causing a localized disruption in lateral cell-to-cell junctions. Cdh1 was the initial substrate identified for HtrA. Subsequently, it was found that desmoglein-2 (Dsg-2) from desmosomes, along with the tight junction proteins occludin and claudin-8, are also targets of *H. pylori* HtrA (Bernegger *et al.*, 2021; Tegtmeyer *et al.*, 2017). Indeed, HtrA activity was identified as the primary factor responsible for the opening of tight and adherens junctions between epithelial cells, while host-derived proteases like matrix metalloproteases or ADAM proteases play only a minor role in *H. pylori* infections (Bernegger *et al.*, 2021).

The shedding of Cdh1 mediated by HtrA and the subsequent disruption of cell–cell adhesions allow *H. pylori* to move through the host epithelial monolayer, allowing the direct interaction with integrin-β1 at the basolateral membrane and facilitate the translocation of the virulence factor cytotoxin-associated gene A (CagA) into host cells via T4SS. Once inside the cytosol, CagA is quickly phosphorylated on tyrosine residues by Src family kinases (SFK) and c-Abl, directly influencing a complex network of signalling pathways that result in epithelial depolarization (Tegtmeyer *et al.*, 2017; Toh & Wilson, 2020).

In an earlier study, the analysis of 1,043 genomes of *H. pylori* identified an SNP in the serine protease HtrA (at position serine/leucine 171) that is strongly associated with gastric cancer. It showed that the mutation from 171S to 171L prompts the formation of HtrA trimers, enhancing proteolytic activity and the cleavage of epithelial junction proteins like occludin

and the tumour-suppressor E-cadherin. They also found that only *H. pylori* with the 171L-type HtrA, not those with 171S-HtrA, cause significant epithelial damage, facilitate the injection of the oncoprotein CagA into epithelial cells, increase NF-κB-mediated inflammation and cell proliferation through the nuclear accumulation of β-catenin, and induce double-strand breaks in host DNA, which collectively lead to malignant transformations (Sharafutdinov *et al.*, 2023). These findings underscore the amino acid changes 171S/L HtrA as a unique bacterial cancer-associated SNP and suggest its potential as a biomarker for predicting risks in *H. pylori* infections (Backert *et al.*, 2024).

1.3.4 Vacuolating cytotoxin A (VacA)

Another major protein toxin secreted by *H. pylori* is vacuolating cytotoxin A (VacA). When VacA toxin binds to host cells via endocytosis, it induces cytoplasmic vacuole formation, causing multiple cellular transformations such as the alteration of membrane permeability, which results in apoptosis (Gobert & Wilson, 2022; Palframan *et al.*, 2012). All *H. pylori* strains contain a single chromosomal *vacA* gene, which encodes a protein about 140-kDa in mass. Following translation and proteolysis, two domains, p33 and p55, are secreted. Both are essential for the toxin to bind to the plasma membrane of cells effectively (Soyfoo *et al.*, 2021). Although nearly all strains isolated from humans possess the *vacA* gene, the capacity to induce cell vacuolization differs significantly from strain to strain (Atherton *et al.*, 1995).

The *vacA* gene has a mosaic-like structure, featuring both conserved and variable allelic sequences. These variable sequences are located in different regions from the N-terminal side, including the signal sequence region, intermediate region, deletion region, mid region and c-region. The difference in vacuolating abilities is determined by the polymorphisms in

the *vacA* gene sequence in these five regions: *s*-region (*s*1 and *s*2), *i*-region (*i*1, *i*2, *i*3), *m*-region (*m*1 and *m*2), *d*-region (*d*1 and *d*2) and *c*-region (*c*1 and *c*2) (Foegeding *et al.*, 2016; Keikha *et al.*, 2020) (Figure 1.5). A variable combination of sequences in these 3 regions leads to multiple alleles and hence determines the degree of vacuolating activity (Figure 1.5) (Atherton *et al.*, 1995; Keikha *et al.*, 2020).

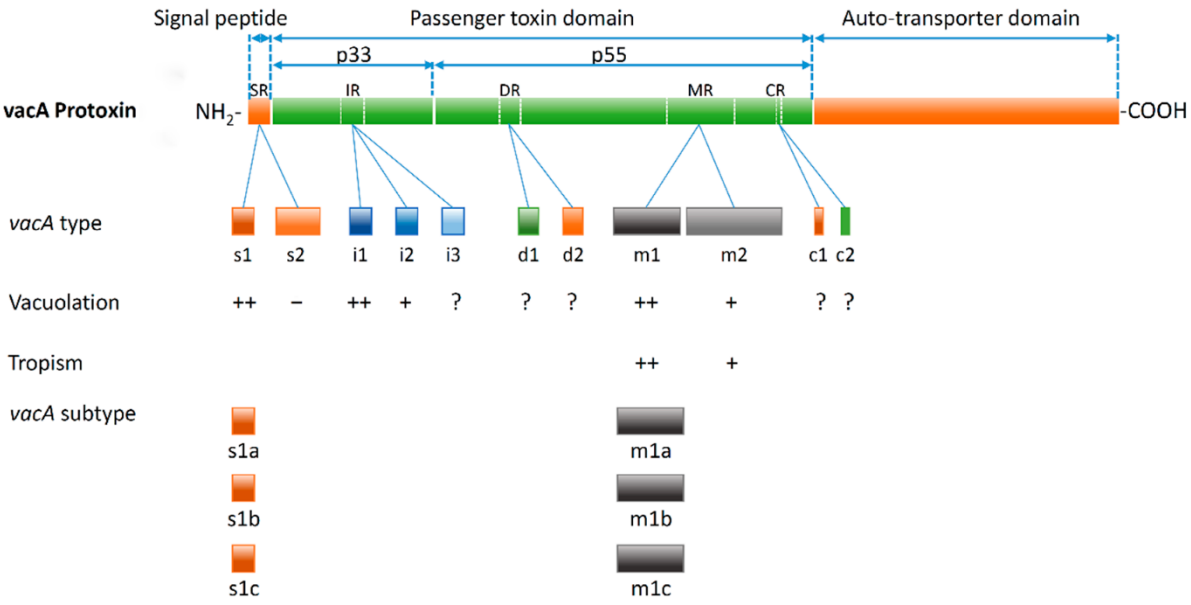


Figure 1.5: The sequence diversity regions of vacA, which are closely linked to the vacuolating activity of H. pylori and clinical outcomes, are located in the s-region, the i-region on the p33 domain, d-region, m-region, and c-region on the p55 domain. *Variations in these regions correspond to differences in vacuolation, specificity, and clinical outcomes (Thi Huyen Trang et al., 2016).*

The *vacA* gene features three regions with high sequence diversity: the signal (*s*), intermediate (*i*), and middle (*m*) regions—that are intricately linked to vacuolating activity and clinical outcomes (Chung *et al.*, 2010; Rhead *et al.*, 2007). The *s*- and *m*-regions are the primary polymorphic areas and are well-established markers for *H. pylori*'s virulence and the likelihood of developing serious diseases. The *s*-region includes signal peptides crucial for

transporting the mature 88 kDa VacA toxin and the amino-terminal residues of p33, known for being a highly hydrophobic domain of VacA. On the other hand, the *m*-region encodes part of the carboxyl-terminal p55 subunit, which plays a key role in facilitating VacA binding to host cells. The *i*-region, positioned between the *s*- and *m*-regions in the carboxyl-terminal half of p33, is the third polymorphic determinant. It appears to interact with the *s* and *m* regions and is identified as a key factor for VacA toxicity. Additionally, the *i*-region is considered a more reliable predictor of disease severity than the *s*- or *m*-regions (Basso *et al.*, 2008; Thi Huyen Trang *et al.*, 2016).

The *s*1, *m*1, and *i*1 types are classified as fully active VacA, associated with a higher risk of developing gastric cancer compared to the *s*2, *m*2, or *i*2 types. Unlike the *s*1 type, the *s*2 forms of VacA do not exhibit detectable vacuolation activity in most *in vitro* assays. Compared to the *m*1/*i*1 types, the *m*2/*i*2 types are significantly less active and are almost non-toxic (Thi Huyen Trang *et al.*, 2016). The *s*1 and *m*1 genotypes have been further divided into three subtypes: *s*1a, *s*1b, *s*1c and *m*1a, *m*1b, *m*1c, respectively (Thi Huyen Trang *et al.*, 2016). In general, vacuolating activity is high in *s*1/*m*1 genotypes, intermediate in *s*1/*m*2 genotypes and absent in *s*2/*m*2 genotypes (Atherton *et al.*, 1995; Keikha *et al.*, 2020).

In 2009, Ogiwara *et al.* (2009) identified the *d*-region as an 81-base pair deletion situated between the *i*- and *m*-regions. They proposed that this region might play a role in the binding of VacA to host gastric cells and its vacuolating activity. The *d*-region was classified into two genotypes: *d*1, which has no deletion or short deletions ranging from 9 to 23 base pairs, and *d*2, which has an 81-base pair deletion. However, the roles of these genotypes in disease remain unclear, and the existing evidence is contradictory (Thi Huyen Trang *et al.*, 2016). Likewise, the *c*-region's function is still unknown; however, the *c*1 genotype (a

deletion of 15 bp in the 3'-end region of *vacA*) has been found to be significantly associated with the risk of gastric cancer (Bakhti *et al.*, 2016).

Research suggests a strong association between the *vacA s*1*m*1 strain and an increased risk of PUD and gastric cancer (Memon *et al.*, 2014). Previous studies revealed that individuals infected with *H. pylori* carrying *vacA s*1 or *m*1 have an increased risk of gastric cancer in Western populations (Matos *et al.*, 2013), whereas *vacA i1* type *H. pylori* infection is linked with higher gastric cancer risk in the Middle Asia and Eastern area (Liu *et al.*, 2016; Sheikh *et al.*, 2018). The presence of the *i*1 allele can influence whether the *s*1*m*2 genotype induces cytotoxic activity (Chauhan *et al.*, 2019). Meanwhile, data from several studies suggested that in regions such as Colombia and Japan, where the prevalence rate of distal gastric cancer is high, most *H. pylori* strains carry *vacA s*1/*m*1 genotypes (Boyanova *et al.*, 2002; Garza-Gonzalez *et al.*, 2004).

1.3.5 Duodenal ulcer promoting gene (*DupA*)

Another virulence factor, called duodenal ulcer promoting gene A (*dupA*) was named for its role in increasing the risk of duodenal ulcer (DU) in *H. pylori*-infected patients and offering some protection against gastric cancer (Hussein *et al.*, 2015). *DupA* encodes a *virB4* ATPase homolog situated in the plasticity region and includes the jhp0917 and jhp0918 genes, which are positioned next to the cag pathogenic island. This gene was found to be homologous to the virulence gene *virB* which encodes a type IV secretion protein in *Agrobacterium tumefaciens* (Alam *et al.*, 2020).

DupA gene is located in the right module of the *tfs4b* ICE, and genes within *tfs3* and *tfs4* are known to be linked to pro-inflammatory activity in the gastric mucosa and an increased risk

of gastroduodenal diseases, hence is recognized as a specific marker for DU (Phuc *et al.*, 2021). The presence of *dupA* alongside its neighbouring T4SS genes in a complete cluster (C1R1) may serve as a more reliable indicator of disease risk than *dupA* alone or in an incomplete cluster (Jung *et al.*, 2012). It is also has been observed to trigger pro-inflammatory cytokines such as IL-8 and IL-12, which contribute to mucosal inflammation and the infiltration of polymorphonuclear leukocytes, resulting in gastritis and duodenal ulcers (Bhattacharjee *et al.*, 2024).

The *in vitro* study demonstrated that certain *tfs4b* T4SS genes (*virB2*, *virB4*, *virB8*, and *virB10*) showed increased expression when exposed to low pH and contact with a human gastric cell line, indicating the role of *tfs4b* in host colonization (Silva *et al.*, 2017). These findings imply that *tfs3* and *tfs4b* might create an alternative T4SS for DNA or effector proteins, like *cag*PAI, and are regarded as virulence factors of *H. pylori*. The distribution of *tfs3* and *tfs4b* ICEs may vary, suggesting that the potential risk of these clusters in gastroduodenal diseases should be assessed within each country and ethnicity (Delahay *et al.*, 2018; Fischer *et al.*, 2014; Waskito *et al.*, 2018).

Phuc *et al.* (2021) demonstrated that in patients with DU, the prevalence of *H. pylori* strains containing the complete *tfs3* ICE was significantly higher compared to those with non-cardia gastric cancer (NCGC). Moreover, the occurrence of strains with both complete *tfs3* ICE and *cag*PAI was notably higher in DU patients than in those with NCGC and chronic gastritis. These findings show that the presence of *cag*PAI and complete *tfs3* increased the risk of DU when compared to NCGC. In Vietnam, a complete *tfs3* ICE cluster was linked to gastroduodenal diseases, although the prevalence of the *dupA*/complete *dupA* cluster was low in Vietnamese strains. Overall, the acquisition of *tfs3*/*4* ICE was common among *H.*

pylori strains in individuals with gastroduodenal disease in Vietnam, and the complete *tfs3* ICE cluster served as a reliable marker for disease severity in the *H. pylori*-infected population.

An intriguing connection between *dupA* and *H. pylori* strains has been found in Asian populations, but not in Western ones, leading to some controversy. Moreover, studies conducted in Belgium, Brazil, China, Iran, and North America yielded conflicting results, suggesting that *dupA*-associated clinical outcomes are more prevalent in Asian countries compared to Western ones (Alam *et al.*, 2020). A meta-analysis carried out in 2021 also showed that *dupA*-positive strains are not significantly linked to gastric cancer risk in Western countries, but there is a strong association with gastric cancer in Asian countries (Karbalaei *et al.*, 2021).

A genotyping analysis of the *dupA* gene from Spain found the prevalence of *dupA* was higher in peptic ulcer than gastric cancer (Fernandez-Gonzalez & Backert, 2014) , whilst the whole genome sequence analysis of *H. pylori* strains isolated from 41 non-atrophic gastritis patients in Switzerland showed that *dupA* was present in 24.4% of the population, and it was not associated with severe gastritis (Imkamp *et al.*, 2019).

However, a study conducted by Zhu *et al.* (2022) in Beijing, China found that the presence of *dupA* was found to be inversely related to the risk of atrophic gastritis. Meanwhile, in Shandong and Guangxi, *dupA*+ strains were more frequently found in patients with PUD compared to those with non-ulcer dyspepsia (NUD), but this association was not seen in other regions in China (Xue *et al.*, 2021).

One study in Guangzhou, China revealed that *dupA*+ in *H. pylori* was not found in precancerous lesions but was more common in erosive gastritis, while *dupA*- *H. pylori* was

prevalent in precancerous lesions. These findings suggest that high *dupA*+ expression in *H. pylori* is linked to a higher risk of erosive gastritis and less disturbance to the gastric microbiome, preserving its richness, indicating that the *dupA* gene should be considered a risk factor for erosive gastritis rather than gastric cancer (Chen *et al.*, 2023b).

1.4 Host genetic factors

In general, the key pathophysiological event in *H. pylori* infection is the induction of an inflammatory response (Israel & Peek, 2018). Since *H. pylori* infection causes damage to the gastric epithelial cells through initiation of chronic inflammation, it is appropriate to consider genes that control this process which may be responsible for the individual genetic predisposition to *H. pylori*-related diseases such as gastric cancer. Hence, apart from the bacterial virulence factors, host genetic factors may also influence the extent of gastritis in *H. pylori*-infected patients.

Primarily, the inflammation that results from this host-bacterial interaction is the main mediator of disease (Robinson *et al.*, 2007). For example, the pro-inflammatory cytokine interleukin (IL)-1β is regarded as a pivotal mediator in host-environment interactions. It was reported that IL-1β is upregulated in the presence of *H. pylori* and responsible for triggering and intensifying the inflammatory reaction after bacterial infection in host cell (Li *et al.*, 2015b). In addition to that, tumour necrosis factor-A (TNFα) is another potent pro-inflammatory cytokine that is involved in a diverse range of signalling pathways including apoptotic pathways, NF-kB activation of inflammation and stress-activated protein kinases (SAPKs) (Zhang & An, 2007). Both IL-1β and TNFα are also effective in inhibiting gastric acid

secretion, hence, these soluble peptide cytokines have been proposed to play key roles in the development of gastric cancer (Shanks & El-Omar, 2009).

The existence of genetic polymorphisms in the *IL-1* gene cluster, such as different *IL1B* alleles may influence the host-bacterial interactions and contribute to more inflammation responses in *H. pylori*-infected patients (Amieva & El–Omar, 2008), and have been associated with increased risk for gastric cancer (Pachathundikandi *et al.*, 2016). A study carried out by El-Omar *et al.* (2000) also revealed that the linkage of high IL-1β genotypes (two polymorphisms in the *IL-1B* and *IL-1RN* genes) with hypochlorhydria and gastric atrophy in a Caucasian population were known to be at increased risk of developing gastric cancer in *H. pylori*-infected patients.

Furthermore, the association between *IL-1* gene cluster polymorphisms and gastric cancer and its precursors has also been established by other studies carried out in Asian and Hispanic populations (Figueiredo *et al.*, 2002; Oliveira *et al.*, 2012; Zhao *et al.*, 2012). Previous studies have also revealed that polymorphisms in TNFα, IL-1B, IL-6, IL-8, and IL-10 genes are strongly linked to peptic ulcer disease (PUD) (Miftahussurur & Yamaoka, 2015), and associated with the risk of gastric cancer (Kang & Choi, 2023).

1.5 Environmental factors

Environmental factors may also contribute to the pathogenesis of *H. pylori*-related diseases. Current smoking has been proven to be the most significant behavioural risk factor for gastric cancer in individuals seropositive for *H. pylori* (Butt *et al*., 2019). A previous study revealed that people who smoke were more likely to develop gastric cardia cancers, as compared to the non-smoker group (Nomura *et al.*, 2012). Moreover, smoking may also

reduce the efficacy of antibiotics, which subsequently increases the likelihood of eradication therapy failures (Yu *et al.*, 2022).

Other factors such as a diet high in salt and preservative intake, may also increase the risk of gastric cancer in *H. pylori*-infected individuals (Balendra *et al.*, 2023). A previous *in vitro* study reported that a high dietary salt condition significantly upregulated the expression of *cagA* gene, in comparison to a regular diet condition, leading to inflammation and enhanced carcinogenesis in *H. pylori*-infected animal model (Gaddy *et al.*, 2013). Interestingly, data from one study suggested that B cell IL-10 secretion was suppressed by smoking and obesity in *H. pylori*-infected subjects and was associated with an increased risk of gastric cancer when compared to non-smoking and non-obese subjects (Li *et al.*, 2015a). Studies have also shown that consuming green vegetables, fruits, and green tea that are high in antioxidants could decrease the risk of gastric cancer (Eusebi *et al.*, 2020; Jeurnink *et al.*, 2012).

In summary, disease outcomes associated with *H. pylori* infection are mediated by the combined effects of bacteria, host, and environment. In addition to these risk factors, multiple evidence has suggested the modulation in immune response also contributes to the pathogenic outcomes of *H. pylori*-related disease. This host immune response risk factor triggered by *H. pylori* will be covered in much detail in section 1.7.

1.6 Treatment for *H. pylori* and antimicrobial resistance

1.6.1 Clinical diagnosis and current treatment recommendations

Studies have shown that eradication therapy of *H. pylori* has been linked to decreased rates of ulcer recurrence and regression of low-grade MALT lymphoma (Gong *et al.*, 2016; Nakamura & Hojo, 2023; Park & Koo, 2014). Eradication of *H. pylori* is also proven to be beneficial in preventing gastric cancer (Wu *et al.*, 2019). Therefore, due to the benefits of eradication therapy, the experts and specialists in the field fully agreed on the recommendations and guidelines.

European guidelines for the diagnosis and treatment of *H. pylori* infection have been published by the Maastricht IV/Florence Consensus (Malfertheiner *et al.*, 2022), as well as Public Health England (McNulty, 2017). All patients with PUD and MALT lymphoma should be tested for *H. pylori*. Public Health England also recommended that patients with uncomplicated dyspepsia be tested for *H. pylori* as part of a test-and-treat strategy (non-invasive). Furthermore, patients with unexplained iron deficiency anaemia and nonsteroidal anti-inflammatory drug (NSAID)-naive patients (who need long-term NSAID therapy) should also be tested for *H. pylori*. In addition to that, the European guidelines (Maastricht) advised that first-degree relatives to patients with gastric cancer be tested for *H. pylori* given that a 50% increased risk of cancer in children and a 3-fold increased risk in siblings.

Guidelines from the UK National Institute for Health and Care Excellence (NICE) recommend an endoscopy for patients over the age of 55 with recent onset and persistent dyspepsia to exclude cancer (National Collaborating Centre for, 2015). Non-invasive patients can be tested with a urea-breath test (UBT) or stool antigen test (SAT), whilst endoscopic patients

can be tested with a rapid urease test. All *H. pylori*-positive patients should be offered eradication therapy, depending on their antibiotic history as each additional course of clarithromycin, metronidazole or quinolone increases resistance risk (McNulty, 2017).

The treatment options for eradicating *H. pylori* include sequential first, second and third-line therapies, as summarized in guidelines published by Public Health England and The Maastricht IV/Florence Consensus (Figure 1.6). Patients who receive triple therapy are normally administered a regimen containing a combination of two antibiotics and a proton pump inhibitor (PPI) for 7 days. Meanwhile, quadruple treatment combines two antibiotics, PPI and bismuth compounds for 10 days. Proton pump inhibitors (PPI) such as omeprazole, lansoprazole or pantoprazole inhibit gastric acid secretion (Nehra *et al.*, 2018) whereas compounds such as bismuth subsalicylate has been shown to work synergistically with antibiotics to reduce the bacterial burden and increase the likelihood of *H. pylori* eradication (Alkim *et al.*, 2017). The choice of therapy depends on previous patient treatments, regional bacterial resistance patterns (if known), local recommendations and drug availability. It is also recommended that if a patient has recently been treated with clarithromycin, the same drug should not be used in their treatment for *H. pylori*.

Figure 1.6 includes the treatment options in Public Health England guidelines; standard triple therapy using clarithromycin is a reasonable initial first-line therapy where local clarithromycin resistance is low. If the first-line treatment fails to eradicate the infection, a second-line treatment is necessary. For patients who have a penicillin allergy, metronidazole is recommended instead of amoxicillin. However, Public Health England guidelines have not been revised for a while, and the UK remains one of the only European countries still using triple therapy as a first-line treatment, while other countries have transitioned to quadruple

therapy as first-line treatment, guided by Maastricht IV/Florence Consensus Report recommendations (Malfertheiner *et al.*, 2024).

The Maastricht IV/Florence consensus recommended quadruple bismuth therapy (PPI + bismuth subsalicylate + two antibiotics) as a first-line treatment for *H*. *pylori* infection among areas with high rates of clarithromycin resistance (Malfertheiner *et al.*, 2022). If patients however have had two failed rounds of antibiotic therapy and are still *H. pylori*-positive, their strains should be isolated, and an antibiotic susceptibility test should be performed. No testing is performed before this stage when many patients have already received two rounds of treatment with inappropriate selections of antibiotics.

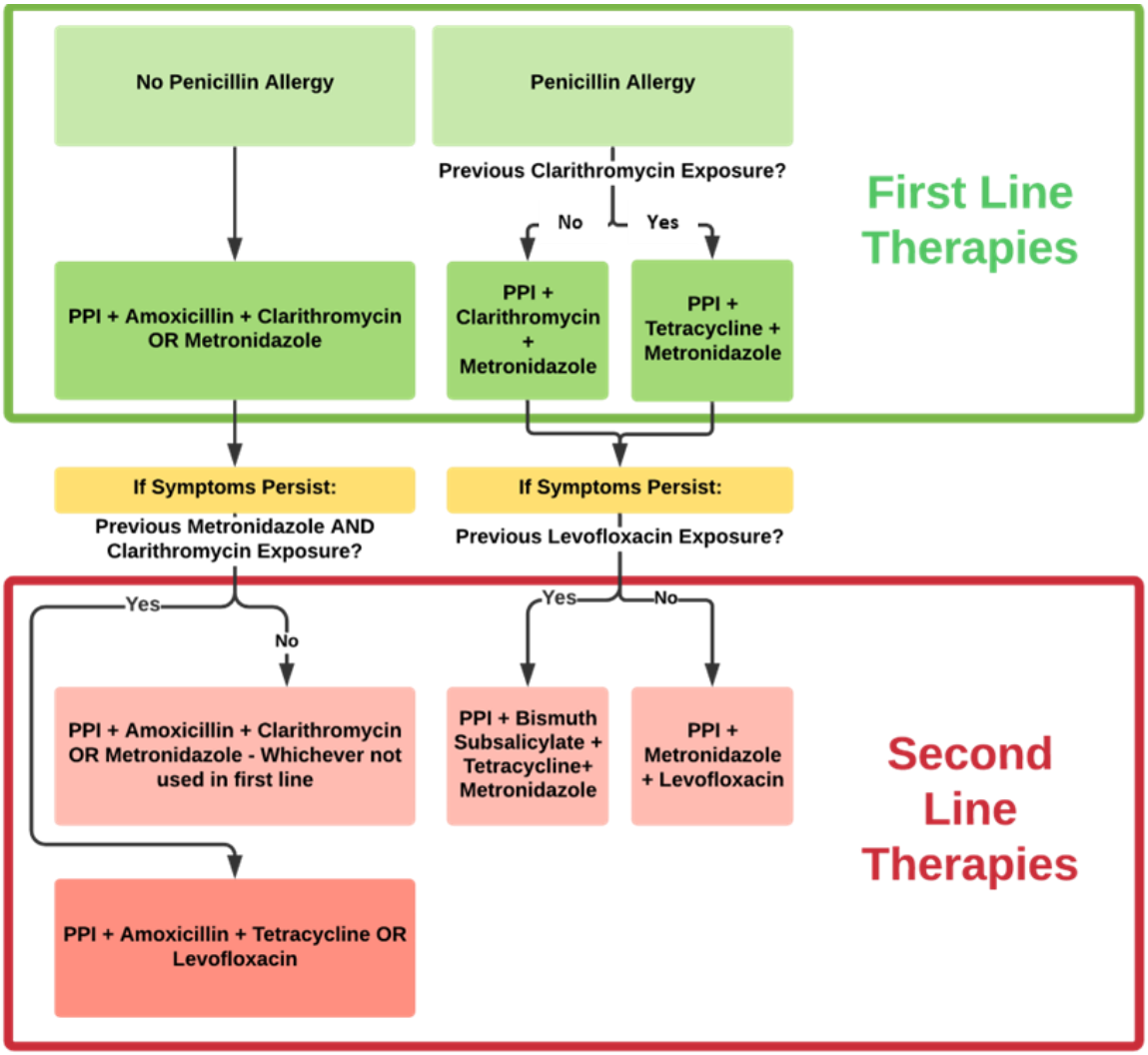


Figure 1.6: Public Health Guidelines for first- and second-line therapies for the treatment of H. pylori. ** PPI = proton pump inhibitor. Reproduced from (McNulty, 2017).*

Meanwhile, in the United States, several professional organisations provide clinical guidelines for diagnosing, treating, and managing *H. pylori* infection, most notably the American College of Gastroenterology (ACG) and the American Gastroenterological Association (AGA). The ACG recommended that treatment for both treatment-naïve patients and those who failed in eradicating *H. pylori* (with an initial PPI-clarithromycin triple therapy) is a 14-day optimized bismuth-based quadruple therapy. This therapy includes taking a PPI twice daily, tetracycline 500 mg four times daily, metronidazole 500 mg three or

four times a day, and bismuth subcitrate or bismuth subsalicylate four times daily for 14 days. Rifabutin-based triple therapy and vonoprazan-amoxicillin dual therapy are suggested as alternative regimens. The guidelines also advise against using a PPI-clarithromycin triple therapy unless antibiotic sensitivity testing confirms clarithromycin sensitivity (Chey *et al.*, 2024).

H. pylori infection is widespread in East Asia, with seroprevalence rates of 44.2% in China, 37.6%-43.2% in Japan, and 51.0% in South Korea. These East Asian countries experience high gastric cancer rates; therefore, the Kyoto Global Consensus report highlighted that *H. pylori* gastritis should be regarded as the primary cause of gastric cancer development. Consequently, the *H. pylori* treatment guidelines in China, Japan, and South Korea have been recently updated based on data specific to each country (Cho & Jin, 2022).

As per the Fifth Chinese Consensus on *H. pylori* Management in 2016, treatment regimens incorporating bismuth salt are suggested as an empirical first-line therapy for *H. pylori* in China (Liu *et al.*, 2018). In 2016, the Japanese Society for *Helicobacter* Research (JSHR) revised its guidelines for the diagnosis and treatment of *H. pylori* infection and improved the effectiveness of treatment by introducing the potassium-competitive acid blocker (P-CAB) in the standard primary eradication therapy (Kato *et al.*, 2019). Meanwhile, in South Korea, the Korean College of *Helicobacter* and Upper Gastrointestinal Research recommended a personalized *H. pylori* eradication strategy, informed by PCR or sequencing for clarithromycin resistance, when a 7-day standard triple therapy is considered as a first-line treatment (Jung *et al.*, 2021).

1.6.2 *H. pylori* eradication failure

Eradication of *H. pylori* has been prescribed as a treatment and a preventative for various gastric-related diseases. The regimens that utilize PPI in combination with several antibiotics such as clarithromycin, metronidazole and amoxicillin have been proven to be successful in eliminating *H. pylori*. However, there has been a notable decline in *H. pylori* eradication success rates in the recent past.

Grading the rates of eradication's effectiveness would help clinicians to rationally identify and compare treatment regimens in patients. In principle, the intention to treat (ITT) cure rate categories are F or unacceptable (80%), D or poor (81-84%), C or fair (85-89%), B or good (90-95%), and A or excellent (95-100%). Ideally, *H. pylori* eradication therapy should be safe, economical, and effective with an eradication rate of greater than 90% (Zhang *et al.*, 2015).

That said, data show that the widely accepted triple combination treatment has lost its efficacy, with an eradication rate ranging from 78.1-79.1% in the United States to 81.5% in the Europe (Nyssen *et al.*, 2021; Shah *et al.*, 2022). One of the key factors for this eradication failure is *H. pylori* antibiotic resistance, especially to clarithromycin and metronidazole which may affect the efficacy of combination therapy against the infection. A meta-analysis study of the relationship between the previous use of antibiotics and the failure of eradication therapy in 3181 *H. pylori*-positive patients who have been administered standard triple therapy was analysed. The overall failure rate of *H. pylori* eradication therapy was 14.8%, with the failure rates increasing significantly in patients with a history of clarithromycin or other macrolides. Of the patients with antibiotics used before

starting *H. pylori* eradication therapy, the failure rate was 35.9% for clarithromycin, 28.8% for other macrolides, and 18.0% for cephalosporins (Lim *et al.*, 2016a).

A systematic review study of 46 randomised controlled trials across America, Europe and Asia revealed that the overall eradication rate of sequential therapy was 84.3% in 5666 patients who had been given a dual therapy of PPI and amoxicillin, followed by a triple therapy of PPI, clarithromycin and nitroimidazole (Gatta *et al.*, 2013). The study also showed that the sequential therapy was able to eradicate 72.8% of the *H. pylori* strains resistant to clarithromycin.

In a large cohort study of adult patients with confirmed diagnosis of *H. pylori* infection in James Paget Hospital in Norfolk, UK, all patients receiving eradication treatments of first and sequential therapies between February 2000 and May 2001 were evaluated. The successes of individual regimens as first, second and third-line therapies were determined. The overall success after one, two and three courses of therapies were 73%, 94% and 98%, respectively (Beales, 2001). The use of PPI with clarithromycin and nitroimidazole as initial therapy has the highest eradication rates failure, hence the need for sequential therapies among the patients.

A meta-analysis of twenty-two studies from nine African countries with a total population of 2163 was reported. This study showed that the pooled eradication rate in Africa was estimated to be 79%. The overall eradication rate is lower than reports from Ethiopia (90%), Nigeria (87%), South Africa (86%), Egypt (82%), and Morocco (82%) and higher than reports from Tanzania (69%), Kenya (68%), and the Ivory Coast (22.3%) (Fekadu *et al.*, 2023).

A prospective study in a tertiary hospital in Tanzania was carried out between 2015 and May 2017. This study investigated the triple therapy treatment failure rate in 210 dyspeptic patients who tested positive for *H. pylori*. The results from this study demonstrated that 35.7% of patients tested positive for *H. pylori* stool antigen test immediately after the completion of treatment of 7 days, and 30.9% of patients still tested positive for *H. pylori* infection even after five weeks of completion of treatment (Jaka *et al.*, 2019).

In Korea, the efficacy of first-line therapy consisting of PPI, amoxicillin and clarithromycin for *H. pylori* infection has decreased over the last 10 years. This downward trend can be seen in one retrospective study, in which 1413 patients were diagnosed with *H. pylori* infection and received 7 days of triple therapy between January 2003 and December 2012. From this study, it was revealed that the annual *H. pylori* eradication rates in the per-protocol (PP) analysis from 2003 to 2012 were 93.5%, 80.0%, 87.2%, 88.5%, 92.0%, 88.3%, 85.7%, 84.1%, 83.7%, and 78.8%, respectively (Kim *et al.*, 2015).

In summary, all these previous studies reflect that the eradication rates of first-line therapies have been declining in different regions of the world. The success rates of standard therapy have fallen below the optimal eradication efficacy. The rise in rates of eradication failure emphasizes the urgent need to look for a new strategy in combating drug-resistant *H. pylori*. To achieve an initial eradication success rate of 90% and higher, therapy should be tailored according to individual antibiotic resistance profiles. This may not only reduce the overuse of antibiotics, but it could also minimise the development of antibiotic-resistant *H. pylori* strains.

1.6.3 Clarithromycin-resistant *H. pylori*

Indisputably, the resistance of *H. pylori* to many frequently used antibiotics has been on the rise worldwide, but there has been a significant increase in the prevalence of resistance to clarithromycin, particularly over the last 20 years (Jonaitis *et al.*, 2023). A meta-analysis study showed that the prevalence of primary clarithromycin resistance was 16-20% in Europe, 23-44% in the Eastern Mediterranean regions, 4-16% in America, with the highest percentage of 30-38% in the Western Pacific regions (Savoldi *et al.*, 2018). Meanwhile, a recent systematic review and meta-analysis study showed that the latest primary resistance prevalence in the Asia-Pacific region was 30% for clarithromycin (Hong *et al.*, 2024).

The increased rates of resistance to clarithromycin have a significant impact on the efficacy of clarithromycin-based regimens, which subsequently contribute to a major factor in *H. pylori* eradication failure. This is especially true for areas where the incidence of primary clarithromycin resistance is high, such as in the Western Pacific regions. An overall cure rate of clarithromycin-containing triple therapy had dropped to less than 80%, in which, clarithromycin resistance in Japan particularly, has fallen to 50% (Shiotani *et al.*, 2017). For this reason, the Maastricht IV/Florence Consensus has recommended that PPI-clarithromycin-based triple therapy should be abandoned in areas where clarithromycin resistance rates are above 15-20% (Malfertheiner *et al.*, 2017).

Antibiotic-resistant bacteria were defined as any bacterial isolates that became resistant to the administered drug with a change from ‘susceptible’ to ‘intermediate’ or ‘resistant’, or from ‘intermediate’ to ‘resistant’ in the susceptibility pattern (Tamma *et al.*, 2012). Infections from multidrug-resistant bacteria cause more than 750,000 deaths worldwide annually, and the number is expected to rise unless critical action is taken. One of the main

causes of antimicrobial resistance (AMR) in bacteria are inappropriate use of antibiotics due to presumptive treatment and lack of guidelines and policies for the use of antibiotics. A report from the World Bank alerted about the AMR that could cause as much damage to the global economy as the 2008 monetary crisis (Jonas *et al.*, 2017).

H. pylori infection has become increasingly difficult to eradicate owing to the drug-resistant *H. pylori* strains worldwide. This threatening situation may not only pose a health hazard to human beings, but it may also cause a significant detrimental impact on the global economy. Hence, a robust network of surveillance systems should be established to trace antibiotic prescribing and resistance profiles in communities, to fight against the AMR at national and international levels. Furthermore, future research should focus on molecular-based genotypic testing for antibiotic resistance and the development of effective novel drugs that will have a high-efficiency rate against multidrug-resistant *H. pylori*.

1.6.4 Mode of action of metronidazole

Metronidazole is a first-line agent for treating anaerobic and microaerophilic bacterial infections, as well as protozoal diseases. It exerts a cytotoxic effect on facultative anaerobes. Clinically, it's employed to treat infections of the reproductive system, gastrointestinal tract, skin, heart, bones, joints, lungs, blood, nervous system, and other areas of the body. It is also used to treat certain sexually transmitted diseases (STDs) (Weir & Le, 2025).

Metronidazole, due to its low molecular weight, readily crosses the cytoplasmic membranes of anaerobic bacteria and protozoa by passive diffusion. Metronidazole is a prodrug that needs to be activated by intracellular reduction of the nitro group attached to the imidazole

ring (Dingsdag & Hunter, 2018). Inside the cell, its nitro group is enzymatically reduced, mediated by the enzyme pyruvate ferredoxin oxidoreductase (PFOR) using ferredoxin or flavodoxin as electron carriers. This reduction generates a highly reactive nitroso radical, which is believed to be the cytotoxic species (Krakovka *et al.*, 2022). The reduction of metronidazole also generates reactive oxygen species (ROS), which may induce DNA strand breaks (both single- and double-stranded), disrupting DNA structure, inhibiting replication and protein synthesis, and ultimately causing microbial cell death (Bhattacharjee, 2022).

The reduction of metronidazole is mainly mediated by oxygen-insensitive NADPH nitroreductase (RdxA), NADPH flavin oxidoreductase (FrxA), and ferredoxin-like enzymes (FdxB) in *H. pylori*. Metronidazole resistance in *H. pylori* is primarily due to decreased drug activation mediated by mutations in *rdxA* gene, which encodes an oxygen-insensitive NADPH nitroreductase (Hasanuzzaman *et al.*, 2024). The *rdxA* activity significantly impacts nitroreductase activity, which is critical for metronidazole antibiotic action (Zhang *et al.*, 2020a). Changes in the *rdxA* gene are the most important factor because they can make *H. pylori* resistant to metronidazole without changing *frxA* (Elbaiomy *et al.*, 2025).

Meanwhile, a secondary resistance mechanism involves mutation of *frxA* gene, which encodes NADPH-flavin oxidoreductase enzyme. When *rdxA i*s already inactivated by mutation, further mutation in *frxA* can compound the resistance, making the bacteria more resistant to metronidazole. Research shows that *frxA* frameshift mutations occur in both metronidazole-resistant and -sensitive strains, and *frxA* mutations are thought to enhance *rdxA*-mediated metronidazole resistance rather than confer metronidazole resistance on their own (Gong *et al.*, 2023). However, metronidazole resistance was also observed in *H. pylori* isolates without the loss of functional RdxA and FrxA, suggesting that other factors are

involved in metronidazole resistance (Lee *et al.*, 2018; Zhang *et al.*, 2020a). Other putative mechanisms of metronidazole resistance in *H. pylori* include mutations in FdxB, ferric uptake regulator (Fur), and enhancement of efflux pump (HefA) protein (Attaran *et al.*, 2017).

1.6.5 Mode of action of clarithromycin

Clarithromycin is a semi-synthetic 14-membered macrolide derived from erythromycin, which is composed of a macrocyclic lactone ring connected to two deoxysugars, cladinose and desosamine (Pradhan *et al.*, 2024). It exhibits enhanced acid stability and favourable pharmacokinetic properties compared to its parent compound, erythromycin, making it suitable for oral administration (Dinos, 2017). Clarithromycin is frequently prescribed for mild to moderate infections caused by susceptible bacteria. Clarithromycin primarily exerts a bacteriostatic effect against many Gram-positive organisms, including *Streptococci*, *Staphylococci*, *Clostridia*, *Corynebacteria*, and *Listeria*, as well as certain Gram-negative pathogens such as *Haemophilus* species, *Moraxella*, and *Neisseria meningitidis*. It also shows activity against *Chlamydia pneumoniae*, *H. pylori*, and various atypical *Mycobacteria* (Bethesda, 2012).

Clarithromycin is a key macrolide antibiotic used in standard triple therapy regimens for the eradication of *H. pylori*. Its primary mode of action involves inhibition of bacterial protein synthesis by binding to the 50S ribosomal subunit, specifically at the *23S rRNA* domain V within the peptidyl transferase centre. *In H. pylori*, this binding site is highly conserved and critical for peptide elongation during translation (Ayaş *et al.*, 2024). Clarithromycin exerts its effect by reversibly binding to nucleotides A2058 and A2059 in the *23S rRNA*, which blocks the exit tunnel of the nascent polypeptide chain. This prevents the addition of new amino acids to the growing polypeptide, thereby halting protein synthesis and leading to bacterial

stasis. This mechanism primarily produces a bacteriostatic effect, though at high concentrations, bactericidal activity may occur (Bortolanza *et al.*, 2020; Krawczyk *et al.*, 2024).

The primary mechanism of clarithromycin resistance in *H. pylori* is mediated by point mutations in the *23S rRNA* gene (which constitutes the RNA component of the large ribosomal unit). These mutations, particularly A2142G, A2142C, and A2143G, interfere with clarithromycin's ability to bind to its ribosomal target (Gareayaghi & Kocazeybek, 2022). Among these, the A2143G mutation is the most frequently observed globally and is associated with high-level resistance, thought to be responsible for the failure of *H. pylori* eradication (Bogiel *et al.*, 2025). Moreover, mutations in the ribosomal protein L22 (*rpl22*) and translation initiation factor IF-2 (*infB*) genes had synergistic effects with mutations in the *23S rRNA* genes, resulting in a higher minimum inhibitory concentration of clarithromycin (Tshibangu-Kabamba & Yamaoka, 2021).

In addition to point mutations, efflux pump mechanisms may also contribute to clarithromycin resistance. Overexpression of efflux transporter genes (e.g., *hefA*, *hefB*, *hefC*) can reduce intracellular concentrations of the antibiotic. However, such mechanisms are considered secondary and play a more supportive rather than primary role in resistance (Rocha *et al.*, 2025).

1.6.6 Factors that make *H. pylori* eradication difficult to treat

The main factors leading to *H. pylori* treatment failure are non-compliance, antibiotic resistance, bacterial factors, disease conditions associated with *H. pylori* infection, and pharmacological properties (Malfertheiner *et al.*, 2003). Patient compliance is vital for

successful *H. pylori* eradication. In a previous study, the *H. pylori* eradication rate was 96% for patients who complied with more than 60% of the prescribed medications. However, the eradication rate fell to 69% for patients who took less than 60% of the medications (Graham *et al.*, 1992). Hence, adherence to the drug regimen is very crucial in determining the successful eradication of *H. pylori* infection and can be improved by physicians carefully explaining the therapeutic regimens to the patients. Other several factors including smoking, diet, treatment duration, dosages, and methods of treatment (e.g., concomitant, hybrid, or sequential therapy) have been perceived to be causal factors of the cure rate in *H. pylori* infection (Uotani *et al.*, 2015).

Pharmacological resistance illustrates a condition where treatment fails due to the decreased susceptibility of bacteria to antimicrobials at the site where the bacterium is residing (Yılmaz & Özcengiz, 2017). This means that the antibiotic may have not achieved the required concentration or duration of exposure to *H. pylori*. *H. pylori* are known to colonize the mucus layer of the stomach and surface epithelial cells. Most antibiotics need to be absorbed into the bloodstream after oral ingestion. Once absorbed, the antibiotic is distributed systemically and then diffuse back across the epithelial barrier into the mucus. Few antibiotics possess this capability because it depends on their pharmacokinetics and ability to cross epithelial barriers in reverse. The lack of direct antibiotic secretion into the GI mucus restricts the number of effective treatments for specific infections (Ejazi *et al.*, 2023).

In addition, it has recently become clear that they can also penetrate the glands and directly interact with specialized epithelial cells deep in the glands (Wizenty *et al.*, 2020). The colonisation of *H. pylori* in these sites enables them to adapt to this unique environment and have different biological behaviours, making them less sensitive to antibiotics.

Therefore, *H. pylori*'s hospitable ecological niches such as gastric antrum and body junction may help them to elude the action of antimicrobials, where the diffusion of antibiotics is limited (Zhang, 2015).

Another principal element is that *H. pylori* reside in the mucus, which forms a protective layer for the stomach lining. The mucus layer consists of an abundance of high-molecular-weight glycoproteins, namely mucins and lipids. As a result of disulphide bonding, the mucins form a high viscoelastic layer that reduces solute diffusion rates, including those of antibiotics, hence only a low proportion of antibiotics may reach the bacterium (Debraekeleer & Remaut, 2018).

Additionally, this special niche is hostile to nearly all other microbes, hence the ability of *H. pylori* to thrive under an extremely low pH gastric environment gives them a survival advantage, where the pH is lower than required for the antibiotics to be effective. This is because most antibiotics are pH dependent, and usually the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of most antibiotics against *H. pylori* are determined at pH 7 or 7.4. For example, the MIC_{90} (mg/ml) of clarithromycin against *H. pylori* at pH 7.5, 6.0 and 5.5 are 0.03, 0.06 and 0.25 mg/ml, respectively (Megraud *et al.*, 2013). The MIC of antibiotics increases at a lower pH, hence causing a loss of antibiotic activity in the acidic gastric environment.

Therefore, the colonisation of *H. pylori* in many parts of stomach are difficult to eradicate, which subsequently may contribute to treatment failure. Another contributing factor that makes the eradication treatment difficult is that *H. pylori* are also relatively slow growers, and since most antibiotics are effective only with actively multiplying organisms, slow growth may provide a higher viability advantage for *H. pylori* (Graham, 1998).

1.7 Host response to *H. pylori*

1.7.1 The innate response to *H. pylori*

It has been well established that the hallmark of the interaction between *H. pylori* and the host immune system is the prolonged infection for years, resulting in a chronic inflammation of the gastric mucosa. To persist within the human host, *H. pylori* has acquired dual strategies to induce inflammation whilst simultaneously tempering the immune response for its survival. The severity of inflammation during *H. pylori* infection is believed to be linked to the regulation of pro-inflammatory molecules such as IL-1, IL-6, IL-8 and TNFα (Dincă *et al.*, 2022).

H. pylori carrying the virulence determinant *cag*PAI induces epithelial cell lines to express a large amount of the pro-inflammatory cytokines such as interleukin-8 (IL-8) that causes inflammation in gastric mucosa (Gobert & Wilson, 2022). In addition to the CagA protein, peptidoglycan peptides which are components of the bacterial cell wall are also translocated into host epithelial cells using the *cag* T4SS. *H. pylori* peptidoglycan is, in turn, recognized by NOD-1, an intracellular pattern recognition receptor (PRR) that senses peptidoglycan and induces NF-κB activation (Figure 1.7) (Oudouhou *et al.*, 2024; Viala *et al.*, 2004).

Once CagA is intracellular, its phosphorylation by MAP kinases (MAPK) consequently induces a signalling cascade, resulting in the activation of transcription factors such as NF-$_{k}$B and AP-1. The activation of the AP-1 and NF-$_{k}$B signalling pathway drives the secretion and upregulation of pro-inflammatory cytokines such as IL-8 which may direct the chronic inflammation toward the pre-cancerous gastric lesions (Figure 1.7) (Backert & Tegtmeyer,

2017; Chattopadhyay *et al.*, 2023). The activation of NF-κB has a dual role: it participates in triggering innate and adaptive immune responses to combat pathogens, while its prolonged activation is associated with the development of inflammation-related diseases, infectious diseases, and cancer (Hu *et al.*, 2016b).

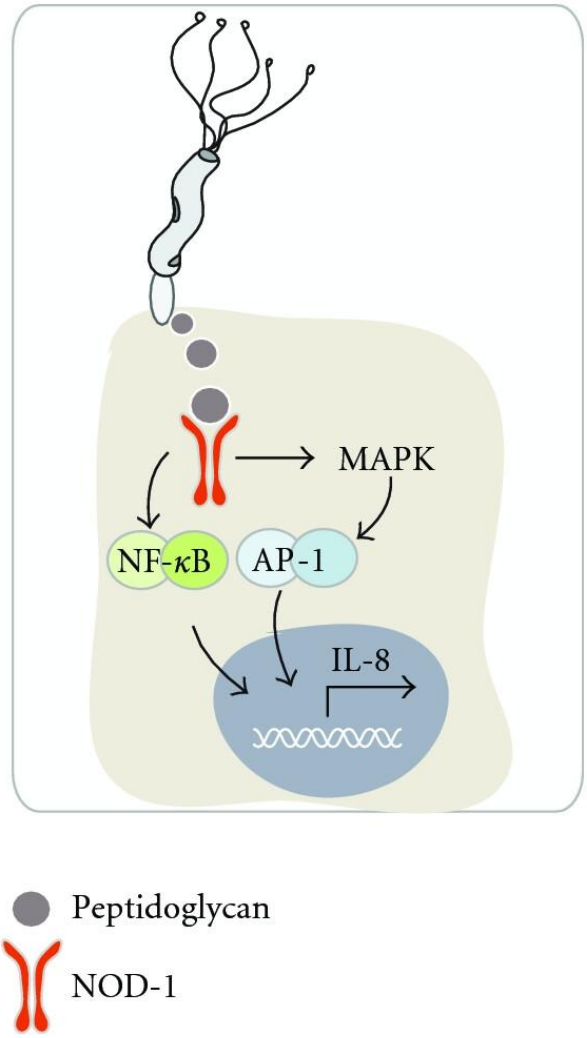


Figure 1.7: The proinflammatory responses induced by H. pylori rely on the bacterium having a functional type IV secretion system (T4SS). *This system delivers effector molecules such as peptidoglycan and the protein CagA into the host cell. Peptidoglycan is recognized by NOD-1 which activates the transcription of proinflammatory genes such as IL8 via the transcription factors NF-κB and AP-1 (Bauer & Meyer, 2011).*

A previous study has reported that in Mongolian gerbils infected with *cagA*-deficient *H. pylori*, less NF-$_k$B activation was present in the gastric mucosa than in gerbils infected with the *cagA* positive wild-type strain, suggesting that CagA plays a role in NF-$_k$B activation *in vivo* (Shibata *et al.*, 2006). Hence, it was apparent that CagA is responsible for the severity of gastric inflammation in *H. pylori*-infected Mongolian gerbils in this study. *cag*PAI-induced epithelial cell IL-8 secretion is an essential precursor of the immune response, and continual IL-8 expression is crucial in the pathogenesis of peptic ulcer and gastric carcinoma (Lin *et al.*, 2020).

1.7.1.1 Tol-like receptors (TLRs)

Another innate immune mechanism for inducing the production of pro-and anti-inflammatory cytokines adopted by the host cell is the activation of pattern recognition receptors (PRRs), in the early detection of *H. pylori*. PRRs detect microbial infections and bacterial components by identifying distinctive molecules known as Pathogen-Associated Molecular Patterns (PAMPs). PAMPs are conserved components that originate from bacteria such as peptidoglycan, lipopolysaccharide (LPS), flagellin, and microbial nucleic acids (Rai *et al.*, 2022). PAMPs bind to integral membrane glycoprotein PRRs on host immune cells, signalling the host cell and initiating microbicidal and pro-inflammatory responses (Eletto *et al.*, 2022). NOD-1, the intracellular receptor stimulated by *cag*A$^+$ strains are an example PRR, and the PAMP it recognises is the peptidoglycan from the *H. pylori* component (Figure 1.7) (Robinson *et al.*, 2007).

One of the best-studied PRRs is Toll-like-receptors (TLRs). Toll-like receptors (TLRs) are innate immune receptors that are present in host epithelial cells and serve as a first line of defence against any foreign agents (Varga & Peek, 2017). They contribute to the modulation

of innate and adaptive immune responses against various pathogens including *H. pylori* (Nemati *et al*., 2017). The binding of TLRs to PAMPs triggers the activation of signalling pathways such as NF-$_k$B that leads to the expression of pro- and anti-inflammatory mediators which subsequently regulate the type and extent of inflammatory response (De Nardo, 2015).

Many previous studies have suggested that TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, and TLR10 contribute to the early immune response against *H. pylori*, leading to the activation of NF-$_k$B and inducing the expression of cytokines (Castaño-Rodríguez *et al.*, 2014; Obonyo *et al.*, 2007; Pachathundikandi *et al.*, 2015). In the *H. pylori*-infected gastric mucosa, the gene expression of TLR2, TLR6, TLR7, TLR8, TLR9, and TLR10 was upregulated by more than two-fold (Nagashima *et al.*, 2015).

For instance, TLR2 recognizes several *H. pylori* components including *cag*PAI, LPS, HSP60, and urease (Smith, 2014). The binding of TLR2 and TLR4 to *H. pylori*-derived components leads to the recruitment of an adapter molecule, MyD88, and then induces the mitogen-activating protein kinase (MAPK) signalling pathway. As a result, the transcription factor NF-$_k$B is activated, stimulating the expression of pro- or anti-inflammatory cytokines and chemokines which may influence the outcome of infections (Li *et al.*, 2013). The net effect of TLR signalling may result in *H. pylori* persistence, elimination, or pathological reactions.

However, *H. pylori* employ masking strategies to prevent canonical ligands from triggering TLRs. These strategies include modifications to LPS and specific flagellin sequences that evade detection by TLR4 and TLR5, respectively (Pachathundikandi *et al.*, 2023). *H. pylori* LPS is unique within its bacterial species as it is recognized by the evolutionarily related TLR members TLR2, TLR4, and TLR10. The molecular variants of LPS are produced through

dephosphorylation by *H. pylori* enzymes, which conceals *H. pylori* LPS from detection by the classical LPS receptor, TLR4. However, TLR2 and TLR10 detect this modified *H. pylori* LPS and trigger anti-inflammatory responses, which might favour *H. pylori* colonization (Pachathundikandi *et al.*, 2023).

Specific mutations in the D1 interaction domain of *H. pylori* flagellin FlaA prevent its flagella from being detected by TLR5. TLR5-binding motifs in two structural T4SS-pilus proteins, CagL and CagY, may benefit the pathogen. The T4SS-pilus can switch between an active ('ON') and inactive ('OFF') status through known recombination mechanisms in the *cagY* gene (Barrozo *et al.*, 2013). This allows *H. pylori* to avoid constant TLR5 activation through an unresponsive FlaA and to adjust the host response using CagL and CagY finely. This study demonstrated the weak recognition of *H. pylori* flagellin by TLR5, and consequently, *H. pylori* flagellin is only a weak inducer of IL-8 expression in gastric epithelial cells (Pachathundikandi *et al.*, 2019).

1.7.1.2 Interleukin-8 (IL-8)

One of the most important pro-inflammatory factors in *H. pylori*-derived carcinogenesis is IL-8 as it is thought to play a key role in the recruitment of lymphocytes and neutrophils in the inflamed gastric mucosa (Robinson *et al.*, 2007). Gastric epithelial cells exposed to *H. pylori* exhibit overexpression of IL-8. This cytokine is also markedly elevated in both the tumour tissue and its surrounding microenvironment, where it plays a crucial role in regulating cell proliferation, angiogenesis, and metastasis (Lee *et al.*, 2013).

IL-8 or CXCL-8 was originally described as a chemokine whose main function is the attraction of a polymorphonuclear inflammatory leukocyte infiltrate acting via chemokine receptor

1/2. Initially identified for its role in inflammation, IL-8 is now recognized as playing significant roles in cancer and the regular movement of immune system cells (Lacalle *et al.*, 2017). Chemokines are structurally categorized into four groups based on the positions of conserved cysteine residues: the CCL, CXCL, XCL, and CX3CL families (Kufareva, 2016). Some function constitutively, while others are active during inflammatory conditions.

Most chemokines can activate several different chemokine receptors, and numerous chemokine receptors can be triggered by various chemokines (Yao *et al.*, 2016). The CXCL family consists of several prominent chemokines responsible for neutrophil migration, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 (IL-8). These chemokines are generated by both immune cells (like neutrophils, macrophages, and T cells) and non-leukocytes (such as epithelial and endothelial cells) in reaction to injury and infection (Atretkhany *et al.*, 2016).

Yamada *et al.* (2013) found that gastric cancers were identified in over 80% of the patients exhibiting elevated levels of IL-8 mRNA expression. This study revealed that both *H. pylori* infection and IL-8 mRNA expression were identified as relative risks for gastric cancer in the population, and consequently, IL-8 mRNA expression could serve as a valuable diagnostic and prognostic risk marker for gastric cancer.

1.8 Antimicrobial peptides in the inflamed gastric mucosa

1.8.1 Human defensins and cathelicidins

Antimicrobial peptides (AMPs) are produced by a vast majority of organisms and are used to protect the host against pathogens (Neshani *et al.*, 2019). They are also known as 'host

defence peptides' where in lower organisms, AMPs constitute a significant part of their defence mechanisms, while in higher eukaryotic cells, they are constitutively expressed or induced as part of the innate and adaptive immune response (Pasupuleti *et al.*, 2012). They are small proteins made up of 12 to 100 amino acid residues and typically carry a positive charge (cationic peptides). Their structure folds into an amphipathic structure, enabling solubility in both water and lipid environments, due to their composition of 40-50% hydrophobic residues. Generally, AMPs are categorised based on their secondary structural features, such as defensins (β-strand peptides connected by disulfide bonds), and cathelicidins (linear α-helical peptides) (Li *et al.*, 2021).

Two major types of AMPs play key roles in humans: defensins and cathelicidins (Hassan *et al.*, 2023). Defensins are among the earliest antimicrobial peptides (AMPs) to be identified and have broad-spectrum antimicrobial properties (Ouellette, 2011). To date, over 100 α-defensin and β-defensin molecules have been identified. However, humans specifically express six distinct α-defensins and at least four β-defensins (Lehrer & Lu, 2012).

Human neutrophils are rich in cationic α-defensin peptides, specifically HNP-1 to HNP-3, and to a lesser extent, HNP-4. Additionally, two α-defensins such as HD-5 and HD-6 are mostly expressed by specialized epithelial cells of the intestines, i.e. the Paneth cells (Wang, 2014). Meanwhile, four types of human β-defensins (hβD 1-4) have been recognised and they are expressed by epithelial cells, macrophages, and monocytes (Duits *et al.*, 2002). Neutrophils and epithelial cells are the main cellular sources of these peptides, but monocytes, macrophages, dendritic cells, and lymphocytes may also produce defensins (Hiemstra, 2006).

Cathelicidins on the other hand are produced by humans and animals in response to various Gram-positive and Gram-negative bacteria. The only known cathelicidin in man is human cationic antibacterial protein 18-kDa (hCAP18) (Vandamme *et al.*, 2012). The C-terminal end of this protein contained a 37-amino acid-long peptide with broad antibacterial activity (Cowland *et al.*, 1995). Later, this peptide was called LL-37, as it starts with two leucines.

High cathelicidin concentrations are typically found at sites of inflammation, where they act as a primary defence against bacteria and other pathogens. Cathelicidins also function as chemokines and can modulate or stimulate immune system cells. They influence both the innate and adaptive immune systems, serving as a link between the two. Expression was found in epithelial cells of the intestine, airway, genitals, ocular surface, skin, and eccrine glands. However, expression in most epithelial tissues is constitutive, but in keratinocytes, it is induced. Besides epithelial cells, cathelicidin is also constitutively expressed in neutrophils, natural killer (NK) cells, and mast cells (Vandamme *et al.*, 2012).

1.8.2 AMP mechanisms of action

Most AMPs share a common feature; the presence of a positive net charge of +2 to +9 due to excessive positively charged amino acids. The net positive charge will determine the mechanism of action since AMPs react electrostatically with negative charges of bacterial cell membranes (i.e., phospholipids), hence increasing the bacterial membrane permeability which may cause cell death (Espeche *et al.*, 2024).

Generally, there are three probable mechanisms of actions exerted by AMPs against the pathogens: (i) AMPs bind to bacterial membrane surface, aggregate to form superstructures and disrupt membrane integrity; (ii) act intracellularly by the inhibition of transcriptional,

translational or other processes; or (iii) target precursors, mechanisms or essential intermediates such as peptidoglycan, LPS and interfering with other biosynthetic pathways (Pero *et al.*, 2017).

Mechanisms of action for all AMPs have not been determined precisely, but it has been postulated that the permeabilization of bacterial cell membranes is a crucial step in defensin-mediated antimicrobial activity. Pore formation on the target cell membrane has been reported to be one of the known antibacterial action mechanisms of human defensins (Rani *et al.*, 2022). One study showed that the formation of pores and shrunken flagella on *H. pylori* cells after being treated with cathelicidin LL-37 has been proved via scanning electron microscopy (Zhang *et al.*, 2016).

In another study, human β-defensins were found to be fused into the bacterial cell membranes and lyse the negatively charged liposomes which are mediated primarily by electrostatic forces and hydrophobic interactions (Sudheendra *et al.*, 2015). The positive charge of the peptide reacts with the negative charge of the bacterial surface and causes the penetration of defensins into the bacterial cell membrane lipids (Mahlapuu *et al.*, 2016). Hence, these findings suggested that one putative mode of action of defensins might involve membrane disruption of the targeted cell by penetrating the lipid bilayer and inducing the permeabilization of the bacterial cell membrane. This event is lethal which may ultimately lead to cell death.

However, pore formation is not the only bactericidal mechanism of defensin. Human β-defensins can inhibit DNA, RNA, or protein synthesis and induce the production of interferon-gamma which may contribute to bacterial eradication (Lei *et al.*, 2019). These peptides penetrate the cell membranes, accumulate inside the bacteria, and block bacterial

functions by interacting with cellular DNA or RNA (Graf & Wilson, 2019). Some AMPs such as cathelicidins can inhibit the synthesis of protein and cell walls, resulting in morphological changes of bacteria and inhibiting cell growth. Bacterial cell walls are eventually perforated, resulting in efflux of cellular contents which may lead to cell death. However, it is also possible that other several hypothesized mechanisms cooperate to induce pathogen death. The same cationic peptides may also probably have more than one mechanism of action (Aisenbrey *et al.*, 2019).

1.8.3 *H. pylori* modulate defensin expression

Typically, human β-defensins 2 and 3 (hβD2 and hβD3) are usually present at low levels under normal physiological conditions but are upregulated when exposed to bacteria or pro-inflammatory cytokines such as IL-1β and TNF-α. On the other hand, hβD1 can be expressed both constitutively and in an inducible manner (Weinberg *et al.*, 2012). Many *in vitro* and *in vivo* studies have shown that *H. pylori* can induce the expression of defensins (Boughan *et al.*, 2006; Grubman *et al.*, 2010; Muhammad *et al.*, 2016a; Otte *et al.*, 2009).

However, *H. pylori* can persistently colonize its host despite inducing the expression of various antimicrobial peptides. For example, hβD3 was found to exhibit potent activity against *H. pylori in vitro* and is stimulated by EGFR signalling in the early stages of infection (Muhammad *et al.*, 2016b). Activation of the EGFR pathway initiates signalling through the p38 mitogen-activated protein (MAP) kinase and the activators of transcription (JAK/STAT) pathways, leading to nuclear signalling and the transcription of hβD3 (Figure 1.8).

Interestingly, during prolonged infection, hβD3 expression was subsequently downregulated by the *H. pylori* CagA (Bauer *et al.*, 2012). When translocated into host cells, CagA binds to

the cellular tyrosine phosphatase SHP-2, which leads to the termination of EGFR activation and its downstream signalling, thereby revoking hβD3 synthesis and enhancing bacterial viability (Figure 1.8) (Bauer *et al.*, 2012).

A recent study revealed that TNFα, IL1β, and IFNγ trigger the release of antimicrobials such as lactotransferrin, lipocalin2, complement component 3, and CXCL9 into the mucus layer. This antimicrobial-rich mucus can partially eradicate *H. pylori*, and the effectiveness of this bactericidal action relies on the concentration of each antimicrobial, and their gene expression is elevated in patients experiencing inflammation and *H. pylori*-related chronic gastritis. However, it is also observed that *H. pylori* infection can decrease the expression of genes encoding antimicrobials that inflammation typically enhances. These observations indicate that regulating antimicrobial secretion in the mucus is vital for epithelial immunity. Nonetheless, pathogens like *H. pylori* can bypass these defences and persist in the mucosa (Vllahu *et al.*, 2024).

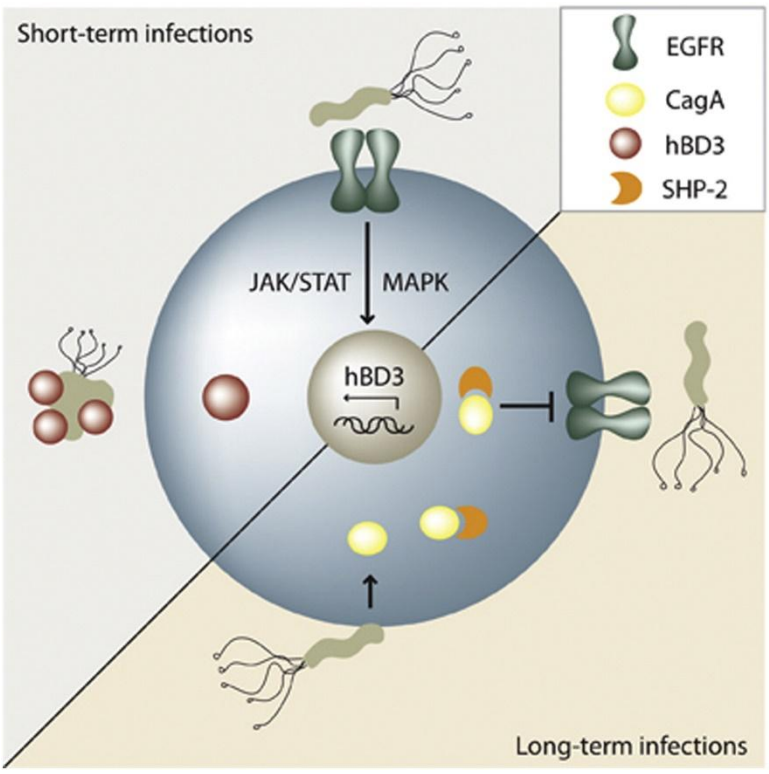
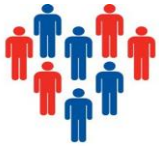


Figure 1.8: CagA abrogates hβD3 expression by the dephosphorylation of EGFR (Bauer et al., 2012)

1.9 Aims of the work

1. To determine the prevalence of *H. pylori* resistance to antibiotics in isolates from patients with and without a previous round of eradication therapy in the UK.
    - To characterise the antimicrobial resistance profiles of *H. pylori* in the UK using antibiotic sensitivity tests.
2. To investigate the host immune response to *H. pylori* infection (*ex vivo*).
    - To determine the relationship between human defensin expression (*DEFB4* and *DEFA5* mRNA) and *H. pylori* infection in gastric antral biopsies of uninfected and infected *H. pylori* patients.
3. To investigate the host immune response and antibiotic eradication to *H. pylori* infection (*in vitro*).
    - To assess the relationship between human defensin protein expression hβD2 and chemokine IL-8 and *H. pylori* infection in gastric cell lines culture infected with *cagA*+ and *cagA*- *H. pylori* strains.
    - To examine the inhibitory effect of macrolides such as clarithromycin and azithromycin on hβD2 and IL-8 production in gastric cell line cultures infected with *cagA*+ and *cagA*- *H. pylori* strains.
4. To further understand antibiotic resistance in *H. pylori* genome assemblies.
    - To predict antimicrobial resistance patterns in *H. pylori* genome assemblies using a database (genotypic) and compare them with MIC results (phenotypic).
    - To locate amino acid substitutions in *H. pylori* PBP1A and PBP2 in amoxicillin-resistant and -sensitive strains using bioinformatics and assess the relationship between amino acid substitution and amoxicillin resistance.

1.10 Experimental process

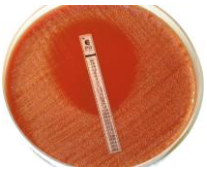


162 *H. pylori* infected patients undergone upper gastrointestinal endoscopy

STEP 1

241 clinical isolates from antrum and corpus

Disc diffusion method



E-test strip method

Total antibiotic resistance profiles of *H. pylori* isolates were characterised

STEP 2

56 clinical isolates from antrum

RT-qPCR

The level of human defensins *DEFB4* and *DEFA5* in *H. pylori* isolates were determined (*ex vivo*)

STEP 3

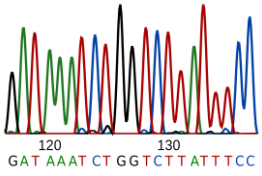
Four clarithromycin-resistant *H. pylori* clinical isolates of *cagA*+ and *cagA*- from the antrum

AGS cells co-cultured with clarithromycin-resistant *H. pylori* isolates

The inhibitory effect of macrolides on hβD2 and IL-8 production in *H. pylori* isolates were examined (*in vitro*)

STEP 4

35 clinical isolates from antrum and corpus



Genomic analysis

Bioinformatics analyses were performed on *H. pylori* genome assemblies

Chapter 2 : Prevalence of *Helicobacter pylori* resistance to antibiotics in isolates from patients with and without a previous round of eradication therapy in the UK

2.1 Introduction

The alarming increase of antibiotic resistance in *H. pylori* threatens the health of people globally. Antibiotic resistance of *H. pylori* is the main driving force of eradication failure (Savoldi *et al.*, 2018). However, the prevalence of *H. pylori* resistance not only varies among countries around the world, but also between local areas of the same country and it changes over time (Mladenova, 2021). Several factors are important in influencing resistance evolution such as patient's compliance, local antibiotic consumption, host factors, virulence factors and migration (Boyanova *et al.*, 2023).

According to clinical guidelines, amoxicillin, metronidazole, levofloxacin, tetracycline and clarithromycin are the most commonly used antibiotics in regimens to treat *H. pylori* (NICE, 2024). Hence, the understanding of *H. pylori* resistance development to these five antibiotics used in eradication therapy is essential to tackle the decline in treatment success rate. Data from a study showed that the resistance rates to metronidazole, levofloxacin and clarithromycin from *H. pylori*-infected paediatric patients obtained from gastric biopsies taken between the year of 2015-2020 increased annually in a linear manner and increased significantly with age (Shu *et al.*, 2022). This study also suggested that the resistance rates to these three antibiotics increased in patients with prior eradication treatment, as compared to patients without prior treatment.

Hence, based on the knowledge from previous studies of antibiotic resistance of *H. pylori* around the globe, it is hypothesized that local antibiotic resistance rates, especially for metronidazole and clarithromycin will also increase in prevalence in the UK. As not many research studies on the prevalence of antibiotic resistance in *H. pylori* isolates have been

done in the UK in recent years, this project aimed to unveil the present situation of drug-resistance in *H. pylori* from infected adult patients attending an endoscopy clinic in Queens Medical Centre in Nottingham. Since this study focused on patients with and without previous rounds of failed eradication therapy, it was intriguing to see if the patients who had undergone failed eradication treatment more commonly were infected with antibiotic resistant *H. pylori* as compared to treatment naïve patients.

Approximately, 40% of adults in the UK are believed to experience chronic dyspeptic symptoms. However, only around 5% of these individuals consult their general practitioner (GP), with just 1% of them being referred for endoscopy (NICE, 2014). Most patients who undergo upper gastrointestinal endoscopy have chronic dyspepsia as their predominant symptom. If they are proven to be *H. pylori* positive, then antibiotic therapy is given. According to National Institute for Health and Care Excellence (2014), the prescribed drugs and endoscopies cost the NHS about £600 million annually, and over-the counter medications cost patients a further £100 million, thus, chronic dyspepsia has significant economic costs. However, due to factors such as the evolving clinical definitions of dyspepsia over the last 30 years, endoscopy is not always readily available, there may be long waiting lists, financial implications, and poor-quality referrals, which have caused both GPs and gastroenterologists to be ambivalent with diagnostic approaches and the management of dyspepsia.

In addition, studies showed that eradication of *H. pylori* may reduce the risk of peptic ulcer disease and stomach cancer (Piscione *et al.*, 2021). Therefore, there has been a debate about how much the clinician can do to reduce the risk of dyspepsia and pre-cancerous lesions in patients, and the best management strategy and cost-effective approaches. Other

questions include whether a limited healthcare resource such as endoscopy should be targeted at certain patients or certain treatments, and if a follow-up routine is necessary to ensure *H. pylori* is eradicated and further development of pre-malignant lesions can be prevented (Banks *et al.*, 2019).

2.1.1 Objectives

The objectives of this chapter were to:

1. Characterise a large collection of *H. pylori* clinical isolates, collected in the Queen's Medical Centre, Nottingham University Hospital, UK, between 2001 and 2018, for their antimicrobial sensitivities.
2. Determine the total antibiotic resistance profiles in *H. pylori* isolates taken from the gastric antrum and corpus of patients.
3. Analyse trends in antibiotic resistance rates of *H. pylori* isolates over time.
4. Compare *H. pylori* antibiotic resistance rates between patient subgroups with and without a previous history of eradication therapy.
5. Investigate if factors such as the gender of patients could predict treatment failure by comparing patients with/without previous eradication therapy.
6. Investigate if factors such as the age of patients could have an impact on the minimum inhibitory concentration (MIC) level of antibiotics in *H. pylori*-resistant isolates.
7. Determine if virulent strains are more likely to harbour antibiotic-resistant *H. pylori* strains.
8. Determine if histopathologic findings of higher severity in gastric biopsies from *H. pylori*-infected patients are associated with treatment failure and examine any

histological changes in returning patients with/without previously failed eradication therapy.

2.2 Materials and Methods

2.2.1 Ethics and clinical samples

A total of 241 *H. pylori* isolates from the Nottingham strain collection were gathered between the years of 2001 – 2018 from patients undergoing a routine upper gastro-intestinal endoscopy at the Queens Medical Centre, Nottingham. All participants were informed about the objectives and purpose of the study, and they were required to provide written consent for the collection of a peripheral blood sample and additional gastric biopsies for the purposes of research, along with anonymised demographic information. This study was approved by the Nottingham Research Ethics Committee for the use of the clinical samples and patient information, prior to commencement of the study; reference number 08/H0408/195.

H. pylori infection status was determined using a rapid biopsy urease test, and confirmed by histology, successful isolation of the strain from biopsy tissue, serology, and PCR. Patients who had taken PPI, antibiotics, or NSAIDs in the 2 weeks prior to the endoscopy, were excluded from this study. A total of 162 patients provided biopsy samples in the study, from which 241 isolates were collected, and characterised for antimicrobial sensitivity. Table 2.1 presents the demographic information of the 162 patients from whom the *H. pylori* isolates were obtained.

Table 2.1 Demographics of the 162 patients from whom the H. pylori isolates were derived.

Gender	Male 50.0% (n=81/162) Female 50.0% (n=81/162)
Age	Mean 53.2 years (range 19-86 years)
Gastro-duodenal disease status identified at endoscopy	Duodenal ulcer 38.9% (n=63/162) Gastric ulcer 11.1% (n=18/162) Gastric cancer 0.6% (n=1/162) Gastritis/duodenitis 6.8% (n=11/162) None 42.6% (n=69/162)
Number of patients who had previous *H. pylori* eradication therapy Number of patients who did not have previous *H. pylori* eradication therapy Previous successful *H. pylori* eradication therapy Previous failed *H. pylori* eradication therapy	25 % (n=39/156) (the data from the six discordant pairs were excluded) 75% (n=117/156) (the data from the six discordant pairs were excluded) 48.7% (n=19/39) 51.3% (n=20/39)
Isolates recovered from antral and corpus gastric biopsies	46 isolates from the antrum only (single site isolates from 46 patients) 37 isolates from the corpus only (single site isolates from 37 patients)

	158 isolates, from both the antrum and corpus (paired isolates from 79 patients) A total of 241 isolates were recovered from 162 patients
Smoking, % (n/N)	Non-Smoker 72% (116/162) Smoker 15% (25/162) Ex-smoker 9% (14/162) Unknown 4% (7/162)
Virulence factor genotype of all isolates, % (n/N)	*cagA* status: Positive 72% (166/232); Negative 28% (66/232) (9 isolates removed due to ambiguous data) *vacA* type: *i*1 66% (152/231); *i*2 34% (79/ 231) (10 isolates removed due to ambiguous data)

2.2.2 *H. pylori* isolation and culture on blood agar plates

H. pylori was isolated from the antrum and/or corpus biopsy areas of the stomach of patients using methods previously described (Garvey *et al.*, 2023; Letley *et al.*, 2003). To isolate samples, biopsy samples taken at endoscopy were immediately transferred to 1 ml of Iso-Sensitest broth (Oxoid, UK) containing 15% (v/v) sterile glycerol (Courtin & Warner, UK). Biopsies were swabbed over a blood agar plate (Blood Agar base No. 2 with 5% horse blood) and the plate was then incubated to see if any *H. pylori* would grow. These were incubated for 72-96 hours (over the weekend) at 37°C, in microaerophilic conditions (86% nitrogen, 6% oxygen, 3% hydrogen and 5% carbon dioxide) in a workstation (MACS VA500, Don Whitley Scientific, UK). After five days, avoiding contaminant growth, single colony isolates were passaged onto fresh blood agar plates containing 5% horse blood and grown in

a microaerophilic condition as before for 48 hours. The colonies and sweeps of *H. pylori* were identified and picked based on morphology and confirmed using Gram staining and urease testing. Colonies from the same patient biopsy sample were pooled. These were suspended in 1 ml of Iso-Sensitest Broth (Oxoid, UK) with 15% (vol/vol) sterile glycerol (Courtin & Warner, UK) for storage at -80°C as frozen stocks.

To prepare frozen strains for testing, 100µl of the frozen stock was spread onto blood agar plates and incubated at 37°C for 48-72 hours in microaerophilic conditions as before. The bacterial growth was then picked with a sterile cotton swab and passaged onto a fresh plate, which was then incubated for a further 24 hours in the same conditions. Sub-cultures were obtained through passage where necessary.

2.2.3 Histopathology

To assess histology scores, two antral and two corporal biopsy samples were taken from each patient and immediately fixed in 6-10% formalin in 0.9% NaCl. The biopsies were embedded in paraffin wax and 4µm sections were then prepared with a microtome, mounted on glass slides, and stained with either toluidine blue, or haematoxylin and eosin for histological slide preparation. Assessment of histopathology was performed by an experienced histopathologist, Dr Abed Zaitoun, who was blinded to all other data. The histopathological assessment of the specimens were performed to evaluate inflammation, neutrophil activity, atrophy, intestinal metaplasia and bacterial density. Each of these five features was graded on a four-point scale (0 = none, 1 = mild, 2 = moderate, 3 = severe) following the Sydney classification. (Dixon *et al.*, 1996). (See Section 2.3.7 for further information on Sydney classification).

2.2.4 Virulence factor typing

H. pylori bacterial virulence factors were typed by PCR from genomic DNA extracted from colony sweeps after culture *in vitro* as mentioned in section 2.2.2. As described previously (Rhead *et al.*, 2007; Winter *et al.*, 2014), PCR genotyping was performed by Joanne Rhead and other members of the *Helicobacter* Research Group to determine *cagA* and *vacA* status of each isolate. A simple PCR typing system was developed based on a conserved forward primer and specific reverse primers to allow rapid identification of the *i*-region cluster C type.

2.2.5 Media preparation for antibiotic susceptibility test

Blood agar was prepared by dissolving 15.6 g of Mueller Hinton agar (Oxoid) in 370 ml deionised water and autoclaved. Bottles of agar were then transferred in a 70°C water bath, cooled slightly, and 30 ml defibrinated horse blood (Thermo Scientific) was added. 30 ml aliquots of the blood agar were poured into sterile petri dishes aseptically and gently to avoid bubble formation. The blood agar plates were allowed to solidify at room temperature, and plates were stored at 4°C and used within 7 days. From each batch of plates made, one was tested for quality control. A suspension of *Escherichia coli* strain DH5-α was prepared in sterile 5 ml of 0.85% saline (suspended to a standard of 3 ± 0.2 on a McFarland Den1 Densitometer) (Grant Instruments, Cambridgeshire). The suspension was used to moisten a sterile cotton swab, and then spread over the surface of the agar and allowed to sink in. A 10 µg amoxicillin disc was placed in the centre of the plate, which was then inverted and incubated for 16 hours at 37°C, under normal atmospheric conditions.

The diameter of the zone of clearing around the disc was then measured in millimetres using callipers. Batches of plates were only used if the zone diameter was 12-20 mm.

2.2.6 Antibiotic sensitivity tests

2.2.6.1 Disc diffusion method

Antibiotic susceptibility testing was performed using the disc diffusion method as described in Lang and García (2004). Twenty-four hours after passaging the *H. pylori* isolates, the growth was suspended in sterile 0.85% saline, up to a standard of 3 ± 0.2 on a McFarland Den1 Densitometer (Grant Instruments, Cambridgeshire). A sterile cotton swab was then used to spread this suspension culture onto 5 prepared plates of Mueller-Hinton agar with 7.5% defibrinated horse blood (Thermo Scientific) and allowed to dry. After drying, one antibiotic disc (either clarithromycin (15µg), amoxicillin (10µg), levofloxacin (5µg), tetracycline (30µg), or metronidazole (5µg) (all Oxoid, UK) was placed firmly onto the centre of the inoculated blood agar plate using sterile forceps. For each set of sensitivity tests performed, a control strain of *H. pylori* NCTC 11637 (ATCC 43504/CCUG 17874) was also plated, and antibiotic discs were applied as described above. Plates were incubated at 37°C under microaerophilic conditions as stated above for 5 days, after which the zones of inhibition were measured in millimetres using callipers. The zone of inhibition was defined as the area around the disc with no bacterial growth (clear zone). All susceptibility tests were duplicated independently by a colleague. If the results were inconsistent or plates were contaminated, the test was repeated.

2.2.6.2 Epsilometer test (E-test)

The minimum inhibitory concentrations (MICs) for clarithromycin, amoxicillin, levofloxacin, tetracycline and metronidazole against *H. pylori* strains were determined using Epsilometer test (E-test) method. E-test is a quantitative technique that comprises a predefined and continuous antibiotic concentration gradient based on the concept of dilution and diffusion principles for susceptibility testing (Behera *et al.*, 2019). The plates were inoculated as described previously (Section 2.2.6.1). The E-test strip (bioMerieux) for each type of antibiotic (1 strip per plate) was applied and placed firmly onto the centre of the inoculated blood agar plate using sterile forceps. For each set of sensitivity tests performed, a control strain of *H. pylori* NCTC 11637 (ATCC 43504/CCUG 17874) was also plated, and an E-test strip was applied as described above. Plates were incubated at 37°C for 5 days under microaerophilic conditions as indicated before. The plates were examined by looking at the interface between the antibiotic concentration on the E-test strip and the *H. pylori* inhibition growth. The MIC is read as the point where the growth inhibition ellipse intersects the MIC scale on the strip. All susceptibility tests were duplicated independently by a colleague. Plates that were overgrown with contamination were discounted and the test was repeated.

2.2.6.3 Determination of drug sensitivity and resistance

The clinical EUCAST zone diameter breakpoints for disc diffusion testing on *H. pylori* strains are not available. Hence, this study used the zone diameter breakpoints from published literature as reference for the interpretation of ‘susceptible’, ‘resistant’ or ‘intermediate’ strains. These breakpoints were validated using E-tests. Table 2.2 shows the disc diffusion interpretation of zone diameters for clarithromycin, amoxicillin, levofloxacin, tetracycline and metronidazole against *H. pylori* strains. Antibiotics such as amoxicillin, clarithromycin,

metronidazole, tetracycline and levofloxacin were included for the susceptibility test in this study because these standard antibiotics are typically used in first-line therapy.

Table 2.2 Diameter of the zone of inhibition (mm), with breakpoint limits indicative of resistance and susceptibility of H. pylori strains to 5 antibiotics.

Antibiotic	**Diameter of the zone of inhibition, with interpretation (mm)**		**Reference**
	Resistant	**Susceptible**	
Clarithromycin (15 µg)	≤28	>28	Alarcón-Millán *et al.* (2016)
Amoxicillin (10 µg)	≤25	>25	Lang and García (2004)
Levofloxacin (5 µg)	<12	≥12	Yu *et al.* (2011)
Tetracycline (30 µg)	<25	>25	Lang and García (2004)
Metronidazole (5 µg)	<16	≥21	Chaves *et al.* (1999)

*For metronidazole, if the measurements were in between the two thresholds, the strain was classified as 'intermediate' resistance/susceptible.

2.2.6.4 Determination of minimal inhibitory concentration (MIC)

The MIC values of antibiotics for *H. pylori* strains should be interpreted as S (susceptible), or R (resistant) by comparing the obtained MIC values of each antibiotic with the recommended EUCAST clinical MIC breakpoints (EUCAST, 2024) as shown in Table 2.3.

Table 2.3 EUCAST clinical MIC (mg/L) breakpoint limits indicative of resistance and susceptibility of H. pylori strains to 5 antibiotics.

Antibiotic	MIC breakpoint (mg/L)	
	Susceptible	Resistant
Clarithromycin	≤0.25	>0.25
Amoxicillin	≤0.125	>0.125
Levofloxacin	≤1	>1
Tetracycline	≤1	>1
Metronidazole	≤8	>8

2.2.7 Data analysis and statistical tests

Data was collected and recorded in Microsoft Excel and statistical analysis was performed using GraphPad Prism Version 10.3.0 (507). Chi-squared tests were used for multiple parameters, whereas a Fisher's exact test was used to analyse frequencies between patients who had or had not previously undergone eradication therapy. Mann-Whitney test was performed to compare the MIC level of antibiotics between two age groups of patients. A P value of < 0.05 was considered statistically significant.

2.3 Results

2.3.1 Antibiotic resistance profiles of *H. pylori* isolates

To date, 241 *H. pylori* isolates from 162 infected patients have been tested for antibiotic susceptibility from the Nottingham strain collection. Of these, 241 strains (125 isolated from the antrum, and 116 from the corpus) from 162 patients have been included in the analysis and the data were analysed based on the origins of the stomach; antrum and corpus. 46 patients yielded an isolate only from the antrum, other isolates were recovered only from the corpus of 37 patients, whilst paired isolates were cultured from both the antrum and corpus of 79 patients.

Of the total 241 isolates, as shown in Table 2.4, 149 were resistant to metronidazole (61.8%), 67 were resistant to clarithromycin (27.8%), 10 were resistant to levofloxacin (4.1%) and 6 were resistant to amoxicillin (2.5%). None of these isolates were found to be resistant to tetracycline.

Furthermore, 77 isolates (31.9%) were sensitive to all the antibiotics, whilst 98 isolates (40.7%) were resistant to one antibiotic. In terms of multiple drug resistance, 58 isolates (24.1%) were resistant to two, and 8 isolates (3.3%) were resistant to three antibiotics. None of them have acquired non-susceptibility to more than three antibiotics.

For dual resistance, 58 isolates (24.1%) were resistant to clarithromycin and metronidazole, and 5 isolates (2.1%) were resistant to levofloxacin and metronidazole. Of isolates resistant to three antibiotics, four isolates (1.7%) were resistant to clarithromycin, amoxicillin, and metronidazole. Meanwhile, two isolates (0.8%) were resistant to clarithromycin,

levofloxacin, and metronidazole, and clarithromycin, amoxicillin, and levofloxacin, respectively. The 8 isolates resistant to three antibiotics originated from 5 patients. Four had previously received eradication therapy, including one who had undergone five failed rounds of therapy. This patient had the same antibiotic resistance profiles for both antrum and corpus isolates that were resistant to clarithromycin, amoxicillin, and metronidazole.

Table 2.4 Antimicrobial resistance profiles of H. pylori isolates to 5 antibiotics.

Antibiotic	Number of Resistant Isolates (n=241)	Percentage
Metronidazole	149	61.8%
Clarithromycin	67	27.8%
Levofloxacin	10	4.1%
Amoxicillin	6	2.5%
Tetracycline	0	0%
No resistance to any antibiotic tested	77	31.9%
Resistance to one antibiotic only	98	40.7%
Resistance to two antibiotics	58	24.1%
Resistance to three antibiotics	8	3.3%
Resistance to >three antibiotics	0	0%
Clarithromycin + Metronidazole only	58	24.1%
Levofloxacin + Metronidazole only	5	2.1%
Clarithromycin + Levofloxacin + Metronidazole	2	0.8%
Clarithromycin + Levofloxacin + Amoxicillin	2	0.8%
Clarithromycin + Amoxicillin + Metronidazole	4	1.7%

*Isolates of *H. pylori* from the stomachs of 162 patients were tested for antibiotic resistance via disc diffusion and E-test.

2.3.2 Resistance profiles of *H. pylori* paired isolates

In this study, a total of 79 patients had paired isolates from both the gastric antrum and corpus. Of these, 73 had identical resistance profiles (92%), and 6 had discordant resistance profiles (7.59%). As for mismatched paired isolates, two of these pairs were differed in metronidazole resistance. For example, one antral isolate was resistant with an MIC of 64 μg/ml, and the corpus isolate was sensitive (MIC 0.19 μg/ml). In another case, the antral isolate was resistant to metronidazole (MIC 192 μg/ml) and clarithromycin (MIC >256 μg/ml), whilst the corpus isolate was sensitive to both antibiotics (MICs 0.047 and 0.5 μg/ml respectively). Three additional pairs differed in clarithromycin resistance, also with large differences in MICs (>256 and 0.016 μg/ml; 0.016 and 1.5 μg/ml; 0.38 and 0.125 μg/ml for antral and corpus isolates respectively). One pair differed in levofloxacin resistance (MICs of 0.25 and 12 μg/ml). Interestingly in one case, the isolate from the corpus biopsy was resistant to metronidazole (MIC >256 μg/ml), clarithromycin (MIC 1.5 μg/ml) and amoxicillin (MIC 0.75 μg/ml), whilst the antral isolate was only resistant to metronidazole (MIC >256 μg/ml).

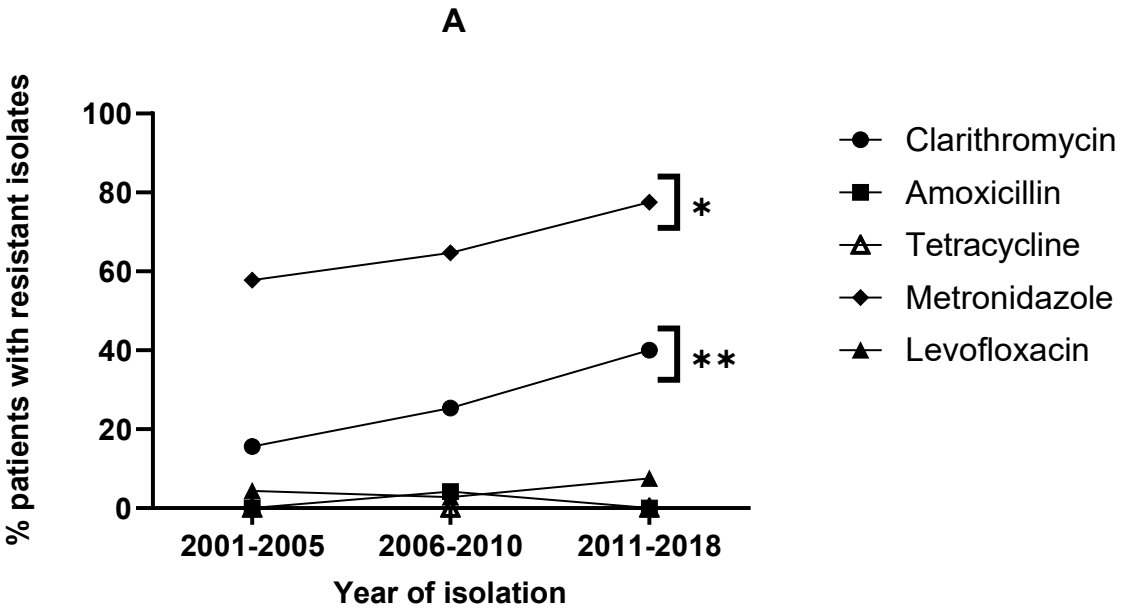
2.3.3 Antibiotic resistance rates of *H. pylori* isolates over time

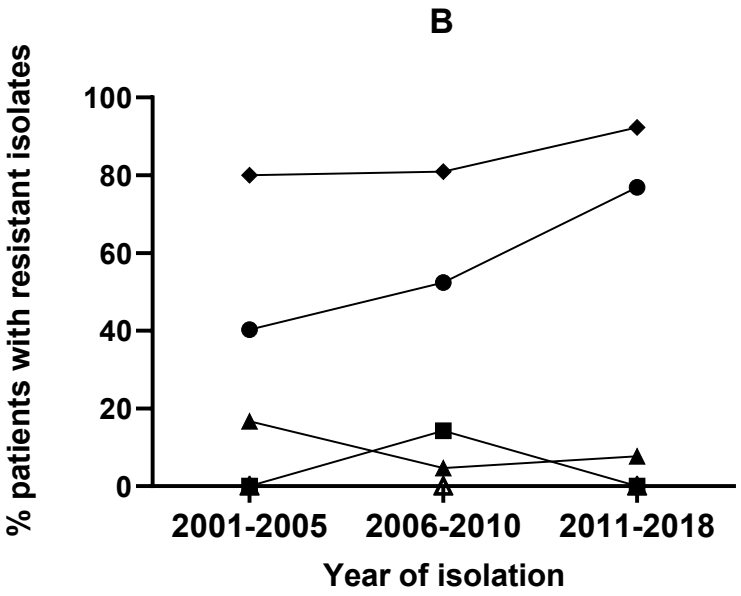
The analysis for identifying antibiotic resistance from the years of 2001-2018 was performed on 156 isolates, (1 isolate per patient), to avoid bias from double counting the 79 pairs of isolates (Figure 2.1A). The data from the six discordant pairs were excluded. Each isolate was categorised by year based on the date that the isolate was recovered from the gastric biopsy and classified into three intervals: 2001-2005 n=45, 2006-2010 n=71, and 2011-2018 n=40. The frequency of patients infected with clarithromycin resistant-*H. pylori* strains

increased significantly from 2001-2005 (15.6%) (7/45), to 40% (16/40) in isolates gathered between 2011 and 2018 (p=0.011) (Chi-square test). As for amoxicillin, the rates of resistance maintained a similar trend for each group of years, from 0% (0/45) to 4.2% (3/71), and decreased to 0% (0/40), respectively. Furthermore, the percentage resistance for levofloxacin resistant-*H. pylori* strains for each group of years were 4.4% (2/45), 2.8% (2/71), and 7.5% (3/40), respectively. On the other hand, the total rates of resistance for metronidazole increased significantly from 57.8% (26/45) in the years of 2001-2005, to 77.5% (31/40) in the years of 2011-2018 (p=0.05) (Chi-square test).

Moreover, the data was further subdivided into categories according to whether the patients had or had not previously undergone *H. pylori* eradication therapy (Figure 2.1B and C). Of those who had previously received eradication therapy (n=39) (Figure 2.1B), the resistance level for clarithromycin increased slightly from 40% (2/5) to 52.4% (11/21) between the intervals of 2001-2005 and 2006-2010 and increased to 76.9% (10/13) amongst strains gathered in 2011-2018. As for metronidazole, the rates of resistance increased slowly between all the group years, from 80% (4/5) in 2001-2005 to 80.9% (17/21) in 2006-2010, and to 92.3% (12/13) in 2011-2018. None of these data showed any significant results.

Meanwhile, of those who had not previously received eradication therapy (n=117) (Figure 2.1C), clarithromycin resistance increased steadily, although not significantly, from 12.5% (5/40) in 2001-2005, to 14.3% (7/49) and 25.9% (7/28), respectively in the year of 2006-2010, and 2011-2018. As for metronidazole, the resistance rates increased from 55% (22/40) to 61.2% (30/49) between the year of 2001-2005 to 2006-2010 and then increased slightly to 67.9% (19/28) in the year of 2011-2018. Again, data based on patients who had not previously received eradication therapy yielded no significant trends.


A
% patients with resistant isolates
100
80
60
40
20
0
*
**
2001-2005
2006-2010
2011-2018
Year of isolation
Clarithromycin
Amoxicillin
Tetracycline
Metronidazole
Levofloxacin


B
% patients with resistant isolates
100
80
60
40
20
0
2001-2005
2006-2010
2011-2018
Year of isolation

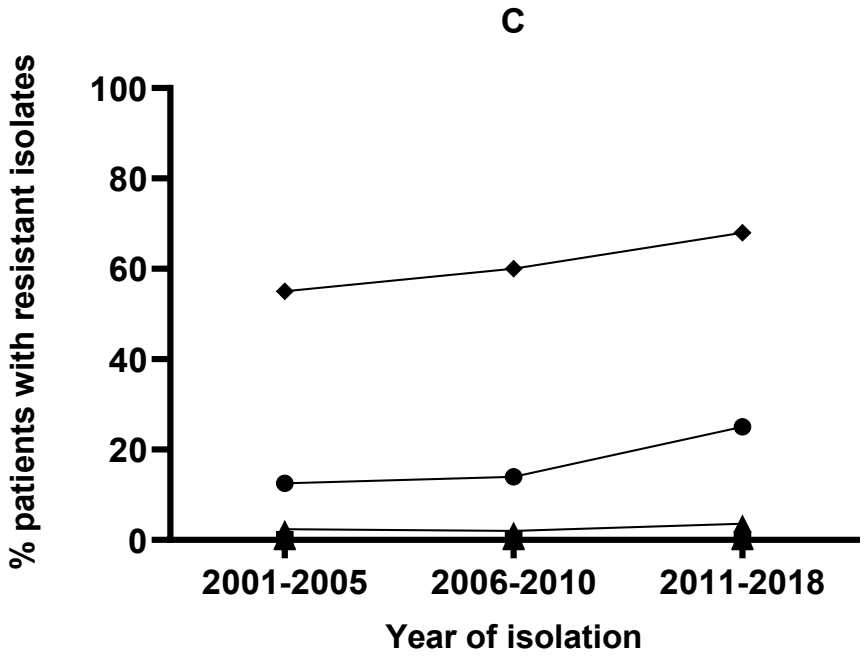


Figure 2.1: The frequencies of patients with resistant isolates, who had or had not previously undertaken H. pylori eradication therapy over the period of the study. *For the whole dataset (A), 2001-5 n=45, 2006-2011 n=71, and 2011-2018 n=40. For the patients with previous eradication therapy (B), 2001-5 n=5, 2006-2011 n=21, and 2011-2018 n=13. For the patients without previous eradication therapy (C), 2001-5 n=40, 2006-2011 n=49, and 2011-2018 n=28. Significant trends in the data were found for metronidazole-resistant ($p=0.05^{*}$) and clarithromycin-resistant ($p=0.011^{**}$) and (Chi-square test) isolates, but only in the whole dataset (A). No significant trends were found for the other antibiotics, or within the subgroup analyses (B & C).*

2.3.4 *H. pylori* antibiotic resistance rates between patients with/without previous eradication therapy

The data (1 sample per patient) were categorised into patients who had and had not previously received *H. pylori* eradication therapy (n=39 and n=117, respectively). The antibiotic resistance profiles of isolates from patients in these categories were compared (Figure 2.2). It was found that clarithromycin resistance was present in 16.2% (19/117) of total isolates from patients who had not previously received eradication therapy and was

significantly present in 59% (23/39) of total isolates from patients who had (p<0.0001) (Fisher's exact test). In metronidazole, the total number of resistant isolates were significantly higher (84.6%) (33/39) in patients with previous eradication therapy than those without previous eradication therapy (60.7%) (71/117) (p=0.006) (Fisher's exact test).

On the other hand, no significant differences have been observed for both amoxicillin- and levofloxacin-resistant isolates in patients with previous eradication therapy and without previous eradication therapy. Only 3 patients in total had amoxicillin-resistant strains, with all having had previous therapy. Seven patients had levofloxacin-resistant isolates, 3 of whom had previously received therapy. No resistance was observed for tetracycline.

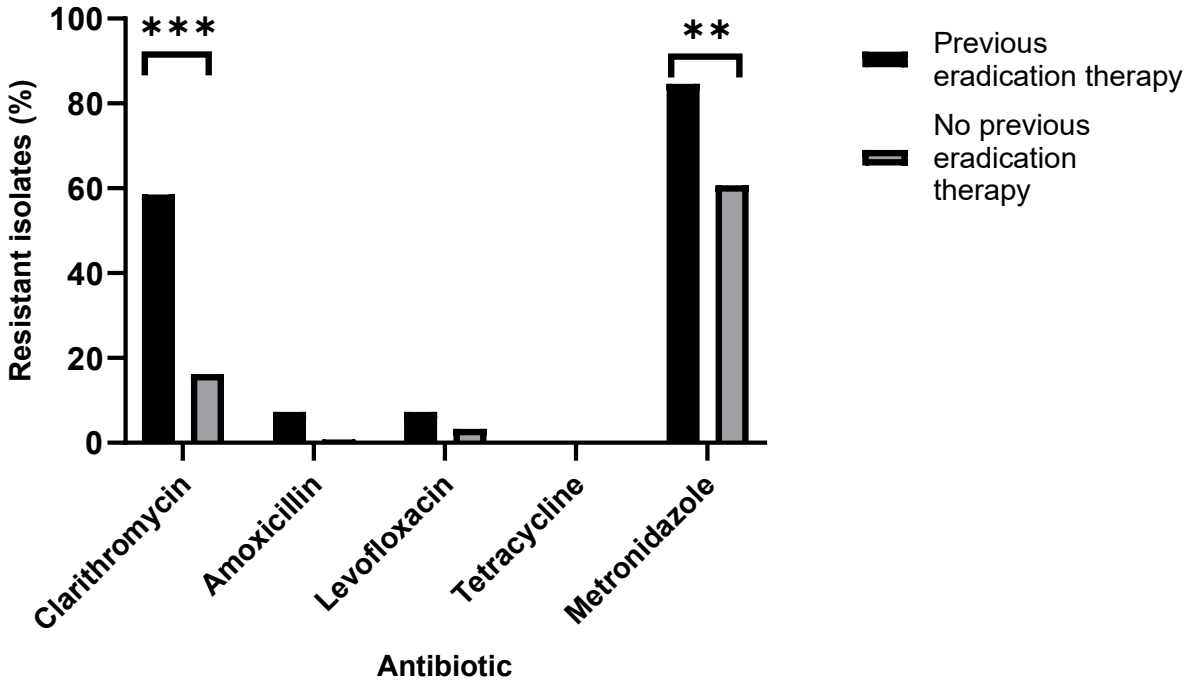


Figure 2.2: The percentage of H. pylori isolates resistant to 5 antibiotics, from patients who had previously received prior H. pylori eradication therapy (n=39) compared to those who had not (n=117). *The frequency of metronidazole-resistant isolates was significantly higher amongst the patients with previous therapy (p=0.006**), and the same was true for strains resistant to clarithromycin (p<0.0001***) (Fisher's exact test).*

2.3.5 Comparisons of gender between patients with/without previous successful eradication therapy

A previous study suggested that the gender of patients may have an impact on the treatment outcomes, and it was found that treatment failure was significantly more common in men compared with women (Furuta *et al.*, 2004). To investigate this, the data (1 sample per patient) were categorised into patients who had (n=39) and had not (n=117) previously received a round of failed *H. pylori* eradication therapy (n=156). The gender of patients in these categories was then compared. However, the percentage distribution of male and female patients was roughly equal for both groups - patients with and without previous successful eradication therapy, (48.72% and 51.28%) (n=19/39 and n=20/39), and (49.57% and 50.43%) (n=58/117 and n=59/117), respectively. Hence, there were no statistically significant differences among male and female patients between these two groups.

2.3.6 Investigation of a relationship between MIC level of antibiotics and patients of different ages

As the prevalence of antibiotic resistance of *H. pylori* has become more common in recent years, it was hypothesised that younger patients would be more likely to be infected with resistant strains, hence the MIC value of antibiotics is prone to be higher. To confirm this, patients were classified into two main age groups: below 60 years old, and over 60 years old.

Statistical analysis showed that the 60 years and below group had higher median MIC level when compared to 60 years and over age group for antibiotics clarithromycin, and

metronidazole. No variations were detected across all age categories in terms of MIC levels of amoxicillin, levolfoxacin, and tetracycline.

Regarding *H. pylori* strains resistant to clarithromycin, it was revealed that the median MIC of isolates from in the 60 years and below age group was significantly higher at 0.1575 mg/L compared to those from the over 60 age group at 0.04350 mg/L (p=0.0299) (Mann-Whitney test) (Figure 2.3). This study also demonstrated that the MIC metronidazole was notably elevated amongst isolates from the 60 years and below age group (44 mg/L), in contrast to those from the over 60 age group, where the median MIC level was 0.315 mg/L (p=0.0132) (Mann-Whitney test) (Figure 2.4).

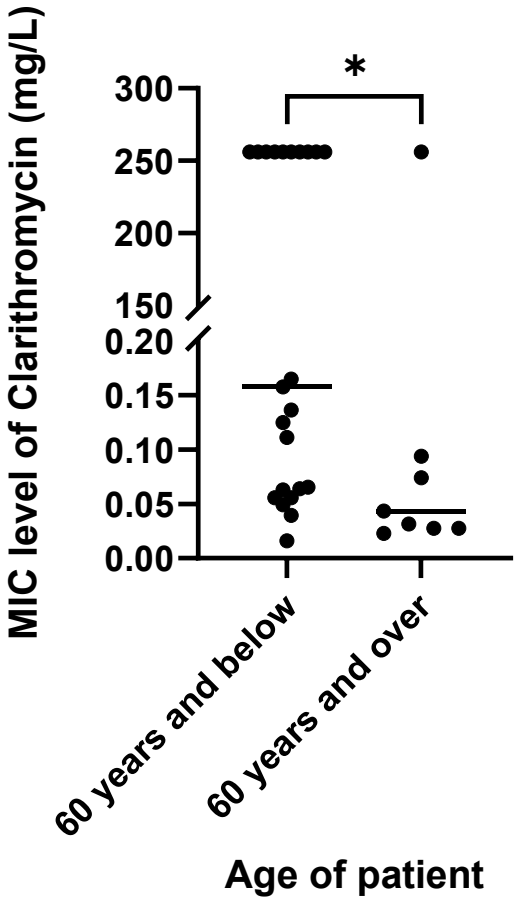


Figure 2.3: MIC levels for clarithromycin (mg/L) in isolates from 32 patients, where p=0.0299 (Mann-Whitney test).*

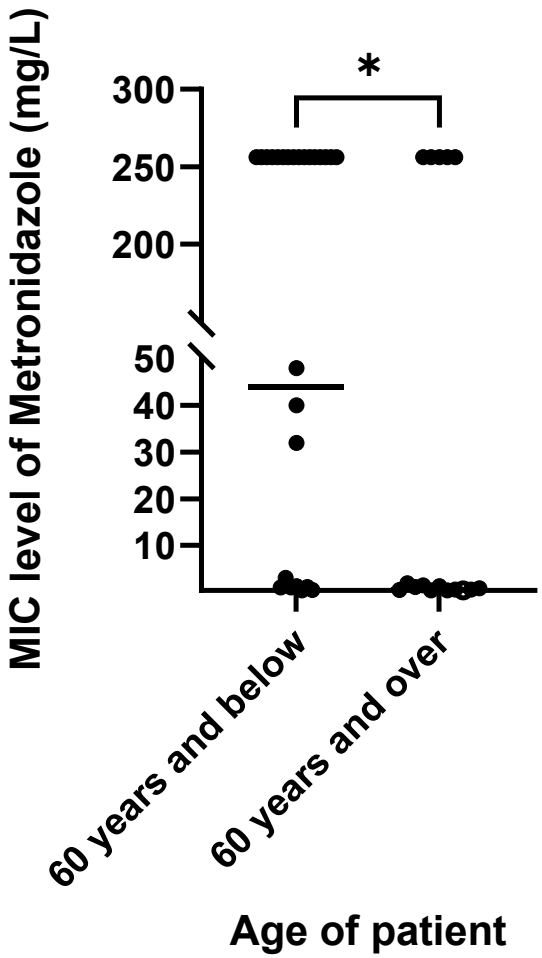


Figure 2.4: Average MIC level of metronidazole (mg/L) in isolates from 70 patients, where p=0.0132 (Mann-Whitney test).*

2.3.7 Comparison of histological features in gastric biopsies from *H. pylori*-infected patients

Of the 244 *H. pylori* gastric biopsies from 162 infected patients gathered, 129 and 115 biopsies removed from antrum and corpus regions of the stomach respectively, were then subjected to histopathological evaluation for determination of 1. inflammation, 2. neutrophil activity, 3. atrophy, 4. intestinal metaplasia and 5. bacterial density scores, performed by pathologists. Biopsies were scored for five histological variables with four severity scores (0=none, 1= mild, 2=moderate, and 3=severe) according to the Sydney classification (Table 2.5).

The histological findings scored for all *H. pylori*-related features showed significant differences between the antrum and corpus. The obtained results (Table 2.5) showed that the inflammation scores were more prominent in the antrum compared to the corpus. Most of gastric biopsies from the antrum appeared to have moderate inflammation (94/129 biopsies; 72.87%), as compared to those from the corpus where 60% (69/115) of them had mild inflammation. This difference was statistically significant ($p<0.0001$) (Chi-square test). Only 9.3% (12/129) of antral and 7.8% (9/115) of corpus biopsies were found to have severe inflammation (score of 3).

In addition, the neutrophil activity scores were higher in the antrum than in corpus, where 69.77% (90/129) of patient's antral biopsies had moderate neutrophil infiltration, whilst 46.96% (54/115) of corpus biopsies had normal neutrophil activity scores (score of 0) ($p<0.0001$) (Chi-square test) (Table 2.5). Severe neutrophil infiltration (score of 3) was seen in 4 out of 129 antrum biopsies, whereas none were detected in the corpus.

Most antral biopsies, 69.53% (89/128), and corpus biopsies, 95.65% (110/115) were scored as normal for mucosal glandular atrophy. Only 29.69% (38/128) and 4.35% (5/115) of antrum and corpus biopsies, respectively, were graded with mild atrophy (Table 2.5). Only one patient (0.78%) has moderate atrophy in the antrum. None were classified as severe for glandular atrophy. Seemingly, almost all of the corpus biopsies (97.39%, 112/115) showed the absence of intestinal metaplasia. This was also true for antral biopsies where 80.62% (104/129) of them were graded as normal (score of 0). Only two out of 129 antrum (1.55%) and none from corpus biopsy were evaluated as a score of 3.

As for the density of *H*. *pylori* overlying the epithelium, the highest density (score of 3) was present in 38.28% (49/128) of biopsies from the antrum, whereas 50.88% (58/114) of corpus biopsies were graded as mild. Also, two out of 128 (1.56%) patient's antrum biopsies were scored as 0; 32.03% (41/128) had a score of 1; and 28.13% (36/128) had a score of 2. Additionally, patient's corpus biopsies (1.56%, 2/128) received a score of 0; 21.05% (24/114) were scored at 2, and 26.32% (30/114) were scored at 3 (Table 2.5).

Table 2.5 The number and percentage prevalence of five variable histological features in gastric biopsies taken from the antrum (n=129) and corpus (n=115) regions from patients.

	Number and percentage of gastric biopsies			
	Inflammation scores			
	0	**1**	**2**	**3**
Antrum **(n=129)**	0; 0%	23; 17.83%	94; 72.87%***	12; 9.30%
Corpus **(n=115)**	0; 0%	69; 60%***	37; 32.17%	9; 7.83%
	Neutrophil activity scores			
	0	**1**	**2**	**3**
Antrum **(n=129)**	18; 13.95%	17; 13.18%	90; 69.77%***	4; 3.10%
Corpus **(n=115)**	54; 46.96%***	17; 14.78%	44; 38.26%	0; 0%
	Atrophy scores			
	0	**1**	**2**	**3**
Antrum **(n=128)**	89; 69.53%	38; 29.69%	1; 0.78%	0; 0%
Corpus **(n=115)**	110; 95.65%	5; 4.35%	0; 0%	0; 0%
	Intestinal Metaplasia scores			
	0	**1**	**2**	**3**
Antrum **(n=129)**	104; 80.62%	16; 12.40%	7; 5.43%	2; 1.55%
Corpus **(n=115)**	112; 97.39%	2; 1.74%	1; 0.87%	0; 0%
	***H. pylori* density scores**			
	0	**1**	**2**	**3**
Antrum **(n=128)**	2; 1.56%	41; 32.03%	36; 28.13%	49; 38.28%
Corpus **(n=114)**	2; 1.75%	58; 50.88%	24; 21.05%	30; 26.32%

*The majority of gastric biopsies from the antrum showed moderate inflammation (94 out of 129 biopsies; 72.87%), whereas 60% (69 out of 115) of the biopsies from the corpus showed mild inflammation ($p < 0.0001$***) (Chi-square test). The neutrophil activity scores were higher in the antrum than in corpus, where 69.77% (90/129) of patient's antral biopsies had moderate neutrophil infiltration, whilst 46.96% (54/115) of corpus biopsies had

normal neutrophil activity scores ($p<0.0001$***) (Chi-square test). Sydney score of 0: absent or normal, 1: mild, 2: moderate, and 3: severe abnormality.

It has been reported that the eradication of *H. pylori* typically leads to the resolution of inflammation, which in some cases can also reduce atrophy and the risk of developing malignancies (Fox & Wang, 2002). However, the histological changes of gastric mucosal lesions after eliminating *H. pylori* remain poorly understood. Thus, to further examine this, the data were then further partitioned according to whether the gastric biopsies were taken from patients with failed eradication therapy (n=60) and patients without failed eradication therapy (n= 184), as shown in Table 2.6.

Based on antral and corpus results, no statistically significant results were found between these two subgroups (patients with failed and without failed eradication therapy). However, statistical analysis showed that 87.10% (27/31) and 63.92% (62/97) of the antrum isolates were categorised as normal for mucosal glandular atrophy for both two subgroups ($p=0.0173$) (Chi-square test). Only 12.9% (4/31) and 35.05% (34/97) of antrum isolates were regarded as mild atrophy for patients with, and without previous rounds of eradication therapy, respectively (Table 2.6).

Table 2.6 The number and percentage prevalence of five variable histological features in gastric biopsies taken from the antrum (n=129) and corpus (n=115) regions, from patients who had previously received prior H. pylori eradication therapy (n=60) compared to those who had not (n=184).

	Antrum				Corpus			
	Inflammation scores							
	0	1	2	3	0	1	2	3
With previous failed eradication therapy	0; 0%	3; 9.68%	27; 87.10%	1; 3.23%	0; 0%	19; 65.52%	10; 34.48%	0; 0%
Without previous failed eradication therapy	0; 0%	21; 21.43%	66; 67.35%	11; 11.22%	0; 0%	50; 58.14%	27; 31.40%	9; 10.47%
	Neutrophil activity scores							
	0	1	2	3	0	1	2	3
With previous failed eradication therapy	2; 6.67%	5; 16.67%	23; 76.67%	0; 0%	14; 48.28%	6; 20.69%	9; 31.03%	0; 0%
Without previous failed eradication therapy	17; 17.89%	12; 12.63%	66; 69.47%	0; 0%	40; 46.51%	11; 12.79%	35; 40.70%	0; 0%
	Atrophy scores							
	0	1	2	3	0	1	2	3
With previous failed eradication therapy	27; 87.10%*	4; 12.9%	0; 0%	0; 0%	28; 96.55%	1; 3.45%	0; 0%	0; 0%
Without previous failed	62; 63.92%*	34; 35.05	1; 1.03%	0; 0%	82; 95.35%	4; 4.65%	0; 0%	0; 0%

eradication therapy								
	Intestinal Metaplasia scores							
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**
With previous failed eradication therapy	30; 96.77%	0; 0%	1; 3.23%	0; 0%	28; 96.55%	0; 0%	1; 3.45%	0; 0%
Without previous failed eradication therapy	74; 75.51%	16; 16.33%	6; 6.12%	2; 2.04%	84; 97.67%	2; 2.33%	0; 0%	0; 0%
	***H. pylori* density scores**							
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**
With previous failed eradication therapy	0; 0%	7; 22.58%	10; 32.26%	14; 45.16%	0; 0%	19; 65.52%	7; 24.14%	3; 10.34%
Without previous failed eradication therapy	0; 0%	30; 31.58%	29; 30.53%	36; 37.89	0; 0%	39; 46.99%	17; 20.48%	27; 32.53%

In the antrum, 87.10% (27 out of 31) and 63.92% (62 out of 97) of the isolates were classified as normal regarding mucosal glandular atrophy across both subgroups (patients with and without previous failed eradication therapy, respectively) (p=0.0173) (Chi-square test). Sydney score of 0: absent or normal, 1: mild, 2: moderate, and 3: severe abnormality.

2.3.8 Patients who donated samples to the study on more than one occasion

According to our Nottingham collection database, there were eight patients (mean age= 46.63 years old, SD= 12.37, and the ratio of male to female 1:3) who donated samples to the study on two occasions. The most common indication for their endoscopy was dyspepsia (n=6) based on the endoscopist's notes. Based on the information given, these returning patients were categorised based on their *H. pylori* status for both visits; patients found to be *H. pylori* positive on both visits, patients who had successfully eradicated their *H. pylori* infection during the second endoscopy, and patients who were not found to have *H. pylori* following two endoscopies. The status of *H. pylori*, disease, and differences in histology scores were analysed to see if there were any changes after the eradication treatment. Prior studies indicated that eradicating *H. pylori* could reduce inflammation, and different durations of follow-up showed varying levels of recovery in atrophic gastritis and intestinal metaplasia (Weng *et al.*, 2021). Therefore, it is crucial to evaluate the time that elapsed between the endoscopies to determine whether histological damage might have worsened without *H. pylori* eradication or improved with successful treatment.

Among them, four patients were found to be *H. pylori* positive on both visits. Some of the indications for the second endoscopy were dyspepsia, resistant *H. pylori* and previous failed eradication therapy. All of them were diagnosed with duodenal ulcer disease (DUD) at the first endoscopy, and three of four had persistent DUD that was still present at the second endoscopy (Table 2.7).

Two *H. pylori*-positive patients (during their first endoscopy) were reported to have successfully eradicated their *H. pylori* infection upon returning for a second endoscopy. One of the patients returned for the second visit due to dyspepsia, whereas the other patient

was found to have reflux esophagitis for the second visit. Both had a diagnosis of DUD during the first endoscopy but later were found to be cleared from the disease following the second endoscopy (Table 2.8).

A further two patients were not found to have a *H. pylori* infection following two endoscopies. The main indication for the initial endoscopy was dyspepsia for both patients, and one of the patients came back due to epigastric pain. However, they showed no signs of any peptic ulcer diseases or reflux esophagitis during endoscopic examination for both visits. However, the other patient was found to have gastric atrophy following the second endoscopy (Table 2.9).

Of the eight returning patients, three had isolates recovered from both the antrum and the corpus, and histology analysis was performed following both endoscopies (Table 2.10). Two patients were reported to be *H. pylori* positive throughout the first and second visits to the clinic, with the first patient (patient ID 295 and 326) showing the same histological scores profiles, whilst the second patient (patient ID 249 and 357) had different histological scores profiles in both corpus and antrum isolates for both check-ups. The second patient had mild atrophy in the antral biopsy on the first visit, but the second endoscopy showed no sign of atrophy after eradication treatment. However, the histological scores for inflammation and neutrophil activity for antrum showed increased scores from mild to moderate following the second endoscopy. Meanwhile, there were no changes detected in the corporal biopsy for both visits except for *H. pylori* density where the score decreased from moderate to mild.

The third patient (patient ID 391 and 478) was tested as *H. pylori*-positive during the first visit but later was found to be *H. pylori*-negative during the second visit, and as expected, this patient showed different histological scores profiles for both corpus and antrum biopsies

for both visits. Surprisingly, the histological analysis for both visits showed that the inflammation and activity scores in the corpus increased from moderate to severe, and normal to moderate, respectively. However, *H. pylori* density score in the corporal biopsy was lessened from severe to mild following the second endoscopy. Meanwhile, there were no changes detected for histological scores in the antrum except for *H. pylori* density where expectedly, the score decreased from mild to normal after the eradication treatment. No atrophy or intestinal metaplasia was detected in this patient.

It is also interesting to note that 39 patients in the study had previously undergone rounds of *H. pylori* eradication therapy based on the endoscopist's notes. The number of previous rounds of therapy was not always indicated, but some patients had undergone multiple rounds of eradication treatment with the highest number was five. For example, Table 2.11 showed a case study of a patient who had five previous rounds of eradication therapy, with dyspeptic symptoms referred from the GP clinic for upper gastrointestinal endoscopy. A rapid urease test indicated that he was positive for *H. pylori* infection, while an endoscopy revealed an acute duodenal ulcer disease accompanied by erosive esophagitis. The antibiotic resistance profiles of *H. pylori* isolates from the gastric antrum and corpus revealed that both isolates were resistant to multiple antibiotics including clarithromycin, metronidazole and amoxicillin. Meanwhile, results of genotyping of virulence genes from gastric biopsies showed that both corpus and antrum regions of the stomach were positive for more virulent *cagA*+ strains, whereas more virulent *vacA i*1 type for corpus and *vacA i*2 for antrum. All histological variables, aside from inflammation where the antrum has a higher score of 3, than in corpus (score of 2), have the same scores for both corpus and antrum (Table 2.11).

Table 2.7 Database of returning patients (H. pylori positive for both visits).

Patient ID	Age and time since the first visit	Gender	Indication for endoscopy	Disease status	Oesophagus
First visit (295)	68 years old	Female	Not stated	Both GUD/DUD	Reflux esophagitis
Second visit (326)	6 months later		Resistant *H. pylori*	Both GUD/DUD	Reflux esophagitis
First visit (410)	48 years old	Female	Dyspepsia	DUD	Normal
Second visit (629)	2 years later		Not stated	DUD	Normal
First visit (249)	41 years old	Male	Dyspepsia and *H. pylori*-positive	DUD	Reflux esophagitis
Second visit (537)	4 years later		Previous failed eradication therapy	DUD	Reflux esophagitis
First visit (680)	35 years old	Female	Previous *H. pylori* eradication	DUD	Reflux esophagitis
Second visit (827)	2.5 years later		Dyspepsia	None	Normal

Table 2.8 Database of returning patients (H. pylori positive on the first visit, but negative during the second visit).

Patient ID	Age	Gender	Indication for endoscopy	Disease status	Oesophagus
First visit (391)	60 years old	Female	Resistant *H. pylori*	DUD	Reflux esophagitis
Second visit (478)	11 months later		Not stated	None	Reflux esophagitis
First visit (443)	31 years old	Female	Not stated	DUD	Normal
Second visit (762)	4 years later		Dyspepsia	None	Normal

Table 2.9 Database of returning patients (H. pylori negative for both visits).

Patient ID	Age	Gender	Indication for endoscopy	Disease status	Oesophagus
First visit (449)	48 years old	Male	Dyspepsia	None	Normal
Second visit (771)	3.5 years later		Epigastric pain	None	Normal
First visit (399)	42 years old	Female	Dyspepsia	None	Normal
Second visit (835)	5 years later		Not stated	Atrophy	Normal

Table 2.10 The Sydney scoring system was used for the characterisation of gastritis.

Patient ID	Corpus					Antrum				
	Inflammation	Activity	Atrophy	Intestinal Metaplasia	*H. pylori* density	Inflammation	Activity	Atrophy	Intestinal Metaplasia	*H. pylori* density
First visit (295)	1	0	0	0	1	2	2	0	0	1
Second visit (326)	1	0	0	0	1	2	2	0	0	1
First visit (249)	1	0	0	0	2	1	1	1	0	2
Second visit (537)	1	0	0	0	1	2	2	0	0	2
First visit (391)	2	0	0	0	3	2	0	0	0	1
Second visit (478)	3	2	0	0	1	2	0	0	0	0

*Five histological variables: inflammation, neutrophil activity, glandular atrophy, intestinal metaplasia, and *H. pylori* density were selected for grading on a simple four-point scale – 0: absent or normal, 1: mild, 2: moderate, and 3: severe abnormality.

Table 2.11 Data on a patient with a medical history of five previous rounds of eradication therapies.

Age	**Gender**	***H. pylori* status**	**Disease status**	**Indication for endoscopy**	**Patient medical history**	**CagA status**	**VacA *i* type**
57	Male	Positive	DUD	Dyspepsia	Five previous courses of *H. pylori* eradication	Positive for both corpus and antrum	VacA *i*2 for antrum, but VacA *i*1 for corpus

	Histological scores				
	Inflammation	Activity	Atrophy	Intestinal Metaplasia	*H. pylori* density
Corpus	2	2	0	0	2
Antrum	3	2	0	0	2

2.3.9 Investigation of a relationship between *H. pylori* antibiotic resistance and the virulence genotype

A previous systematic review and meta-analysis revealed that *H. pylori* virulence factors such as *cagA* and *vacA s*1*m*1 play crucial roles in determining the clinical outcomes and eradication success rates (Karbalaei *et al.*, 2022). Therefore, this study examined the possible link between *cagA* and *vacA* genotypes and the antibiotic resistance seen in *H. pylori* clinical isolates. In the present study, the isolates were categorised as either *cagA*-negative (n=66) or *cagA*-positive (n=166), with nine isolates removed from the analysis due to ambiguous *cagA* status, leaving n=232.

Overall, the proportions of antibiotic-resistant isolates in the *cagA*-positive group were very similar to the *cagA*-negative group (Table 2.12). There were no statistically significant differences. It was found that the percentage of clarithromycin-resistant in *cagA*-positive and *cagA*-negative *H. pylori* isolates were similar; 25.9% and 30.3%, respectively. In amoxicillin and levofloxacin, the percentage of resistant *cagA*-positive isolates was found to be 3% and 2.4%, and the percentage of resistant *cagA*-negative isolates was found to be 0% and 3%, respectively. As for metronidazole, 60.2% of the total number of *cagA*-positive isolates were resistant, whereas 65.2% accounted for the total number of *cagA*-negative isolates.

Table 2.12 The number and percentage prevalence of antibiotic resistance in cagA-positive and cagA-negative H. pylori isolates.

	Number and percentage of antibiotic-resistant isolates				
	Clarithromycin	**Amoxicillin**	**Levofloxacin**	**Tetracycline**	**Metronidazole**
***cagA*+ (n=166)**	43; 25.9%	5; 3.0%	4; 2.4%	0; 0%	100; 60.2%
***cagA*- (n=66)**	20; 30.3%	0; 0%	2; 3.0%	0; 0%	43; 65.2%

Meanwhile, the *H. pylori* isolates were also classified into *vacA i*1 (n=152) and *vacA i*2 (n=79), with ten isolates removed from the analysis due to unclear genotypic data, leaving n=231 (Table 2.13). Overall, the results showed that for each antibiotic, there was a slightly higher percentage of *vacA i*2 isolates that were antibiotic-resistant compared to *vacA i*1 ones. It was found that for clarithromycin, amoxicillin, levofloxacin, and metronidazole, each had a higher percentage of resistant isolates that had the *vacA i*2 genotype, with 34%, 5%, 4%, and 72% resistant isolates, each, respectively. The *vacA i*1 genotyped isolates had a lower percentage of isolates that were resistant to the aforementioned antibiotics, with 24%, 1%, 3%, and 66% isolates identified as resistant, respectively. Comparable results to the analysis of *cagA* positive and negative strains, no statistically significant results were found in *vacA* strains in this study.

Table 2.13 The number and percentage prevalence of antibiotic resistance in vacA i1 and vacA i2 H. pylori isolates.

	Number and percentage of antibiotic-resistant isolates				
	Clarithromycin	**Amoxicillin**	**Levofloxacin**	**Tetracycline**	**Metronidazole**
***vacA i*1 (n=152)**	37; 24%	2; 1%	4; 3%	0; 0%	100; 66%
***vacA i*2 (n=79)**	27; 34%	4; 5%	3; 4%	0; 0%	57; 72%

2.4 Discussion

2.4.1 A high prevalence of *H. pylori* resistance to clarithromycin and metronidazole was observed

Among the *H. pylori* isolates investigated in this study, the overall rates of resistance to metronidazole were surprisingly high: 61.8% (149/241) of the total number of *H. pylori* strains tested were resistant to metronidazole. Metronidazole is known as a broad-spectrum antibiotic drug that is frequently used as prescribed antibiotic in the UK to treat common cold, flu, stomach and intestinal infections caused by a wide variety of bacterial infections and parasites. According to Thompson *et al.* (2022), metronidazole was the second highest antibiotic prescribed by dental practitioners which accounted for 28.4% of all oral antibiotics in 2017, as this drug is used as an effective antibiotic in oral and dental infections as first-line treatment. Hence, the improper use of this inexpensive drug may contribute to the increase in metronidazole resistance in the community, and therefore an elevated level of metronidazole-resistant *H. pylori* strains was observed in this study.

The present metronidazole resistance (61.8%) prevalence found in this study is comparable to the 69.2% (50-79%) resistance rate to metronidazole reported in a recent study by Mégraud *et al.* (2023) across the US and Europe, though previous studies in Bangor between 2000-2003 reported metronidazole resistance rates of 29% to 40% (Elviss *et al.*, 2004). Meanwhile, McNulty *et al.* (2012) found 88% resistance rate to metronidazole in one study in London. However, in an East London subpopulation, resistance rates varied according to ethnic origins; 37% for UK-born, 67% for non-UK-born and 90% for patients from the Bangladeshi community (Banatvala *et al.*, 1994). Surprisingly, the European study proved that

ethnic origin was significantly linked with resistance to metronidazole (Glupczynski *et al.*, 2001).

As for clarithromycin, the second most widely used antibiotic in *H. pylori* eradication therapy, the rate of resistance in clarithromycin for this study (27.8%) was similar to the 21.4% rate for clarithromycin-resistant *H. pylori* strains in Europe (Megraud *et al.*, 2021). However, the rates of resistance in clarithromycin are still lower in Europe, compared to other regions in Asia such as India (58.8%) and China (46.54%) (Ghotaslou *et al.*, 2015). However, a recent systematic review and meta-analysis study found that the latest primary resistance rate for clarithromycin in the Asia-Pacific region is 30% (Hong *et al.*, 2024), aligning with current findings.

The differences between the resistance rates in clarithromycin may reflect the variation in antibiotic usage between continents and countries. This is possibly due to the total consumption of macrolides which has been decreased (-15.5%) over the past 5 years in the UK (Agency, 2023b) and other parts of European countries (Control, 2019). Whereas the inappropriate use of macrolides in many developing countries in Asia is high (Nepal & Bhatta, 2018).

Levofloxacin resistance was identified in 4.1% of total *H. pylori* isolates in this study. In the UK, the data for levofloxacin-resistant *H. pylori* is scarce. Meanwhile, one 2012 UK study found that levofloxacin resistance rates of between 1-17%, depending on the region where the testing took place (McNulty *et al.*, 2012), and the WHO European region data estimated the rates of levofloxacin resistance in *H. pylori* strains is 15.8% (McNulty *et al.*, 2012). It was apparent that levofloxacin resistance rates obtained in this study were in the lower range of resistance rates found in the previous studies in the UK and Europe. This is probably

because levofloxacin is unfavourable as a first-line treatment and its general usage has been decreasing between 2017 and 2021 in the UK (Agency, 2023a) due to the overall efficacy and safety data which suggests that levofloxacin may cause serious side effects including hepatotoxicity, cardiac arrhythmia, severe skin reactions and tendon rupture (MHRA, 2012).

In the present study, the amoxicillin resistance rate was found to be 2.5%, and this finding is similar to the < 3% reported by (McNulty *et al.*, 2012) in the UK. The average prevalence rate of amoxicillin resistance in *H. pylori* strains in Europe is still very low, 0.2% (ranging from 0 to 1.7%), indicating that amoxicillin resistance is still not yet a problem in European countries (Megraud *et al.*, 2021). On the other hand, none of *H. pylori* isolates were found to be resistant to tetracycline in this study, which is consistent with the previous report that resistance to tetracycline is as low as 0.5% in the UK, or even absent in most countries (Megraud *et al.*, 2021; Savoldi *et al.*, 2018). Such findings suggest that, compared to other antibiotics, all of *H. pylori* isolates tested in this study were susceptible to tetracycline, and this antibiotic was not affected by resistance which indicates the importance of this drug in eradicating *H. pylori* strains.

As for dual resistance, clarithromycin and metronidazole are the antibiotics most frequently used with amoxicillin in the first-line eradication therapy, thus it was not uncommon to see that the rate of dual-resistance for clarithromycin and metronidazole is 24.1% (58/241) in this study. This finding was in line with a study by Goni *et al.* (2022) where dual antibiotic resistance was seen in 21.8% of patients in Germany. However, the multi-drug-resistant prevalence obtained in this study is higher than 8.4% of dual-resistant *H. pylori* strains identified in the previous study in England and Wales between the year of 2000-2005 (Chisholm & Owen, 2009), and notably, higher than any other regions in Europe (15%)

(Savoldi *et al.*, 2018). The prevalence of dual resistance is possibly due to the failure of previous eradication therapy that used both clarithromycin and metronidazole, which may then harbour double resistance in *H. pylori* strains (Heep *et al.*, 2000).

2.4.2 Different antibiotic resistance profiles of *H. pylori* paired isolates taken from the gastric antrum and corpus of the same patients

Of the paired isolates taken from both antrum and corpus of the same patient, 92% (73/79) had identical resistance profiles, whereas 7.59% (6/79) had different profiles. These antibiotic profiles differences between the paired isolates taken from antrum and corpus may be indicative that there is more than one strain of *H. pylori* present in the individuals' stomach. Although the presence of multiple *H. pylori* strains in a single patient has been reported from previous studies, the rates of prevalence vary among studies (Ayala *et al.*, 2011; Farzi *et al.*, 2015) . The frequency of discordant resistance profiles in paired of isolates found in this study is slightly lower than most previous reports. For example, Selgrad *et al.* (2014) found that 10 out of 66 patients (15.2%) had different antibiotic profiles in both isolates. In the same context, Seo *et al.* (2019) reported a rate of 12.5% (10 from 80 patients) for multiple *H. pylori* strains with different antibiotic profiles.

Furthermore, the high discordant antibiotic susceptibility to clarithromycin and metronidazole between the antrum and corpus biopsies found in this study agreed with a previous study (Selgrad *et al.*, 2014). It is also noteworthy that half of the patients who had isolates with discordant resistance profiles in this study had previously undergone *H. pylori* eradication therapy. This showed that metronidazole and clarithromycin have been widely used as first-line therapy in *H. pylori* eradication treatment. It is thought that previous

antibiotic therapy can change the genetic profiles of the stomach microflora through horizontal gene transfer (HGT), which contribute to the emergence of multidrug-resistant pathogens (Kent *et al.*, 2020). Therefore, the presence of *H. pylori* strains with different resistance spectrums in the same patient is likely to cause treatment failure and increase resistant strains selection (Goni *et al.*, 2022).

Previous studies revealed that extensive allelic diversity was found amongst populations within the antrum and corpus regions of each patient's stomach (Wilkinson *et al.*, 2022). In this recent study, it was noted that most patients were originally infected with a single strain, and the variations observed within and among different regions were likely a result of nonsynonymous mutations spreading over time. Another study identified region-specific diversity in the gastric area and suggested that antibiotic treatment could significantly impact the population structure of *H. pylori* in the stomach (Ailloud *et al.*, 2019).

2.4.3 Increasing trends of resistance for clarithromycin and metronidazole in *H. pylori* isolates over time

This study also showed a general trend of increased overall resistance for clarithromycin and metronidazole in *H. pylori* isolates over the period of 2001 until 2018. The resistance rates of clarithromycin increased significantly between the group year of 2001-2005 (15.6%) to 2011-2018 (40%). This comes as no surprise because in the time period 2000–2010, global macrolide consumption increased by 19% (Van Boeckel *et al.*, 2014), hence the sharp increase in clarithromycin's rate of resistances was observed between two-year groups of this study. Similarly, rate of resistance for metronidazole was also increased significantly during the year of (2001-2005) (57.8%) to (2011-2018) (77.5%) in this study. A study in

England and Wales over a six-year period (2000-2005) is also consistent with the current findings that the rates of resistance to metronidazole and clarithromycin increased in both areas (Chisholm *et al.*, 2007).

The current findings also confirmed previous reports by (Lu *et al.*, 2019) and (Lee *et al.*, 2019), although one meta-analysis study showed that there were no significant changes observed for clarithromycin and metronidazole resistance rates between 2006-2016 for the European region (Savoldi *et al.*, 2018). In contrast, another European study carried out previously reported that *H. pylori* resistance to clarithromycin decreased from 36.65% in 2009 to 24.38% in 2014. However, increased clarithromycin resistance has been observed in Asia, from 15.28% in 2009 to 32.46% in 2014, most likely due to the higher macrolide consumption in this region (Ghotaslou *et al.*, 2015).

2.4.4 Patients with previous *H. pylori* eradication therapy had higher clarithromycin and metronidazole resistance rates in their isolates compared to patients who had not received therapy

This chapter also confirmed the hypothesis that patients who had previously undergone *H. pylori* eradication therapy, had higher clarithromycin and metronidazole resistance rates in their colonising strains when compared to patients who had not previously undergone eradication therapy. As expected, the analysis of antibiotic profiles between these two categories of patients throughout the whole period of this study (2001-2018) revealed that the total number of clarithromycin-resistant isolates in patients who had a previous eradication treatment were significantly higher than those who never had any previous eradication treatment in the past. This was consistent with the previous study reported by

(Bluemel *et al.*, 2020) where prior eradication treatment was significantly associated with individual carriage of clarithromycin-resistant *H. pylori* strains. Previous macrolide treatment could exert selective pressure on an existing *H. pylori* infection, selecting for strains that are macrolide-resistant and influencing *H. pylori* susceptibility for years after treatment (McMahon *et al.*, 2003). Likewise, Boltin *et al.* (2015) have associated the outpatient use of long-acting macrolide with clarithromycin resistance.

As predicted, the total number of metronidazole-resistant isolates were significantly higher in patients with previous eradication therapy than those without previous eradication therapy. These findings agree with another report that showed the secondary resistance to metronidazole and clarithromycin were higher among patients who failed in previous *H. pylori* eradication therapy (Liu *et al.*, 2019), and a further study has linked metronidazole-resistant *H. pylori* isolates to previous use of metronidazole (O'Connor *et al.*, 2010). A meta-analysis study reported by Savoldi *et al.* (2018) also revealed that the resistance rates in *H. pylori* strains are higher in previously treated individuals than naïve patients for almost all included antibiotics. Previous studies have demonstrated a strong correlation between antibiotic consumption and the development of resistant bacteria strains (Serwecińska, 2020). Secondary resistance to an antibiotic is mainly caused by prior exposure to that antibiotic, and hence, the results found in this study were foreseeable as exposure to any prior metronidazole-containing treatment is significantly associated with increased metronidazole resistance.

2.4.5 Gender was not found to be associated with the treatment outcomes

This study found no significant gender differences in the effects of *H. pylori* eradication treatment. This finding is consistent with the previous studies carried out in France (Broutet *et al.*, 2003) and Italy (Perri *et al.*, 2001), where they found no association between gender with *H. pylori* eradication therapy outcome, although one large cohort study in the UK carried out on 1064 of *H. pylori*-infected patients found that women were more likely to have metronidazole-resistant strains than male patients (Parsons *et al.*, 2001), and one study in Germany found that female sex was a risk factor for clarithromycin resistance (Bluemel *et al.*, 2020).

Interestingly, other reports showed women were more likely to have a failure in eradication than men (Chang *et al.*, 2019; Lim *et al.*, 2016b; Saracino *et al.*, 2020), and gender was a predictive factor of *H. pylori* eradication failure in developing countries (Queiroz *et al.*, 2002). Antibiotic resistance rates found in this study also do not appear to be related to cigarette smoking although previous reports have demonstrated that smoking is a significant independent factor predicting treatment failure of *H. pylori* (Camargo *et al.*, 2007; Suzuki *et al.*, 2006).

2.4.6 Patients aged 60 years and below showed a higher likelihood of harbouring *H. pylori* strains resistant to clarithromycin and metronidazole

A previous study in the UK demonstrated that metronidazole resistance was higher in patients of younger ages and decreased in patients over 60 years old (Parsons *et al.*, 2001). This seems to be in line with the findings in this study where metronidazole resistance was significantly higher in the 60 years old and below age group than in those over 60 years of

age. Studies in the United States confirmed this current finding and established a link between metronidazole resistance rate and young age (Meyer *et al.*, 2002; Osato *et al.*, 2001b). Equally, another study in Spain also revealed that the prevalence of metronidazole-resistant strains was lower among patients aged ≥ 70 years old (Morilla *et al.*, 2019). However, one previous study has shown no significant age differences associated with the prevalence of antibiotic-resistant strains in individuals infected with *H. pylori* (Boyanova *et al.*, 2017).

Similarly, clarithromycin resistance was also discovered to be more common in patients below than 60 years old in the present study. This finding was similar to the previous studies which showed that a higher prevalence of clarithromycin-resistant strains was observed in patients aged below 40 years old (Wong *et al.*, 2003), and younger than 50 years old (Saracino *et al.*, 2020). However, previous reports regarding the effect of age on antibiotic resistance are conflicting (Agudo *et al.*, 2010; Bluemel *et al.*, 2020; Mamori *et al.*, 2010; Miendje Deyi *et al.*, 2011), due to differences in other patient factors among studies, such as therapy compliance, alcohol consumption, and smoking.

2.4.7 Higher histological scores for pathological characteristics were observed in the antrum compared to the corpus

The study detected higher histological scores for chronic inflammation (mild to severe) in comparison to glandular atrophy (normal to moderate) and intestinal metaplasia (normal to moderate), for both corpus and antrum. However, in general, the histological scores for chronic inflammation, neutrophil activity, glandular atrophy, intestinal metaplasia, and *H. pylori* density were significantly higher in the antrum than in the corpus. One previous study

confirmed the result of this finding (Chitapanarux *et al.*, 2021). A previous study in the UK also found that the histological scores for chronic inflammation, neutrophil activity, glandular atrophy, and intestinal metaplasia were higher in the antrum than in the corpus (Bodger *et al.*, 2001). Meanwhile, another study carried out by Kim *et al.* (2008) showed that the presence of atrophy and intestinal metaplasia was higher in the antrum than in the corpus, whilst Carvalho *et al.* (2012) showed that gastric mucosal inflammation caused by *H. pylori* is more severe in the antrum than in the corpus.

Chronic gastritis associated with *H. pylori* infection is believed to be more prevalent and more severe in the antrum than in the corpus (Bayerdörffer *et al.*, 1992). It has been postulated that *H. pylori* first colonize the antrum, where they elicit an inflammatory response consisting of neutrophils, eosinophils, and mononuclear cells. It is thought that the inflammation initiated by *H. pylori* bacteria typically starts in the antrum of the stomach before progressing to other areas, causing atrophic changes and the spread of pre-cancerous cells (Piazuelo *et al.*, 2021), towards the corpus where the bacterial biomass is smaller, and the mucosal cellular immune responses are less prominent (Rauws *et al.*, 1988). This seems that the nature of the inflammatory response to *H. pylori* infection differs between the antrum and corpus.

Another possible explanation is that the most common type of *H. pylori* gastritis is antral predominant, which is an early stage of infection with only minimal corpus involvement due to higher acid output by suppressing somatostatin release and increasing gastrin release from the G cells in the gastric antrum (Calam & Baron, 2001). This type of gastritis, where inflammation is predominant in the antrum, relatively sparing the corpus, is most common in individuals with duodenal ulcers. Most patients visiting the clinic exhibit antral-

predominant gastritis, which could potentially account for the increased levels of inflammation observed in the antrum as opposed to the corpus in gastric biopsies. By contrast, corpus-predominant gastritis is the less frequent type of gastritis and is associated with a high risk of gastric cancer (Watari *et al.*, 2014).

2.4.8 Eradication therapy may have long-lasting benefits in the regression of inflammation and pre-cancerous lesions in *H. pylori* patients

Some *H. pylori*-infected patients may achieve successful eradication, while others might not after receiving *H. pylori* eradication therapy. If eradication is effective, it may take some time for the inflammation in the stomach to regress. This pilot study showed that one of the two returning patients who were found to be *H. pylori* positive on the first and second endoscopy had the same histological scores profiles in both corpus and antrum. A stability of inflammation, neutrophil activity, and *H. pylori* density scores observed in the gastric mucosa could be due to the short six-month gap between both check-ups.

However, the other patient (patient ID 249 and 357) who regressed from having a mild atrophy into normal mucosa (no atrophy) in the antral biopsy, and *H. pylori* density score decreased from moderate to mild in the corpus was possibly due to the delay of 4 years following the second endoscopy. This finding indicates that eradication over a substantial amount of time is suggested to be effective in improving the development of atrophy. A previous study confirmed this result, showing significant improvement in atrophy in both the antrum and corpus one year after eradicating *H. pylori* (Kodama *et al.*, 2021). A study in France also supported this finding where they found a regression in 16% of patients with gastric pre-cancerous lesions, where those patients regressed into normal mucosa and non-

atrophic gastritis during the mean follow-ups of 5.5 years with the minimal 6 months check-ups (Chapelle *et al.*, 2020). Meanwhile, Kodama *et al.* (2012) demonstrated that inflammation, activity, and atrophy scores for both antrum and corpus were significantly reduced between six months to 6 years after eradication. Hence, whether patients have similar or different levels of clinical pathological characteristics after eradication depends on the duration of antibiotic exposure and the severity of initial lesions (Dinis-Ribeiro *et al.*, 2004).

To sum up, both *H. pylori* eradication treatment and successful clearance of the infection demonstrated a long-term beneficial effect on the progression of inflammation and the pre-cancerous lesions. As a first step, the implementation of guidelines on the management of individuals at high risk should be considered, even in countries such as the UK, where the overall risk of gastric cancer is low. Therefore, improving the early diagnosis of pre-cancerous lesions through follow-up is the key to strategies for improving patient's treatment plans.

2.5 Limitations and Future work

The findings of this study are important as there is presently limited antibiotic susceptibility testing conducted on *H. pylori* isolates in the UK, due to the difficulty in the requirement for endoscopy to collect isolates and the related costs involved. This is despite recommendations that testing for sensitivity to *H. pylori* should be conducted in cases where patients have limited antibiotic options due to hypersensitivity, have undergone two unsuccessful antibiotic treatment courses in the past, or when local resistance rates are known to be high (England, 2019). Therefore, the absence of local resistance rate data in the

UK increases the likelihood of patients receiving first-line therapies that may not be effective, as indicated by our research findings. Improper additional courses of treatment may be necessary to eliminate the infection, potentially leading to complications on the patient such as increased exposure to antibiotic side effects and ongoing symptoms.

There has been a recent recommendation to conduct testing for clarithromycin-resistant *H. pylori* in patients before prescribing the medication (Malfertheiner *et al.*, 2022). However, such tests are not currently performed routinely in the UK. Thus, there is a need to enhance the testing of *H. pylori* isolates in the UK, particularly in cases where clarithromycin is being considered for treatment. A recent study carried out in France has shown that stool-based PCR tests have a high level of precision in identifying clarithromycin resistance. When comparing the Amplidiag *H. pylori* +ClariR stool PCR test to E-Tests, the sensitivity was found to be 98.4% with a specificity of 100% (Pichon *et al.*, 2020). These non-invasive testing techniques hold great potential in enhancing the treatment options.

One of the major limitations of this patient-based study is the reliability of the information provided during the visit. At times, the personal information and medical history details, including past treatments received, are reliant on the patient's responses to the endoscopist's questions. For instance, patients do not always remember that they have taken NSAID drugs, e.g. in a cold remedy, and GPs would not be able to give information on over-the-counter medications. Additionally, another barrier to the study is the lack of data access on previous antibiotic consumption by patients from GPs. Patient information on the antibiotic regimen for eradication therapy, and any antibiotics taken during the relevant period to target any infection, should be considered to make more reliable MIC data

interpretations. Thus, any future research will require permission from the relevant authority to access information from GP's medical records.

As this study exclusively included patients visiting a specialized clinic for symptom evaluation, these individuals may not accurately reflect the general population. Moreover, the participants recruited were primarily those referred by GPs, who may represent more challenging cases in primary care settings, potentially explaining the elevated levels of antibiotic resistance observed in this research.

Furthermore, since treating this infection necessitates the use of multiple antibiotics in triple or quadruple therapy, this study could have been enhanced by examining cross-resistance or synergistic interactions by testing combinations of the medications. This study solely focused on the susceptibility of the isolates to individual antibiotic, rather than the combination of antibiotics. Therefore, future studies should include *in vitro* synergy testing of multiple antibiotics against *H. pylori*-positive isolates by checkerboard and time-kill assays.

An additional crucial aspect would have been to isolate individual colonies of *H. pylori* from the biopsies rather than a 'sweep' or whole population culture, combined together. These individual colonies could have differed genetically and in their susceptibility to antimicrobial agents. Hence, this could affect the accuracy of antibiotic resistance profiles of an individual as this person may carry more than one single strain of *H. pylori.* Due to this reason, future studies will involve isolating a single colony from the pooled culture of *H. pylori* isolates.

2.6 Chapter summary

- The high prevalence of resistance in *H. pylori* isolates to metronidazole and clarithromycin were demonstrated in this study.
- There was no resistance to tetracycline, and the incidences of amoxicillin and levofloxacin resistance were minimal.
- Different antibiotic resistance profiles of *H. pylori* paired isolates taken from the antrum and corpus of some patients were observed.
- Increasing trends of resistance for clarithromycin and metronidazole in *H. pylori* isolates over time were demonstrated.
- The rates of resistance to clarithromycin and metronidazole were notably higher in isolates obtained from individuals who had undergone eradication therapy compared to those who had not.
- Younger patients (60 years old and below) showed a greater possibility of carrying *H. pylori* strains resistant to clarithromycin and metronidazole than older patients (60 years and over).
- Higher histological scores for pathological characteristics were observed in the antrum than in the corpus of gastric biopsies.
- It was also suggested that the prolonged eradication treatment of *H. pylori* may regress the inflammation and reduce the severity of pre-cancerous lesions such as atrophic gastritis in infected patients.
- Therefore, enhancing the early detection of pre-cancerous lesions through regular follow-up is crucial so that patients will have the best chance for successful treatment.

Chapter 3 : *Ex vivo* relationship between human defensins expression and *H. pylori* infection

3.1 Introduction

The previous chapter demonstrated that *H. pylori*'s resistance to common antibiotics indicates its adaptation strategies in the bacterium's long-term survival within its host. Thus, it is crucial to investigate the host's immune response to *H. pylori* infection to further elucidate how *H. pylori* can evade host innate immunity and persistently colonize the human gut. Several studies have revealed the molecular mechanisms that explain the innate immune response of the host against pathogens, as in aiding the host in resisting pathogen colonization on epithelial mucosal surfaces (Chu *et al.*, 2012; Miani *et al.*, 2018; Ramanan *et al.*, 2014). The mucosal lining of gastric epithelial cells is constantly exposed to a variety of microorganisms. In reaction to this exposure, the epithelial surfaces generate a wide array of antimicrobial proteins (AMPs) that can directly eliminate or hinder the growth of pathogenic bacteria (Pero *et al.*, 2019b). Therefore, AMPs are essential for the gastrointestinal mucosa's innate defence and are typically synthesized in response to inflammation or infection. Numerous AMPs have been described to have bactericidal activity against *H. pylori*, including members of the defensin family and the cathelicidin LL-37 (Otte *et al.*, 2009; Rogoll *et al.*, 2011; Zhang *et al.*, 2016).

As mentioned previously, AMPs can be categorized into three primary groups: α-defensins, β-defensins, and cathelicidins, and the distinction between α- and β-defensins lies in the specific locations of their disulfide bonds (Linn *et al.*, 2023). Both β-defensins and α-defensins also can function as chemoattractants for immune cells (Shelley *et al.*, 2020). Previous studies showed that in patients infected with *H. pylori* and in human gastric epithelial cells infected *in vitro*, increased levels of human β-defensin 2, 3, and 4 have been observed in the gastric mucosa (Allaker & Kapas, 2003; Boughan *et al.*, 2006; Isomoto *et al.*,

2004; Otte *et al.*, 2009; Patel *et al.*, 2013). However, this chapter will only focus on human β-defensin 2 (hβD2), encoded by the *DEFB4* gene, which is found in a variety of epithelial tissues and plays an essential role in inflammatory responses (Johansen *et al.*, 2016). *DEFB4* is thought to be ubiquitously expressed in gastric epithelial cells, but the expression seems to be upregulated in relation to *H. pylori* infection (Pero *et al.*, 2019a). Therefore, to examine the hypothesis that *DEFB4* are involved in the mucosal response to *H. pylori*, the expression of the gene was measured in both uninfected and *H. pylori*-infected antral gastric biopsies in this study.

Meanwhile, the α-defensin human defensin 5 (HD-5), encoded by the *DEFA5* gene is expressed in the Paneth cells prenatally (Mallow *et al.*, 1996) in the upper gastrointestinal tract, and these cells are exocrine serous glandular cells at the base of small intestinal crypts in the duodenum, jejunum, and the terminal ileum (Lisitsyn *et al.*, 2012). However, little is currently known about the local expression patterns of defensins along the human gastrointestinal tract, aside from the fact that they are produced by Paneth cells located exclusively in the small intestine. Hence, there may be variations in the composition of enteric defensins throughout the length of the small intestine, potentially affecting the local microbiota (Nakamura *et al.*, 2016). Furthermore, limited data exists regarding the possible involvement of alpha-defensin originating from gastric epithelial cells upon *H. pylori* infection, and the precise mechanisms involved in regulating *DEFA5* gene expression in the context of *H. pylori* infection remain unclear in many studies. Therefore, this chapter's main aim was to examine the relationship of the AMP gene, *DEFA5* relating to *H. pylori* infection in gastric biopsy samples from uninfected and *H. pylori*-infected patients.

Several review studies have shown the expression of defensins in multiple types of cancer, such as colon cancer, lung cancer, and renal cell carcinomas, implying a potential involvement of defensins in the onset and advancement of cancer (Droin *et al.*, 2009; Ghosh *et al.*, 2019). Defensins also play a role in the progression of gastric cancer, and the previous report indicates that the expression of *DEFA5* was significantly downregulated in human gastric cancer (Wu *et al.*, 2021). However, the functions of HD-5 in gastric cancer formation and progression are yet to be determined. As mentioned previously, *H. pylori* is widely recognized for causing chronic gastric inflammation, which can advance to atrophy, metaplasia, dysplasia, and, gastric cancer (Jaroenlapnopparat *et al.*, 2022). Therefore, to understand how *H. pylori* infection contributes to the development of gastric cancer and to elucidate the role of defensins in this process, it is essential to investigate the relationship between defensin expression and chronic inflammation that leads to gastric cancer.

The histological degrees of neutrophil and mononuclear cellular infiltration have previously been associated with *DEFB4* concentration in gastric juice in the corpus, but not in the antrum (Isomoto *et al.*, 2005). Hence, this study aimed to examine the association of *DEFB4* expression with the degree of inflammation, glandular atrophy, and intestinal metaplasia (IM) scores (as assessed by histopathology scoring described in Section 2.2.3) in 32 gastric antral biopsies of *H. pylori*-infected patients. Meanwhile, Soylu *et al.* (2008) showed that there was a significant correlation between α-defensin expression and increased levels of neutrophil density and chronic inflammation in gastric biopsies of dyspeptic paediatric patients. However, the relationship between *DEFA5* gene expression and neutrophil and mononuclear cells in adult patients is not currently understood. Hence, this study sought to explore the relationship between *DEFA5* expression and histological pathology in adult patients with *H. pylori* infection.

The regulation pathway of β defensin expression seems to be modulated in an NF-κB and *cag*PAI-dependent manner (Boughan *et al.*, 2006; Mustapha *et al.*, 2014; Patel *et al.*, 2013). A previous review study demonstrated the significance of *cagPAI* of *H. pylori* in the induction of human β-defensin-2 (Pero *et al.*, 2019b). The expression of the *DEFB4* gene is also said to be influenced by protein complexes that act as transcription factor, specifically NF-κB and AP-1. It was postulated that these complexes bind to the *DEFB4* gene promoter, which is essential for activating the gene responsible for hβD2 induction (Steubesand *et al.*, 2009; Tomita *et al.*, 2002). The increased expression of defensin in mammalian species during infection and inflammation indicates that these peptide antibiotics may play roles in antimicrobial defence at mucosal sites (Dutta & Das, 2016; Silwal *et al.*, 2021) . However, at present, no previous studies have established a connection between the expression of the *DEFA5* gene and the virulence factors of *H. pylori*, as per the most recent data. Thus, it is of interest to see if *H. pylori* infection and its virulence factors have impact on altering the *DEFB4* and *DEFA5* gene expression levels in gastric biopsies of infected patients carrying both *cag*A and *vac*A genes.

AMP expression is said to be influenced by acute or chronic infections, as well as interactions with pathogens (Linn *et al.*, 2023). A previous review study has shown that the release of defensins serves as a warning sign in the progression of upper gastrointestinal diseases (Wu *et al.*, 2022). Bauer *et al.* (2013) showed in their study that human beta-defensin-2 levels were observed to increase in response to *H. pylori* infection, and this upregulation was associated with the severity of gastritis. Furthermore, patients with gastric or duodenal ulcers proved to have higher levels of *DEFB4* expression compared to individuals with healthy stomachs (Pero *et al.*, 2017). However, the impact of defensin such as *DEFA5* on peptic ulcer diseases is not yet clear, and research is still lacking to understand

the extent of its effect. Thus, this chapter seeks to explore the possible correlation between *DEFB4* and *DEFA5* gene expression, and peptic ulcer diseases in individuals infected with *H. pylori*.

3.1.1 Objectives

The objectives of this chapter were to:

1. Investigate the expression of *DEFB4* and *DEFA5* in *H. pylori* infection in *ex vivo* gastric antral biopsies.
2. Determine the relationship between *DEFB4* and *DEFA5* expression levels and pathology.
3. Assess the link between bacterial virulence factors and gastric mucosal *DEFB4* and *DEFA5* mRNA expression.
4. Examine the relationship between *DEFB4* and *DEFA5* expression levels and the disease status of *H. pylori*-infected patients.

3.2 Materials and Methods

3.2.1 Purification of RNA from gastric biopsies

Gastric antral biopsies of patients for RNA analysis were preserved in RNAlater (Sigma-Aldrich, UK) as described previously (Patel *et al.*, 2013). Thawed biopsies were transferred to RLT lysis buffer containing β-mecaptoethanol (350 µl) and homogenised for 30 seconds using a T8 Ultra rotor-stator homogeniser (IKA, Werke & Co. Freiburg, Germany). Nucleic acids were then extracted using the RNeasy Mini Protocol - for purification of total RNA from animal cells, as per the manufacturer's instructions (RNeasy minikit, Qiagen). Briefly, DNA was first isolated by passing the tissue homogenate through a QIAshredder spin column (Qiagen), and then through a RNeasy spin column to selectively isolate RNA. 50 µl of RNase-free water (Qiagen) was added to elute the RNA. All eluates were assayed for their RNA concentration, using NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, USA). The quality of extracted nucleic acid was assessed by the A_{260}/A_{280} ratio to evaluate RNA purity. The acceptable values between 1.8 and 2.0 suggest the RNA was free from contamination (Chong *et al.*, 2020), and any samples that were of poor quality were discarded.

DNase treatment was not performed on RNA extracted from human gastric biopsies prior to cDNA synthesis, as the RT-qPCR assays targeted eukaryotic mRNA. Unlike bacterial RNA, eukaryotic mRNA is spliced and lacks introns, making it distinguishable from genomic DNA. Primers were designed to span exon–exon junctions, ensuring specific amplification of cDNA rather than any contaminating genomic DNA. Additionally, no reverse transcriptase (no-RT) controls were included in the qPCR runs to confirm the absence of gDNA contamination,

thereby validating the specificity of the amplification and compensating for the lack of DNase treatment.

3.2.2 cDNA synthesis

cDNA synthesis from 1000 ng of total RNA was performed using Superscript II reverse transcriptase (Invitrogen), as per the manufacturer's instructions. Each tube contained 1 µl solution of oligo $(dT)_{12-18}$ primers at 500 ng/µl; 1 µl of deoxyribonucleotide (dNTP) mix at 10mM each (Promega); 3 µl of RNA template per tube at 33.3 ng/µl; and 7 µl of RNase-free H_2O (Invitrogen). After heating for 5 minutes at 65°C, the sample was placed on ice for 2 minutes. 4 µl of 5X first strand buffer, 2 µl of 0.1 M Dithiothreitol (DTT) and 1 µl RNaseOUT™ (all by Invitrogen), were added to each tube. After being heated for 2 minutes at 42°C, 1 µl of Superscript™ II Reverse Transcriptase (Invitrogen) or 1 µl of RNase-free H_2O (as negative reverse transcriptase control) were added to each tube. The samples were incubated at 42°C for 50 minutes followed by inactivation at 70°C for 15 minutes. The cDNA samples were stored at -20°C.

3.2.3 Real-time quantitative polymerase chain reaction (PCR) for human defensin gene expression

Real-time PCR was carried out on 2 µl of the cDNA template (prepared previously described in section 3.2.2), with target and reference primers (3 µl for each forward and reverse primers) (Sigma Aldrich), on the Rotor-Gene 3000 (Corbett Research, Cambridge, UK), using 12.5 µl of SYBR Green qPCR kit (GRI, Braintree, UK). Reactions mixtures were made up to 25 µl with nuclease-free water. The RT-qPCR was carried out over 45 cycles, with a denaturing phase of 15 seconds at 95°C; an annealing phase of 30 seconds at 60°C; and an extension

phase of 30 seconds at 72°C. A no template control (NTC) for each primer set, was included in each run and a commercial universal human cDNA (United States Biological) at 2.5 ng/µl was used as a positive control in all assays. The negative control samples (produced in the absence of reverse transcriptase from each RNA samples) were tested in parallel to ensure that products are not amplified from contaminating genomic DNA.

The Qiagen primer designs (*GAPDH*, *DEFB4,* and *DEFA5*) were adopted directly from those constructed by the group's previous research, and a published paper (Table 3.1). These primers were used for the quantification of human *DEFB4* and *DEFA5* expression and gene expression was normalised using Qiagen *GAPDH* primers. The primer sequences were analysed using the Netprimer web tool: www.premierbiosoft.com/netprimer to predict the melting temperatures (Tm), and to detect their potential to form any secondary structures, such as hairpins, self-dimers or repeat tracts, that could affect the PCR efficiency. Reaction efficiencies were measured with a standard curve developed using a 4-fold dilution series of a commercial human cDNA (United States Biological). The efficiencies of each PCR were calculated from the gradients of cDNA input plotted against cycle threshold (Ct) values. Reaction efficiencies for each primer were determined; 1.94 (*DEFB4*), 1.81 (*DEFA5*), and 1.89 (*GAPDH*) (See Appendix 1).

Table 3.1 Primer pair sequences used for RT-qPCR reactions

Primer	Primer Sequence	Product size (bp)	References
GAPDH Forward Reverse	 CCACATCGCTCAGACACCAT GGCAACAATATCCACTTTACCAGAGT	114 bp	Patel *et al.* (2013)
DEFB4 Forward Reverse	 CTGATGCCTCTTCCAGGTGTTT GAGACCACAGGTGCCAATTTG	123 bp	Patel *et al.* (2013)
DEFA5 Forward Reverse	 CCATCCTTGCTGCCATTCTC TCGGCAATAGCAGGTGGC	191 bp	Vordenbäumen *et al.* (2010b)

3.2.4 Relative quantification of gene expression using RT-qPCR

A pool of samples from *H*. *pylori*-negative donors were produced and this control was included in each run, so that the test gene expression of the samples could be analysed against as a comparator, providing a fold-difference. For this uninfected comparator, cDNA was synthesized from purified RNA pooled and extracted from biopsies by 10 *H. pylori* negative patients. Samples were run in duplicate, and the results were analysed according to the Pfaffl method (Pfaffl, 2001). Relative defensin gene expression levels were determined by normalising against *GAPDH* mRNA levels, and data were presented as a fold difference in comparison to the reference sample obtained from *H*. *pylori*-negative donors.

Relative gene expression:

Pfaffl method: $\frac{(E_{target})^{\Delta Ct\ target\ (control-sample)}}{(E_{ref})^{\Delta Ct\ ref\ (control-sample)}}$

*The relative expression ratio (*R*) of a target gene is calculated based on *E* (*efficiency*) and the Ct (cycle threshold) differences of an unknown sample versus a control (comparator) and expressed in comparison to a reference gene (*GAPDH*).

3.2.5 Histopathology

Assessment of histopathology on gastric antral biopsies was evaluated as previously mentioned in section 2.2.3.

3.2.6 Virulence factor typing

The clinical isolates of *H. pylori* taken from gastric biopsies were genotyped for *cagA* and *vacA i*-region as previously described in section 2.2.4.

3.2.7 Data analysis and statistical tests

Data were collected and recorded in Microsoft Excel. Statistical analysis and scatter plots were prepared using GraphPad Prism Version 10.3.0 (507). All statistical tests of unpaired data were carried out using the Mann-Whitney test to compare two independent groups. Meanwhile, the Kruskal-Wallis test with Dunn's post hoc test was applied to the pairwise comparisons with multiple variables. A P value of < 0.05 was considered as statistically significant.

3.3 Results

3.3.1 *H. pylori* clinical isolates used

For the analysis of *DEFB4* and *DEFA5* mRNA expression in relation to *H. pylori* infection, gastric biopsy samples were initially collected from the same cohort of patients. A total of 32 *H. pylori*-infected and 24 uninfected biopsy samples were analysed for *DEFB4* expression (Table 3.2). However, during the *DEFA5* qPCR analysis, 1 infected and 3 uninfected samples failed to yield valid amplification results despite repeated attempts. These samples were therefore excluded from the *DEFA5* expression analysis, resulting in a final dataset of 31 *H. pylori*-infected and 21 uninfected biopsies (Table 3.2). The slight reduction in sample numbers for *DEFA5* expression analysis compared to *DEFB4* was probably due to technical limitations in qPCR amplification, such as insufficient RNA quality or quantity, degradation, or target gene expression levels below the detection threshold.

For the analysis investigating the relationship between *DEFB4* mRNA expression and *H. pylori* virulence determinants (*cagA* and *vacA* genotypes), a subset of samples from the main expression cohort was used. Although 32 *H. pylori*-infected gastric biopsy samples were analysed for *DEFB4* expression, only 15 of these had corresponding data on *H. pylori* virulence status (Table 3.2). This was due to the unavailability or incomplete genotyping of some isolates, which resulted from factors such as insufficient DNA yield, poor quality, or prior omission from molecular profiling workflows. Meanwhile, 24 uninfected samples with previously established *cagA* and *vacA* status were included as controls for comparative purposes (Table 3.2).

Similarly, for the investigation of *DEFA5* mRNA expression in relation to *H. pylori* virulence factors, 22 *H. pylori*-infected and 21 uninfected gastric biopsy samples were analysed (Table 3.2). As with the *DEFB4* analysis, the difference in sample number compared to the total number of biopsies assessed for *DEFA5* expression (31 infected and 21 uninfected) was due to the limited availability of paired genotyping data. Only samples with both *DEFA5* expression values and confirmed *H. pylori* virulence status were included in this subset analysis.

Table 3.2 Table of H. pylori clinical isolates used

Experiment	*H. pylori*-infected antral gastric biopsy (number of isolates)	Uninfected antral gastric biopsy (number of isolates)
3.3.2 Investigation of a relationship between *DEFB4* expression and *H. pylori* infection	32	24
3.3.3 Investigation of a relationship between *DEFA5* expression and *H. pylori* infection	31	21
3.3.4 Investigation of an association of *DEFB4* expression with pathology	32	-

3.3.5 Investigation of an association of *DEFA5* expression with pathology	31	-
3.3.6 Investigation of a relationship between *DEFB4* expression and virulence determinants of *H. pylori*	15	24
3.3.7 Investigation of a relationship between *DEFA5* expression and virulence determinants of *H. pylori*	22	21
3.3.8 Investigation of an association between *DEFB4* expression and disease status	32	-
3.3.9 Investigation of an association between *DEFA5* expression and disease status	31	-

3.3.2 Investigation of a relationship between *DEFB4* expression and *H. pylori* infection

To test the hypothesis that *DEFB4* is involved in the mucosal response to *H. pylori*, the expression of the gene was quantified in uninfected and *H. pylori*-infected antral gastric

biopsies. It was found that *DEFB4* mRNA expression level was 17-fold higher in gastric antral biopsies from 32 *H. pylori*-infected compared with 24 uninfected controls (p<0.0001) (Mann-Whitney test) (Figure 3.1). Median defensin expression levels for infected and uninfected biopsies were 13.85 and 0.8250, respectively, in which the control biopsies expressed *DEFB4* mRNA at low levels, providing further evidence for its constitutive nature. Hence, this suggests that the expression of *DEFB4* is significantly higher in patients with *H. pylori*-positive antral biopsies compared to uninfected patients.

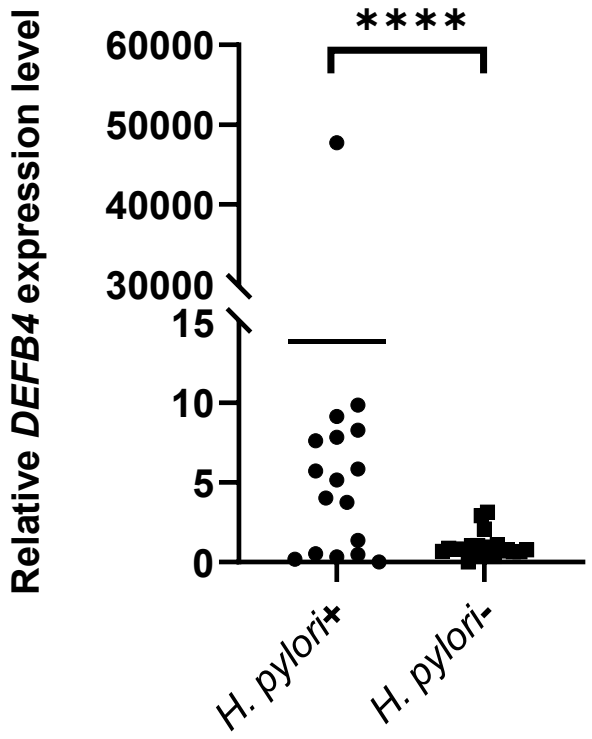


Figure 3.1: Relative DEFB4 mRNA expression in gastric antral biopsies from 32 H. pylori infected and 24 uninfected donors*. The expression of DEFB4 was significantly higher (p<0.0001****) (Mann-Whitney test) in infected patients than in uninfected patients. The expression of DEFB4 was normalised against GAPDH and compared to a pooled negative control sample extracted from biopsies of 10 H. pylori negative patients.*

3.3.3 Investigation of a relationship between *DEFA5* expression and *H. pylori* infection

To further investigate whether *H. pylori* infection in the gastric mucosa has an impact on the expression level of *DEFA5*, the mRNA expression level of *DEFA5* was determined in 21 uninfected and 31 *H. pylori*-infected antral gastric biopsies.

This study however showed that the relative level of *DEFA5* mRNA expression in samples of gastric antral biopsies was significantly downregulated (*P*=0.0029) (Mann-Whitney test) by 76-fold in patients with *H. pylori*-positive (median=0.022) (n=31) when compared with uninfected patients (median=1.68) (n=21) (Figure 3.2).

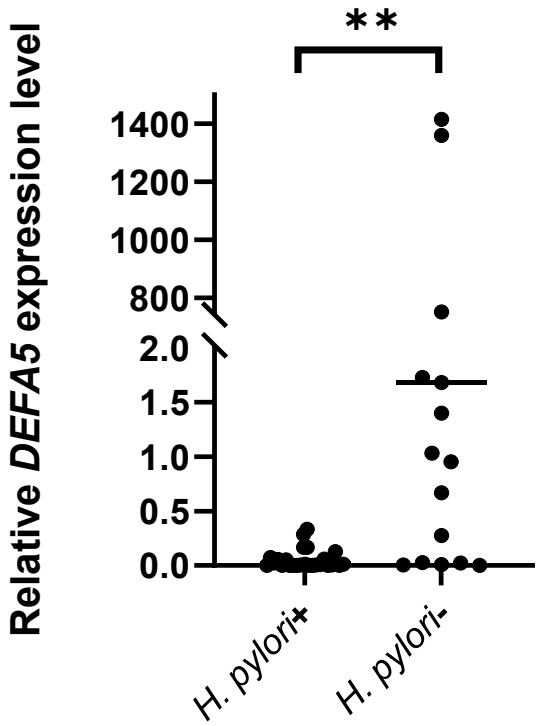


Figure 3.2: Relative DEFA5 mRNA expression in gastric antral biopsies from 31 H. pylori infected and 21 uninfected donors*. The expression of DEFA5 is significantly lower (p=0.0029**) (Mann-Whitney test) in infected patients than in uninfected patients. The*

expression of DEFA5 was normalised against GAPDH and compared to a pooled negative control sample extracted from biopsies of 10 H. pylori negative patients.

3.3.4 Investigation of an association of *DEFB4* expression with pathology

This study aimed to examine the association of *DEFB4* expression with the degree of inflammation, glandular atrophy, and intestinal metaplasia (IM) scores (as assessed by histopathology scoring described in Section 2.2.3) in 32 gastric antral biopsies of *H. pylori*-infected patients. Expression levels were stratified based on histological inflammation scores, and it was found that the defensin expression level was not significantly associated with the severity of the inflammation (For score 1: median=203.8, n=6, IQR=6.058-14288; for score 2: median=8.270, n=21, IQR=2.135-61.22; and for score 3: median=34.45, n=4, IQR=9.69-1433). The same was also observed for neutrophil activity score where *DEFB4* expression was not significantly correlated to neutrophil activity in the gastric mucosa of infected patients (for score 0: median=19.23, n=4, IQR=0.695-2361; for score 1: 403.4, n=6, IQR=3.055-3631; and for score 2: 13.5, n=22, IQR=5.57-59.63. No samples were found to have a score of 3 for neutrophilic infiltration).

DEFB4 expression levels were measured in 32 antral gastric biopsy samples and stratified according to the presence or absence of glandular atrophy, and whether gastric tissue has intestinal metaplasia (IM), or no IM, as there were only a few samples to compare statistically. Interestingly, it was revealed that *DEFB4* gene expression for the presence of atrophy was more than fifty-fold higher (median=408.1, n=13, IQR= 23.11-4466) (p=0.0059) (Mann-Whitney test) than those without atrophy (median=7.620, n=19, IQR=3.74-21.63) (Figure 3.3). Furthermore, it was revealed that *DEFB4* gene expression level was not

significantly related to the presence of IM (presence: median=37.09, n=9, IQR=6.385-1597; absence of IM: median=17.86, n=23, IQR=3.74-408.1) (Mann-Whitney).

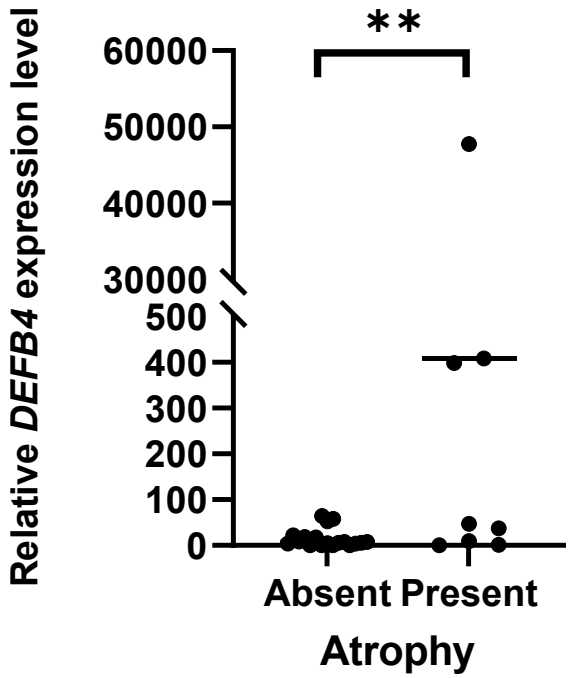


Figure 3.3: The association between DEFB4 expression and atrophy in gastric antral biopsies from 32 H. pylori-infected donors*. The expression of DEFB4 was significantly higher (p=0.0059**) (Mann-Whitney test) in patients with the presence of atrophy than those with no atrophy.*

3.3.5 Investigation of an association of *DEFA5* expression with pathology

To explore the potential association between *DEFA5* and its correlation with the degree of inflammation, neutrophil infiltration, atrophy, and intestinal metaplasia, the levels of defensin expression were compared in 31 gastric biopsies from *H. pylori*-infected patients for whom histopathological data was also accessible. Similar to *DEFB4* gene expression, there were no statistical differences observed between *DEFA5* mRNA expression and the

severity of histopathological changes in inflammation of gastric biopsies in infected patients (for score 1: median=0.333, n=5, IQR=0.013-17.94; for score 2: median=0.0075, n=22, IQR=0.00044-0.1683; and for score 3: median=0.0335, n=4, IQR=0.00475-0.229). However, statistical analysis revealed that the median *DEFA5* expression level was significantly higher for the samples with a neutrophil activity score of 1 (median=12.97, n=4, IQR=2.571-303.8) than a score of 2 (median=0.0115, n=22, IQR=0.00088-0.1480). No samples were found to have a score of 3 for neutrophilic infiltration. For samples with a score of 0, the median was 0.006, n=5, IQR=0.0032-24.70.

In the same manner as *DEFB4* gene expression, it was found that the expression of the *DEFA5* gene in individuals with atrophy was more than forty times higher (median=0.1115, n=12, IQR= 0.015-19.06) (p=0.0062, Mann-Whitney test) than those without atrophy (median=0.0027, n=20, IQR=0.00048-0.1113) (see Figure 3.4). However, it was observed that the level of *DEFA5* gene expression was not significantly associated with the presence of intestinal metaplasia (presence: median=0.0085, n=6, IQR=0.00043-12.47; absence of IM: median=0.016, n=26, IQR=0.002-2.453) (Mann-Whitney test) (Figure 3.5).

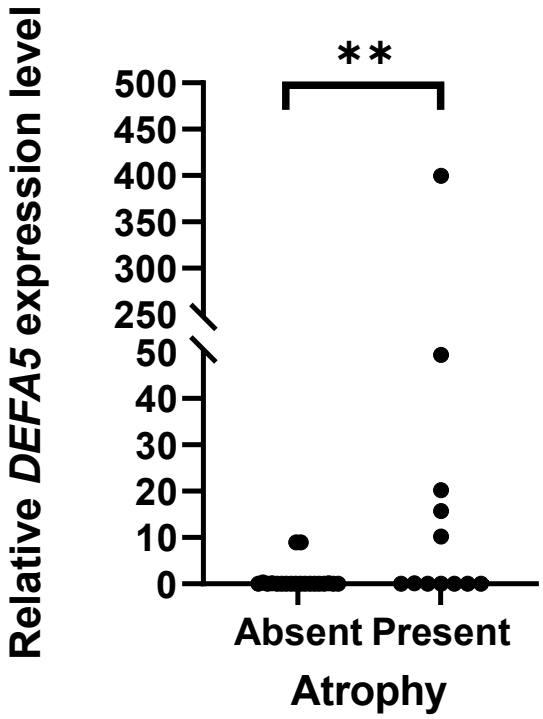


Figure 3.4: The association between DEFA5 expression and atrophy in gastric antral biopsies from 31 H. pylori-infected donors*. The expression of DEFA5 was significantly higher (p=0.0062**) (Mann-Whitney test) in patients with the presence of atrophy than those with no atrophy.*

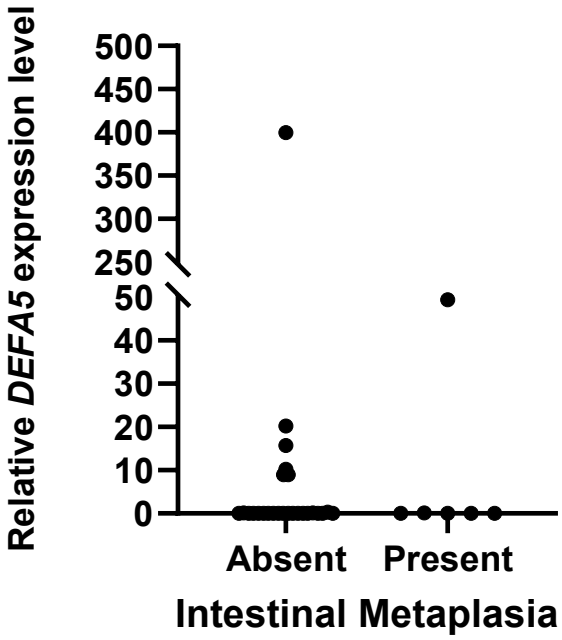


Figure 3.5: The association between DEFA5 expression and intestinal metaplasia in gastric antral biopsies from 31 H. pylori-infected donors*. No significant differences were found in the expression of DEFA5 between these two groups (Mann-Whitney test).*

3.3.6 Investigation of a relationship between *DEFB4* expression and virulence determinants of *H. pylori*

This study characterised the influence of *H. pylori* virulence factors on human defensin expression levels in *H. pylori*-infected patients. The *DEFB4* expression levels in 15 antral gastric tissue of infected patients were determined, for which strain genotype data was also available, and stratified according to the *cagA* status and *vacA* alleic type. It was found that *DEFB4* mRNA expression level was significantly higher in gastric antral biopsies from 11 *H. pylori*-infected patients carrying *cagA*$^{+}$ strains compared to 24 uninfected controls (p=0.0316) (Kruskal-Wallis test) (Dunn's multiple comparisons test) (Figure 3.6). However, there were no significant differences between *DEFB4* mRNA expression in gastric antral

biopsies from patients infected with *H. pylori* with the virulent *vacA i*1 genotype compared to uninfected patients (Figure 3.6).

Overall, *DEFB4* expression was significantly associated with *cagA* positive status in the colonising strain. Increased levels of *DEFB4* were also observed for samples from patients infected with *cagA*+ strains compared to *cagA*-, and the same was true for *vacA* strains, where *vacA i*1 had a higher *DEFB4* mRNA level than *vacA i*2 (Figure 3.6). However, these differences were not statistically significant.

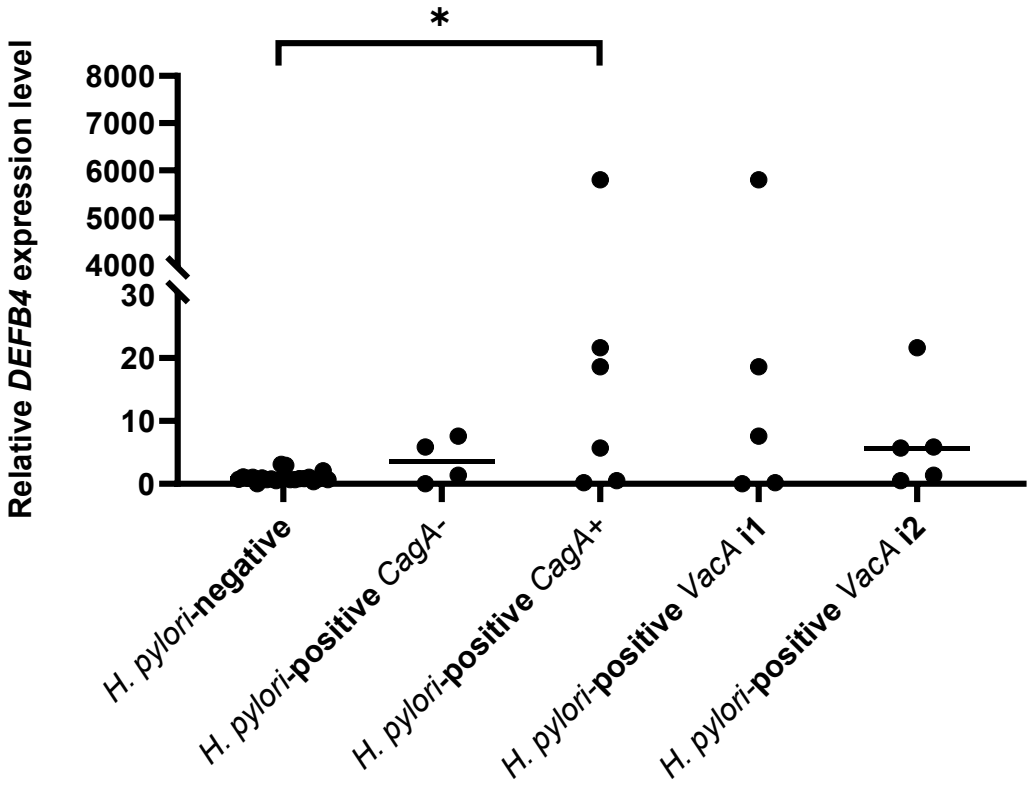


Figure 3.6: The association between bacterial virulence factors and DEFB4 expression ex vivo. *DEFB4 expression levels were measured in antral tissue samples of H. pylori-positive patients carrying CagA+ (n=11), CagA- (n=4), VacA i1 (n=10), VacA i2 (n=5) and H. pylori-negative patients (n=24). The expression of DEFB4 was significantly higher in infected CagA+ patients (p=0.0316*) (Kruskal-Wallis test) (Dunn's multiple comparisons test) than those with uninfected patients.*

3.3.7 Investigation of a relationship between *DEFA5* expression and virulence determinants of *H. pylori*

The study aimed to examine the correlation between *H. pylori* virulence factors and the expression levels of human α-defensin-5 in the gastric mucosa. Therefore, *DEFA5* expression was analysed in 22 antral gastric biopsies from patients infected with *H. pylori*. The patients were categorized based on their *cagA* status and *vacA* allelic type. In general, *DEFA5* expression did not show any significant differences with respect to the *cagA* and *vacA* status of the clinical strains in *H. pylori*-positive individuals.

However, the highest level of *DEFA5* was observed in *H. pylori*-negative samples compared to all other groups. Higher level of *DEFA5* were also noted in samples infected with *cagA*+ strains compared to *cagA*- strains as well as for *vacA* strains where *vacA i*1 exhibited a higher *DEFA5* mRNA level than *vacA i*2 (Figure 3.7).

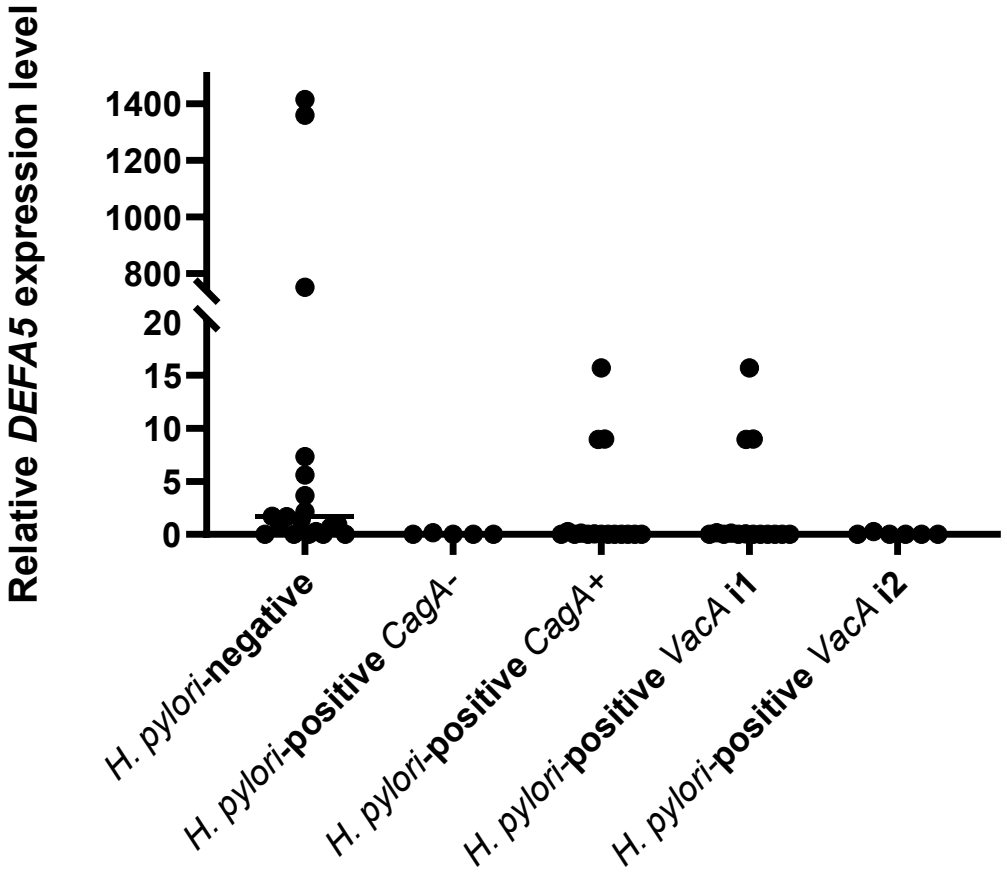


Figure 3.7: The association between bacterial virulence factors and DEFA5 expression ex vivo. *DEFA5 expression levels were measured in antral tissue samples of H. pylori-positive patients carrying CagA+ (n=17), CagA- (n=5), VacA i1 (n=16), VacA i2 (n=6) and H. pylori-negative patients (n=21). No significant differences were found in the expression of DEFA5 between these groups (Kruskal-Wallis test) (Dunn's multiple comparisons test).*

3.3.8 Investigation of an association between *DEFB4* expression and disease status

Samples were collected from the antrum of infected patients, and disease status was available for 32 patients, of which 15 presented with peptic ulcer disease (such as gastric and duodenal ulcers) and 17 with no disease status. In general, much higher *DEFB4* expression levels were measured in the antrum of patients with peptic ulcer disease, compared to those without any disease, although not statistically significant. Overall, no

statistical differences were observed for *DEFB4* expression with regards to patients with differing disease status (PUD: median= 47.27, IQR=8.27-2909; No disease: median=5.85, IQR=2.135-50.74) (Figure 3.8).

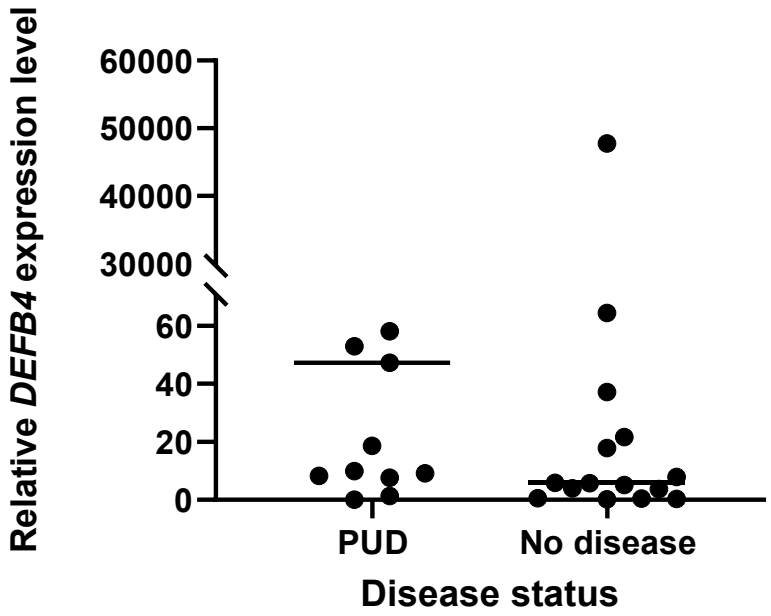


Figure 3.8: The association between disease status and DEFB4 expression ex vivo. *DEFB4 expression levels were measured in antral tissue samples of patients with peptic ulcer disease (PUD) (n=15) and patients with no disease status (n=17). No significant differences were found in the expression of DEFB4 between these two groups (Mann-Whitney test).*

3.3.9 Investigation of an association between *DEFA5* expression and disease status

As no patients in the current study were found to have gastric cancer, the relationship between *DEFA5* expression and *H. pylori*-associated gastroduodenal ulcer disease was investigated in 31 gastric antral biopsies of *H. pylori*-infected patients. Overall, higher *DEFA5* expression levels were detected in the antrum of patients with peptic ulcer disease compared to those without the disease. However, it was found that *DEFA5* expression was

not significantly related to the disease status (PUD: median= 0.0415, n=16, IQR=0.0052-6.8;

No disease: median=0.0061, n=15, IQR=0.002-0.1680) (Figure 3.9).

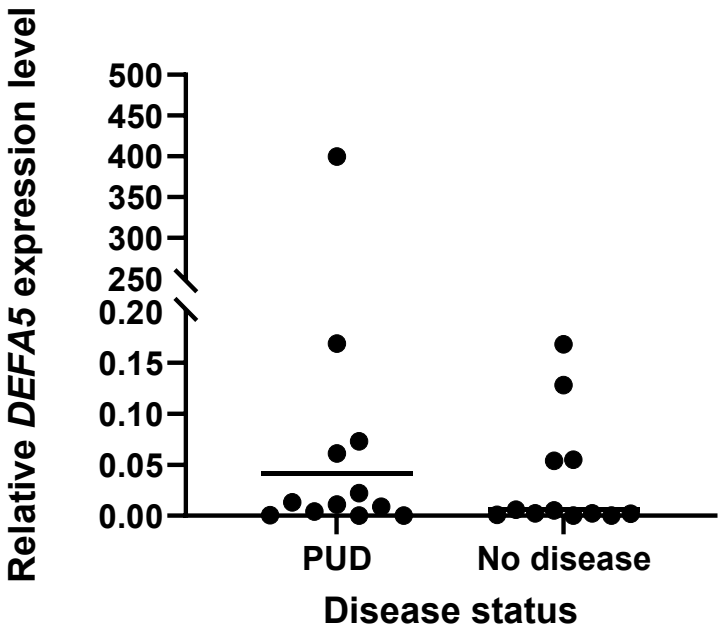


Figure 3.9: The association between disease status and DEFA5 expression ex vivo. *DEFA5 expression levels were measured in antral tissue samples of patients with peptic ulcer disease (PUD) (n=16) and patients with no disease status (n=15). No significant differences were found in the expression of DEFA5 between these two groups (Mann-Whitney test).*

3.4 Discussion

3.4.1 *DEFB4* expression was upregulated during *H. pylori* infection in *ex vivo* human gastric biopsies

As previously mentioned, the gastric epithelium can produce and release a diverse range of antimicrobial peptides, among which are defensins including human beta-defensin 2 (hβD2). hβD2 is encoded by the *DEFB4* gene, and this β-defensin group of AMPs have antimicrobial activity against *H. pylori* (Pero *et al.*, 2017). There is a consensus that the β-defensin hβD1 is expressed continuously in the upper gastrointestinal tract regardless of pathogen exposure (Kagnoff, 2014), while the expression of hβD2 is minimal or non-existent, unless they are induced (Kalus *et al.*, 2009; Kiehne *et al.*, 2005; Shelley *et al.*, 2020).

In this study, a significantly increased *DEFB4* mRNA expression level was observed in gastric biopsies from the antrum of *H. pylori*-infected patients compared to uninfected patients. This confirmed a previous report by Patel *et al.* (2013). Interestingly, another study by Nuding *et al.* (2013) reported an upregulation in *DEFB4* expression using different primer sequences for amplification of defensin gene than those used in the current study. In a study by Vordenbäumen *et al.* (2010a) involving paediatric individuals infected with *H. pylori*, it was also demonstrated that the levels of hβD2 expression were notably elevated compared to a control group of healthy individuals. Also, they discovered that biopsies indicating mucosal inflammation in the absence of *H. pylori* infection (non-*H. pylori*) exhibited significantly decreased levels of hβD2 in antral biopsies when compared to the *H. pylori*-infected group, which confirmed that *H. pylori* infection is known to significantly upregulate hβD2 production. Hence, the evidence from current and previous studies

supported the hypothesis that *DEFB4* expression is induced as a mucosal immune response to *H. pylori* infection.

Infection with *H. pylori* may weaken the immune system of the host, allowing it to colonize persistently in the stomach, hence triggering the epithelium of the stomach to secrete *DEFB4* for early control of the infection. O'Neil *et al.* (2000) showed that the activation of *DEFB4* was dependent on the direct interaction of live *H. pylori* with the epithelium. This study shows an involvement of the peptide in the innate host defence against infection and inflammation. The increased expression of hβD2 observed in current and previous studies may indicate a protective reaction by the gastric epithelium aimed at restricting *H. pylori* infection.

Despite the expression of *DEFB4* in gastric epithelial cells, which has antimicrobial properties against *H. pylori*, it does not seem to prevent or eliminate chronic *H. pylori* infection in humans (Hamanaka *et al.*, 2001). One theory suggests that *DEFB4* may exert greater activity against competing (commensal) bacteria in the gastric environment, potentially creating a niche that facilitates the colonization of *H. pylori* (Hornsby *et al.*, 2008). The decreased efficacy against *H. pylori* in comparison to other microorganisms, might result in changes to the composition of the gastric microbiota during host infection (Pero *et al.*, 2017). Moreover, the discovery in rhesus macaques which revealed that *DEFB4* expression is influenced by the *H. pylori* virulence factor, *cag*PAI, implies that this cunning pathogen might manipulate the host's defensin response to create a favourable environment in the gastric region for its survival (Hornsby *et al.*, 2008).

3.4.2 *DEFA5* was downregulated during *H. pylori* infection in *ex vivo* human gastric biopsies

In this study, human α-defensin 5 expression was significantly downregulated or was of low abundance (relative median=0.022) in infected patients, when compared with uninfected patients (relative median=1.68). This aligns with a prior study conducted by Wehkamp *et al*. (2003), which indicated that *H. pylori* did not induce the gastric expression of human α-defensin 5 but did affect the expression of human α-defensin 6. However, Isomoto *et al.* (2004) found that the levels of human α-defensins 1-3 in gastric juice were notably elevated in patients with *H. pylori* infection compared to those without the infection, and suggested that the elevation of α-defensin levels were secondary to *H. pylori* associated gastric inflammation.

The low levels of *DEFA5* expression observed in both current and previous studies may be attributed to the fact that human α-defensins are notably more abundant in duodenal biopsies compared to those obtained from the gastric mucosa (Frye *et al.*, 2000; Vordenbäumen *et al.*, 2009). In contrast, the expression of hβD2 was significantly expressed in the stomach, compared to duodenum (Vordenbäumen *et al.*, 2009), which explains the notable amount of hβD2 expression observed in the gastric mucosa in this study. Both Chung and Raffatellu (2019), and (Linn *et al.*, 2023) demonstrated that mRNA expression of *DEFA5* was predominantly present in the duodenum, jejunum and terminal ileum (reflecting the high abundance of Paneth cells in these particular regions), but it is not detected in the epithelial cells of the normal stomach and colon, as these tissues lack Paneth cells (Cunliffe, 2003).

3.4.3 *DEFB4* expression was associated with atrophy in infected patients

In this study, *DEFB4* expression was not associated with the intensity of inflammation in gastric antral biopsies, which was consistent with past study by Patel *et al.* (2013). Similarly, Vordenbäumen *et al.* (2010a) found no observed association between mRNA expression of defensins and the histological grading of polymorphonuclear or mononuclear cell infiltration at any of the biopsy sites in *H. pylori*-infected paediatric patients. Therefore, it could be speculated that the expression of hβD2 may not be solely attributed to inflammation alone (Vordenbäumen *et al.*, 2010a). The expression of hβD2 was said to be increased in response to infection (Duits *et al.*, 2003; Sørensen *et al.*, 2005), pro-inflammatory cytokines (including IL-1β, IL-17, TNFα, and IL-22) (Braff *et al.*, 2005; Mulcahy *et al.*, 2016), and injury (Shelley *et al.*, 2020).

Contrastingly, a study in the UK conducted by Taha *et al.* (2005b) demonstrated that the antral histological scores of neutrophilic infiltrations were correlated positively with the expression of *DEFB4* gene. Meanwhile, Isomoto *et al.* (2005) showed that hβD2 expression in gastric juice showed a positive correlation with the activity and chronic inflammation scores in the corpus region, but not in the antrum. Additionally, Wehkamp *et al*. (2003) demonstrated a strong correlation between hβD2 and the occurrence of neutrophilic inflammation in both the antrum and corpus. The inconsistency in current and previous findings might be due to major differences in study design and case definition, differences in the expression pattern of *DEFB4* in the sites from which specimens of healthy and diseased tissue were obtained, or differences in the methods used to quantify *DEFB4* expression (Jaradat *et al.*, 2013).

Another plausible reason for these conflicting results between studies may be due to significant copy number variations (CNVs) in the *DEFB4* gene, resulting in variations in the modulation of antimicrobial immunity, which might consequently influence the expression of defensin genes. Several single nucleotide polymorphisms (SNPs) and the copy number of polymorphism have been described for *DEFB4* gene (Abe *et al.*, 2013; Bentley *et al.*, 2010; James *et al.*, 2018), which showed that *DEFB4* mRNA expression is significantly correlated with *DEFB4* copy number. The variation in the number of repeat units among individuals within the population, along with probable sequence differences among copies, indicate that CNVs of defensins may influence the modulation of defensin expression and its functionality (Hardwick *et al.*, 2011; Jansen *et al.*, 2009). As a result, a genetic factor has been linked to the production of the hβD2 peptide, and these significant variations in the structural genome of humans are especially relevant to diseases where defensins may be implicated in their pathology.

Current findings showed that *DEFB4* gene expression was correlated positively with glandular atrophy, but not with IM. On the other hand, Isomoto *et al.* (2005) showed that there were no significant correlations between hβD2 and both scores of histological parameters. It would be intriguing to investigate the correlation between premalignant pathologies and *DEFB4* expression in gastric tissue. This investigation could reveal whether the overexpression of the gene observed in this study is a contributing factor to the advancement of the disease to a cancerous stage. However, little is known about *DEFB4* gene expression and its role in carcinogenesis, although there is evidence suggesting a potential role for defensins in gastric cancer.

As mentioned previously, it has been proposed that *H. pylori* infection initiates a series of events starting with chronic inflammation that, over years in susceptible individuals, leads to atrophic gastritis, intestinal metaplasia (IM), dysplasia, and eventually gastric adenocarcinoma (Uemura *et al.*, 2001). Chronic atrophic gastritis can lead to the loss of specialized gastric glands (Crafa *et al.*, 2018) and loss of parietal cells normally involved in the production of gastric acid and intrinsic factors (Castellana *et al.*, 2024). It is important to note that the antrum of the human stomach contains a combination of oxyntic and antral glands, where oxyntic glands include large numbers of acid-secreting parietal cells, and the antral glands consist of foveolar surface and mucin-expressing deep mucous cells (Engevik *et al.*, 2020). Parietal cells play a crucial role in secreting gastric acid, which helps in food digestion, mineral absorption, and protection against harmful bacteria (Engevik *et al.*, 2020). However, the parietal loss during gastric atrophy may reduce various secreted signals that regulate the growth and differentiation of gastric progenitor cells, which contribute to the progression to metaplasia (Fox & Wang, 2007).

H. pylori can modulate gastric acid secretion in multiple ways. One of them is reducing acid secretion directly by disrupting the expression of proton pumps (H^+, K^+-ATPase) in parietal cells and indirectly by triggering neural pathways that stimulate somatostatin while suppressing histamine, gastrin, and acid production (Smolka & Schubert, 2017). By influencing proton pump expression, *H. pylori* can control the quantity and variety of microbiota within the intestinal lumen (Yao & Smolka, 2019). Since the colonization of gut microbes must survive the hostile acidic environment of the stomach, alterations in gastric acid secretion caused by *H. pylori* could influence gut microbial homeostasis. The disruption of this microbial balance, known as dysbiosis, has been recognized as having significant effects on human health (Sommer & Bäckhed, 2013). A decrease in gastric acid secretion

raises the likelihood of bacterial overgrowth and affects the makeup of microorganisms in the intestines or oral cavity including those organisms' causing disease (Martinsen *et al.*, 2005). Studies indicate that *H. pylori* are the predominant species in the stomach, accounting for 72-99% of sequencing readouts, and significantly impacts the composition of the gastric microbiota, (Andersson *et al.*, 2008; Bik *et al.*, 2006), where an increase in the relative abundance of *Acidobacteria*, *Spirochetes*, and *Proteobacteria*, and a decrease in *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were observed in *H. pylori*-infected patients (Maldonado-Contreras *et al*., 2010). Similarly, an increase in *Streptococcus* and a reduction in *Prevotella* have been discovered in patients with atrophic gastritis compared to control subjects (Engstrand & Lindberg, 2013).

The stomach protects against microbes by maintaining a low gastric pH and secreting AMPs and mucins from epithelial cells. Normal gastric mucosa primarily generates neutral mucin, a glycoprotein of gastric mucus that forms an epithelial barrier expressing *MUC1*, *MUC5AC*, and *MUC6* (Battista *et al.*, 2021). A previous study has shown a significant absence of *MUC1* in *H. pylori* gastritis patients (Vinall *et al.*, 2002), and the under expression of *MUC1*, *MUC5AC*, and *MUC6* in patients with IM (Goldenring *et al.*, 2010; Hibdon & Samuelson, 2018; Radyk *et al.*, 2018). Therefore, the combined effect of physiological changes of gastric mucosa atrophy such as reduced acid and mucin secretion, and changes in gastric pH leads to dysbiosis which promotes the persistence of bacteria usually killed by the adverse environment of the stomach (Pero *et al.*, 2019b). It was postulated that the changes in the gut microbiota induced by *H. pylori* may be linked to the progression of the illness (Pero *et al.*, 2019b) and influence the production of defensins (Dolara *et al.*, 2005), which explains the observed association between defensins and atrophy in *H. pylori*-infected patients in this study. For example, previous studies have shown that the *E. coli*

Nissle 1917 strain induces the strongest expression of β-defensin *in vitro* (Steubesand *et al.*, 2009; Wada *et al.*, 1999), hence proving that other bacteria may also contribute to the production of defensins in gastric mucosal cells.

3.4.4 *DEFA5* expression was associated with atrophy in infected patients

In addition to the roles of *DEFA5* in innate immunity, studies show that *DEFA5* has been linked to various physiological and pathological processes, including tumorigenesis (Li *et al.*, 2020; Nomura *et al.*, 2013; Wiens & Smith, 2015). The expression of *DEFA5* in gastric cancer was first reported by Takenawa *et al.* (2004), and in their study, they found that *DEFA5* exhibited significant upregulation in high-grade adenomas (pre-cancerous lesions) and downregulation in adenocarcinomas. These results indicate that *DEFA5* gene expression reflects the molecular characteristics of gastric tumours with varying histological classifications, and this gene may have a particular function in adenoma formation. The upregulation of *DEFA5* gene in pre-cancerous cells may act as a tumour suppressor, and the downregulation of *DEFA5* gene expression which was observed in cancer cells, may promote cancer cell invasion and proliferation (Wu *et al.*, 2021). A previous study has shown a notable decrease in the expression of *DEFA5* in human gastric cancer when compared with the normal samples. This study also demonstrated that when *DEFA5* was synthetically overexpressed in gastric cancer cell lines SGC7901 and BGC823, it led to reduced cell proliferation and a decrease in colony-forming ability (Wu *et al.*, 2021).

Hence, the reported significant upregulation of *DEFA5* gene expression in gastric mucosal atrophy observed in this study suggests the tumour suppressive properties of the *DEFA5* gene in the preliminary stages of gastric carcinogenesis. Likewise, another study also showed that the overexpression of *DEFA5* was found to significantly inhibit the proliferation

and ability to form colonies in colon cancer cell lines, and the growth of tumours in nude mice was also reduced when *DEFA5* was overexpressed (Qiao *et al.*, 2021). It was proposed that *DEFA5* binds directly to the subunits of the PI3K complex, thereby reducing downstream signalling and resulting in slower cell growth and metastasis. Thus, *DEFA5* exhibits inhibitory effects on the growth of colon cancer cells and has the potential to function as a tumour suppressor in colon cancer (Qiao *et al.*, 2021).

Another previous study also showed a significant increase in the expression levels of *DEFA5* in colorectal cancer tissues, compared to normal tissues, which suggests that *DEFA5* might be used as a potential specific tumour marker, and help with better prognosis of colorectal cancer (Zhao *et al.*, 2023). Overall, a mechanism is activated that upregulates the expression of *DEFA5* gene and promotes cancer progression. Nevertheless, it is important to note that the relationship between increased defensin expression and carcinogenesis is complex and still not fully understood. Further investigation is necessary to fully elucidate the mechanisms by which *DEFA5* affects tumour cell invasion.

3.4.5 *DEFB4* expression was associated with *H. pylori* virulence determinants

In this study, it is evident that the expression of the *DEFB4* gene increased during *H. pylori* infection, and *ex vivo* assessment also indicated that higher levels of *DEFB4* were detected in samples infected with *cagA*+ strains compared to *H. pylori*-negative strains. These findings support the previous research by Hornsby *et al.* (2008) who revealed that strains that carry the *cagA* gene have been shown to stimulate higher production of β-defensin 2 compared to strains without *cagA*. Additionally, O'Neil *et al.* (2000) demonstrated the upregulation of *DEFB4* mRNA in AGS cells co-cultured with *cagA*+ *vacA*+ *H. pylori* strain. It was proposed that this enhanced defensin response may make *cagA*-positive strains more

susceptible to eradication from the host as it appears that antibiotics are more effective in eradicating *cagA*-positive strains than *cagA*-negative strains (Broutet *et al.*, 2001).

A previous study has also demonstrated that *DEFB4* expression increased in the presence of *H. pylori* infection, in a *cag*PAI-dependent and NF-κB- mediated manner (George *et al.*, 2003; Hornsby *et al.*, 2008). As mentioned, it is now established that hβD2 is not expressed constitutively, but can be triggered following exposure to bacteria, lipopolysaccharide, and other pro-inflammatory cytokines such as TNFα and IL-1β (King *et al.*, 2002). The *cag*PAI of *H. pylori* was demonstrated to transport peptidoglycan via the Type IV Secretion System, triggering recognition by the pathogen detection molecule NOD1 (Allison *et al.*, 2009). Consequently, the activated NOD1 signalling pathway results in activation of both transcription factors of NF-$_k$B and AP-1, which subsequently leads to the expression of defensin *DEFB4* mRNA (Allison *et al.*, 2009; Grubman *et al.*, 2010).

This discovery was corroborated by another study conducted by Wada *et al.* (2001) who demonstrated that deletion or mutation of the NF-$_k$B site at -208 prevented the activation of the hβD2 promoter. The activation of the NF-kB site in the hβD2 promoter gene was observed only in *H. pylori* strains that possessed a *cag*PAI gene. Hence, this confirmed the hypothesis that *cagA* gene is required in the modulation of hβD2 expression, and involved in the regulated host gastric epithelial cell, in response to *H. pylori* infection.

Nevertheless, the expression of hβD2 is not unique to *cag*PAI-positive *H. pylori*, as other bacteria like *Salmonella* can also stimulate and trigger the production of hβD2 in human intestinal epithelial cells (O'Neil *et al.*, 1999). Several other types of bacteria, such as *Vibrio cholerae*, *Bacteroides fragilis*, and *Pseudomonas aeruginosa* also may regulate the synthesis

of hβD2 (Huang, 2014; Shirin *et al.*, 2011; Yoon *et al.*, 2010). Therefore, it is challenging to ascertain if this upregulation is influenced by *H. pylori* or other gut-dwelling pathogens.

3.4.6 *DEFA5* expression was not associated with *H. pylori* virulence determinants

In this study, there was no significant correlation found between *DEFA5* expression and the *cagA* or *vacA* virulence factors. Currently, based on the latest information available, there have been no prior studies determining the link between *DEFA5* gene expression and the virulence factors of *H. pylori*. However, Hase *et al.* (2003a) study on human cathelicidin *LL-37* showed that *cagA*+ and *vacA*+ strains led to increased expression of *LL-37*, whilst an isogenic Δ*cag*E mutant of *H. pylori* did not stimulate *LL-37* production in human gastric epithelial cell line. This suggests that *cagA* gene plays a crucial role in regulating the production of *LL-37* in epithelial cells, and the components of the *cag*PAI are crucial for activating the transcription factor NF-κB by *H. pylori*, in which the promoter region of *LL-37* contains a potential NF-κB-binding site (Hase *et al.*, 2003a).

3.4.7 *DEFB4* expression was not associated with the disease status of patients

As discussed, defensins are induced by the host immune response as the initial barrier against *H. pylori* infection. However, to survive, most of pathogenic bacteria have developed counter measures to reduce the effectiveness of AMPs, and in many cases, resistance to AMPs indicates strong virulence factors (Peschel, 2002). General mechanisms include lack of recognition by the host (i.e. evasion), bacteria-mediated downregulation of AMP expression in host cells, and expression of factors that prevent the effectiveness of AMPs (Wehkamp *et*

al., 2005a). For that reason, it is of interest to determine the roles of defensin, particularly hβD2 in interacting with pathogenic bacteria, and the mechanism which contributes to the pathogenesis of gastroduodenal diseases. In this study, elevated levels of *DEFB4* expression were observed in the antrum of patients with PUD when compared to those without any disease, although the difference was not statistically significant likely due to low sample numbers.

Epidemiological studies showed a strong association between *H. pylori* infection and duodenal and gastric ulcers (Pero *et al.*, 2017). Individuals diagnosed with either gastric or duodenal ulcers exhibited higher levels of hβD2 expression compared to individuals with a completely healthy stomach (Nishi *et al.*, 2005; Taha *et al.*, 2005a). The same pattern was observed in patients with gastritis. This indicates that the higher level of hβD2 found in peptic ulcer disease patients signifies inflammatory changes in the gastric mucosa triggered by *H. pylori* infection, and a role of defensin for host protection towards systemic delivery of pathogen. This current discovery may be linked to the idea of natural antibiotics being expressed, as defensin can be induced to maximize their antimicrobial effectiveness in response to infection or inflammation (King *et al.*, 2002).

Undoubtedly, both α- and β-defensin gene families provide an antimicrobial barrier against microorganisms in the gastrointestinal tract. Research has firmly established that disruptions in barrier function play a significant role in the development of inflammatory bowel disease (IBD), a diverse range of inflammatory conditions affecting the digestive system (Bouma & Strober, 2003). Several studies have indicated elevated levels of hβD2 expression in individuals diagnosed with ulcerative colitis (UC) (O'Neil *et al.*, 1999; Wehkamp *et al.*, 2003), however, they were downregulated in patients with Crohn's disease

(CD) (Aldhous *et al.*, 2009; Tollin *et al.*, 2003). These differentially expressed defensins in UC and CD in response to colonic location and inflammation, may reflect the dysregulated innate immunity and disease pathogenesis in IBD (Aldhous *et al.*, 2009). It has also been proposed that higher levels of hβD2 in individuals with IBD may contribute to the secondary effect of barrier disruption.

Thus, it is increasingly evident that changes in the expression of β-defensin genes take place during gastrointestinal infections and inflammation. It is hypothesized that there is a dynamic interaction between the luminal contents (including both beneficial and harmful microbes), the gastrointestinal epithelium, and lamina propria cells, resulting in the adjustment of the host's innate immune response. This adjustment plays a crucial role in the initial colonization and invasion of microbes at the mucosal surface (Dommett *et al.*, 2005).

3.4.8 *DEFA5* expression was not associated with the disease status of patients

In this study, no significant association between *DEFA5* expression and patients with PUDs was observed, consistent with a prior study conducted in the UK by Taha *et al.* (2005b), in which both studies explored the *DEFA5* expression levels in the gastric epithelial cells of human stomach. However, as mentioned, Paneth cells produce alpha-defensins in the small intestine, hence changes in the release of enteric alpha-defensins have been observed in the intestinal epithelium of individuals affected by IBD (Lisitsyn *et al.*, 2012).

Wehkamp *et al.* (2005b) demonstrated that patients with Crohn's disease affecting the ileum have reduced levels of Paneth cell alpha-defensins HD-5, in comparison to the control groups. Similarly, another research conducted in the UK also found that the concentrations

of Paneth cell HD-5 in the effluent of ileostomy patients with Crohn's disease were lower than in the control group (Elphick *et al.*, 2008). Interestingly, a previous study of NOD2-deficient mice indicates a decrease in the expression of human α-defensin equivalents in Paneth cells, known as cryptdins in mice, and the decrease in expression is linked to more susceptibility to ingested *Listeria monocytogenes*, a Gram-positive bacterial pathogen (Kobayashi *et al.*, 2005). Another study has also demonstrated that the NOD2 genotype has an impact on the ileal microbiome in Crohn's disease (Li *et al.*, 2019). Hence, it can be conjectured that the NOD2 genotype could be responsible for Paneth cell defensin expression, and mutations in NOD2 may alter defensin expression (Wehkamp & Stange, 2020). It appears that the reduction in Paneth cell α-defensin in the ileum could potentially play a significant role in the pathogenesis of ileal Crohn's disease by disrupting the local commensal microbiome and compromised intestinal mucosal integrity (Wehkamp & Stange, 2020).

In general, reduced levels of antimicrobial peptides could be viewed as a potential underlying mechanism of intestinal barrier dysfunction in individuals with gastrointestinal diseases. Paneth cells are considered crucial for innate immunity in the intestinal tract (Selsted & Ouellette, 2005). As a result, mutations of Paneth cell function, resulting in reduced expression or effectiveness of antimicrobial peptides, could have adverse effects on the intestinal defence against microorganisms in the lumen. This consequently could exacerbate barrier dysfunction and advance disease progress in infectious and inflammatory disorders (Wehkamp *et al.*, 2004).

Interestingly, human alpha-defensin-5 was found to inhibit exotoxins produced by hypervirulent strains of *Clostridium difficile* in human cells, implying that defensins could

also serve as a protective mechanism against certain clostridial glycosylating cytotoxins (Giesemann *et al.*, 2008; Korbmacher *et al.*, 2020). *C. difficile* is a nosocomial pathogen that can lead to toxin-mediated *C. difficile* infections (CDIs), with clinical manifestations varying from asymptomatic cases to diarrhoea, pseudomembranous colitis, severe fulminant colitis, and potentially death (Lessa *et al.*, 2012). This human defensin shows promise as a pharmacological agent for treating or preventing *C. difficile* associated disease (CDAD) and may play a beneficial role as a first line of host defence against invading pathogens, alongside its newly identified capability to neutralize these protein toxins (Korbmacher *et al.*, 2020).

Nevertheless, little is currently known regarding the consistency of *DEFA5* expression along the human intestinal tract, aside from the well-known fact that they are expressed by Paneth cells which are specific to the small intestine (Sankaran-Walters *et al.*, 2017). It was hypothesized that the differences in *DEFA5* expression levels observed may be attributed to the anatomical site, as well as by conditions such as gastritis, peptic ulcers, and *H. pylori* infection (Taha *et al.*, 2005a). Thus, it is possible to speculate that the differences in the composition of human enteric defensins along the length of the small intestine might have an impact on the composition of local microbiota in gut niches (Nakamura *et al.*, 2016).

3.5 Limitations and Future work

Samples in this research were obtained from patients undergoing a routine upper gastrointestinal endoscopy, who were referred for examination primarily due to symptoms of dyspepsia. Therefore, the *H. pylori* negative control might not accurately reflect an uninfected "normal" control group. The reason for this is that endoscopy is an invasive procedure that poses risks of bleeding and infection for patients, making it unsuitable for

healthy controls. Also, the sample size was small, so decreases the statistical significance of the analysis. Despite these limitations, significant differences in both α and β defensin levels were observed between individuals positive and negative for *H. pylori*.

As mentioned, variations found in defensin levels between current and previous studies could be due to differences in the samples assessed. As the present study collected gastric biopsy samples, other studies gathered gastric juice and samples from various regions of the gastrointestinal tract, such as the small intestine. Also, *H. pylori*-infected patients were stratified based on the presence of disease (PUD or no disease) observed during the endoscopy procedure. Nevertheless, tissue specimens were not obtained from the duodenum, despite the stratification of patients according to the presence of duodenal ulcers. Hence, this leaves the possibility that the immune response in the duodenum may not reflect those in the stomach.

Moreover, a significant drawback of this study is its focus on *ex vivo* investigations without incorporating *in vitro* studies. Although this was not covered in the current chapter, however, it is crucial to validate the current results by expanding to include *in vitro* studies in future research, and previous studies have shown that defensins were expressed in human gastric carcinoma cell lines when co-cultured with *H. pylori* (Muhammad *et al.*, 2016a; Patel *et al.*, 2013). The *in vitro* validation of *ex vivo* results might eliminate any potential confounding variables present in human tissue samples.

As previously stated, host genetics, specifically polymorphisms in the defensin gene such as *DEFB*4 plays a significant role in determining the outcome of *H. pylori* infection (Hollox *et al.*, 2003). While not addressed in the present study, it would be intriguing to explore how these factors might influence defensin expression levels and their relation to the observed

association between pathology, bacterial virulence factors, and diseases in the gastric mucosa. Interestingly, one study showed that some genetic polymorphisms in *DEFB4* may affect defensin expression in epithelial tissues and can play a role in drug susceptibility, enabling the prediction of appropriate dosage and drug for the treatment of patients with specific genotype (Suleiman *et al.*, 2021). Therefore, future studies should investigate how *DEFB4* polymorphism influences defensin expression levels in the gastric mucosa and their interaction with *H. pylori* virulence factors. Furthermore, integrating host genotyping with microbial and histopathological data may reveal how defensin variants influence disease progression and drug response in *H. pylori* infection, thereby supporting personalised therapy.

Another previous study investigated the expression of the *DEFB4* gene and DNA methylation changes to the defensin promoter region in the gastric mucosa of *H. pylori*-infected patients and a control group. Results revealed that *DEFB4* gene methylation levels at the 4 CpG sites were overall higher in *H*. *pylori*-negative samples than in *H*. *pylori*-positive samples. This study also showed that the specific demethylation observed in two CpG sites (-825 and -786) on the *DEFB4* promoter region could be related to the active transcription pattern of the gene in *H*. *pylori*-positive gastritis (Pero *et al.*, 2019a). Hence, gene polymorphisms and the analysis of epigenetic regulation from defensin expression during *H. pylori*-associated gastric tumorigenesis should be included in a future study to further reveal the mechanisms regarding resistance and susceptibility of *H. pylori* against defensins which might favour the detection of targets for new eradication therapeutics.

3.6 Chapter summary

- *DEFB4* expression was upregulated, whilst *DEFA5* expression was downregulated during *H. pylori* infection in the human stomach *ex vivo*.
- *DEFB4* and *DEFA5* expressions were associated with atrophic gastritis in *H. pylori*-infected patients.
- *DEFB4* was linked to *H. pylori* virulence factors, whereas *DEFA5* showed no connection to *H. pylori* virulence factors.
- *DEFB4* and *DEFA5* expressions were not related to the gastroduodenal disease status of patients.

Chapter 4 : *In vitro* relationship between the host immune response (IL-8 and HβD2) and antibiotic eradication in *H. pylori* infection

4.1 Introduction

It is now understood that human defensins are modulated upon *H. pylori* infection, based on the findings of the *ex vivo* studies in the previous chapter and previously published data. Defensins are known to be crucial in linking the innate and adaptive immune responses and may act as signalling molecules within the immune system (Lehrer, 2004). As a proinflammatory mediator, defensins chemotactically recruit leukocytes and trigger the expression and release of pro-inflammatory cytokines such as tumour necrosis factor TNFα and IL-8 (Phan *et al.*, 2018). One key characteristic of *H. pylori* infection in the gastric mucosa is the release of various pro-inflammatory cytokines, many of which are produced by gastric epithelial cells. For example, a study showed that when gastric epithelial cells are infected with *H. pylori*, host cytokines such as TNFα and several interleukins are released to trigger inflammation and modulate the immune response (Morningstar-Wright *et al.*, 2022). These cytokines significantly contribute to developing gastroduodenal diseases linked to *H. pylori* infection (Alzahrani *et al.*, 2014). Therefore, factors that influence cytokine responses could contribute to either susceptibility to or protection against diseases associated with *H. pylori* (Tsai & Hsu, 2017). Hence, this chapter aimed to determine the interaction between pro-inflammatory cytokines such as TNFα with the IL-8 response, and defensin stimulation during *H. pylori* infection *in vitro*.

In innate immunity, invading microbes are detected by pattern recognition receptors that identify pathogen-associated molecular patterns (PAMPs) on microbial surfaces. Toll-like receptors (TLRs) are a type of these receptors. TLRs bind to PAMPs on extracellular bacteria, including lipopolysaccharides, flagellin, and lipoproteins. When these receptors engage with their ligands, they induce the production of inflammatory cytokines like interleukin IL-β1

and TNFα, leading to acute inflammation. This process activates pro-inflammatory NF-κB signalling, promotes the expression of antimicrobial agents such as defensins, and generates reactive oxygen and nitrogen species that help eliminate invading bacteria (Andrés *et al.*, 2022). Therefore, this study explored the impact of the pro-inflammatory cytokine TNFα on the bacterial density in a human gastric adenocarcinoma cell line (AGS) that is co-infected with *H. pylori*. The objective was to see whether TNFα aids in controlling bacterial infection by decreasing bacterial load or if it fosters a more favourable environment for *H. pylori* persistence.

A prior study examined cytokine production patterns in gastric mucosal biopsies from patients with dyspepsia who were either *H. pylori*-positive or *H. pylori*-negative (Holck *et al.*, 2003). The findings indicated an increased expression of IL-8, IL-10, and interferon-γ (IFN-γ) in those with *H. pylori* infection, and a significant positive correlation was observed between these cytokines and the bacterial load. Meanwhile, one study reported a significant positive correlation between serum levels of IL-9 and bacterial counts in *Mycobacterium bovis* infection in cattle (Khalid *et al.*, 2022). This study identified cytokines as potential biomarkers and distinguished between *M. bovis*-infected animals and those vaccinated with Bacillus Calmette-Guerin (BCG) for tuberculosis. Therefore, the present study also addressed whether pro-inflammatory cytokines such as TNFα may influence bacterial density and evaluate their potential as biomarkers for *H. pylori* diagnostics.

Studies on patients infected with *H. pylori* and in animal models have shown that *H. pylori* strains possessing a *cag*PAI and an operational type IV secretion system (T4SS) which triggers the expression and secretion of IL-8 in the gastric mucosa (Wiedemann *et al.*, 2016). Moreover, Cha *et al.* (2015) revealed that the upregulation of IL-8 was observed when

human gastric adenocarcinoma cell lines (AGS cells) were co-infected with *H. pylori*, and prolonged exposure to these cytokines can result in cell proliferation and an increased risk of DNA replication errors, consequently fostering tumour development by inhibiting cell autophagy and apoptosis (Chen *et al.*, 2020; Duan *et al.*, 2016). The overexpression of IL-8 significantly plays a crucial role in the advancement of gastric cancer, and high IL-8 levels might be a marker of poor prognosis in this disease, hence targeting IL-8 could be a promising approach for treating this disease (Lee *et al.*, 2013).

A previous study has also described that pro-inflammatory cytokine such as TNFα potently stimulated IL-8 mRNA expression in human gastric epithelial cells (O'Hara *et al.*, 2009). Both *H. pylori* infection and TNFα induced IL-8 expression have been shown to be mediated by the activation of transcription factor NF-κB in gastric epithelial cells (Bae *et al.*, 2014; Osawa *et al.*, 2002), and inhibition of this pathway led to a halt in IL-8 production and triggered apoptosis in cells (Osawa *et al.*, 2002). Therefore, this study aimed to explore the response of IL-8 in gastric epithelial cell lines during infection with *H. pylori* and to examine whether there are substantial differences between TNFα cytokine-stimulated treatment and unstimulated AGS cells in the ability to induce IL-8 gene expression. Thus, understanding these inflammatory mediators' roles in response to *H. pylori* infection could improve our knowledge of gastric carcinogenesis mechanisms. Additionally, this insight may reveal new treatment possibilities for gastric cancer, particularly with the rise of novel biological therapies that target a wide range of cytokines.

The previous chapter has shown that *ex vivo*, human defensin gene *DEFB4* was found to be expressed in gastric biopsies from *H. pylori*-infected patients. In the past, further analysis using human gastric cancer cell lines revealed that defensins were either constitutively

expressed in gastric epithelial cells or induced when infected with *H. pylori* (Hase *et al.*, 2003b; Ohara *et al.*, 2004). Therefore, conducting an *in vitro* cell culture with *H. pylori* strains to measure defensin secretion levels and comparing the results with previous *ex vivo* findings would be intriguing. It was also hypothesized that certain inflammatory cytokines, such as TNFα and IL-1β, might induce the secretion of defensins (Johansen *et al.*, 2016). However, most research on the stimulation of TNFα in defensin production has been performed in cell lines other than gastric epithelial cells, such as those from the human keratinocyte, airway, gingival, and corneal epithelial cells (Albanesi *et al.*, 2007; Harder *et al.*, 2000; Mahanonda *et al.*, 2009; Narayanan *et al.*, 2003). Currently, a limited number of studies have investigated hβD2 protein secretion in gastric epithelial cells following TNFα stimulation. Therefore, one of the goals of this chapter was also to determine if hβD2 exhibits differential expression in the gastric epithelial cell lines with and without H. *pylori* infection and to explore whether proinflammatory cytokine TNFα can influence the expression of hβD2 in the cell lines.

Clarithromycin is a commonly used macrolide antibiotic for the eradication of *H. pylori* infection and resistance to this antibiotic is a major cause of treatment failure (Kocsmár *et al.*, 2021). The increased resistance rates of clarithromycin against *H. pylori* clinical isolates have been noted in the second chapter of the thesis. Despite the high resistance rates of *H. pylori* to clarithromycin, doctors continue to prescribe this antibiotic because it has been shown to alleviate infection symptoms in patients. This is likely due to their immunomodulatory and anti-inflammatory effects, in addition to their antibacterial activity (Pollock & Chalmers, 2021). Among the commonly used macrolides, azithromycin,

clarithromycin, and erythromycin, are considered to have the most potent immunomodulatory effects (Rubin & Henke, 2004).

Studies have shown that macrolides inhibit inflammatory signalling and suppress the production of several pro-inflammatory cytokines and chemokines in the lung tissue (Bosnar *et al.*, 2009). It was also shown that azithromycin decreases IL-6 and IL-8 in human corneal epithelial cells (Li *et al.*, 2010), possibly by suppressing the transcription factor NF- κB or AP-1 (Alzolibani & Zedan, 2012), and reducing neutrophil activity (Tamaoki *et al.*, 2004). However, the anti-inflammatory properties of these substances have been extensively researched in chronic inflammatory airway diseases *in vitro* and also in bronchoalveolar lavage fluid (Tamaoki *et al.*, 2004) since macrolides have been well-established as an adjunctive treatment to β-lactam antibiotics in pulmonary diseases (Nau & Tauber, 2008). Furthermore, the effect of macrolides on defensin protein expression in gastric epithelial cells infected with *H. pylori* infection was unknown.

Hence this chapter aimed to investigate the inhibitory effects of macrolides, particularly on IL-8 and hβD2 protein expression, in gastric epithelial cells infected with *H. pylori* isolates. The Maastricht V/Florence Consensus report advised that clarithromycin-based triple therapy should not be empirically used as the first-line treatment if the local resistance rate exceeds 15% (Malfertheiner *et al.*, 2017). Therefore, understanding how macrolides affect IL-8 and defensin production in response to *H. pylori* infection is essential as this knowledge could determine whether the inclusion of macrolides in the standard triple therapy remains necessary in an area with high rates of clarithromycin which may help to improve patient outcomes and reduce the symptoms.

4.1.1 Objectives

The objectives of this chapter were to:

1. Determine the effects of the pro-inflammatory cytokine TNFα on bacterial load in AGS cells co-infected with *H. pylori* strains 60190 (*cag*PAI positive), and Tx30a (*cag*PAI negative) *in vitro.*
2. Measure the IL-8 response to *H. pylori* infection, with and without cytokine TNFα stimulation, in AGS cells infected with *H. pylori* strains of different virulence properties *in vitro*.
3. Determine the defensin hβD2 secretion in response to *H. pylori* infection, with and without TNFα stimulation, in AGS cells infected with *H. pylori* strains of different virulence properties *in vitro*.
4. Examine the inhibitory effects of macrolides such as clarithromycin and azithromycin on IL-8 production in AGS cells co-infected with *cagA*+ and *cagA*- *H. pylori* strains *in vitro*.
5. Determine the inhibitory effects of macrolides such as clarithromycin and azithromycin on hβD2 concentrations in AGS cell lines co-infected with *cagA*+ and *cagA*- *H. pylori* strains *in vitro*.

4.2 Materials and Methods

4.2.1 *H. pylori* strains and culture

Clarithromycin-resistant *H. pylori* clinical isolates of *cagA*$^{+}$ and *cagA*$^{-}$ status were isolated from human gastric biopsies. Both clinical isolates and laboratory strains were cultured on blood agar plates as described in section 2.2.2. Laboratory and clinical strains used are listed in Table 4.1. and Table 4.2.

Table 4.1 H. pylori laboratory strains used.

***H. pylori* strain**	**Relevant strain characteristics**	**Reference**
Tx30a	Wild-type (ATCC 51932) *cag*PAI^{-}	Leunk *et al.* (1988)
60190	Wild-type (ATCC 49503) *cag*PAI^{+}	Leunk *et al.* (1988)

Table 4.2 Clarithromycin-resistant H. pylori clinical strains used and the minimum inhibitory concentrations of clarithromycin (MIC).

***H. pylori* strain**	**Relevant strain characteristics**	**MIC values (mg/L)**	**Reference**
779a	Nottingham collection strain *cagA*$^{-}$	12	(Garvey *et al.*, 2023)
802a	Nottingham collection strain *cagA*$^{-}$	128-256	(Garvey *et al.*, 2023)
863a	Nottingham collection strain *cagA*$^{+}$	>256	(Garvey *et al.*, 2023)
873a	Nottingham collection strain *cagA*$^{+}$	64-128	(Garvey *et al.*, 2023)

*MICs denoting resistance are >0.25 mg/L (EUCAST, 2024).

4.2.2 Culture of the AGS human gastric adenocarcinoma cell line

4.2.2.1 Recovering frozen cell lines stocks

A frozen vial of AGS gastric epithelial cells, originally derived from a gastric adenocarcinoma (European Collection of Authenticated Cell Cultures 89090402), was briefly placed into warm water at about 30-35°C until just thawed. The thawed cells were carefully pipetted into the sterile universal tube containing pre-warmed F-12 Ham's nutrient mixture medium (Sigma-Aldrich, UK), supplemented with 1% L-glutamine (Sigma-Aldrich, UK) and 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich, UK). The universal tube was topped up to 25 ml with the medium and centrifuged at 200 x *g* for 5 minutes. The supernatant was poured into the

waste bottle, the universal tube was flicked to resuspend the cell pellet, and 25 ml of medium was refilled into the tube. The universal tube was centrifuged again at 200 x *g* for 5 minutes, so the cells had two washes. The supernatant was poured off into a waste bottle and the cell pellet was resuspended in 10 ml medium and two 5 ml aliquots were transferred into T75 flasks. The flasks were incubated horizontally at 37°C in a 5% CO_2 humidified incubator. The growth of cells was observed daily until the cells were confluent.

4.2.2.2 Passage of growing cells

Once the cells were fully confluent, the flasks were seeded to maintain the culture. The medium was poured into a waste bottle, and 10-20 ml of sterile phosphate-buffered saline (PBS) was added to each confluent flask. The flask was gently rocked to wash the adhered cells, and the PBS was discarded into the waste bottle. The PBS wash was repeated to remove traces of serum that may inhibit the trypsin in the next step and then treated with 2 ml of pre-warmed Trypsin-EDTA (Invitrogen) to release cells from the flask. 10 ml of F-12 medium was added to the cells to wash away any remaining Trypsin-EDTA once the cells had detached under the microscope and transferred to a universal tube. The flask was rinsed with a 10 ml wash medium and added to the same universal. Cells were collected by centrifugation at 200 x *g* for 5 minutes, and the supernatant was poured into a waste bottle. The tube was flicked to resuspend the pellet and 25 ml of medium was added. The centrifugation was repeated so the cells had two washes in total. The supernatant was discarded, and the cell pellet was resuspended in 10 ml of medium and added to fresh T75 flasks. The flasks were incubated horizontally at 37°C in a 5% CO_2 humidified incubator. The growth of cells was monitored, and the medium was replaced every 2-3 days as required until the cells reached confluency and required passage again.

4.2.3 Co-culture assays

4.2.3.1 AGS cell quantification

The day before a co-culture assay, all cells were cultured as described in section 4.2.2.2, trypsinized, and seeded into flat-bottomed 24-well tissue culture plates at 1 x 10^5 cells/well. 1 volume of AGS cells was stained with 3 volumes of Trypan blue solution (0.4 % w/v; Sigma) and quantified using a haemocytometer. The culture plates were then incubated at 37°C in a 5% CO_2 humidified incubator for 24 hours.

4.2.3.2 H. pylori cell quantification using spectrophotometer

H. pylori isolates were cultured on blood agar plates as described in section 2.2.2. Twenty-four hours after passaging the *H. pylori* isolates, the bacteria were suspended in 6 ml of sterile F-12 medium. 200 µl of bacterial suspension was placed in a disposable cuvette, and 800 µl of the medium was added to make a 1/5 dilution. The concentration of *H. pylori* cells in a suspension was estimated spectrophotometrically using Pharmacia Novaspec II, where an OD_{600} of 1.0 contains 1 x 10^9 *H. pylori* colony forming units (CFUs/ml) (determined previously in the group). For a multiplicity of infection (MOI) of 100 bacteria per cell, each well should contain 1 x 10^5 AGS cells and 1 x 10^7 bacteria.

4.2.3.3 AGS cell co-culture with H. pylori isolates

AGS cells at 80% confluence had the medium replaced with a bacterial suspension at an MOI 100, MOI 20, or F-12 Ham's nutrient mixture medium alone (as a control). AGS cells were infected with *cagA*$^+$ and *cagA*$^-$ strains and incubated for 24 hours in a 5% CO_2 humidified

incubator. The cell-free supernatants were collected after 24 hours for further analysis, or stored at -80°C.

4.2.3.4 H. pylori cell quantification using viable cell count assay

A viable cell count assay was used to calculate the bacterial colony forming units (CFUs)/ml in the inoculum which was added to the AGS cells. Therefore, a 10-fold dilution series of bacterial suspension was created in sterile F-12 media, and the CFUs were determined by spotting 10 µl volumes of the diluted suspensions 10^{-1} to 10^{-8} onto blood agar plates. Four 10 µl spots were plated for each dilution, and CFUs were enumerated once colonies were visible, usually after 4 days of incubation (which were incubated as described in section 2.2.2). The CFUs per ml in the inoculum that was added to the cells were counted.

CFU/ml= mean count from replicate 10 µl spots X 100 X the dilution factor

4.2.4 Determination of the inhibitory effect of macrolides on IL-8 and hβD2 production *in vitro*

4.2.4.1 Antibiotic and cytokine assays

AGS cells were cultured and infected with *H. pylori* as described in section 4.2.3. For antibiotic treatment, 10 µg/ml of clarithromycin and azithromycin were added at the time of infection, respectively to clinical isolates of clarithromycin-resistant *H. pylori cagA*$^{+}$ and *cagA*$^{-}$ strains. As for cytokine treatment, commercial recombinant protein TNFα (Peprotech) was added at 50 ng/ml concentration at the time of infection. Untreated cultures and uninfected AGS cytokine-stimulated controls were included as controls. After 24 hours of culture, supernatants were harvested by pipetting and used to assess IL-8 and HBD-2

secretion levels. All co-culture assays were performed in duplicate and three independent experiment trials were conducted using identical conditions.

4.2.4.2 ELISA assays

IL-8 levels were assayed using a human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen), and HBD-2 levels were assayed using a human BD-2 Standard TMB ELISA development kit (Peprotech), according to the manufacturer's instructions. A standard curve was included on each plate and samples were measured in duplicate. Optical densities were measured using a microplate reader (LabSystems iEMS reader MF) at 450nm and 620nm. The cytokine and defensin concentration in each sample was calculated using the standard curves. All ELISA assays were performed in duplicate. The range of detection was 2 – 250 pg/ml for IL-8 and 12 - 1500 pg/ml for hβD2.

4.2.5 Data analysis and statistical tests

Data were collected and recorded in Microsoft Excel and statistical analysis was performed using GraphPad Prism Version 10.3.0 (507). All analyses of *in vitro* data were tested using the Mann-Whitney test to compare two independent groups. Meanwhile, the Kruskal-Wallis test with Dunn's post hoc test was applied to the pairwise comparisons with multiple variables in the experiment of the inhibitory effect of macrolides on IL-8 and defensin production *in vitro*. A P value of < 0.05 was considered statistically significant.

4.3 Results

4.3.1 The effect of pro-inflammatory cytokine stimulation on bacterial load *in vitro*

AGS gastric epithelial cells were infected with *H. pylori* strains 60190 (*cag*PAI positive) or Tx30a *(cag*PAI negative) (MOI= 100 and 20), in the presence or absence of TNFα stimulation. Each experimental condition was performed with two replicates, on three separate occasions to minimize error. Cells were incubated for 24 hours, after which the number of bacterial CFUs was determined. Stimulation with TNFα did not change bacterial CFU counts in comparison to the unstimulated control (Table 4.3).

Table 4.3 The effect of cytokine stimulation (TNFα) on bacterial load when cultured with human gastric epithelial cells.

	Average CFU count/ml after 24 hours	
AGS cells cultured with	***H. pylori* 60190 MOI 100**	***H. pylori* 60190 MOI 20**
H. pylori	$2.21 \times 10^7 \pm 0.432$	$2.14 \times 10^7 \pm 0.472$
H. pylori +TNFα	$4.16 \times 10^7 \pm 1.096$	$2.643 \times 10^7 \pm 1.093$
P value	0.2619	0.3290
AGS cells cultured with	***H. pylori* Tx30a MOI 100**	***H. pylori* Tx30a MOI 20**
H. pylori	$1.83 \times 10^7 \pm 0.356$	$1.58 \times 10^7 \pm 0.741$
H. pylori +TNFα	$2.30 \times 10^7 \pm 0.729$	$2.23 \times 10^7 \pm 0.446$
P value	0.5528	0.1645

*AGS cells were stimulated with 50 ng/ml of TNFα and infected with either *H. pylori* strain 60190 or Tx30a (MOI=100 and 20). After 24 hours, the average number of colony-forming units (CFU/ml) in six replicates was determined. Differences in CFU counts between cells cultured in the absence or presence of cytokine were not statistically significant (Mann-Whitney test). No bacterial growth was observed in uninfected AGS controls.

4.3.2 The response of IL-8 secretion in *H. pylori* infection with cytokine stimulation *in vitro*

AGS cells were cultured with *H. pylori* strain 60190 at MOIs of 100 and 20 in the presence or absence of TNFα stimulation, and the IL-8 response was measured by ELISA. Uninfected and unstimulated AGS cells were used as negative controls. In this study, an increase in IL-8 secretion was observed in AGS cells in the presence of TNFα (an 82-fold increase compared to the negative control) ($p<0.0001$) (Mann-Whitney test) (Figure 4.1). IL-8 responses were also induced in *H. pylori*-infected cells without TNFα. However, the greatest responses were observed in the infected cells (MOI 100) also stimulated by TNFα. Two-fold increased IL-8 responses were induced with TNFα-stimulated infected AGS cells (*H. pylori* MOI 100 and MOI 20) compared to *H. pylori*-infected cells without TNFα treatment ($p=0.0001$ and $p=0.033$ respectively) (Figure 4.1). In general, *H. pylori*-infected AGS cells responded to TNFα treatment by secreting higher concentrations of IL-8.

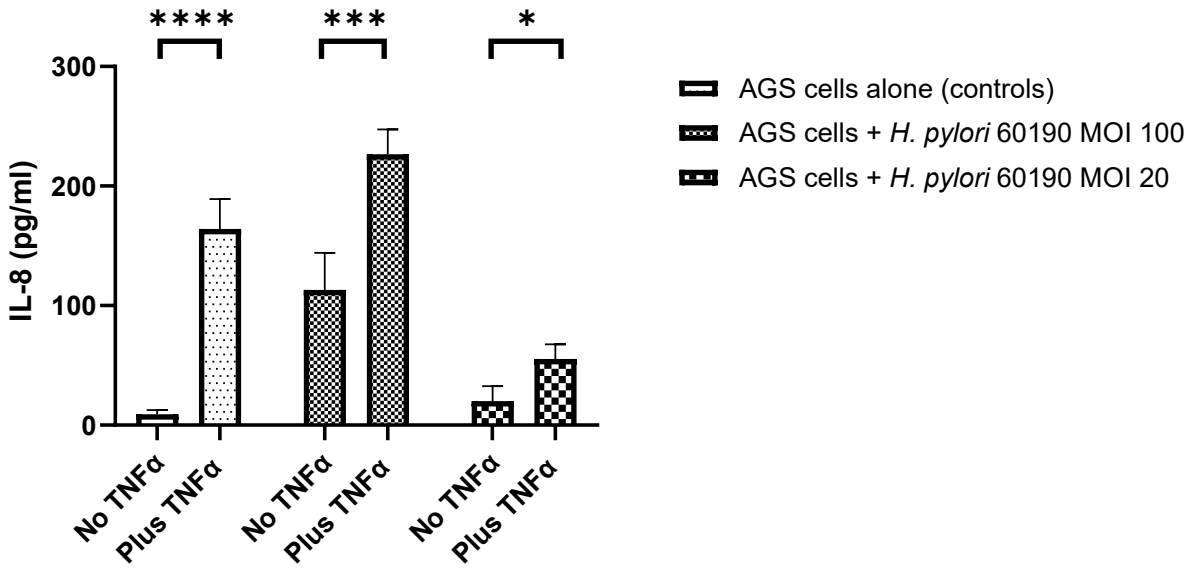


Figure 4.1: IL-8 secretion levels in response to cytokine stimulation of human gastric epithelial cells infected with H. pylori. *AGS cells were cultured with H. pylori strain 60190 (MOI 100 and MOI 20) in the presence or absence of cytokine TNFα (50 ng/ml). ELISA measured IL-8 response to TNFα after 24 hours in two replicate wells per condition. The data shown are from three independent experiments. Uninfected AGS cells with and without the TNFα stimulation were included for each experiment. The concentrations of IL-8 were significantly higher after TNFα stimulation compared to unstimulated controls (p=0.033*) (p=0.0001***) (p<0.0001****) (Mann-Whitney test).*

4.3.3 The hβD2 response to *H. pylori* infection and cytokine stimulation *in vitro*

This study also explored the hβD2 response to *H. pylori* infection and cytokine stimulation. Therefore, AGS cells were cultured with *H. pylori* strain 60190 (wild-type, *cag*PAI^{+}) at MOIs of 100 and 20, both with and without TNFα stimulation. Uninfected AGS cells served as negative controls. It was shown that hβD2 expression was minimally induced in unstimulated, uninfected AGS cells (Figure 4.2). However, it was significantly upregulated in the presence of *H. pylori* and TNFα, corroborating the previous chapter's finding that hβD2 expression levels increase upon *H. pylori* infection, and hβD2 expression is inducible in these cells, rather than constitutive.

As with IL-8, the most pronounced responses were seen in infected cells (MOI 100) stimulated with TNFα. HβD2 expression was induced in TNFα-stimulated infected AGS cells (*H. pylori* at MOI 100 and MOI 20) by a 1-fold and 2-fold increase, respectively, compared to the unstimulated *H. pylori*-infected cells (p=0.0079) (p=0.0018) (Mann-Whitney test) (Figure 4.2). An elevation in hβD2 secretion was also noted in uninfected AGS cells when exposed to TNFα, showing a 2-fold increase compared to the unstimulated control (p=0.0001) (Mann-Whitney test) (Figure 4.2).

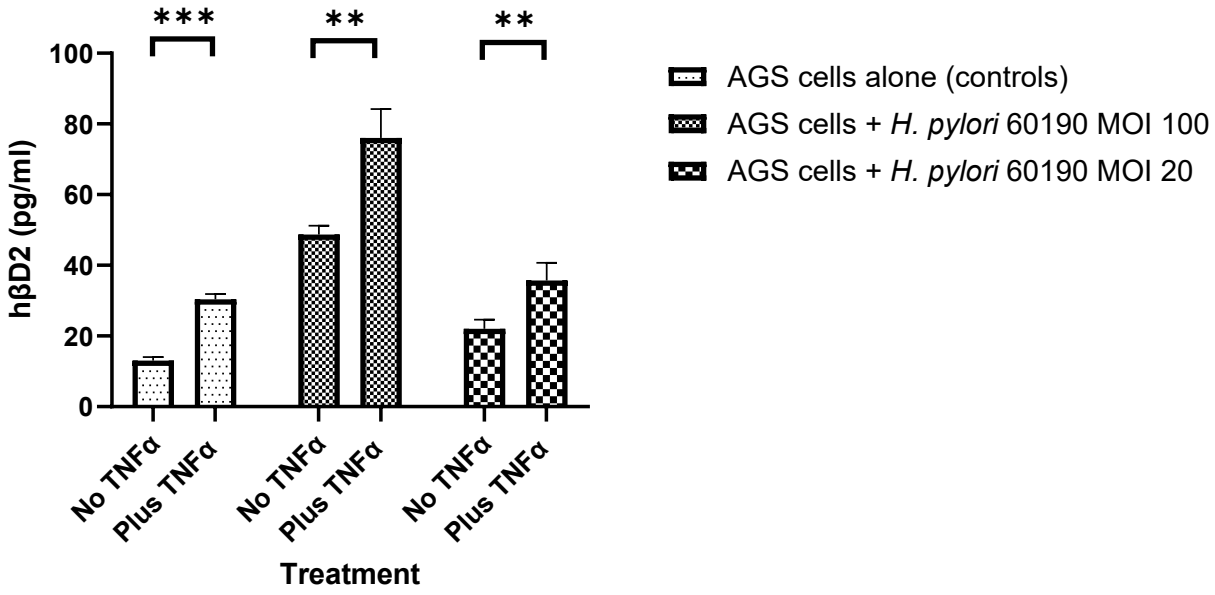


Figure 4.2: hβD2 secretion levels in response to cytokine stimulation of human gastric epithelial cells infected with H. pylori. *AGS cells were cultured with H. pylori strain 60190 (at MOI 100 and MOI 20) in the presence or absence of TNFα (50 ng/ml). ELISA was used to measure the HBD-2 response to TNFα after 24 hours, with all conditions tested in two replicate wells. The data shown are from three independent experiments. Uninfected AGS cells with and without TNFα stimulation were included as controls for each experiment. The secretion of hβD2 is significantly higher after cytokine stimulation compared to unstimulated controls (p=0.0079**) (p=0.0018**) (p=0.0001***) (Mann-Whitney test).*

4.3.4 Determination of the inhibitory effect of macrolides on IL-8 production *in vitro*

To investigate whether macrolide antibiotics such as clarithromycin and azithromycin can inhibit the epithelial cell inflammatory response to *H. pylori*, AGS cells were co-cultured with *cagA*$^{+}$ and *cagA*$^{-}$ clinical isolates of clarithromycin-resistant *H. pylori*. Two clinical isolates of each *cagA* genotype were incubated with AGS cells, in the presence and absence of 10 µg/ml clarithromycin or azithromycin. The control groups consisted of uninfected AGS cells with and without antibiotic treatments, TNFα-stimulated AGS cells without *H. pylori* infection (with and without antibiotic treatments), and uninfected AGS cells with the solvents used to dilute the antibiotics (ethanol for azithromycin and dimethyl sulfoxide, DMSO for clarithromycin) (Figure 4.3). The levels of IL-8 were significantly elevated in the treatment with TNFα alone compared to the untreated group, the uninfected group treated with 10 µg/ml of clarithromycin only, the uninfected group treated with 10 µg/ml of azithromycin, the TNFα treatment with clarithromycin only, the TNFα treatment with azithromycin, the DMSO treatment, and the ethanol treatment ($p=0.0022$) (Mann-Whitney test), respectively.

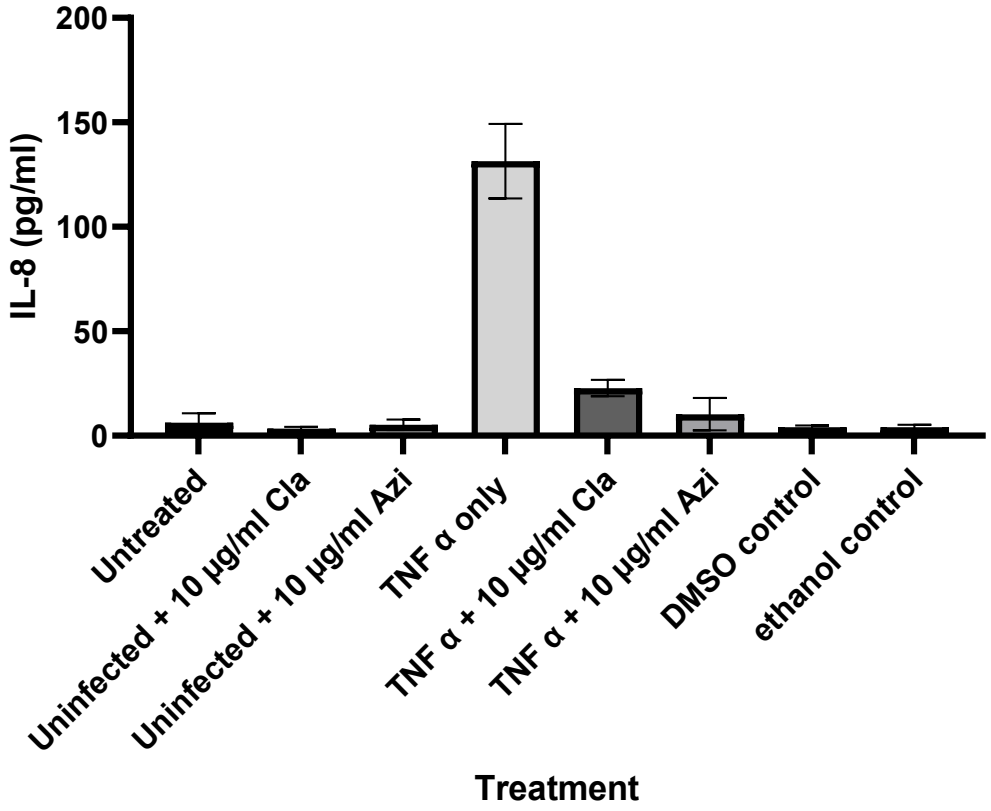


Figure 4.3: IL-8 secretion levels in response to controls. *AGS cells were cultured under these conditions and incorporated into each experiment. ELISA was used to measure the IL-8 response to TNFα after 24 hours, with all conditions tested in two replicate wells. The data shown are from three independent experiments. The concentrations of IL-8 were significantly higher in TNFα only treatment compared to all the treatments (p=0.0022) (Mann-Whitney test). Cla=clarithromycin, Azi=azithromycin, and DMSO=dimethyl sulfoxide.*

The AGS cells were then co-cultured with all *H. pylori* clinical isolates and laboratory strains using MOIs of 100 and 20. It was observed that AGS cells infected with the control strain 60190 at an MOI of 100 produced IL-8 at levels 4.4 times higher than those infected with the Tx30a strain at the same MOI, with a p-value of 0.0022 (Mann-Whitney test), indicating statistical significance. As expected, the IL-8 production by AGS cells infected with *cagA*-positive clinical isolates 863A and 873A at MOIs of 100 and treated with 10 µg/ml of clarithromycin was reduced by 1.89-fold and 2.7-fold (p=0.0022) (Kruskal-Wallis test)

(Dunn's multiple comparisons test), in comparison to infected cells in the absence of clarithromycin (Figure 4.4). It was further confirmed that IL-8 production by AGS cells infected with *cagA*-positive clinical isolates 863A and 873A at MOIs of 20, in the presence of 10 μg/ml of clarithromycin, was reduced by 2-fold, and 2.7-fold (p=0.0051) (Kruskal-Wallis test) (Dunn's multiple comparisons test) respectively, compared to AGS cells co-cultured in the absence of clarithromycin (Figure 4.5).

When treated with 10 μg/ml of azithromycin, IL-8 production by AGS cells infected with *cagA*-positive clinical isolates 863A at MOIs of 100 showed no change, whilst a significant reduction by 2.25 (p=0.015) (Kruskal-Wallis test) (Dunn's multiple comparisons test) was observed when infected with clinical isolate 873A at MOI 100, compared to the untreated counterparts (Figure 4.4). When these isolates were tested at an MOI of 20, there were no significant differences when azithromycin was present in the 863A cultures, but a significant reduction in IL-8 over 2.7-fold (p=0.0204) (Kruskal-Wallis test) (Dunn's multiple comparisons test) was demonstrated for the 873A isolate. (Figure 4.5).

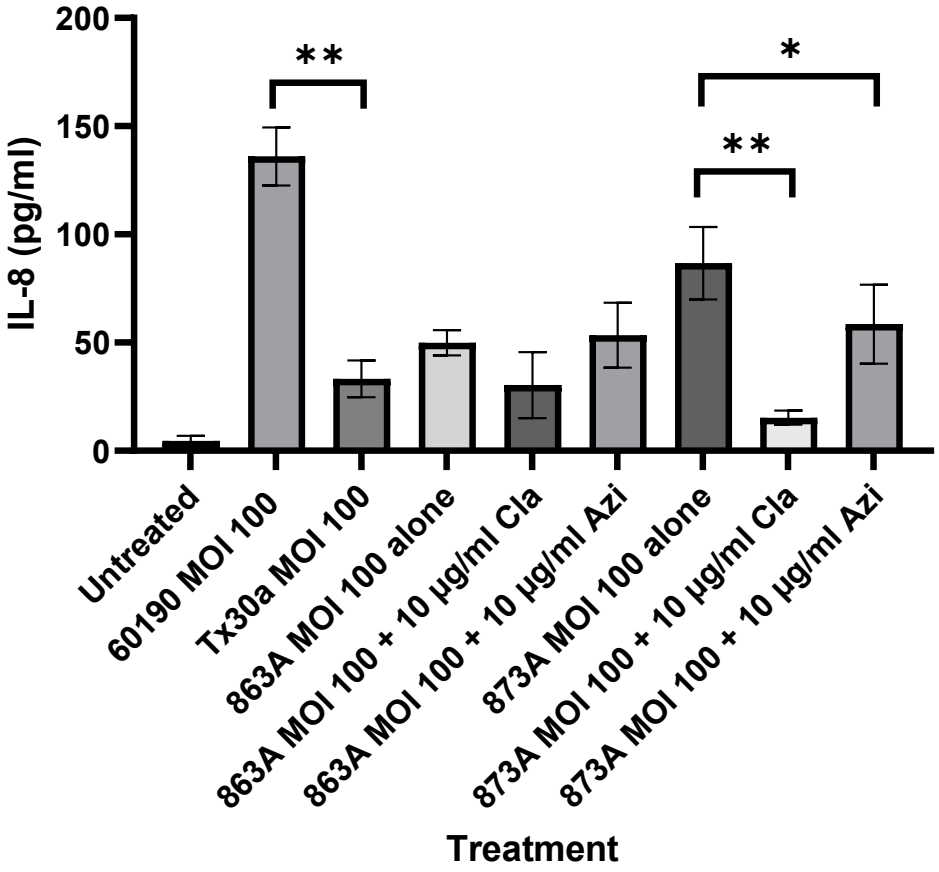


Figure 4.4: IL-8 secretion levels in response to antibiotics treatment of human gastric epithelial cells infected with H. pylori. AGS cells were cultured with two H. pylori cagA⁺ strains (MOI 100), with and without antibiotic treatments (10 µg/ml). *ELISA measured IL-8 response to antibiotics after 24 hours in two replicates. The data shown are from three independent experiments. All controls were included for each experiment. The levels of IL-8 secretion were significantly reduced when macrolides were added to AGS cells co-cultured with 873A MOI 100 strain compared to without antibiotic (p=0.015*) (p=0.0022**) (Kruskal-Wallis test) (Dunn's multiple comparisons test). The concentration of IL-8 response was also significantly higher in lab strain 60190 MOI 100 than Tx30a MOI 100 (p=0.0022**) (Mann-Whitney test). Cla=clarithromycin, and Azi=azithromycin.*

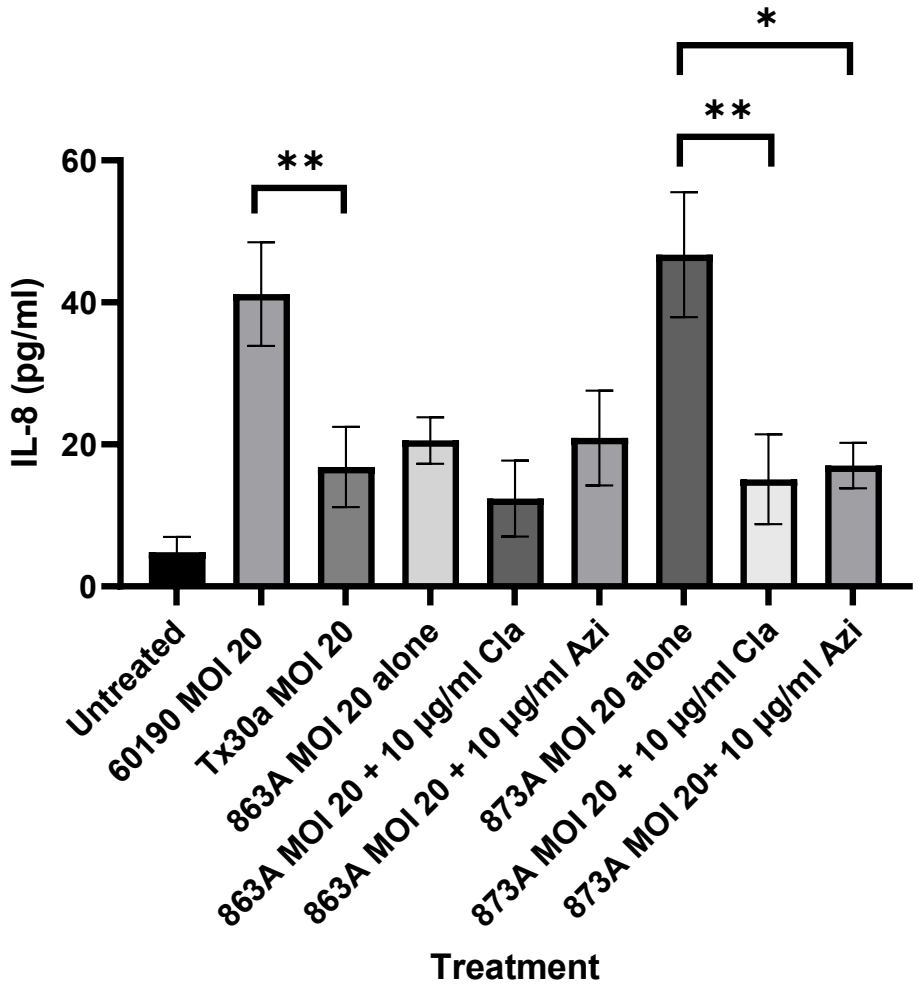


Figure 4.5: IL-8 secretion levels in response to antibiotics treatment of human gastric epithelial cells infected with H. pylori. AGS cells were cultured with two H. pylori cagA⁺ strains (MOI 20), with and without antibiotic treatments (10 µg/ml). *ELISA measured IL-8 response to antibiotics after 24 hours in two replicates. The data shown are from three independent experiments. All controls were included for each experiment. The levels of IL-8 secretion were significantly reduced when macrolides were added to AGS cells co-cultured with 873A MOI 20 strain compared to without antibiotic (p=0.0204*) (p=0.0051**) (Kruskal-Wallis test) (Dunn's multiple comparisons test). The concentration of IL-8 response was also significantly higher in lab strain 60190 MOI 20 than Tx30a MOI 20 (p=0.0022**) (Mann-Whitney test). Cla=clarithromycin, and Azi=azithromycin.*

AGS cells were subsequently cultured with two clinical isolates of clarithromycin-resistant *H. pylori* that were *cagA*-negative. Unexpectedly, administering 10 µg/ml of clarithromycin and azithromycin resulted in no significant changes in IL-8 production for *cagA*-negative isolates when compared to untreated co-infected AGS cells at MOIs of 100 and 20 (Figure 4.6 and

4.7). In contrast, adding 10 µg/ml of azithromycin significantly decreased IL-8 secretion levels in AGS cells co-cultured with the 802A MOI 100 strain, compared to when no antibiotic was used (p=0.0356) (Kruskal-Wallis test) (Dunn's multiple comparisons test) (Figure 4.6).

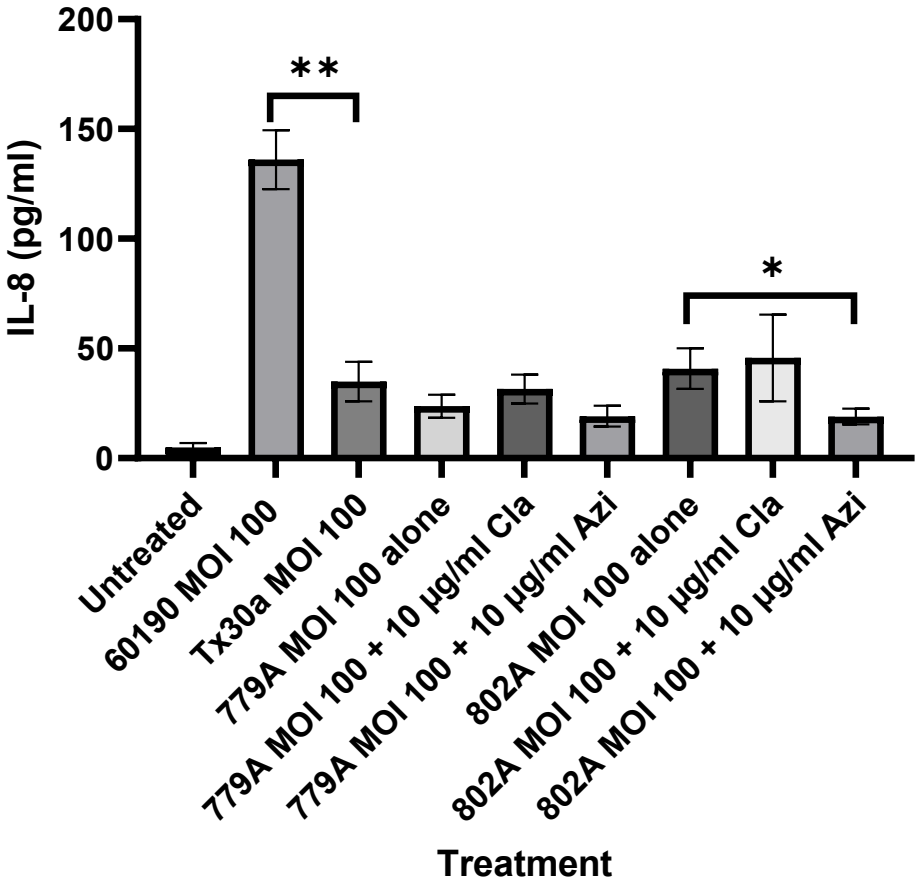


Figure 4.6: IL-8 secretion levels in response to antibiotics treatment of human gastric epithelial cells infected with H. pylori. AGS cells were cultured with two H. pylori cagA$^-$ strains (MOI 100), with and without antibiotics treatments (10 µg/ml). *ELISA measured IL-8 response to antibiotics after 24 hours in two replicates. The data shown are from three independent experiments. All controls were included for each experiment. The levels of IL-8 secretion were significantly reduced when azithromycin was added to AGS cells co-cultured with 802A MOI 100 strain compared to without antibiotic (p=0.0356*) (Kruskal-Wallis test) (Dunn's multiple comparisons test). The concentration of IL-8 response was also significantly higher in lab strain 60190 MOI 100 than Tx30a MOI 100 (p=0.0022**) (Mann-Whitney test). Cla=clarithromycin, and Azi=azithromycin.*

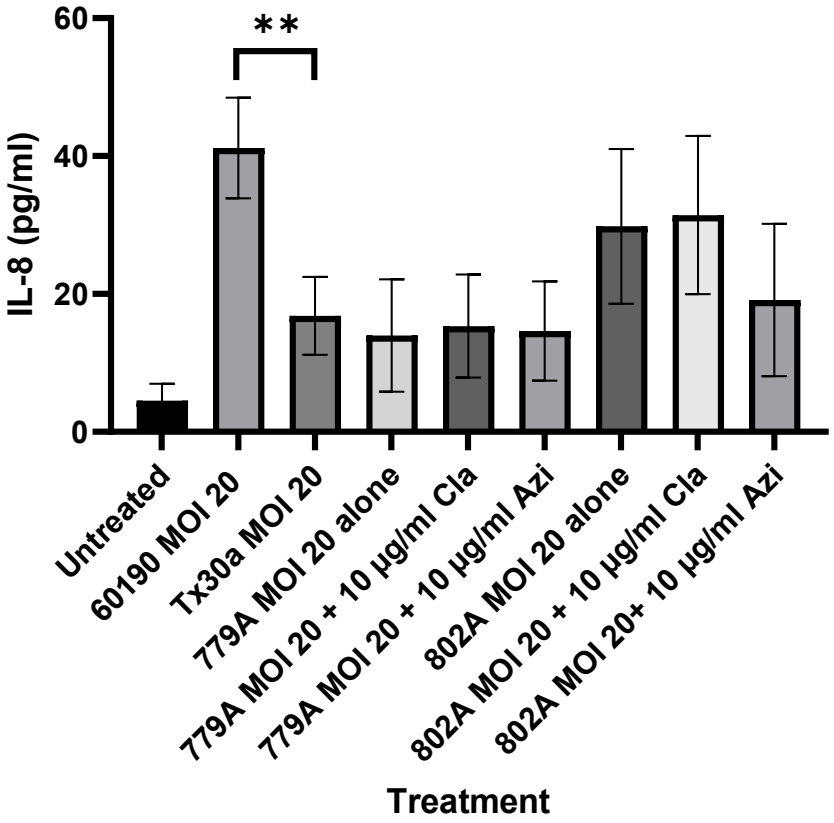


Figure 4.7: IL-8 secretion levels in response to antibiotics treatment of human gastric epithelial cells infected with H. pylori. AGS cells were cultured with two H. pylori cagA$^-$ strains (MOI 20), with and without antibiotic treatments (10 µg/ml). *ELISA measured IL-8 response to antibiotics after 24 hours in two replicates. The data shown are from three independent experiments. All controls were included for each experiment. The concentration of IL-8 response was significantly higher in lab strain 60190 MOI 20 than Tx30a MOI 20 (p=0.0022**) (Mann-Whitney test). Cla=clarithromycin, and Azi=azithromycin.*

4.3.5 Determination of the inhibitory effect of macrolide on hβD2 production *in vitro*

To examine whether antibiotics like clarithromycin and azithromycin can suppress the defensin levels upon *H. pylori* infection, AGS cells were co-cultured with clinical isolates of clarithromycin-resistant *H. pylori*, both *cagA*+ and *cagA*-. AGS cells were co-infected with two clinical isolates from each genotype and then treated with 10 μg/ml of either clarithromycin or azithromycin. The control groups consisted of uninfected AGS cells with and without antibiotic treatment, TNFα-stimulated AGS cells without *H. pylori* infection (both with and without antibiotic treatment), and uninfected AGS cells treated with the solvents used for antibiotic dilution (Figure 4.8). It was revealed that hβD2 protein levels were significantly higher in the TNFα-only treatment compared to the untreated group, the uninfected group treated solely with 10 μg/ml of clarithromycin, the uninfected group treated with 10 μg/ml of azithromycin, the TNFα treatment combined with clarithromycin, the TNFα treatment combined with azithromycin, as well as the DMSO and ethanol treatments ($p=0.0022$, Mann-Whitney test). Specifically, treating with TNFα alone resulted in a notable rise in hβD2 levels.

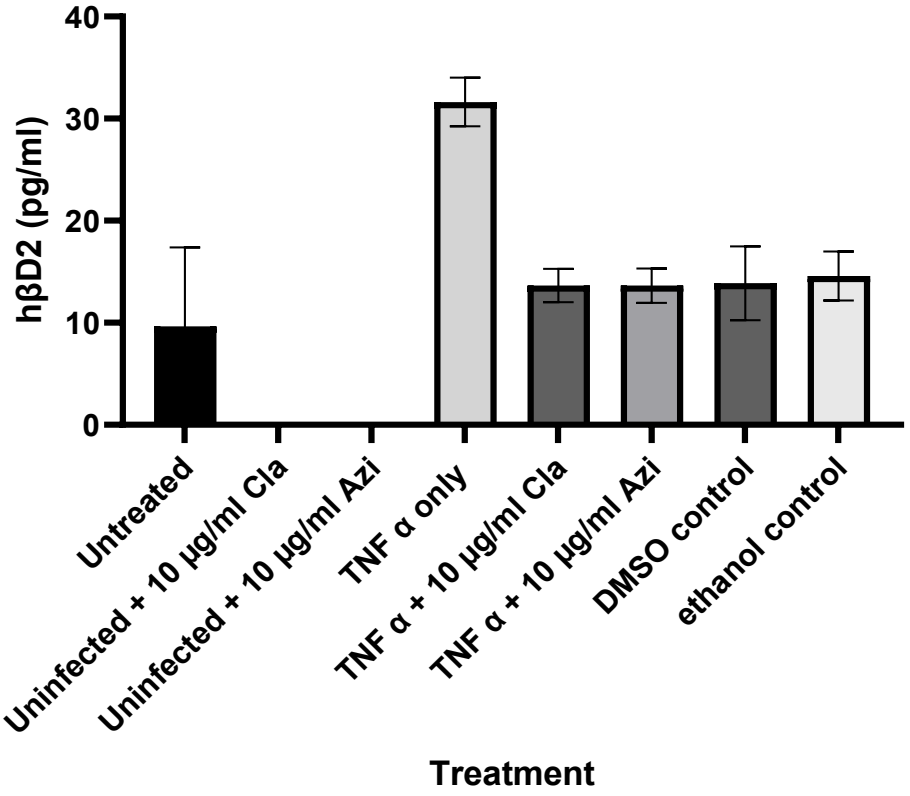


Figure 4.8: hβD2 secretion levels in response to controls. *AGS cells were cultured under these conditions and incorporated into each experiment. ELISA was used to measure the hβD2 response to TNFα after 24 hours, with all conditions tested in two replicate wells. The data shown are from three independent experiments. The concentrations of hβD2 were significantly higher in TNFα only treatment compared to all the treatments (p=0.0022) (Mann-Whitney test). Cla=clarithromycin, Azi=azithromycin, and DMSO=dimethyl sulfoxide.*

AGS cells were subsequently infected with the *H. pylori* laboratory strains 60190 and Tx30a, as well as all clinical isolates and laboratory strains, using MOIs of 100 and 20. This study found that AGS cells infected with the control strain 60190 at an MOI of 100 produced hβD2 at levels 2.2 times greater than those infected with the Tx30a strain at the same MOI (Figure 4.9). This result was statistically significant, with a p-value of 0.0022 (Mann-Whitney test).

The results indicated that macrolides reduced hβD2 production in response to all *H. pylori* clinical isolates. Specifically, AGS cells infected with *cagA*-positive isolates 863A at an MOI of

100 and 873A at an MOI of 100, when treated with 10 µg/ml of clarithromycin, showed a 1.6-fold and 2-fold reduction, respectively, compared to AGS cells co-cultured with the clinical isolates 863A MOI 100 and 873A MOI 100 (p=0.0222) (Kruskal-Wallis test) (Dunn's multiple comparisons test) in the absence of clarithromycin (Figure 4.9). Further analysis confirmed that hβD2 production in AGS cells treated with clinical isolates 863A MOI 20 and 873A MOI 20, each with 10 µg/ml of clarithromycin, was reduced by 2.7-fold and 3-fold, respectively, compared to AGS cells co-cultured with clinical isolates 863A MOI 20 (p=0.0061) and 873A MOI 20 alone (p=0.0056) (Kruskal Wallis test) (Dunn's multiple comparisons test) in the absence of clarithromycin (Figure 4.10).

Likewise, treatment with 10 µg/ml of azithromycin resulted in a 2-fold and 2.9-fold reduction in hβD2 production in AGS cells infected with *cagA*-positive clinical isolates 863A MOI 100 (p=0.0074) and 873A MOI 100 (p=0.0047) (Kruskal-Wallis test) (Dunn's multiple comparisons test), respectively, compared to the corresponding untreated controls (Figure 4.9). *H. pylori cagA*-positive isolate 863A and 873A at an MOI of 20 when treated with azithromycin exhibited a decrease of 2-fold and 3-fold, compared to AGS cells co-cultured solely with the isolate (p=0.0174) (p=0.0188) (Kruskal-Wallis test) (Dunn's multiple comparisons test) in the absence of azithromycin, respectively (Figure 4.10).

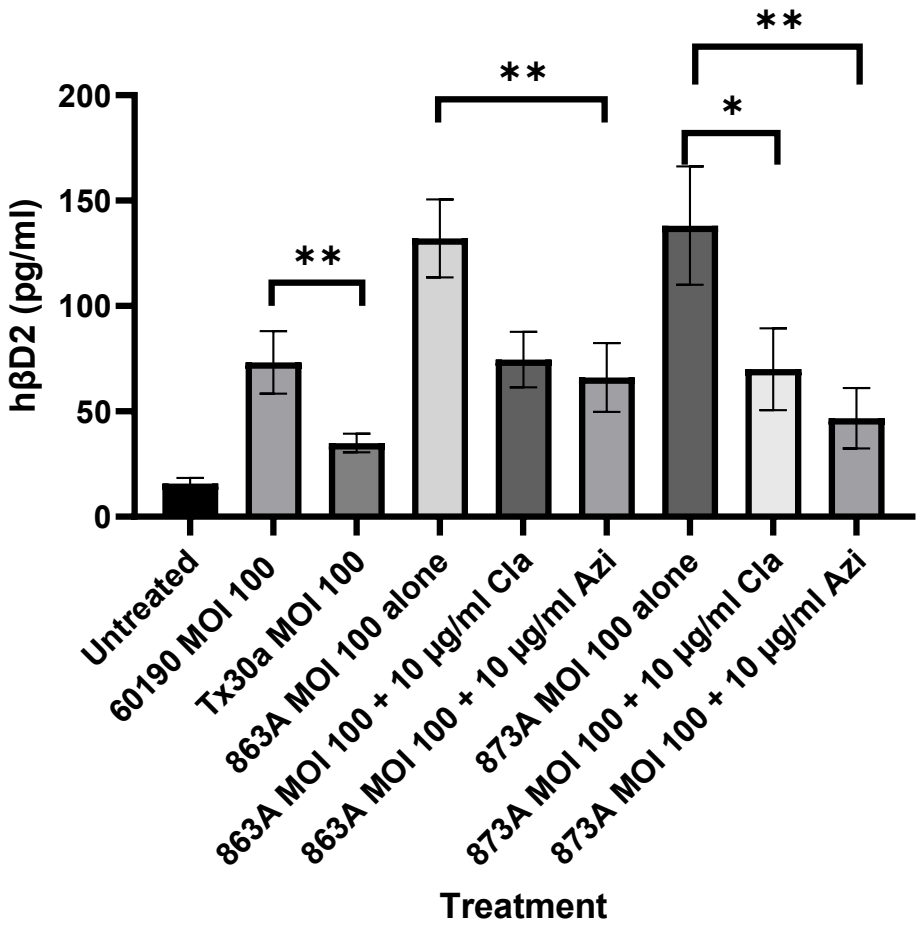


Figure 4.9: hβD2 secretion levels in response to antibiotics treatment of human gastric epithelial cells infected with H. pylori. AGS cells were cultured with two H. pylori cagA$^+$ strains (MOI 100), with and without antibiotic treatments (10 µg/ml). *ELISA measured hβD2 response to antibiotics after 24 hours in two replicates. All controls were included for each experiment. The data shown are from three independent experiments. The level of hβD2 secretion in AGS cells co-cultured with 863A MOI 100 alone is significantly higher than its counterparts when treated with azithromycin (p=0.0074**) (Kruskal-Wallis test) (Dunn's multiple comparisons test), while the level of hβD2 secretion in AGS cells co-cultured with 873A MOI 100 alone is significantly higher than the ones treated with clarithromycin (p=0.0222*) and azithromycin (p=0.0047**) (Kruskal-Wallis test) (Dunn's multiple comparisons test). The concentration of hβD2 response was significantly higher in lab strain 60190 MOI 100 than Tx30a MOI 100 (p=0.0022**) (Mann-Whitney test) Cla=clarithromycin, and Azi=azithromycin.*

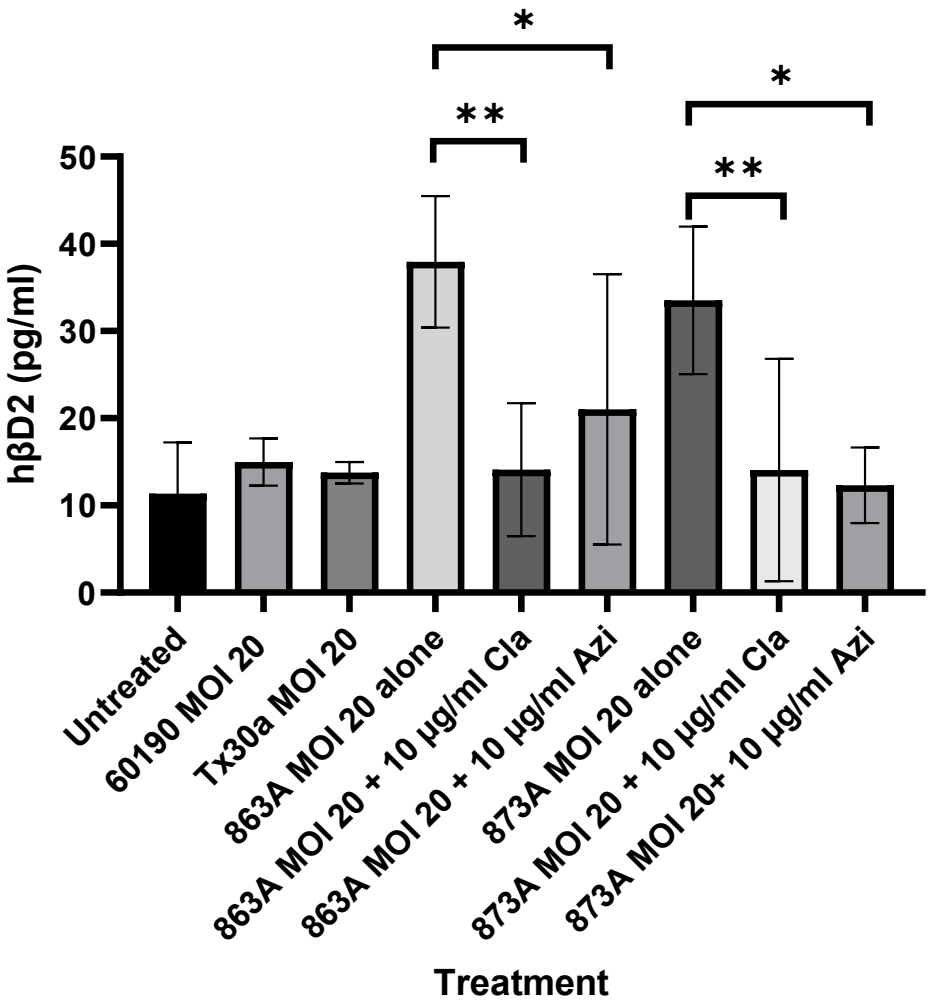


Figure 4.10: hβD2 secretion levels in response to antibiotics treatment of human gastric epithelial cells infected with H. pylori. AGS cells were cultured with two H. pylori cagA⁺ strains (MOI 20), with and without antibiotic treatments (10 µg/ml). *ELISA measured hβD2 response to antibiotics after 24 hours in two replicates. All controls were included for each experiment. The data shown are from three independent experiments. The level of hβD2 was significantly reduced when clarithromycin was added to AGS cells co-cultured with 863A and 873A MOI 20 alone compared to without antibiotic (p=0.0061**) (p=0.0056**) (Kruskal Wallis test) (Dunn's multiple comparisons test), respectively. The level of hβD2 secretion was also significantly reduced when azithromycin was added to the AGS cells co-cultured with 863A MOI 20 and 873A MOI 20 alone than the ones without antibiotic (p=0.0174*) (p=0.0188*) (Kruskal-Wallis test) (Dunn's multiple comparisons test), respectively. Cla=clarithromycin, and Azi=azithromycin.*

AGS cells were then cultured with two *cagA*-negative, clarithromycin-resistant *H. pylori* clinical isolates. The results demonstrated that treatment with 10 μg/ml of clarithromycin reduced hβD2 production in response to both isolates. There was a 2.1-fold reduction for isolate 779A MOI of 100 ($p=0.0035$) (Kruskal-Wallis test) (Dunn's multiple comparisons test) and a 1.9-fold reduction for isolate 802A ($p=0.0011$) (Kruskal-Wallis test) (Dunn's multiple comparisons test) at an MOI of 100, in comparison to infected AGS cells in the absence of clarithromycin (Figure 4.11). Similarly, treating AGS cells infected with 779A at an MOI of 20 with 10 μg/ml of clarithromycin led to a 1.6-fold reduction in hβD2 secretion. For cells infected with 802A at the same MOI, hβD2 secretion decreased modestly by 1.1-fold compared to untreated cells (Figure 4.12).

When AGS cells infected with *cagA*-negative clinical isolates 779A at an MOI of 100 and 802A at an MOI of 100 were treated with 10 μg/ml of azithromycin, hβD2 production exhibited a slight reduction by 1.3-fold and 1.2-fold, respectively, in comparison to the infected cells without azithromycin (Figure 4.11). As expected, the *cagA*-negative *H. pylori* isolates 779A and 802A at an MOI of 20 also showed a 3.38-fold ($p=0.0073$) and 2.6-fold ($p=0.0010$) (Kruskal-Wallis test) (Dunn's multiple comparisons test) reduction compared to the infected cells without azithromycin (Figure 4.12).

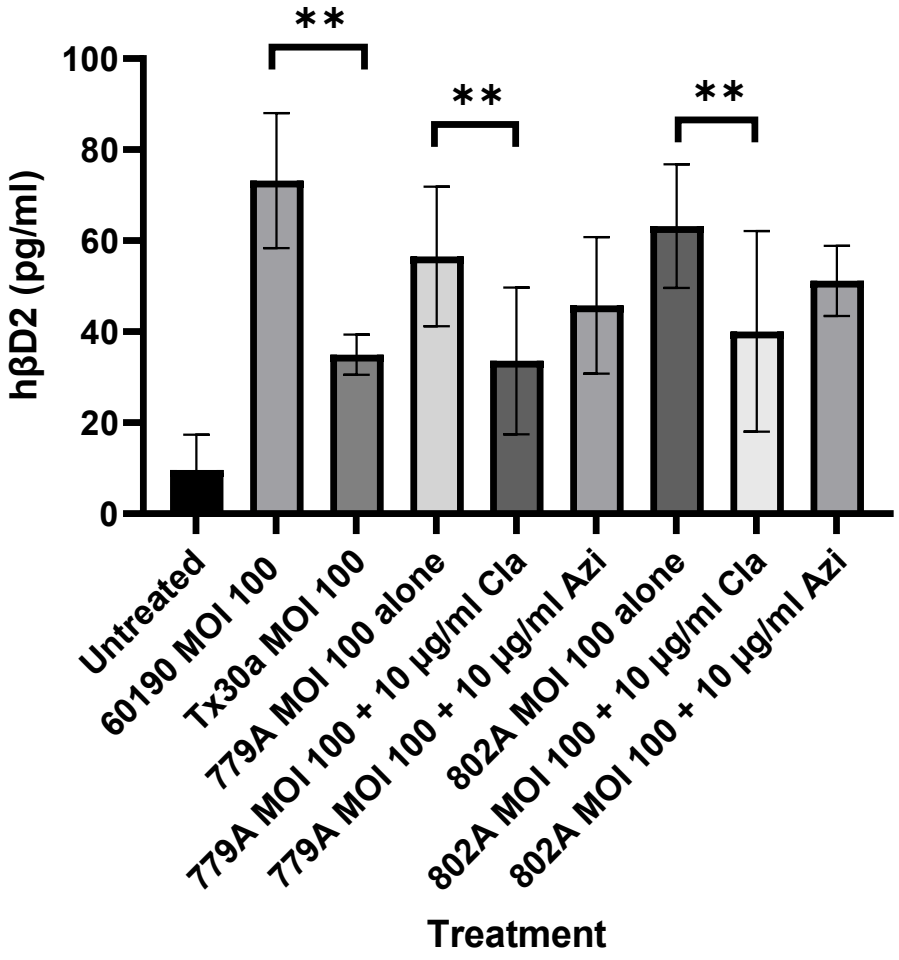


Figure 4.11: hβD2 secretion levels in response to antibiotics treatment of human gastric epithelial cells infected with H. pylori. AGS cells were cultured with two H. pylori cagA$^-$ strains (MOI 100), with and without antibiotics treatments (10 µg/ml). *ELISA measured hβD2 response to antibiotics after 24 hours in two replicates. All controls were included for each experiment. The data shown are from three independent experiments. The level of hβD2 was significantly reduced when clarithromycin was added to AGS cells co-cultured with 779A and 802A MOI 20 compared to without antibiotic (p=0.0035**) (p=0.0011**) (Kruskal Wallis test) (Dunn's multiple comparisons test), respectively. The concentration of hβD2 response was significantly higher in lab strain 60190 MOI 100 than Tx30a MOI 100 (p=0.0022**) (Mann-Whitney test) Cla=clarithromycin, and Azi=azithromycin.*

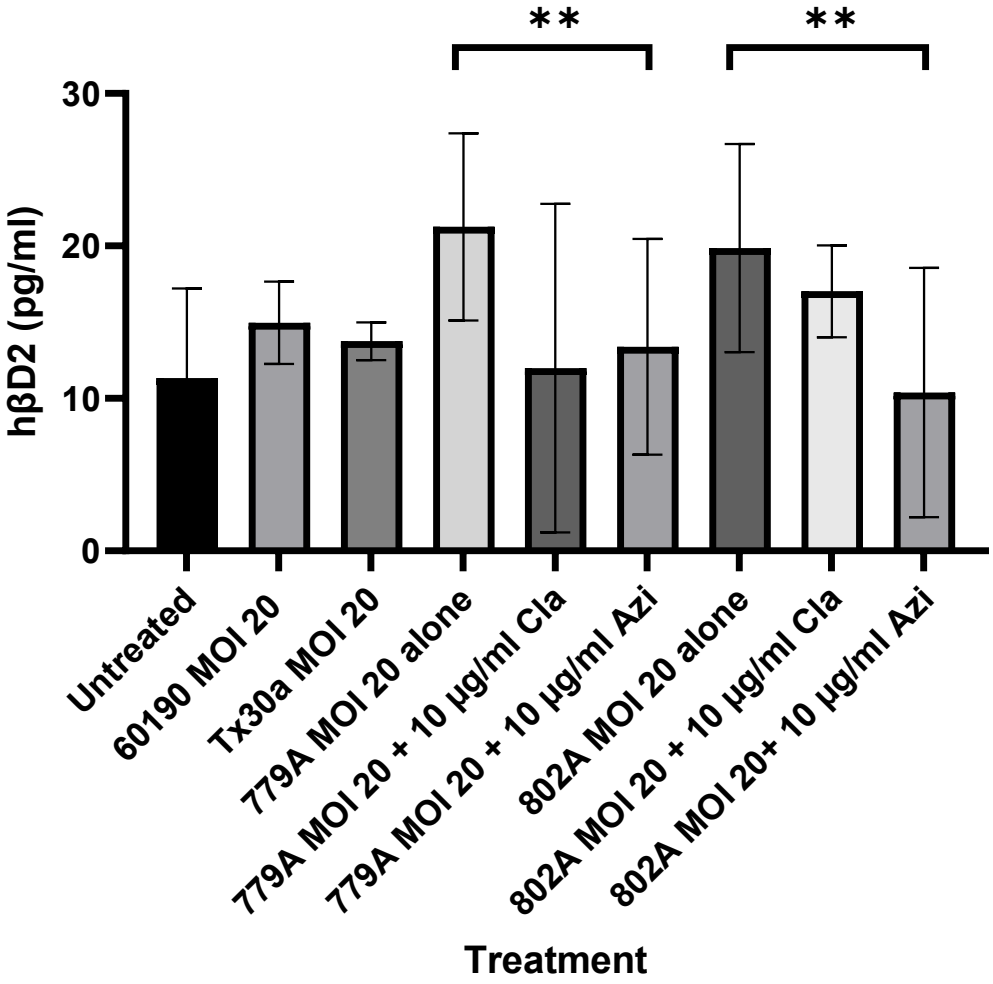


Figure 4.12: hβD2 secretion levels in response to antibiotics treatment of human gastric epithelial cells infected with H. pylori. AGS cells were cultured with two H. pylori cagA⁻ strains (MOI 20), with and without antibiotic treatments (10 µg/ml). *ELISA measured hβD2 response to antibiotics after 24 hours in two replicates. All controls were included for each experiment. The data shown are from three independent experiments. The level of hβD2 was significantly reduced when azithromycin was added to AGS cells co-cultured with 779A and 802A MOI 20 compared to without antibiotic (p=0.0073**) (p=0.0010**) (Kruskal Wallis test) (Dunn's multiple comparisons test), respectively. Cla=clarithromycin, and Azi=azithromycin.*

4.4 Discussion

4.4.1 Addition of TNFα did not have an impact on *H. pylori* density

In this study, stimulation of *H. pylori*-infected AGS cells with a high dose of TNFα did not change bacterial CFU counts compared to the unstimulated controls. This was surprising because a previous *in vitro* study showed that TNFα was correlated with *H. pylori* density (Lehmann *et al.*, 2002). A prior *ex vivo* study also revealed that the expression of TNFα mRNA was significantly elevated in the gastric mucosa of paediatric patients infected with *H. pylori* compared to those who were uninfected, and this increase in TNFα corresponded with bacterial density (Michalkiewicz *et al.*, 2015). Meanwhile, a previous study investigated the colonisation capability of the *H. pylori* strain in the stomachs of both wild-type mice and those with a TNFα gene knockout. The density of *H. pylori* colonising the stomachs of *TNFα*−/− mice was greater than wild-type mice. These results indicate that TNFα might protect against *H. pylori* infection (Yamamoto *et al.*, 2004).

TNFα strongly activates NF-κB, which initiates the transcription of genes that support inflammation and antibacterial defence. NF-κB stimulates the production of cytokines, chemokines, and antibacterial immune cells like macrophages and neutrophils, along with effectors such as defensins and reactive oxygen and nitrogen species, assisting in the elimination of bacteria. Although this inflammatory response effectively limits bacterial survival, it can also damage tissue and heighten the risk of *H. pylori*-related diseases, including PUD and gastric cancer (Saha *et al.*, 2022; Wang *et al.*, 2017).

A past study demonstrated that T cell lines obtained from antral biopsies of ten patients infected with *H. pylori* released TNFα in concentrations ranging from 30 to 4000 pg/ml

(Lehmann *et al.*, 2002). In the current study, the use of 50 ng/ml concentration of TNFα in AGS cells co-cultured with *H. pylori* may not be physiological to human conditions because under normal and healthy conditions, the circulating TNFα levels in human plasma are typically much lower, often in the range of generally between 1-10 pg/ml for a baseline level (Quarta *et al.*, 2022). During inflammation or infection, particularly in chronic infections or diseases like *H. pylori*-induced gastric ulcers or gastritis, TNFα levels can increase significantly, but even during inflammation, concentration usually does not reach as high as 50 ng/ml. Elevated TNFα levels in inflammatory conditions such as gastritis are typically in the range of tens to hundreds of pg/ml (Yang *et al.*, 2024).

Initially, the higher concentration of TNFα was used in this experimental setting to induce a strong inflammatory response or to mimic a pathological condition where TNFα is highly upregulated, such as severe inflammation like in gastritis, PUD or gastric cancer induced by *H. pylori*. This was based on a previous study where the AGS cells were stimulated for 4 hours with TNFα of 20 ng/ml and the expression of hβD2 mRNA was markedly increased following the TNFα stimulation and upon *H. pylori* infection (O'Neil *et al.*, 2000). However, in chronic infections like *H. pylori* gastritis, the immune response, including TNFα release does not always eradicate bacteria and reduce its density, but may instead lead to persistent infection and gastric inflammation. Therefore, if the goal is to replicate more typical human responses to *H. pylori*, lower concentrations of TNFα might be appropriate, and hence, using a range of TNFα concentrations to assess a dose-dependent response in future research would be beneficial.

An experimental factor that might have inhibited the response to TNFα stimulation is possibly the use of serum-rich media. In this study, cells were maintained and cultured in an

F-12 ham medium supplemented with Foetal Bovine Serum (FBS). Both serum and medium have been demonstrated to promote increased growth rates of *H. pylori* (Douraghi *et al.*, 2010; Sainsus *et al.*, 2008), thus, as a result, the culture conditions could have accelerated *H. pylori* growth, potentially masking the epithelial cell response to cytokine stimulation. FBS is widely used as a growth supplement in the *in vitro* cultivation of animal and human cells primarily because it is rich in both micro- and macronutrients, as well as growth factors required for cell attachment, proliferation, and maintenance (Subbiahanadar Chelladurai *et al.*, 2021). Furthermore, the selection of incubation time might also be important. In this study, the incubation period was set to 24 hours, while Lehmann *et al.* (2002) removed the supernatant after 18 hours. This difference in the incubation period could affect cytokine stimulation of gastric epithelial cells and consequently impact bacterial density at earlier times.

H. pylori is known to trigger inflammation in the gastric mucosa, prompting host cells such as gastric epithelial cells (including AGS cells) to release a range of pro-inflammatory cytokines, including TNFα, as a component of the immune response to the infection. During *H. pylori* infection, TNFα might play an essential role in promoting bacteria colonization and inflammation by efficiently inhibiting gastric epithelial cell renewal and promoting gastric epithelial cell apoptosis (Lv *et al.*, 2018). Although AGS cells and other gastric epithelial cells can generate TNFα at very low concentrations when exposed to *H. pylori*, the primary producers of TNFα in gastric tissue during infection are the immune and inflammatory cells that infiltrate the area, especially macrophages and T lymphocytes. These cells are drawn to the infection site and play a vital role in the cytokine-driven

immune response that contributes to the inflammation and tissue damage seen in *H. pylori*-associated gastritis and related conditions (Chen *et al.*, 2018a).

The signalling pathways triggered by TNFα and by *H. pylori* differ significantly in several aspects, including the types of receptors they engage, the downstream signalling cascades they utilize, and the resulting biological effects. Although there is some overlap in pathways, like the activation of NF-κB and MAPK pathways, the underlying mechanisms that drive these responses vary (Chen *et al.*, 2022; Tavares & Pathak, 2018). TNFα mainly facilitates systemic immune responses, such as inflammation and apoptosis. In contrast, *H. pylori* infection, particularly with *cag*PAI^{+}, triggers more intricate signalling pathways involving direct bacterial effector proteins like CagA, which enhance inflammation, promote cell survival, and may contribute to tumour development in the gastric epithelium (Al-Mathkour *et al.*, 2023; Zhang *et al.*, 2024b).

TNFα primarily communicates through two receptors: tumour necrosis factor receptor 1 (TNFR1) and tumour necrosis factor receptor 2 (TNFR2). TNFR1 is the main receptor responsible for initiating pro-inflammatory and apoptotic responses and is found on most cell types. In contrast, TNFR2 plays a bigger role in regulating immune cells, especially T cells, and is associated with anti-apoptotic or immune-modulatory effects (Alshevskaya *et al.*, 2022). When TNFR1 is activated, it recruits the TNF receptor-associated death domain (TRADD), triggering the IκB kinase complex (IKK complex). This process causes the breakdown of the inhibitory protein IκB, leading to the activation of NF-κB transcription factors, such as p65/p50 dimers. Once activated, NF-κB moves into the nucleus and promotes the transcription of pro-inflammatory genes, including cytokines like IL-6, IL-1β, TNF-α, and chemokines like IL-8 (Barnabei *et al.*, 2021). Additionally, TNFα can activate the

MAPK pathway, which includes JNK, p38, and ERK, influencing cell survival, differentiation, or apoptosis depending on the situation. TNFR1 activation may also trigger apoptosis via the caspase cascade, involving caspase-8 and caspase-3, resulting in programmed cell death under certain conditions (Yang *et al.*, 2020).

In Yang *et al.* (2024)'s experimental study, they detected elevated TNFα levels in gastritis compared to normal patients, with significantly higher levels observed in *H. pylori*-associated gastritis than in *H. pylori*-independent gastritis. This finding is consistent with the recognized role of TNFα as a key mediator of systemic inflammation and acute phase reactions in the context of gastritis (Fond *et al.*, 2014; Güzel *et al.*, 2016). It was also proven that TNFα rises in response to macrophages being stimulated by *H. pylori* lipopolysaccharide, hence plays a vital role in the inflammatory reactions to *H. pylori* (Moradipour *et al.*, 2018). The importance of TNFα in gastritis is highlighted by its role in recruiting and activating monocytes, macrophages, and neutrophils, as well as its significantly elevated levels in patients with *H. pylori* infection compared to healthy individuals (Yang *et al.*, 2024).

A previous study uncovered a particularly interesting finding. Patients with *H. pylori*-infected nodular gastropathy (NG) exhibited a higher bacterial load in the gastric corpus and significantly lower levels of TNFα compared to non-NG-infected patients (Mansilla-Vivar *et al.*, 2020). NG is an inflammatory condition of the gastric mucosa, characterized by the endoscopic appearance of multiple millimetres-sized protrusions (Mansilla-Vivar *et al.*, 2020). The observation of increased bacterial load without a corresponding rise in mucosal inflammatory cytokine responses in *H. pylori*-infected individuals with NG suggests a general suppression of immune responses or an active immune evasion strategy employed by *H.*

pylori. These findings imply that the increased bacterial load in NG might lead to reduced immune detection, facilitated by *H. pylori*'s immune evasion mechanisms in NG patients (Mansilla-Vivar *et al.*, 2020).

4.4.2 IL-8 production was significantly increased during *H. pylori* infection of AGS cells with TNFα stimulation *in vitro*

In this study, there was strong evidence that the production of IL-8 in AGS cells was significantly increased upon *H. pylori* infection, and this was consistent with previous *in vitro* studies (Beswick *et al.*, 2005; Domínguez-Martínez *et al.*, 2023; Eftang *et al.*, 2012; Fazeli *et al.*, 2016; Nemidkanam *et al.*, 2024). Current findings also showed that AGS cells, both uninfected and infected with *H. pylori* responded to cytokine treatment, and the levels of IL-8 production were significantly elevated in the presence of TNFα, which was in line with the previous study (Fan *et al.*, 1995). A past study also demonstrated that *H. pylori* and TNFα produced a dose-dependent increase in IL-8 production (Beales & Calam, 1997). Meanwhile, Zachrisson *et al.* (2001) showed that TNFα also triggered the release of IL-8 from human duodenal mucosa biopsies.

Tumour necrosis factor receptor-associated factor 6 (TRAF6) and TNFα are two important components in the immune system, particularly involved in inflammation, cell survival, and immune responses. TRAF6 is an adaptor protein that plays a crucial role in the signal transduction pathways that mediate immune and inflammatory responses. It is part of a family of TRAF proteins that interact with receptors of the TNF receptor superfamily and other receptors involved in innate immunity, such as the IL-1 receptor (Dhillon *et al.*, 2019). TRAF6 is activated upon binding to its receptors, leading to its oligomerization and the

activation of downstream signalling pathways, primarily through the activation of kinases like transforming growth factor beta-activated kinase 1 (TAK1) and the subsequent activation of NF-κB, MAPKs, and other transcription factors. This process leads to the production of pro-inflammatory cytokines, such as TNFα, IL-8, and IL-1β (Wang *et al.*, 2023).

The interaction of CagA with host signalling pathways creates a complex interplay with TNFα signalling, particularly through the NF-κB pathway and other pro-inflammatory cascades. When TNFα binds to its receptor (TNFR1 and TNFR2), it recruits TRAF6 to the receptor complex. This leads to the activation of downstream signalling cascades, including the NF-kB and MAPK pathways (Shi & Sun, 2018). Meanwhile, *H. pylori* induce the activation of NF-κB via CagA-dependent and independent mechanisms. After injection into cells, CagA can interact with the TRAF6 and TAK1, and initiates aberrant poly-ubiquitination of TAK1, activating IKK and NF-kB inflammatory response (Hatakeyama, 2017).

Both *H. pylori* CagA and TNFα signalling induced NF-κB activation, hence the result is a synergistic increase in the transcription of inflammatory genes. This amplifies the production of pro-inflammatory cytokines such as TNFα, IL-6, and chemokines (e.g., IL-8), enhancing immune cell recruitment (Chen *et al.*, 2023a; Chen *et al.*, 2022; Teng *et al.*, 2020). Both signalling also stimulate the production of reactive oxygen and nitrogen species (ROS/RNS). TNFα enhances ROS production via NADPH oxidase activation, while CagA disrupts mitochondrial function, increasing oxidative stress. Elevated ROS levels contribute to tissue damage and mutation, linking to *H. pylori*-associated carcinogenesis (Al-Roub *et al.*, 2023; Bhosale *et al.*, 2022; Woo *et al.*, 2023).

The convergence of CagA+ *H. pylori* and TNFα signalling creates a pro-inflammatory microenvironment, driving gastritis and enhancing mucosal damage. Sustained activation of

NF-κB and MAPK pathways promotes cell proliferation, inhibits apoptosis, and increases DNA damage through oxidative stress, elevating the risk of gastric cancer. Therefore, targeting intersections between *H. pylori* CagA and TNFα signalling (e.g., NF-κB inhibitors, ROS scavengers, or TNFα blockers) could mitigate inflammation and reduce cancer risk while preserving host defence (Chaithongyot *et al.*, 2021; Sah *et al.*, 2023).

4.4.3 hβD2 production was significantly increased during *H. pylori* infection of AGS cells with TNFα stimulation *in vitro*

In this study, it was observed that the level of hβD2 protein expression in gastric epithelial cells infected with *H. pylori* was upregulated compared to uninfected cells, thereby confirming the findings of the previous chapter. Like IL-8 production, the stimulation of TNFα seemed to have great effects on defensin expression where a significant elevation in hβD2 protein level was observed in AGS cells, both uninfected and those infected with *H. pylori,* in the presence of TNFα. This is consistent with previous reports where secretion of hβD2 was induced by *H. pylori* infection (Uehara *et al.*, 2003) and by TNFα treatment in many epithelial cells such as human airway epithelial cells (Harder *et al.*, 2000; Kao *et al.*, 2004), gingival epithelial cells (Joly *et al.*, 2005), and human corneal epithelial cells (McDermott *et al.*, 2003). Meanwhile, Kubler *et al.* (2009) found the levels of *DEFB4* mRNA were positively correlated with inflammatory cytokine TNFα in individuals with active Crohn's disease whilst Wehkamp *et al.* (2003) demonstrated no association of *DEFB4* expression with that of TNFα in Crohn's disease patients. Hence, these findings indicate that the regulation of hβD2 likely depends on the pro-inflammatory actions of TNFα activation, potentially suggesting the common signalling pathway for defensin and cytokine regulation.

Defensin expression is proposed to be regulated by various cytokines, for example, Joly *et al.* (2005) showed that significant synergistic effects were observed in hβD2 expression when primary inducers such as IL-1β and TNFα were combined, leading to the involvement of multiple signalling pathways in their expression and regulation. Extensive literature details the complex regulation of defensins, driven by transcription factors initialized by pro-inflammatory cytokines or signalling pathways associated with the activation of PRRs such as through TLRs (Birchler *et al.*, 2001; Hertz *et al.*, 2003) and MAPK pathways (Krisanaprakornkit *et al.*, 2002; McDermott *et al.*, 2003; Shi *et al.*, 2014).

Furthermore, the promoter regions of defensin genes include the binding sites for key cellular transcription factors such as AP-1 and NF-κB, which are known to also be activated following infection of gastric epithelial cells with *cag*PAI+ *H. pylori* (Jang *et al.*, 2004; Shi *et al.*, 2014). Therefore, in living organisms, the β-defensin response to *H. pylori* inflammation or infection is presumably initiated by a complex interplay and cooperation among these pathways. However, the details of the mechanisms controlling the inducible expression of defensin particularly concerning the host-derived factors involved remain unclear, and further research is needed (Wozniak *et al.*, 2024).

4.4.4 Macrolides downregulated IL-8 production by infected AGS cells *in vitro*

In this study, generally, both macrolides were observed to suppress the production of IL-8 in AGS cells co-cultured with clarithromycin-resistant *H. pylori cagA*$^+$ and *cagA*$^-$ strains, and suppression was more pronounced with the *cagA*$^+$ strains. Previous *in vitro* studies supported this finding showing that clarithromycin effectively reduce IL-8 expression in *H. pylori*-infected cells (Nemidkanam *et al.*, 2024). Park *et al.* (2005) also demonstrated that IL-8 production induced by *H. pylori* was higher than the control, and these values decreased

when macrolide was added to the culture, although they were not statistically significant. Regarding other epithelial cell lines, macrolides such as azithromycin have also demonstrated an ability to downregulate the mRNA expression of TNFα and IL-8 in airway epithelial cells of cystic fibrosis patients (Cigana *et al.*, 2007; Cigana *et al.*, 2006).

In the current study, it is also important to note that the inhibitory effect of clarithromycin and azithromycin on IL-8 production varies, and studies showed that the immunomodulatory effects appear to differ among various macrolides (Zimmermann *et al.*, 2018). Previous studies showed that azithromycin was less commonly linked to alterations in measured immunological markers (such as cytokines and chemokines) compared to the other macrolides (Criqui *et al.*, 2000; Čulić *et al.*, 2002). However, past *in vitro* studies indicated that clarithromycin exhibits less immunomodulatory activity compared to other macrolides, for instance, clarithromycin demonstrated a significantly weaker effect in decreasing IL-6 production by human macrophages compared to erythromycin (Sato *et al.*, 2007). While both clarithromycin and azithromycin can inhibit IL-8 production, the degree and consistency of this effect can vary based on factors like drug concentration, cell type, and the inflammatory context. Azithromycin is often seen as having stronger anti-inflammatory effects in some cases, but clarithromycin might have different effects depending on the setting (Matsuo *et al.*, 2019; Zhang *et al.*, 2023). Hence, the reporting discrepancies between the degree of inflammatory responses observed in this study and other previous studies might suggest a varying immunomodulatory impact of different macrolides on cytokine levels.

Apart from inhibiting pathogen virulence factor production and protein synthesis by binding to the 50S ribosomal unit, macrolides have been proposed to have several other

mechanisms, including effects on the host immune response (Zimmermann *et al.*, 2018). However, the mechanisms responsible for the non-antimicrobial effects of macrolides are not as clearly understood. It is postulated that the mechanisms by which macrolides exert their anti-inflammatory effects are multifaceted (Alzolibani & Zedan, 2012). One of the most commonly and consistently observed immunomodulatory effects of macrolides is the reduction of neutrophilic inflammation. This reduction in neutrophils and suppression of their function results in lower levels of neutrophil elastase and IL-8, which leads to decreased tissue damage (Cheung *et al.*, 2010).

Furthermore, the presence of *cagA* may influence the differences in the suppressive effects of macrolides on *H. pylori* strains. *CagA*+ *H. pylori* strains induce more severe inflammation, which might influence how macrolides interact with the host immune system. In this study, it was observed that the immune modulation by macrolides had a greater impact on patients infected with *cagA*+ compared to *cagA*- *H. pylori* strains. This aligns with a previously mentioned hypothesis, which proposes that more virulent strains provoke a stronger inflammatory response, increasing blood flow to the infection site. This, in turn, enhances antibiotic delivery and the likelihood of successful eradication (Khan *et al.*, 2012). Another possible explanation is that a more virulent strain of *H. pylori* might replicate more rapidly, making it more susceptible to antibiotics that work by inhibiting bacterial replication (Sugimoto & Yamaoka, 2009).

It has been recognized that *H. pylori* infection results in the activation of NF-κB, which subsequently leads to IL-8 expression. However, there has been ongoing debate about whether the activation of the NF-κB pathway due to *H. pylori* infection is solely dependent on CagA (Martinelli *et al.*, 2023). Sokolova and Naumann (2017) demonstrated that *H. pylori*

strains possessing *cag*PAI genes induced NF-κB and IL-8 expression more effectively than *cag*PAI-negative strains.

Meanwhile, an *in vitro* study by Peng *et al.* (2014) revealed that clarithromycin effectively reduced IL-8 expression and suppressed the *H. pylori*-induced activation of NF-κB, regardless of CagA expression. Conversely, Nemidkanam *et al.* (2024) demonstrated that clarithromycin significantly reduced IL-8 expression, although it did not notably inhibit NF-κB expression. Furthermore, previous *in vitro* studies proved that macrolides lower cytokine levels by inhibiting NF-κB and AP-1 in human bronchial epithelial cells (Desaki *et al.*, 2000; Miyanohara *et al.*, 2000).

Therefore, future studies should include studying the effects of macrolides on key signalling pathways like NF-kB, MAPK and AP-1, and assessing the potential mechanism behind the suppressive effects of macrolides. For instance, treated cell lines with macrolides at varying concentrations and time intervals can be used, alongside positive controls such as TNFα to activate the NF-kB pathway. Western blotting could then be utilized to assess the relative intensities of phosphorylated key proteins in these pathways within the nucleus following macrolide treatment, compared to untreated controls. Alternatively, the CRISPR-Cas9 or siRNA-mediated techniques can be employed to knock down crucial genes in the signalling pathways (like MAPK1 for the ERK signalling pathway). After a successful knockout, the cell lines would be treated with the macrolides, and the suppressive effects of macrolides could be evaluated by measuring pathway activation in wild type versus knockout cells through Western blotting (Xu *et al.*, 2024; Zhong *et al.*, 2024).

4.4.5 Macrolides downregulated hβD2 production by infected AGS cells *in vitro*

The current study found that macrolides decreased hβD2 production in response to all *H. pylori* clinical isolates tested. However, there have been few published studies investigating the inhibitory effects of macrolides, particularly on hβD2 expression, in gastric epithelial cells infected with *H. pylori* isolates. Macrolides have been used or studied primarily for the treatment of chronic inflammatory lung diseases, such as diffuse pan bronchiolitis, cystic fibrosis, chronic obstructive pulmonary disease, and asthma (López-Boado & Rubin, 2008). A previous study has shown that macrolide treatment significantly reduced the levels of hβD2 in the bronchoalveolar lavage (BAL) fluid of patients suffering from diffuse pan bronchiolitis (Hiratsuka *et al.*, 2003). On the other hand, erythromycin was found to significantly elevate the production of hβD2 mRNA and protein levels in human airway epithelial cells (Ishizawa *et al.*, 2005). This study showed that although macrolides impact the epithelial barrier and ciliary function positively, this effect might be secondary to reducing airway inflammation.

Meanwhile, another previous study revealed that exposure to human neutrophil peptide-1 (HNP-1) or LPS from *Pseudomonas aeruginosa* elevates the levels of mucin *MUC5AC* mRNA and protein in bronchial epithelial cells (Ishimoto *et al.*, 2008). Furthermore, co-stimulation with both HNP-1 and LPS results in an additive increase in mucin production and adding macrolides such as azithromycin and clarithromycin inhibited the overproduction of mucin MUC5AC. These findings indicate that defensins play a role in excessive mucus production in patients with respiratory tract infections, and macrolides directly mitigate these effects by disrupting intracellular signal transduction (Ishimoto *et al.*, 2008).

In this study, the MIC values for clarithromycin-resistant *H. pylori* isolates cultured in the AGS cell lines were 12 to 256 µg/ml. Even though a clarithromycin concentration of 10 µg/ml was added to the co-cultured AGS cells, this low dose effectively reduced the production of IL-8 and defensin. This finding proves that although a low dose of clarithromycin will not be effective as an antibacterial agent, it may still serve as an anti-inflammatory or immunomodulatory agent (Pollock & Chalmers, 2021). In pulmonary practice, the extended use of macrolide therapy at lower dosages has gained popularity for treating patients with chronic inflammatory conditions, such as non-cystic fibrosis, bronchiectasis, and sinusitis due to its anti-inflammatory properties in addition to its antimicrobial effects (Altenburg *et al.*, 2010).

Overall, macrolide antibiotics have been reported to exhibit numerous immunomodulatory effects. These include reducing prolonged inflammation (Parnham *et al.*, 2023), lowering airway mucus secretion (Haghi *et al.*, 2015), inhibiting bacterial biofilm formation (Moshynets *et al.*, 2022), decreasing the production of cytokines (Fan *et al.*, 2017), preventing neutrophil activation and mobilization (Vanaudenaerde *et al.*, 2007), promoting neutrophil apoptosis (Fischer *et al.*, 2013), and blocking the activation of nuclear transcription factors (Aghai *et al.*, 2007).

A previous study showed that macrolides like azithromycin could also potentially reduce viral load when combined with hydroxychloroquine in patients with mild COVID-19 (Rosenberg *et al.*, 2020). Moreover, azithromycin, erythromycin, and clarithromycin were discovered to inhibit the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α, in influenza and rhinovirus-modelled infections (Batiha *et al.*, 2021). The anti-inflammatory mechanism of macrolides involves inhibiting the activation of NF-κB in cell

nuclei and transcription reduction, which makes it a promising candidate drug for COVID-19 treatment, as much of the disease's pathology is driven by inflammation (Rashad *et al.*, 2021).

4.5 Limitations and Future work

One major limitation of our study, as with most studies employing co-culture of *H. pylori* with gastric epithelial cell lines, is that the cells were derived from gastric tumours. Hence, the gene expression from these cell lines may not resemble that typically occurring in the normal gastric mucosa *in vivo*. Despite this, gastric cancer cells in culture are valuable for providing an initial wide gene expression profile associated with *H. pylori* infection, and specific genes involved in several pathways can then be examined in more detail *in vivo* (Lim *et al.*, 2003). Therefore, the development of human gastric organoids has garnered significant attention for its potential to address these limitations.

Organoids are cell aggregates cultured in three-dimensions that grow with similar characteristics as their tissue-of-origin. This novel model of self-renewing gastric epithelium derived from adult stem cells from primary tissues, embryonic stem cells, or induced pluripotent stem cells can differentiate into various stomach lineages and more accurately represents the gastric epithelium than commonly used cell lines. Due to their self-renewal and proliferative capacity, organoids can be sustained in culture for extended periods and, in many instances, can be expanded indefinitely (Seidlitz *et al.*, 2021). Unlike gastric cancer cell lines, the organoids model also permits the parallel cultivation of normal and cancerous gastric cells from the same patients. Such capabilities will facilitate the development of

patient-derived disease models, drug screening, gastric stem cell research, and studies of host-pathogen interactions (Bartfeld *et al.*, 2015).

Numerous reviews have focused on gastric organoids, especially in the context of studying gastric cancer and *H. pylori* pathogenesis, as well as the creation of human gastric organoids (Eicher *et al.*, 2018; Idowu *et al.*, 2022; Traulsen *et al.*, 2021). Past literature has highlighted progress in techniques and new findings in the field of cancer initiation (Aguilar *et al.*, 2021; Traulsen *et al.*, 2021).

Gastric organoid cultures seem to more closely resemble the *in vivo* situation compared to gastric epithelial cell lines when examining the interaction of *H. pylori* with the apical-junctional complex. Uotani *et al.* (2019) observed that the epithelial morphology and IL-8 expression were akin to those found in *in vivo* model studies. This study also discovered that, unlike previous findings with gastric cancer cell lines (Backert & Naumann, 2010; Brandt *et al.*, 2005), IL-8 expression was unaffected by *H. pylori* virulence factors like CagA. The wildtype *H. pylori* strain TN2GF4 led to higher IL-8 expression in AGS and MKN28 cells compared to a *cag*PAI deletion mutant; however, no difference in IL-8 expression was noted in gastroid monolayers infected with both strains. Additionally, clinical isolates missing virulence factors such as the *cag*PAI, OipA, VacA, and BabA increased IL-8 expression in gastroid monolayers but did not alter IL-8 levels in MKN28 cells. These findings suggest that *H. pylori* might have varying impacts on cancer patients compared to non-cancer patients.

However, a limitation is that the gastric organoids used were derived from only one healthy individual and were developed solely from the antrum, rather than the corpus, which is predominantly impacted by gastric cancer (Miehlke *et al.*, 1998). Therefore, additional research is needed using organoids from both regions of the stomach in both cancer and

non-cancer patients, along with studies on the roles of different cell types present in the antrum and corpus.

Organoid cultures have demonstrated the capability to replicate the increased expression of inflammatory cytokines in response to *H. pylori* infection, as observed in both mucosoid cultures and 3D spheroid models (Boccellato *et al.*, 2019). Sebrell *et al.* (2019) also found that CXCL2, and CXCL8 were upregulated in human 3D spheroids after 3 hours of infection, with CXL8 secretion being higher in spheroids infected with $CagA^+$ strains compared to those with CagA-deficient strains, consistent with findings in human gastric epithelial cell lines (Tran *et al.*, 2017). Therefore, future research should replace *H. pylori*-infected cell line tissue cultures with the new gastric organoid models.

Future studies also need to include TNFα-stimulated AGS cells with the solvents used to dilute antibiotics (ethanol and DMSO) as one of the control groups. These are important to rule out solvent effects on AGS cells viability, as they would show if the reduction in IL-8 and defensin was probably due to the solvents killing the AGS cells co-cultured with clinical isolates of *H. pylori*. It has been reported that DMSO can be employed without toxic effects at concentrations less than 0.6% for HepG2, MDA-MB-231, MCF-7, and VNBRCA1 human cancer cell lines. Ethanol had no toxic effects on these cell lines at concentrations below 1.25% (Nguyen *et al.*, 2020). In the present study, ethanol and DMSO were used at approximately 0.00254%, indicating that the solvent concentrations for the experiment on the inhibitory effect of macrolides on IL-8 and defensin production were very low and not likely to be significantly cytotoxic to the cells.

The current design of these co-culture experiments makes it impractical to incorporate a significantly larger number of bacterial strains and mutants, multiple parallel controls, or to

perform complex pre-treatments. Ideally, it would be interesting to characterise the signalling pathways that underlie different host cytokine responses to various stimulation upon infection with *H. pylori* in gastric epithelial cells. For example, profiling the TLR and transcription factor responses to various bacterial mutants and conditions aims to understand better the mechanism by which *H. pylori* induce immune tolerance via the TLR signalling pathway, which is still not fully elucidated.

A recent study by Zhang *et al.* (2024a) observed that TLR6 levels and inflammatory cytokines in human gastric epithelial cell lines initially increased and then decreased during *H. pylori* treatment both *in vitro* and *in vivo*. They also found that enhancing the TLR6 expression boosted the levels of IL-1β and IL-8 in gastric epithelial cells, which in turn recruited neutrophils and diminished *H. pylori* colonization in the gastric mucosa of gerbils. This study also discovered that chronic infection with *H. pylori* diminishes the sensitivity of TLR6 to bacterial components and modulates the expression of inflammatory cytokines in gastric epithelial cells via TLR6/JNK signalling. These promising findings indicate that TLR6 could be a potential candidate for immunotherapy in *H. pylori* infections, as TLR6 agonists significantly reduced the inflammation caused by *H. pylori* infection both *in vitro* and *in vivo* (Zhang *et al.*, 2024a).

4.6 Chapter summary

- No effect on *H*. *pylori* density was found when TNFα was added to co-cultures with AGS cells.
- IL-8 production was significantly increased during *H. pylori* infection of AGS cells with TNFα stimulation *in vitro.*
- Defensin hβD2 expression was significantly upregulated during *H. pylori* infection of AGS cells with TNFα stimulation *in vitro*.
- Macrolides were found to downregulate the IL-8 production induced by *H. pylori* infection of AGS cells *in vitro,* and the observed effects were greater for *cagA*+ strains than *cagA*- strains.
- Macrolides were also found to reduce the secretion of defensin hβD2 in response to *H. pylori* infection of AGS *in vitro*.

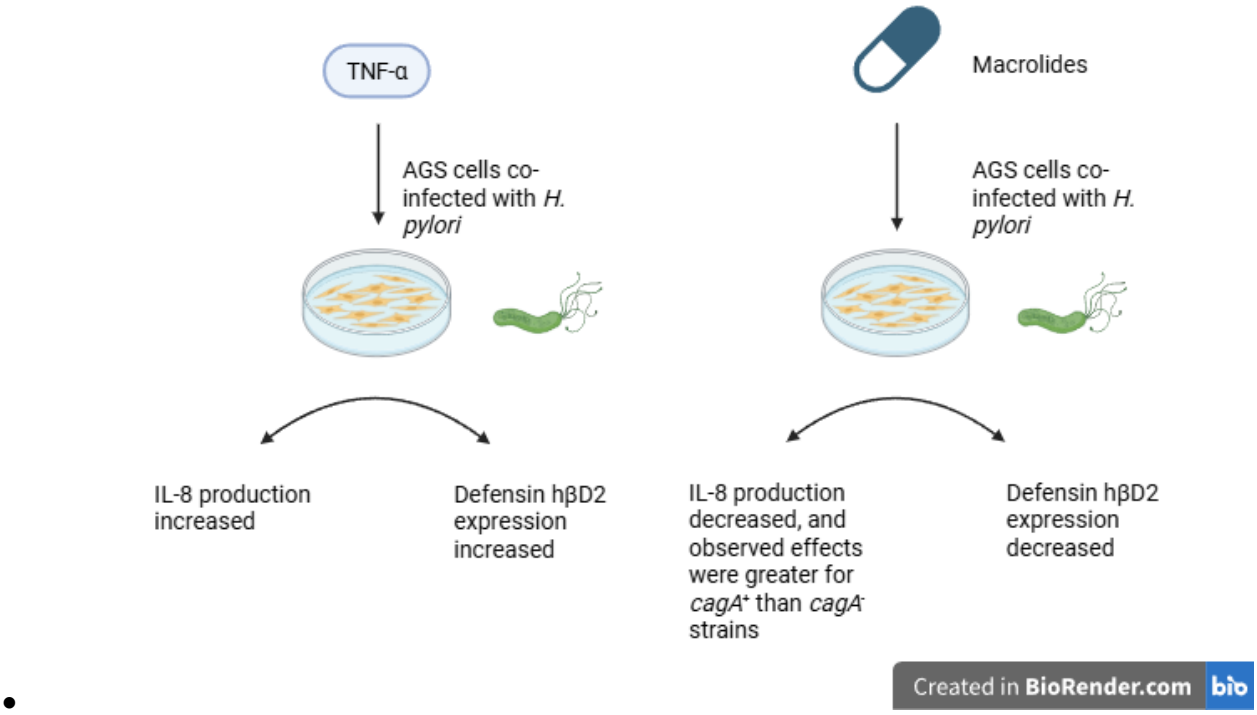


Figure 4.13 Schematic representation of H. pylori–host interactions under TNFα stimulation and the modulatory effects of macrolides on inflammatory and defensin responses

Chapter 5 : Whole-genome sequencing analysis of antimicrobial resistance in *H. pylori* clinical isolates

5.1 Introduction

Bacteria employ two primary genetic strategies to adapt to antibiotic exposure. The first involves acquiring resistance genes through horizontal transfer of foreign DNA, while the second involves mutations in genes related to the drugs' mechanisms of action (Munita & Arias, 2016; Tuan *et al.*, 2019). *H. pylori*'s antimicrobial resistance is primarily due to point mutations. Other mechanisms include reduced permeability from increased outer membrane protein expression or porin loss, efflux pump transporter overexpression, and biofilm formation (Liu *et al.*, 2022; Marques *et al.*, 2020).

The genomic diversity of *H. pylori* plays a vital role in linking genetic components to antibiotic resistance. A well-documented intrinsic resistance mechanism in *H. pylori* indicates that resistance mainly arises from mutations in antimicrobial target genes. In particular, the single-nucleotide mutations in the target genes for clarithromycin (*23S rRNA*), metronidazole (*rdxA* and *frxA*), and levofloxacin (*gyrA*) allow *H. pylori* to evade these antibiotics by inhibiting bacterial protein synthesis, hindering the intracellular reduction-activation of metronidazole, and blocking DNA replication and transcription in bacteria, respectively (Alba *et al.*, 2017; Gong & Yuan, 2018).

Traditional methods for testing antibiotic susceptibility such as disk diffusion and E-test techniques face several challenges and limitations. Culturing *H. pylori* is time-consuming since it grows slowly and requires a special tri-gas incubator. This entire process is laborious as it can take several weeks, with a culture success rate from gastric biopsies of only about 50%–70%. Additionally, most domestic hospital laboratories do not offer these tests, and routine susceptibility testing for *H. pylori* is generally not performed in clinical settings,

which often leads to empirical treatment. This approach can result in treatment failure and an increasing resistance rate (Chey *et al.*, 2017; Hu *et al.*, 2023).

In the last ten years, several molecular methods, such as PCR, Sanger sequencing, and whole genome sequencing (WGS), have been utilized to detect both known and novel markers for quickly predicting phenotypic antimicrobial resistance. Some tests are commercially available and less expensive, e.g. the clarithromycin and levofloxacin stool PCR test (Fan *et al.*, 2024; Mommersteeg *et al.*, 2023). A high throughput multiplex genetic detection assay (HMGA) for detection, semi-quantification, and virulence genotyping of *H. pylori* from non-invasive oral samples has been also validated as a reliable diagnostic tool for detection, semi-quantification, and virulence genotyping of *H. pylori* (Chi *et al.*, 2023). Other tests can be used directly with biopsy tissues without the need to isolate *H. pylori* (Pittie *et al.*, 2024; Tran *et al.*, 2024), but similar resistance genes and mutations may be present on other organisms in the gastric microbiota. Due to the marked genetic diversity of *H. pylori* and the significant variation in resistance genotypes across different geographical areas, identifying new resistance patterns and interpreting the results is quite challenging.

Since the early 21st century, the development of next-generation sequencing (NGS) technologies has provided a comprehensive, cost-effective, and rapid tool (with a turnaround time of 24 to 72 hours) for disease surveillance, predicting drug resistance, and conducting evolutionary analysis of infectious diseases. PacBio real-time sequencing (Pacific Bioscience Inc., CA, USA) and Oxford Nanopore sequencing (Oxford Nanopore Technologies Ltd., UK) are third generation "direct" sequencing techniques that do not require an amplification step. At present, Illumina short reads and PacBio sequencing are the most used methods for sequencing bacterial genomes (Saracino *et al.*, 2021).

The NGS of the whole genome approaches have proven valuable in offering a thorough understanding of bacterial genotypes and are effective for detecting new genetic factors that contribute to the pathogen's antimicrobial resistance (Badr *et al.*, 2021). Different NGS approaches were used, either for WGS (Watanabe *et al.*, 2021), addressing, for instance, the correlation between genotypic markers and phenotype (Lang *et al.*, 2021; Schubert *et al.*, 2024), or antibiotic-resistance biomarker genes (Starkova *et al.*, 2024). A comparison between NGS-based stool susceptibility testing and gastric biopsy culture demonstrated that the NGS approach showed high concordance in detecting resistance to clarithromycin, levofloxacin, and amoxicillin (Bonilla *et al.*, 2023).

One recent study examined the antimicrobial susceptibility and short-read genome sequences of *H. pylori* isolates from patients with varying severities of gastritis. The comparative genome analysis identified genes linked to efflux pumps, restriction-modification systems, phages, insertion sequences, and virulence factors like the cytotoxin genes *cagA* and *vacA*. The study also found that the *H. pylori* isolates from different gastritis severities displayed variations in antimicrobial susceptibility and genome content. This information is valuable for developing targeted eradication treatment regimens for different gastritis types. Furthermore, they found that *H. pylori* isolates were phylogenetically distant from each other but were closely related to other lineages circulating in the country. Therefore, understanding the genetic and phenotypic diversity of *H. pylori* across regions is also crucial for shaping health policies that can effectively manage and prevent diseases locally and nationally (Alvarez-Aldana *et al.*, 2024).

In this study, WGS analysis were performed to detect mutations in antibiotic-resistance and sensitive *H. pylori* strains from infected patients, an area that has rarely been explored in *H.*

pylori research. This chapter also aimed to investigate the correlation between the occurrence of point mutations and amino acid changes in the target genes (genotypic) and phenotypic antibiotic susceptibility testing results previously done and assessed the agreement between them.

5.1.1 Varying global *H. pylori* amoxicillin resistance rates

The prevalence of amoxicillin resistance in *H. pylori* reported in various countries shows significant variation. Table 5.1 summarizes the findings on amoxicillin resistance from studies identified through a literature search for this thesis. In Europe, resistance of *H. pylori* to amoxicillin remains minimal, at 1.01% (Table 5.1). According to Megraud *et al.* (2021), the average prevalence of amoxicillin resistance in *H. pylori* strains in Europe remains very low at 0.2%, with a range from 0 to 1.7%. This indicates that amoxicillin resistance is not yet a significant issue in European countries. Conversely, McNulty *et al*. (2012) found a resistance rate of under 3% in the UK, which aligns with the current results of this study.

Amoxicillin resistance rates are higher in Asia, with Ghotaslou *et al.* (2015) and De Francesco *et al.* (2004) documenting rates of 24% and 11.6%, respectively. However, there is considerable variation in resistance across different Asian countries. For instance, Bhutan has reported no resistance (Vilaichone *et al.*, 2020). Additionally, significant discrepancies exist in reported resistance rates within the same country; for example, three separate studies in Taiwan found resistance rates of 1%, 34.1%, and 36.1% (Hu *et al.*, 2007; Hung *et al.*, 2009; Kuo *et al.*, 2021).

Africa has the highest observed resistance rates to amoxicillin. In Cameroon, a study revealed that 85.6% of strains were resistant to the antibiotic (Ndip *et al.*, 2008). Similarly,

high resistance rates were reported in the Democratic Republic of Congo and Nigeria, with 34.3% and 33.3% of *H. pylori* strains showing resistance, respectively (Harrison *et al.*, 2017; Tshibangu-Kabamba *et al.*, 2020).

Table 5.1 A summary of the reported amoxicillin resistance rates of H. pylori by country.

Country	Amoxicillin resistance rate (%)	Method	Defined resistance breakpoint (µg/ml)	Reference
Europe	**1.0**			
Bulgaria	1.1	Agar dilution	≥0.5	Boyanova *et al.* (2009)
Norway	0	E-test	>1	Larsen *et al.* (2013)
Spain	0	E-test	>0.125	Macías-García *et al.* (2017)
Sweden	0	Agar dilution	≥0.5	Storskrubb *et al.* (2006)
UK	2	E-test	>0.5	McNulty *et al.* (2012)
UK	3	E-test and disc diffusion	>0.125 and ≤25 mm	Garvey *et al.* (2023)
Asia	**12.8**			
Bhutan	0	E-test	>0.125	Vilaichone *et al.* (2020)
Cambodia	9.1	Agar dilution	>0.125	Tuan *et al.* (2019)
China	9	E-test	≥1000	Wang *et al.* (2019)
China	4.4	E-test	>0.12	Zhang *et al.* (2015)
India	17.6	Agar dilution	>0.12	Gehlot *et al.* (2016)

Iran	4	E-test	>0.125	Attaran *et al.* (2021)
Japan	13	E-test	≥0.1	Kageyama *et al.* (2019)
Japan	15.4	Agar dilution	≥0.5	Rimbara *et al.* (2008)
Mongolia	11.9	Agar dilution	≥0.25	Azzaya *et al.* (2020)
South Korea	6	Agar dilution	>0.5	Kim and Kim (2013)
South Korea	4.5	Agar dilution	≥0.5	Hwang *et al.* (2010)
Taiwan	36.1	E-test	>8	Hu *et al.* (2007)
Taiwan	1	E-test	≥0.5	Hung *et al.* (2009)
Taiwan	34.1	E-test	>0.12	Kuo *et al.* (2021)
Vietnam	25.7	E-test	≥0.125	Tran *et al.* (2022b)
Africa	**31.1**			
Cameroon	85.6	Disc diffusion and agar dilution	>8	Ndip *et al.* (2008)
Democratic Republic of Congo	34.3	Agar dilution	≥0.25	Tshibangu-Kabamba *et al.* (2020)
Nigeria	33.3	E-test	>0.125	Harrison *et al.* (2017)
Senegal	0	E-test	>0.5	Seck *et al.* (2013)
South Africa	2.5	Disc diffusion and agar dilution	≥1	Tanih *et al.* (2010)
North America	**5.0**			
Cuba	0	E-test	≥1	Llanes *et al.* (2010)

Honduras	10	E-test	>0.125	Ortiz *et al.* (2019)
Mexico	1.8	Agar dilution	>0.125	Camorlinga-Ponce *et al.* (2020)
USA	6.4	Agar dilution	>0.125	Hulten *et al.* (2021)
USA	7	E-test	>0.125	Saranathan *et al.* (2020)
South America	**9.5**			
Argentina	1	Agar dilution	≥0.5	Matteo *et al.* (2008)
Brazil	1.9	E-test	>1	Picoli *et al.* (2014)
Chile	2.3	E-test	>2	Otth *et al.* (2011)
Peru	32.9	E-test	>0.125	Boehnke *et al.* (2017)

*Amoxicillin resistance rate refers to the percentage of *H. pylori* strains which were resistant to amoxicillin in each study. Average rates for each continent are in bold. Method refers to the method used to determine the minimum inhibitory concentration (MIC) of amoxicillin.

The reasons for such significant variations remain unclear, and no correlation is evident between the method of MIC determination and resistance rates (Table 5.1). Previous research has demonstrated that E-Test strips, broth microdilution, and disc diffusion assays are all comparable in assessing amoxicillin susceptibility in *H. pylori* (McNulty *et al.*, 2002). Therefore, discrepancies might arise from broader variations across different laboratories. Differences in growth conditions and sample preparation could lead to varying results. As illustrated in Table 5.1, different studies utilized different breakpoints to define amoxicillin resistance, which could account for some of the observed variations.

The variations in resistance rates between countries and continents are likely influenced by the differing levels of antibiotic consumption. Previous research has demonstrated a strong correlation between the consumption of macrolides and clarithromycin resistance in *H. pylori* (Megraud *et al*., 2021), and a similar pattern may occur with amoxicillin. The availability of over-the-counter antibiotics could lead to increased amoxicillin resistance. For instance, a study in Nigeria discovered that 86% of respondents used non-prescribed antibiotics, with penicillin being the most frequently purchased class (Badger-Emeka *et al.*, 2018). Another study in Sub-Saharan Africa found that amoxicillin was the most commonly purchased antibiotic without a prescription (Belachew *et al.*, 2021). This could account for the higher resistance rates observed in some African countries. In contrast, in many European countries like the UK, amoxicillin can only be obtained with a prescription, which likely contributes to lower resistance rates.

In general, the resistance rate of *H. pylori* to amoxicillin is notably lower compared to many other bacterial species (Hrbacek *et al.*, 2020; Nagy *et al.*, 2011). This significantly lower resistance rate could be due to the infrequent acquisition of beta-lactamase genes by *H. pylori*. Although it was once believed that *H. pylori* could not acquire beta-lactamases, one study has identified an amoxicillin-resistant strain of *H. pylori* that possesses a beta-lactamase enzyme (Tseng *et al.*, 2009). Nonetheless, it is thought that the production of beta-lactamases is not a major factor in the resistance of *H. pylori* to amoxicillin.

5.1.2 Penicillin-binding proteins in *H. pylori*

Penicillin-binding proteins (PBPs), which serve as the targets for beta-lactam antibiotics like amoxicillin, can be classified into two primary categories: high molecular mass (HMM) and low molecular mass (LMM). The HMM PBPs are believed to be the principal targets for beta-

lactams and can themselves be subdivided into two classes. These are bifunctional Class A PBPs that possess both glycosyltransferase and transpeptidase activities, and Class B PBPs that solely exhibit transpeptidase activity (Macheboeuf *et al.*, 2006). The transpeptidase domain is situated at the C-terminus of the HMM PBPs, whereas in Class A PBPs, the glycosyltransferase domain is found at the N-terminus (Sauvage *et al.*, 2008). The transpeptidase domain, which is the binding site for beta-lactams, is primarily the focus when searching for mutations associated with resistance. This penicillin-binding domain consists of two sub-domains: a 5-stranded beta-sheet and an alpha-helical domain (Figure 5.1). The active site is situated at the interface of these sub-domains and contains widely conserved amino acid motifs. These motifs are SxxK, which includes the active serine and is consistently found at the N-terminus of alpha-helix 2; SxN, positioned between alpha-helices 4 and 5; and KTG(T/S) (Harris *et al.*, 2000; Sauvage *et al.*, 2008).

The three conserved motifs in the active site serve distinct roles in maintaining the functionality of PBPs. The D-Ala-D-Ala sequence of peptidoglycan engages with the PBP active site, positioning the amide group of the second-to-last D-Ala near the asparagine side chain of the SxN motif and the beta-sheet that lines the site. The terminal D-Ala interacts with the conserved KTG(T/S) motif. The SxN and KTG(T/S) motifs have important roles in the positioning of the D-Ala-D-Ala substrate, while the glycine of KTG(T/S) is important as bulkier amino acids could block entry to the active site (Sauvage *et al.*, 2008). The active serine in the SxxK motif is acylated by the penultimate D-Ala of a donor peptidoglycan pentapeptide, leading to the release of the terminal D-Ala. This acylated enzyme then interacts with a second pentapeptide chain, known as the acceptor, which triggers the transpeptidase activity (Catherwood *et al.*, 2020).

The number of PBPs present in a bacterium varies by species, with *H. pylori* having been reported to possess up to 9 (Harris *et al.*, 2000). PBP1A, 2, and 3 are HMM PBPs with amoxicillin showing a binding affinity for all of them (Attaran *et al.*, 2021). However, in amoxicillin-resistant *H. pylori* strains, it is the reduced affinity to PBP1A that is observed (Okamoto *et al.*, 2002). Studies have indicated that amino acid changes in the *PBP1A* gene are the primary reason for amoxicillin resistance in *H. pylori* (Gerrits *et al*., 2006).

5.1.3 Amoxicillin-resistant penicillin-binding proteins

Beta-lactam antibiotics mimic the D-Ala-D-Ala segment of peptidoglycan. They form a covalent bond with serine in the active site, preventing peptidoglycan from accessing it and thus hindering cross-linking. Molecular modelling of *H. pylori* PBP1A revealed that the access pathway to the active site is quite narrow, and mutations within this pathway are linked to amoxicillin resistance. Changes in the residues within either the active site or the access pathway might affect amoxicillin's ability to interact with the active serine (Attaran *et al.*, 2021).

Research on *Streptococcus pneumoniae* revealed that the active site is located within a narrow, tunnel-like formation. Alterations at the entrance of this active site changed its polarity and accessibility, and these mutations were associated with beta-lactam-resistant strains (Contreras-Martel *et al.*, 2006). Mutations found in PBPs of resistant *S. pneumoniae* caused a conformational change to a loop at the entrance to the active site which in turn reduced the antibiotic binding (Carapito *et al.*, 2006). A similar phenomenon occurs in *Neisseria gonorrhoeae*, where the insertion of an aspartate residue on a loop close to the PBP active site results in decreased affinity for beta-lactams (Fedarovich *et al.*, 2014). Other resistance-associated mutations in *N. gonorrhoeae* PBPs resulted in more subtle structural

alterations; nevertheless, these changes increased the flexibility near the active site. This enhanced flexibility is believed to affect beta-lactam binding ability or acylation (Powell *et al.*, 2009). This highlights the complexity of mutations responsible for beta-lactam resistance. Therefore, this study aimed to identify the amino acid changes in *H. pylori* PBP1A and PBP2 in strains resistant and sensitive to amoxicillin and evaluate the association between these amino acid changes and amoxicillin resistance.

5.1.4 Objectives

The objectives of this chapter were to:

1. Analyse Illumina MiSeq genome sequence data from 35 clinical isolates of *H. pylori*.
2. Identify point mutations and amino acid changes in antibiotic-resistant and sensitive *H. pylori* strains from infected patients.
3. Explore the relationship between point mutations and amino acid changes in target genes (genotypic) and antibiotic susceptibility testing results (phenotypic) and evaluate how well they align.
4. Determine the amino acid substitutions in *H. pylori* PBP1A and PBP2 in amoxicillin-resistant and -sensitive strains and assess the relationship between amino acid substitution and amoxicillin resistance.
5. Predict the 3D structure of *H. pylori* 26695 strain's PBP1A and PBP2 using I-TASSER software.
6. Map the amino acid mutations in *H. pylori* 26695 strain's PBP1A on the 3D structure using AlphaFold2.

5.2 Materials & Methods

5.2.1 *H. pylori* clinical isolates used

Table 5.2 Table of H. pylori clinical isolates used

Experiment	*H. pylori* clinical isolates used
Genomic extraction and whole genome sequencing for prediction of antimicrobial resistance patterns	35 *H. pylori* clinical isolates from 19 infected patients in the UK (extracted by Mrs Joanne Rhead, and sequenced by Dr Jonathan Thomas) were assembled, curated, and annotated. The genotypic analysis using Comprehensive Antibiotic Resistance (CARD) databases for antibiotic resistance was carried out on 36 genome assemblies (where 35 genome assemblies were produced from 35 *H. pylori* clinical isolates, and one genome assembly from *H. pylori* 26695 reference strain), and compared with phenotypic tests (E-test) done previously.
Analysis of PBP amino acid substitution in clinical *H. pylori* strains	16 *H. pylori* clinical isolates from 11 infected patients in the UK were extracted by Mrs Joanne Rhead and sequenced by Dr Jonathan Thomas. Two strains from patients in the USA were sequenced, and their amoxicillin susceptibility was determined by (Saranathan *et al.*, 2020).

	Two strains from patients in Cambodia were sequenced, and their amoxicillin susceptibility was determined by (Tuan *et al.*, 2019). All strains were assembled, curated, and annotated. The *H. pylori* 26695 strain was used as the template to protein BLAST (Basic Local Alignment Search Tool) all the clinical strains against to identify any substitutions in the amino acid sequence.

5.2.2 Genomic extraction and whole genome sequencing

A total of 35 *H. pylori* isolates from 19 infected patients were tested for antibiotic susceptibility from the Nottingham strain collection and selected for whole genome sequencing (Table 5.2). Among these, paired isolates from both the antrum and corpus were collected from 16 patients, isolates from two patients were obtained exclusively from the corpus, and one patient had an isolate only from the antrum. Genome sequences of these clinical *H. pylori* strains with known antibiotic susceptibility profiles (from antibiotic sensitivity tests – see Chapter 2) were obtained as previously described (Wilkinson, 2019). Genomic DNA was extracted by Mrs Joanne Rhead using the QIAmp DNA Mini Kit (QIAGEN, Netherlands) as per the manufacturer's instructions. Dr Jonathan Thomas at Nottingham Trent University performed whole genome sequencing on 35 clinical isolates using the Illumina MiSeq platform (Illumina, USA).

5.2.3 Quality control and trimming of sequencing reads

5.2.3.1 Quality control using FASTQC

After Illumina sequencing, the data generated raw sequence reads in a FASTQ format to display quality scores for each of the sequenced nucleotides (Galaxy, 2018). These Illumina paired-end sequence reads (Liu et al., 2020) were assessed for their quality using a tool called FastQC on the Galaxy online bioinformatics platform: https://usegalaxy.org/ (Liu *et al*., 2020).

5.2.3.2 Trimming of Illumina reads

Following the removal of Illumina sequencing adapters, the resulting reads were further trimmed for quality and length using Sickle (Galaxy version 1.33.2), based on a quality (Q) cut-off score and retain high-quality sequencing data (Joshi & Fass, 2011). A quality cut-off score of 30 was chosen to improve data quality and remove low-quality bases and adapters. To account for the potential trimming of adapter sequences and to maximize the number of processed reads while removing very short reads, those of less than 50 bases in length were removed from the dataset.

The curated reads were analysed using FastQC (Galaxy version 0.74 + galaxy1) (Andrews, 2010) to confirm the expected trimming outcomes. Specifically, this included removing sequencing adapters, ensuring base quality greater than 30, and maintaining a length distribution between 50 and 250 bases. Separate reports were generated for each set of forward and reverse reads and manually reviewed for each sample.

5.2.4 Whole genome assembly of *H. pylori* isolates

Curated sequencing reads were *de-novo* assembled using SPAdes version 3.15.5 + galaxy2 (Bankevich *et al.*, 2012). Spades is a *de novo* genome assembler that uses short read sets as input, and the assembly method is applied based on de Bruijn graphs (Compeau *et al.*, 2011). Paired-end data is handled by reverse complementing one of the sequencing mates and assembling both together as a read pair, where resulting graphs show the reverse complement at their edge. The genome assembly tool combines the reads into larger regions called contigs (Gurevich *et al.*, 2013).

5.2.5 Curation of assembled genomes

Each sample's assembled contigs were subsequently evaluated using the QUAST tool version 5.2.0 + galaxy1 (Gurevich *et al.*, 2013), which provides statistics on assembly quality. QUAST presents assembly statistics such as the total number of contigs, largest contig, total length, N50 (a value indicating the minimum contig size such that contigs of this size or larger cover half of the genome), GC content (%), and mismatches (Gurevich *et al.*, 2013). The expected genome size of *H. pylori* is approximately 1.68 Mb, and a variation of ± 1.4 kb was accepted. This adjustment was necessary as it exclusively used high-quality reads (Q score ≥ 30) for genome assembly, which can lead to a slightly smaller genome size compared to using all available sequencing reads. This approach was adopted to ensure that the assembled contigs were of high quality for subsequent analysis (Wilkinson, 2019).

5.2.6 Whole genome annotation

Whole genome annotation is the process of identifying features of interest within a collection of genomic DNA sequences and labelling them with useful information (Seemann,

2014). The complete genome annotation was done using the Prokka tool (Galaxy Version 1.14.6+galaxy1), designed for rapid prokaryotic genome annotation, and produced standard output files that require only minor tweaking to submit to GenBank. Prokka was executed in 'compliant' mode to generate an output file in Genbank standard file format. Additional taxonomic details, including kingdom, genus, and species identifiers, were embedded into the Genbank file produced by Prokka. To confirm whether the *de novo* genome assemblies were *H. pylori* strains, the presence of *cagA* and *vacA* genes (Argent *et al.*, 2008) was determined visually by finding regions of DNA sequence similarity among the largest contigs for each *H. pylori* strain.

5.2.7 Antimicrobial resistance prediction using genome sequence data

This study used an online database, Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/home) (Alcock *et al.*, 2020) to predict antimicrobial resistance in 35 *H. pylori* clinical isolates. The genomes assembled in FASTA file format were utilized as inputs for the database. This database was originally compiled for other more commonly researched organisms, e.g. *E. coli, Pseudomonas aeruginosa*, and therefore some *H. pylori* AMR genes may not be included.

5.2.8 Analysis of PBP amino acid substitutions in clinical *H. pylori* strains

5.2.8.1 Clinical H. pylori sequences

H. pylori genome sequences of 16 clinical strains of known amoxicillin susceptibility profiles were obtained as described in section 5.2.2 and four strains were accessed from published data (Table 5.2). Two strains from patients in the USA were sequenced and their amoxicillin susceptibility was determined by Saranathan *et al.* (2020). Two strains from patients in

Cambodia were sequenced and amoxicillin susceptibility was determined by Tuan *et al.* (2019). A summary of the strains accessed, their accession number, and amoxicillin status is shown in Table 5.3.

Table 5.3 A summary of the H. pylori strains analysed.

Strain	Origin	Accession number	Amoxicillin resistance status
45a	UK	-	Susceptible
45c	UK	-	Susceptible
295a	UK	-	Susceptible
295c	UK	-	Susceptible
439a	UK	-	Susceptible
444a	UK	-	Susceptible
495a	UK	-	Susceptible
732a	UK	-	Susceptible
120a	UK	-	Resistant
120c	UK	-	Resistant
308a	UK	-	Resistant
308c	UK	-	Resistant
335c	UK	-	Resistant
350a	UK	-	Resistant
350c	UK	-	Resistant
391c	UK	-	Resistant
MHP11	USA	SAMN12783476	Resistant
MHP06	USA	SAMN12783471	Susceptible
KH0141	Cambodia	SAMN12011831	Resistant

KH0097	Cambodia	SAMN12011820	Susceptible

*Sequences were obtained from studies with patients from the UK, Cambodia (Tuan *et al*., 2019), or the USA (Saranathan *et al*., 2020). The reported amoxicillin resistance status of Cambodian and American strains was obtained from their respective studies. UK strains were sequenced, and amoxicillin susceptibility was determined as previously described in Section 5.2.1.

5.2.8.2 Analysis of PBP amino acid mutations

The largest contigs from genome assemblies were submitted to the Pathosystems Resource Integration Center (PATRIC) (https://www.bv-brc.org/), where the comprehensive genome analysis tool annotated the sequences using RASTtk (Gillespie *et al.*, 2011). The PBP1 was annotated as a multi-modular transpeptidase transglycosylase, and PBP2 was identified as Peptidoglycan D, D-transpeptidase MrdA. The amino acid FASTA files for each strain's PBP2 were located using the PATRIC interface. The *H. pylori* 26695 strain (Accession number: SAMN02603761) was used as the template to protein BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) the clinical strains against to identify any substitutions in the amino acid sequence. To visualise the substitutions, FASTA sequences were compared using the Geneious Prime version 2024.0.7 software with the *H. pylori* 26695 strain used as the reference (https://www.geneious.com/).

5.2.9 3D Structure prediction of *H. pylori* 26695 strain PBP1A and PBP2

A 3D model of the PBP1A and PBP2 from the reference strain 26695 (GenBank accession number: AFV42789.1) was predicted using I-TASSER software: https://zhanggroup.org/D-I-TASSER/ (Zhang, 2008). The model was generated by inputting the FASTA amino acid sequence without applying any constraints or templates.

5.2.10 Mapping amino acid substitutions on the 3D structure

H. pylori 26695 PBP1A protein folding was predicted with AlphaFold2 (Jumper *et al.*, 2021; Skolnick *et al.*, 2021) (https://deepmind.com/research/open-source/alphafold) using the default setting. The top-rated predicted structure was visualised using UCSF ChimeraX (Pettersen *et al.*, 2021). The substitutions identified as most associated with amoxicillin resistance were mapped.

5.2.11 Statistical analysis

The correlation between the types of mutations in antibiotic resistance genes and phenotypic resistance in *H. pylori* strains was investigated using Fisher's exact test. The agreement between antibiotic-resistant phenotypes and genotypes was evaluated using accordance rates and the kappa consistency test. A P-value <0.05 was considered to denote statistical significance.

5.3 Results

5.3.1 Comparative analysis between the phenotypic and genotypic detection of antibiotic resistance among *H. pylori* isolates

Genotypic analysis using CARD databases for antibiotic resistance was carried out on genome assemblies from 36 *H. pylori* strains and compared with phenotypic tests (E-test) done previously. Table 5.4 shows the correlation between phenotypic and genotypic antibiotic resistance *in H. pylori*. There was strong concordance between phenotypic and genotypic resistance for levofloxacin, with *gyrA* genotypes effectively distinguishing strain susceptibility to these antibiotics (kappa coefficient of 1) (Table 5.4). In contrast, the agreement between phenotypic and genotypic resistance for amoxicillin, clarithromycin, and metronidazole was unsatisfactory (kappa coefficients of 0.000, 0.000, and -0.379, respectively).

The correlation between the types of mutations in antibiotic resistance genes and phenotypic resistance in *H. pylori* strains is presented in Table 5.5. The most common mutation locus for clarithromycin resistance was A2144G and C1707T in *23S rRNA* genes, with 100% (9/9) of the resistant strains carrying mutation at these points, whilst the prevalence of the A2147G mutation (89%) (8/9) was significantly distinct between the clarithromycin-sensitive and clarithromycin-resistant strains ($p<0.0001$) (Fisher's exact test) (Table 5.5).

The amino acid changes E572G and S49H in *PBP2* were most frequent in amoxicillin-resistant strains with prevalences of 100% (8/8) for both mutations. As for *PBP3*, 100% (1/1) of amoxicillin-resistant strains had an amino acid change at F490Y, whereas an amino acid

change at A541T was found in 66% (4/6) of amoxicillin-sensitive strains (Table 5.5). Meanwhile, all levofloxacin-resistant strains (2/2) had an amino acid change D91N in the *gyrA* gene. Overall, amino acid alterations did not show any significant differences between sensitive and resistant strains for both amoxicillin and levofloxacin.

Analysis of the *rdxA* gene mutations related to metronidazole resistance, revealed that the amino acid change D59N was most frequent in metronidazole-resistant strains with a prevalence of 100% (14/14), followed by C49T, T31E, S108A, A118T, and R16C with a prevalence of 86% (12/14), 36% (5/14), 28% (4/14), 21% (3/14), 7% (1/14), respectively. However, none of these amino acid changes showed any significant differences between sensitive and resistant strains except for A118S (p=0.041) (Fisher's exact test) (Table 5.5).

Moreover, the amino acid changes R90K and Y62D in the *frxA* gene were found in all metronidazole-resistant strains, with a prevalence of 100% (8/8) for both. Half of metronidazole-resistant strains (4/8) exhibited the A85V amino acid changes. The K64N, H97T, and V71 amino acid changes were present in 25% (2/8), 13% (1/8), and 13% (1/8) of the resistant strains, respectively. It was also shown that the prevalence of the A85V and R90K amino acid changes were significantly different between metronidazole-sensitive and metronidazole-resistant strains (p=0.03) (Fisher's exact test) (Table 5.5).

Interestingly, the majority of patients with paired gastric biopsies (14 out of 16) exhibited more synonymous amino acid changes between the paired antrum and corpus samples. Only two patients (2 out of 16) displayed non-synonymous amino acid changes. In one of these cases, the patient had distinct amino acid changes in the PBP2 in the paired antrum and corpus samples, and in the other case, the patient had different amino acid changes in the FrxA and RdxA proteins in the paired antrum and corpus samples.

Table 5.4 Agreement between the phenotypic and genotypic resistance.

		Phenotypic resistance				
Antibiotics	**Genotypic resistance**	Sensitive	Resistant	Accordance rate	Kappa coefficient (95% CI)	Cohen's Kappa interpretation
Amoxicillin	Sensitive	0	0	22.22%	0.000 (-0.000 to 0.000)	No agreement
	Resistant	28	8			
Clarithromycin	Sensitive	0	0	25%	0.000 (0.000 to 0.000)	Slight agreement
	Resistant	27	9			
Metronidazole	Sensitive	0	9	44.44%	-0.379 (-0.539 to -0.220)	No agreement
	Resistant	11	16			
Levofloxacin	Sensitive	34	0	100%	1.000 (1.000 to 1.000) *	Perfect agreement
	Resistant	0	2			

*A strong agreement between phenotypic and genotypic resistance to levofloxacin, with *gyrA* genotypes accurately identifying strain susceptibility to the antibiotic (kappa coefficient of 1*).

Table 5.5 Correlation of mutation types (SNPs) of resistance genes 23S rRNA, amino acid changes in PBP2, PBP3, GyrA, RdxA, and FrxA proteins, and phenotypic resistance in H. pylori.

Mutation type	Resistant phenotype		*P* value
	Resistant, n (%)	Sensitive, n (%)	
Clarithromycin (*23S rRNA*)	9	27	
A2144G	9 (100%)	27 (100%)	>0.999
A2147G	8 (89%)	0 (0.00%)	<0.0001***
C1707T	9 (100%)	27 (100%)	>0.999
Amoxicillin (PBP2 and PBP3)			
PBP2	8	28	
E572G	8 (100%)	27 (96%)	>0.999
S494H	8 (100%)	28 (100%)	>0.999
PBP3	1	6	
A541T	0 (0%)	4 (66%)	0.555
F490Y	1 (100%)	2 (33%)	0.541
Levofloxacin (GyrA)	2	0	
D91N	2 (100%)	0	>0.999
Metronidazole (RdxA and FrxA)			
RdxA	14	13	
A118S	0	4 (31%)	0.041*
A118T	3 (21%)	6 (46%)	0.237
C49T	12 (86%)	11 (84%)	>0.999
D59N	14 (100%)	11 (85%)	0.222
R16C	1 (7%)	0 (0%)	>0.999

S108A	4 (28%)	2 (15%)	0.648
T31E	5 (36%)	7 (54%)	0.450
FrxA	8	9	
A85V	4 (50%)	0 (0%)	0.030*
H97T	1 (13%)	2 (22%)	>0.999
K64N	2 (25%)	1 (11%)	0.577
R16H	0 (0%)	2 (22%)	0.471
R90K	8 (100%)	4 (44%)	0.030*
V71	1 (13%)	4 (44%)	0.294
Y62D	8 (100%)	7 (78%)	0.471

5.3.2 Amino acid substitutions in *H. pylori* PBP1A and PBP2

Table 5.6 and Table 5.7 summarizes all the strains analysed, the number of substitutions, location of substitutions, and whether the amino acid change is conservative, i.e. change is to an amino acid with similar biochemical properties. On average, susceptible strains had 11.8 amino acid substitutions per strain in the PBP1A protein (Table 5.6), whereas resistant strains had 13.6 (Table 5.7). Meanwhile, susceptible strains showed 13.3 amino acid substitutions per strain in the entire PBP2 protein, compared to 12.8 in resistant strains.

There was also an increase in non-conservative substitutions, i.e. major amino acid changes with 4.5 and 8 substitutions per strain for susceptible and resistant strains in the PBP1A protein, respectively. Meanwhile, susceptible strains displayed an average of 8 amino acid substitutions across the entire PBP2 protein per strain, whereas resistant strains exhibited an average of 7.4 substitutions.

Beta-lactams bind to the transpeptidase (TP) domain, therefore the prevalence of substitutions in this domain was also analysed. On average, 7 substitutions per strain were seen in the TP domain of PBP1A-resistant strains compared to 5.2 in susceptible strains. Major non-conservative amino acid changes were found on average 3.1 times in the TP domain of PBP1A-resistant strains compared to 1.5 times in susceptible strains. Furthermore, resistant strains exhibited an average of 4.1 substitutions per strain in the TP domain of the PBP2 protein, while susceptible strains showed an average of 4.0 substitutions. Additionally, non-conservative amino acid changes occurred on average 1.7 times in the TP domain of PBP2-resistant strains, compared to 1.5 times in susceptible strains. Overall, there was an increase in amino acid substitutions in resistant strains compared to susceptible strains.

Table 5.6 A summary of the substitutions found in the PBP1A and PBP2 amino acid sequences of amoxicillin-susceptible clinical H. pylori strains compared to the reference genome H. pylori 26695.

	PBP1A					PBP2				
Strain	**Identities (%)**	**Positives (%)**	**Gaps (%)**	**Substitution**	**Conservative**	**Identities (%)**	**Positives (%)**	**Gaps (%)**	**Substitution**	**Conservative**
45a	648/659 (98%)	654/659 (99%)	0/659 (0%)	V16I	+	573/588 (97%)	579/588 (98%)	0/588 (0%)	A21V	-
				M17I	+				N55D	+
				A46V	-				F63L	-
				F125L	-				D83E	+
				E266K	+				I90V	+
				D272N	+				A128I	-
				D336E	+				T129A	-
				S338N	+				A145V	-
				S543R	-				L224P	-
				I643T	-				I254V	+
				S653G	-				H326N	+
									H344R	-

									L415F	-
									I508V	+
									H539Q	-
45c	648/659 (98%)	654/659 (99%)	0/659 (0%)	V16I	+	573/588 (97%)	579/588 (98%)	0/588 (0%)	A21V	-
				M17I	+				N55D	+
				A46V	-				F63L	-
				F125L	-				D83E	+
				E266K	+				I90V	+
				D272N	+				A128I	-
				D336E	+				T129A	-
				S338N	+				A145V	-
				S543R	-				L224P	-
				I643T	-				I254V	+
				S653G	-				H326N	+
									H344R	-
									L415F	-

									I508V	+
									H539Q	-
295a	648/662 (98%)	654/662 (98%)	3/662 (0%)	V16I	+	575/588 (98%)	581/588 (98%)	0/588 (0%)	A21V	-
				V45I	+				N55D	+
				F125L	-				D61H	-
				323_324insD	-				D83E	+
				S417T	+				N106D	+
				D479E	+				F124L	-
				M515I	+				A135T	-
				D535N	+				A145V	-
				S543R	-				V210I	+
				592_595insG	-				L224P	-
				593_596insG	-				H344R	-
				T644A	-				K466Q	+
				D651G	-				I508V	+
				R656P	-					

295c	648/662 (98%)	654/662 (98%)	3/662 (0%)	V16I V45I F125L 323_324insD S417T D479E M515I D535N S543R 592_595insG 593_596insG T644A D651G R656P	+ + - - + + + + - - - - - -	575/588 (98%)	581/588 (98%)	0/588 (0%)	A21V N55D D61H D83E N106D F124L A135T A145V V210I L224P H344R K466Q I508V	- + - + + - - - + - - + +
439a	654/659 (99%)	657/659 (99%)	0/659 (0%)	M17I F125L	+ -	578/588 (98%)	583/588 (99%)	0/588 (0%)	A21V G78R	- -

				S255F D535N S543N	- + +				D83E A145V L224P D258N H326N H344R K466Q I508V	+ - - + + - + +
444a	654/659 (99%)	658/659 (99%)	0/659 (0%)	K107R F125L I148L D508E K617R	+ - + + +	574/588 (98%)	579/588 (98%)	0/588 (0%)	A21I V50I D83E K93T T102A F124L P132S V168M	- + + - - - - +

									L224P T227A H326N H344R T396M I508V	- - + - - +
495a	649/659 (98%)	658/659 (99%)	0/659 (0%)	V16I M17I F125L D336E T492S D508E M515I D535N S543N	+ + - + + + + + +	578/588 (98%)	581/588 (98%)	0/588 (0%)	A21V D83E P132S L133F A145V Y182H R209H L224P H344R	- + - - - + - - -

				I552V	+				I508V	+
732a	651/659 (99%)	657/659 (99%)	0/659 (0%)	V16I M17I F125L D324N E406A D479E M515I D535N	+ + - + - + + +	577/588 (98%)	580/588 (98%)	0/588 (0%)	A21V D61N E89G P132S A135T A145V L146R V168M L224P H344R I508V	- + - - - - - + - - +
MHP06	637/659 (97%)	652/659 (98%)	0/659 (0%)	V16I M17I I22V I24V	+ + + +	568/588 (97%)	576/588 (97%)	0/588 (0%)	N3S F16V A21I V50I	+ - - +

				G44S	-				D61H	-
				I101V	+				T66I	-
				F125L	-				D83E	+
				I148L	+				I90M	+
				G242S	-				S99P	-
				N322D	+				P132S	-
				E406A	-				A145V	-
				S417T	+				L146R	-
				V469M	+				R209H	-
				N504D	+				L224P	-
				D508N	+				T227A	-
				V509I	+				D258N	+
				T511I	-				H344R	-
				V532A	-				N464H	+
				I541V	+				K466Q	+
				S589G	-				I508V	+

				T659S	+					
				R649K	+					
KH0097	642/660 (97%)	654/660 (99%)	1/660 (0%)	M17I	+	576/588 (98%)	581/588 (98%)	0/588 (0%)	A21V	-
				F125L	-				D61H	-
				M129I	+				D83E	+
				I148L	+				N106T	-
				G242S	-				F124L	-
				I1259T	-				A135T	-
				E406K	+				V223M	+
				D479E	+				L224P	-
				N504D	+				H326N	+
				D508N	+				H344R	-
				V509I	+				K466Q	+
				M515I	+				I508V	+
				D535N	+					
				S543N	+					

				595_596insS K648P R649K R656P	- - + -					

*Substitutions were determined using the protein BLAST. Identities refer to the number of identical amino acids in the sequence, positives include identities plus conservative amino acid changes where the amino acids have similar biochemical properties.

Table 5.7 A summary of the substitutions found in the PBP1A and PBP2 amino acid sequences of amoxicillin-resistant clinical H. pylori strains compared to the reference genome H. pylori 26695.

	PBP1A					PBP2				
Strain	**Identities (%)**	**Positives (%)**	**Gaps (%)**	**Substitution**	**Conservative**	**Identities (%)**	**Positives (%)**	**Gaps (%)**	**Substitution**	**Conservative**
120a	649/660 (98%)	656/660 (99%)	1/660 (0%)	V16I	+	576/588 (98%)	584/588 (99%)	0/588 (0%)	N55D	+
				M17I	+				E68K	+
				A69V	-				D83E	+
				K107R	+				A145V	-
				F125L	-				K202R	+
				I148L	+				L224P	-
				D508E	+				D293N	+
				V532A	-				H326N	+
				D535N	+				H344R	-
				I563V	+				T352I	-
				593_594insG	-				V424I	+
									K466Q	+

120c	649/660 (98%)	656/660 (99%)	1/660 (0%)	V16I M17I A69V K107R F125L I148L D508E V532A D535N I563V 593_594insG	+ + - + - + + - + + -	576/588 (98%)	584/588 (99%)	0/588 (0%)	N55D E68K D83E A145V K202R L224P D293N H326N H344R T352I V424I K466Q	+ + + - + - + + - - + +
308a	645/660 (98%)	652/660 (98%)	1/660 (0%)	A21V F125L V267A D324N	- - - +	576/588 (98%)	580/588 (98%)	0/588 (0%)	A21V N55D G78R D83E	- + - +

				V374L	+				P132S	-
				E406A	-				A135T	-
				N504D	+				L224P	-
				D508N	+				H326N	+
				V509I	+				H344R	-
				D535N	+				V367A	-
				S543N	+				I508V	+
				N562Y	-				M530T	-
				T592A	-					
				593_594insG	-					
				R607H	-					
308c	645/660 (98%)	652/660 (98%)	1/660 (0%)	A21V	-	576/588 (98%)	580/588 (98%)	0/588 (0%)	A21V	-
				F125L	-				N55D	+
				V267A	-				G78R	-
				D324N	+				D83E	+
				V374L	+				P132S	-

				E406A	-				A135T	-
				N504D	+				L224P	-
				D508N	+				H326N	+
				V509I	+				H344R	-
				D535N	+				V367A	-
				S543N	+				I508V	+
				N562Y	-				M530T	-
				T592A	-					
				593_594insG	-					
				R607H	-					
335c	641/660 (97%)	650/660 (98%)	1/660 (0%)	V16I	+	570/588 (97%)	574/588 (98%)	0/588 (0%)	N3S	+
				M17I	+				F16V	-
				F125L	-				A21I	-
				I148L	+				D61H	-
				G242S	-				T66I	-
				323_324insD	-				G78R	-

				D336E	+				D83E	+
				E406A	-				F94L	-
				S417T	+				P132S	-
				T503A	-				A135T	-
				N504D	+				A145V	-
				D508N	+				L146R	-
				V509I	+				R209H	-
				T511V	-				L224P	-
				V532A	-				T227A	-
				S589G	-				D258N	+
				T593A	-				H344R	-
				S603P	-				I508V	+
				R649K	+					
350a	650/660 (98%)	657/660 (98%)	1/660 (0%)	M17I	+	574/588 (98%)	581/588 (98%)	0/588 (0%)	A21V	-
				F125L	-				N55D	+
				V250I	+				G78R	-

				323_324insN	-				D83E	+
				D479E	+				I90M	+
				L530I	+				A128I	-
				D535N	+				T129A	-
				R546H	-				P132S	-
				I552V	+				V222I	+
				A611S	+				L224P	-
									D258N	+
									H344R	-
									K466Q	+
									I508V	+
350c	650/660 (98%)	657/660 (99%)	1/660 (0%)	M17I	+	574/588 (98%)	581/588 (98%)	0/588 (0%)	A21V	-
				F125L	-				N55D	+
				V250I	+				G78R	-
				323_324insN	-				D83E	+
				D479E	+				I90M	+

				L530I D535N R546H I552V A611S	+ + - + +				A128I T129A P132S V222I L224P D258N H344R K466Q I508V	- - - + - + - + +
391c	648/660 (98%)	654/660 (99%)	1/660 (0%)	V16I M17I V45I F125L I148L G242S 323_324insD	+ + + - + - -	578/588 (98%)	582/588 (98%)	0/588 (0%)	A21V N55D G78R D83E A128T T129A L224P	- + - + - - -

				D479E	+				H326N	+
				D535N	+				H344R	-
				S543R	-				I508V	+
				T593A	-					
				G595S	-					
MHP11	644/660 (98%)	652/660 (98%)	1/660 (0%)	V16I	+	578/588 (98%)	581/588 (98%)	0/588 (0%)	A21V	-
				M17I	+				N55D	+
				F125L	-				D83E	+
				I259T	-				K93T	-
				E323D	+				T102V	-
				D324E	+				A128I	-
				324_325insY	-				T129A	-
				D337E	+				L224P	-
				E407T	-				H344R	-
				S417T	+				I508V	+
				D509E	+					

				V532A D535N S589G T593A G595S	- + - - -					
KH0141	642/659 (97%)	651/659 (98%)	0/659 (0%)	M17V I79V K107R F125L I148L T197A I259T D324N E406K V469M F473L	+ + + - + - - + + + -	574/588 (98%)	579/588 (98%)	0/588 (0%)	A21V D61H T66L D83E I90M F124L A135T A145V V222I V223M L224P	- - - + + - - - + + -

				D508E	+				H344R	-
				D535N	+				A499V	-
				S589G	-				I508V	+
				T593G	-					
				G595S	-					
				R627H	-					

*Substitutions were determined using the protein BLAST. Identities refer to the number of identical amino acids in the sequence, positives include identities plus conservative amino acid changes where the amino acids have similar biochemical properties.

5.3.3 3D-Modelling of PBP1A and PBP2 in *H. pylori* 26695 strain

Using the I-TASSER software a 3D model of the PBP1A and PBP2 protein in 26695 *H. pylori* strain was produced from the reported FASTA sequence (Figure 5.1-5.4). The location of the active site serine at amino acid positions 368 and 311 are shown in purple in Figures 5.2 and 5.4, respectively. As expected, the active serine is within the transpeptidase domain and at the end of an alpha helix structure. From analysing the 3D structure, and the amino acid sequence data, the positions of the 3 conserved active site residues were found to be $SxxK_{368-371}$, $SxN_{433-435}$, and $KTGT_{555-558}$ in PBP1A, and $SxxK_{379-382}$, $SxN_{366-368}$, and $KTGT_{513-516}$ in PBP2. The C-Score is a confidence score in the quality of the model ranging from -5 to +2, where the higher the number the higher the confidence of the model. Models with a C-score of >-1.5 are thought to be reliable. For the 3D structure of PBP1A and PBP2, the C-score was -0.96 and -0.13, respectively.

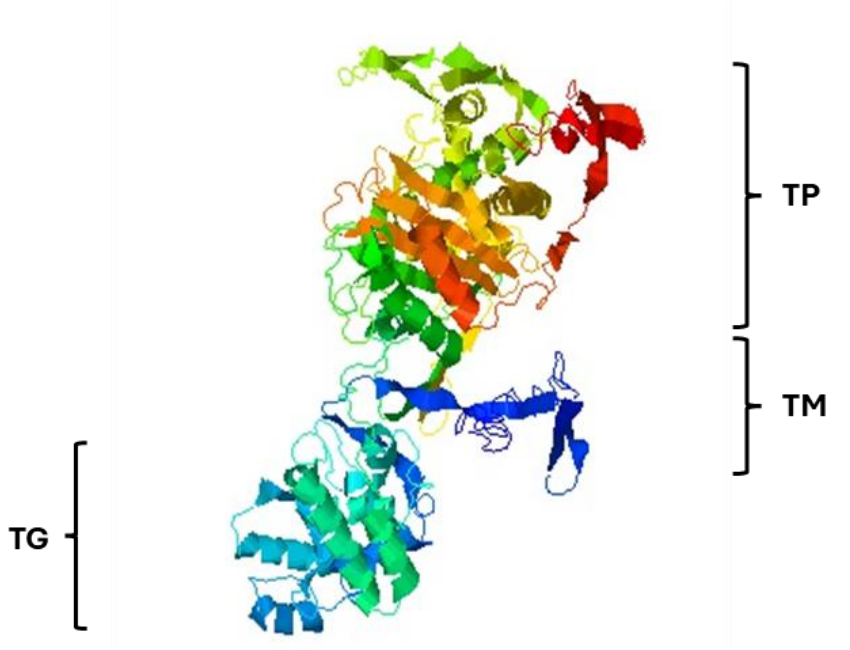


Figure 5.1: The 3D structure of penicillin-binding protein 1A (PBP1A) of Helicobacter pylori 26695 strain, created using I-TASSER. *(A) The transpeptidase domain (TP), transglycosylase domain (TG), and transmembrane domain (TM) are labelled.*

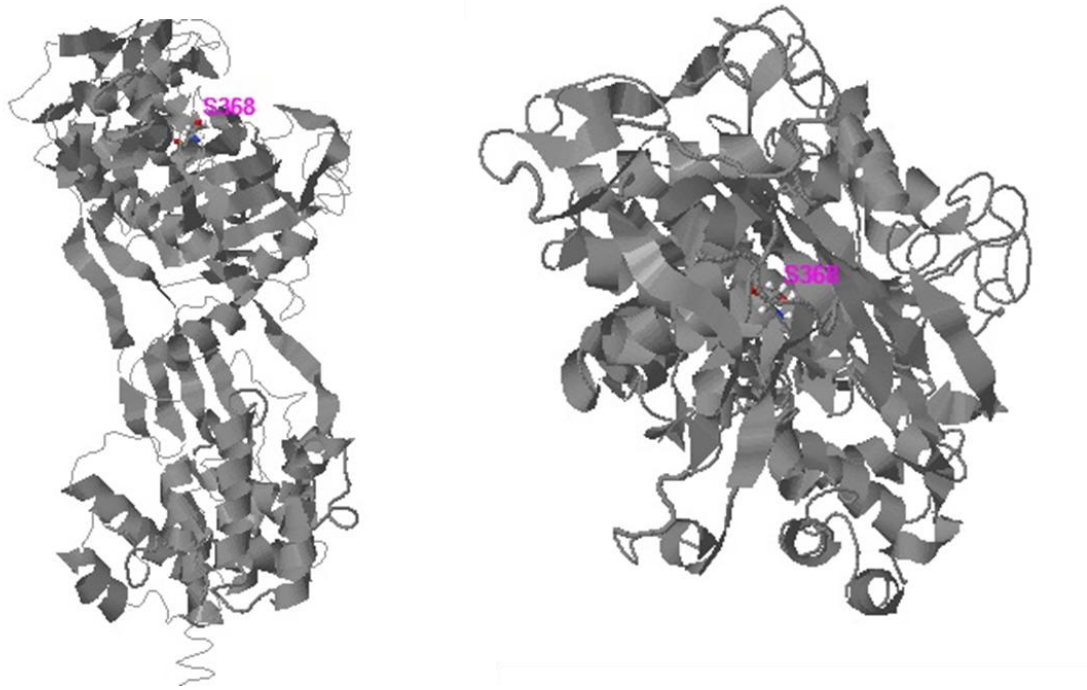


Figure 5.2:The location of the active site serine in penicillin-binding protein 1A (PBP1A) of Helicobacter pylori 26695 strain, created using I-TASSER. *The active serine is highlighted in purple and is located in the transpeptidase domain (S368) and at the end of an alpha helix as expected.*

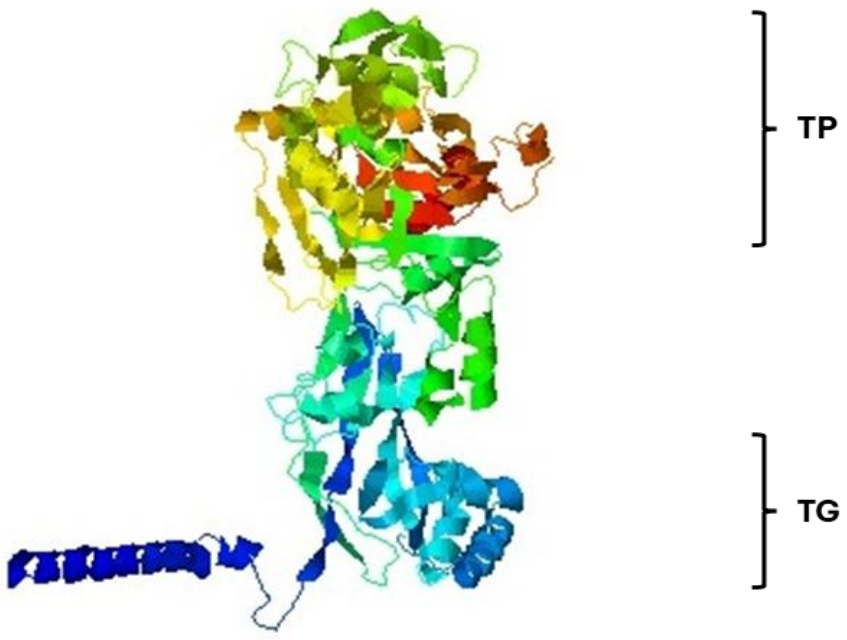


Figure 5.3: The 3D structure of penicillin-binding protein 2 (PBP2) of Helicobacter pylori 26695 strain, created using I-TASSER. *(A) The transpeptidase domain (TP), and transglycosylase domain (TG) are labelled.*

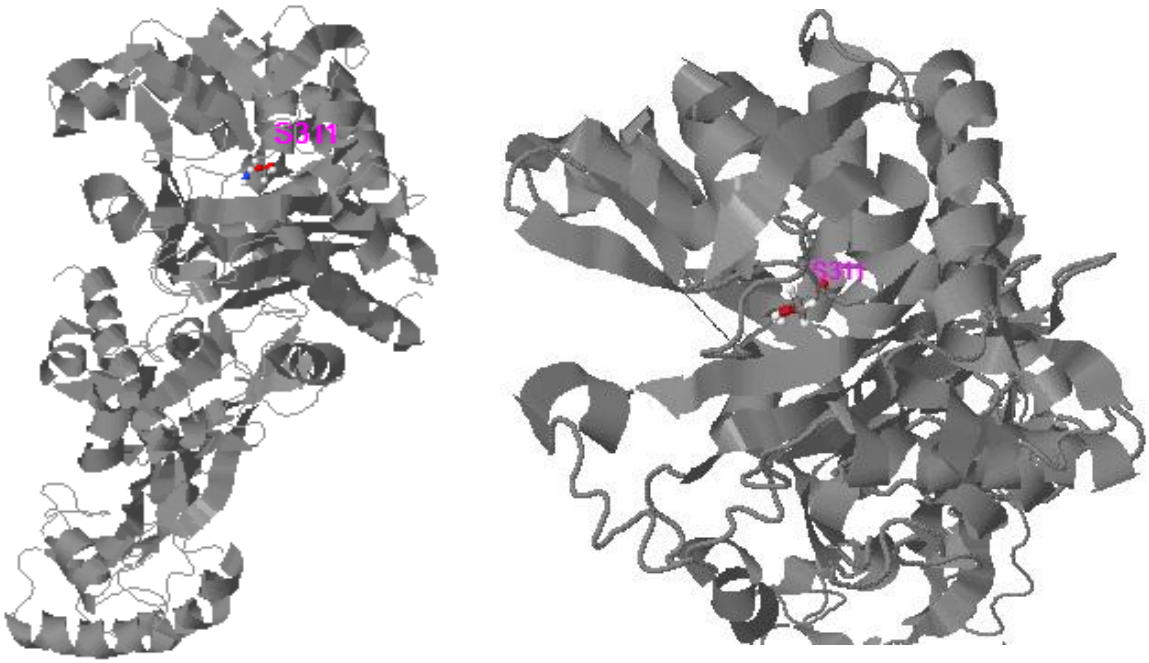


Figure 5.4: The location of the active site serine in penicillin-binding protein 2 (PBP2) of Helicobacter pylori 26695 strain, created using I-TASSER. *The active serine is highlighted in purple and is located in the transpeptidase domain (S311) and at the end of an alpha helix as expected.*

5.3.4 The relationship between amino acid substitutions in PBPs and amoxicillin resistance

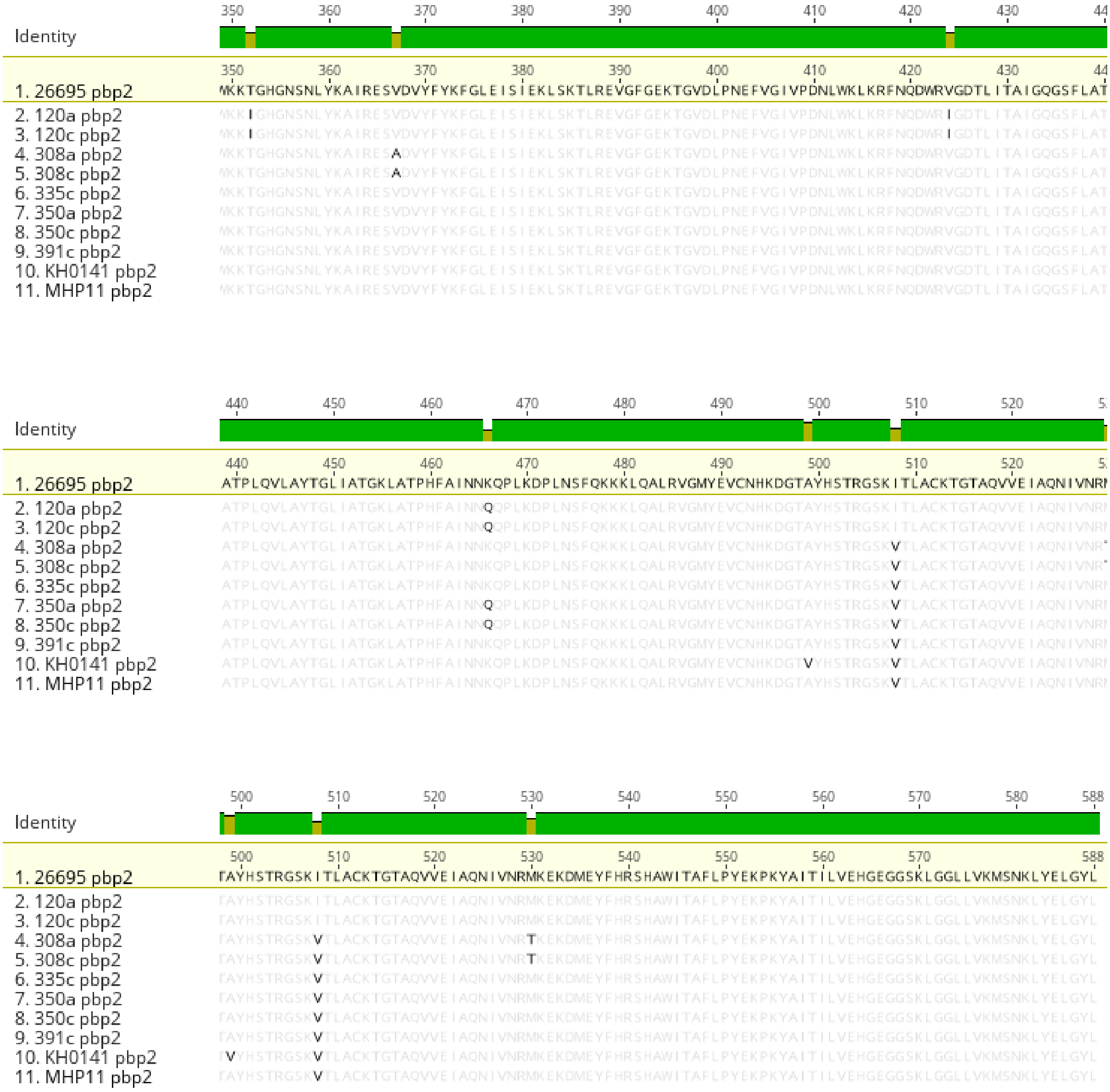
The Geneious Prime version 2024.0.7 software was used to visualise the location of the substitutions in the PBP1A (Figure 5.5) and PBP2 (Figure 5.6) amino acid sequence of resistant strains. From these figures, we can see highly variable regions in the TP domain between PBP1A amino acids 320-325, 500-510, and 590-600 in resistant strains with much less variability seen in PBP2 protein. Variable regions were seen scattered between PBP2 amino acids 55-90, 125-145, and 220-227 in resistant strains (Figure 5.6).

While the alignments created by Geneious allow a quick assessment of variable regions across multiple strains. There were discrepancies in these alignments when compared to those produced by protein BLAST. For example, in UK strain 120a, the Geneious alignment reports a glycine insertion at position 595 in PBP1A protein (Figure 5.5), however BLAST reports this as a glycine insertion at position 593 (Table 5.7). BLAST alignments retain the reference genome amino acid position and therefore any reported substitution positions are as determined from BLAST alignments.

Figure 5.5: The amino acid sequence alignment of PBP1A from amoxicillin-resistant H. pylori strains.

*The reference strain 26695 is highlighted in yellow. The identity refers to the % of identical residues. Green = 100% identity, Brown = 30-99% identity, Red = <30% identity.

Figure 5.6: The amino acid sequence alignment of PBP2 from amoxicillin-resistant H. pylori strains.

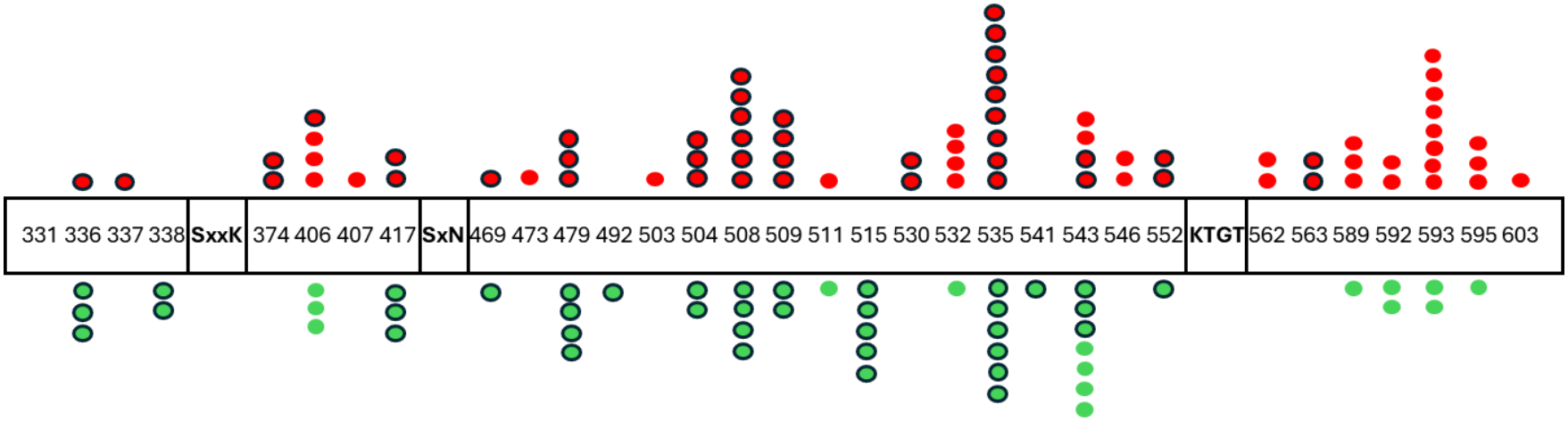


*The reference strain 26695 is highlighted in yellow. The identity refers to the % of identical residues. Green = 100% identity, Brown = 30-99% identity, Red = <30% identity.

A summary of the amino acid substitutions within the TP domain, as determined from the BLAST results, is shown in Figure 5.7 and Figure 5.8. Several amino acid positions are highly associated with resistance, mainly at positions between 546-603 in PBP1A protein (Figure 5.7). For example, the valine at position 374, leucine at position 530, arginine at position 546, asparagine at position 562, and isoleucine at position 563 and were substituted in 2/10 resistant strains respectively, with no changes at these positions seen in susceptible strains. Substitution of the aspartic acid at position 337, glutamic acid at position 407, phenylalanine at position 473, threonine at position 503, and serine at position 603 was reported in one resistant strain and none of the susceptible strains. 4/10 resistant strains also have the V532A substitution compared to only 1/10 susceptible strains.

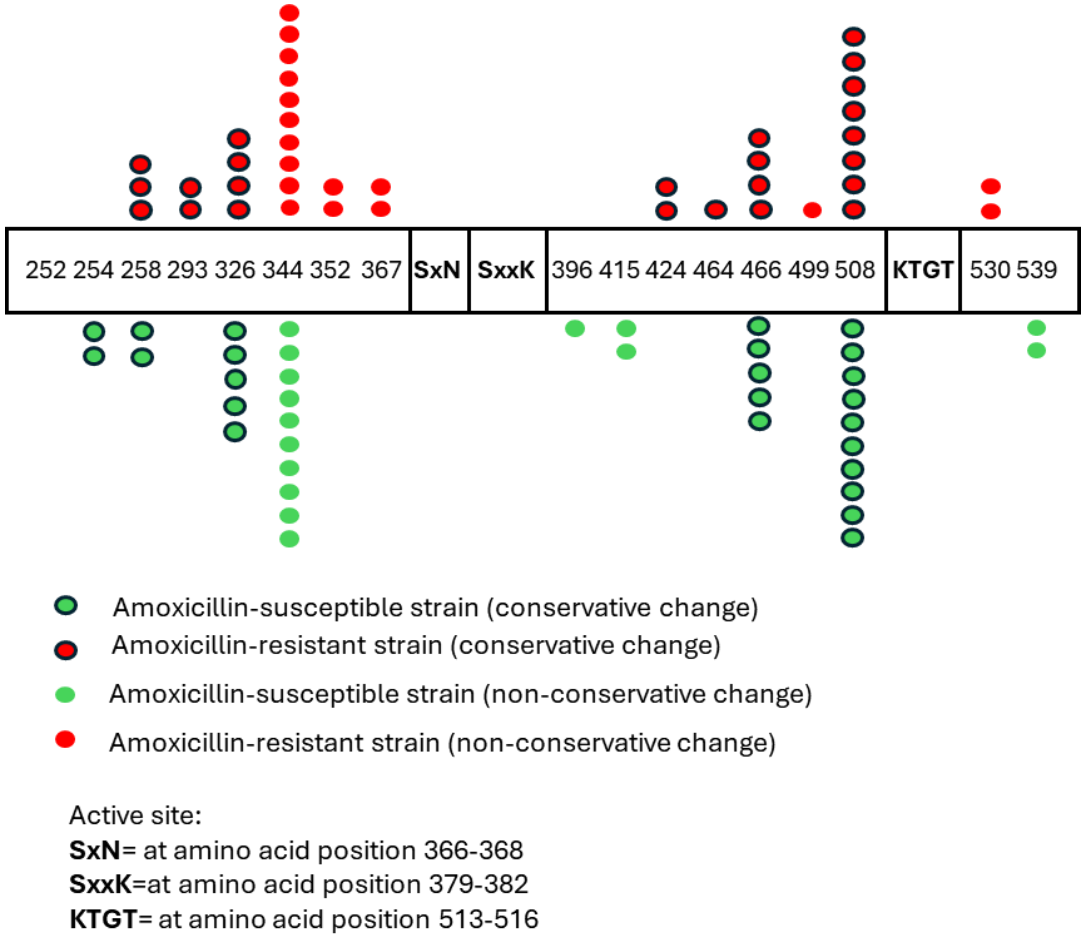
Meanwhile, the amino acid substitutions in the transpeptidase domain of PBP2 were quite similar for both susceptible and resistant strains (Figure 5.8). Interestingly, the substitution of histidine at position 344 was present in all 20 susceptible and resistant strains. 10/10 and 4/10 resistant strains also had the I508V and K466Q substitution compared to only 8/10 and 4/10 susceptible strains, respectively. Substitutions of the aspartic acid at position 293, threonine at position 352, valine at position 367 and 424, and methionine at position 530 were observed in two resistant strains and none of the susceptible strains.

Figure 5.7: Summary of the position of amino acid substitutions in the transpeptidase domain of PBP1A of 20 clinical H. pylori strains as compared to reference strain 26695.



*Red circles = a substitution at this position in resistant strains, green circles = a substitution at this position in susceptible strains, solid lines = conservative change (i.e. to an amino acid with similar biochemical properties), no lines = major amino acid change.

Figure 5.8: Summary of the position of amino acid substitutions in the transpeptidase domain of PBP2 of 20 clinical H. pylori strains as compared to reference strain 26695.



*Red circles = a substitution at this position in resistant strains, green circles = a substitution at this position in susceptible strains, solid lines = conservative change (i.e. to an amino acid with similar biochemical properties), no lines = major amino acid change.

5.3.5 Amino acid changes and their positions on the 3D structure of PBP1A

The amino acids at positions 473, 589, 593, and 595 were mapped to the predicted 3D structure using AlphaFold 2 and USCF ChimeraX. The location of the 3 active sites were also visualised. The results of the 3D mapping indicated that the positions of substitutions highly associated with amoxicillin resistance are adjacent to or near the active sites (Figure 5.9), suggesting substitutions at these sites may cause structural changes which reduce the binding of affinity or access of amoxicillin to the active site.

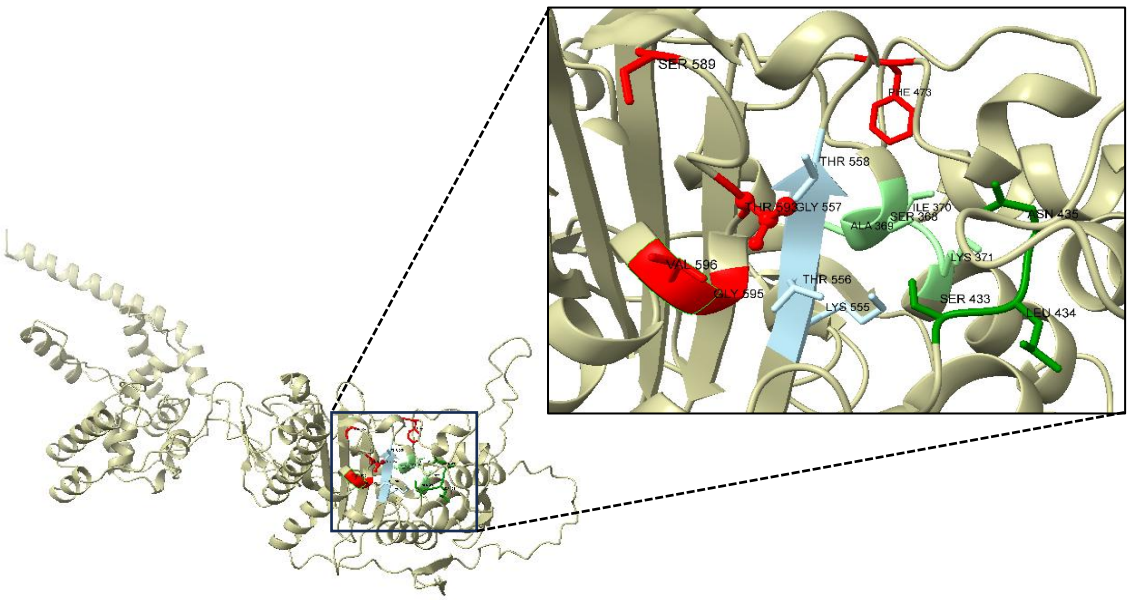


Figure 5.9: The position of amoxicillin resistance associated substitutions mapped against the predicted H. pylori 26695 PBP1A 3D structure. *The folding of H. pylori 26695 PBP1A was predicted with AlphaFold2 (Skolnick et al., 2021) using default settings. The top-rated predicted structure was visualised using UCSF ChimeraX (Pettersen et al., 2021). The position of the 3 reported active sites, $SAIK_{368-371}$, $SLN_{433-435}$, and $KTGT_{555-558}$ are shown in different shades of green. The active serine is that at position 368. The positions with resistance associated substitutions, P_{473}, S_{589}, T_{593}, G_{595}, are highlighted in red.*

5.4 Discussion

5.4.1 Comparative analysis between the phenotypic and genotypic detection of antibiotic resistance among *H. pylori* isolates

The mechanism of drug resistance in *H. pylori* is thought to be due to mutations located in the chromosome (Lauener *et al.*, 2019). The peptidyl-transferase region in the domain V of 23S rRNA is responsible for the binding of antibiotics; therefore, point mutations in this region result in the inhibition of binding of macrolide antibiotics and ribosomal subunits, leading to bacterial resistance to macrolides (Anis *et al.*, 2021; Tran *et al.*, 2019). In this study, 89% of clarithromycin-resistant strains had common mutation locus A2147G, consistent with prior studies conducted in Switzerland which showed that A2147G point mutation were present in 70% of clarithromycin-resistant isolates (Lauener *et al.*, 2019). It has also been demonstrated that the A2142G and A2143G point mutations are primarily responsible for *H. pylori* resistance to clarithromycin (De Francesco *et al.*, 2010; Marques *et al.*, 2020; Park *et al.*, 2018a). Meanwhile, point mutations at other positions (such as C2147G, A2144G/T, and A2115G) show geographic variation, and are associated with the clarithromycin resistance phenotype (Kocazeybek *et al.*, 2019; Marques *et al.*, 2020; Ng *et al.*, 2020).

In the current study, the A2147G locus was found in almost all clarithromycin-resistant strains, and not in every sensitive strain, however, there was a lack of agreement between genetic and phenotypic resistance. Therefore, the A2147G mutation in the *23S rRNA* gene identified in this study may not be a reliable indicator for predicting phenotypic antibiotic sensitivity. In contrast, a study carried out by Cambau *et al.* (2009) revealed that the

presence of A2147G mutation in clarithromycin-resistant strains was predictive, where a genotypic test GenoType HelicoDR was concordant (concordance scores were 0.96) with a phenotypic MIC test.

A recent study reported new macrolide resistance mechanisms in *H. pylori*-resistant strains using next-generation sequencing. The study discovered a novel mutation in the genes responsible for encoding the sulfite exporter TauE/SafE family protein and the DUF874 family protein, which is associated with resistance or cross-resistance to clarithromycin. These mutations were identified in the *H. pylori* genome for the first time, emphasizing their potential as targets for further advanced studies (Ayaş *et al.*, 2024).

It is widely recognized that the primary reason for *H. pylori* resistance to metronidazole is the inactivation of reductase-encoding genes, which is caused by frame mutations, insertions, or deletions in the *rdxA* and *frxA* genes (Camorlinga-Ponce *et al.*, 2021; Vital *et al.*, 2022). In this study, the A85V amino acid substitution in the FrxA protein appears to be a potential drug resistance biomarker, found exclusively in half of metronidazole-resistant isolates, and consistent with a previous study (Saranathan *et al.*, 2020). The described amino acid substitutions at D59N, R90K, Y62D also have been found in all metronidazole-resistant strains, observed in this study and other studies (Cortez Nunes *et al.*, 2023; Nemr *et al.*, 2022; Tshibangu-Kabamba & Yamaoka, 2021). Meanwhile, in this study, other amino acid changes such as A118T, C49T, S108A, T31E, H97T, K64N, and V71 were present in both sensitive and resistant strains.

However, the correlation between phenotypic and genotypic resistance to metronidazole demonstrated in this study was poor, as also described by others (Xiong *et al.*, 2023; Zhong *et al.*, 2021). Thus, it is not a surprise the low concordance found with molecular and

phenotypic detection, considering the large number of mutations in the *rdxA*. Moreover, all 36 clinical isolates were phenotypically tested for metronidazole resistance, and only 6 of them exhibited metronidazole MICs greater than 256 µg/ml. This means that presence of amino acid changes in RdxA and FrxA did not consistently correlate with high MICs, suggesting that other possible mechanisms of resistance such as the overexpression of *TolC* homologous genes that upregulates efflux pump activity might also play a role in resistance to metronidazole (Hashemi *et al.*, 2019).

Levofloxacin is a fluoroquinolone antibiotic that acts on bacterial DNA gyrase, a crucial enzyme for DNA replication and transcription (Correia *et al.*, 2017). DNA gyrase is composed of two subunits, A and B, which are encoded by the *gyrA* and *gyrB* genes, respectively (Matsuzaki *et al.*, 2010). Amino acid substitutions D91N and D91G occur in the quinolone resistance determining region (QRDR region) of the DNA gyrase enzyme, where quinolone antibiotics typically bind. These mutations hinder levofloxacin's ability to attach to the enzyme, diminishing the drug's inhibitory impact (Correia *et al.*, 2017). Numerous studies have consistently associated these mutations with decreased levofloxacin susceptibility (Lok *et al.*, 2020; Zhang *et al.*, 2020b).

In this study, all levofloxacin-resistant strains exhibited a mutual amino acid substitution D91N in GyrA, with MIC values of >32 µg/mL. These findings are supported by earlier studies which identified amino acid variations at positions 87 and 91 of GyrA that are associated with high MIC values for levofloxacin (Hanafiah *et al.*, 2019; López-Gasca *et al.*, 2018; Tuan *et al.*, 2019). None of the levofloxacin-sensitive strains possess this amino acid substitution, and there was a high level of agreement between phenotypic and genotypic resistance for levofloxacin (kappa= 1), hence it could be speculated that D91N might be a key point of

mutation and detecting resistance to levofloxacin by this resistance genotype is an applicable alternative method. This result aligns with earlier studies showing that the D91N amino acid substitution in levofloxacin-resistant strains, identified through PCR, consistently matched the results of phenotypic antibiotic susceptibility testing. This suggests that analysing this genotype of *H. pylori* antibiotic resistance can be useful in designing eradication therapies (Xiong *et al.*, 2023; Zhong *et al.*, 2021).

Overall, there was a discrepancy between phenotypic and genotypic resistance to clarithromycin, metronidazole, and amoxicillin, but not for levofloxacin. Researchers from different parts of the worlds have explored both phenotypic and genotypic methods, leading to a diverse range of results. Similarly, Mascellino *et al.* (2020) reported a strong genotype-phenotype correlation in *H. pylori* strains resistant to levofloxacin, but not in those resistant to clarithromycin. Whilst Tuan *et al.* (2019) observed a strong link between genotype and phenotype for clarithromycin, a good correlation for levofloxacin and amoxicillin, and no correlation for metronidazole. Meanwhile, a study carried out in China revealed that there was a strong association between phenotypic and genotypic resistance for levofloxacin and clarithromycin, whereas the relationship was weak for amoxicillin and metronidazole (Hu *et al.*, 2023).

In general, these findings provide a basis for further resistance-associated research. Therefore, it is necessary to further validate molecular methods used to characterize resistant genes, as this allows for the identification of the most effective antibiotics for each patient based on the genetic makeup of the infecting *H. pylori* strain, leading to more personalized and targeted treatment. This approach could reduce the use of broad-

spectrum antibiotics and avoid unnecessary treatments that are ineffective against resistant strains.

5.4.2 The relationship between amino acid substitutions in PBPs and amoxicillin resistance in *H. pylori* isolates

It is also worth noting that this study did not find any beta-lactamase genes among the genes resistant to amoxicillin, although Tseng *et al.* (2009) found that a high-level amoxicillin-resistant clinical strain of *H. pylori* 3778 possesses a beta-lactamase bla_{TEM-1} gene. Surprisingly, a recent study also showed that the beta-lactam resistance gene $bla_{TEM-181}$ was found in seven genomes from Colombia (Alvarez-Aldana *et al.*, 2024). While beta-lactamase genes have been identified in some *H. pylori* strains, they are not usually present in all amoxicillin-resistant strains. The more commonly observed resistance mechanisms involve mutations in PBPs. The presence of beta-lactamase genes might also depend on geographic and clinical factors, as resistance patterns can vary regionally (Bush & Bradford, 2020).

Amoxicillin resistance in *H. pylori* is complex and cannot be attributed to specific amino acid changes found in all resistant strains. Although the exact substitutions leading to resistance remain unidentified, this study has pinpointed positions within the transpeptidase domain, particularly certain amino acids, that are strongly linked to resistance in positions 469, 473, 543, 562, 589, 593, and 595, in the *H. pylori* 26695 PBP1A sequence. Several other studies have identified substitutions at these positions that are associated with amoxicillin resistance in *H. pylori* (Attaran *et al.*, 2021; Qureshi *et al.*, 2011; Tran *et al.*, 2022a; Tshibangu-Kabamba *et al.*, 2020). None of these positions are located within the conserved transpeptidase active sites. Changes within these motifs are probably not viable since they

would impact both the peptidoglycan and beta-lactam affinities. This would hinder the peptidoglycan cross-linking function of the transpeptidase domain. Maintaining a delicate balance is crucial between a mutation that prevents the antibiotic from binding and one that still maintains the protein's biological function. If the mutation alters the protein to the point of losing its activity, it offers no real benefit to the bacterium.

Rimbara *et al.* (2008) proposed that mutations in *PBP1A*, *PBP2*, and *PBP3* have synergistic effects on amoxicillin resistance. Meanwhile, a molecular analysis of PBP1A of *H. pylori* study carried out in the Malaysian population revealed a total of 21 variants of amino acids with three of them (K403X, S405I, and E406K) located in or near the PBP-motif ($SKN_{402-404}$) (Ng *et al.*, 2024). Current findings appear to support this, showing that the highly resistant strains 308a and 308c have mutations near the SxxK, SxN, and KTG motifs of PBP1A, and PBP2. This indicates that there is large heterogeneity in amoxicillin resistance, and that the resistance mechanisms in different isolates cannot be attributed to just one amino acid change in PBP motifs (Domanovich-Asor *et al.*, 2021). In this study, it was discovered that resistant strains possess a greater number of amino acid substitutions than susceptible strains in PBP1A. It was hypothesized that strains with more amino acid substitutions in the transpeptidase domain would exhibit a higher MIC. However, the data from the USA study does not support this. Strain MHP11, for instance, has the highest amoxicillin MIC despite having fewer substitutions of the USA strains studied (Saranathan *et al.*, 2020). This suggests that it is the presence of a small number of specific mutations can cause amoxicillin resistance in *H. pylori*.

When examining the predicted 3D structure of the PBP1A protein in the *H. pylori* strain 26695, the amino acid substitutions closely linked to resistance are positioned next to or

near the three conserved active sites. Consequently, substitutions at this location might lead to changes in the active site's conformation, potentially hindering amoxicillin's ability to covalently bond with the site or altering access to it. These findings highlight the significance of mapping mutational changes on the 3D structure, as substitutions can impact resistance even if they are not adjacent in the 2D sequence.

Attaran *et al.* (2021) identified five access tunnels in *H. pylori*'s PBP1A through which amoxicillin can reach the active serine. They proposed that changes in the amino acids lining these tunnels might block the drug's ability to access or bind to the active site. Among the amino acid positions closely linked to resistance discussed in this study, those at positions 589, 593, and 595 are located along these tunnels. These substitutions could lead to structural alterations that block or reduce amoxicillin's access to the active site. Gerrits *et al.* (2006) also noted that amino acid changes near the binding sites in PBP1A can contribute to resistance.

In other organisms, such as *Streptococcus pneumoniae*, it has been noted that amino acid mutations in PBPs often lead to resistance to beta-lactam antibiotics (Maurer *et al.*, 2008). Smith and Klugman (2005) found that reversing amino acid mutations at several sites in beta-lactam resistant strains resulted in decreased resistance, with six specific mutations collectively contributing to the resistant phenotype. Likewise, Laible and Hakenbeck (1991) discovered that five distinct combinations of mutations could result in beta-lactam resistance in *S. pneumoniae*. This combination pathway to resistance is similar to what was observed in *H. pylori* and reported in this chapter.

A study carried out by Varghese *et al.* (2020) showed that amino acid substitutions were detected in just one of the three active sites of one of the three *PBP* genes (*PBP2B, PBP2X,*

and *PBP1A*). Consequently, the *PBP* genes, in the absence of the major substitutions typically linked to penicillin resistance, resulted in a slight increase in the penicillin MIC to between 0.06 and 2.0 μg/mL, indicating resistance based on meningeal breakpoints. Meanwhile Li *et al.* (2016) discovered that higher β-lactam MICs were closely linked to specific PBP types determined by sequence patterns in the transpeptidase domains (TPDs) of the three key penicillin-binding proteins (PBPs): PBP1A, PBP2B, and PBP2X. Therefore, the PBP transpeptidase signatures are strong predictors of MIC levels for various β-lactam antibiotics in clinical pneumococcal isolates, offering a precise alternative to traditional phenotypic susceptibility testing.

5.5 Limitations and Future work

When conducting a study using WGS, several limitations can arise, particularly when dealing with a small sample size. In this study, a small sample size may not be able to detect genetic patterns associated with antibiotic resistance and may not fully represent the diversity of *H. pylori* strains in the study population, leading to biased results, which can affect the reliability of the findings. Furthermore, WGS relies on comparison to existing reference genomes for proper assembly and variant calling. In this study, limited published sequence data is available for *H. pylori*-resistant strains, therefore missing or incomplete reference genomes can lead to errors in identifying novel mutations or resistance mechanisms.

However, the initial findings gained in this study can be useful for future research that may aim at performing culture-independent WGS directly from clinical specimens for the prediction of phenotypic drug susceptibility in *H. pylori*. It may also be a reliable way to obtain antibiotic susceptibility results for *H. pylori* and overcome the limitation of the small sample in an unsuccessful culture. A recent study has already successfully applied WGS

directly on gastric biopsies for the detection of *H. pylori*. This study identified novel variants (five *CYP2C19* homozygous extensive metabolizers and three *CYP3A4* intermediate metabolizers) and designed a custom next-generation sequencing panel for personalized *H. pylori* eradication treatment (NGS-PHET) which targeted the regions for amoxicillin, clarithromycin, metronidazole, tetracycline and levofloxacin-resistance in *H. pylori* and PPI metabolism (Min *et al*., 2024). This helps to perform eradication treatment quickly and effectively in most patients with antibiotic-resistant *H. pylori* strains and is also useful in research to find novel antibiotic-resistance gene candidates.

The incorporation of artificial intelligence (AI) and machine learning (ML) techniques into AMR research enhances the abilities for predicting outbreaks, classifying lineages, assessing risk, and identifying markers of pathogenicity or host specificity (Singh *et al.*, 2024). One study showed that WGS combined with an ML approach produced diagnostic models with greater sensitivity and specificity for identifying antibiotic resistance patterns in *H. pylori* strains (Yu *et al.*, 2023).

Techniques like deep learning (DL) that utilizes artificial neural networks (ANN) have also been used to encode single nucleotide polymorphisms (SNPs) into formats that improve the identification of resistance mechanisms (Popa *et al.*, 2022). Brincat and Hofmann (2022) developed a text-mining system that utilizes DL algorithms to expedite the curation process. These algorithms can replace manual curation by analysing vast amounts of literature to identify genes associated with antibiotic resistance. This newly developed text-mining algorithm was the first attempt to detect antibiotic resistance genes in *H. pylori*, using the nitroimidazole antibiotic group as a case study. The results indicated that out of 28

identified genes, they found that 23 should be added to knowledge databases because they could serve as potential candidates for research on *H. pylori* antibiotic resistance.

AI-driven analysis of WGS data enables rapid identification of resistant strains and streamlining decisions on effective antibiotics for diagnostic improvement. It can also offer tailored antibiotic regimens for personalized medicine and help in monitoring the emerging resistance trends globally. Therefore, new approaches using AI and ML based on WGS data should be considered in future studies for the detection of novel resistance markers and to predict the treatment outcomes with higher accuracy which may improve the management of antibiotic resistance in *H. pylori* eradication therapy.

WGS analysis using the CARD database can detect the resistant population even at sub-inhibitory concentrations compared to phenotypic tests, which primarily identify susceptible bacteria. The E-test may not identify all resistant strains, likely because colonies are randomly selected from the culture media for testing. This random selection means that both susceptible and resistant colonies might be chosen, but there is a greater chance of picking susceptible colonies. Mixed *H. pylori* infections have been observed in patients, with both wild type and resistant strains present in a single gastric region simultaneously (Mascellino *et al.*, 2020). A previous study showed that the genotypic resistance testing using PCR was the only means to reveal resistance to clarithromycin and levofloxacin when live bacteria were absent (where only inactive coccoid forms were found), which could lead to a resistance underestimation (Mascellino *et al.*, 2020). Several studies conducted in Germany have also shown a preference for genotypic over phenotypic methods when assessing primary or secondary resistance to clarithromycin or levofloxacin, to tailor specific therapies for that region (Bluemel *et al.*, 2020; Wueppenhorst *et al.*, 2013).

However, the potential contribution of active efflux mechanisms was not explored in this study. It was thought that four conserved RND (Resistance-Nodulation-Division) families of efflux pump transporters might contribute to macrolide resistance in *H. pylori* by lowering the intracellular concentration of the antimicrobial agent (Marques *et al.*, 2020; Saracino *et al.*, 2021). Studies also showed that the overexpression of the *hefA* gene promotes antibiotic efflux via the efflux pump system, contributing to the development of multi-drug resistance in *H. pylori* strains (Liu *et al.*, 2008). Additionally, the transporters HP0939, HP0497, and HP0471 play a role in the intrinsic multidrug resistance of *H. pylori* by increasing drug efflux, which significantly promotes the expression of *hefA* and *gluP* in these resistant strains (Cai *et al.*, 2020). Nevertheless, the role they play in the intrinsic antibiotic resistance of *H. pylori* would require transcriptional analyses of the genes encoding drug efflux systems (for instance, using a meta-transcriptomics approach), which was outside the scope of this research. Therefore, future studies should include the study on the multi-drug efflux pump genes of *H. pylori* with the combined action of point mutations and amino acid changes in the target genes to further elucidate the resistance mechanism in *H. pylori* resistant isolates.

5.6 Chapter summary

- The known and some novel point mutations and amino acid substitutions in the target genes and proteins in *H. pylori* isolates were demonstrated in this study.
- The A2147G point mutation in *23S rRNA* was significantly linked to *H. pylori* strains that exhibit phenotypic resistance to clarithromycin.
- The A85V and R90K amino acid changes in the *frxA* gene were significantly associated with *H. pylori* strains that show phenotypic resistance to metronidazole.
- There was an inconsistency between genotype-to-phenotype resistance correlation for clarithromycin, metronidazole, and amoxicillin, but not for levofloxacin.
- There was an increase in amino acid substitutions in PBP1A in resistant strains compared to susceptible strains.
- The amino acid substitutions in *H. pylori* 26695 PBP1A that are strongly linked to amoxicillin resistance are located near the active sites. This implies that these substitutions might induce structural changes that decrease amoxicillin's binding affinity or access to the active site.

Chapter 6： Discussion

6.1 (Chapter 2): Prevalence of *H. pylori* resistance to antibiotics in isolates from patients with and without a previous round of eradication therapy

This study hypothesized that *H. pylori* antibiotic resistance rates may be rising in the UK, reflecting the global trend of increased resistance, particularly to metronidazole and clarithromycin. Such a rise in prevalence would mirror *H. pylori* eradication failure rates. It is crucial to evaluate the antibiotic combinations used in future *H. pylori* therapies to ensure their effectiveness. This study analysed the local antibiotic resistance profiles in *H. pylori* isolates from gastric biopsies of GP-referred endoscopy patients in Nottingham and explored the resistance mechanisms.

Current and past studies indicated that there is a high prevalence and increasing trend of resistance to clarithromycin and metronidazole in *H. pylori* isolates in the UK, supporting the hypothesis (Garvey *et al.*, 2023; McNulty *et al.*, 2012). Nevertheless, as mentioned previously, one meta-analysis study found no significant changes in resistance rates for these antibiotics between 2006 and 2016 in Europe (Savoldi *et al.*, 2018), and another earlier European study reported a decrease in *H. pylori* resistance to clarithromycin between the year 2009 to 2014 (Ghotaslou *et al.*, 2015). However, it was clarified that the discrepancies might be due to variations in the methods used for antibiotic susceptibility tests (agar dilution, disc diffusion, and E-test). Disc diffusion and E-test strips were used in this thesis, and due to the higher cost of the E-test method compared to the traditional antibiotic disc diffusion approach, we aimed to standardise the disc method and evaluate its effectiveness against E-tests. We conducted a side-by-side comparison of 30 isolates,

examining the categorical outcomes (resistant, sensitive, and intermediate). The findings showed complete agreement between the two methods for clarithromycin, amoxicillin, and levofloxacin; however, discrepancies arose with metronidazole (Garvey *et al.*, 2023). The disc diffusion test results for metronidazole were deemed unreliable, hence the isolates had to be re-tested using E-tests.

Another standard antimicrobial susceptibility testing for *H. pylori* is the agar dilution method. This technique involves adding various concentrations of antimicrobials, typically in a serial dilution, into blood agar plates, but they are difficult to perform routinely (Li *et al.*, 2022). One study evaluated the reliability of the E-test and disk diffusion compared to the agar dilution method for testing the antimicrobial susceptibility of *H. pylori*. They found that metronidazole displayed a strong correlation with both the E-test and disk diffusion methods, whilst clarithromycin had a moderate correlation with these methods. However, amoxicillin and tetracycline showed a weak correlation with both the E-test and disk diffusion methods (Ogata *et al.*, 2014).

Preparing numerous fresh blood agar plates for agar dilution susceptibility testing is labour-intensive and time-consuming. While this approach is efficient for testing a large batch of isolates for antibiotic resistance surveillance, it is not practical for regularly handling a small number of clinical isolates (Best *et al.*, 2003). Studies indicate that the resistance rate of *H. pylori* to metronidazole is higher when detected using the E-test compared to the agar dilution method (Glupczynski *et al.*, 2002; Miftahussurur *et al.*, 2020). This discrepancy may be partly due to varying time intervals between preparing the medium and conducting the susceptibility tests, which affect the redox potential, a key factor for metronidazole reduction (Mégraud & Lehours, 2007). The agreement between the E-test and agar dilution

method for amoxicillin, clarithromycin, quinolones, and tetracycline is excellent (Glupczynski *et al.*, 2002; Osato *et al.*, 2001a). Consequently, the E-test can serve as an alternative to the agar dilution method for testing clinical isolates from individual patients. Both EUCAST and The British Society for Antimicrobial Chemotherapy (BSAC) recommend the E-test method for determining *H. pylori* susceptibility to antibiotics (Smith *et al.*, 2014). However, the high cost of E-test strips restricts their widespread use in clinical settings.

Overall, the E-test method is a currently recommended phenotypic method because it provides a quantitative result, better standardization, and sensitivity to low concentration, making it an accurate choice for testing a fastidious organism like *H. pylori*. As a result, the E-test, despite being more costly, is preferred over the other methods for *H. pylori* susceptibility testing (Mishra *et al.*, 2006).

Another significant finding of this chapter is that the resistance rates to clarithromycin and metronidazole were substantially higher in isolates from individuals who had received eradication therapy compared to those who had not. As expected, this is possibly due to the selective pressure of previous treatment exposures of *H. pylori* to antibiotics, which can lead to the survival of resistant strains. When patients undergo treatment, especially with antibiotics like clarithromycin and metronidazole, susceptible strains are often eliminated, allowing resistant strains to survive and proliferate. This was evidenced by our finding which showed that six out of 79 patients had different antibiotic resistance profiles for the antral and corporal gastric biopsies. Different strains may coexist within the same individual; hence this genetic diversity can lead to variations in resistance profiles, which can be more pronounced in different gastric sites.

Another potential explanation for this discordant resistance profile in the antrum and corpus in the same patients could be related to mutations, as *H. pylori* can acquire genetic mutations that confer resistance to antibiotics during treatment exposure, thereby increasing the likelihood of such mutations developing and persisting in the bacterial population. The genetic diversity of *H. pylori* is crucial in linking genetic elements to antibiotic resistance. Consequently, a study on the relationship between the presence of point mutations in the target genes and the phenotypic antibiotic susceptibility testing was conducted and examined in Chapter 5. However, it would be better to perform deep sequencing of isolates (>100 times coverage) in the future study, so that resistance genes and mutations can be detected from low copy numbers in diverse *H. pylori* populations (Wilkinson *et al.*, 2022).

Current and previous findings have also shown a significant improvement in atrophy within 1 to 4 years following *H. pylori* eradication therapy. Atrophic gastritis, a precursor to gastric cancer, is partially reversible after *H. pylori* eradication. Long-term follow-up studies indicate significant improvements in gastric mucosal atrophy after bacterial eradication, especially when treated before the condition becomes severe (Kodama *et al.*, 2021). The eradication treatment combined with long-term surveillance offers substantial benefits in reducing inflammation and regressing pre-cancerous lesions in patients with *H. pylori*. However, even after eradication, patients with significant pre-cancerous changes require monitoring as long-term surveillance may identify cases of incomplete regression or early signs of malignancy. The earlier the intervention, the more pronounced the benefits, underscoring the importance of timely detection and treatment of *H. pylori* infection (Kodama *et al*., 2021).

H. pylori eradication is also known to reduce the occurrence of gastric cancer in asymptomatic individuals and in those who have undergone endoscopic gastric cancer resection (Lee *et al.*, 2016; Sugano, 2019). It is therefore necessary to recruit more patients for follow-up endoscopies and histological assessment to evaluate the extent of regression in atrophic gastritis and intestinal metaplasia following *H. pylori* eradication for future research. Hence, tailored eradication therapy for *H. pylori* infection, based on an individual's antibiotic resistance profile is important for minimizing the risk of treatment failure, and in reducing the incidence of related diseases, such as peptic ulcers, gastric atrophy and gastric cancer, thereby benefiting public health. These findings emphasize the importance of managing *H. pylori*, not just for treating infections, but also for maintaining long-term gastrointestinal health and preventing cancer.

6.2 (Chapter 3): *Ex vivo* relationship between human defensin expression and *H. pylori* infection

Chapter 2 illustrated that the resistance of *H. pylori* to commonly used antibiotics reflects its adaptation strategies for long-term survival within its host. *H. pylori* continuously inhabit the human stomach despite the body's inflammatory responses to the infection. Therefore, it is essential to study the host's immune response to *H. pylori* infection to better understand how *H. pylori* can evade host innate immunity and persistently inhabit the human gut. Gaining a deeper insight into the cellular processes involved is vital for identifying the factors that influence these responses, which can help inform the development of new therapeutic approaches. The host immune response employs various strategies to directly kill bacteria, including the secretion of AMPs. Infection with *H. pylori* causes increased

expression of several AMPs, such as defensins, which have shown bactericidal effects against the bacterium; however, the infection persists (Pero *et al.*, 2019b).

Chapter 3 investigated the human immune response to *H. pylori* based on the gastric biopsies of infected and uninfected patients, by stratifying defensin concentrations with pathology, bacterial virulence factors, and the extent of gastric disease, and quantifying defensin levels mRNA expression. It was hypothesized *H. pylori* infection would result in altered defensin expression in gastric epithelial cells which may provide a potential biomarker for *H. pylori*-related gastric diseases (Linn *et al.*, 2023). Therefore, we aimed to measure mRNA expression levels of *DEFB4* and *DEFA5* in the gastric mucosa.

Most of the published literature indicates that *DEFB4* levels rise in the gastric mucosa of patients infected with *H. pylori* (Nuding *et al*., 2013; Patel *et al*., 2013), and current studies have validated this association. Elevated expression of *DEFB4* may be part of the host's response to *H. pylori* infection, potentially helping to control bacterial growth and colonization. Furthermore, we also observed a decrease in *DEFA5* mRNA levels in gastric biopsies from patients infected with *H. pylori* compared to uninfected controls. *DEFA5* is primarily produced by Paneth cells and its direct role in gastric mucosa during *H. pylori* infection is less well studied, and it is believed to contribute to the overall mucosal defence (Linn *et al.*, 2023). *DEFA5* expression is constitutive, highlighting its crucial role in innate defence against infections. Consequently, *H. pylori* may have evolved strategies to reduce the expression of this antimicrobial peptide, facilitating persistent infection.

We also found a significant link between *DEFB4* and *DEFA5* mRNA levels and gastric atrophy in infected patients. Hence, this study could determine whether the observed overexpression of the genes is a factor in the disease's progression towards gastric

adenocarcinoma. Emerging evidence suggests that defensins may have a significant impact, especially in the case of gastric cancer, although our understanding of defensin gene expression and its exact role in cancer development is limited (Wu *et al.*, 2021). Understanding the roles of defensins in the context of *H. pylori* infection could have implications for developing new therapeutic strategies aimed at enhancing mucosal defence or modulating the immune response to reduce *H. pylori*-related pathologies. Thus, one possible outcome of this research is the discovery of prognostic tumour markers for assessing the pre-cancerous lesions or the risk of *H. pylori*-related diseases especially gastric cancer. This potential diagnostic approach could assist in early detection of pre-malignant lesions in *H. pylori*-infected patients (which is usually asymptomatic), along with a personalized eradication therapy based on individual's antibiotic resistance profile that helps to regress the inflammation and atrophy in patients as mentioned in Chapter 2.

The finding that both *DEFB4* and *DEFA5* mRNA expression were markedly upregulated and downregulated, respectively, in gastric mucosa from infected individuals was expected. *H. pylori* can modulate defensin expression through several mechanisms. For example, the activation of the NOD1 signalling pathway, which is dependent on the *cag*PAI, activates both NF-kB and AP-1 transcription factors, leading to the expression of defensin *DEFB4* mRNA (Allison *et al.*, 2009; Grubman *et al.*, 2010). We found that higher *DEFB4* levels were observed in samples infected with *cagA*+ strains compared to *cagA*- strains. Similarly, for *vacA* strains, *DEFB4* mRNA levels were slightly higher in *vacA i*1 strains than in *vacA i*2 strains, though this difference was not statistically significant, likely due to the small sample size.

It would also be intriguing to collect gut microbiome data in future research, as the microbiota has been shown to influence defensin production (Puértolas-Balint & Schroeder, 2023). Their expression is tightly modulated by microbial cues, particularly in the gut. For instance, Paneth cells located in the small intestine generate α-defensins, and their function can be affected by microbial signals like LPS and bacterial metabolites (Armbruster *et al.*, 2017). Short-chain fatty acids (SCFAs), generated through the fermentation process by gut microbes, have been demonstrated to modulate the production of defensins and epithelial barrier function (Sato *et al.*, 2024). Meanwhile, *L. plantarum* ZS2058 is reported to enhance host defence peptides like pBD2 and PG1-5, thereby strengthening the intestinal barrier function (Ghosh *et al.*, 2021).

However, dysbiosis (imbalance in the gut microbiome) can alter defensin expression, which may contribute to susceptibility to infections, inflammation, or conditions like IBD. Studies showed that *H. pylori* eradication therapy can also lead to gut dysbiosis and promote the selection of drug-resistant species within the gut microbiota. This process can further enhance single-drug resistance (SDR) and multiple-drug resistance (MDR) mechanisms in other microbial species (Tshibangu-Kabamba & Yamaoka, 2021). Since the gut microbiota can potentially transfer resistance genes from harmless microbes to harmful ones and influence host biological functions, minimizing antibiotic resistance genes and maintaining the intrinsic gut microbiota composition could improve the success rate of *H. pylori* eradication and lessen unintended consequences.

Therefore, incorporating probiotics into the eradication process would effectively complement antibiotic treatments. A review study has reported that adding probiotics to antibiotic treatments could moderately reduce drug-related side effects, enhance antibiotic

efficacy and increase positive treatment outcomes in *H. pylori*-infected patients (Nabavi-Rad *et al.*, 2022). A randomized placebo-controlled study has reported that *Lactobacillus reuteri* increases the eradication rate of *H. pylori* following a 14-day high-dose PPI and bismuth-containing quadruple therapy (Poonyam *et al.*, 2019) while *Clostridium butyricum* MIYAIRI 588 significantly improve the *H. pylori* eradication rate in standard triple therapy (Mukai *et al.*, 2020).

The close relationship between gastrointestinal microbiota and host health, combined with changes in the microbiome and decreased alpha diversity during therapeutic interventions, indicates a significant role of the host microbiota in the side effects of *H. pylori* treatment (Ye *et al.*, 2020). Taking probiotics during eradication treatment can help maintain the host's indigenous microbiota, support microbial balance restoration, and reestablish the natural equilibrium of bacteria in the gastrointestinal tract (Handa *et al.*, 2020; Valdes *et al.*, 2018). Therefore, future studies should include elucidating the gut microbiome composition and diversity because alterations in microbial populations or variations might occur as either a cause or consequence of *H. pylori* infection and antibiotic treatment. Studies also should include long-term follow-up studies to assess the effectiveness of probiotics in eradicating *H. pylori* and the complex interplay between gut microbiota and host health.

Research into therapies that enhance defensin activity and restore a balanced microbiome is a promising approach for improving eradication rates, especially in antibiotic-resistant infections. Probiotic strains such as *L. reuteri* and *S. boulardii* have been found to enhance the effectiveness of antibiotics by stimulating defensin production and suppressing *H. pylori* growth (Liang *et al.*, 2022). Additionally, the consumption of prebiotic fibres supports beneficial microbes, which in turn promote defensin expression and maintain epithelial

health (So *et al.*, 2024). Therefore, combination therapies such as antibiotics, antimicrobial peptides (with microbiome-restoring interventions), and adjunctive therapies such as vitamin D and zinc supplementation (dietary interventions) could provide alternatives to conventional antibiotics. Another alternative future therapeutic strategy is using advanced diagnostic tools like microbiome sequencing to tailor therapies that address specific microbial imbalances caused by *H. pylori*. This personalized defensin-boosting therapy is based on an individual's genetic polymorphisms that influence defensin expression. Further research and clinical trials will be essential to translate these ideas into effective therapies.

6.3 (Chapter 4): *In vitro* relationship between the host immune response (IL-8 and hβD2) and antibiotic eradication in *H. pylori* infection

It is now established that human defensins are regulated following *H. pylori* infection as evidenced by *ex vivo* studies in Chapter 3 and earlier studies (Nuding *et al.*, 2013; Patel *et al.*, 2013). Therefore, in Chapter 4, it would have been interesting to conduct an *in vitro* cell culture with *H. pylori* strains to measure defensin secretion levels and compare these results with prior *ex vivo* findings. However, Chapter 4 only measured the protein level of defensin, hence, it would have been an interesting addition to perform similar defensin mRNA quantification from the co-culture assays to accompany the mRNA data obtained from gastric biopsies of patients in Chapter 3. Future studies should include total RNA isolation from the pellets of AGS cells co-cultured with *H. pylori* and qPCR quantification to allow for meaningful comparisons of defensin expression levels between *in vitro* and *ex vivo* findings.

Defensins play a vital role in the innate immune system and acting as pro-inflammatory mediators, defensins attract leukocytes and stimulate the production and release of pro-inflammatory cytokines such as TNFα and IL-8 (Phan *et al.*, 2018). A hallmark of *H. pylori* infection in the gastric mucosa is the release of several pro-inflammatory cytokines, such as IL-8, exacerbating inflammation and contributing to gastric pathology. One major consequence of *H. pylori* activating host epithelial signalling pathways is the disturbance of essential mechanisms in the host's immune responses, with weakened immunity playing a role in the chronic persistence of the infection. Hence, these cytokines are essential in the development of gastroduodenal diseases linked to *H. pylori* infection. Therefore, factors that impact cytokine responses could affect one's susceptibility to or protection against diseases associated with *H. pylori*.

Based on the initial findings on defensins, exploring the link between human defensin expression levels (hβD1, hβD2, hβD3, and hβD4) and the success rate of eradication in the future would be intriguing. This could involve measuring defensin expression in gastric biopsy samples and asking patients to return to the clinic a month later to verify their *H. pylori* status using stool antigen tests or urea breath tests. This could help determine whether defensins play a crucial role in aiding antibiotic eradication and evaluate if patients with the highest defensin expression are more likely to achieve successful eradication. Additionally, it could explore whether this is linked to patients' defensin gene polymorphisms, which may predispose some individuals to express higher levels of AMPs. If a positive association with eradication success is confirmed, perhaps it might lead to the development of therapies aimed at enhancing defensin expression in the stomach.

A promising concept is a treatment strategy that combines conventional antibiotics with a targeted approach to enhance endogenous antimicrobial responses. This could be achieved by using liposomal formulations of compounds, such as bacterial LPS from non-pathogenic strains like *E. coli*, that can locally activate NF-kB without triggering inflammation, thereby increasing defensin expression in gastric epithelial cells. A potential topical treatment that operates within the stomach alongside eradication therapy could be administered to patients to boost defensin expression, reducing *H. pylori* colonization densities and increasing the bacteria's susceptibility to antibiotics by compromising their membranes. Future research should also include pre-clinical studies to test these liposomal formulations in animal models of *H. pylori* infection to assess defensin induction, colonization density, and mucosal health.

The current findings revealed that macrolides mediate a reduction in defensin expression and therefore, a therapy that can heal ulcers quickly while *H. pylori* is eradicated would be ideal. Perhaps a phased therapy approach for patients with severe *H. pylori* disease, where this strategy would target the unique needs of different stages of the disease: initial bacterial eradication, followed by mucosal healing and prevention of recurrence might be beneficial. The initial phase involves utilizing highly effective antibiotic treatments, such as quadruple therapy (including bismuth, a proton pump inhibitor, tetracycline, and metronidazole), to effectively eliminate *H. pylori* or using liposomal antibiotics to enhance delivery to the gastric lining and reduce systemic side effects. The proposed second phase would focus on clearing any remaining *H. pylori* and promoting early healing by employing defensin-enhanced therapies (like liposomal NF-kB activators derived from bacteria) and mild anti-inflammatory agents to decrease excessive inflammation and encourage mucosal repair. The final phase would involve incorporating dietary and probiotic support, such as

Lactobacillus or *Bifidobacterium*, to restore the balance of gut microbiota, along with lower doses of defensin-stimulating agents to maintain ongoing mucosal defence and prevent reinfection.

In Chapter 4, how pro-inflammatory cytokines like TNFα interact with the IL-8 response and the stimulation of defensins during *in vitro H. pylori* infection was explored. TNFα is a potent promoter of inflammation, and it is well documented that TNFα binds to its receptor and triggers a cascade that leads to the activation of pro-inflammatory signalling NF-kB and the expression of defensin and IL-8 expression (Chen *et al.*, 2022; Tavares & Pathak, 2018). In the current studies, IL-8 and hβD2 expression were significantly upregulated during *H. pylori* infection with recombinant TNFα stimulation *in vitro*, confirming the previous findings. However, it was surprising to discover that there was no link between TNFα and the density of *H. pylori.* In the literature, the concentration of TNFα used in experiments to examine its effects on bacterial load in AGS cell line co-cultures can vary, but generally, concentrations in the range of 10 to 100 ng/mL are commonly used *in vitro* for studies involving TNFα (Eliesen *et al.*, 2022; Kim *et al.*, 2018; Li *et al.*, 2023a; Wolczyk *et al.*, 2016). In this current study, a concentration of 50 ng/ml of TNFα was used to investigate its effect on bacterial load. Therefore, it would be useful to consider multiple concentrations of TNFα to determine a dose-dependent response in future studies.

The interaction between TNFα, *H. pylori,* and the inflammatory response in gastric cells has been addressed in the early part of Chapter 4. As the current study's main interest was to evaluate the antibiotics used in *H. pylori* eradication therapy (Chapter 2), we sought to elucidate how the roles of macrolides have been shown to inhibit the activation of NF-kB and subsequently reduce the expression of IL-8, providing a dual benefit in treating *H. pylori*

infections. Macrolides such as clarithromycin are well known to have anti-inflammatory properties beyond their antibacterial effects (Pollock & Chalmers, 2021). Despite the high levels of resistance that *H. pylori* exhibit to clarithromycin (observed in Chapter 2), clinicians still prescribe this antibiotic because it has been proven to relieve infection symptoms in patients.

The inclusion of macrolides, such as clarithromycin, in standard triple therapy for *H. pylori* infection has been debated, especially in areas with high rates of clarithromycin resistance. In regions where resistance is prevalent, the efficacy of clarithromycin can be significantly reduced, leading to treatment failure, as demonstrated in Chapter 2. This raises the question of whether alternative regimens should be prioritized. Many developed countries such as the EU and the USA now use quadruple therapy with bismuth compounds and including tetracycline to replace clarithromycin (Chey *et al.*, 2024; Malfertheiner *et al.*, 2022). In this chapter, it was confirmed that macrolides reduce IL-8 and hβD2 defensin levels during an *H. pylori* infection *in vitro*, supporting our hypothesis. Therefore, it could be deduced that macrolides can have anti-inflammatory properties that may help alleviate symptoms in *H. pylori* patients, and this knowledge is essential as whether the inclusion of macrolides in the standard triple therapy remains necessary in an area with high rates of clarithromycin, as proposed in The Maastricht VI/Florence Consensus report (Malfertheiner *et al.*, 2022). Ultimately, the information gained from this study such as patient's antibiotic resistance patterns and the connection between the host immune response and antibiotic eradication in *H. pylori* infection may guide clinicians in selecting the most effective therapy. This could lead to better patient outcomes, improved eradication rates, and enhanced symptom relief.

6.4 (Chapter 5): Whole-genome sequencing analysis of antimicrobial resistance in *H. pylori* clinical isolates

Traditional methods for testing antibiotic susceptibility, such as disk diffusion and E-test techniques, present several challenges and limitations**.** For example, a lack of reproducibility and comparability across laboratories and countries due to biological variability, as well as differences in technical staff training and standards (Boolchandani *et al.*, 2019; Khan *et al.*, 2019). This often results in empirical treatment, which can lead to treatment failure and an increasing resistance rate**.** Ideally, susceptibility testing should occur at the initial diagnosis of *H. pylori* infection. However, because susceptibility testing is impractical for clinical use, so it is typically performed only after an initial treatment failure or in regions with known high rates of clarithromycin resistance (Malfertheiner *et al.*, 2017). To address this issue, molecular techniques for identifying resistance have become an appealing option.

H. pylori infection is characterized by significant genomic diversity on a global scale, within local settings, and in individual patients. Research so far has explored this diversity through DNA fingerprinting, gene-specific analysis, and more recently, whole genome sequencing of multiple single colonies isolates from human, and animal infection models. These efforts have improved our understanding of *H. pylori*'s genetic diversity and its possible role in host adaptation, disease progression, and persistence. However, due to limited sample sizes, the complete picture of *H. pylori* diversity at the population level remains unclear (Wilkinson *et al.*, 2022).

In Chapter 5, a comparative analysis between the phenotypic and genotypic detection of antibiotic resistance among *H. pylori* clinical isolates was investigated. This study identified

common point mutations in the target gene *23S rRNA*, and amino acid changes in RdxA, FrxA, GyrA, as well as PBP1A, PBP2, and PBP3 in *H. pylori* isolates. The A2147G point mutation in *23S rRNA* was strongly associated with *H. pylori* strains showing phenotypic resistance to clarithromycin whilst the A85 and R90K amino acid changes in the FrxA protein were significantly linked to *H. pylori* strains demonstrating phenotypic resistance to metronidazole. Knowing point mutations and amino acid substitutions in target genes and proteins helps to elucidate the mechanisms by which *H. pylori* strains develop resistance to antibiotics. This can also serve as a predictive tool for determining antibiotic susceptibility, allowing for more accurate treatment decisions.

However, there was a lack of consistency between the genotype and phenotype resistance correlation for clarithromycin, metronidazole, and amoxicillin, but this discrepancy did not occur with levofloxacin in this study. Other researchers from different parts of the world have also explored both phenotypic and genotypic methods, leading to variations in their findings (Xiong *et al.*, 2023; Zhong *et al.*, 2021). While antibiotic resistance in *H. pylori* isolates was predicted using Comprehensive Antibiotic Resistance Database (CARD) in this study, PCR-based validation could also be carried out. The use of CARD can have limitations. For example, the resistance mechanisms catalogued in CARD may not fully capture the unique genetic and phenotypic diversity of *H. pylori*. Different strains may harbour distinct mutations or resistance genes that are not well represented in the database, and as *H. pylori* evolves, new resistance genes/mechanisms may emerge that are not yet documented in CARD. While CARD includes a broad range of bacteria and resistance mechanisms, the data specific to *H. pylori* might be less comprehensive. Therefore, this can lead to discrepancies between expected and actual resistance profiles.

Furthermore, *in silico* AMR predictions may also suffer from high false-susceptible (false-negative) and/or false-resistant (false-positive) rates. In resistance phenotyping, a false-negative result is considered a very major error (VME), and a false-positive result is a major error (ME). A VME might result in the use of an ineffective therapeutic agent for treatment, leading to treatment failure; an ME might limit therapeutic options and complicate treatment (Stoesser *et al.*, 2013; Zankari *et al.*, 2017). Meanwhile, Pesesky *et al.* (2016) highlighted that the WGS-AST comparison carried out on Enterobacteriaceae using CARD demonstrated poorer overall performance with reduced specificity and higher ME rates.

One large-scale study with a highly diverse dataset of thousands of bacterial isolates from multiple species, locations, and times illustrated that CARD may not yield optimal results to predict resistance phenotypes from single markers, and several limitations in genetic phenotype prediction still exist. To achieve FDA requirements for clinical microbiology diagnostic testing below 3% MEs and 1.5% VMEs, current AMR databases need to be further curated and expanded, provide AMR marker annotations per antibiotic rather than antibiotic class and employ combinations of individual AMR markers selected for optimal diagnostic performance in experimentally validated multivariate panels (Mahfouz *et al.*, 2020).

In Chapter 5, a detailed examination of the PBP1A and PBP2 amino acid sequences from both amoxicillin-resistant and susceptible *H. pylori* strains revealed several substitutions strongly linked to resistance. Mapping these substitutions onto the 3D structure of PBP1A showed that they are all located close to the active site. This indicates that amoxicillin resistance in *H. pylori* could be attributed to structural alterations that potentially decrease the drug's access to or affinity for the active site. The work presented in this chapter

suggests that resistance to amoxicillin is probably due to the accumulation of multiple mutations, unlike the specific DNA point mutations strongly linked to clarithromycin resistance. As a result, it is hypothesized that a rapid molecular-based detection method is unlikely to determine if strains are susceptible to amoxicillin accurately.

In summary, despite these limitations, WGS remains a valuable tool for understanding antibiotic resistance. Its integration with other methods, including phenotypic testing and epidemiological studies, can help provide a more comprehensive picture of resistance in *H. pylori* bacterial populations. Perhaps machine learning (ML) and artificial intelligence (AI) techniques could also be highly effective in identifying *H. pylori* genes associated with AMR and could significantly enhance diagnostic tests based on biopsy-derived WGS. By integrating ML with WGS-based diagnostics, it could become feasible to develop rapid, precise, and highly personalised eradication therapies for *H. pylori* as AI models can analyse the genome obtained from a biopsy to predict resistance profiles for multiple antibiotics significantly faster than traditional culture-based methods. Based on the predicted resistance profiles, the system can recommend the optimal combination of antibiotics to maximize eradication success while minimizing side effects and resistance development. These algorithms could also account for local resistance trends, host factors, and prior treatment history to tailor therapies.

Future research into the mutational causes of amoxicillin resistance in *H. pylori* should involve analysing a larger number of sequences to gain a deeper understanding of the frequency of PBP1A and PBP2 amino acid substitutions and their connection to amoxicillin resistance. It is crucial to continue monitoring the amoxicillin resistance background of *H. pylori* to ensure the sustained effectiveness of amoxicillin-based treatments. The

development of a computational pipeline to analyse the structural effects of these substitutions would also enhance our understanding of their role in the development of amoxicillin resistance. This will help to provide a detailed understanding of how specific mutations in target genes impact its function and resistance mechanisms, potentially informing the development of targeted therapeutic strategies to combat *H pylori* infections.

6.5 Conclusion

This study provides several novel insights into the antibiotic resistance patterns, host immune responses, and molecular mechanisms involved in *H. pylori* infection. These findings significantly contribute to the understanding of antimicrobial resistance dynamics and host-pathogen interactions within the UK population.

Firstly, a high prevalence of resistance to metronidazole and clarithromycin was demonstrated in *H. pylori* clinical isolates. Notably, different resistance profiles were observed between paired isolates obtained from the antrum and corpus of six patients, indicating the potential for intra-host heterogeneity. Temporal analysis also revealed an increasing trend of resistance to both clarithromycin and metronidazole over the years. Resistance rates were significantly higher in isolates from patients with a history of eradication therapy compared to treatment-naïve individuals. Moreover, patients aged 60 years and below were more likely to harbour strains resistant to clarithromycin and metronidazole, suggesting possible demographic influences on resistance acquisition. These findings underscore the importance of conducting routine antibiotic susceptibility testing prior to treatment to prevent ineffective regimens. Recognizing intra-host heterogeneity and temporal resistance trends can lead to more accurate diagnosis and treatment selection, helping to prevent treatment failures and reduce the risk of resistance spreading.

This contributes to global antimicrobial stewardship and improves patient outcomes through tailored therapy.

Histopathological evaluation revealed significantly higher inflammatory and pathological scores in the antrum compared to the corpus. Importantly, the study suggests that prolonged eradication therapy may reduce gastric inflammation and contribute to the regression of precancerous lesions such as atrophic gastritis. These findings highlight the potential benefit of sustained *H. pylori* management in modulating disease progression. In this context, early detection and regular follow-up for precancerous lesions are emphasized as essential components of patient care to ensure better outcomes. By linking eradication therapy with histological improvements, this study supports evidence-based guidelines promoting early and aggressive *H. pylori* treatment to reduce cancer risk, particularly in high-incidence regions.

At the molecular level, novel patterns of host antimicrobial peptide expression were identified. During *H. pylori* infection, human defensin *DEFB4* expression was significantly upregulated, while *DEFA5* expression was downregulated in *ex vivo* gastric tissues. Both defensins were associated with atrophic gastritis, however, only *DEFB4* expression was correlated with the presence of *H. pylori* virulence factors, such as *cagA*, indicating its potential role in host response to more virulent strains. These findings offer promising biomarkers for predicting mucosal immune responses and may help identify patients at risk of progressing to more severe gastric pathology. This contributes to the growing field of host biomarker research in *H. pylori* infection, which can enhance early diagnosis, stratify disease risk, and facilitate the development of targeted immunotherapies.

In vitro experiments using AGS gastric epithelial cells demonstrated that *H. pylori* infection, in the presence of TNFα co-stimulation, significantly enhanced IL-8 and human defensin hβD2 expression. Furthermore, macrolide antibiotics were found to attenuate both IL-8 production and hβD2 expression induced by *H. pylori*, with more pronounced effects observed in infections with *cagA*-positive strains. This suggests a potential immunomodulatory role of macrolides in addition to their antimicrobial action. The discovery that macrolides may modulate host immune responses opens new possibilities for adjunctive treatment strategies, where antibiotics not only eradicate bacteria but also reduce harmful inflammation. This dual benefit could be particularly useful in managing chronic or refractory *H. pylori*-associated gastritis, thus enhancing treatment outcomes.

Genomic analysis revealed several known and novel point mutations and amino acid substitutions in resistance-associated genes. The A2147G mutation in the *23S rRNA* gene was strongly associated with phenotypic resistance to clarithromycin. For metronidazole resistance, the A85V and R90K amino acid changes in the *frxA* gene were significantly correlated with resistant strains. An inconsistency in genotype-to-phenotype correlation was noted for clarithromycin, metronidazole, and amoxicillin resistance, but not for levofloxacin. Additionally, resistant strains exhibited a greater number of amino acid substitutions in the penicillin-binding protein PBP1A, particularly near the active sites, which are hypothesized to alter the protein structure and reduce amoxicillin binding affinity. These findings enrich the understanding of genetic resistance mechanisms in *H. pylori*, offering new molecular targets for diagnostic assays and surveillance tools. Identifying specific mutations associated with resistance can aid in the development of rapid molecular diagnostics, enabling personalized antibiotic selection and minimizing trial-and-error

approaches in therapy. This aligns with the goals of precision medicine and could help combat the rising tide of antimicrobial resistance.

Overall, this study provides important new insights into the resistance mechanisms of *H. pylori*, host immune responses, and the potential benefits of early therapeutic intervention and long-term follow-up in managing *H. pylori*-associated diseases. These findings offer valuable guidance for personalized treatment strategies, better disease risk stratification, and improved patient care, while also contributing to the global research efforts aimed at eradicating *H. pylori* and reducing the gastric cancer burden.

Chapter 7 : Bibliography

Abe, S., Miura, K., Kinoshita, A., Mishima, H., Miura, S., Yamasaki, K., Hasegawa, Y., Higashijima, A., Jo, O., Sasaki, K., Yoshida, A., Yoshiura, K.-i., & Masuzaki, H. (2013). Copy number variation of the antimicrobial-gene, defensin beta 4, is associated with susceptibility to cervical cancer. *Journal of Human Genetics*, *58*(5), 250-253. https://doi.org/10.1038/jhg.2013.7

Agency, U. H. S. (2023a). *English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) report 2022 to 2023*. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1118730/ESPAUR-report-2021-2022-annexe.pdf

Agency, U. H. S. (2023b). *English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) Report 2022 to 2023*. U. H. S. Agency. https://www.gov.uk/government/publications/english-surveillance-programme-antimicrobial-utilisation-and-resistance-espaur-report

Aghai, Z. H., Kode, A., Saslow, J. G., Nakhla, T., Farhath, S., Stahl, G. E., Eydelman, R., Strande, L., Leone, P., & Rahman, I. (2007). Azithromycin Suppresses Activation of Nuclear Factor-kappa B and Synthesis of Pro-inflammatory Cytokines in Tracheal Aspirate Cells From Premature Infants. *Pediatric Research*, *62*(4), 483-488. https://doi.org/10.1203/PDR.0b013e318142582d

Agudo, S., Pérez-Pérez, G., Alarcón, T., & López-Brea, M. (2010). High prevalence of clarithromycin-resistant *Helicobacter pylori* strains and risk factors associated with resistance in Madrid, Spain. *J Clin Microbiol*, *48*(10), 3703-3707. https://doi.org/10.1128/jcm.00144-10

Aguilar, C., Alves da Silva, M., Saraiva, M., Neyazi, M., Olsson, I. A. S., & Bartfeld, S. (2021). Organoids as host models for infection biology - a review of methods. *Exp Mol Med*, *53*(10), 1471-1482. https://doi.org/10.1038/s12276-021-00629-4

Ailloud, F., Didelot, X., Woltemate, S., Pfaffinger, G., Overmann, J., Bader, R. C., Schulz, C., Malfertheiner, P., & Suerbaum, S. (2019). Within-host evolution of *Helicobacter pylori* shaped by niche-specific adaptation, intragastric migrations and selective sweeps. *Nature Communications*, *10*(1), 2273. https://doi.org/10.1038/s41467-019-10050-1

Aisenbrey, C., Marquette, A., & Bechinger, B. (2019). The Mechanisms of Action of Cationic Antimicrobial Peptides Refined by Novel Concepts from Biophysical Investigations. In K. Matsuzaki (Ed.), *Antimicrobial Peptides: Basics for Clinical Application* (pp. 33-64). Springer Singapore. https://doi.org/10.1007/978-981-13-3588-4_4

Aktaş, D., Bağırova, M., Allahverdiyev, A. M., Abamor, E. Ş., Safarov, T., & Kocazeybek, B. S. (2020). Resuscitation of the *Helicobacter pylori* Coccoid Forms by Resuscitation Promoter Factor Obtained from *Micrococcus Luteus*. *Current Microbiology*, *77*, 2093 - 2103.

Al-Mathkour, M. M., Zhu, S., Cao, L., Khalafi, S., Chen, Z., Poveda, J., Peng, D., Lu, H., Soutto, M., Hu, T., McDonalnd, O., Zaika, A. I., & El-Rifai, W. (2023). Abstract 5972: CDK1 bridges NF-kB and b-catenin signaling in response to *H. pylori* infection in gastric tumorigenesis. *Cancer Research*.

Al-Roub, A. A., Akhter, N., Al-Rashed, F., Wilson, A., Alzaid, F., Al-Mulla, F., Sindhu, S., & Ahmad, R. (2023). TNFα induces matrix metalloproteinase-9 expression in monocytic cells through ACSL1/JNK/ERK/NF-kB signaling pathways. *Scientific Reports*, *13*.

Alam, J., Sarkar, A., Karmakar, B. C., Ganguly, M., Paul, S., & Mukhopadhyay, A. K. (2020). Novel virulence factor dupA of *Helicobacter pylori* as an important risk determinant for disease manifestation: An overview. *World J Gastroenterol*, *26*(32), 4739-4752. https://doi.org/10.3748/wjg.v26.i32.4739

Alarcón-Millán, J., Fernández-Tilapa, G., Cortés-Malagón, E. M., Castañón-Sánchez, C. A., De Sampedro-Reyes, J., Cruz-del Carmen, I., Betancourt-Linares, R., & Román-Román, A. (2016). Clarithromycin resistance and prevalence of *Helicobacter pylori* virulent genotypes in patients from Southern México with chronic gastritis. *Infection, Genetics and Evolution*, *44*, 190-198. https://doi.org/https://doi.org/10.1016/j.meegid.2016.06.044

Alba, C., Blanco, A., & Alarcón, T. (2017). Antibiotic resistance in *Helicobacter pylori*. *Curr Opin Infect Dis*, *30*(5), 489-497. https://doi.org/10.1097/qco.0000000000000396

Albanesi, C., Fairchild, H. R., Madonna, S., Scarponi, C., De Pità, O., Leung, D. Y. M., & Howell, M. D. (2007). IL-4 and IL-13 Negatively Regulate TNF-α- and IFN-γ-Induced β-Defensin Expression through STAT-6, Suppressor of Cytokine Signaling (SOCS)-1, and SOCS-31. *The Journal of Immunology*, *179*(2), 984-992. https://doi.org/10.4049/jimmunol.179.2.984

Alcock, B. P., Raphenya, A. R., Lau, T. T. Y., Tsang, K. K., Bouchard, M., Edalatmand, A., Huynh, W., Nguyen, A. V., Cheng, A. A., Liu, S., Min, S. Y., Miroshnichenko, A., Tran, H. K., Werfalli, R. E., Nasir, J. A., Oloni, M., Speicher, D. J., Florescu, A., Singh, B., . . . McArthur, A. G. (2020). CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Res*, *48*(D1), D517-d525. https://doi.org/10.1093/nar/gkz935

Aldhous, M. C., Noble, C. L., & Satsangi, J. (2009). Dysregulation of human beta-defensin-2 protein in inflammatory bowel disease. *PLoS One*, *4*(7), e6285. https://doi.org/10.1371/journal.pone.0006285

Alkim, H., Koksal, A. R., Boga, S., Sen, I., & Alkim, C. (2017). Role of Bismuth in the Eradication of *Helicobacter pylori*. *American Journal of Therapeutics*, *24*(6), e751-e757. https://doi.org/10.1097/mjt.0000000000000389

Allaker, R. P., & Kapas, S. (2003). Adrenomedullin Expression by Gastric Epithelial Cells in Response to Infection. *Clinical and Vaccine Immunology*, *10*(4), 546-551. https://doi.org/doi:10.1128/CDLI.10.4.546-551.2003

Allison, C. C., Kufer, T. A., Kremmer, E., Kaparakis, M., & Ferrero, R. L. (2009). *Helicobacter pylori* Induces MAPK Phosphorylation and AP-1 Activation via a NOD1-Dependent Mechanism1. *The Journal of Immunology*, *183*(12), 8099-8109. https://doi.org/10.4049/jimmunol.0900664

Alshevskaya, A., Zhukova, J., Kireev, F., Lopatnikova, J., Evsegneeva, I., Demina, D., Nepomniashchikch, V., Gladkikh, V., Karaulov, A., & Sennikov, S. (2022). Redistribution of TNF Receptor 1 and 2 Expression on Immune Cells in Patients with Bronchial Asthma. *Cells*, *11*(11). https://doi.org/10.3390/cells11111736

Altenburg, J., de Graaff, C. S., van der Werf, T. S., & Boersma, W. G. (2010). Immunomodulatory Effects of Macrolide Antibiotics – Part 2: Advantages and Disadvantages of Long-Term, Low-Dose Macrolide Therapy. *Respiration*, *81*(1), 75-87. https://doi.org/10.1159/000320320

Alvarez-Aldana, A., Ikhimiukor, O. O., Guaca-González, Y. M., Montoya-Giraldo, M., Souza, S. S. R., Buiatte, A. B. G., & Andam, C. P. (2024). Genomic insights into the antimicrobial resistance and virulence of *Helicobacter pylori* isolates from gastritis patients in Pereira, Colombia. *BMC Genomics*, *25*(1), 843. https://doi.org/10.1186/s12864-024-10749-6

Alzahrani, S., Lina, T. T., Gonzalez, J., Pinchuk, I. V., Beswick, E. J., & Reyes, V. E. (2014). Effect of *Helicobacter pylori* on gastric epithelial cells. *World J Gastroenterol*, *20*(36), 12767-12780. https://doi.org/10.3748/wjg.v20.i36.12767

Alzolibani, A. A., & Zedan, K. (2012). Macrolides in Chronic Inflammatory Skin Disorders. *Mediators of Inflammation*, *2012*(1), 159354. https://doi.org/https://doi.org/10.1155/2012/159354

Amieva, M. R., & El–Omar, E. M. (2008). Host-Bacterial Interactions in *Helicobacter pylori* Infection. *Gastroenterology*, *134*(1), 306-323. https://doi.org/https://doi.org/10.1053/j.gastro.2007.11.009

Andersson, A. F., Lindberg, M., Jakobsson, H., Bäckhed, F., Nyrén, P., & Engstrand, L. (2008). Comparative Analysis of Human Gut Microbiota by Barcoded Pyrosequencing. *PLoS One*, *3*(7), e2836. https://doi.org/10.1371/journal.pone.0002836

Andrés, C. M. C., Pérez de la Lastra, J. M., Juan, C. A., Plou, F. J., & Pérez-Lebeña, E. (2022). The Role of Reactive Species on Innate Immunity. *Vaccines*, *10*(10), 1735. https://www.mdpi.com/2076-393X/10/10/1735

Andrews, S. (2010). *FastQC - A Quality Control application for FastQ files*. Retrieved 20/8/2024 from https://www.bioinformatics.babraham.ac.uk/index.html

Anis, S., Farooqi, S. R., & Niaz, S. K. (2021). Characterization of Domain V Mutations in Clinical Isolates of *Helicobacter pylori* in Pakistan and Their Effect on Clarithromycin MIC. *Infect Drug Resist*, *14*, 3393-3403. https://doi.org/10.2147/idr.S306878

Ansari, S., & Yamaoka, Y. (2017). Survival of *Helicobacter pylori* in gastric acidic territory. *Helicobacter*, *22*(4). https://doi.org/10.1111/hel.12386

Areia, M., Carvalho, R., Cadime, A. T., Rocha Gonçalves, F., & Dinis-Ribeiro, M. (2013). Screening for Gastric Cancer and Surveillance of Premalignant Lesions: a Systematic Review of Cost-Effectiveness Studies. *Helicobacter*, *18*(5), 325-337. https://doi.org/https://doi.org/10.1111/hel.12050

Argent, R. H., Thomas, R. J., Aviles-Jimenez, F., Letley, D. P., Limb, M. C., El-Omar, E. M., & Atherton, J. C. (2008). Toxigenic *Helicobacter pylori* infection precedes gastric hypochlorhydria in cancer relatives, and H. pylori virulence evolves in these families. *Clin Cancer Res*, *14*(7), 2227-2235. https://doi.org/10.1158/1078-0432.Ccr-07-2022

Armbruster, N. S., Stange, E. F., & Wehkamp, J. (2017). In the Wnt of Paneth Cells: Immune-Epithelial Crosstalk in Small Intestinal Crohn's Disease. *Front Immunol*, *8*, 1204. https://doi.org/10.3389/fimmu.2017.01204

Atherton, J. C. (2006). The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annu Rev Pathol*, *1*, 63-96. https://doi.org/10.1146/annurev.pathol.1.110304.100125

Atherton, J. C., & Blaser, M. J. (2009). Coadaptation of *Helicobacter pylori* and humans: ancient history, modern implications. *J Clin Invest*, *119*(9), 2475-2487. https://doi.org/10.1172/jci38605

Atherton, J. C., Cao, P., Peek, R. M., Tummuru, M. K. R., Blaser, M. J., & Cover, T. L. (1995). Mosaicism in Vacuolating Cytotoxin Alleles of *Helicobacter pylori*: ASSOCIATION OF SPECIFIC vacA TYPES WITH CYTOTOXIN PRODUCTION AND PEPTIC ULCERATION (∗). *Journal of Biological Chemistry*, *270*(30), 17771-17777. https://doi.org/10.1074/jbc.270.30.17771

Atretkhany, K. S. N., Drutskaya, M. S., Nedospasov, S. A., Grivennikov, S. I., & Kuprash, D. V. (2016). Chemokines, cytokines and exosomes help tumors to shape inflammatory microenvironment [Review]. *Pharmacology and Therapeutics*, *168*, 98-112. https://doi.org/10.1016/j.pharmthera.2016.09.011

Attaran, B., Falsafi, T., & 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*(7), 1163-1170. https://doi.org/10.3748/wjg.v23.i7.1163

Attaran, B., Salehi, N., Ghadiri, B., Esmaeili, M., Kalateh, S., Tashakoripour, M., Eshagh Hosseini, M., & Mohammadi, M. (2021). The penicillin binding protein 1A of *Helicobacter pylori*, its amoxicillin binding site and access routes. *Gut Pathog*, *13*(1), 43. https://doi.org/10.1186/s13099-021-00438-0

Ayala, G., Galván-Portillo, M., Chihu, L., Fierros, G., Sánchez, A., Carrillo, B., Román, A., López-Carrillo, L., & Silva-Sánchez, J. (2011). Resistance to antibiotics and characterization of *Helicobacter pylori* strains isolated from antrum and body from adults in Mexico. *Microb Drug Resist*, *17*(2), 149-155. https://doi.org/10.1089/mdr.2010.0154

Ayaş, M., Oktem-Okullu, S., Özcan, O., Kocagöz, T., & Gürol, Y. (2024). Exploring the Molecular Mechanisms of Macrolide Resistance in Laboratory Mutant *Helicobacter pylori*. *Antibiotics (Basel)*, *13*(5). https://doi.org/10.3390/antibiotics13050396

Azzaya, D., Gantuya, B., Oyuntsetseg, K., Davaadorj, D., Matsumoto, T., Akada, J., & Yamaoka, Y. (2020). High Antibiotic Resistance of *Helicobacter pylori* and Its Associated Novel Gene Mutations among the Mongolian Population. *Microorganisms*, *8*(7). https://doi.org/10.3390/microorganisms8071062

Backert, S., Bernegger, S., Skórko-Glonek, J., & Wessler, S. (2018). Extracellular HtrA serine proteases: An emerging new strategy in bacterial pathogenesis. *Cellular Microbiology*, *20*(6), e12845. https://doi.org/https://doi.org/10.1111/cmi.12845

Backert, S., & Naumann, M. (2010). What a disorder: proinflammatory signaling pathways induced by *Helicobacter pylori*. *Trends Microbiol*, *18*(11), 479-486. https://doi.org/10.1016/j.tim.2010.08.003

Backert, S., & Tegtmeyer, N. (2017). Type IV Secretion and Signal Transduction of *Helicobacter pylori* CagA through Interactions with Host Cell Receptors. *Toxins*, *9*(4), 115. https://www.mdpi.com/2072-6651/9/4/115

Backert, S., Tegtmeyer, N., Horn, A. H. C., Sticht, H., & Linz, B. (2024). Two remarkable serine/leucine polymorphisms in *Helicobacter pylori*: functional importance for serine protease HtrA and adhesin BabA. *Cell Commun Signal*, *22*(1), 250. https://doi.org/10.1186/s12964-024-01635-5

Bacon, C. M., Du, M. Q., & Dogan, A. (2007). Mucosa-associated lymphoid tissue (MALT) lymphoma: a practical guide for pathologists. *J Clin Pathol*, *60*(4), 361-372. https://doi.org/10.1136/jcp.2005.031146

Badger-Emeka, L. I., Emeka, P. M., & Okosi, M. (2018). Evaluation of the extent and reasons for increased non-prescription antibiotics use in a University town, Nsukka Nigeria. *Int J Health Sci (Qassim)*, *12*(4), 11-17.

Badr, M. T., Omar, M., & Häcker, G. (2021). Comprehensive Integration of Genome-Wide Association and Gene Expression Studies Reveals Novel Gene Signatures and Potential Therapeutic Targets for *Helicobacter pylori*-Induced Gastric Disease. *Front Immunol*, *12*, 624117. https://doi.org/10.3389/fimmu.2021.624117

Bae, M., Jang, S., Lim, J. W., Kang, J., Bak, E. J., Cha, J. H., & Kim, H. (2014). Protective effect of Korean Red Ginseng extract against *Helicobacter pylori*-induced gastric inflammation in Mongolian gerbils. *J Ginseng Res*, *38*(1), 8-15. https://doi.org/10.1016/j.jgr.2013.11.005

Bakhti, S. Z., Latifi-Navid, S., Mohammadi, S., Zahri, S., Bakhti, F. S., Feizi, F., Yazdanbod, A., & Siavoshi, F. (2016). Relevance of *Helicobacter pylori* vacA 3'-end Region Polymorphism to Gastric Cancer. *Helicobacter*, *21*(4), 305-316. https://doi.org/https://doi.org/10.1111/hel.12284

Balendra, V., Amoroso, C., Galassi, B., Esposto, J., Bareggi, C., Luu, J., Scaramella, L., & Ghidini, M. (2023). High-Salt Diet Exacerbates *H. pylori* Infection and Increases Gastric Cancer Risks. *Journal of Personalized Medicine*, *13*(9), 1325. https://www.mdpi.com/2075-4426/13/9/1325

Banatvala, N., Davies, G. R., Abdi, Y., Clements, L., Rampton, D. S., Hardie, J. M., & Feldman, R. A. (1994). High prevalence of *Helicobacter pylori* metronidazole resistance in migrants to east London: relation with previous nitroimidazole exposure and gastroduodenal disease. *Gut*, *35*(11), 1562-1566. https://doi.org/10.1136/gut.35.11.1562

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol*, *19*(5), 455-477. https://doi.org/10.1089/cmb.2012.0021

Banks, M., Graham, D., Jansen, M., Gotoda, T., Coda, S., di Pietro, M., Uedo, N., Bhandari, P., Pritchard, D. M., Kuipers, E. J., Rodriguez-Justo, M., Novelli, M. R., Ragunath, K., Shepherd, N., & Dinis-Ribeiro, M. (2019). British Society of Gastroenterology guidelines on the diagnosis and management of patients at risk of gastric adenocarcinoma. *Gut*, *68*(9), 1545-1575. https://doi.org/10.1136/gutjnl-2018-318126

Banks, P. M. (2007). Gastrointestinal lymphoproliferative disorders. *Histopathology*, *50*(1), 42-54. https://doi.org/https://doi.org/10.1111/j.1365-2559.2006.02571.x

Barnabei, L., Laplantine, E., Mbongo, W., Rieux-Laucat, F., & Weil, R. (2021). NF-κB: At the Borders of Autoimmunity and Inflammation [Review]. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.716469

Barrozo, R. M., Cooke, C. L., Hansen, L. M., Lam, A. M., Gaddy, J. A., Johnson, E. M., Cariaga, T. A., Suarez, G., Peek, R. M., Jr., Cover, T. L., & Solnick, J. V. (2013). Functional Plasticity in the

Type IV Secretion System of *Helicobacter pylori*. *PLOS Pathogens*, *9*(2), e1003189. https://doi.org/10.1371/journal.ppat.1003189

Bartfeld, S., Bayram, T., van de Wetering, M., Huch, M., Begthel, H., Kujala, P., Vries, R., Peters, P. J., & Clevers, H. (2015). *In vitro* expansion of human gastric epithelial stem cells and their responses to bacterial infection. *Gastroenterology*, *148*(1), 126-136.e126. https://doi.org/10.1053/j.gastro.2014.09.042

Bashir, S. K., & Khan, M. B. (2023). Overview of *Helicobacter pylori* Infection, Prevalence, Risk Factors, and Its Prevention. *Advanced Gut & Microbiome Research*, *2023*(1), 9747027. https://doi.org/https://doi.org/10.1155/2023/9747027

Basso, D., Zambon, C. F., Letley, D. P., Stranges, A., Marchet, A., Rhead, J. L., Schiavon, S., Guariso, G., Ceroti, M., Nitti, D., Rugge, M., Plebani, M., & Atherton, J. C. (2008). Clinical Relevance of *Helicobacter pylori cagA* and *vacA* Gene Polymorphisms. *Gastroenterology*, *135*(1), 91-99. https://doi.org/https://doi.org/10.1053/j.gastro.2008.03.041

Batiha, G. E.-S., Zayed, M. A., Awad, A. A., Shaheen, H. M., Mustapha, S., Herrera-Calderon, O., Pagnossa, J. P., Algammal, A. M., Zahoor, M., Adhikari, A., Pandey, I., Elazab, S. T., Rengasamy, K. R. R., Cruz-Martins, N., & Hetta, H. F. (2021). Management of SARS-CoV-2 Infection: Key Focus in Macrolides Efficacy for COVID-19 [Review]. *Frontiers in Medicine*, *8*. https://doi.org/10.3389/fmed.2021.642313

Battista, S., Ambrosio, M. R., Limarzi, F., Gallo, G., & Saragoni, L. (2021). Molecular Alterations in Gastric Preneoplastic Lesions and Early Gastric Cancer. *International Journal of Molecular Sciences*, *22*(13), 6652. https://www.mdpi.com/1422-0067/22/13/6652

Bauer, B., & Meyer, T. F. (2011). The Human Gastric Pathogen *Helicobacter pylori* and Its Association with Gastric Cancer and Ulcer Disease. *Ulcers*, *2011*(1), 340157. https://doi.org/https://doi.org/10.1155/2011/340157

Bauer, B., Pang, E., Holland, C., Kessler, M., Bartfeld, S., & Meyer, Thomas F. (2012). The *Helicobacter pylori* Virulence Effector CagA Abrogates Human β-Defensin 3 Expression via Inactivation of EGFR Signaling. *Cell Host & Microbe*, *11*(6), 576-586. https://doi.org/https://doi.org/10.1016/j.chom.2012.04.013

Bauer, B., Wex, T., Kuester, D., Meyer, T., & Malfertheiner, P. (2013). Differential expression of human beta defensin 2 and 3 in gastric mucosa of *Helicobacter pylori*-infected individuals. *Helicobacter*, *18*(1), 6-12. https://doi.org/10.1111/hel.12000

Bayerdörffer, E., Lehn, N., Hatz, R., Mannes, G. A., Oertel, H., Sauerbruch, T., & Stolte, M. (1992). Difference in expression of *Helicobacter pylori* gastritis in antrum and body. *Gastroenterology*, *102*(5), 1575-1582. https://doi.org/10.1016/0016-5085(92)91716-h

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

Beales, I. L. P., & Calam, J. (1997). STIMULATION OF IL-8 PRODUCTION IN HUMAN GASTRIC EPITHELIAL CELLS BY*HELICOBACTER PYLORI*, IL-1β AND TNF-α REQUIRES TYROSINE KINASE ACTIVITY, BUT NOT PROTEIN KINASE C. *Cytokine*, *9*(7), 514-520. https://doi.org/https://doi.org/10.1006/cyto.1996.0195

Behera, B., Anil Vishnu, G. K., Chatterjee, S., Sitaramgupta V, V. S. N., Sreekumar, N., Nagabhushan, A., Rajendran, N., Prathik, B. H., & Pandya, H. J. (2019). Emerging technologies for antibiotic susceptibility testing. *Biosensors and Bioelectronics*, *142*, 111552. https://doi.org/https://doi.org/10.1016/j.bios.2019.111552

Belachew, S. A., Hall, L., & Selvey, L. A. (2021). Non-prescription dispensing of antibiotic agents among community drug retail outlets in Sub-Saharan African countries: a systematic review and meta-analysis. *Antimicrobial Resistance & Infection Control*, *10*(1), 13. https://doi.org/10.1186/s13756-020-00880-w

Bentley, R. W., Pearson, J., Gearry, R. B., Barclay, M. L., McKinney, C., Merriman, T. R., & Roberts, R. L. (2010). Association of Higher *DEFB4* Genomic Copy Number With Crohn's Disease. *The*

American Journal of Gastroenterology, *105*(2), 354-359. https://doi.org/https://doi.org/10.1038/ajg.2009.582

Bernegger, S., Vidmar, R., Fonovic, M., Posselt, G., Turk, B., & Wessler, S. (2021). Identification of Desmoglein-2 as a novel target of *Helicobacter pylori* HtrA in epithelial cells. *Cell Commun Signal*, *19*(1), 108. https://doi.org/10.1186/s12964-021-00788-x

Best, L. M., Haldane, D. J., Keelan, M., Taylor, D. E., Thomson, A. B., Loo, V., Fallone, C. A., Lyn, P., Smaill, F. M., Hunt, R., Gaudreau, C., Kennedy, J., Alfa, M., Pelletier, R., & Veldhuyzen Van Zanten, S. J. (2003). Multilaboratory comparison of proficiencies in susceptibility testing of *Helicobacter pylori* and correlation between agar dilution and E test methods. *Antimicrob Agents Chemother*, *47*(10), 3138-3144. https://doi.org/10.1128/aac.47.10.3138-3144.2003

Beswick, E. J., Das, S., Pinchuk, I. V., Adegboyega, P., Suarez, G., Yamaoka, Y., & Reyes, V. E. (2005). *Helicobacter pylori*-Induced IL-8 Production by Gastric Epithelial Cells Up-Regulates CD74 Expression1. *The Journal of Immunology*, *175*(1), 171-176. https://doi.org/10.4049/jimmunol.175.1.171

Bethesda. (2012). LiverTox: Clinical and Research Information on Drug-Induced Liver Injury. . In *LiverTox*. National Institute of Diabetes and Digestive and Kidney Diseases. https://www.ncbi.nlm.nih.gov/books/NBK547886/

Bhattacharjee, A., Sahoo, O. S., Sarkar, A., Bhattacharya, S., Chowdhury, R., Kar, S., & Mukherjee, O. (2024). Infiltration to infection: key virulence players of *Helicobacter pylori* pathogenicity. *Infection*, *52*(2), 345-384. https://doi.org/10.1007/s15010-023-02159-9

Bhattacharjee, M. K. (2022). Antibiotics That Inhibit Nucleic Acid Synthesis. In M. K. Bhattacharjee (Ed.), *Chemistry of Antibiotics and Related Drugs* (pp. 125-148). Springer International Publishing. https://doi.org/10.1007/978-3-031-07582-7_5

Bhosale, P. B., Abusaliya, A., Kim, H. H., Ha, S. E., Park, M., Jeong, S. H., Vetrivel, P., Heo, J. D., Kim, J.-A., Won, C., Kim, H.-W., & Kim, G. (2022). Apigetrin Promotes TNFα-Induced Apoptosis, Necroptosis, G2/M Phase Cell Cycle Arrest, and ROS Generation through Inhibition of NF-κB Pathway in Hep3B Liver Cancer Cells. *Cells*, *11*.

Bik, E. M., Eckburg, P. B., Gill, S. R., Nelson, K. E., Purdom, E. A., Francois, F., Perez-Perez, G., Blaser, M. J., & Relman, D. A. (2006). Molecular analysis of the bacterial microbiota in the human stomach. *Proceedings of the National Academy of Sciences*, *103*(3), 732-737. https://doi.org/doi:10.1073/pnas.0506655103

Birchler, T., Seibl, R., Büchner, K., Loeliger, S., Seger, R., Hossle, J. P., Aguzzi, A., & Lauener, R. P. (2001). Human Toll-like receptor 2 mediates induction of the antimicrobial peptide human beta-defensin 2 in response to bacterial lipoprotein. *European Journal of Immunology*, *31*(11), 3131-3137. https://doi.org/https://doi.org/10.1002/1521-4141(200111)31:11<3131::AID-IMMU3131>3.0.CO;2-G

Bluemel, B., Goelz, H., Goldmann, B., Grüger, J., Hamel, H., Loley, K., Ludolph, T., Meyer, J., Miehlke, S., Mohr, A., Tüffers, K., Usadel, H., Wagner, S., Wenzel, H., Wiemer, L., Vorreiter, J., Eisele, B., Hofreuter, D., & Glocker, E. O. (2020). Antimicrobial resistance of *Helicobacter pylori* in Germany, 2015 to 2018. *Clin Microbiol Infect*, *26*(2), 235-239. https://doi.org/10.1016/j.cmi.2019.06.007

Boccellato, F., Woelffling, S., Imai-Matsushima, A., Sanchez, G., Goosmann, C., Schmid, M., Berger, H., Morey, P., Denecke, C., Ordemann, J., & Meyer, T. F. (2019). Polarised epithelial monolayers of the gastric mucosa reveal insights into mucosal homeostasis and defence against infection. *Gut*, *68*(3), 400-413. https://doi.org/10.1136/gutjnl-2017-314540

Bodger, K., Wyatt, J. I., & Heatley, R. V. (2001). Variation in serum pepsinogens with severity and topography of *Helicobacter pylori*-associated chronic gastritis in dyspeptic patients referred for endoscopy. *Helicobacter*, *6*(3), 216-224. https://doi.org/10.1046/j.1523-5378.2001.00031.x

Boehnke, K. F., Valdivieso, M., Bussalleu, A., Sexton, R., Thompson, K. C., Osorio, S., Novoa Reyes, I., Crowley, J. J., Baker, L. H., & Xi, C. (2017). Antibiotic resistance among *Helicobacter pylori*

clinical isolates in Lima, Peru. *Infect Drug Resist*, *10*, 85-90. https://doi.org/10.2147/idr.S123798

Bogiel, T., Szaflarska-Popławska, A., & Krawczyk, A. (2025). Prevalence and Variability of *Helicobacter pylori* Clarithromycin Resistance Mutations in Pediatric Patients in Poland: A Genotypic Analysis Using the Bosphore Genotyping Kit. *Antibiotics*, *14*(4), 352. https://www.mdpi.com/2079-6382/14/4/352

Boltin, D., Ben-Zvi, H., Perets, T. T., Kamenetsky, Z., Samra, Z., Dickman, R., & Niv, Y. (2015). Trends in secondary antibiotic resistance of *Helicobacter pylori* from 2007 to 2014: has the tide turned? *J Clin Microbiol*, *53*(2), 522-527. https://doi.org/10.1128/jcm.03001-14

Bongkotvirawan, P., Aumpan, N., Pornthisarn, B., Chonprasertsuk, S., Siramolpiwat, S., Bhanthumkomol, P., Nunanun, P., Issariyakulkarn, N., Mahachai, V., Pawa, K. K., & Vilaichone, R.-k. (2024). Predictive Factors Associated with Survival in Female Gastric Cancer Patients in Southeast Asia. *Women's Health Reports*, *5*(1), 178-185. https://doi.org/10.1089/whr.2023.0069

Bonilla, S., Goldsmith, J., Mitchell, P., & Bousvaros, A. (2023). *Helicobacter pylori* Antimicrobial Resistance Using Next-Generation Sequencing in Stool Samples in a Pediatric Population. *J Pediatr Gastroenterol Nutr*, *77*(5), 623-627. https://doi.org/10.1097/mpg.0000000000003908

Boolchandani, M., D'Souza, A. W., & Dantas, G. (2019). Sequencing-based methods and resources to study antimicrobial resistance. *Nat Rev Genet*, *20*(6), 356-370. https://doi.org/10.1038/s41576-019-0108-4

Bortolanza, M., Santos-Lobato, B. L., Nascimento, G. C., & Del-Bel, E. (2020). Chapter 29 - Management with antibiotics in Parkinson's disease. In C. R. Martin & V. R. Preedy (Eds.), *Diagnosis and Management in Parkinson's Disease* (pp. 491-510). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-815946-0.00029-6

Bosnar, M., Bošnjak, B., Čužić, S., Hrvačić, B., Marjanović, N., Glojnarić, I., Čulić, O., Parnham, M. J., & Haber, V. E. (2009). Azithromycin and Clarithromycin Inhibit Lipopolysaccharide-Induced Murine Pulmonary Neutrophilia Mainly through Effects on Macrophage-Derived Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-1β. *Journal of Pharmacology and Experimental Therapeutics*, *331*(1), 104-113. https://doi.org/10.1124/jpet.109.155838

Boughan, P. K., Argent, R. H., Body-Malapel, M., Park, J.-H., Ewings, K. E., Bowie, A. G., Ong, S. J., Cook, S. J., Sorensen, O. E., Manzo, B. A., Inohara, N., Klein, N. J., Nuñez, G., Atherton, J. C., & Bajaj-Elliott, M. (2006). Nucleotide-binding Oligomerization Domain-1 and Epidermal Growth Factor Receptor: CRITICAL REGULATORS OF β-DEFENSINS DURING *HELICOBACTER PYLORI* INFECTION *. *Journal of Biological Chemistry*, *281*(17), 11637-11648. https://doi.org/10.1074/jbc.M510275200

Bouma, G., & Strober, W. (2003). The immunological and genetic basis of inflammatory bowel disease [Review]. *Nature Reviews Immunology*, *3*(7), 521-533. https://doi.org/10.1038/nri1132

Boyanova, L., Gergova, G., Markovska, R., Kandilarov, N., Davidkov, L., Spassova, Z., & Mitov, I. (2017). Primary *Helicobacter pylori* resistance in elderly patients over 20 years: A Bulgarian study. *Diagn Microbiol Infect Dis*, *88*(3), 264-267. https://doi.org/10.1016/j.diagmicrobio.2017.05.001

Boyanova, L., Hadzhiyski, P., Gergova, R., & Markovska, R. (2023). Evolution of *Helicobacter pylori* Resistance to Antibiotics: A Topic of Increasing Concern. *Antibiotics (Basel)*, *12*(2). https://doi.org/10.3390/antibiotics12020332

Boyanova, L., Ilieva, J., Gergova, G., Spassova, Z., Nikolov, R., Davidkov, L., Evstatiev, I., Kamburov, V., Katsarov, N., & Mitov, I. (2009). Evaluation of clinical and socio-demographic risk factors for antibacterial resistance of *Helicobacter pylori* in Bulgaria. *J Med Microbiol*, *58*(Pt 1), 94-100. https://doi.org/10.1099/jmm.0.003855-0

Boyanova, L., Mentis, A., Gubina, M., Rozynek, E., Gosciniak, G., Kalenic, S., Göral, V., Kupcinskas, L., Kantarçeken, B., Aydin, A., Archimandritis, A., Dzierżanowska, D., Vcev, A., Ivanova, K., Marina, M., Mitov, I., Petrov, P., Özden, A., & Popova, M. (2002). The status of antimicrobial resistance of *Helicobacter pylori* in eastern Europe. *Clinical Microbiology and Infection*, *8*(7), 388-396. https://doi.org/https://doi.org/10.1046/j.1469-0691.2002.00435.x

Braff, M. H., Bardan, A., Nizet, V., & Gallo, R. L. (2005). Cutaneous Defense Mechanisms by Antimicrobial Peptides. *Journal of Investigative Dermatology*, *125*(1), 9-13. https://doi.org/10.1111/j.0022-202X.2004.23587.x

Brandt, S., Kwok, T., Hartig, R., König, W., & Backert, S. (2005). NF-kappaB activation and potentiation of proinflammatory responses by the *Helicobacter pylori* CagA protein. *Proc Natl Acad Sci U S A*, *102*(26), 9300-9305. https://doi.org/10.1073/pnas.0409873102

Bridge, D. R., Blum, F. C., Jang, S., Kim, J., Cha, J.-H., & Merrell, D. S. (2017). Creation and Initial Characterization of Isogenic *Helicobacter pylori* CagA EPIYA Variants Reveals Differential Activation of Host Cell Signaling Pathways. *Scientific Reports*, *7*(1), 11057. https://doi.org/10.1038/s41598-017-11382-y

Brincat, A., & Hofmann, M. (2022). Automated extraction of genes associated with antibiotic resistance from the biomedical literature. *Database*, *2022*. https://doi.org/10.1093/database/baab077

Broutet, N., Marais, A., Lamouliatte, H., Mascarel, A. d., Samoyeau, R., Salamon, R., & Mégraud, F. (2001). cagA Status and Eradication Treatment Outcome of Anti-*Helicobacter pylori* Triple Therapies in Patients with Nonulcer Dyspepsia. *Journal of Clinical Microbiology*, *39*(4), 1319-1322. https://doi.org/doi:10.1128/jcm.39.4.1319-1322.2001

Broutet, N., Tchamgoué, S., Pereira, E., Lamouliatte, H., Salamon, R., & Mégraud, F. (2003). Risk factors for failure of *Helicobacter pylori* therapy--results of an individual data analysis of 2751 patients. *Aliment Pharmacol Ther*, *17*(1), 99-109. https://doi.org/10.1046/j.1365-2036.2003.01396.x

Bush, K., & Bradford, P. A. (2020). Epidemiology of β-Lactamase-Producing Pathogens. *Clinical Microbiology Reviews*, *33*(2), 10.1128/cmr.00047-00019. https://doi.org/doi:10.1128/cmr.00047-19

Butt, J., Varga, M. G., Wang, T., Tsugane, S., Shimazu, T., Zheng, W., Abnet, C. C., Yoo, K.-Y., Park, S. K., Kim, J., Jee, S. H., Qiao, Y.-l., Shu, X.-O., Waterboer, T., Pawlita, M., & Epplein, M. (2019). Smoking, *Helicobacter Pylori* Serology, and Gastric Cancer Risk in Prospective Studies from China, Japan, and Korea. *Cancer Prevention Research*, *12*(10), 667-674. https://doi.org/10.1158/1940-6207.Capr-19-0238

Cai, Y., Wang, C., Chen, Z., Xu, Z., Li, H., Li, W., & Sun, Y. (2020). Transporters HP0939, HP0497, and HP0471 participate in intrinsic multidrug resistance and biofilm formation in *Helicobacter pylori* by enhancing drug efflux. *Helicobacter*, *25*(4), e12715. https://doi.org/10.1111/hel.12715

Calam, J., & Baron, J. H. (2001). ABC of the upper gastrointestinal tract: Pathophysiology of duodenal and gastric ulcer and gastric cancer. *Bmj*, *323*(7319), 980-982. https://doi.org/10.1136/bmj.323.7319.980

Camargo, M. C., Piazuelo, M. B., Mera, R. M., Fontham, E. T., Delgado, A. G., Yepez, M. C., Ceron, C., Bravo, L. E., Bravo, J. C., & Correa, P. (2007). Effect of smoking on failure of *H. pylori* therapy and gastric histology in a high gastric cancer risk area of Colombia. *Acta Gastroenterol Latinoam*, *37*(4), 238-245.

Cambau, E., Allerheiligen, V., Coulon, C., Corbel, C., Lascols, C., Deforges, L., Soussy, C. J., Delchier, J. C., & Megraud, F. (2009). Evaluation of a new test, genotype HelicoDR, for molecular detection of antibiotic resistance in *Helicobacter pylori*. *J Clin Microbiol*, *47*(11), 3600-3607. https://doi.org/10.1128/jcm.00744-09

Camorlinga-Ponce, M., Gómez-Delgado, A., Aguilar-Zamora, E., Torres, R. C., Giono-Cerezo, S., Escobar-Ogaz, A., & Torres, J. (2020). Phenotypic and Genotypic Antibiotic Resistance

Patterns in *Helicobacter pylori* Strains From Ethnically Diverse Population in México. *Front Cell Infect Microbiol*, *10*, 539115. https://doi.org/10.3389/fcimb.2020.539115

Camorlinga-Ponce, M., Gómez-Delgado, A., Aguilar-Zamora, E., Torres, R. C., Giono-Cerezo, S., Escobar-Ogaz, A., & Torres, J. (2021). Phenotypic and Genotypic Antibiotic Resistance Patterns in *Helicobacter pylori* Strains From Ethnically Diverse Population in México [Original Research]. *Frontiers in Cellular and Infection Microbiology*, *10*. https://doi.org/10.3389/fcimb.2020.539115

Carapito, R., Chesnel, L., Vernet, T., & Zapun, A. (2006). Pneumococcal beta-lactam resistance due to a conformational change in penicillin-binding protein 2x. *J Biol Chem*, *281*(3), 1771-1777. https://doi.org/10.1074/jbc.M511506200

Cardos, I. A., Zaha, D. C., Sindhu, R. K., & Cavalu, S. (2021). Revisiting Therapeutic Strategies for *H. pylori* Treatment in the Context of Antibiotic Resistance: Focus on Alternative and Complementary Therapies. *Molecules*, *26*.

Carvalho, M. A., Machado, N. C., Ortolan, E. V., & Rodrigues, M. A. (2012). Upper gastrointestinal histopathological findings in children and adolescents with nonulcer dyspepsia with *Helicobacter pylori* infection. *J Pediatr Gastroenterol Nutr*, *55*(5), 523-529. https://doi.org/10.1097/MPG.0b013e3182618136

Castaño-Rodríguez, N., Kaakoush, N. O., & Mitchell, H. M. (2014). Pattern-Recognition Receptors and Gastric Cancer [Review]. *Frontiers in Immunology*, *5*. https://doi.org/10.3389/fimmu.2014.00336

Castellana, C., Eusebi, L. H., Dajti, E., Iascone, V., Vestito, A., Fusaroli, P., Fuccio, L., D'Errico, A., & Zagari, R. M. (2024). Autoimmune Atrophic Gastritis: A Clinical Review. *Cancers*, *16*(7), 1310. https://www.mdpi.com/2072-6694/16/7/1310

Catherwood, A. C., Lloyd, A. J., Tod, J. A., Chauhan, S., Slade, S. E., Walkowiak, G. P., Galley, N. F., Punekar, A. S., Smart, K., Rea, D., Evans, N. D., Chappell, M. J., Roper, D. I., & Dowson, C. G. (2020). Substrate and Stereochemical Control of Peptidoglycan Cross-Linking by Transpeptidation by *Escherichia coli* PBP1B. *Journal of the American Chemical Society*, *142*(11), 5034-5048. https://doi.org/10.1021/jacs.9b08822

Cha, B., Lim, J. W., & Kim, H. (2015). Jak1/Stat3 is an upstream signaling of NF-κB activation in *Helicobacter pylori*-induced IL-8 production in gastric epithelial AGS cells. *Yonsei Med J*, *56*(3), 862-866. https://doi.org/10.3349/ymj.2015.56.3.862

Chaithongyot, S., Jantaree, P., Sokolova, O., & Naumann, M. (2021). NF-κB in Gastric Cancer Development and Therapy. *Biomedicines*, *9*(8). https://doi.org/10.3390/biomedicines9080870

Chang, W.-L., Yeh, Y.-C., & Sheu, B.-S. (2018). The impacts of *H. pylori* virulence factors on the development of gastroduodenal diseases. *Journal of Biomedical Science*, *25*(1), 68. https://doi.org/10.1186/s12929-018-0466-9

Chang, Y. W., Ko, W. J., Oh, C. H., Park, Y. M., Oh, S. J., Moon, J. R., Cho, J. H., Kim, J. W., & Jang, J. Y. (2019). Clarithromycin resistance and female gender affect *Helicobacter pylori* eradication failure in chronic gastritis. *Korean J Intern Med*, *34*(5), 1022-1029. https://doi.org/10.3904/kjim.2018.054

Chapelle, N., Péron, M., Quénéhervé, L., Bourget, A., Leroy, M., Touchefeu, Y., Cauchin, E., Coron, E., Mosnier, J. F., & Matysiak-Budnik, T. (2020). Long-Term Follow-up of Gastric Precancerous Lesions in a Low GC Incidence Area. *Clin Transl Gastroenterol*, *11*(12), e00237. https://doi.org/10.14309/ctg.0000000000000237

Chattopadhyay, I., Gundamaraju, R., & Rajeev, A. (2023). Diversification and deleterious role of microbiome in gastric cancer. *Cancer Reports*, *6*(11), e1878. https://doi.org/https://doi.org/10.1002/cnr2.1878

Chauhan, N., Tay, A. C. Y., Marshall, B. J., & Jain, U. (2019). *Helicobacter pylori* VacA, a distinct toxin exerts diverse functionalities in numerous cells: An overview. *Helicobacter*, *24*(1), e12544. https://doi.org/10.1111/hel.12544

Chaves, S., Gadanho, M., Tenreiro, R., & Cabrita, J. (1999). Assessment of metronidazole susceptibility in *Helicobacter pylori*: statistical validation and error rate analysis of breakpoints determined by the disk diffusion test. *J Clin Microbiol*, *37*(5), 1628-1631. https://doi.org/10.1128/jcm.37.5.1628-1631.1999

Chen, B., Liu, X., Yu, P., Xie, F., Kwan, J. S. H., Chan, W. N., Fang, C., Zhang, J., Cheung, A. H. K., Chow, C., Leung, G. W. M., Leung, K. T., Shi, S., Zhang, B., Wang, S., Xu, D., Fu, K., Wong, C. C., Wu, W. K. K., . . . Kang, W. (2023a). *H. pylori*-induced NF-κB-PIEZO1-YAP1-CTGF axis drives gastric cancer progression and cancer-associated fibroblast-mediated tumour microenvironment remodelling. *Clin Transl Med*, *13*(11), e1481. https://doi.org/10.1002/ctm2.1481

Chen, H.-Y., Hu, Y., Lu, N.-H., & Zhu, Y. (2020). Caudal type homeoboxes as a driving force in *Helicobacter pylori* infection-induced gastric intestinal metaplasia. *Gut Microbes*, *12*(1), 1809331. https://doi.org/10.1080/19490976.2020.1809331

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2018a). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, *9*(6), 7204-7218. https://doi.org/10.18632/oncotarget.23208

Chen, M., Huang, X., Gao, M., Yang, Z., Fang, Z., Wei, J., & Wu, B. (2022). *Helicobacter pylori* promotes inflammatory factor secretion and lung injury through VacA exotoxin-mediated activation of NF-κB signaling. *Bioengineered*, *13*(5), 12760-12771. https://doi.org/10.1080/21655979.2022.2071011

Chen, M. Y., He, C. Y., Meng, X., & Yuan, Y. (2013). Association of *Helicobacter pylori babA2* with peptic ulcer disease and gastric cancer. *World J Gastroenterol*, *19*(26), 4242-4251. https://doi.org/10.3748/wjg.v19.i26.4242

Chen, R., Li, Y., Chen, X., Chen, J., Song, J., Yang, X., Ye, L., Wu, Z., Xie, P., Zhong, Q., Yang, R., & Wu, J. (2023b). *dupA+H. pylori* reduces diversity of gastric microbiome and increases risk of erosive gastritis [Original Research]. *Frontiers in Cellular and Infection Microbiology*, *13*. https://doi.org/10.3389/fcimb.2023.1103909

Chen, X., Xia, C., Li, Q., Jin, L., Zheng, L., & Wu, Z. (2018b). Comparisons Between Bacterial Communities in Mucosa in Patients With Gastric Antrum Ulcer and a Duodenal Ulcer. *Front Cell Infect Microbiol*, *8*, 126. https://doi.org/10.3389/fcimb.2018.00126

Chen, Y. C., Malfertheiner, P., Yu, H. T., Kuo, C. L., Chang, Y. Y., Meng, F. T., Wu, Y. X., Hsiao, J. L., Chen, M. J., Lin, K. P., Wu, C. Y., Lin, J. T., O'Morain, C., Megraud, F., Lee, W. C., El-Omar, E. M., Wu, M. S., & Liou, J. M. (2024). Global Prevalence of *Helicobacter pylori* Infection and Incidence of Gastric Cancer Between 1980 and 2022. *Gastroenterology*, *166*(4), 605-619. https://doi.org/10.1053/j.gastro.2023.12.022

Cheung, P. S., Si, E. C., & Hosseini, K. (2010). Anti-inflammatory Activity of Azithromycin as Measured by Its NF-κB Inhibitory Activity. *Ocular Immunology and Inflammation*, *18*(1), 32-37. https://doi.org/10.3109/09273940903359725

Chey, W. D., Howden, C. W., Moss, S. F., Morgan, D. R., Greer, K. B., Grover, S., & Shah, S. C. (2024). ACG Clinical Guideline: Treatment of *Helicobacter pylori* Infection. *Official journal of the American College of Gastroenterology | ACG*, *119*(9), 1730-1753. https://doi.org/10.14309/ajg.0000000000002968

Chey, W. D., Leontiadis, G. I., Howden, C. W., & Moss, S. F. (2017). ACG Clinical Guideline: Treatment of *Helicobacter pylori* Infection. *Am J Gastroenterol*, *112*(2), 212-239. https://doi.org/10.1038/ajg.2016.563

Chi, W., Wang, S., Liu, T., Jiang, W., Ding, L., Miao, Y., Yang, F., Zhang, J., Ji, D., Xiao, Z., Zhu, H., Wu, Y., Bao, Z., Zhao, H., & Wang, S. (2023). A rapid and high-throughput multiplex genetic detection assay for detection, semi-quantification and virulence genotyping of *Helicobacter pylori* in non-invasive oral samples [Original Research]. *Frontiers in Cellular and Infection Microbiology*, *13*. https://doi.org/10.3389/fcimb.2023.1267288

Chisholm, S. A., & Owen, R. J. (2009). Frequency and molecular characteristics of ciprofloxacin- and rifampicin-resistant *Helicobacter pylori* from gastric infections in the UK. *J Med Microbiol*, *58*(Pt 10), 1322-1328. https://doi.org/10.1099/jmm.0.011270-0

Chisholm, S. A., Teare, E. L., Davies, K., & Owen, R. J. (2007). Surveillance of primary antibiotic resistance of *Helicobacter pylori* at centres in England and Wales over a six-year period (2000-2005). *Euro Surveill*, *12*(7), E3-4. https://doi.org/10.2807/esm.12.07.00721-en

Chitapanarux, T., Jesadaporn, P., Chitapanarux, N., & Lertprasertsuke, N. (2021). Chronic gastritis according to age and *Helicobacter pylori* in Thailand: histopathological patterns. *Scand J Gastroenterol*, *56*(3), 228-233. https://doi.org/10.1080/00365521.2020.1869820

Cho, J. H., & Jin, S. Y. (2022). Current guidelines for *Helicobacter pylori* treatment in East Asia 2022: Differences among China, Japan, and South Korea. *World J Clin Cases*, *10*(19), 6349-6359. https://doi.org/10.12998/wjcc.v10.i19.6349

Chong, G. O., Han, H. S., Lee, S. D., & Lee, Y. H. (2020). Improvement in RNA quantity and quality in cervico-vaginal cytology. *Virol J*, *17*(1), 8. https://doi.org/10.1186/s12985-020-1282-x

Chu, H., Pazgier, M., Jung, G., Nuccio, S. P., Castillo, P. A., de Jong, M. F., Winter, M. G., Winter, S. E., Wehkamp, J., Shen, B., Salzman, N. H., Underwood, M. A., Tsolis, R. M., Young, G. M., Lu, W., Lehrer, R. I., Bäumler, A. J., & Bevins, C. L. (2012). Human α-defensin 6 promotes mucosal innate immunity through self-assembled peptide nanonets. *Science*, *337*(6093), 477-481. https://doi.org/10.1126/science.1218831

Chung, C., Olivares, A., Torres, E., Yilmaz, O., Cohen, H., & Perez-Perez, G. (2010). Diversity of VacA intermediate region among *Helicobacter pylori* strains from several regions of the world. *J Clin Microbiol*, *48*(3), 690-696. https://doi.org/10.1128/jcm.01815-09

Chung, L. K., & Raffatellu, M. (2019). G.I. pros: Antimicrobial defense in the gastrointestinal tract. *Semin Cell Dev Biol*, *88*, 129-137. https://doi.org/10.1016/j.semcdb.2018.02.001

Cigana, C., Assael, B. M., & Melotti, P. (2007). Azithromycin selectively reduces tumor necrosis factor alpha levels in cystic fibrosis airway epithelial cells. *Antimicrob Agents Chemother*, *51*(3), 975-981. https://doi.org/10.1128/aac.01142-06

Cigana, C., Nicolis, E., Pasetto, M., Assael, B. M., & Melotti, P. (2006). Anti-inflammatory effects of azithromycin in cystic fibrosis airway epithelial cells. *Biochem Biophys Res Commun*, *350*(4), 977-982. https://doi.org/10.1016/j.bbrc.2006.09.132

Compeau, P. E., Pevzner, P. A., & Tesler, G. (2011). How to apply de Bruijn graphs to genome assembly. *Nat Biotechnol*, *29*(11), 987-991. https://doi.org/10.1038/nbt.2023

Contreras-Martel, C., Job, V., Di Guilmi, A. M., Vernet, T., Dideberg, O., & Dessen, A. (2006). Crystal structure of penicillin-binding protein 1a (PBP1a) reveals a mutational hotspot implicated in beta-lactam resistance in *Streptococcus pneumoniae*. *J Mol Biol*, *355*(4), 684-696. https://doi.org/10.1016/j.jmb.2005.10.030

Control, E. C. f. D. P. a. (2019). *Antimicrobial consumption in the EU/EEA, annual epidemiological report for 2018*. E. C. f. D. P. a. Control. https://www.ecdc.europa.eu/sites/default/files/documents/Antimicrobial-consumption-EU-EEA.pdf

Correa, P., Fontham, E. T. H., Bravo, J. C., Bravo, L. E., Ruiz, B., Zarama, G., Realpe, J. L., Malcom, G. T., Li, D., Johnson, W. D., & Mera, R. (2000). Chemoprevention of Gastric Dysplasia: Randomized Trial of Antioxidant Supplements and Anti-*Helicobacter pylori* Therapy. *JNCI: Journal of the National Cancer Institute*, *92*(23), 1881-1888. https://doi.org/10.1093/jnci/92.23.1881

Correia, S., Poeta, P., Hébraud, M., Capelo, J. L., & Igrejas, G. (2017). Mechanisms of quinolone action and resistance: where do we stand? *Journal of Medical Microbiology*, *66*(5), 551-559. https://doi.org/https://doi.org/10.1099/jmm.0.000475

Cortez Nunes, F., Taillieu, E., Letra Mateus, T., Teixeira, S., Haesebrouck, F., & Amorim, I. (2023). Molecular Detection of Metronidazole and Tetracycline Resistance Genes in *Helicobacter*

pylori-Like Positive Gastric Samples from Pigs. *Antibiotics*, *12*(5), 906. https://www.mdpi.com/2079-6382/12/5/906
Cover, T. L. (2012). Role of *Helicobacter pylori* CagL in modulating gastrin expression. *Gut*, *61*(7), 965-966. https://doi.org/10.1136/gutjnl-2012-302142
Cover, T. L., & Blaser, M. J. (2009). *Helicobacter pylori* in health and disease. *Gastroenterology*, *136*(6), 1863-1873. https://doi.org/10.1053/j.gastro.2009.01.073
Cowland, J. B., Johnsen, A. H., & Borregaard, N. (1995). hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. *FEBS Letters*, *368*(1), 173-176. https://doi.org/https://doi.org/10.1016/0014-5793(95)00634-L
Crafa, P., Russo, M., Miraglia, C., Barchi, A., Moccia, F., Nouvenne, A., Leandro, G., Meschi, T., De' Angelis, G. L., & Di Mario, F. (2018). From Sidney to OLGA: an overview of atrophic gastritis. *Acta Biomed*, *89*(8-s), 93-99. https://doi.org/10.23750/abm.v89i8-S.7946
Criqui, G., Solomon, C., Welch, B., Ferrando, R., Boushey, H., & Balmes. (2000). Effects of azithromycin on ozone-induced airway neutrophilia and cytokine release. *European Respiratory Journal*, *15*(5), 856-862. https://doi.org/10.1034/j.1399-3003.2000.15e08.x
Čulić, O., Eraković, V., Čepelak, I., Barišić, K., Brajša, K., Ferenčić, Ž., Galović, R., Glojnarić, I., Manojlović, Z., Munić, V., Novak-Mirčetić, R., Pavičić-Beljak, V., Sučić, M., Veljača, M., Žanić-Grubišić, T., & Parnham, M. J. (2002). Azithromycin modulates neutrophil function and circulating inflammatory mediators in healthy human subjects. *European Journal of Pharmacology*, *450*(3), 277-289. https://doi.org/https://doi.org/10.1016/S0014-2999(02)02042-3
Cunliffe, R. N. (2003). α-Defensins in the gastrointestinal tract. *Molecular Immunology*, *40*(7), 463-467. https://doi.org/https://doi.org/10.1016/S0161-5890(03)00157-3
De Francesco, V., Zullo, A., Hassan, C., Della Valle, N., Pietrini, L., Minenna, M. F., Winn, S., Monno, R., Stoppino, V., Morini, S., Panella, C., & Ierardi, E. (2004). The prolongation of triple therapy for *Helicobacter pylori* does not allow reaching therapeutic outcome of sequential scheme: a prospective, randomised study. *Dig Liver Dis*, *36*(5), 322-326. https://doi.org/10.1016/j.dld.2003.12.015
De Francesco, V., Zullo, A., Ierardi, E., Giorgio, F., Perna, F., Hassan, C., Morini, S., Panella, C., & Vaira, D. (2010). Phenotypic and genotypic *Helicobacter pylori* clarithromycin resistance and therapeutic outcome: benefits and limits. *J Antimicrob Chemother*, *65*(2), 327-332. https://doi.org/10.1093/jac/dkp445
De Nardo, D. (2015). Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine*, *74*(2), 181-189. https://doi.org/https://doi.org/10.1016/j.cyto.2015.02.025
Debraekeleer, A., & Remaut, H. (2018). Future Perspective for Potential *Helicobacter Pylori* Eradication Therapies. *Future Microbiology*, *13*(6), 671-687. https://doi.org/10.2217/fmb-2017-0115
Delahay, R. M., Croxall, N. J., & Stephens, A. D. (2018). Phylogeographic diversity and mosaicism of the *Helicobacter pylori* tfs integrative and conjugative elements. *Mob DNA*, *9*, 5. https://doi.org/10.1186/s13100-018-0109-4
Desaki, M., Takizawa, H., Ohtoshi, T., Kasama, T., Kobayashi, K., Sunazuka, T., Omura, S., Yamamoto, K., & Ito, K. (2000). Erythromycin suppresses nuclear Factor-κB and activator protein-1 activation in human bronchial epithelial cells [Article]. *Biochemical and Biophysical Research Communications*, *267*(1), 124-128. https://doi.org/10.1006/bbrc.1999.1917
Dhillon, B., Aleithan, F., Abdul-Sater, Z., & Abdul-Sater, A. A. (2019). The Evolving Role of TRAFs in Mediating Inflammatory Responses [Mini Review]. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.00104
Dincă, A. L., Meliț, L. E., & Mărginean, C. O. (2022). Old and New Aspects of *H. pylori*-Associated Inflammation and Gastric Cancer. *Children*, *9*(7), 1083. https://www.mdpi.com/2227-9067/9/7/1083

Dingsdag, S. A., & Hunter, N. (2018). Metronidazole: an update on metabolism, structure-cytotoxicity and resistance mechanisms. *J Antimicrob Chemother*, *73*(2), 265-279. https://doi.org/10.1093/jac/dkx351

Dinis-Ribeiro, M., & Kuipers, E. J. (2020). How to Manage a Patient With Gastric Intestinal Metaplasia: An International Perspective. *Gastroenterology*, *158*(6), 1534-1537. https://doi.org/10.1053/j.gastro.2020.01.008

Dinis-Ribeiro, M., Lopes, C., da Costa-Pereira, A., Guilherme, M., Barbosa, J., Lomba-Viana, H., Silva, R., & Moreira-Dias, L. (2004). A follow up model for patients with atrophic chronic gastritis and intestinal metaplasia. *J Clin Pathol*, *57*(2), 177-182. https://doi.org/10.1136/jcp.2003.11270

Dinos, G. P. (2017). The macrolide antibiotic renaissance. *British Journal of Pharmacology*, *174*(18), 2967-2983. https://doi.org/https://doi.org/10.1111/bph.13936

Dixon, M. F. (2001). Pathology of Gastritis and Peptic Ulceration. In *Helicobacter pylori* (pp. 457-469). https://doi.org/https://doi.org/10.1128/9781555818005.ch38

Dixon, M. F., Genta, R. M., Yardley, J. H., & Correa, P. (1996). Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol*, *20*(10), 1161-1181. https://doi.org/10.1097/00000478-199610000-00001

Dolara, P., Luceri, C., De Filippo, C., Femia, A. P., Giovannelli, L., Caderni, G., Cecchini, C., Silvi, S., Orpianesi, C., & Cresci, A. (2005). Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutat Res*, *591*(1-2), 237-246. https://doi.org/10.1016/j.mrfmmm.2005.04.022

Domanovich-Asor, T., Motro, Y., Khalfin, B., Craddock, H. A., Peretz, A., & Moran-Gilad, J. (2021). Genomic Analysis of Antimicrobial Resistance Genotype-to-Phenotype Agreement in *Helicobacter pylori*. *Microorganisms*, *9*(1), 2. https://www.mdpi.com/2076-2607/9/1/2

Domínguez-Martínez, D. A., Fontes-Lemus, J. I., García-Regalado, A., Juárez-Flores, Á., & Fuentes-Pananá, E. M. (2023). IL-8 Secreted by Gastric Epithelial Cells Infected with *Helicobacter pylori* CagA Positive Strains Is a Chemoattractant for Epstein–Barr Virus Infected B Lymphocytes. *Viruses*, *15*(3), 651. https://www.mdpi.com/1999-4915/15/3/651

Dommett, R., Zilbauer, M., George, J. T., & Bajaj-Elliott, M. (2005). Innate immune defence in the human gastrointestinal tract. *Molecular Immunology*, *42*(8), 903-912. https://doi.org/https://doi.org/10.1016/j.molimm.2004.12.004

Douraghi, M., Kashani, S. S., Zeraati, H., Esmaili, M., Oghalaie, A., & Mohammadi, M. (2010). Comparative evaluation of three supplements for *Helicobacter pylori* growth in liquid culture. *Curr Microbiol*, *60*(4), 254-262. https://doi.org/10.1007/s00284-009-9534-4

Droin, N., Hendra, J. B., Ducoroy, P., & Solary, E. (2009). Human defensins as cancer biomarkers and antitumour molecules. *J Proteomics*, *72*(6), 918-927. https://doi.org/10.1016/j.jprot.2009.01.002

Duan, Y., Gao, Y., Zhang, J., Chen, Y., Jiang, Y., Ji, J., Zhang, J., Chen, X., Yang, Q., Su, L., Zhang, J., Liu, B., Zhu, Z., Wang, L., & Yu, Y. (2016). Mitochondrial aldehyde dehydrogenase 2 protects gastric mucosa cells against DNA damage caused by oxidative stress. *Free Radic Biol Med*, *93*, 165-176. https://doi.org/10.1016/j.freeradbiomed.2016.02.001

Duits, L. A., Nibbering, P. H., van Strijen, E., Vos, J. B., Mannesse-Lazeroms, S. P. G., van Sterkenburg, M. A. J. A., & Hiemstra, P. S. (2003). Rhinovirus increases human β-defensin-2 and -3 mRNA expression in cultured bronchial epithelial cells. *FEMS Immunology & Medical Microbiology*, *38*(1), 59-64. https://doi.org/10.1016/s0928-8244(03)00106-8

Duits, L. A., Ravensbergen, B., Rademaker, M., Hiemstra, P. S., & Nibbering, P. H. (2002). Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. *Immunology*, *106*(4), 517-525. https://doi.org/10.1046/j.1365-2567.2002.01430.x

Dutta, P., & Das, S. (2016). Mammalian Antimicrobial Peptides: Promising Therapeutic Targets Against Infection and Chronic Inflammation. *Curr Top Med Chem*, *16*(1), 99-129. https://doi.org/10.2174/1568026615666150703121819

Eftang, L. L., Esbensen, Y., Tannæs, T. M., Bukholm, I. R. K., & Bukholm, G. (2012). Interleukin-8 is the single most up-regulated gene in whole genome profiling of *H. pylori* exposed gastric epithelial cells. *BMC Microbiology*, *12*(1), 9. https://doi.org/10.1186/1471-2180-12-9

Eicher, A. K., Berns, H. M., & Wells, J. M. (2018). Translating Developmental Principles to Generate Human Gastric Organoids. *Cell Mol Gastroenterol Hepatol*, *5*(3), 353-363. https://doi.org/10.1016/j.jcmgh.2017.12.014

Ejazi, S. A., Louisthelmy, R., & Maisel, K. (2023). Mechanisms of Nanoparticle Transport across Intestinal Tissue: An Oral Delivery Perspective. *ACS Nano*, *17*(14), 13044-13061. https://doi.org/10.1021/acsnano.3c02403

El-Omar, E. M., Carrington, M., Chow, W.-H., McColl, K. E. L., Bream, J. H., Young, H. A., Herrera, J., Lissowska, J., Yuan, C.-C., Rothman, N., Lanyon, G., Martin, M., Fraumeni, J. F., & Rabkin, C. S. (2000). Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature*, *404*(6776), 398-402. https://doi.org/10.1038/35006081

Elbaiomy, R. G., Luo, X., Guo, R., Deng, S., Du, M., El-Sappah, A. H., Bakeer, M., Azzam, M. M., Elolimy, A. A., Madkour, M., Li, Z., & Zhang, Z. (2025). Antibiotic resistance in *Helicobacter pylori*: a genetic and physiological perspective. *Gut Pathogens*, *17*(1), 35. https://doi.org/10.1186/s13099-025-00704-5

Eletto, D., Mentucci, F., Voli, A., Petrella, A., Porta, A., & Tosco, A. (2022). *Helicobacter pylori* Pathogen-Associated Molecular Patterns: Friends or Foes? *International Journal of Molecular Sciences*, *23*(7), 3531. https://www.mdpi.com/1422-0067/23/7/3531

Eliesen, G. A. M., Fransen, M., Kooijman, N., van den Broek, P. H. H., Russel, F. G. M., & Greupink, R. (2022). Effects of tumor necrosis factor on undifferentiated and syncytialized placental choriocarcinoma BeWo cells. *Toxicology in Vitro*, *80*, 105327. https://doi.org/https://doi.org/10.1016/j.tiv.2022.105327

Elphick, D., Liddell, S., & Mahida, Y. R. (2008). Impaired luminal processing of human defensin-5 in Crohn's disease: persistence in a complex with chymotrypsinogen and trypsin. *Am J Pathol*, *172*(3), 702-713. https://doi.org/10.2353/ajpath.2008.070755

Elviss, N. C., Owen, R. J., Xerry, J., Walker, A. M., & Davies, K. (2004). *Helicobacter pylori* antibiotic resistance patterns and genotypes in adult dyspeptic patients from a regional population in North Wales. *J Antimicrob Chemother*, *54*(2), 435-440. https://doi.org/10.1093/jac/dkh343

Engevik, A. C., Kaji, I., & Goldenring, J. R. (2020). The Physiology of the Gastric Parietal Cell. *Physiological Reviews*, *100*(2), 573-602. https://doi.org/10.1152/physrev.00016.2019

Test and treat for *Helicobacter pylori* (HP) in dyspepsia, (2019). https://www.gov.uk/government/publications/helicobacter-pylori-diagnosis-and-treatment

Engstrand, L., & Lindberg, M. (2013). *Helicobacter pylori* and the gastric microbiota. *Best Practice & Research Clinical Gastroenterology*, *27*(1), 39-45. https://doi.org/https://doi.org/10.1016/j.bpg.2013.03.016

Espeche, J. C., Varas, R., Maturana, P., Cutro, A. C., Maffía, P. C., & Hollmann, A. (2024). Membrane permeability and antimicrobial peptides: Much more than just making a hole. *Peptide Science*, *116*(1), e24305. https://doi.org/https://doi.org/10.1002/pep2.24305

EUCAST. (2024). *Clinical breakpoints and dosing of antibiotics*. European Comittee on Antimicrobial Susceptibility Testing. Retrieved 2/4/2024 from https://www.eucast.org/clinical_breakpoints

Eusebi, L. H., Telese, A., Marasco, G., Bazzoli, F., & Zagari, R. M. (2020). Gastric cancer prevention strategies: A global perspective. *Journal of Gastroenterology and Hepatology*, *35*(9), 1495-1502. https://doi.org/https://doi.org/10.1111/jgh.15037

Fan, C. J., Li, Z., Zhai, L. L., Wang, H., Zhao, X. L., Xie, D. L., Cai, Y., Huang, K., Bai, Q. X., Ding, H. O., & Cheng, J. P. (2024). Diagnostic accuracy of a real-time PCR assay for detection of

Helicobacter pylori and resistance to clarithromycin and levofloxacin directly from stool. *Eur Rev Med Pharmacol Sci*, *28*(12), 3836-3840. https://doi.org/10.26355/eurrev_202406_36460

Fan, L.-C., Lin, J.-L., Yang, J.-W., Mao, B., Lu, H.-W., Ge, B.-X., Choi, A. M. K., & Xu, J.-F. (2017). Macrolides protect against *Pseudomonas aeruginosa* infection via inhibition of inflammasomes. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *313*(4), L677-L686. https://doi.org/10.1152/ajplung.00123.2017

Fan, X. G., Chua, A., Fan, X. J., & Keeling, P. W. (1995). Increased gastric production of interleukin-8 and tumour necrosis factor in patients with *Helicobacter pylori* infection. *Journal of Clinical Pathology*, *48*(2), 133-136. https://doi.org/10.1136/jcp.48.2.133

Farzi, N., Malekian, T., Alebouyeh, M., Vaziri, F., & Zali, M. R. (2015). Genotype Diversity and Quasispecies Development of *Helicobacter pylori* in a Single Host. *Jpn J Infect Dis*, *68*(4), 351. https://doi.org/10.7883/yoken.JJID.2015.E002

Fazeli, Z., Alebouyeh, M., Rezaei Tavirani, M., Azimirad, M., & Yadegar, A. (2016). *Helicobacter pylori* CagA induced interleukin-8 secretion in gastric epithelial cells. *Gastroenterol Hepatol Bed Bench*, *9*(Suppl1), S42-s46.

Fedarovich, A., Cook, E., Tomberg, J., Nicholas, R. A., & Davies, C. (2014). Structural effect of the Asp345a insertion in penicillin-binding protein 2 from penicillin-resistant strains of *Neisseria gonorrhoeae*. *Biochemistry*, *53*(48), 7596-7603. https://doi.org/10.1021/bi5011317

Fekadu, S., Engiso, H., Seyfe, S., Iizasa, H., Godebo, A., Deyno, S., & Yoshiyama, H. (2023). Effectiveness of eradication therapy for *Helicobacter pylori* infection in Africa: a systematic review and meta-analysis. *BMC Gastroenterology*, *23*(1), 55. https://doi.org/10.1186/s12876-023-02707-5

Fernandez-Gonzalez, E., & Backert, S. (2014). DNA transfer in the gastric pathogen *Helicobacter pylori*. *J Gastroenterol*, *49*(4), 594-604. https://doi.org/10.1007/s00535-014-0938-y

Figueiredo, C. u., Machado, J. C., Pharoah, P., Seruca, R., Sousa, S. n., Carvalho, R., Capelinha, A. F., Quint, W., Caldas, C., van Doorn, L.-J., Carneiro, F. t., & Sobrinho-Simões, M. (2002). *Helicobacter pylori* and Interleukin 1 Genotyping: An Opportunity to Identify High-Risk Individuals for Gastric Carcinoma. *JNCI: Journal of the National Cancer Institute*, *94*(22), 1680-1687. https://doi.org/10.1093/jnci/94.22.1680

Fischbach, W., & Malfertheiner, P. (2018). *Helicobacter Pylori* Infection. *Dtsch Arztebl Int*, *115*(25), 429-436. https://doi.org/10.3238/arztebl.2018.0429

Fischer, C. D., Beatty, J. K., Duquette, S. C., Morck, D. W., Lucas, M. J., & Buret, A. G. (2013). Direct and Indirect Anti-Inflammatory Effects of Tulathromycin in Bovine Macrophages: Inhibition of CXCL-8 Secretion, Induction of Apoptosis, and Promotion of Efferocytosis. *Antimicrobial Agents and Chemotherapy*, *57*(3), 1385-1393. https://doi.org/doi:10.1128/aac.01598-12

Fischer, W., Breithaupt, U., Kern, B., Smith, S. I., Spicher, C., & Haas, R. (2014). A comprehensive analysis of *Helicobacter pylori* plasticity zones reveals that they are integrating conjugative elements with intermediate integration specificity. *BMC Genomics*, *15*, 310. https://doi.org/10.1186/1471-2164-15-310

Foegeding, N. J., Caston, R. R., McClain, M. S., Ohi, M. D., & Cover, T. L. (2016). An Overview of *Helicobacter pylori* VacA Toxin Biology. *Toxins (Basel)*, *8*(6). https://doi.org/10.3390/toxins8060173

Fond, G., Hamdani, N., Kapczinski, F., Boukouaci, W., Drancourt, N., Dargel, A., Oliveira, J., Le Guen, E., Marlinge, E., Tamouza, R., & Leboyer, M. (2014). Effectiveness and tolerance of anti-inflammatory drugs' add-on therapy in major mental disorders: a systematic qualitative review. *Acta Psychiatr Scand*, *129*(3), 163-179. https://doi.org/10.1111/acps.12211

Fox, J. G. (2002). The non-*H pylori helicobacters*: their expanding role in gastrointestinal and systemic diseases. *Gut*, *50*(2), 273-283. https://doi.org/10.1136/gut.50.2.273

Fox, J. G., & Wang, T. C. (2002). *Helicobacter pylori* infection: pathogenesis. *Curr Opin Gastroenterol*, *18*(1), 15-25. https://doi.org/10.1097/00001574-200201000-00004

Fox, J. G., & Wang, T. C. (2007). Inflammation, atrophy, and gastric cancer. *J Clin Invest*, *117*(1), 60-69. https://doi.org/10.1172/jci30111

Frye, M., Bargon, J., Lembcke, B., Wagner, T. O., & Gropp, R. (2000). Differential expression of human alpha- and beta-defensins mRNA in gastrointestinal epithelia. *Eur J Clin Invest*, *30*(8), 695-701. https://doi.org/10.1046/j.1365-2362.2000.00696.x

Fujimoto, S., Olaniyi Ojo, O., Arnqvist, A., Yih Wu, J., Odenbreit, S., Haas, R., Graham, D. Y., & Yamaoka, Y. (2007). *Helicobacter pylori* BabA Expression, Gastric Mucosal Injury, and Clinical Outcome. *Clinical Gastroenterology and Hepatology*, *5*(1), 49-58. https://doi.org/https://doi.org/10.1016/j.cgh.2006.09.015

Furuta, T., Shirai, N., Xiao, F., El-omar, E. M., Rabkin, C. S., Sugimura, H., Ishizaki, T., & Ohashi, K. (2004). Polymorphism of interleukin-1β affects the eradication rates of *Helicobacter pylori* by triple therapy. *Clinical Gastroenterology and Hepatology*, *2*(1), 22-30. https://doi.org/10.1016/S1542-3565(03)00288-X

Gaddy, J. A., Radin, J. N., Loh, J. T., Zhang, F., Washington, M. K., Peek, R. M., Algood, H. M. S., & Cover, T. L. (2013). High Dietary Salt Intake Exacerbates *Helicobacter pylori*-Induced Gastric Carcinogenesis. *Infection and Immunity*, *81*(6), 2258-2267. https://doi.org/doi:10.1128/iai.01271-12

Galaxy, F. i. (2018). Galaxy Australia Training. Retrieved 20/8/2024 from https://galaxy-au-training.github.io/tutorials/modules/fastqc/

Gareayaghi, N., & Kocazeybek, B. (2022). Detection of A2143G, A2142C, and A2142G Point Mutations with Real-Time PCR in Stool Specimens from Children Infected with *Helicobacter pylori*. *Diagnostics (Basel)*, *12*(9). https://doi.org/10.3390/diagnostics12092119

Garvey, E., Rhead, J., Suffian, S., Whiley, D., Mahmood, F., Bakshi, N., Letley, D., White, J., Atherton, J., Winter, J. A., & Robinson, K. (2023). High incidence of antibiotic resistance amongst isolates of *Helicobacter pylori* collected in Nottingham, UK, between 2001 and 2018. *Journal of Medical Microbiology*, *72*(11). https://doi.org/https://doi.org/10.1099/jmm.0.001776

Garza-Gonzalez, E., Bosques-Padilla, F. J., Tijerina-Menchaca, R., & Perez-Perez, G. I. (2004). Characterisation of *Helicobacter pylori* isolates from the north-eastern region of Mexico. *Clinical Microbiology and Infection*, *10*(1), 41-45. https://doi.org/https://doi.org/10.1111/j.1469-0691.2004.00731.x

Gatta, L., Vakil, N., Vaira, D., & Scarpignato, C. (2013). Global eradication rates for *Helicobacter pylori* infection: systematic review and meta-analysis of sequential therapy. *BMJ : British Medical Journal*, *347*, f4587. https://doi.org/10.1136/bmj.f4587

Gehlot, V., Mahant, S., Mukhopadhyay, A. K., Das, K., De, R., Kar, P., & Das, R. (2016). Antimicrobial susceptibility profiles of *Helicobacter pylori* isolated from patients in North India. *J Glob Antimicrob Resist*, *5*, 51-56. https://doi.org/10.1016/j.jgar.2015.09.009

George, J. T., Boughan, P. K., Karageorgiou, H., & Bajaj-Elliott, M. (2003). Host anti-microbial response to *Helicobacter pylori* infection. *Mol Immunol*, *40*(7), 451-456. https://doi.org/10.1016/s0161-5890(03)00158-5

Gerrits, M. M., Godoy, A. P., Kuipers, E. J., Ribeiro, M. L., Stoof, J., Mendonça, S., van Vliet, A. H., Pedrazzoli, J., Jr., & Kusters, J. G. (2006). Multiple mutations in or adjacent to the conserved penicillin-binding protein motifs of the penicillin-binding protein 1A confer amoxicillin resistance to *Helicobacter pylori*. *Helicobacter*, *11*(3), 181-187. https://doi.org/10.1111/j.1523-5378.2006.00398.x

Ghosh, S., Whitley, C. S., Haribabu, B., & Jala, V. R. (2021). Regulation of Intestinal Barrier Function by Microbial Metabolites. *Cell Mol Gastroenterol Hepatol*, *11*(5), 1463-1482. https://doi.org/10.1016/j.jcmgh.2021.02.007

Ghosh, S. K., McCormick, T. S., & Weinberg, A. (2019). Human Beta Defensins and Cancer: Contradictions and Common Ground. *Front Oncol*, *9*, 341. https://doi.org/10.3389/fonc.2019.00341

Ghotaslou, R., Leylabadlo, H. E., & Asl, Y. M. (2015). Prevalence of antibiotic resistance in *Helicobacter pylori*: A recent literature review. *World J Methodol*, *5*(3), 164-174. https://doi.org/10.5662/wjm.v5.i3.164

Giesemann, T., Guttenberg, G., & Aktories, K. (2008). Human α-Defensins Inhibit *Clostridium difficile* Toxin B. *Gastroenterology*, *134*(7), 2049-2058. https://doi.org/https://doi.org/10.1053/j.gastro.2008.03.008

Gillespie, J. J., Wattam, A. R., Cammer, S. A., Gabbard, J. L., Shukla, M. P., Dalay, O., Driscoll, T., Hix, D., Mane, S. P., Mao, C., Nordberg, E. K., Scott, M., Schulman, J. R., Snyder, E. E., Sullivan, D. E., Wang, C., Warren, A., Williams, K. P., Xue, T., . . . Sobral, B. W. (2011). PATRIC: the comprehensive bacterial bioinformatics resource with a focus on human pathogenic species. *Infect Immun*, *79*(11), 4286-4298. https://doi.org/10.1128/iai.00207-11

Glupczynski, Y., Broutet, N., Cantagrel, A., Andersen, L. P., Alarcon, T., López-Brea, M., & Mégraud, F. (2002). Comparison of the E test and agar dilution method for antimicrobial suceptibility testing of *Helicobacter pylori*. *Eur J Clin Microbiol Infect Dis*, *21*(7), 549-552. https://doi.org/10.1007/s10096-002-0757-6

Glupczynski, Y., Mégraud, F., Lopez-Brea, M., & Andersen, L. P. (2001). European multicentre survey of *in vitro* antimicrobial resistance in *Helicobacter pylori*. *Eur J Clin Microbiol Infect Dis*, *20*(11), 820-823. https://doi.org/10.1007/s100960100611

Gobert, A. P., & Wilson, K. T. (2022). Induction and Regulation of the Innate Immune Response in *Helicobacter pylori* Infection. *Cellular and Molecular Gastroenterology and Hepatology*, *13*(5), 1347-1363. https://doi.org/https://doi.org/10.1016/j.jcmgh.2022.01.022

Goldenring, J. R., Nam, K. T., Wang, T. C., Mills, J. C., & Wright, N. A. (2010). Spasmolytic polypeptide-expressing metaplasia and intestinal metaplasia: time for reevaluation of metaplasias and the origins of gastric cancer. *Gastroenterology*, *138*(7), 2207-2210, 2210.e2201. https://doi.org/10.1053/j.gastro.2010.04.023

Gong, E. J., Ahn, J. Y., Jung, H. Y., Park, H., Ko, Y. B., Na, H. K., Jung, K. W., Kim do, H., Lee, J. H., Choi, K. D., Song, H. J., Lee, G. H., & Kim, J. H. (2016). *Helicobacter pylori* Eradication Therapy Is Effective as the Initial Treatment for Patients with *H. pylori*-Negative and Disseminated Gastric Mucosa-Associated Lymphoid Tissue Lymphoma. *Gut Liver*, *10*(5), 706-713. https://doi.org/10.5009/gnl15510

Gong, Y., & Yuan, Y. (2018). Resistance mechanisms of *Helicobacter pylori* and its dual target precise therapy. *Crit Rev Microbiol*, *44*(3), 371-392. https://doi.org/10.1080/1040841x.2017.1418285

Gong, Y., Zhai, K., Sun, L., He, L., Wang, H., Guo, Y., & Zhang, J. (2023). RdxA Diversity and Mutations Associated with Metronidazole Resistance of *Helicobacter pylori*. *Microbiol Spectr*, *11*(2), e0390322. https://doi.org/10.1128/spectrum.03903-22

Goni, E., Tammer, I., Schütte, K., Thon, C., Jechorek, D., Mahajan, U. M., Vasapolli, R., Macke, L., Aulinger, B., Selgrad, M., Link, A., Malfertheiner, P., & Schulz, C. (2022). The influence of gastric atrophy on *Helicobacter pylori* antibiotics resistance in therapy-naïve patients. *Front Microbiol*, *13*, 938676. https://doi.org/10.3389/fmicb.2022.938676

Graf, M., & Wilson, D. N. (2019). Intracellular Antimicrobial Peptides Targeting the Protein Synthesis Machinery. In K. Matsuzaki (Ed.), *Antimicrobial Peptides: Basics for Clinical Application* (pp. 73-89). Springer Singapore. https://doi.org/10.1007/978-981-13-3588-4_6

Graham, D. Y. (1998). Antibiotic resistance in *Helicobacter pylori*: Implications for therapy. *Gastroenterology*, *115*(5), 1272-1277. https://doi.org/https://doi.org/10.1016/S0016-5085(98)70100-3

Graham, D. Y. (2014). History of *Helicobacter pylori*, duodenal ulcer, gastric ulcer and gastric cancer. *World J Gastroenterol*, *20*(18), 5191-5204. https://doi.org/10.3748/wjg.v20.i18.5191

Graham, D. Y., Lew, G. M., Malaty, H. M., Evans, D. G., Evans, D. J., Jr., Klein, P. D., Alpert, L. C., & Genta, R. M. (1992). Factors influencing the eradication of *Helicobacter pylori* with triple therapy. *Gastroenterology*, *102*(2), 493-496. https://doi.org/10.1016/0016-5085(92)90095-g

Grubman, A., Kaparakis, M., Viala, J., Allison, C., Badea, L., Karrar, A., Boneca, I. G., Le Bourhis, L., Reeve, S., Smith, I. A., Hartland, E. L., Philpott, D. J., & Ferrero, R. L. (2010). The innate immune molecule, NOD1, regulates direct killing of *Helicobacter pylori* by antimicrobial peptides. *Cell Microbiol*, *12*(5), 626-639. https://doi.org/10.1111/j.1462-5822.2009.01421.x

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics*, *29*(8), 1072-1075. https://doi.org/10.1093/bioinformatics/btt086

Güzel, M., Sönmez, M. F., Baştuğ, O., Aras, N. F., Öztürk, A. B., Küçükaydın, M., & Turan, C. (2016). Effectiveness of lycopene on experimental testicular torsion. *Journal of Pediatric Surgery*, *51*(7), 1187-1191. https://doi.org/https://doi.org/10.1016/j.jpedsurg.2015.11.008

Haghi, M., Saadat, A., Zhu, B., Colombo, G., King, G., Young, P. M., & Traini, D. (2015). Immunomodulatory Effects of a Low-Dose Clarithromycin-Based Macrolide Solution Pressurised Metered Dose Inhaler. *Pharmaceutical Research*, *32*(6), 2144-2153. https://doi.org/10.1007/s11095-014-1605-y

Hamanaka, Y., Nakashima, M., Wada, A., Ito, M., Kurazono, H., Hojo, H., Nakahara, Y., Kohno, S., Hirayama, T., & Sekine, I. (2001). Expression of human β-defensin 2 (hBD-2) in*Helicobacter pylori* induced gastritis: antibacterial effect of hBD-2 against *Helicobacter pylori*. *Gut*, *49*(4), 481-487. https://doi.org/10.1136/gut.49.4.481

Hanafiah, A., Binmaeil, H., Raja Ali, R. A., Mohamed Rose, I., & Lopes, B. S. (2019). Molecular characterization and prevalence of antibiotic resistance in *Helicobacter pylori* isolates in Kuala Lumpur, Malaysia. *Infection and Drug Resistance*, *12*(null), 3051-3061. https://doi.org/10.2147/IDR.S219069

Handa, O., Naito, Y., Osawa, M., Murao, T., Matsumoto, H., Umegaki, E., & Shiotani, A. (2020). Nutrients and probiotics: current trends in their use to eradicate *Helicobacter pylori*. *J Clin Biochem Nutr*, *67*(1), 26-28. https://doi.org/10.3164/jcbn.20-51

Harder, J., Meyer-Hoffert, U., Teran, L. M., Schwichtenberg, L., Bartels, J., Maune, S., & Schröder, J. M. (2000). Mucoid Pseudomonas aeruginosa, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *Am J Respir Cell Mol Biol*, *22*(6), 714-721. https://doi.org/10.1165/ajrcmb.22.6.4023

Hardwick, R. J., Machado, L. R., Zuccherato, L. W., Antolinos, S., Xue, Y., Shawa, N., Gilman, R. H., Cabrera, L., Berg, D. E., Tyler-Smith, C., Kelly, P., Tarazona-Santos, E., & Hollox, E. J. (2011). A worldwide analysis of beta-defensin copy number variation suggests recent selection of a high-expressing *DEFB103* gene copy in East Asia. *Hum Mutat*, *32*(7), 743-750. https://doi.org/10.1002/humu.21491

Harris, A. G., Hazell, S. L., & Netting, A. G. (2000). Use of digoxigenin-labelled ampicillin in the identification of penicillin-binding proteins in *Helicobacter pylori*. *J Antimicrob Chemother*, *45*(5), 591-598. https://doi.org/10.1093/jac/45.5.591

Harrison, U., Fowora, M. A., Seriki, A. T., Loell, E., Mueller, S., Ugo-Ijeh, M., Onyekwere, C. A., Lesi, O. A., Otegbayo, J. A., Akere, A., Ndububa, D. A., Adekanle, O., Anomneze, E., Abdulkareem, F. B., Adeleye, I. A., Crispin, A., Rieder, G., Fischer, W., Smith, S. I., & Haas, R. (2017). *Helicobacter pylori* strains from a Nigerian cohort show divergent antibiotic resistance rates and a uniform pathogenicity profile. *PLoS One*, *12*(5), e0176454. https://doi.org/10.1371/journal.pone.0176454

Hasanuzzaman, M., Bang, C. S., & Gong, E. J. (2024). Antibiotic Resistance of *Helicobacter pylori*: Mechanisms and Clinical Implications. *J Korean Med Sci*, *39*(4), e44. https://doi.org/10.3346/jkms.2024.39.e44

Hase, K., Murakami, M., Iimura, M., Cole, S. P., Horibe, Y., Ohtake, T., Obonyo, M., Gallo, R. L., Eckmann, L., & Kagnoff, M. F. (2003a). Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against *Helicobacter pylori*. *Gastroenterology*, *125*(6), 1613-1625. https://doi.org/https://doi.org/10.1053/j.gastro.2003.08.028

Hase, K., Murakami, M., Iimura, M., Cole, S. P., Horibe, Y., Ohtake, T., Obonyo, M., Gallo, R. L., Eckmann, L., & Kagnoff, M. F. (2003b). Expression of *LL-37* by human gastric epithelial cells as a potential host defense mechanism against *Helicobacter pylori*. *Gastroenterology*, *125*(6), 1613-1625. https://doi.org/10.1053/j.gastro.2003.08.028

Hashemi, S. J., Sheikh, A. F., Goodarzi, H., Yadyad, M. J., Seyedian, S. S., Aslani, S., & Assarzadegan, M.-A. (2019). Genetic basis for metronidazole and clarithromycin resistance in *Helicobacter pylori* strains isolated from patients with gastroduodenal disorders. *Infection and Drug Resistance*, *12*(null), 535-543. https://doi.org/10.2147/IDR.S192942

Hassan, M., Flanagan, T. W., Kharouf, N., Bertsch, C., Mancino, D., & Haikel, Y. (2023). Antimicrobial Proteins: Structure, Molecular Action, and Therapeutic Potential. *Pharmaceutics*, *15*(1), 72. https://www.mdpi.com/1999-4923/15/1/72

Hatakeyama, M. (2004). Oncogenic mechanisms of the *Helicobacter pylori* CagA protein. *Nature Reviews Cancer*, *4*(9), 688-694. https://doi.org/10.1038/nrc1433

Hatakeyama, M. (2017). Structure and function of *Helicobacter pylori* CagA, the first-identified bacterial protein involved in human cancer. *Proc Jpn Acad Ser B Phys Biol Sci*, *93*(4), 196-219. https://doi.org/10.2183/pjab.93.013

Hayashi, T., Senda, M., Suzuki, N., Nishikawa, H., Ben, C., Tang, C., Nagase, L., Inoue, K., Senda, T., & Hatakeyama, M. (2017). Differential Mechanisms for SHP2 Binding and Activation Are Exploited by Geographically Distinct *Helicobacter pylori* CagA Oncoproteins. *Cell Reports*, *20*(12), 2876-2890. https://doi.org/10.1016/j.celrep.2017.08.080

Heep, M., Kist, M., Strobel, S., Beck, D., & Lehn, N. (2000). Secondary resistance among 554 isolates of *Helicobacter pylori* after failure of therapy. *Eur J Clin Microbiol Infect Dis*, *19*(7), 538-541. https://doi.org/10.1007/s100960000288

Hertz, C. J., Wu, Q., Porter, E. M., Zhang, Y. J., Weismüller, K.-H., Godowski, P. J., Ganz, T., Randell, S. H., & Modlin, R. L. (2003). Activation of Toll-Like Receptor 2 on Human Tracheobronchial Epithelial Cells Induces the Antimicrobial Peptide Human β Defensin-2 1. *The Journal of Immunology*, *171*(12), 6820-6826. https://doi.org/10.4049/jimmunol.171.12.6820

Hibdon, E. S., & Samuelson, L. C. (2018). Cellular Plasticity in the Stomach: Insights Into the Cellular Origin of Gastric Metaplasia. *Gastroenterology*, *154*(4), 801-803. https://doi.org/10.1053/j.gastro.2018.02.001

Hiemstra, P. S. (2006). DEFENSINS. In G. J. Laurent & S. D. Shapiro (Eds.), *Encyclopedia of Respiratory Medicine* (pp. 7-10). Academic Press. https://doi.org/https://doi.org/10.1016/B0-12-370879-6/00110-1

Hiratsuka, T., Mukae, H., Iiboshi, H., Ashitani, J., Nabeshima, K., Minematsu, T., Chino, N., Ihi, T., Kohno, S., & Nakazato, M. (2003). Increased concentrations of human β-defensins in plasma and bronchoalveolar lavage fluid of patients with diffuse panbronchiolitis. *Thorax*, *58*(5), 425-430. https://doi.org/10.1136/thorax.58.5.425

Holck, S., Nørgaard, A., Bennedsen, M., Permin, H., Norn, S., & Andersen, L. P. (2003). Gastric mucosal cytokine responses in *Helicobacter pylori*-infected patients with gastritis and peptic ulcers. Association with inflammatory parameters and bacteria load. *FEMS Immunology & Medical Microbiology*, *36*(3), 175-180. https://doi.org/10.1016/s0928-8244(03)00028-2

Holleczek, B., Schöttker, B., & Brenner, H. (2020). *Helicobacter pylori* infection, chronic atrophic gastritis and risk of stomach and esophagus cancer: Results from the prospective population-based ESTHER cohort study. *International Journal of Cancer*, *146*(10), 2773-2783. https://doi.org/https://doi.org/10.1002/ijc.32610

Hollox, E. J., Armour, J. A. L., & Barber, J. C. K. (2003). Extensive Normal Copy Number Variation of a β-Defensin Antimicrobial-Gene Cluster. *The American Journal of Human Genetics*, *73*(3), 591-600. https://doi.org/10.1086/378157

Hong, T.-C., El-Omar, E. M., Kuo, Y.-T., Wu, J.-Y., Chen, M.-J., Chen, C.-C., Fang, Y.-J., Leow, A. H. R., Lu, H., Lin, J.-T., Tu, Y.-K., Yamaoka, Y., Wu, M.-S., Liou, J.-M., Hong, T.-C., El-Omar, E. M., Kuo, Y.-T., Wu, J.-Y., Chen, M.-J., . . . Chang, Y.-Y. (2024). Primary antibiotic resistance of

Helicobacter pylori in the Asia-Pacific region between 1990 and 2022: an updated systematic review and meta-analysis. *The Lancet Gastroenterology & Hepatology*, *9*(1), 56-67. https://doi.org/https://doi.org/10.1016/S2468-1253(23)00281-9

Hornsby, Huff, J. L., Kays, R. J., Canfield, D. R., Bevins, C. L., & Solnick, J. V. (2008). *Helicobacter pylori* induces an antimicrobial response in rhesus macaques in a cag pathogenicity island-dependent manner. *Gastroenterology*, *134*(4), 1049-1057. https://doi.org/10.1053/j.gastro.2008.01.018

Hrbacek, J., Cermak, P., & Zachoval, R. (2020). Current Antibiotic Resistance Trends of Uropathogens in Central Europe: Survey from a Tertiary Hospital Urology Department 2011-2019. *Antibiotics (Basel)*, *9*(9). https://doi.org/10.3390/antibiotics9090630

Hu, C. T., Wu, C. C., Lin, C. Y., Cheng, C. C., Su, S. C., Tseng, Y. H., & Lin, N. T. (2007). Resistance rate to antibiotics of *Helicobacter pylori* isolates in eastern Taiwan. *J Gastroenterol Hepatol*, *22*(5), 720-723. https://doi.org/10.1111/j.1440-1746.2006.04743.x

Hu, Q., Zhang, Y., Zhang, X., & Fu, K. (2016a). Gastric mucosa-associated lymphoid tissue lymphoma and *Helicobacter pylori* infection: a review of current diagnosis and management. *Biomarker Research*, *4*(1), 15. https://doi.org/10.1186/s40364-016-0068-1

Hu, S., Zhou, Y., Deng, Y., Bo, Y., Chen, X., Yang, W., Shi, R., Zhao, W., Hou, Z., Hu, J., Liu, J., Zhang, X., Yong, H., Wang, P., Li, F., Qi, H., Wang, X., Jin, L., Cui, T., . . . Gao, H. (2023). Characteristics of phenotypic antibiotic resistance of *Helicobacter pylori* and its correlation with genotypic antibiotic resistance: A retrospective study in Ningxia. *Helicobacter*, *28*(3), e12960. https://doi.org/https://doi.org/10.1111/hel.12960

Hu, Y., Liu, J.-P., Zhu, Y., & Lu, N.-H. (2016b). The Importance of Toll-like Receptors in NF-κB Signaling Pathway Activation by *Helicobacter pylori* Infection and the Regulators of this Response. *Helicobacter*, *21*(5), 428-440. https://doi.org/https://doi.org/10.1111/hel.12292

Huang, F. C. (2014). Differential regulation of interleukin-8 and human beta-defensin 2 in *Pseudomonas aeruginosa*-infected intestinal epithelial cells. *BMC Microbiol*, *14*, 275. https://doi.org/10.1186/s12866-014-0275-6

Huang, J. Q., Zheng, G. F., Sumanac, K., Irvine, E. J., & Hunt, R. H. (2003). Meta-Analysis of the Relationship between cagA Seropositivity and Gastric Cancer [Article]. *Gastroenterology*, *125*(6), 1636-1644. https://doi.org/10.1053/j.gastro.2003.08.033

Huang, T. T., Cao, Y. X., & Cao, L. (2024). Novel therapeutic regimens against *Helicobacter pylori*: an updated systematic review. *Front Microbiol*, *15*, 1418129. https://doi.org/10.3389/fmicb.2024.1418129

Huang, Y., Wang, Q. L., Cheng, D. D., Xu, W. T., & Lu, N. H. (2016). Adhesion and Invasion of Gastric Mucosa Epithelial Cells by *Helicobacter pylori*. *Front Cell Infect Microbiol*, *6*, 159. https://doi.org/10.3389/fcimb.2016.00159

Hulten, K. G., Lamberth, L. B., Kalfus, I. N., & Graham, D. Y. (2021). National and Regional US Antibiotic Resistance to *Helicobacter pylori*: Lessons From a Clinical Trial. *Gastroenterology*, *161*(1), 342-344.e341. https://doi.org/10.1053/j.gastro.2021.03.045

Humans, I. W. G. o. t. E. o. C. R. t. (1994). *Schistosomes, Liver Flukes and Helicobacter pylori* (Vol. 61). International Agency for Research on Cancer. https://www.ncbi.nlm.nih.gov/books/NBK487782/

Hung, K. H., Sheu, B. S., Chang, W. L., Wu, H. M., Liu, C. C., & Wu, J. J. (2009). Prevalence of primary fluoroquinolone resistance among clinical isolates of *Helicobacter pylori* at a University Hospital in Southern Taiwan. *Helicobacter*, *14*(1), 61-65. https://doi.org/10.1111/j.1523-5378.2009.00655.x

Hussein, N. R., Tunjel, I., Majed, H. S., Yousif, S. T., Aswad, S. I., & Assafi, M. S. (2015). Duodenal ulcer promoting gene 1 (*dupA1*) is associated with A2147G clarithromycin-resistance mutation but not interleukin-8 secretion from gastric mucosa in Iraqi patients. *New Microbes and New Infections*, *6*, 5-10. https://doi.org/https://doi.org/10.1016/j.nmni.2015.02.005

Hwang, T. J., Kim, N., Kim, H. B., Lee, B. H., Nam, R. H., Park, J. H., Lee, M. K., Park, Y. S., Lee, D. H., Jung, H. C., & Song, I. S. (2010). Change in Antibiotic Resistance of *Helicobacter pylori* Strains and the Effect of A2143G Point Mutation of *23S rRNA* on the Eradication of *H. pylori* in a Single Center of Korea. *Journal of Clinical Gastroenterology*, *44*(8), 536-543. https://doi.org/10.1097/MCG.0b013e3181d04592

Idowu, S., Bertrand, P. P., & Walduck, A. K. (2022). Homeostasis and Cancer Initiation: Organoids as Models to Study the Initiation of Gastric Cancer. *Int J Mol Sci*, *23*(5). https://doi.org/10.3390/ijms23052790

Imkamp, F., Lauener, F. N., Pohl, D., Lehours, P., Vale, F. F., Jehanne, Q., Zbinden, R., Keller, P. M., & Wagner, K. (2019). Rapid Characterization of Virulence Determinants in *Helicobacter pylori* Isolated from Non-Atrophic Gastritis Patients by Next-Generation Sequencing. *J Clin Med*, *8*(7). https://doi.org/10.3390/jcm8071030

Ishimoto, H., Mukae, H., Sakamoto, N., Amenomori, M., Kitazaki, T., Imamura, Y., Fujita, H., Ishii, H., Nakayama, S., Yanagihara, K., & Kohno, S. (2008). Different effects of telithromycin on MUC5AC production induced by human neutrophil peptide-1 or lipopolysaccharide in NCI-H292 cells compared with azithromycin and clarithromycin. *Journal of Antimicrobial Chemotherapy*, *63*(1), 109-114. https://doi.org/10.1093/jac/dkn427

Ishizawa, K., Suzuki, T., Yamaya, M., Jia, Y. X., Kobayashi, S., Ida, S., Kubo, H., Sekizawa, K., & Sasaki, H. (2005). Erythromycin increases bactericidal activity of surface liquid in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*, *289*(4), L565-573. https://doi.org/10.1152/ajplung.00316.2004

Isomoto, H., Mukae, H., Ishimoto, H., Date, Y., Nishi, Y., Inoue, K., Wada, A., Hirayama, T., Nakazato, M., & Kohno, S. (2004). Elevated Concentrations of α-Defensins in Gastric Juice of Patients with *Helicobacter pylori* Infection. *Official journal of the American College of Gastroenterology | ACG*, *99*(10). https://journals.lww.com/ajg/fulltext/2004/10000/elevated_concentrations_of___defensins_in_gastric.11.aspx

Isomoto, H., Mukae, H., Ishimoto, H., Nishi, Y., Wen, C. Y., Wada, A., Ohnita, K., Hirayama, T., Nakazato, M., & Kohno, S. (2005). High concentrations of human beta-defensin 2 in gastric juice of patients with *Helicobacter pylori* infection. *World J Gastroenterol*, *11*(31), 4782-4787. https://doi.org/10.3748/wjg.v11.i31.4782

Israel, D. A., & Peek, R. M. (2018). Chapter 63 - Mechanisms of *Helicobacter pylori*-Induced Gastric Inflammation. In H. M. Said (Ed.), *Physiology of the Gastrointestinal Tract (Sixth Edition)* (pp. 1517-1545). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-809954-4.00063-3

Jaka, H., Mueller, A., Kasang, C., & Mshana, S. E. (2019). Predictors of triple therapy treatment failure among *H. pylori* infected patients attending at a tertiary hospital in Northwest Tanzania: a prospective study. *BMC Infectious Diseases*, *19*(1), 447. https://doi.org/10.1186/s12879-019-4085-1

James, C. P., Bajaj-Elliott, M., Abujaber, R., Forya, F., Klein, N., David, A. L., Hollox, E. J., & Peebles, D. M. (2018). Human beta defensin (HBD) gene copy number affects HBD2 protein levels: impact on cervical bactericidal immunity in pregnancy. *European Journal of Human Genetics*, *26*(3), 434-439. https://doi.org/10.1038/s41431-017-0061-7

Jang, B.-C., Lim, K.-J., Paik, J.-H., Kwon, Y.-K., Shin, S.-W., Kim, S.-C., Jung, T.-Y., Kwon, T. K., Cho, J.-W., Baek, W.-K., Kim, S.-P., Suh, M.-H., & Suh, S.-I. (2004). Up-regulation of human β-defensin 2 by interleukin-1β in A549 cells: involvement of PI3K, PKC, p38 MAPK, JNK, and NF-κB. *Biochemical and Biophysical Research Communications*, *320*(3), 1026-1033. https://doi.org/https://doi.org/10.1016/j.bbrc.2004.06.049

Jansen, P. A. M., Rodijk-Olthuis, D., Hollox, E. J., Kamsteeg, M., Tjabringa, G. S., de Jongh, G. J., van Vlijmen-Willems, I. M. J. J., Bergboer, J. G. M., van Rossum, M. M., de Jong, E. M. G. J., den Heijer, M., Evers, A. W. M., Bergers, M., Armour, J. A. L., Zeeuwen, P. L. J. M., & Schalkwijk, J.

(2009). β-Defensin-2 Protein Is a Serum Biomarker for Disease Activity in Psoriasis and Reaches Biologically Relevant Concentrations in Lesional Skin. *PLoS One*, *4*(3), e4725. https://doi.org/10.1371/journal.pone.0004725

Jaradat, S. W., Hoder-Przyrembel, C., Cubillos, S., Krieg, N., Lehmann, K., Piehler, S., Sigusch, B. W., & Norgauer, J. (2013). Beta-defensin-2 Genomic Copy Number Variation and Chronic Periodontitis. *Journal of Dental Research*, *92*(11), 1035-1040. https://doi.org/10.1177/0022034513504217

Jaroenlapnopparat, A., Bhatia, K., & Coban, S. (2022). Inflammation and Gastric Cancer. *Diseases*, *10*(3). https://doi.org/10.3390/diseases10030035

Jeurnink, S. M., Büchner, F. L., Bueno-de-Mesquita, H. B., Siersema, P. D., Boshuizen, H. C., Numans, M. E., Dahm, C. C., Overvad, K., Tjønneland, A., Roswall, N., Clavel-Chapelon, F., Boutron-Ruault, M. C., Morois, S., Kaaks, R., Teucher, B., Boeing, H., Buijsse, B., Trichopoulou, A., Benetou, V., . . . Gonzalez, C. A. (2012). Variety in vegetable and fruit consumption and the risk of gastric and esophageal cancer in the European prospective investigation into cancer and nutrition. *International Journal of Cancer*, *131*(6), E963-E973. https://doi.org/https://doi.org/10.1002/ijc.27517

Ji, X., Sun, Z., Wu, H., Zhang, J., Liu, S., Cao, X., Wang, B., Wang, F., Zhang, Y., Li, B., Feng, J., & Zhao, H. (2024). More powerful dysregulation of *Helicobacter pylori* East Asian-type CagA on intracellular signalings. *BMC Microbiology*, *24*(1), 467. https://doi.org/10.1186/s12866-024-03619-4

Johansen, C., Bertelsen, T., Ljungberg, C., Mose, M., & Iversen, L. (2016). Characterization of TNF-α– and IL-17A–Mediated Synergistic Induction of *DEFB4* Gene Expression in Human Keratinocytes through IκBζ. *Journal of Investigative Dermatology*, *136*(8), 1608-1616. https://doi.org/https://doi.org/10.1016/j.jid.2016.04.012

Joly, S., Organ, C. C., Johnson, G. K., McCray, P. B., Jr., & Guthmiller, J. M. (2005). Correlation between beta-defensin expression and induction profiles in gingival keratinocytes. *Mol Immunol*, *42*(9), 1073-1084. https://doi.org/10.1016/j.molimm.2004.11.001

Jonaitis, P., Nyssen, O. P., Saracino, I. M., Fiorini, G., Vaira, D., Pérez-Aísa, Á., Tepes, B., Castro-Fernandez, M., Pabón-Carrasco, M., Keco-Huerga, A., Voynovan, I., Lucendo, A. J., Lanas, Á., Martínez-Domínguez, S. J., Almajano, E. A., Rodrigo, L., Vologzanina, L., Brglez Jurecic, N., Denkovski, M., . . . Jonaitis, L. (2023). Comparison of the management of *Helicobacter pylori* infection between the older and younger European populations. *Sci Rep*, *13*(1), 17235. https://doi.org/10.1038/s41598-023-43287-4

Jonas, O. B., Irwin, A., Berthe, Frank Cesar Jean., & Le Gall, F. G., Marquez, Patricio. (2017). *Drug-resistant infections: a threat to our economic future*. http://documents.worldbank.org/curated/en/323311493396993758/final-report

Joshi, N., & Fass, J. (2011). *Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files*. Retrieved 20/8/2024 from https://github.com/najoshi/sickle

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., . . . Hassabis, D. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature*, *596*(7873), 583-589. https://doi.org/10.1038/s41586-021-03819-2

Jung, H. K., Kang, S. J., Lee, Y. C., Yang, H. J., Park, S. Y., Shin, C. M., Kim, S. E., Lim, H. C., Kim, J. H., Nam, S. Y., Shin, W. G., Park, J. M., Choi, I. J., Kim, J. G., & Choi, M. (2021). Evidence-Based Guidelines for the Treatment of *Helicobacter pylori* Infection in Korea 2020. *Gut Liver*, *15*(2), 168-195. https://doi.org/10.5009/gnl20288

Jung, S. W., Sugimoto, M., Shiota, S., Graham, D. Y., & Yamaoka, Y. (2012). The intact dupA cluster is a more reliable *Helicobacter pylori* virulence marker than dupA alone. *Infect Immun*, *80*(1), 381-387. https://doi.org/10.1128/iai.05472-11

Kageyama, C., Sato, M., Sakae, H., Obayashi, Y., Kawahara, Y., Mima, T., Matsushita, O., Yokota, K., Mizuno, M., & Okada, H. (2019). Increase in antibiotic resistant *Helicobacter pylori* in a University Hospital in Japan. *Infect Drug Resist*, *12*, 597-602. https://doi.org/10.2147/idr.S196452

Kagnoff, M. F. (2014). The intestinal epithelium is an integral component of a communications network. *J Clin Invest*, *124*(7), 2841-2843. https://doi.org/10.1172/jci75225

Kalali, B., Mejías-Luque, R., Javaheri, A., & Gerhard, M. (2014). *H. pylori* Virulence Factors: Influence on Immune System and Pathology. *Mediators of Inflammation*, *2014*(1), 426309. https://doi.org/https://doi.org/10.1155/2014/426309

Kalus, A. A., Fredericks, L. P., Hacker, B. M., Dommisch, H., Presland, R. B., Kimball, J. R., & Dale, B. A. (2009). Association of a genetic polymorphism (-44 C/G SNP) in the human *DEFB1* gene with expression and inducibility of multiple β-defensins in gingival keratinocytes. *BMC Oral Health*, *9*(1), 21. https://doi.org/10.1186/1472-6831-9-21

Kang, J. M., & Choi, Y. (2023). Host Factor: Genetic Polymorphism. In N. Kim (Ed.), *Helicobacter pylori* (pp. 121-131). Springer Nature Singapore. https://doi.org/10.1007/978-981-97-0013-4_7

Kao, C.-Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R. W., & Wu, R. (2004). IL-17 Markedly Up-Regulates β-Defensin-2 Expression in Human Airway Epithelium via JAK and NF-κB Signaling Pathways1. *The Journal of Immunology*, *173*(5), 3482-3491. https://doi.org/10.4049/jimmunol.173.5.3482

Kao, C.-Y., Sheu, B.-S., & Wu, J.-J. (2016). *Helicobacter pylori* infection: An overview of bacterial virulence factors and pathogenesis. *Biomedical Journal*, *39*(1), 14-23. https://doi.org/https://doi.org/10.1016/j.bj.2015.06.002

Karbalaei, M., Hussein, N. R., & Keikha, M. (2021). Predicting the role of dupA-positive *Helicobacter pylori* strains in severe gastrointestinal disorders: An updated meta-analysis. *Gene Reports*, *24*, 101263. https://doi.org/https://doi.org/10.1016/j.genrep.2021.101263

Karbalaei, M., Talebi Bezmin Abadi, A., & Keikha, M. (2022). Clinical relevance of the *cagA* and *vacA s1m1* status and antibiotic resistance in *Helicobacter pylori*: a systematic review and meta-analysis. *BMC Infectious Diseases*, *22*(1), 573. https://doi.org/10.1186/s12879-022-07546-5

Kato, M., Ota, H., Okuda, M., Kikuchi, S., Satoh, K., Shimoyama, T., Suzuki, H., Handa, O., Furuta, T., Mabe, K., Murakami, K., Sugiyama, T., Uemura, N., & Takahashi, S. i. (2019). Guidelines for the management of *Helicobacter pylori* infection in Japan: 2016 Revised Edition. *Helicobacter*, *24*(4), e12597. https://doi.org/https://doi.org/10.1111/hel.12597

Kayali, S., Manfredi, M., Gaiani, F., Bianchi, L., Bizzarri, B., Leandro, G., Di Mario, F., & De' Angelis, G. L. (2018). *Helicobacter pylori*, transmission routes and recurrence of infection: state of the art. *Acta Biomed*, *89*(8-s), 72-76. https://doi.org/10.23750/abm.v89i8-S.7947

Keikha, M., Ali-Hassanzadeh, M., & Karbalaei, M. (2020). Association of *Helicobacter pylori vacA* genotypes and peptic ulcer in Iranian population: a systematic review and meta-analysis. *BMC Gastroenterology*, *20*(1), 266. https://doi.org/10.1186/s12876-020-01406-9

Keilberg, D., & Ottemann, K. M. (2016). How *Helicobacter pylori* senses, targets and interacts with the gastric epithelium. *Environmental Microbiology*, *18*(3), 791-806. https://doi.org/https://doi.org/10.1111/1462-2920.13222

Kent, A. G., Vill, A. C., Shi, Q., Satlin, M. J., & Brito, I. L. (2020). Widespread transfer of mobile antibiotic resistance genes within individual gut microbiomes revealed through bacterial Hi-C. *Nature Communications*, *11*(1), 4379. https://doi.org/10.1038/s41467-020-18164-7

Khalid, H., van Hooij, A., Connelley, T. K., Geluk, A., & Hope, J. C. (2022). Protein Levels of Pro-Inflammatory Cytokines and Chemokines as Biomarkers of *Mycobacterium bovis* Infection and BCG Vaccination in Cattle. *Pathogens*, *11*(7), 738. https://www.mdpi.com/2076-0817/11/7/738

Khan, A., Farooqui, A., Manzoor, H., Akhtar, S. S., Quraishy, M. S., & Kazmi, S. U. (2012). Antibiotic resistance and *cagA* gene correlation: a looming crisis of *Helicobacter pylori*. *World J Gastroenterol*, *18*(18), 2245-2252. https://doi.org/10.3748/wjg.v18.i18.2245

Khan, Z. A., Siddiqui, M. F., & Park, S. (2019). Current and Emerging Methods of Antibiotic Susceptibility Testing. *Diagnostics*, *9*(2), 49. https://www.mdpi.com/2075-4418/9/2/49

Kiehne, K., Brunke, G., Meyer, D., Harder, J., & Herzig, K.-H. (2005). Oesophageal defensin expression during Candida infection and reflux disease. *Scandinavian Journal of Gastroenterology*, *40*(5), 501-507. https://doi.org/10.1080/00365520510015386

Kienesberger, S., Perez-Perez, G. I., Olivares, A. Z., Bardhan, P., Sarker, S. A., Hasan, K. Z., Sack, R. B., & Blaser, M. J. (2018). When is *Helicobacter pylori* acquired in populations in developing countries? A birth-cohort study in Bangladeshi children. *Gut Microbes*, *9*(3), 252-263. https://doi.org/10.1080/19490976.2017.1421887

Kim, B. J., & Kim, J. G. (2013). Substitutions in penicillin-binding protein 1 in amoxicillin-resistant *Helicobacter pylori* strains isolated from Korean patients. *Gut Liver*, *7*(6), 655-660. https://doi.org/10.5009/gnl.2013.7.6.655

Kim, M., Jung, K., Kim, I.-S., Lee, I.-S., Ko, Y., Shin, J. E., & Park, K. I. (2018). TNF-α induces human neural progenitor cell survival after oxygen–glucose deprivation by activating the NF-κB pathway. *Experimental & Molecular Medicine*, *50*(4), 1-14. https://doi.org/10.1038/s12276-018-0033-1

Kim, N., Park, Y. S., Cho, S. I., Lee, H. S., Choe, G., Kim, I. W., Won, Y. D., Park, J. H., Kim, J. S., Jung, H. C., & Song, I. S. (2008). Prevalence and risk factors of atrophic gastritis and intestinal metaplasia in a Korean population without significant gastroduodenal disease. *Helicobacter*, *13*(4), 245-255. https://doi.org/10.1111/j.1523-5378.2008.00604.x

Kim, S. E., Park, M. I., Park, S. J., Moon, W., Choi, Y. J., Cheon, J. H., Kwon, H. J., Ku, K. H., Yoo, C. H., Kim, J. H., Lee, G. W., & Song, S. E. (2015). Trends in *Helicobacter pylori* eradication rates by first-line triple therapy and related factors in eradication therapy. *Korean J Intern Med*, *30*(6), 801-807. https://doi.org/10.3904/kjim.2015.30.6.801

King, A. E., Fleming, D. C., Critchley, H. O. D., & Kelly, R. W. (2002). Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells. *Molecular Human Reproduction*, *8*(4), 341-349. https://doi.org/10.1093/molehr/8.4.341

Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nuñez, G., & Flavell, R. A. (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science*, *307*(5710), 731-734. https://doi.org/10.1126/science.1104911

Kocazeybek, B., Sakli, M. K., Yuksel, P., Demirci, M., Caliskan, R., Sarp, T. Z., Saribas, S., Demiryas, S., Kalayci, F., Cakan, H., Uysal, H. K., Gareayaghi, N., Ergin, S., Erzin, Y. Z., Bal, K., Tascı, İ., & Tokman, H. B. (2019). Comparison of new and classical point mutations associated with clarithromycin resistance in *Helicobacter pylori* strains isolated from dyspeptic patients and their effects on phenotypic clarithromycin resistance. *J Med Microbiol*, *68*(4), 566-573. https://doi.org/10.1099/jmm.0.000944

Kocsmár, É., Buzás, G. M., Szirtes, I., Kocsmár, I., Kramer, Z., Szijártó, A., Fadgyas-Freyler, P., Szénás, K., Rugge, M., Fassan, M., Kiss, A., Schaff, Z., Röst, G., & Lotz, G. (2021). Primary and secondary clarithromycin resistance in *Helicobacter pylori* and mathematical modeling of the role of macrolides. *Nat Commun*, *12*(1), 2255. https://doi.org/10.1038/s41467-021-22557-7

Kodama, M., Murakami, K., Okimoto, T., Sato, R., Uchida, M., Abe, T., Shiota, S., Nakagawa, Y., Mizukami, K., & Fujioka, T. (2012). Ten-year prospective follow-up of histological changes at five points on the gastric mucosa as recommended by the updated Sydney system after *Helicobacter pylori* eradication. *J Gastroenterol*, *47*(4), 394-403. https://doi.org/10.1007/s00535-011-0504-9

Kodama, M., Okimoto, T., Mizukami, K., Hirashita, Y., Wada, Y., Fukuda, M., Matsunari, O., Okamoto, K., Ogawa, R., Fukuda, K., Kudo, Y., Kawahara, Y., & Murakami, K. (2021). Gastric mucosal changes, and sex differences therein, after *Helicobacter pylori* eradication: A long-term prospective follow-up study. *J Gastroenterol Hepatol*, *36*(8), 2210-2216. https://doi.org/10.1111/jgh.15477

Korbmacher, M., Fischer, S., Landenberger, M., Papatheodorou, P., Aktories, K., & Barth, H. (2020). Human α-Defensin-5 Efficiently Neutralizes Clostridioides difficile Toxins TcdA, TcdB, and CDT. *Front Pharmacol*, *11*, 1204. https://doi.org/10.3389/fphar.2020.01204

Krakovka, S., Ribacke, U., Miyamoto, Y., Eckmann, L., & Svärd, S. (2022). Characterization of Metronidazole-Resistant Giardia intestinalis Lines by Comparative Transcriptomics and Proteomics. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.834008

Krawczyk, S. J., Leśniczak-Staszak, M., Gowin, E., & Szaflarski, W. (2024). Mechanistic Insights into Clinically Relevant Ribosome-Targeting Antibiotics. *Biomolecules*, *14*(10). https://doi.org/10.3390/biom14101263

Krisanaprakornkit, S., Kimball, J. R., & Dale, B. A. (2002). Regulation of Human β-Defensin-2 in Gingival Epithelial Cells: The Involvement of Mitogen-Activated Protein Kinase Pathways, But Not the NF-κB Transcription Factor Family1. *The Journal of Immunology*, *168*(1), 316-324. https://doi.org/10.4049/jimmunol.168.1.316

Kubler, I., KOSLOWSKI, M. J., GERSEMANN, M., FELLERMANN, K., BEISNER, J., BECKER, S., ROTHFUSS, K., HERRLINGER, K. R., STANGE, E. F., & WEHKAMP, J. (2009). Influence of standard treatment on ileal and colonic antimicrobial defensin expression in active Crohn's disease. *Alimentary Pharmacology & Therapeutics*, *30*(6), 621-633. https://doi.org/https://doi.org/10.1111/j.1365-2036.2009.04070.x

Kufareva, I. (2016). Chemokines and their receptors: insights from molecular modeling and crystallography. *Current Opinion in Pharmacology*, *30*, 27-37. https://doi.org/https://doi.org/10.1016/j.coph.2016.07.006

Kuipers, E. J., Nelis, G. F., Klinkenberg-Knol, E. C., Snel, P., Goldfain, D., Kolkman, J. J., Festen, H. P. M., Dent, J., Zeitoun, P., Havu, N., Lamm, M., & Walan, A. (2004). Cure of *Helicobacter pylori* infection in patients with reflux oesophagitis treated with long term omeprazole reverses gastritis without exacerbation of reflux disease: results of a randomised controlled trial. *Gut*, *53*(1), 12-20. https://doi.org/10.1136/gut.53.1.12

Kuo, C.-J., Lee, C.-H., Chang, M.-L., Lin, C.-Y., Lin, W.-R., Su, M.-Y., Chiu, C.-H., Tseng, C.-N., Wu, Y.-S., Chiu, C.-T., & Lai, C.-H. (2021). Multidrug resistance: The clinical dilemma of refractory *Helicobacter pylori* infection. *Journal of Microbiology, Immunology and Infection*, *54*(6), 1184-1187. https://doi.org/https://doi.org/10.1016/j.jmii.2021.03.006

Kusters, J. G., Gerrits, M. M., Van Strijp, J. A., & Vandenbroucke-Grauls, C. M. (1997). Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. *Infect Immun*, *65*(9), 3672-3679. https://doi.org/10.1128/iai.65.9.3672-3679.1997

Kusters, J. G., van Vliet, A. H., & Kuipers, E. J. (2006a). Pathogenesis of *Helicobacter pylori* infection. *Clin Microbiol Rev*, *19*(3), 449-490. https://doi.org/10.1128/cmr.00054-05

Kusters, J. G., Vliet, A. H. M. v., & Kuipers, E. J. (2006b). Pathogenesis of *Helicobacter pylori* Infection. *Clinical Microbiology Reviews*, *19*(3), 449-490. https://doi.org/doi:10.1128/cmr.00054-05

Kwok, T., Zabler, D., Urman, S., Rohde, M., Hartig, R., Wessler, S., Misselwitz, R., Berger, J., Sewald, N., König, W., & Backert, S. (2007). *Helicobacter* exploits integrin for type IV secretion and kinase activation. *Nature*, *449*(7164), 862-866. https://doi.org/10.1038/nature06187

Lacalle, R. A., Blanco, R., Carmona-Rodríguez, L., Martín-Leal, A., Mira, E., & Mañes, S. (2017). Chapter Five - Chemokine Receptor Signaling and the Hallmarks of Cancer. In L. Galluzzi (Ed.), *International Review of Cell and Molecular Biology* (Vol. 331, pp. 181-244). Academic Press. https://doi.org/https://doi.org/10.1016/bs.ircmb.2016.09.011

Laible, G., & Hakenbeck, R. (1991). Five independent combinations of mutations can result in low-affinity penicillin-binding protein 2x of *Streptococcus pneumoniae*. *J Bacteriol*, *173*(21), 6986-6990. https://doi.org/10.1128/jb.173.21.6986-6990.1991

Lanas, A., & Chan, F. K. L. (2017). Peptic ulcer disease. *The Lancet*, *390*(10094), 613-624. https://doi.org/10.1016/S0140-6736(16)32404-7

Lang, J., Zhu, R., Sun, X., Zhu, S., Li, T., Shi, X., Sun, Y., Yang, Z., Wang, W., Bing, P., He, B., & Tian, G. (2021). Evaluation of the MGISEQ-2000 Sequencing Platform for Illumina Target Capture Sequencing Libraries. *Front Genet*, *12*, 730519. https://doi.org/10.3389/fgene.2021.730519

Lang, L., & García, F. (2004). Comparison of E-test and disk diffusion assay to evaluate resistance of *Helicobacter pylori* isolates to amoxicillin, clarithromycin, metronidazole and tetracycline in Costa Rica. *Int J Antimicrob Agents*, *24*(6), 572-577. https://doi.org/10.1016/j.ijantimicag.2004.07.009

Larsen, A. L., Ragnhildstveit, E., Moayeri, B., Eliassen, L., & Melby, K. K. (2013). Resistance rates of metronidazole and other antibacterials in *Helicobacter pylori* from previously untreated patients in Norway. *Apmis*, *121*(4), 353-358. https://doi.org/10.1111/apm.12009

Lauener, F. N., Imkamp, F., Lehours, P., Buissonnière, A., Benejat, L., Zbinden, R., Keller, P. M., & Wagner, K. (2019). Genetic Determinants and Prediction of Antibiotic Resistance Phenotypes in *Helicobacter pylori*. *J Clin Med*, *8*(1). https://doi.org/10.3390/jcm8010053

Lee, A., Dixon, M. F., Danon, S. J., Kuipers, E., Mégraud, F., Larsson, H., & Mellgård, B. (1995). Local acid production and *Helicobacter pylori*: a unifying hypothesis of gastroduodenal disease. *Eur J Gastroenterol Hepatol*, *7*(5), 461-465.

Lee, J. Y., Kim, N., Nam, R. H., In Choi, S., Lee, J. W., & Lee, D. H. (2019). Primary and secondary antibiotic resistance of *Helicobacter pylori* in Korea from 2003 to 2018. *Helicobacter*, *24*(6), e12660. https://doi.org/10.1111/hel.12660

Lee, K. E., Khoi, P. N., Xia, Y., Park, J. S., Joo, Y. E., Kim, K. K., Choi, S. Y., & Jung, Y. D. (2013). *Helicobacter pylori* and interleukin-8 in gastric cancer. *World J Gastroenterol*, *19*(45), 8192-8202. https://doi.org/10.3748/wjg.v19.i45.8192

Lee, S. M., Kim, N., Kwon, Y. H., Nam, R. H., Kim, J. M., Park, J. Y., Lee, Y. S., & Lee, D. H. (2018). *rdxA*, *frxA*, and efflux pump in metronidazole-resistant *Helicobacter pylori*: Their relation to clinical outcomes. *J Gastroenterol Hepatol*, *33*(3), 681-688. https://doi.org/10.1111/jgh.13906

Lee, Y. C., Chiang, T. H., Chou, C. K., Tu, Y. K., Liao, W. C., Wu, M. S., & Graham, D. Y. (2016). Association Between *Helicobacter pylori* Eradication and Gastric Cancer Incidence: A Systematic Review and Meta-analysis. *Gastroenterology*, *150*(5), 1113-1124.e1115. https://doi.org/10.1053/j.gastro.2016.01.028

Lehmann, F. S., Terracciano, L., Carena, I., Baeriswyl, C., Drewe, J., Tornillo, L., De Libero, G., & Beglinger, C. (2002). *In situ* correlation of cytokine secretion and apoptosis in *Helicobacter pylori*-associated gastritis. *Am J Physiol Gastrointest Liver Physiol*, *283*(2), G481-488. https://doi.org/10.1152/ajpgi.00422.2001

Lehrer, R. I. (2004). Primate defensins. *Nat Rev Microbiol*, *2*(9), 727-738. https://doi.org/10.1038/nrmicro976

Lehrer, R. I., & Lu, W. (2012). α-Defensins in human innate immunity. *Immunological Reviews*, *245*(1), 84-112. https://doi.org/https://doi.org/10.1111/j.1600-065X.2011.01082.x

Lei, J., Sun, L., Huang, S., Zhu, C., Li, P., He, J., Mackey, V., Coy, D. H., & He, Q. (2019). The antimicrobial peptides and their potential clinical applications. *Am J Transl Res*, *11*(7), 3919-3931.

Lessa, F. C., Gould, C. V., & McDonald, L. C. (2012). Current status of *Clostridium difficile* infection epidemiology. *Clin Infect Dis*, *55 Suppl 2*(Suppl 2), S65-70. https://doi.org/10.1093/cid/cis319

Letley, D. P., Rhead, J. L., Twells, R. J., Dove, B., & Atherton, J. C. (2003). Determinants of Non-toxicity in the Gastric Pathogen *Helicobacter pylori* *. *Journal of Biological Chemistry*, *278*(29), 26734-26741. https://doi.org/https://doi.org/10.1074/jbc.M304071200

Leung, W. K., Wong, I. O. L., Cheung, K. S., Yeung, K. F., Chan, E. W., Wong, A. Y. S., Chen, L., Wong, I. C. K., & Graham, D. Y. (2018). Effects of *Helicobacter pylori* Treatment on Incidence of Gastric Cancer in Older Individuals. *Gastroenterology*, *155*(1), 67-75. https://doi.org/https://doi.org/10.1053/j.gastro.2018.03.028

Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G., & Morgan, D. R. (1988). Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J Med Microbiol*, *26*(2), 93-99. https://doi.org/10.1099/00222615-26-2-93

Li, D.-Q., Zhou, N., Zhang, L., Ma, P., & Pflugfelder, S. C. (2010). Suppressive Effects of Azithromycin on Zymosan-Induced Production of Proinflammatory Mediators by Human Corneal Epithelial Cells. *Investigative Ophthalmology & Visual Science*, *51*(11), 5623-5629. https://doi.org/10.1167/iovs.09-4992

Li, E., Zhang, Y., Tian, X., Wang, X., Gathungu, G., Wolber, A., Shiekh, S. S., Sartor, R. B., Davidson, N. O., Ciorba, M. A., Zhu, W., Nelson, L. M., Robertson, C. E., & Frank, D. N. (2019). Influence of Crohn's disease related polymorphisms in innate immune function on ileal microbiome. *PLoS One*, *14*(2), e0213108. https://doi.org/10.1371/journal.pone.0213108

Li, G., Wulan, H., Song, Z., Paik, P. A., Tsao, M. L., Goodman, G. M., MacEachern, P. T., Downey, R. S., Jankowska, A. J., Rabinowitz, Y. M., Learch, T. B., Song, D. Z., Yuan, J. J., Zheng, S., & Zheng, Z. (2015a). Regulatory B Cell Function Is Suppressed by Smoking and Obesity in *H. pylori*-Infected Subjects and Is Correlated with Elevated Risk of Gastric Cancer. *PLoS One*, *10*(7), e0134591. https://doi.org/10.1371/journal.pone.0134591

Li, H., Shen, M., & Wang, S. (2024). Current therapies and progress in the treatment of advanced gastric cancer. *Front Oncol*, *14*, 1327055. https://doi.org/10.3389/fonc.2024.1327055

Li, H., Shen, Y., Song, X., Tang, X., Hu, R., Marshall, B. J., Tang, H., & Benghezal, M. (2022). Need for standardization and harmonization of *Helicobacter pylori* antimicrobial susceptibility testing. *Helicobacter*, *27*(2), e12873. https://doi.org/https://doi.org/10.1111/hel.12873

Li, J., Lee, D. S., & Madrenas, J. (2013). Evolving Bacterial Envelopes and Plasticity of TLR2-Dependent Responses: Basic Research and Translational Opportunities [Review]. *Frontiers in Immunology*, *4*. https://doi.org/10.3389/fimmu.2013.00347

Li, J., Li, X., Song, J., Yan, B., Rock, S. A., Jia, J., Liu, J., Wang, C., Weiss, T., Weiss, H. L., Gao, T., Alam, A., & Evers, B. M. (2020). Absence of neurotensin attenuates intestinal dysbiosis and inflammation by maintaining Mmp7/α-defensin axis in diet-induced obese mice [Article]. *FASEB Journal*, *34*(6), 8596-8610. https://doi.org/10.1096/fj.201902374RR

Li, S., Wang, Y., Xue, Z., Jia, Y., Li, R., He, C., & Chen, H. (2021). The structure-mechanism relationship and mode of actions of antimicrobial peptides: A review. *Trends in Food Science & Technology*, *109*, 103-115. https://doi.org/https://doi.org/10.1016/j.tifs.2021.01.005

Li, W., Liu, Q., Shi, J., Xu, X., & Xu, J. (2023a). The role of TNF-α in the fate regulation and functional reprogramming of mesenchymal stem cells in an inflammatory microenvironment. *Front Immunol*, *14*, 1074863. https://doi.org/10.3389/fimmu.2023.1074863

Li, X., Liu, S., Luo, J., Liu, A., Tang, S., Liu, S., Yu, M., & Zhang, Y. (2015b). *Helicobacter pylori* induces IL-1β and IL-18 production in human monocytic cell line through activation of NLRP3 inflammasome via ROS signaling pathway. *Pathogens and Disease*, *73*(4). https://doi.org/10.1093/femspd/ftu024

Li, Y., Choi, H., Leung, K., Jiang, F., Graham, D. Y., & Leung, W. K. (2023b). Global prevalence of *Helicobacter pylori* infection between 1980 and 2022: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol*, *8*(6), 553-564. https://doi.org/10.1016/s2468-1253(23)00070-5

Li, Y., Metcalf, B. J., Chochua, S., Li, Z., Gertz, R. E., Walker, H., Hawkins, P. A., Tran, T., Whitney, C. G., McGee, L., & Beall, B. W. (2016). Penicillin-Binding Protein Transpeptidase Signatures for Tracking and Predicting β-Lactam Resistance Levels in *Streptococcus pneumoniae*. *mBio*, *7*(3), 10.1128/mbio.00756-00716. https://doi.org/doi:10.1128/mbio.00756-16

Liang, B., Yuan, Y., Peng, X., Liu, X.-L., Hu, X., & Xing, D. M. (2022). Current and future perspectives for *Helicobacter pylori* treatment and management: From antibiotics to probiotics. *Frontiers in Cellular and Infection Microbiology*, *12*.

Lim, J. W., Kim, H., & Kim, K. H. (2003). Cell adhesion-related gene expression by *Helicobacter pylori* in gastric epithelial AGS cells. *The International Journal of Biochemistry & Cell Biology*, *35*(8), 1284-1296. https://doi.org/https://doi.org/10.1016/S1357-2725(03)00051-7

Lim, S. G., Park, R. W., Shin, S. J., Yoon, D., Kang, J. K., Hwang, J. C., Kim, S. S., Kim, J. H., & Lee, K. M. (2016a). The relationship between the failure to eradicate *Helicobacter pylori* and previous antibiotics use. *Digestive and Liver Disease*, *48*(4), 385-390. https://doi.org/https://doi.org/10.1016/j.dld.2015.12.001

Lim, S. G., Park, R. W., Shin, S. J., Yoon, D., Kang, J. K., Hwang, J. C., Kim, S. S., Kim, J. H., & Lee, K. M. (2016b). The relationship between the failure to eradicate *Helicobacter pylori* and previous antibiotics use. *Dig Liver Dis*, *48*(4), 385-390. https://doi.org/10.1016/j.dld.2015.12.001

Lin, A. S., Dooyema, S. D. R., Frick-Cheng, A. E., Harvey, M. L., Suarez, G., Loh, J. T., McDonald, W. H., McClain, M. S., Peek, R. M., & Cover, T. L. (2020). Bacterial Energetic Requirements for *Helicobacter pylori* Cag Type IV Secretion System-Dependent Alterations in Gastric Epithelial Cells. *Infection and Immunity*, *88*(2), 10.1128/iai.00790-00719. https://doi.org/doi:10.1128/iai.00790-19

Linn, O., Menges, B., Lammert, F., Weber, S. N., & Krawczyk, M. (2023). Altered Expression of Antimicrobial Peptides in the Upper Gastrointestinal Tract of Patients with Diabetes Mellitus. *Nutrients*, *15*(3), 754. https://www.mdpi.com/2072-6643/15/3/754

Liou, J. M., Malfertheiner, P., Lee, Y. C., Sheu, B. S., Sugano, K., Cheng, H. C., Yeoh, K. G., Hsu, P. I., Goh, K. L., Mahachai, V., Gotoda, T., Chang, W. L., Chen, M. J., Chiang, T. H., Chen, C. C., Wu, C. Y., Leow, A. H., Wu, J. Y., Wu, D. C., . . . El-Omar, E. M. (2020). Screening and eradication of *Helicobacter pylori* for gastric cancer prevention: the Taipei global consensus. *Gut*, *69*(12), 2093-2112. https://doi.org/10.1136/gutjnl-2020-322368

Lisitsyn, N. A., Bukurova, Y. A., Nikitina, I. G., Krasnov, G. S., Sykulev, Y., & Beresten, S. F. (2012). Enteric alpha defensins in norm and pathology. *Annals of Clinical Microbiology and Antimicrobials*, *11*(1), 1. https://doi.org/10.1186/1476-0711-11-1

Liu, D. S., Wang, Y. H., Zhu, Z. H., Zhang, S. H., Zhu, X., Wan, J. H., Lu, N. H., & Xie, Y. (2019). Characteristics of *Helicobacter pylori* antibiotic resistance: data from four different populations. *Antimicrob Resist Infect Control*, *8*, 192. https://doi.org/10.1186/s13756-019-0632-1

Liu, M., Hu, Z., Wang, C., & Zhang, Y. (2023). The TLR/MyD88 signalling cascade in inflammation and gastric cancer: the immune regulatory network of *Helicobacter pylori*. *Journal of Molecular Medicine*, *101*(7), 767-781. https://doi.org/10.1007/s00109-023-02332-5

Liu, T., Chen, C.-Y., Chen-Deng, A., Chen, Y.-L., Wang, J.-Y., Hou, Y.-I., & Lin, M.-C. (2020). Joining Illumina paired-end reads for classifying phylogenetic marker sequences. *BMC Bioinformatics*, *21*(1), 105. https://doi.org/10.1186/s12859-020-3445-6

Liu, W. Z., Xie, Y., Lu, H., Cheng, H., Zeng, Z. R., Zhou, L. Y., Chen, Y., Wang, J. B., Du, Y. Q., & Lu, N. H. (2018). Fifth Chinese National Consensus Report on the management of *Helicobacter pylori* infection. *Helicobacter*, *23*(2), e12475. https://doi.org/10.1111/hel.12475

Liu, X., He, B., Cho, W. C., Pan, Y., Chen, J., Ying, H., Wang, F., Lin, K., Peng, H., & Wang, S. (2016). A systematic review on the association between the *Helicobacter pylori vacA i* genotype and gastric disease. *FEBS Open Bio*, *6*(5), 409-417. https://doi.org/https://doi.org/10.1002/2211-5463.12046

Liu, Y., Wang, S., Yang, F., Chi, W., Ding, L., Liu, T., Zhu, F., Ji, D., Zhou, J., Fang, Y., Zhang, J., Xiang, P., Zhang, Y., & Zhao, H. (2022). Antimicrobial resistance patterns and genetic elements associated with the antibiotic resistance of *Helicobacter pylori* strains from Shanghai. *Gut Pathogens*, *14*(1), 14. https://doi.org/10.1186/s13099-022-00488-y

Liu, Z. Q., Zheng, P. Y., & Yang, P. C. (2008). Efflux pump gene *hefA* of *Helicobacter pylori* plays an important role in multidrug resistance. *World J Gastroenterol*, *14*(33), 5217-5222. https://doi.org/10.3748/wjg.14.5217

Llanes, R., Soria, C., Nagashima, S., Kobayashi, N., Gala, A., Guzmán, D., Feliciano, O., Valdés, L., Gutiérrez, O., Fernández, H., Llop, A., & Wada, A. (2010). Phenotypic and genetic characterization of antimicrobial profiles of *Helicobacter pylori* strains in Cuba. *J Health Popul Nutr*, *28*(2), 124-129. https://doi.org/10.3329/jhpn.v28i2.4881

Lok, C. H., Zhu, D., Wang, J., Ren, Y. T., Jiang, X., Li, S. J., & Zhao, X. Y. (2020). Phenotype and Molecular Detection of Clarithromycin and Levofloxacin Resistance in *Helicobacter pylori* Clinical Isolates in Beijing. *Infect Drug Resist*, *13*, 2145-2153. https://doi.org/10.2147/idr.S249370

López-Boado, Y. S., & Rubin, B. K. (2008). Macrolides as immunomodulatory medications for the therapy of chronic lung diseases. *Current Opinion in Pharmacology*, *8*(3), 286-291. https://doi.org/https://doi.org/10.1016/j.coph.2008.01.010

López-Gasca, M., Peña, J., García-Amado, M. A., Michelangeli, F., & Contreras, M. (2018). Point Mutations at *gyrA* and *gyrB* Genes of Levofloxacin-Resistant *Helicobacter pylori* Isolates in the Esophageal Mucosa from a Venezuelan Population. *Am J Trop Med Hyg*, *98*(4), 1051-1055. https://doi.org/10.4269/ajtmh.17-0478

Lu, H. H., Lai, F. P., Lo, H. Y., Sheu, B. S., & Yang, Y. J. (2019). Increasing antimicrobial resistance to clarithromycin and metronidazole in pediatric *Helicobacter pylori* infection in southern Taiwan: A comparison between two decades. *Helicobacter*, *24*(5), e12633. https://doi.org/10.1111/hel.12633

Lv, Y.-p., Teng, Y.-s., Mao, F.-y., Peng, L.-s., Zhang, J.-y., Cheng, P., Liu, Y.-g., Kong, H., Wang, T.-t., Wu, X.-l., Hao, C.-j., Chen, W., Yang, S.-m., Zhao, Y.-l., Han, B., Ma, Q., Zou, Q.-m., & Zhuang, Y. (2018). *Helicobacter pylori*-induced *IL-33* modulates mast cell responses, benefits bacterial growth, and contributes to gastritis. *Cell Death & Disease*, *9*(5), 457. https://doi.org/10.1038/s41419-018-0493-1

Macheboeuf, P., Contreras-Martel, C., Job, V., Dideberg, O., & Dessen, A. (2006). Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. *FEMS Microbiol Rev*, *30*(5), 673-691. https://doi.org/10.1111/j.1574-6976.2006.00024.x

Macías-García, F., Llovo-Taboada, J., Díaz-López, M., Bastón-Rey, I., & Domínguez-Muñoz, J. E. (2017). High primary antibiotic resistance of *Helicobacter Pylori* strains isolated from dyspeptic patients: A prevalence cross-sectional study in Spain. *Helicobacter*, *22*(6). https://doi.org/10.1111/hel.12440

Mahanonda, R., Sa-Ard-Iam, N., Eksomtramate, M., Rerkyen, P., Phairat, B., Schaecher, K. E., Fukuda, M. M., & Pichyangkul, S. (2009). Cigarette smoke extract modulates human β-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *Journal of Periodontal Research*, *44*(4), 557-564. https://doi.org/https://doi.org/10.1111/j.1600-0765.2008.01153.x

Mahfouz, N., Ferreira, I., Beisken, S., von Haeseler, A., & Posch, A. E. (2020). Large-scale assessment of antimicrobial resistance marker databases for genetic phenotype prediction: a systematic review. *Journal of Antimicrobial Chemotherapy*, *75*(11), 3099-3108. https://doi.org/10.1093/jac/dkaa257

Mahlapuu, M., Håkansson, J., Ringstad, L., & Björn, C. (2016). Antimicrobial Peptides: An Emerging Category of Therapeutic Agents [Review]. *Frontiers in Cellular and Infection Microbiology*, *6*. https://doi.org/10.3389/fcimb.2016.00194

Maldonado-Contreras, A., Goldfarb, K. C., Godoy-Vitorino, F., Karaoz, U., Contreras, M., Blaser, M. J., Brodie, E. L., & Dominguez-Bello, M. G. (2010). Structure of the human gastric bacterial community in relation to *Helicobacter pylori* status. *The ISME Journal*, *5*(4), 574-579. https://doi.org/10.1038/ismej.2010.149

Malfertheiner, P., Camargo, M. C., El-Omar, E., Liou, J.-M., Peek, R., Schulz, C., Smith, S. I., & Suerbaum, S. (2023). *Helicobacter pylori* infection. *Nature Reviews Disease Primers*, *9*(1), 19. https://doi.org/10.1038/s41572-023-00431-8

Malfertheiner, P., Megraud, F., O'Morain, C. A., Gisbert, J. P., Kuipers, E. J., Axon, A. T., Bazzoli, F., Gasbarrini, A., Atherton, J., Graham, D. Y., Hunt, R., Moayyedi, P., Rokkas, T., Rugge, M.,

Selgrad, M., Suerbaum, S., Sugano, K., & El-Omar, E. M. (2017). Management of *Helicobacter pylori* infection-the Maastricht V/Florence Consensus Report. *Gut*, *66*(1), 6-30. https://doi.org/10.1136/gutjnl-2016-312288

Malfertheiner, P., Megraud, F., Rokkas, T., Gisbert, J. P., Liou, J. M., Schulz, C., Gasbarrini, A., Hunt, R. H., Leja, M., O'Morain, C., Rugge, M., Suerbaum, S., Tilg, H., Sugano, K., & El-Omar, E. M. (2022). Management of *Helicobacter pylori* infection: the Maastricht VI/Florence consensus report. *Gut*. https://doi.org/10.1136/gutjnl-2022-327745

Malfertheiner, P., Peitz, U., & Treiber, G. (2003). What Constitutes Failure for *Helicobacter pylori* Eradication Therapy? *Canadian Journal of Gastroenterology and Hepatology*, *17*(B), 241508. https://doi.org/https://doi.org/10.1155/2003/241508

Malfertheiner, P., Schulz, C., & Hunt, R. H. (2024). *Helicobacter pylori* Infection: A 40-Year Journey through Shifting the Paradigm to Transforming the Management. *Digestive Diseases*, *42*(4), 299-308. https://doi.org/10.1159/000538079

Mallow, E. B., Harris, A., Salzman, N., Russell, J. P., DeBerardinis, R. J., Ruchelli, E., & Bevins, C. L. (1996). Human enteric defensins: Gene structure and developmental expression [Article]. *Journal of Biological Chemistry*, *271*(8), 4038-4045. https://doi.org/10.1074/jbc.271.8.4038

Mamori, S., Higashida, A., Kawara, F., Ohnishi, K., Takeda, A., Senda, E., Ashida, C., & Yamada, H. (2010). Age-dependent eradication of *Helicobacter pylori* in Japanese patients. *World J Gastroenterol*, *16*(33), 4176-4179. https://doi.org/10.3748/wjg.v16.i33.4176

Mansilla-Vivar, R., Serrano, C. A., Palma, C., Vera, M., Hernandez, C., Pizarro, M., Torres, J., Harris, P. R., Fuentes-López, E., Riquelme, A., & Espino, A. (2020). High *Helicobacter pylori* Bacterial Load and Low Cytokine Expression Levels Are Associated with Nodular Gastropathy. *Dig Dis Sci*, *65*(2), 565-575. https://doi.org/10.1007/s10620-019-05769-2

Marques, A. T., Vítor, J. M. B., Santos, A., Oleastro, M., & Vale, F. F. (2020). Trends in *Helicobacter pylori* resistance to clarithromycin: from phenotypic to genomic approaches. *Microb Genom*, *6*(3). https://doi.org/10.1099/mgen.0.000344

Martinelli, G., Fumagalli, M., Piazza, S., Maranta, N., Genova, F., Sperandeo, P., Sangiovanni, E., Polissi, A., Dell'Agli, M., & De Fabiani, E. (2023). Investigating the Molecular Mechanisms Underlying Early Response to Inflammation and *Helicobacter pylori* Infection in Human Gastric Epithelial Cells. *International Journal of Molecular Sciences*, *24*(20), 15147. https://www.mdpi.com/1422-0067/24/20/15147

Martinsen, T. C., Bergh, K., & Waldum, H. L. (2005). Gastric juice: a barrier against infectious diseases. *Basic Clin Pharmacol Toxicol*, *96*(2), 94-102. https://doi.org/10.1111/j.1742-7843.2005.pto960202.x

Mascellino, M. T., Oliva, A., Miele, M. C., De Angelis, M., Bruno, G., & Severi, C. (2020). Secondary Antibiotic Resistance, Correlation between Genotypic and Phenotypic Methods and Treatment in *Helicobacter pylori* Infected Patients: A Retrospective Study. *Antibiotics*, *9*(9), 549. https://www.mdpi.com/2079-6382/9/9/549

Matos, J. I., de Sousa, H. A. C., Marcos-Pinto, R., & Dinis-Ribeiro, M. (2013). *Helicobacter pylori* CagA and VacA genotypes and gastric phenotype: a meta-analysis. *European Journal of Gastroenterology & Hepatology*, *25*(12), 1431-1441. https://doi.org/10.1097/MEG.0b013e328364b53e

Matsunari, O., Miftahussurur, M., Shiota, S., Suzuki, R., Vilaichone, R.-k., Uchida, T., Ratanachu-ek, T., Tshering, L., Mahachai, V., & Yamaoka, Y. (2016). Rare *Helicobacter pylori* Virulence Genotypes in Bhutan. *Scientific Reports*, *6*(1), 22584. https://doi.org/10.1038/srep22584

Matsuo, J., Sakai, K., Okubo, T., & Yamaguchi, H. (2019). *Chlamydia pneumoniae* enhances Interleukin 8 (IL-8) production with reduced azithromycin sensitivity under hypoxia. *Apmis*, *127*, 131 - 138.

Matsuzaki, J., Suzuki, H., Tsugawa, H., Nishizawa, T., & Hibi, T. (2010). Homology model of the DNA gyrase enzyme of *Helicobacter pylori*, a target of quinolone-based eradication therapy. *J Gastroenterol Hepatol*, *25 Suppl 1*, S7-10. https://doi.org/10.1111/j.1440-1746.2010.06245.x

Matteo, M. J., Granados, G., Olmos, M., Wonaga, A., & Catalano, M. (2008). *Helicobacter pylori* amoxicillin heteroresistance due to point mutations in PBP-1A in isogenic isolates. *Journal of Antimicrobial Chemotherapy*, *61*(3), 474-477. https://doi.org/10.1093/jac/dkm504

Maurer, P., Koch, B., Zerfaß, I., Krauß, J., van der Linden, M., Frère, J.-M., Contreras-Martel, C., & Hakenbeck, R. (2008). Penicillin-binding Protein 2x of *Streptococcus pneumoniae*: Three New Mutational Pathways for Remodelling an Essential Enzyme into a Resistance Determinant. *Journal of Molecular Biology*, *376*(5), 1403-1416. https://doi.org/https://doi.org/10.1016/j.jmb.2007.12.058

McDermott, A. M., Redfern, R. L., Zhang, B., Pei, Y., Huang, L., & Proske, R. J. (2003). Defensin Expression by the Cornea: Multiple Signalling Pathways Mediate IL-1β Stimulation of hBD-2 Expression by Human Corneal Epithelial Cells. *Investigative Ophthalmology & Visual Science*, *44*(5), 1859-1865. https://doi.org/10.1167/iovs.02-0787

McMahon, B. J., Hennessy, T. W., Bensler, J. M., Bruden, D. L., Parkinson, A. J., Morris, J. M., Reasonover, A. L., Hurlburt, D. A., Bruce, M. G., Sacco, F., & Butler, J. C. (2003). The relationship among previous antimicrobial use, antimicrobial resistance, and treatment outcomes for *Helicobacter pylori* infections. *Ann Intern Med*, *139*(6), 463-469. https://doi.org/10.7326/0003-4819-139-6-200309160-00008

Test and treat for *Helicobacter pylori* (HP) in Dyspepsia: Quick Reference Guide for Primary Care, (2017). https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/828593/HP_Quick_Reference_Guide_v18.0_August_2019_change_highlighted.pdf

McNulty, C., Owen, R., Tompkins, D., Hawtin, P., McColl, K., Price, A., Smith, G., & Teare, L. (2002). *Helicobacter pylori* susceptibility testing by disc diffusion. *J Antimicrob Chemother*, *49*(4), 601-609. https://doi.org/10.1093/jac/49.4.601

McNulty, C. A., Lasseter, G., Shaw, I., Nichols, T., D'Arcy, S., Lawson, A. J., & Glocker, E. (2012). Is *Helicobacter pylori* antibiotic resistance surveillance needed and how can it be delivered? *Aliment Pharmacol Ther*, *35*(10), 1221-1230. https://doi.org/10.1111/j.1365-2036.2012.05083.x

Medina-Franco, H., Germes, S. S., & Maldonado, C. L. (2007). Prognostic Factors in Primary Gastric Lymphoma. *Annals of Surgical Oncology*, *14*(8), 2239-2245. https://doi.org/10.1245/s10434-006-9244-0

Megraud, F., Bruyndonckx, R., Coenen, S., Wittkop, L., Huang, T. D., Hoebeke, M., Bénéjat, L., Lehours, P., Goossens, H., & Glupczynski, Y. (2021). *Helicobacter pylori* resistance to antibiotics in Europe in 2018 and its relationship to antibiotic consumption in the community. *Gut*, *70*(10), 1815-1822. https://doi.org/10.1136/gutjnl-2021-324032

Megraud, F., Coenen, S., Versporten, A., Kist, M., Lopez-Brea, M., Hirschl, A. M., Andersen, L. P., Goossens, H., & Glupczynski, Y. (2013). *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut*, *62*(1), 34-42. https://doi.org/10.1136/gutjnl-2012-302254

Mégraud, F., Graham, D. Y., Howden, C. W., Trevino, E., Weissfeld, A., Hunt, B., Smith, N., Leifke, E., & Chey, W. D. (2023). Rates of Antimicrobial Resistance in *Helicobacter pylori* Isolates From Clinical Trial Patients Across the US and Europe. *Am J Gastroenterol*, *118*(2), 269-275. https://doi.org/10.14309/ajg.0000000000002045

Mégraud, F., & Lehours, P. (2007). *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin Microbiol Rev*, *20*(2), 280-322. https://doi.org/10.1128/cmr.00033-06

Memon, A. A., Hussein, N. R., Miendje Deyi, V. Y., Burette, A., & Atherton, J. C. (2014). Vacuolating cytotoxin genotypes are strong markers of gastric cancer and duodenal ulcer-associated *Helicobacter pylori* strains: a matched case-control study. *J Clin Microbiol*, *52*(8), 2984-2989. https://doi.org/10.1128/jcm.00551-14

Meyer, J. M., Silliman, N. P., Wang, W., Siepman, N. Y., Sugg, J. E., Morris, D., Zhang, J., Bhattacharyya, H., King, E. C., & Hopkins, R. J. (2002). Risk factors for *Helicobacter pylori*

resistance in the United States: the surveillance of *H. pylori* antimicrobial resistance partnership (SHARP) study, 1993-1999. *Ann Intern Med*, *136*(1), 13-24. https://doi.org/10.7326/0003-4819-136-1-200201010-00008
MHRA. (2012). Drug Safety Update *6*(2). Retrieved 6/4/2024, from http://www.sefap.it/web/upload/con185631.pdf
Miani, M., Le Naour, J., Waeckel-Enée, E., Verma, S. c., Straube, M., Emond, P., Ryffel, B., van Endert, P., Sokol, H., & Diana, J. (2018). Gut Microbiota-Stimulated Innate Lymphoid Cells Support β-Defensin 14 Expression in Pancreatic Endocrine Cells, Preventing Autoimmune Diabetes. *Cell Metabolism*, *28*(4), 557-572.e556. https://doi.org/10.1016/j.cmet.2018.06.012
Michalkiewicz, J., Helmin-Basa, A., Grzywa, R., Czerwionka-Szaflarska, M., Szaflarska-Poplawska, A., Mierzwa, G., Marszalek, A., Bodnar, M., Nowak, M., & Dzierzanowska-Fangrat, K. (2015). Innate Immunity Components and Cytokines in Gastric Mucosa in Children with *Helicobacter pylori* Infection. *Mediators of Inflammation*, *2015*(1), 176726. https://doi.org/https://doi.org/10.1155/2015/176726
Miehlke, S., Hackelsberger, A., Meining, A., Hatz, R., Lehn, N., Malfertheiner, P., Stolte, M., & Bayerdörffer, E. (1998). Severe expression of corpus gastritis is characteristic in gastric cancer patients infected with *Helicobacter pylori*. *Br J Cancer*, *78*(2), 263-266. https://doi.org/10.1038/bjc.1998.475
Miendje Deyi, V. Y., Bontems, P., Vanderpas, J., De Koster, E., Ntounda, R., Van den Borre, C., Cadranel, S., & Burette, A. (2011). Multicenter survey of routine determinations of resistance of *Helicobacter pylori* to antimicrobials over the last 20 years (1990 to 2009) in Belgium. *J Clin Microbiol*, *49*(6), 2200-2209. https://doi.org/10.1128/jcm.02642-10
Miftahussurur, M., Fauzia, K. A., Nusi, I. A., Setiawan, P. B., Syam, A. F., Waskito, L. A., Doohan, D., Ratnasari, N., Khomsan, A., Adnyana, I. K., Akada, J., & Yamaoka, Y. (2020). E-test versus agar dilution for antibiotic susceptibility testing of *Helicobacter pylori*: a comparison study. *BMC Research Notes*, *13*(1), 22. https://doi.org/10.1186/s13104-019-4877-9
Miftahussurur, M., & Yamaoka, Y. (2015). *Helicobacter pylori* virulence genes and host genetic polymorphisms as risk factors for peptic ulcer disease. *Expert Review of Gastroenterology & Hepatology*, *9*(12), 1535-1547. https://doi.org/10.1586/17474124.2015.1095089
Min, B.-J., Seo, M.-E., Bae, J. H., Kim, J. W., & Kim, J. H. (2024). Development and validation of next-generation sequencing panel for personalized *Helicobacter pylori* eradication treatment targeting multiple species [Original Research]. *Frontiers in Cellular and Infection Microbiology*, *14*. https://doi.org/10.3389/fcimb.2024.1379790
Mishra, K. K., Srivastava, S., Garg, A., & Ayyagari, A. (2006). Antibiotic Susceptibility of *Helicobacter pylori* Clinical Isolates: Comparative Evaluation of Disk-Diffusion and E-Test Methods. *Current Microbiology*, *53*(4), 329-334. https://doi.org/10.1007/s00284-006-0143-1
Mitchell, H., & Katelaris, P. (2016). Epidemiology, clinical impacts and current clinical management of *Helicobacter pylori* infection. *Med J Aust*, *204*(10), 376-380. https://doi.org/10.5694/mja16.00104
Miura, M., Ohnishi, N., Tanaka, S., Yanagiya, K., & Hatakeyama, M. (2009). Differential oncogenic potential of geographically distinct *Helicobacter pylori* CagA isoforms in mice. *International Journal of Cancer*, *125*(11), 2497-2504. https://doi.org/https://doi.org/10.1002/ijc.24740
Miyanohara, T., Ushikai, M., Matsune, S., Ueno, K., Katahira, S., & Kurono, Y. (2000). Effects of Clarithromycin on Cultured Human Nasal Epithelial Cells and Fibroblasts. *The Laryngoscope*, *110*(1), 126-131. https://doi.org/https://doi.org/10.1097/00005537-200001000-00023
Mladenova, I. (2021). Clinical Relevance of *Helicobacter pylori* Infection. *J Clin Med*, *10*(16). https://doi.org/10.3390/jcm10163473
Mommersteeg, M. C., Nieuwenburg, S. A. V., Wolters, L. M. M., Roovers, B., van Vuuren, H. A. J., Verhaar, A. P., Bruno, M. J., Kuipers, E. J., Peppelenbosch, M. P., Spaander, M. C. W., & Fuhler, G. M. (2023). The use of non-invasive stool tests for verification of *Helicobacter pylori*

eradication and clarithromycin resistance. *United European Gastroenterol J*, *11*(9), 894-903. https://doi.org/10.1002/ueg2.12473

Moradipour, A., Khosravi, A., & Piri, F. (2018). Fecal *Helicobacter pylori* glmM and *16S rRNA* genes correlate with serum TNF-α and IL-1β cytokine fluctuations. *Acta Microbiol Immunol Hung*, *65*(4), 489-499. https://doi.org/10.1556/030.65.2018.030

Morilla, A. M., Álvarez-Argüelles, M. E., Duque, J. M., Armesto, E., Villar, H., & Melón, S. (2019). Primary antimicrobial resistance rates and prevalence of *Helicobacter pylori* infection in the north of Spain. A 13-year retrospective study. *Gastroenterol Hepatol*, *42*(8), 476-485. https://doi.org/10.1016/j.gastrohep.2019.05.002

Morningstar-Wright, L., Czinn, S. J., Piazuelo, M. B., Banerjee, A., Godlewska, R., & Blanchard, T. G. (2022). The TNF-Alpha Inducing Protein is Associated With Gastric Inflammation and Hyperplasia in a Murine Model of *Helicobacter pylori* Infection [Original Research]. *Frontiers in Pharmacology*, *13*. https://doi.org/10.3389/fphar.2022.817237

Moshynets, O. V., Baranovskyi, T. P., Cameron, S., Iungin, O. S., Pokholenko, I., Jerdan, R., Kamyshnyi, A., Krikunov, A. A., Potochilova, V. V., Rudnieva, K. L., & Spiers, A. J. (2022). Azithromycin possesses biofilm–inhibitory activity and potentiates non-bactericidal colistin methanesulfonate (CMS) and polymyxin B against *Klebsiella pneumonia*. *PLoS One*, *17*(7), e0270983. https://doi.org/10.1371/journal.pone.0270983

Muhammad, J. S., Zaidi, S. F., Zhou, Y., Sakurai, H., & Sugiyama, T. (2016a). Novel epidermal growth factor receptor pathway mediates release of human β-defensin 3 from *Helicobacter pylori*-infected gastric epithelial cells. *Pathogens and Disease*, *74*(3). https://doi.org/10.1093/femspd/ftv128

Muhammad, J. S., Zaidi, S. F., Zhou, Y., Sakurai, H., & Sugiyama, T. (2016b). Novel epidermal growth factor receptor pathway mediates release of human β-defensin 3 from *Helicobacter pylori*-infected gastric epithelial cells. *Pathog Dis*, *74*(3). https://doi.org/10.1093/femspd/ftv128

Mukai, R., Handa, O., Suyama, Y., Majima, A., & Naito, Y. (2020). Effectiveness of including probiotics to *Helicobacter pylori* eradication therapies. *J Clin Biochem Nutr*, *67*(1), 102-104. https://doi.org/10.3164/jcbn.20-37

Mulcahy, M. E., Leech, J. M., Renauld, J. C., Mills, K. H., & McLoughlin, R. M. (2016). Interleukin-22 regulates antimicrobial peptide expression and keratinocyte differentiation to control *Staphylococcus aureus* colonization of the nasal mucosa. *Mucosal Immunology*, *9*(6), 1429-1441. https://doi.org/10.1038/mi.2016.24

Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. In *Virulence Mechanisms of Bacterial Pathogens* (pp. 481-511). https://doi.org/https://doi.org/10.1128/9781555819286.ch17

Mustapha, P., Paris, I., Garcia, M., Tran, C. T., Cremniter, J., Garnier, M., Faure, J.-P., Barthes, T., Boneca, I. G., Morel, F., Lecron, J.-C., Burucoa, C., & Bodet, C. (2014). Chemokines and Antimicrobial Peptides Have a cag-Dependent Early Response to *Helicobacter pylori* Infection in Primary Human Gastric Epithelial Cells. *Infection and Immunity*, *82*(7), 2881-2889. https://doi.org/doi:10.1128/iai.01517-13

Nabavi-Rad, A., Sadeghi, A., Asadzadeh Aghdaei, H., Yadegar, A., Smith, S. M., & Zali, M. R. (2022). The double-edged sword of probiotic supplementation on gut microbiota structure in *Helicobacter pylori* management. *Gut Microbes*, *14*(1), 2108655. https://doi.org/10.1080/19490976.2022.2108655

Nagashima, H., Iwatani, S., Cruz, M., Jiménez Abreu, J. A., Uchida, T., Mahachai, V., Vilaichone, R.-k., Graham, D. Y., & Yamaoka, Y. (2015). Toll-like Receptor 10 in *Helicobacter pylori* Infection. *The Journal of Infectious Diseases*, *212*(10), 1666-1676. https://doi.org/10.1093/infdis/jiv270

Nagy, E., Urbán, E., & Nord, C. E. (2011). Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe: 20 years of experience. *Clin Microbiol Infect*, *17*(3), 371-379. https://doi.org/10.1111/j.1469-0691.2010.03256.x

Nakamura, K., Sakuragi, N., Takakuwa, A., & Ayabe, T. (2016). Paneth cell α-defensins and enteric microbiota in health and disease. *Biosci Microbiota Food Health*, *35*(2), 57-67. https://doi.org/10.12938/bmfh.2015-019

Nakamura, S., & Hojo, M. (2023). Diagnosis and Treatment for Gastric Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma. *Journal of Clinical Medicine*, *12*(1), 120. https://www.mdpi.com/2077-0383/12/1/120

Narayanan, S., Miller, W. L., & McDermott, A. M. (2003). Expression of Human β-Defensins in Conjunctival Epithelium: Relevance to Dry Eye Disease. *Investigative Ophthalmology & Visual Science*, *44*(9), 3795-3801. https://doi.org/10.1167/iovs.02-1301

National Collaborating Centre for, C. (2015). National Institute for Health and Care Excellence: Clinical Guidelines. In *Suspected Cancer: Recognition and Referral*. National Institute for Health and Care Excellence (NICE)

Nau, R., & Tauber, C. S. (2008). Immunomodulatory Properties of Antibiotics. *Current Molecular Pharmacology*, *1*(1), 68-79. https://doi.org/http://dx.doi.org/10.2174/1874467210801010068

Naumann, M., Sokolova, O., Tegtmeyer, N., & Backert, S. (2017). *Helicobacter pylori*: A Paradigm Pathogen for Subverting Host Cell Signal Transmission. *Trends Microbiol*, *25*(4), 316-328. https://doi.org/10.1016/j.tim.2016.12.004

Ndip, R. N., Malange Takang, A. E., Ojongokpoko, J. E., Luma, H. N., Malongue, A., Akoachere, J. F., Ndip, L. M., MacMillan, M., & Weaver, L. T. (2008). *Helicobacter pylori* isolates recovered from gastric biopsies of patients with gastro-duodenal pathologies in Cameroon: current status of antibiogram. *Trop Med Int Health*, *13*(6), 848-854. https://doi.org/10.1111/j.1365-3156.2008.02062.x

Nehra, A. K., Alexander, J. A., Loftus, C. G., & Nehra, V. (2018). Proton Pump Inhibitors: Review of Emerging Concerns. *Mayo Clinic Proceedings*, *93*(2), 240-246. https://doi.org/https://doi.org/10.1016/j.mayocp.2017.10.022

Nemati, M., Larussa, T., Khorramdelazad, H., Mahmoodi, M., & Jafarzadeh, A. (2017). Toll-like receptor 2: An important immunomodulatory molecule during *Helicobacter pylori* infection. *Life Sciences*, *178*, 17-29. https://doi.org/https://doi.org/10.1016/j.lfs.2017.04.006

Nemidkanam, V., Banlunara, W., & Chaichanawongsaroj, N. (2024). *Kaempferia parviflora* Extracellular Vesicle Loaded with Clarithromycin for the Treatment of *Helicobacter pylori* Infection. *International Journal of Nanomedicine*, *19*(null), 1967-1983. https://doi.org/10.2147/IJN.S444686

Nemr, N., Kishk, R. M., Salem, A., Abdalla, M. O., Abd El-Fadeal, N. M., & Soliman, N. M. (2022). Genotypic detection of metronidazole and clarithromycin resistance in dyspeptic patients with *Helicobacter pylori. Environmental Science and Pollution Research*, *29*(27), 41467-41474. https://doi.org/10.1007/s11356-021-18198-4

Nepal, G., & Bhatta, S. (2018). Self-medication with Antibiotics in WHO Southeast Asian Region: A Systematic Review. *Cureus*, *10*(4), e2428. https://doi.org/10.7759/cureus.2428

Neshani, A., Zare, H., Akbari Eidgahi, M. R., Hooshyar Chichaklu, A., Movaqar, A., & Ghazvini, K. (2019). Review of antimicrobial peptides with anti-*Helicobacter pylori* activity. *Helicobacter*, *24*(1), e12555. https://doi.org/https://doi.org/10.1111/hel.12555

Ng, H. K., Chua, K. H., Kee, B. P., Chuah, K. H., Por, L. Y., & Puah, S. M. (2024). Genetic variations of penicillin-binding protein 1A: insights into the current status of amoxicillin-based regimens for *Helicobacter pylori* eradication in Malaysia. *J Med Microbiol*, *73*(5). https://doi.org/10.1099/jmm.0.001832

Ng, H. K., Goh, K. L., Chuah, K. H., Thalha, A. M., Kee, B. P., Por, L. Y., Chua, K. H., & Puah, S. M. (2020). Sequencing-based detection of *23S rRNA* domain V mutations in treatment-naïve *Helicobacter pylori* patients from Malaysia. *J Glob Antimicrob Resist*, *23*, 345-348. https://doi.org/10.1016/j.jgar.2020.10.012

Nguyen, J., Kotilea, K., Bontems, P., & Miendje Deyi, V. Y. (2023). *Helicobacter pylori* Infections in Children. *Antibiotics*, *12*.

Nguyen, S. T., Nguyen, H. T.-L., & Truong, K. D. (2020). Comparative cytotoxic effects of methanol, ethanol and DMSO on human cancer cell lines. *Biomedical Research and Therapy*, *7*, 3855-3859.

Nguyen, T. H., Ho, T. T. M., Nguyen-Hoang, T.-P., Qumar, S., Pham, T. T. D., Bui, Q. N., Bulach, D., Nguyen, T.-V., & Rahman, M. (2021). The endemic *Helicobacter pylori* population in Southern Vietnam has both South East Asian and European origins. *Gut Pathogens*, *13*(1), 57. https://doi.org/10.1186/s13099-021-00452-2

NICE. (2014). *Dyspepsia and gastrooesophageal reflux disease: investigation and management of dyspepsia, symptoms suggestive of gastro-oesophageal reflux disease, or both*. Retrieved 2/4/2024 from https://www.nice.org.uk/guidance/cg184/evidence/full-guideline-pdf-193203757

NICE. (2024). *Helicobacter pylori infection*. Retrieved 2/4/2024 from https://bnf.nice.org.uk/treatment-summaries/helicobacter-pylori-infection/

Nishi, Y., Isomoto, H., Mukae, H., Ishimoto, H., Wen, C. Y., Wada, A., Ohnita, K., Mizuta, Y., Murata, I., Hirayama, T., Nakazato, M., & Kohno, S. (2005). Concentrations of alpha- and beta-defensins in gastric juice of patients with various gastroduodenal diseases. *World J Gastroenterol*, *11*(1), 99-103. https://doi.org/10.3748/wjg.v11.i1.99

Nishikawa, H., & Hatakeyama, M. (2017). Sequence Polymorphism and Intrinsic Structural Disorder as Related to Pathobiological Performance of the *Helicobacter pylori* CagA Oncoprotein. *Toxins*, *9*. https://doi.org/10.3390/toxins9040136

Nomura, A. M. Y., Wilkens, L. R., Henderson, B. E., Epplein, M., & Kolonel, L. N. (2012). The association of cigarette smoking with gastric cancer: the multiethnic cohort study. *Cancer Causes & Control*, *23*(1), 51-58. https://doi.org/10.1007/s10552-011-9854-0

Nomura, Y., Tanabe, H., Moriichi, K., Igawa, S., Ando, K., Ueno, N., Kashima, S., Tominaga, M., Goto, T., Inaba, Y., Ito, T., Ishida-Yamamoto, A., Fujiya, M., & Kohgo, Y. (2013). Reduction of E-cadherin by human defensin-5 in esophageal squamous cells [Article]. *Biochemical and Biophysical Research Communications*, *439*(1), 71-77. https://doi.org/10.1016/j.bbrc.2013.08.026

Nuding, S., Gersemann, M., Hosaka, Y., Konietzny, S., Schaefer, C., Beisner, J., Schroeder, B. O., Ostaff, M. J., Saigenji, K., Ott, G., Schaller, M., Stange, E. F., & Wehkamp, J. (2013). Gastric Antimicrobial Peptides Fail to Eradicate *Helicobacter pylori* Infection Due to Selective Induction and Resistance. *PLoS One*, *8*(9), e73867. https://doi.org/10.1371/journal.pone.0073867

Nyssen, O. P., Bordin, D., Tepes, B., Pérez-Aisa, Á., Vaira, D., Caldas, M., Bujanda, L., Castro-Fernandez, M., Lerang, F., Leja, M., Rodrigo, L., Rokkas, T., Kupcinskas, L., Pérez-Lasala, J., Jonaitis, L., Shvets, O., Gasbarrini, A., Simsek, H., Axon, A. T. R., . . . Gisbert, J. P. (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*(1), 40-54. https://doi.org/10.1136/gutjnl-2020-321372

O'Connor, A., Taneike, I., Nami, A., Fitzgerald, N., Murphy, P., Ryan, B., O'Connor, H., Qasim, A., Breslin, N., & O'Moráin, C. (2010). *Helicobacter pylori* resistance to metronidazole and clarithromycin in Ireland. *Eur J Gastroenterol Hepatol*, *22*(9), 1123-1127. https://doi.org/10.1097/MEG.0b013e328338e43d

O'Hara, A. M., Bhattacharyya, A., Bai, J., Mifflin, R. C., Ernst, P. B., Mitra, S., & Crowe, S. E. (2009). Tumor necrosis factor (TNF)-alpha-induced IL-8 expression in gastric epithelial cells: role of reactive oxygen species and AP endonuclease-1/redox factor (Ref)-1. *Cytokine*, *46*(3), 359-369. https://doi.org/10.1016/j.cyto.2009.03.010

O'Neil, D. A., Cole, S. P., Martin-Porter, E., Housley, M. P., Liu, L., Ganz, T., & Kagnoff, M. F. (2000). Regulation of human beta-defensins by gastric epithelial cells in response to infection with

Helicobacter pylori or stimulation with interleukin-1. *Infect Immun*, *68*(9), 5412-5415. https://doi.org/10.1128/iai.68.9.5412-5415.2000

O'Rourke, J. L. (2008). Gene expression profiling in *Helicobacter*-induced MALT lymphoma with reference to antigen drive and protective immunization. *Journal of Gastroenterology and Hepatology*, *23*(s2), S151-S156. https://doi.org/https://doi.org/10.1111/j.1440-1746.2008.05553.x

O'Neil, D. A., Porter, E. M., Elewaut, D., Anderson, G. M., Eckmann, L., Ganz, T., & Kagnoff, M. F. (1999). Expression and Regulation of the Human β-Defensins hBD-1 and hBD-2 in Intestinal Epithelium1. *The Journal of Immunology*, *163*(12), 6718-6724. https://doi.org/10.4049/jimmunol.163.12.6718

O'Toole, P. W., Lane, M. C., & Porwollik, S. (2000). *Helicobacter pylori* motility. *Microbes and Infection*, *2*(10), 1207-1214. https://doi.org/https://doi.org/10.1016/S1286-4579(00)01274-0

Obonyo, M., Sabet, M., Cole, S. P., Ebmeyer, J., Uematsu, S., Akira, S., & Guiney, D. G. (2007). Deficiencies of Myeloid Differentiation Factor 88, Toll-Like Receptor 2 (TLR2), or TLR4 Produce Specific Defects in Macrophage Cytokine Secretion Induced by *Helicobacter pylori*. *Infection and Immunity*, *75*(5), 2408-2414. https://doi.org/doi:10.1128/iai.01794-06

Ogata, S. K., Gales, A. C., & Kawakami, E. (2014). Antimicrobial susceptibility testing for *Helicobacter pylori* isolates from Brazilian children and adolescents: comparing agar dilution, E-test, and disk diffusion. *Braz J Microbiol*, *45*(4), 1439-1448. https://doi.org/10.1590/s1517-83822014000400039

Ogiwara, H., Sugimoto, M., Ohno, T., Vilaichone, R. K., Mahachai, V., Graham, D. Y., & Yamaoka, Y. (2009). Role of deletion located between the intermediate and middle regions of the *Helicobacter pylori vacA* gene in cases of gastroduodenal diseases. *J Clin Microbiol*, *47*(11), 3493-3500. https://doi.org/10.1128/jcm.00887-09

Ohara, T., Morishita, T., Suzuki, H., Masaoka, T., Nagata, H., & Hibi, T. (2004). Pathophysiological role of human beta-defensins 2 in gastric mucosa. *Int J Mol Med*, *14*(6), 1023-1027.

Ohno, T., Vallström, A., Rugge, M., Ota, H., Graham, D. Y., Arnqvist, A., & Yamaoka, Y. (2011). Effects of Blood Group Antigen–Binding Adhesin Expression during *Helicobacter pylori* Infection of Mongolian Gerbils. *The Journal of Infectious Diseases*, *203*(5), 726-735. https://doi.org/10.1093/infdis/jiq090

Okamoto, T., Yoshiyama, H., Nakazawa, T., Park, I. D., Chang, M. W., Yanai, H., Okita, K., & Shirai, M. (2002). A change in PBP1 is involved in amoxicillin resistance of clinical isolates of *Helicobacter pylori*. *J Antimicrob Chemother*, *50*(6), 849-856. https://doi.org/10.1093/jac/dkf140

Oliveira, J. G., Duarte, M. C., & Silva, A. E. (2012). IL-1ra anti-inflammatory cytokine polymorphism is associated with risk of gastric cancer and chronic gastritis in a Brazilian population, but the TNF-β pro-inflammatory cytokine is not. *Molecular Biology Reports*, *39*(7), 7617-7625. https://doi.org/10.1007/s11033-012-1596-x

Ong, L.-L., & Lin, C.-H. (2024). Adhesion, infection, and therapeutic treatment of *Helicobacter pylori*: a review on current aspects and future promise. *Discover Applied Sciences*, *6*(6), 323. https://doi.org/10.1007/s42452-024-05923-0

Oppong, P., Majumdar, D., Atherton, J., & Bebb, J. (2015). *Helicobacter pylori* infection and peptic ulcers. *Medicine*, *43*(4), 215-222. https://doi.org/https://doi.org/10.1016/j.mpmed.2015.01.008

Ortiz, V., Estevez-Ordonez, D., Montalvan-Sanchez, E., Urrutia-Argueta, S., Israel, D., Krishna, U. S., Romero-Gallo, J., Wilson, K. T., Peek, R. M., Dominguez, R., & Morgan, D. R. (2019). *Helicobacter pylori* antimicrobial resistance and antibiotic consumption in the low-resource Central America setting. *Helicobacter*, *24*(4), e12595. https://doi.org/10.1111/hel.12595

Osato, M. S., Reddy, R., Reddy, S. G., Penland, R. L., & Graham, D. Y. (2001a). Comparison of the Etest and the NCCLS-approved agar dilution method to detect metronidazole and clarithromycin resistant *Helicobacter pylori*. *Int J Antimicrob Agents*, *17*(1), 39-44. https://doi.org/10.1016/s0924-8579(00)00320-4

Osato, M. S., Reddy, R., Reddy, S. G., Penland, R. L., Malaty, H. M., & Graham, D. Y. (2001b). Pattern of primary resistance of *Helicobacter pylori* to metronidazole or clarithromycin in the United States. *Arch Intern Med*, *161*(9), 1217-1220. https://doi.org/10.1001/archinte.161.9.1217

Osawa, Y., Nagaki, M., Banno, Y., Brenner, D. A., Asano, T., Nozawa, Y., Moriwaki, H., & Nakashima, S. (2002). Tumor necrosis factor alpha-induced interleukin-8 production via NF-kappaB and phosphatidylinositol 3-kinase/Akt pathways inhibits cell apoptosis in human hepatocytes. *Infect Immun*, *70*(11), 6294-6301. https://doi.org/10.1128/iai.70.11.6294-6301.2002

Otte, J.-M., Neumann, H. M., Brand, S., Schrader, H., Schmidt, W. E., & Schmitz, F. (2009). Expression of beta-defensin 4 is increased in human gastritis. *European Journal of Clinical Investigation*, *39*(2), 126-138. https://doi.org/https://doi.org/10.1111/j.1365-2362.2008.02071.x

Otth, L., Wilson, M., Fernández, H., Otth, C., Toledo, C., Cárcamo, V., Rivera, P., & Ruiz, L. (2011). Isolation of *Helicobacter pylori* in gastric mucosa and susceptibility to five antimicrobial drugs in Southern chile. *Braz J Microbiol*, *42*(2), 442-447. https://doi.org/10.1590/s1517-83822011000200005

Oudouhou, F., Morin, C., Bouin, M., Gaudreau, C., & Baron, C. (2024). Inhibition of the type IV secretion system from antibiotic-resistant *Helicobacter pylori* clinical isolates supports the potential of Cagα as an anti-virulence target. *Canadian Journal of Microbiology*, *70*(4), 119-127. https://doi.org/10.1139/cjm-2023-0168 %M 38176008

Ouellette, A. J. (2011). Paneth cell α-defensins in enteric innate immunity. *Cellular and Molecular Life Sciences*, *68*(13), 2215-2229. https://doi.org/10.1007/s00018-011-0714-6

Pachathundikandi, S. K., Lind, J., Tegtmeyer, N., El-Omar, E. M., & Backert, S. (2015). Interplay of the Gastric Pathogen *Helicobacter pylori* with Toll-Like Receptors. *BioMed Research International*, *2015*(1), 192420. https://doi.org/https://doi.org/10.1155/2015/192420

Pachathundikandi, S. K., Müller, A., & Backert, S. (2016). Inflammasome Activation by *Helicobacter pylori* and Its Implications for Persistence and Immunity. In S. Backert (Ed.), *Inflammasome Signaling and Bacterial Infections* (pp. 117-131). Springer International Publishing. https://doi.org/10.1007/978-3-319-41171-2_6

Pachathundikandi, S. K., Tegtmeyer, N., Arnold, I. C., Lind, J., Neddermann, M., Falkeis-Veits, C., Chattopadhyay, S., Brönstrup, M., Tegge, W., Hong, M., Sticht, H., Vieth, M., Müller, A., & Backert, S. (2019). T4SS-dependent TLR5 activation by *Helicobacter pylori* infection. *Nature Communications*, *10*(1), 5717. https://doi.org/10.1038/s41467-019-13506-6

Pachathundikandi, S. K., Tegtmeyer, N., & Backert, S. (2023). Masking of typical TLR4 and TLR5 ligands modulates inflammation and resolution by *Helicobacter pylori*. *Trends in Microbiology*, *31*(9), 903-915. https://doi.org/https://doi.org/10.1016/j.tim.2023.03.009

Palframan, S. L., Kwok, T., & Gabriel, K. (2012). Vacuolating cytotoxin A (VacA), a key toxin for *Helicobacter pylori* pathogenesis [Review]. *Frontiers in Cellular and Infection Microbiology*, *2*. https://doi.org/10.3389/fcimb.2012.00092

Park, C. G., Kim, S., Lee, E. J., Jeon, H. S., & Han, S. (2018a). Clinical relevance of point mutations in the *23S rRNA* gene in *Helicobacter pylori* eradication: A prospective, observational study. *Medicine (Baltimore)*, *97*(33), e11835. https://doi.org/10.1097/md.0000000000011835

Park, J. B., & Koo, J. S. (2014). *Helicobacter pylori* infection in gastric mucosa-associated lymphoid tissue lymphoma. *World J Gastroenterol*, *20*(11), 2751-2759. https://doi.org/10.3748/wjg.v20.i11.2751

Park, J. S., Jun, J. S., Seo, J. H., Youn, H. S., & Rhee, K. H. (2021). Changing prevalence of *Helicobacter pylori* infection in children and adolescents. *Clin Exp Pediatr*, *64*(1), 21-25. https://doi.org/10.3345/cep.2019.01543

Park, J. Y., Forman, D., Waskito, L. A., Yamaoka, Y., & Crabtree, J. E. (2018b). Epidemiology of *Helicobacter pylori* and CagA-Positive Infections and Global Variations in Gastric Cancer. *Toxins (Basel)*, *10*(4). https://doi.org/10.3390/toxins10040163

Park, J. Y., Kim, H. Y., Lee, J. Y., Kim, K. H., Jang, M. K., Lee, J. H., Yoo, J. Y., Han, D. S., & Hahm, J. S. (2005). Macrolide-affected Toll-like receptor 4 expression from *Helicobacter pylori*-infected monocytes does not modify interleukin-8 production. *FEMS Immunology & Medical Microbiology*, *44*(2), 171-176. https://doi.org/10.1016/j.femsim.2005.01.007

Parnham, M. J., Norris, V., Kricker, J. A., Gudjonsson, T., & Page, C. P. (2023). Chapter Four - Prospects for macrolide therapy of asthma and COPD. In W. S. F. Wong (Ed.), *Advances in Pharmacology* (Vol. 98, pp. 83-110). Academic Press. https://doi.org/https://doi.org/10.1016/bs.apha.2023.03.002

Parsons, H. K., Carter, M. J., Sanders, D. S., Winstanley, T., & Lobo, A. J. (2001). *Helicobacter pylori* antimicrobial resistance in the United Kingdom: the effect of age, sex and socio-economic status. *Aliment Pharmacol Ther*, *15*(9), 1473-1478. https://doi.org/10.1046/j.1365-2036.2001.01068.x

Pasupuleti, M., Schmidtchen, A., & Malmsten, M. (2012). Antimicrobial peptides: key components of the innate immune system. *Critical Reviews in Biotechnology*, *32*(2), 143-171. https://doi.org/10.3109/07388551.2011.594423

Patel, S. R., Smith, K., Letley, D. P., Cook, K. W., Memon, A. A., Ingram, R. J., Staples, E., Backert, S., Zaitoun, A. M., Atherton, J. C., & Robinson, K. (2013). *Helicobacter pylori* downregulates expression of human β-defensin 1 in the gastric mucosa in a type IV secretion-dependent fashion. *Cell Microbiol*, *15*(12), 2080-2092. https://doi.org/10.1111/cmi.12174

Peng, Y.-C., Ho, S.-P., Shyu, C.-L., Chang, C.-S., & Huang, L.-R. (2014). Clarithromycin modulates *Helicobacter pylori*-induced activation of nuclear factor-κB through classical and alternative pathways in gastric epithelial cells. *Clinical and Experimental Medicine*, *14*(1), 53-59. https://doi.org/10.1007/s10238-012-0217-2

Pero, R., Angrisano, T., Brancaccio, M., Falanga, A., Lombardi, L., Natale, F., Laneri, S., Lombardo, B., Galdiero, S., & Scudiero, O. (2019a). Beta-defensins and analogs in *Helicobacter pylori* infections: mRNA expression levels, DNA methylation, and antibacterial activity. *PLoS One*, *14*(9), e0222295. https://doi.org/10.1371/journal.pone.0222295

Pero, R., Brancaccio, M., Laneri, S., De Biasi, M.-G., Lombardo, B., & Scudiero, O. (2019b). A Novel View of Human *Helicobacter pylori* Infections: Interplay between Microbiota and Beta-Defensins. *Biomolecules*, *9*(6), 237. https://www.mdpi.com/2218-273X/9/6/237

Pero, R., Coretti, L., Nigro, E., Lembo, F., Laneri, S., Lombardo, B., Daniele, A., & Scudiero, O. (2017). β-Defensins in the Fight against *Helicobacter pylori*. *Molecules*, *22*(3). https://doi.org/10.3390/molecules22030424

Perri, F., Villani, M. R., Festa, V., Quitadamo, M., & Andriulli, A. (2001). Predictors of failure of *Helicobacter pylori* eradication with the standard 'Maastricht triple therapy'. *Aliment Pharmacol Ther*, *15*(7), 1023-1029. https://doi.org/10.1046/j.1365-2036.2001.01006.x

Peschel, A. (2002). How do bacteria resist human antimicrobial peptides? *Trends Microbiol*, *10*(4), 179-186. https://doi.org/10.1016/s0966-842x(02)02333-8

Pesesky, M. W., Hussain, T., Wallace, M., Patel, S., Andleeb, S., Burnham, C. D., & Dantas, G. (2016). Evaluation of Machine Learning and Rules-Based Approaches for Predicting Antimicrobial Resistance Profiles in Gram-negative Bacilli from Whole Genome Sequence Data. *Front Microbiol*, *7*, 1887. https://doi.org/10.3389/fmicb.2016.01887

Pettersen, E. F., Goddard, T. D., Huang, C. C., Meng, E. C., Couch, G. S., Croll, T. I., Morris, J. H., & Ferrin, T. E. (2021). UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci*, *30*(1), 70-82. https://doi.org/10.1002/pro.3943

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*, *29*(9), e45. https://doi.org/10.1093/nar/29.9.e45

Phan, T. K., Lay, F. T., & Hulett, M. D. (2018). Importance of phosphoinositide binding by human β-defensin 3 for Akt-dependent cytokine induction. *Immunology & Cell Biology*, *96*(1), 54-67. https://doi.org/https://doi.org/10.1111/imcb.1017

Phuc, B. H., Tuan, V. P., Dung, H. D. Q., Binh, T. T. T., Tung, P. H., Tri, T. D., Thuan, N. P. M., Van Khien, V., Trang, T. T. T., Akada, J., Matsumoto, T., & Yamaoka, Y. (2021). *Helicobacter pylori* type 4 secretion systems as gastroduodenal disease markers. *Scientific Reports*, *11*.

Piazuelo, M. B., Bravo, L. E., Mera, R. M., Camargo, M. C., Bravo, J. C., Delgado, A. G., Washington, M. K., Rosero, A., Garcia, L. S., Realpe, J. L., Cifuentes, S. P., Morgan, D. R., Peek, R. M., Jr., Correa, P., & Wilson, K. T. (2021). The Colombian Chemoprevention Trial: 20-Year Follow-Up of a Cohort of Patients With Gastric Precancerous Lesions. *Gastroenterology*, *160*(4), 1106-1117.e1103. https://doi.org/10.1053/j.gastro.2020.11.017

Pichon, M., Pichard, B., Barrioz, T., Plouzeau, C., Croquet, V., Fotsing, G., Chéron, A., Vuillemin, É., Wangermez, M., Haineaux, P. A., Vasseur, P., Thiebault, Q., Lefèvre, C., de Singly, A., Cremniter, J., Broutin, L., Michaud, A., Silvain, C., & Burucoa, C. (2020). Diagnostic Accuracy of a Noninvasive Test for Detection of *Helicobacter pylori* and Resistance to Clarithromycin in Stool by the Amplidiag H. pylori+ClariR Real-Time PCR Assay. *J Clin Microbiol*, *58*(4). https://doi.org/10.1128/jcm.01787-19

Picoli, S. U., Mazzoleni, L. E., Fernández, H., De Bona, L. R., Neuhauss, E., Longo, L., & Prolla, J. C. (2014). Resistance to amoxicillin, clarithromycin and ciprofloxacin of *Helicobacter pylori* isolated from Southern Brazil patients. *Rev Inst Med Trop Sao Paulo*, *56*(3), 197-200. https://doi.org/10.1590/s0036-46652014000300003

Piscione, M., Mazzone, M., Di Marcantonio, M. C., Muraro, R., & Mincione, G. (2021). Eradication of *Helicobacter pylori* and Gastric Cancer: A Controversial Relationship [Review]. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.630852

Pittie, G., Laurent, T., Radermacher, J., Herens, S., Boeras, A., & Ho, G. (2024). Detection by Real-Time PCR of *Helicobacter pylori* and Clarithromycin Resistance Compared to Histology on Gastric Biopsies. *Microorganisms*, *12*(11), 2192. https://www.mdpi.com/2076-2607/12/11/2192

Pollock, J., & Chalmers, J. D. (2021). The immunomodulatory effects of macrolide antibiotics in respiratory disease. *Pulmonary Pharmacology & Therapeutics*, *71*, 102095. https://doi.org/https://doi.org/10.1016/j.pupt.2021.102095

Poonyam, P., Chotivitayatarakorn, P., & Vilaichone, R. K. (2019). High Effective of 14-Day High-Dose PPI- Bismuth-Containing Quadruple Therapy with Probiotics Supplement for *Helicobacter Pylori* Eradication: A Double Blinded-Randomized Placebo-Controlled Study. *Asian Pac J Cancer Prev*, *20*(9), 2859-2864. https://doi.org/10.31557/apjcp.2019.20.9.2859

Popa, S. L., Pop, C., Dita, M. O., Brata, V. D., Bolchis, R., Czako, Z., Saadani, M. M., Ismaiel, A., Dumitrascu, D. I., Grad, S., David, L., Cismaru, G., & Padureanu, A. M. (2022). Deep Learning and Antibiotic Resistance. *Antibiotics (Basel)*, *11*(11). https://doi.org/10.3390/antibiotics11111674

Powell, A. J., Tomberg, J., Deacon, A. M., Nicholas, R. A., & Davies, C. (2009). Crystal Structures of Penicillin-binding Protein 2 from Penicillin-susceptible and -resistant Strains of *Neisseria gonorrhoeae* Reveal an Unexpectedly Subtle Mechanism for Antibiotic Resistance *. *Journal of Biological Chemistry*, *284*(2), 1202-1212. https://doi.org/10.1074/jbc.M805761200

Pradhan, B. L., Lodhi, L., Dey, K. K., & Ghosh, M. (2024). Analyzing atomic scale structural details and nuclear spin dynamics of four macrolide antibiotics: erythromycin, clarithromycin, azithromycin, and roxithromycin [10.1039/D4RA00718B]. *RSC Advances*, *14*(25), 17733-17770. https://doi.org/10.1039/D4RA00718B

Puértolas-Balint, F., & Schroeder, B. O. (2023). Intestinal α-Defensins Play a Minor Role in Modulating the Small Intestinal Microbiota Composition as Compared to Diet. *Microbiology Spectrum*, *11*(3), e00567-00523. https://doi.org/doi:10.1128/spectrum.00567-23

Qiao, Q., Bai, R., Song, W., Gao, H., Zhang, M., Lu, J., Hong, M., Zhang, X., Sun, P., Zhang, Q., & Zhao, P. (2021). Human α-defensin 5 suppressed colon cancer growth by targeting PI3K pathway. *Experimental Cell Research*, *407*(2), 112809. https://doi.org/https://doi.org/10.1016/j.yexcr.2021.112809

Quarta, S., Massaro, M., Carluccio, M. A., Calabriso, N., Bravo, L., Sarria, B., & García-Conesa, M.-T. (2022). An Exploratory Critical Review on TNF-α as a Potential Inflammatory Biomarker Responsive to Dietary Intervention with Bioactive Foods and Derived Products. *Foods*, *11*(16), 2524. https://www.mdpi.com/2304-8158/11/16/2524

Queiroz, D. M., Dani, R., Silva, L. D., Santos, A., Moreira, L. S., Rocha, G. A., Corrêa, P. R., Reis, L. F., Nogueira, A. M., Alvares Cabral, M. M., Esteves, A. M., & Tanure, J. (2002). Factors associated with treatment failure of *Helicobacter pylori* infection in a developing country. *J Clin Gastroenterol*, *35*(4), 315-320. https://doi.org/10.1097/00004836-200210000-00007

Qureshi, N., Li, P., & Gu, Q. (2019). Probiotic therapy in *Helicobacter pylori* infection: a potential strategy against a serious pathogen? *Appl Microbiol Biotechnol*, *103*(4), 1573-1588. https://doi.org/10.1007/s00253-018-09580-3

Qureshi, N. N., Morikis, D., & Schiller, N. L. (2011). Contribution of Specific Amino Acid Changes in Penicillin Binding Protein 1 to Amoxicillin Resistance in Clinical *Helicobacter pylori* isolates. *Antimicrobial Agents and Chemotherapy*, *55*(1), 101-109. https://doi.org/doi:10.1128/aac.00545-10

Radyk, M. D., Burclaff, J., Willet, S. G., & Mills, J. C. (2018). Metaplastic Cells in the Stomach Arise, Independently of Stem Cells, via Dedifferentiation or Transdifferentiation of Chief Cells. *Gastroenterology*, *154*(4), 839-843.e832. https://doi.org/10.1053/j.gastro.2017.11.278

Rai, V., Mathews, G., & Agrawal, D. K. (2022). Translational and Clinical Significance of DAMPs, PAMPs, and PRRs in Trauma-induced Inflammation. *Arch Clin Biomed Res*, *6*(5), 673-685. https://doi.org/10.26502/acbr.50170279

Ramanan, D., Tang, Mei S., Bowcutt, R., Loke, P. n., & Cadwell, K. (2014). Bacterial Sensor Nod2 Prevents Inflammation of the Small Intestine by Restricting the Expansion of the Commensal Bacteroides vulgatus. *Immunity*, *41*(2), 311-324. https://doi.org/10.1016/j.immuni.2014.06.015

Rani, L., Arora, A., Majhi, S., Mishra, A., & Mallajosyula, S. S. (2022). Experimental and simulation studies reveal mechanism of action of human defensin derivatives. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1864*(2), 183824. https://doi.org/https://doi.org/10.1016/j.bbamem.2021.183824

Rashad, A., Nafady, A., Hassan, M. H., Mansour, H., Taya, U., Bazeed, S. E. S., Aref, Z. F., Sayed, M. A. A., Nafady-Hego, H., & Abdelmaksoud, A. A. (2021). Therapeutic efficacy of macrolides in management of patients with mild COVID-19. *Scientific Reports*, *11*(1), 16361. https://doi.org/10.1038/s41598-021-95900-z

Rauws, E. A., Langenberg, W., Houthoff, H. J., Zanen, H. C., & Tytgat, G. N. (1988). *Campylobacter pyloridis*-associated chronic active antral gastritis. A prospective study of its prevalence and the effects of antibacterial and antiulcer treatment. *Gastroenterology*, *94*(1), 33-40.

Rhead, J. L., Letley, D. P., Mohammadi, M., Hussein, N., Mohagheghi, M. A., Eshagh Hosseini, M., & Atherton, J. C. (2007). A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology*, *133*(3), 926-936. https://doi.org/10.1053/j.gastro.2007.06.056

Rimbara, E., Noguchi, N., Kawai, T., & Sasatsu, M. (2008). Mutations in penicillin-binding proteins 1, 2 and 3 are responsible for amoxicillin resistance in *Helicobacter pylori*. *Journal of Antimicrobial Chemotherapy*, *61*(5), 995-998. https://doi.org/10.1093/jac/dkn051

Robinson, K., Argent, R. H., & Atherton, J. C. (2007). The inflammatory and immune response to *Helicobacter pylori* infection. *Best Pract Res Clin Gastroenterol*, *21*(2), 237-259. https://doi.org/10.1016/j.bpg.2007.01.001

Robinson, K., & Atherton, J. C. (2021). The Spectrum of *Helicobacter*-Mediated Diseases. *Annu Rev Pathol*, *16*, 123-144. https://doi.org/10.1146/annurev-pathol-032520-024949

Rocha, G. R., Lemos, F. F. B., Silva, L. G. O., Luz, M. S., Correa Santos, G. L., Rocha Pinheiro, S. L., Calmon, M. S., & de Melo, F. F. (2025). Overcoming antibiotic-resistant *Helicobacter pylori* infection: Current challenges and emerging approaches. *World J Gastroenterol*, *31*(10), 102289. https://doi.org/10.3748/wjg.v31.i10.102289

Rogoll, D., Schauber, J., Mheta, K. K., Stich, A., & Scheppach, W. (2011). Differential Cathelicidin Expression in Duodenal and Gastric Biopsies from Tanzanian and German Patients. *PLoS One*, *6*(7), e22049. https://doi.org/10.1371/journal.pone.0022049

Rosenberg, E. S., Dufort, E. M., Udo, T., Wilberschied, L. A., Kumar, J., Tesoriero, J., Weinberg, P., Kirkwood, J., Muse, A., DeHovitz, J., Blog, D. S., Hutton, B., Holtgrave, D. R., & Zucker, H. A. (2020). Association of Treatment With Hydroxychloroquine or Azithromycin With In-Hospital Mortality in Patients With COVID-19 in New York State. *Jama*, *323*(24), 2493-2502. https://doi.org/10.1001/jama.2020.8630

Rubin, B. K., & Henke, M. O. (2004). Immunomodulatory activity and effectiveness of macrolides in chronic airway disease. *Chest*, *125*(2 Suppl), 70s-78s. https://doi.org/10.1378/chest.125.2_suppl.70s

Sagaert, X., Van Cutsem, E., De Hertogh, G., Geboes, K., & Tousseyn, T. (2010). Gastric MALT lymphoma: a model of chronic inflammation-induced tumor development. *Nature Reviews Gastroenterology & Hepatology*, *7*(6), 336-346. https://doi.org/10.1038/nrgastro.2010.58

Sah, D. K., Arjunan, A., Lee, B., & Jung, Y. D. (2023). Reactive Oxygen Species and *H. pylori* Infection: A Comprehensive Review of Their Roles in Gastric Cancer Development. *Antioxidants (Basel)*, *12*(9). https://doi.org/10.3390/antiox12091712

Saha, K., Sarkar, D., Khan, U., Karmakar, B. C., Paul, S., Mukhopadhyay, A. K., Dutta, S., & Bhattacharya, S. (2022). Capsaicin Inhibits Inflammation and Gastric Damage during *H pylori* Infection by Targeting NF-kB–miRNA Axis. *Pathogens*, *11*.

Sainsus, N., Cattori, V., Lepadatu, C., & Hofmann-Lehmann, R. (2008). Liquid culture medium for the rapid cultivation of *Helicobacter pylori* from biopsy specimens. *Eur J Clin Microbiol Infect Dis*, *27*(12), 1209-1217. https://doi.org/10.1007/s10096-008-0567-6

Sankaran-Walters, S., Hart, R., & Dills, C. (2017). Guardians of the Gut: Enteric Defensins. *Front Microbiol*, *8*, 647. https://doi.org/10.3389/fmicb.2017.00647

Saracino, I. M., Fiorini, G., Zullo, A., Pavoni, M., Saccomanno, L., & Vaira, D. (2020). Trends in Primary Antibiotic Resistance in *H. pylori* Strains Isolated in Italy between 2009 and 2019. *Antibiotics (Basel)*, *9*(1). https://doi.org/10.3390/antibiotics9010026

Saracino, I. M., Pavoni, M., Zullo, A., Fiorini, G., Lazzarotto, T., Borghi, C., & Vaira, D. (2021). Next Generation Sequencing for the Prediction of the Antibiotic Resistance in *Helicobacter pylori*: A Literature Review. *Antibiotics (Basel)*, *10*(4). https://doi.org/10.3390/antibiotics10040437

Saranathan, R., Levi, M. H., Wattam, A. R., Malek, A., Asare, E., Behin, D. S., Pan, D. H., Jacobs, W. R., Jr., & Szymczak, W. A. (2020). *Helicobacter pylori* Infections in the Bronx, New York: Surveying Antibiotic Susceptibility and Strain Lineage by Whole-Genome Sequencing. *J Clin Microbiol*, *58*(3). https://doi.org/10.1128/jcm.01591-19

Sato, Y., Kanayama, M., Nakajima, S., Hishida, Y., & Watanabe, Y. (2024). Sialyllactose Enhances the Short-Chain Fatty Acid Production and Barrier Function of Gut Epithelial Cells via Nonbifidogenic Modification of the Fecal Microbiome in Human Adults. *Microorganisms*, *12*.

Sato, Y., Kaneko, K., & Inoue, M. (2007). Macrolide antibiotics promote the LPS-induced upregulation of prostaglandin E receptor EP2 and thus attenuate macrolide suppression of IL-6 production. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *76*(3), 181-188. https://doi.org/https://doi.org/10.1016/j.plefa.2006.12.005

Sauvage, E., Kerff, F., Terrak, M., Ayala, J. A., & Charlier, P. (2008). The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol Rev*, *32*(2), 234-258. https://doi.org/10.1111/j.1574-6976.2008.00105.x

Savoldi, A., Carrara, E., Graham, D. Y., Conti, M., & Tacconelli, E. (2018). Prevalence of Antibiotic Resistance in *Helicobacter pylori*: A Systematic Review and Meta-analysis in World Health Organization Regions. *Gastroenterology*, *155*(5), 1372-1382.e1317. https://doi.org/10.1053/j.gastro.2018.07.007

Schubert, J. P., Tay, A., Lee, K. H., Leong, L. E. X., Rayner, C. K., Warner, M. S., Roberts-Thomson, I. C., Costello, S. P., & Bryant, R. V. (2024). Genomic analysis of in Australia: Antimicrobial resistance, phylogenetic patterns, and virulence factors. *Journal of Gastroenterology and Hepatology*, *39*(9), 1869-1875. https://doi.org/https://doi.org/10.1111/jgh.16636

Sebrell, T. A., Hashimi, M., Sidar, B., Wilkinson, R. A., Kirpotina, L., Quinn, M. T., Malkoç, Z., Taylor, P. J., Wilking, J. N., & Bimczok, D. (2019). A Novel Gastric Spheroid Co-culture Model Reveals Chemokine-Dependent Recruitment of Human Dendritic Cells to the Gastric Epithelium. *Cell Mol Gastroenterol Hepatol*, *8*(1), 157-171.e153. https://doi.org/10.1016/j.jcmgh.2019.02.010

Seck, A., Burucoa, C., Dia, D., Mbengue, M., Onambele, M., Raymond, J., & Breurec, S. (2013). Primary antibiotic resistance and associated mechanisms in *Helicobacter pylori* isolates from Senegalese patients. *Ann Clin Microbiol Antimicrob*, *12*, 3. https://doi.org/10.1186/1476-0711-12-3

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics*, *30*(14), 2068-2069. https://doi.org/10.1093/bioinformatics/btu153

Seidlitz, T., Koo, B.-K., & Stange, D. E. (2021). Gastric organoids—an in vitro model system for the study of gastric development and road to personalized medicine. *Cell Death & Differentiation*, *28*(1), 68-83. https://doi.org/10.1038/s41418-020-00662-2

Selgrad, M., Tammer, I., Langner, C., Bornschein, J., Meißle, J., Kandulski, A., Varbanova, M., Wex, T., Schlüter, D., & Malfertheiner, P. (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*(43), 16245-16251. https://doi.org/10.3748/wjg.v20.i43.16245

Selsted, M. E., & Ouellette, A. J. (2005). Mammalian defensins in the antimicrobial immune response. *Nat Immunol*, *6*(6), 551-557. https://doi.org/10.1038/ni1206

Seo, J. W., Park, J. Y., Shin, T.-S., & Kim, J. G. (2019). The analysis of virulence factors and antibiotic resistance between *Helicobacter pylori* strains isolated from gastric antrum and body. *BMC Gastroenterology*, *19*(1), 140. https://doi.org/10.1186/s12876-019-1062-5

Serwecińska, L. (2020). Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment and to Public Health. *Water*, *12*(12), 3313. https://www.mdpi.com/2073-4441/12/12/3313

Shah, S., Hubscher, E., Pelletier, C., Jacob, R., Vinals, L., & Yadlapati, R. (2022). *Helicobacter pylori* infection treatment in the United States: clinical consequences and costs of eradication treatment failure. *Expert Review of Gastroenterology & Hepatology*, *16*(4), 341-357. https://doi.org/10.1080/17474124.2022.2056015

Shanks, A.-M., & El-Omar, E. M. (2009). *Helicobacter pylori* infection, host genetics and gastric cancer. *Journal of Digestive Diseases*, *10*(3), 157-164. https://doi.org/https://doi.org/10.1111/j.1751-2980.2009.00380.x

Sharafutdinov, I., Tegtmeyer, N., Linz, B., Rohde, M., Vieth, M., Tay, A. C., Lamichhane, B., Tuan, V. P., Fauzia, K. A., Sticht, H., Yamaoka, Y., Marshall, B. J., & Backert, S. (2023). A single-nucleotide polymorphism in *Helicobacter pylori* promotes gastric cancer development. *Cell Host Microbe*, *31*(8), 1345-1358.e1346. https://doi.org/10.1016/j.chom.2023.06.016

Sheikh, A. F., Yadyad, M. J., Goodarzi, H., Hashemi, S. J., Aslani, S., Assarzadegan, M. A., & Ranjbar, R. (2018). CagA and vacA allelic combination of *Helicobacter pylori* in gastroduodenal disorders. *Microb Pathog*, *122*, 144-150. https://doi.org/10.1016/j.micpath.2018.06.023

Shelley, J. R., Davidson, D. J., & Dorin, J. R. (2020). The Dichotomous Responses Driven by β-Defensins [Review]. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.01176

Shi, J. H., & Sun, S. C. (2018). Tumor Necrosis Factor Receptor-Associated Factor Regulation of Nuclear Factor κB and Mitogen-Activated Protein Kinase Pathways. *Front Immunol*, *9*, 1849. https://doi.org/10.3389/fimmu.2018.01849

Shi, N., Jin, F., Zhang, X., Clinton, S. K., Pan, Z., & Chen, T. (2014). Overexpression of human β-defensin 2 promotes growth and invasion during esophageal carcinogenesis. *Oncotarget*, *5*(22), 11333-11344. https://doi.org/10.18632/oncotarget.2416

Shibata, W., Hirata, Y., Maeda, S., Ogura, K., Ohmae, T., Yanai, A., Mitsuno, Y., Yamaji, Y., Okamoto, M., Yoshida, H., Kawabe, T., & Omata, M. (2006). CagA protein secreted by the intact type IV secretion system leads to gastric epithelial inflammation in the Mongolian gerbil model. *The Journal of Pathology*, *210*(3), 306-314. https://doi.org/https://doi.org/10.1002/path.2040

Shiotani, A., Lu, H., Dore, M. P., & Graham, D. Y. (2017). Treating *Helicobacter pylori* effectively while minimizing misuse of antibiotics. *Cleve Clin J Med*, *84*(4), 310-318. https://doi.org/10.3949/ccjm.84a.14110

Shirin, T., Rahman, A., Danielsson, Å., Uddin, T., Bhuyian, T. R., Sheikh, A., Qadri, S. S., Qadri, F., & Hammarström, M. L. (2011). Antimicrobial peptides in the duodenum at the acute and convalescent stages in patients with diarrhea due to *Vibrio cholerae O1* or enterotoxigenic *Escherichia coli* infection. *Microbes Infect*, *13*(12-13), 1111-1120. https://doi.org/10.1016/j.micinf.2011.06.014

Shu, X., Ye, D., Hu, C., Peng, K., Zhao, H., Li, H., & Jiang, M. (2022). Alarming antibiotics resistance of *Helicobacter pylori* from children in Southeast China over 6 years. *Sci Rep*, *12*(1), 17754. https://doi.org/10.1038/s41598-022-21661-y

Silva, B., Nunes, A., Vale, F. F., Rocha, R., Gomes, J. P., Dias, R., & Oleastro, M. (2017). The expression of *Helicobacter pylori tfs* plasticity zone cluster is regulated by pH and adherence, and its composition is associated with differential gastric IL-8 secretion. *Helicobacter*, *22*(4). https://doi.org/10.1111/hel.12390

Silwal, P., Paik, S., Kim, J. K., Yoshimori, T., & Jo, E. K. (2021). Regulatory Mechanisms of Autophagy-Targeted Antimicrobial Therapeutics Against Mycobacterial Infection. *Front Cell Infect Microbiol*, *11*, 633360. https://doi.org/10.3389/fcimb.2021.633360

Singh, A., Rani, P. S., Bandsode, V., Nyambero, M., Qumar, S., & Ahmed, N. (2024). Drivers of virulence and antimicrobial resistance in Gram-negative bacteria in different settings: A genomic perspective. *Infection, Genetics and Evolution*, *124*, 105666. https://doi.org/https://doi.org/10.1016/j.meegid.2024.105666

Skolnick, J., Gao, M., Zhou, H., & Singh, S. (2021). AlphaFold 2: Why It Works and Its Implications for Understanding the Relationships of Protein Sequence, Structure, and Function. *Journal of Chemical Information and Modeling*, *61*(10), 4827-4831. https://doi.org/10.1021/acs.jcim.1c01114

Smith, A. M., & Klugman, K. P. (2005). Amino acid mutations essential to production of an altered PBP 2X conferring high-level beta-lactam resistance in a clinical isolate of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother*, *49*(11), 4622-4627. https://doi.org/10.1128/aac.49.11.4622-4627.2005

Smith, S. M. (2014). Role of Toll-like receptors in *Helicobacter pylori* infection and immunity. *World J Gastrointest Pathophysiol*, *5*(3), 133-146. https://doi.org/10.4291/wjgp.v5.i3.133

Smith, S. M., O'Morain, C., & McNamara, D. (2014). Antimicrobial susceptibility testing for *Helicobacter pylori* in times of increasing antibiotic resistance. *World J Gastroenterol*, *20*(29), 9912-9921. https://doi.org/10.3748/wjg.v20.i29.9912

Smolka, A. J., & Schubert, M. L. (2017). *Helicobacter pylori*-Induced Changes in Gastric Acid Secretion and Upper Gastrointestinal Disease. In N. Tegtmeyer & S. Backert (Eds.), *Molecular Pathogenesis and Signal Transduction by Helicobacter pylori* (pp. 227-252). Springer International Publishing. https://doi.org/10.1007/978-3-319-50520-6_10

So, Y. J., Park, O.-J., Kwon, Y., Im, J., Lee, D., Yun, S. H., Cho, K., Yun, C.-H., & Han, S. H. (2024). *Bacillus subtilis* Induces Human Beta Defensin-2 Through its Lipoproteins in Human Intestinal Epithelial Cells. *Probiotics and antimicrobial proteins*.

Sokolova, O., & Naumann, M. (2017). NF-κB Signaling in Gastric Cancer. *Toxins*, *9*(4), 119. https://www.mdpi.com/2072-6651/9/4/119

Sommer, F., & Bäckhed, F. (2013). The gut microbiota — masters of host development and physiology. *Nature Reviews Microbiology*, *11*(4), 227-238. https://doi.org/10.1038/nrmicro2974

Sørensen, O. E., Thapa, D. R., Rosenthal, A., Liu, L., Roberts, A. A., & Ganz, T. (2005). Differential Regulation of β-Defensin Expression in Human Skin by Microbial Stimuli1. *The Journal of Immunology*, *174*(8), 4870-4879. https://doi.org/10.4049/jimmunol.174.8.4870

Soyfoo, D. M., Doomah, Y. H., Xu, D., Zhang, C., Sang, H.-M., Liu, Y.-Y., Zhang, G.-X., Jiang, J.-X., & Xu, S.-F. (2021). New genotypes of *Helicobacter Pylori* VacA *d*-region identified from global strains. *BMC Molecular and Cell Biology*, *22*(1), 4. https://doi.org/10.1186/s12860-020-00338-2

Soylu, O. B., Ozturk, Y., & Ozer, E. (2008). α-Defensin Expression in the Gastric Tissue of Children with *Helicobacter pylori*–Associated Chronic Gastritis: An Immunohistochemical Study. *Journal of Pediatric Gastroenterology and Nutrition*, *46*(4), 474-477. https://doi.org/10.1097/MPG.0b013e31815a9923

Starkova, D., Gladyshev, N., Polev, D., Saitova, A., Egorova, S., & Svarval, A. (2024). First insight into the whole genome sequence variations in clarithromycin resistant *Helicobacter pylori* clinical isolates in Russia. *Scientific Reports*, *14*(1), 20108. https://doi.org/10.1038/s41598-024-70977-4

Steubesand, N., Kiehne, K., Brunke, G., Pahl, R., Reiss, K., Herzig, K. H., Schubert, S., Schreiber, S., Fölsch, U. R., Rosenstiel, P., & Arlt, A. (2009). The expression of the beta-defensins hBD-2 and hBD-3 is differentially regulated by NF-kappaB and MAPK/AP-1 pathways in an *in vitro* model of *Candida esophagitis*. *BMC Immunol*, *10*, 36. https://doi.org/10.1186/1471-2172-10-36

Stoesser, N., Batty, E. M., Eyre, D. W., Morgan, M., Wyllie, D. H., Del Ojo Elias, C., Johnson, J. R., Walker, A. S., Peto, T. E., & Crook, D. W. (2013). Predicting antimicrobial susceptibilities for *Escherichia coli* and *Klebsiella pneumoniae* isolates using whole genomic sequence data. *J Antimicrob Chemother*, *68*(10), 2234-2244. https://doi.org/10.1093/jac/dkt180

Storskrubb, T., Aro, P., Ronkainen, J., Wreiber, K., Nyhlin, H., Bolling-Sternevald, E., Talley, N. J., Engstrand, L., & Agréus, L. (2006). Antimicrobial susceptibility of *Helicobacter pylori* strains in a random adult Swedish population. *Helicobacter*, *11*(4), 224-230. https://doi.org/10.1111/j.1523-5378.2006.00414.x

Subbiahanadar Chelladurai, K., Selvan Christyraj, J. D., Rajagopalan, K., Yesudhason, B. V., Venkatachalam, S., Mohan, M., Chellathurai Vasantha, N., & Selvan Christyraj, J. R. S. (2021). Alternative to FBS in animal cell culture - An overview and future perspective. *Heliyon*, *7*(8). https://doi.org/10.1016/j.heliyon.2021.e07686

Sudheendra, U. S., Dhople, V., Datta, A., Kar, R. K., Shelburne, C. E., Bhunia, A., & Ramamoorthy, A. (2015). Membrane disruptive antimicrobial activities of human β-defensin-3 analogs. *European Journal of Medicinal Chemistry*, *91*, 91-99. https://doi.org/https://doi.org/10.1016/j.ejmech.2014.08.021

Suerbaum, S., & Michetti, P. (2002). *Helicobacter pylori* Infection. *New England Journal of Medicine*, *347*(15), 1175-1186. https://doi.org/doi:10.1056/NEJMra020542

Sugano, K. (2019). Effect of *Helicobacter pylori* eradication on the incidence of gastric cancer: a systematic review and meta-analysis. *Gastric Cancer*, *22*(3), 435-445. https://doi.org/10.1007/s10120-018-0876-0

Sugimoto, M., & Yamaoka, Y. (2009). Virulence factor genotypes of *Helicobacter pylori* affect cure rates of eradication therapy. *Arch Immunol Ther Exp (Warsz)*, *57*(1), 45-56. https://doi.org/10.1007/s00005-009-0007-z

Suleiman, A. A., Shaban, S. A., & Al-Chalabi, R. (2021). Genetic polymorphism relationship of four SNP in beta defensins genes 1 and 2 with susceptibility and effect of triple therapy of *Helicobacter pylori* infection in Iraq. *Gene Reports*, *25*, 101366. https://doi.org/https://doi.org/10.1016/j.genrep.2021.101366

Suzuki, T., Matsuo, K., Ito, H., Sawaki, A., Hirose, K., Wakai, K., Sato, S., Nakamura, T., Yamao, K., Ueda, R., & Tajima, K. (2006). Smoking increases the treatment failure for *Helicobacter pylori* eradication. *Am J Med*, *119*(3), 217-224. https://doi.org/10.1016/j.amjmed.2005.10.003

Taglialegna, A. (2023). When *Helicobacter pylori* spells gastric cancer. *Nature Reviews Microbiology*, *21*(10), 628-628. https://doi.org/10.1038/s41579-023-00959-2

Taha, A. S., Faccenda, E., Angerson, W. J., Balsitis, M., & Kelly, R. W. (2005a). Gastric epithelial anti-microbial peptides--histological correlation and influence of anatomical site and peptic ulcer disease. *Dig Liver Dis*, *37*(1), 51-56. https://doi.org/10.1016/j.dld.2004.07.019

Taha, A. S., Faccenda, E., Angerson, W. J., Balsitis, M., & Kelly, R. W. (2005b). Gastric epithelial anti-microbial peptides—histological correlation and influence of anatomical site and peptic ulcer disease. *Digestive and Liver Disease*, *37*(1), 51-56. https://doi.org/https://doi.org/10.1016/j.dld.2004.07.019

Takahashi-Kanemitsu, A., Knight, C. T., & Hatakeyama, M. (2020). Molecular anatomy and pathogenic actions of *Helicobacter pylori* CagA that underpin gastric carcinogenesis. *Cellular & Molecular Immunology*, *17*(1), 50-63. https://doi.org/10.1038/s41423-019-0339-5

Takenawa, H., Kurosaki, M., Enomoto, N., Miyasaka, Y., Kanazawa, N., Sakamoto, N., Ikeda, T., Izumi, N., Sato, C., & Watanabe, M. (2004). Differential gene-expression profiles associated with gastric adenoma. *British Journal of Cancer*, *90*(1), 216-223. https://doi.org/10.1038/sj.bjc.6601399

Tamaoki, J., Kadota, J., & Takizawa, H. (2004). Clinical implications of the immunomodulatory effects of macrolides. *The American Journal of Medicine Supplements*, *117*(9, Supplement 1), 5-11. https://doi.org/https://doi.org/10.1016/j.amjmed.2004.07.023

Tamma, P. D., Cosgrove, S. E., & Maragakis, L. L. (2012). Combination Therapy for Treatment of Infections with Gram-Negative Bacteria. *Clinical Microbiology Reviews*, *25*(3), 450-470. https://doi.org/doi:10.1128/cmr.05041-11

Tanih, N. F., Okeleye, B. I., Naidoo, N., Clarke, A. M., Mkwetshana, N., Green, E., Ndip, L. M., & Ndip, R. N. (2010). Marked susceptibility of South African *Helicobacter pylori* strains to ciprofloxacin and amoxicillin: clinical implications. *S Afr Med J*, *100*(1), 49-52.

Tavares, R., & Pathak, S. K. (2018). Induction of TNF, CXCL8 and IL-1β in macrophages by *Helicobacter pylori* secreted protein HP1173 occurs via MAP-kinases, NF-κB and AP-1 signaling pathways. *Microb Pathog*, *125*, 295-305. https://doi.org/10.1016/j.micpath.2018.09.037

Tegtmeyer, N., Wessler, S., Necchi, V., Rohde, M., Harrer, A., Rau, T. T., Asche, C. I., Boehm, M., Loessner, H., Figueiredo, C., Naumann, M., Palmisano, R., Solcia, E., Ricci, V., & Backert, S. (2017). *Helicobacter pylori* Employs a Unique Basolateral Type IV Secretion Mechanism for CagA Delivery. *Cell Host Microbe*, *22*(4), 552-560.e555. https://doi.org/10.1016/j.chom.2017.09.005

Tempera, P. J., Michael, M., Tageldin, O., & Hasak, S. (2022). Gastric Cancer Due to Chronic *H. pylori* Infection: What We Know and Where We Are Going. *Diseases*, *10*(3), 57. https://www.mdpi.com/2079-9721/10/3/57

Teng, Y.-s., Cang, B., Mao, F.-y., Chen, W., Cheng, P., Peng, L.-s., Luo, P., Lu, D.-s., You, N., Zou, Q., & Yuan, Z. (2020). Expression of ETS1 in gastric epithelial cells positively regulate inflammatory response in *Helicobacter pylori*-associated gastritis. *Cell Death & Disease*, *11*.

Thi Huyen Trang, T., Thanh Binh, T., & Yamaoka, Y. (2016). Relationship between vacA Types and Development of Gastroduodenal Diseases. *Toxins*, *8*(6), 182. https://www.mdpi.com/2072-6651/8/6/182

Thompson, C. J., Vu, V. H., Leckband, D. E., & Schwartz, D. K. (2021). Cadherin cis and trans interactions are mutually cooperative. *Proc Natl Acad Sci U S A*, *118*(10). https://doi.org/10.1073/pnas.2019845118

Thompson, W., Teoh, L., Hubbard, C. C., Marra, F., Patrick, D. M., Mamun, A., Campbell, A., & Suda, K. J. (2022). Patterns of dental antibiotic prescribing in 2017: Australia, England, United States, and British Columbia (Canada). *Infection Control & Hospital Epidemiology*, *43*(2), 191-198. https://doi.org/10.1017/ice.2021.87

Toh, J. W. T., & Wilson, R. B. (2020). Pathways of Gastric Carcinogenesis, *Helicobacter pylori* Virulence and Interactions with Antioxidant Systems, Vitamin C and Phytochemicals. *International Journal of Molecular Sciences*, *21*(17), 6451. https://www.mdpi.com/1422-0067/21/17/6451

Tohidpour, A. (2016). CagA-mediated pathogenesis of *Helicobacter pylori*. *Microbial Pathogenesis*, *93*, 44-55. https://doi.org/https://doi.org/10.1016/j.micpath.2016.01.005

Tollin, M., Bergman, P., Svenberg, T., Jörnvall, H., Gudmundsson, G. H., & Agerberth, B. (2003). Antimicrobial peptides in the first line defence of human colon mucosa. *Peptides*, *24*(4), 523-530. https://doi.org/10.1016/s0196-9781(03)00114-1

Tomita, T., Nagase, T., Ohga, E., Yamaguchi, Y., Yoshizumi, M., & Ouchi, Y. (2002). Molecular mechanisms underlying human beta-defensin-2 gene expression in a human airway cell line (LC2/ad). *Respirology*, *7*(4), 305-310. https://doi.org/10.1046/j.1440-1843.2002.00415.x

Tran, C. T., Garcia, M., Garnier, M., Burucoa, C., & Bodet, C. (2017). Inflammatory signaling pathways induced by *Helicobacter pylori* in primary human gastric epithelial cells. *Innate Immun*, *23*(2), 165-174. https://doi.org/10.1177/1753425916681077

Tran, T. T., Nguyen, A. T., Quach, D. T., Pham, D. T.-H., Cao, N. M., Nguyen, U. T.-H., Dang, A. N.-T., Tran, M. A., Quach, L. H., Tran, K. T., Le, N. Q., Ung, V. V., Vo, M. N.-Q., Nguyen, D. T., Ngo, K. D., Tran, T. L., & Nguyen, V. T. (2022a). Emergence of amoxicillin resistance and identification of novel mutations of the *pbp1A* gene in *Helicobacter pylori* in Vietnam. *BMC Microbiology*, *22*(1), 41. https://doi.org/10.1186/s12866-022-02463-8

Tran, T. T., Nguyen, A. T., Quach, D. T., Pham, D. T., Cao, N. M., Nguyen, U. T., Dang, A. N., Tran, M. A., Quach, L. H., Tran, K. T., Le, N. Q., Ung, V. V., Vo, M. N., Nguyen, D. T., Ngo, K. D., Tran, T. L., & Nguyen, V. T. (2022b). Emergence of amoxicillin resistance and identification of novel mutations of the *pbp1A* gene in *Helicobacter pylori* in Vietnam. *BMC Microbiol*, *22*(1), 41. https://doi.org/10.1186/s12866-022-02463-8

Tran, V. H., Ha, T. M. T., Le, P. T. Q., Phan, T. N., & Tran, T. N. H. (2019). Characterisation of point mutations in domain V of the *23S rRNA* gene of clinical *Helicobacter pylori* strains and clarithromycin-resistant phenotype in central Vietnam. *J Glob Antimicrob Resist*, *16*, 87-91. https://doi.org/10.1016/j.jgar.2018.09.012

Tran, V. H., Nguyen, T. M. N., Le, P. T. Q., Nguyen, T. H. T., Nguyen, T. C. L., & Ha, T. M. T. (2024). Current status of *Helicobacter pylori* resistance to clarithromycin and levofloxacin in Vietnam: Results from molecular analysis of gastric biopsy specimens. *Journal of Global Antimicrobial Resistance*, *36*, 76-82. https://doi.org/https://doi.org/10.1016/j.jgar.2023.12.026

Traulsen, J., Zagami, C., Daddi, A. A., & Boccellato, F. (2021). Molecular modelling of the gastric barrier response, from infection to carcinogenesis. *Best Pract Res Clin Gastroenterol*, *50-51*, 101737. https://doi.org/10.1016/j.bpg.2021.101737

Tsai, H.-F., & Hsu, P.-N. (2017). Modulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis by *Helicobacter pylori* in immune pathogenesis of gastric mucosal damage. *Journal of Microbiology, Immunology and Infection*, *50*(1), 4-9. https://doi.org/https://doi.org/10.1016/j.jmii.2016.01.002

Tseng, Y. S., Wu, D. C., Chang, C. Y., Kuo, C. H., Yang, Y. C., Jan, C. M., Su, Y. C., Kuo, F. C., & Chang, L. L. (2009). Amoxicillin resistance with beta-lactamase production in *Helicobacter pylori*. *Eur J Clin Invest*, *39*(9), 807-812. https://doi.org/10.1111/j.1365-2362.2009.02166.x

Tshibangu-Kabamba, E., Ngoma-Kisoko, P. J., Tuan, V. P., Matsumoto, T., Akada, J., Kido, Y., Tshimpi-Wola, A., Tshiamala-Kashala, P., Ahuka-Mundeke, S., Ngoy, D. M., Disashi-Tumba, G., & Yamaoka, Y. (2020). Next-Generation Sequencing of the Whole Bacterial Genome for Tracking Molecular Insight into the Broad-Spectrum Antimicrobial Resistance of *Helicobacter pylori* Clinical Isolates from the Democratic Republic of Congo. *Microorganisms*, *8*(6). https://doi.org/10.3390/microorganisms8060887

Tshibangu-Kabamba, E., & Yamaoka, Y. (2021). *Helicobacter pylori* infection and antibiotic resistance — from biology to clinical implications. *Nature Reviews Gastroenterology & Hepatology*, *18*(9), 613-629. https://doi.org/10.1038/s41575-021-00449-x

Tuan, V. P., Narith, D., Tshibangu-Kabamba, E., Dung, H. D. Q., Viet, P. T., Sokomoth, S., Binh, T. T., Sokhem, S., Tri, T. D., Ngov, S., Tung, P. H., Thuan, N. P. M., Truc, T. C., Phuc, B. H., Matsumoto, T., Fauzia, K. A., Akada, J., Trang, T. T. H., & Yamaoka, Y. (2019). A Next-Generation Sequencing-Based Approach to Identify Genetic Determinants of Antibiotic Resistance in Cambodian *Helicobacter pylori* Clinical Isolates. *J Clin Med*, *8*(6). https://doi.org/10.3390/jcm8060858

Uehara, N., Yagihashi, A., Kondoh, K., Tsuji, N., Fujita, T., Hamada, H., & Watanabe, N. (2003). Human beta-defensin-2 induction in *Helicobacter pylori*-infected gastric mucosal tissues: antimicrobial effect of overexpression. *J Med Microbiol*, *52*(Pt 1), 41-45. https://doi.org/10.1099/jmm.0.04985-0

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N., & Schlemper, R. J. (2001). *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med*, *345*(11), 784-789. https://doi.org/10.1056/NEJMoa001999

Uotani, T., Miftahussurur, M., & Yamaoka, Y. (2015). Effect of bacterial and host factors on *Helicobacter pylori* eradication therapy. *Expert Opinion on Therapeutic Targets*, *19*(12), 1637-1650. https://doi.org/10.1517/14728222.2015.1073261

Uotani, T., Murakami, K., Uchida, T., Tanaka, S., Nagashima, H., Zeng, X. L., Akada, J., Estes, M. K., Graham, D. Y., & Yamaoka, Y. (2019). Changes of tight junction and interleukin-8 expression using a human gastroid monolayer model of *Helicobacter pylori* infection. *Helicobacter*, *24*(3), e12583. https://doi.org/10.1111/hel.12583

Valdes, A. M., Walter, J., Segal, E., & Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *Bmj*, *361*, k2179. https://doi.org/10.1136/bmj.k2179

Van Boeckel, T. P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A., & Laxminarayan, R. (2014). Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lancet Infect Dis*, *14*(8), 742-750. https://doi.org/10.1016/s1473-3099(14)70780-7

Vanaudenaerde, B. M., Wuyts, W. A., Geudens, N., Dupont, L. J., Schoofs, K., Smeets, S., Van Raemdonck, D. E., & Verleden, G. M. (2007). Macrolides Inhibit IL17-induced IL8 and 8-isoprostane Release from Human Airway Smooth Muscle Cells. *American Journal of Transplantation*, *7*(1), 76-82. https://doi.org/https://doi.org/10.1111/j.1600-6143.2006.01586.x

Vandamme, D., Landuyt, B., Luyten, W., & Schoofs, L. (2012). A comprehensive summary of *LL-37*, the factotum human cathelicidin peptide. *Cellular Immunology*, *280*(1), 22-35. https://doi.org/https://doi.org/10.1016/j.cellimm.2012.11.009

Varga, M. G., & Peek, R. M. (2017). DNA Transfer and Toll-like Receptor Modulation by *Helicobacter pylori*. In N. Tegtmeyer & S. Backert (Eds.), *Molecular Pathogenesis and Signal Transduction by Helicobacter pylori* (pp. 169-193). Springer International Publishing. https://doi.org/10.1007/978-3-319-50520-6_8

Varghese, R., Neeravi, A., Subramanian, N., Baskar, P., Anandhan, K., & Veeraraghavan, B. (2020). Analysis of Amino Acid Sequences of Penicillin-Binding Proteins 1a, 2b, and 2x in Invasive *Streptococcus pneumoniae* Nonsusceptible to Penicillin Isolated from Children in India. *Microbial Drug Resistance*, *27*(3), 311-319. https://doi.org/10.1089/mdr.2020.0204

Viala, J., Chaput, C., Boneca, I. G., Cardona, A., Girardin, S. E., Moran, A. P., Athman, R., Mémet, S., Huerre, M. R., Coyle, A. J., DiStefano, P. S., Sansonetti, P. J., Labigne, A., Bertin, J., Philpott, D. J., & Ferrero, R. L. (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol*, *5*(11), 1166-1174. https://doi.org/10.1038/ni1131

Vilaichone, R.-k., Aumpan, N., Ratanachu-ek, T., Uchida, T., Tshering, L., Mahachai, V., & Yamaoka, Y. (2020). Population-based study of *Helicobacter pylori* infection and antibiotic resistance in Bhutan. *International Journal of Infectious Diseases*, *97*, 102-107. https://doi.org/https://doi.org/10.1016/j.ijid.2020.05.077

Vinall, L. E., King, M., Novelli, M., Green, C. A., Daniels, G., Hilkens, J., Sarner, M., & Swallow, D. M. (2002). Altered expression and allelic association of the hypervariable membrane mucin *MUC1* in *Helicobacter pylori* gastritis. *Gastroenterology*, *123*(1), 41-49. https://doi.org/https://doi.org/10.1053/gast.2002.34157

Vital, J. S., Tanoeiro, L., Lopes-Oliveira, R., & Vale, F. F. (2022). Biomarker Characterization and Prediction of Virulence and Antibiotic Resistance from *Helicobacter pylori* Next Generation Sequencing Data. *Biomolecules*, *12*(5), 691. https://www.mdpi.com/2218-273X/12/5/691

Vllahu, M., Voli, A., Licursi, V., Zagami, C., D'Amore, A., Traulsen, J., Woelffling, S., Schmid, M., Crickley, R., Lisle, R., Link, A., Tosco, A., Meyer, T. F., & Boccellato, F. (2024). Inflammation promotes stomach epithelial defense by stimulating the secretion of antimicrobial peptides in the mucus. *Gut Microbes*, *16*(1), 2390680. https://doi.org/10.1080/19490976.2024.2390680

Vordenbäumen, S., Pilic, D., Otte, J.-M., Schmitz, F., & Schmidt-Choudhury, A. (2009). Defensins Are Differentially Expressed With Respect to the Anatomic Region in the Upper Gastrointestinal Tract of Children. *Journal of Pediatric Gastroenterology and Nutrition*, *49*(1), 139-142. https://doi.org/https://doi.org/10.1097/MPG.0b013e31818f0a51

Vordenbäumen, S., Pilic, D., Otte, J.-M., Schmitz, F., & Schmidt-Choudhury, A. (2010a). Defensin-mRNA Expression in the Upper Gastrointestinal Tract Is Modulated in Children With Celiac Disease and *Helicobacter pylori*–positive Gastritis. *Journal of Pediatric Gastroenterology and Nutrition*, *50*(6), 596-600. https://doi.org/https://doi.org/10.1097/MPG.0b013e3181cd26cd

Vordenbäumen, S., Pilic, D., Otte, J. M., Schmitz, F., & Schmidt-Choudhury, A. (2010b). Defensin-mRNA expression in the upper gastrointestinal tract is modulated in children with celiac disease and *Helicobacter pylori*-positive gastritis. *J Pediatr Gastroenterol Nutr*, *50*(6), 596-600. https://doi.org/10.1097/MPG.0b013e3181cd26cd

Wada, A., Mori, N., Oishi, K., Hojo, H., Nakahara, Y., Hamanaka, Y., Nagashima, M., Sekine, I., Ogushi, K.-i., Niidome, T., Nagatake, T., Moss, J., & Hirayama, T. (1999). Induction of Human β-Defensin-2 mRNA Expression by *Helicobacter pylori* in Human Gastric Cell Line MKN45 Cells on cag Pathogenicity Island. *Biochemical and Biophysical Research Communications*, *263*(3), 770-774. https://doi.org/https://doi.org/10.1006/bbrc.1999.1452

Wada, A., Ogushi, K., Kimura, T., Hojo, H., Mori, N., Suzuki, S., Kumatori, A., Se, M., Nakahara, Y., Nakamura, M., Moss, J., & Hirayama, T. (2001). *Helicobacter pylori*-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappaB. *Cell Microbiol*, *3*(2), 115-123. https://doi.org/10.1046/j.1462-5822.2001.00096.x

Wang, D., Guo, Q., Yuan, Y., & Gong, Y. (2019). The antibiotic resistance of *Helicobacter pylori* to five antibiotics and influencing factors in an area of China with a high risk of gastric cancer. *BMC Microbiol*, *19*(1), 152. https://doi.org/10.1186/s12866-019-1517-4

Wang, F., Mao, Z., Liu, D., Yu, J., Wang, Y., Ye, W., Lin, D., Zhou, N., & Xie, Y. (2017). Overexpression of Tim-3 reduces *Helicobacter pylori*-associated inflammation through TLR4/NFκB signaling in vitro. *Mol Med Rep*, *15*(5), 3252-3258. https://doi.org/10.3892/mmr.2017.6346

Wang, G. (2014). Human Antimicrobial Peptides and Proteins. *Pharmaceuticals*, *7*(5), 545-594. https://www.mdpi.com/1424-8247/7/5/545

Wang, H., Zhao, M., Shi, F., Zheng, S., Xiong, L., & Zheng, L. (2023). A review of signal pathway induced by virulent protein CagA of *Helicobacter pylori* [Review]. *Frontiers in Cellular and Infection Microbiology*, *13*. https://doi.org/10.3389/fcimb.2023.1062803

Waskito, L. A., Miftahussurur, M., Lusida, M. I., Syam, A. F., Suzuki, R., Subsomwong, P., Uchida, T., Hamdan, M., Nasronudin, & Yamaoka, Y. (2018). Distribution and clinical associations of integrating conjugative elements and cag pathogenicity islands of *Helicobacter pylori* in Indonesia. *Sci Rep*, *8*(1), 6073. https://doi.org/10.1038/s41598-018-24406-y

Watanabe, Y., Oikawa, R., Kodaka, Y., Sato, Y., Ono, S., Kenmochi, T., Suzuki, H., Futagami, S., Kato, M., Yamamoto, H., & Itoh, F. (2021). Cancer-related genetic variants of *Helicobacter pylori* strains determined using gastric wash-based whole-genome analysis with single-molecule real-time technology. *Int J Cancer*, *148*(1), 178-192. https://doi.org/10.1002/ijc.33257

Watari, J., Chen, N., Amenta, P. S., Fukui, H., Oshima, T., Tomita, T., Miwa, H., Lim, K. J., & Das, K. M. (2014). *Helicobacter pylori* associated chronic gastritis, clinical syndromes, precancerous lesions, and pathogenesis of gastric cancer development. *World J Gastroenterol*, *20*(18), 5461-5473. https://doi.org/10.3748/wjg.v20.i18.5461

Weber, D. M., Dimopoulos, M. A., Anandu, D. P., Pugh, W. C., & Steinbach, G. (1994). Regression of gastric lymphoma of mucosa-associated lymphoid tissue with antibiotic therapy for *Helicobacter pylori*. *Gastroenterology*, *107*(6), 1835-1838. https://doi.org/https://doi.org/10.1016/0016-5085(94)90828-1

Wehkamp, J., Fellermann, K., Herrlinger, K. R., Bevins, C. L., & Stange, E. F. (2005a). Mechanisms of Disease: defensins in gastrointestinal diseases. *Nature Clinical Practice Gastroenterology & Hepatology*, *2*(9), 406-415. https://doi.org/10.1038/ncpgasthep0265

Wehkamp, J., Harder, J., Weichenthal, M., Mueller, O., Herrlinger, K. R., Fellermann, K., Schroeder, J. M., & Stange, E. F. (2003). Inducible and Constitutive β-Defensins Are Differentially Expressed in Crohn's Disease and Ulcerative Colitis. *Inflammatory Bowel Diseases*, *9*(4), 215-223. https://doi.org/10.1097/00054725-200307000-00001

Wehkamp, J., Harder, J., Weichenthal, M., Schwab, M., Schäffeler, E., Schlee, M., Herrlinger, K. R., Stallmach, A., Noack, F., Fritz, P., Schröder, J. M., Bevins, C. L., Fellermann, K., & Stange, E. F. (2004). NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal α-defensin expression [Article]. *Gut*, *53*(11), 1658-1664. https://doi.org/10.1136/gut.2003.032805

Wehkamp, J., Salzman, N. H., Porter, E., Nuding, S., Weichenthal, M., Petras, R. E., Shen, B., Schaeffeler, E., Schwab, M., Linzmeier, R., Feathers, R. W., Chu, H., Lima, H., Jr., Fellermann, K., Ganz, T., Stange, E. F., & Bevins, C. L. (2005b). Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A*, *102*(50), 18129-18134. https://doi.org/10.1073/pnas.0505256102

Wehkamp, J., & Stange, E. F. (2020). An Update Review on the Paneth Cell as Key to Ileal Crohn's Disease [Review]. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.00646

Weinberg, A., Jin, G., Sieg, S., & McCormick, T. S. (2012). The Yin and Yang of Human Beta-Defensins in Health and Disease [Review]. *Frontiers in Immunology*, *3*. https://doi.org/10.3389/fimmu.2012.00294

Weir, C. B., & Le, J. K. (2025). Metronidazole. In *StatPearls*. StatPearls Publishing

Weng, C. Y., Xu, J. L., Sun, S. P., Wang, K. J., & Lv, B. (2021). *Helicobacter pylori* eradication: Exploring its impacts on the gastric mucosa. *World J Gastroenterol*, *27*(31), 5152-5170. https://doi.org/10.3748/wjg.v27.i31.5152

Wiedemann, T., Hofbaur, S., Loell, E., & Rieder, G. (2016). A C-Terminal Coiled-Coil Region of CagL is Responsible for *Helicobacter Pylori*-Induced Il-8 Expression. *Eur J Microbiol Immunol (Bp)*, *6*(3), 186-196. https://doi.org/10.1556/1886.2016.00020

Wiens, M. E., & Smith, J. G. (2015). Alpha-defensin HD5 inhibits furin cleavage of human papillomavirus 16 L2 to block infection [Article]. *Journal of Virology*, *89*(5), 2866-2874. https://doi.org/10.1128/JVI.02901-14

Wilkinson, D. J. (2019). *High throughput genomic analysis of Helicobacter pylori within-host diversity* Nottingham Trent University]. chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://irep.ntu.ac.uk/id/eprint/39527/1/THESIS_FINAL_Jan_2020_all_changes_accepted%20(1).pdf

Wilkinson, D. J., Dickins, B., Robinson, K., & Winter, J. A. (2022). Genomic diversity of *Helicobacter pylori* populations from different regions of the human stomach. *Gut Microbes*, *14*(1), 2152306. https://doi.org/10.1080/19490976.2022.2152306

Winter, J. A., Letley, D. P., Cook, K. W., Rhead, J. L., Zaitoun, A. A., Ingram, R. J., Amilon, K. R., Croxall, N. J., Kaye, P. V., Robinson, K., & Atherton, J. C. (2014). A role for the vacuolating cytotoxin, VacA, in colonization and *Helicobacter pylori*-induced metaplasia in the stomach. *J Infect Dis*, *210*(6), 954-963. https://doi.org/10.1093/infdis/jiu154

Wizenty, J., Tacke, F., & Sigal, M. (2020). Responses of gastric epithelial stem cells and their niche to *Helicobacter pylori* infection. *Ann Transl Med*, *8*(8), 568. https://doi.org/10.21037/atm.2020.02.178

Wolczyk, D., Zaremba-Czogalla, M., Hryniewicz-Jankowska, A., Tabola, R., Grabowski, K., Sikorski, A. F., & Augoff, K. (2016). TNF-α promotes breast cancer cell migration and enhances the concentration of membrane-associated proteases in lipid rafts. *Cellular Oncology*, *39*(4), 353-363. https://doi.org/10.1007/s13402-016-0280-x

Wong, W. M., Gu, Q., Wang, W. H., Fung, F. M., Berg, D. E., Lai, K. C., Xia, H. H., Hu, W. H., Chan, C. K., Chan, A. O., Yuen, M. F., Hui, C. K., Lam, S. K., & Wong, B. C. (2003). Effects of primary metronidazole and clarithromycin resistance to *Helicobacter pylori* on omeprazole, metronidazole, and clarithromycin triple-therapy regimen in a region with high rates of metronidazole resistance. *Clin Infect Dis*, *37*(7), 882-889. https://doi.org/10.1086/377206

Woo, J., Seo, H. J., Lee, J.-y., Lee, I., Jeon, K. S., Kim, B., & Lee, K. (2023). Polypropylene nanoplastic exposure leads to lung inflammation through p38-mediated NF-κB pathway due to mitochondrial damage. *Particle and Fibre Toxicology*, *20*.

Wozniak, W., Sechet, E., Kwon, Y.-J., Aulner, N., Navarro, L., & Sperandio, B. (2024). Identification of human host factors required for beta-defensin-2 expression in intestinal epithelial cells upon a bacterial challenge. *Scientific Reports*, *14*(1), 15442. https://doi.org/10.1038/s41598-024-66568-y

Wroblewski, L. E., Peek, R. M., Jr., & Wilson, K. T. (2010). *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. *Clin Microbiol Rev*, *23*(4), 713-739. https://doi.org/10.1128/cmr.00011-10

Wu, E., Zhu, J., Ma, Z., Tuo, B., Terai, S., Mizuno, K., Li, T., & Liu, X. (2022). Gastric alarmin release: A warning signal in the development of gastric mucosal diseases [Review]. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.1008047

Wu, J.-Y., Lee, Y.-C., & Graham, D. Y. (2019). The eradication of *Helicobacter pylori* to prevent gastric cancer: a critical appraisal. *Expert Review of Gastroenterology & Hepatology*, *13*(1), 17-24. https://doi.org/10.1080/17474124.2019.1542299

Wu, Z., Ding, Z., Cheng, B., & Cui, Z. (2021). The inhibitory effect of human *DEFA5* in growth of gastric cancer by targeting BMI1. *Cancer Sci*, *112*(3), 1075-1083. https://doi.org/10.1111/cas.14827

Wueppenhorst, N., Stueger, H. P., Kist, M., & Glocker, E. O. (2013). High secondary resistance to quinolones in German *Helicobacter pylori* clinical isolates. *J Antimicrob Chemother*, *68*(7), 1562-1566. https://doi.org/10.1093/jac/dkt061

Xiong, M., Aljaberi, H. S. M., Ansari, N. K., Sun, Y., Yin, S., Nasifu, L., Sun, H., Xu, T., Pan, Y., Nie, Z., Liu, C., Zhang, Z., Jiang, Z., Wang, S., & He, B. (2023). Phenotype and genotype analysis for *Helicobacter pylori* antibiotic resistance in outpatients: a retrospective study. *Microbiology Spectrum*, *11*(5), e00550-00523. https://doi.org/doi:10.1128/spectrum.00550-23

Xu, X., Feng, J., Wang, X., Zeng, X., Luo, Y., He, X., Yang, M., Lv, T., Feng, Z., Bao, L., Zhao, L., Huang, D., & Huang, Y. (2024). Mitochondrial GRIM19 Loss Induces Liver Fibrosis through NLRP3/IL33 Activation via Reactive Oxygen Species/NF-κB Signaling. *J Clin Transl Hepatol*, *12*(6), 539-550. https://doi.org/10.14218/jcth.2023.00562

Xue, Z., Yang, H., Su, D., Song, X., Deng, X., Yu, C., Sun, C., He, L., You, Y., Gong, Y., Fan, D., Sun, L., Han, X., Fan, R., Zhang, M., Yan, X., Qian, J., & Zhang, J. (2021). Geographic distribution of the *cagA*, *vacA*, *iceA*, *oipA* and *dupA* genes of *Helicobacter pylori* strains isolated in China. *Gut Pathogens*, *13*(1), 39. https://doi.org/10.1186/s13099-021-00434-4

Yamada, S., Kato, S., Matsuhisa, T., Makonkawkeyoon, L., Yoshida, M., Chakrabandhu, T., Lertprasertsuk, N., Suttharat, P., Chakrabandhu, B., Nishiumi, S., Chongraksut, W., & Azuma, T. (2013). Predominant mucosal IL-8 mRNA expression in non-cagA Thais is risk for gastric cancer. *World J Gastroenterol*, *19*(19), 2941-2949. https://doi.org/10.3748/wjg.v19.i19.2941

Yamamoto, T., Kita, M., Ohno, T., Iwakura, Y., Sekikawa, K., & Imanishi, J. (2004). Role of Tumor Necrosis Factor-Alpha and Interferon-Gamma in *Helicobacter pylori* Infection. *Microbiology and Immunology*, *48*(9), 647-654. https://doi.org/https://doi.org/10.1111/j.1348-0421.2004.tb03474.x

Yamaoka, Y. (2010). Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nature Reviews Gastroenterology & Hepatology*, *7*(11), 629-641. https://doi.org/10.1038/nrgastro.2010.154

Yang, C. L., Zeng, Y. D., Hu, Z. X., & Liang, H. (2020). PCSK9 promotes the secretion of pro-inflammatory cytokines by macrophages to aggravate H/R-induced cardiomyocyte injury via activating NF-κB signalling. *Gen Physiol Biophys*, *39*(2), 123-134. https://doi.org/10.4149/gpb-2019057

Yang, D. C., Blair, K. M., Taylor, J. A., Petersen, T. W., Sessler, T., Tull, C. M., Leverich, C. K., Collar, A. L., Wyckoff, T. J., Biboy, J., Vollmer, W., & Salama, N. R. (2019). A Genome-Wide *Helicobacter pylori* Morphology Screen Uncovers a Membrane-Spanning Helical Cell Shape Complex. *Journal of Bacteriology*, *201*(14), 10.1128/jb.00724-00718. https://doi.org/doi:10.1128/jb.00724-18

Yang, W. J., Zhao, H. P., Yu, Y., Wang, J. H., Guo, L., Liu, J. Y., Pu, J., & Lv, J. (2023). Updates on global epidemiology, risk and prognostic factors of gastric cancer. *World J Gastroenterol*, *29*(16), 2452-2468. https://doi.org/10.3748/wjg.v29.i16.2452

Yang, X.-T., Niu, P.-Q., Li, X.-F., Sun, M.-M., Wei, W., Chen, Y.-Q., & Zheng, J.-Y. (2024). Differential cytokine expression in gastric tissues highlights *Helicobacter pylori*'s role in gastritis. *Scientific Reports*, *14*(1), 7683. https://doi.org/10.1038/s41598-024-58407-x

Yao, M., Brummer, G., Acevedo, D., & Cheng, N. (2016). Chapter Eight - Cytokine Regulation of Metastasis and Tumorigenicity. In D. R. Welch & P. B. Fisher (Eds.), *Advances in Cancer Research* (Vol. 132, pp. 265-367). Academic Press. https://doi.org/https://doi.org/10.1016/bs.acr.2016.05.005

Yao, X., & Smolka, A. J. (2019). Gastric Parietal Cell Physiology and *Helicobacter pylori*-Induced Disease. *Gastroenterology*, *156*(8), 2158-2173. https://doi.org/10.1053/j.gastro.2019.02.036

Ye, Q., Shao, X., Shen, R., Chen, D., & Shen, J. (2020). Changes in the human gut microbiota composition caused by *Helicobacter pylori* eradication therapy: A systematic review and meta-analysis. *Helicobacter*, *25*(4), e12713. https://doi.org/10.1111/hel.12713

Yılmaz, Ç., & Özcengiz, G. (2017). Antibiotics: Pharmacokinetics, toxicity, resistance and multidrug efflux pumps. *Biochemical Pharmacology*, *133*, 43-62. https://doi.org/https://doi.org/10.1016/j.bcp.2016.10.005

Yoon, Y. M., Lee, J. Y., Yoo, D., Sim, Y. S., Kim, Y. J., Oh, Y. K., Kang, J. S., Kim, S., Kim, J. S., & Kim, J. M. (2010). *Bacteroides fragilis* enterotoxin induces human beta-defensin-2 expression in intestinal epithelial cells via a mitogen-activated protein kinase/I kappaB kinase/NF-kappaB-dependent pathway. *Infect Immun*, *78*(5), 2024-2033. https://doi.org/10.1128/iai.00118-10

Yu, C., Li, L., Chen, W., Jiao, Y., Yang, N., Yang, E., Zhang, J., Chen, L., & Li, Y. (2011). Levofloxacin susceptibility testing for *Helicobacter pylori* in China: comparison of E-test and disk diffusion method. *Helicobacter*, *16*(2), 119-123. https://doi.org/10.1111/j.1523-5378.2011.00820.x

Yu, J., Jia, Y., Yu, Q., Lin, L., Li, C., Chen, B., Zhong, P., Lin, X., Li, H., Sun, Y., Zhong, X., He, Y., Huang, X., Lin, S., & Pan, Y. (2023). Deciphering complex antibiotic resistance patterns in *Helicobacter pylori* through whole genome sequencing and machine learning. *Front Cell Infect Microbiol*, *13*, 1306368. https://doi.org/10.3389/fcimb.2023.1306368

Yu, J., Yang, P., Qin, X., Li, C., Lv, Y., & Wang, X. (2022). Impact of smoking on the eradication of *Helicobacter pylori*. *Helicobacter*, *27*(1), e12860. https://doi.org/https://doi.org/10.1111/hel.12860

Yuan, X. Y., Yan, J. J., Yang, Y. C., Wu, C. M., Hu, Y., & Geng, J. L. (2017). *Helicobacter pylori* with East Asian-type *cagPAI* genes is more virulent than strains with Western-type in some *cagPAI* genes. *Braz J Microbiol*, *48*(2), 218-224. https://doi.org/10.1016/j.bjm.2016.12.004

Zachrisson, K., Neopikhanov, V., Wretlind, B., & Uribe, A. (2001). MITOGENIC ACTION OF TUMOUR NECROSIS FACTOR-ALPHA AND INTERLEUKIN-8 ON EXPLANTS OF HUMAN DUODENAL MUCOSA. *Cytokine*, *15*(3), 148-155. https://doi.org/https://doi.org/10.1006/cyto.2001.0917

Zamani, M., Ebrahimtabar, F., Zamani, V., Miller, W. H., Alizadeh-Navaei, R., Shokri-Shirvani, J., & Derakhshan, M. H. (2018). Systematic review with meta-analysis: the worldwide prevalence of *Helicobacter pylori* infection. *Alimentary Pharmacology & Therapeutics*, *47*(7), 868-876. https://doi.org/https://doi.org/10.1111/apt.14561

Zankari, E., Allesøe, R., Joensen, K. G., Cavaco, L. M., Lund, O., & Aarestrup, F. M. (2017). PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. *J Antimicrob Chemother*, *72*(10), 2764-2768. https://doi.org/10.1093/jac/dkx217

Zhang, J.-M., & An, J. (2007). Cytokines, Inflammation, and Pain. *International Anesthesiology Clinics*, *45*(2), 27-37. https://doi.org/10.1097/AIA.0b013e318034194e

Zhang, L., Wu, W. K. K., Gallo, R. L., Fang, E. F., Hu, W., Ling, T. K. W., Shen, J., Chan, R. L. Y., Lu, L., Luo, X. M., Li, M. X., Chan, K. M., Yu, J., Wong, V. W. S., Ng, S. C., Wong, S. H., Chan, F. K. L., Sung, J. J. Y., Chan, M. T. V., & Cho, C. H. (2016). Critical Role of Antimicrobial Peptide Cathelicidin for Controlling *Helicobacter pylori* Survival and Infection. *The Journal of Immunology*, *196*(4), 1799-1809. https://doi.org/10.4049/jimmunol.1500021

Zhang, M. (2015). High antibiotic resistance rate: A difficult issue for *Helicobacter pylori* eradication treatment. *World J Gastroenterol*, *21*(48), 13432-13437. https://doi.org/10.3748/wjg.v21.i48.13432

Zhang, S., Wang, X., Wise, M. J., He, Y., Chen, H., Liu, A., Huang, H., Young, S., Tay, C. Y., Marshall, B. J., Li, X., & Chua, E. G. (2020a). Mutations of *Helicobacter pylori* RdxA are mainly related to the phylogenetic origin of the strain and not to metronidazole resistance. *J Antimicrob Chemother*, *75*(11), 3152-3155. https://doi.org/10.1093/jac/dkaa302

Zhang, T., Chen, Z., Zheng, H., Cheng, R., Lian, B., Zhu, C., Cui, W., & Tang, H. (2023). Antibacterial and Anti-inflammatory Effects of Clarithromycin-Loaded Poly(l-Lactide) Membrane in Rabbit Postoperation Model of Chronic Rhinosinusitis. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery*.

Zhang, X., He, Y., Zhang, X., Fu, B., Song, Z., Wang, L., Fu, R., Lu, X., Xing, J., Lv, J., Guo, M., Huo, X., Liu, X., Lu, J., Du, X., Ge, Z., Chen, Z., & Li, C. (2024a). Sustained exposure to *Helicobacter pylori* induces immune tolerance by desensitizing TLR6. *Gastric Cancer*, *27*(2), 324-342. https://doi.org/10.1007/s10120-023-01461-7

Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics*, *9*, 40. https://doi.org/10.1186/1471-2105-9-40

Zhang, Y., Wen, Y., Xiao, Q., Zheng, W., Long, G., Chen, B., Shu, X., & Jiang, M. (2020b). Mutations in the Antibiotic Target Genes Related to Clarithromycin, Metronidazole and Levofloxacin Resistance in *Helicobacter pylori* Strains from Children in China. *Infect Drug Resist*, *13*, 311-322. https://doi.org/10.2147/idr.S235615

Zhang, Y., Yao, Y., Wei, J., & Zhang, Z. (2024b). miR-338-5p regulated the NF-κB/MAPK pathway to alleviate inflammation and oxidative stress by targeting IL-6 in rats with atrial fibrillation. *3 Biotech*, *14*(7), 182. https://doi.org/10.1007/s13205-024-04024-4

Zhang, Y. X., Zhou, L. Y., Song, Z. Q., Zhang, J. Z., He, L. H., & Ding, Y. (2015). Primary antibiotic resistance of *Helicobacter pylori* strains isolated from patients with dyspeptic symptoms in Beijing: a prospective serial study. *World J Gastroenterol*, *21*(9), 2786-2792. https://doi.org/10.3748/wjg.v21.i9.2786

Zhao, J. D., Geng, P. L., Li, Z. Q., Cui, S., Zhao, J. H., Wang, L. J., Li, J. Z., Ji, F. X., Li, G. Y., Shen, G. S., Lin, M. Z., Shen, C. F., & Cao, C. Z. (2012). Associations between interleukin-1 polymorphisms and gastric cancers among three ethnicities. *World J Gastroenterol*, *18*(47), 7093-7099. https://doi.org/10.3748/wjg.v18.i47.7093

Zhao, X., Lu, M., Liu, Z., Zhang, M., Yuan, H., Dan, Z., Wang, D., Ma, B., Yang, Y., Yang, F., Sun, R., Li, L., & Dang, C. (2023). Comprehensive analysis of alfa defensin expression and prognosis in human colorectal cancer [Original Research]. *Frontiers in Oncology*, *12*. https://doi.org/10.3389/fonc.2022.974654

Zhong, C., Wang, S., Xia, L., Yang, X., Fang, L., Zhang, X., Wang, M., Zhao, H., Wang, G., Wu, J., Guo, R., Zhong, M., & Gohda, E. (2024). The tubulin polymerization inhibitor gambogenic acid induces myelodysplastic syndrome cell apoptosis through upregulation of Fas expression mediated by the NF-κB signaling pathway. *Cancer Biol Ther*, *25*(1), 2427374. https://doi.org/10.1080/15384047.2024.2427374

Zhong, Z., Zhang, Z., Wang, J., Hu, Y., Mi, Y., He, B., Zhang, Y., Zhang, X., Xia, X., Huang, H., Lai, Y., Lin, M., Su, C., Zhang, Z., Wu, Z., Lu, L., Zhang, B., Huang, S., Zhong, C., . . . Gao, H. (2021). A retrospective study of the antibiotic-resistant phenotypes and genotypes of *Helicobacter pylori* strains in China. *Am J Cancer Res*, *11*(10), 5027-5037.

Zhu, X., Zhu, C., Zhao, Y., Liu, X., Sa, R., Wang, Y., Wu, K., & Su, J. (2022). Prevalence of *Helicobacter pylori* Virulence Genes and Their Association with Chronic Gastritis in Beijing, China. *Current Microbiology*, *80*(1), 33. https://doi.org/10.1007/s00284-022-03135-6

Zimmermann, P., Ziesenitz, V. C., Curtis, N., & Ritz, N. (2018). The Immunomodulatory Effects of Macrolides—A Systematic Review of the Underlying Mechanisms [Systematic Review]. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.00302

Chapter 8 : Appendix

8.1 : Appendix 1

Determination of qPCR efficiencies for *GAPDH* gene was performed using standard curves from a serial dilution of commercial human cDNA (US Biological) against Ct value. The R^2 value was 0.997, which is close to 1, and thus illustrates a good straight line fit (Figure 1). The threshold was calculated by the Rotorgene software as 0.146. The reaction efficiency was 1.89, and the Pfaffl equation used to calculate the efficiencies, was:

$$E = 10^{[-1/slope]},$$

where E is reaction efficiency, and slope (m) is the gradient from the standard curve (Pfaffl, 2001).

Table 1: Descriptive statistics for each qPCR assay used; determined from standard curves using serially diluted cDNA.

Target Gene	Linearity (R^2)	Threshold Level	Efficiency (E)
GAPDH	0.997	0.146	1.888
DEFB4	0.969	0.277	1.935
DEFA5	0.965	0.289	1.814

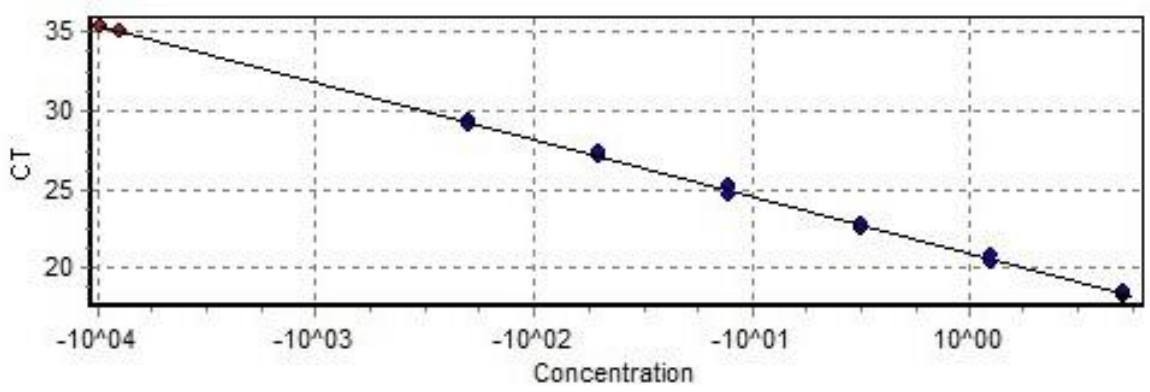


Figure 1: Efficiency plot for *GAPDH*. A four-fold dilution series was carried out in triplicate using *GAPDH* primers and positive control cDNA.

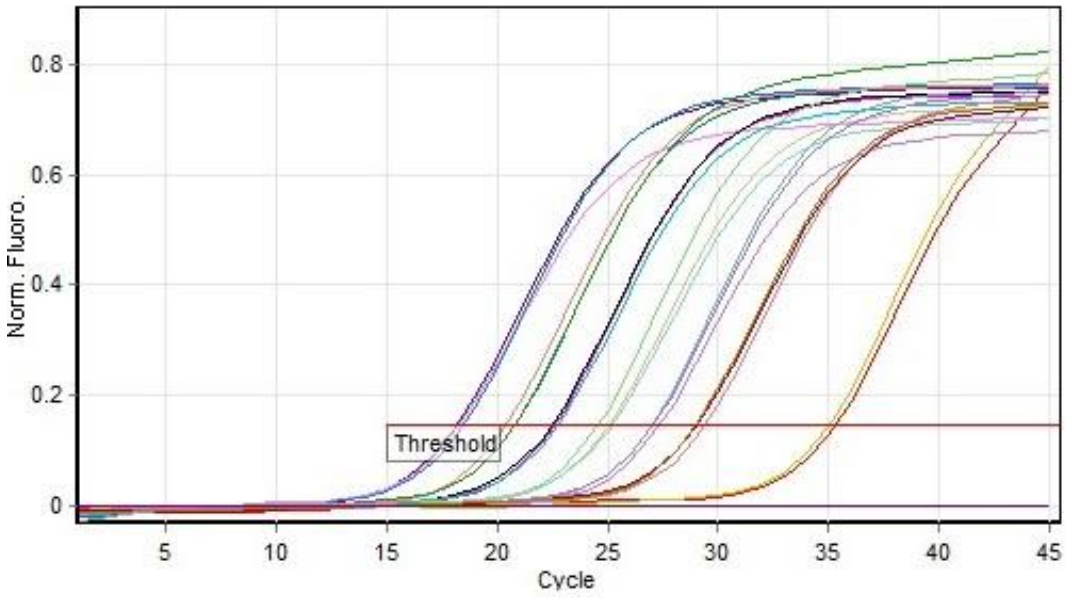


Figure 2: Amplification plot of cycle number against fluorescence for *GAPDH*. The threshold is shown, as calculated via the corresponding efficiency plot (Figure 1).

Figure 2 shows the fluorescence from the cDNA dilutions crossing the threshold, before reaching the exponential phase. The Ct values are plotted to produce the efficiency graph. The same assays were then used to calculate reaction efficiencies and threshold values for human defensins *DEFB4* and *DEFA5*.

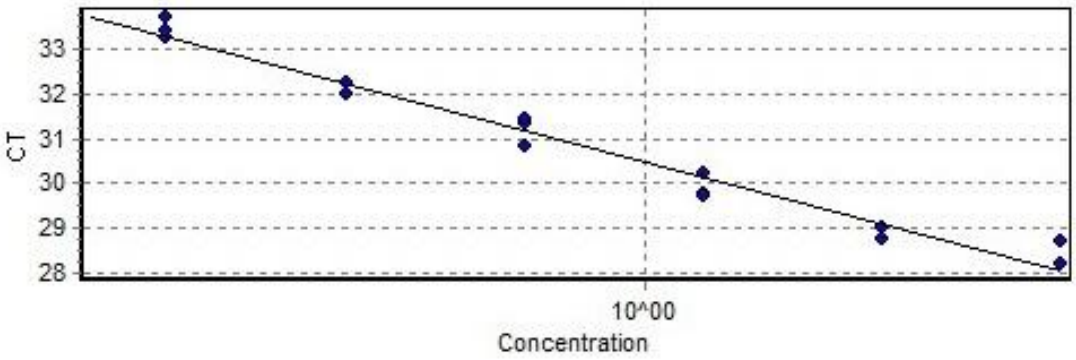


Figure 3: Efficiency plot for *DEFB4*. A four-fold dilution series was carried out in triplicate using *DEFB4* primers and positive control cDNA.

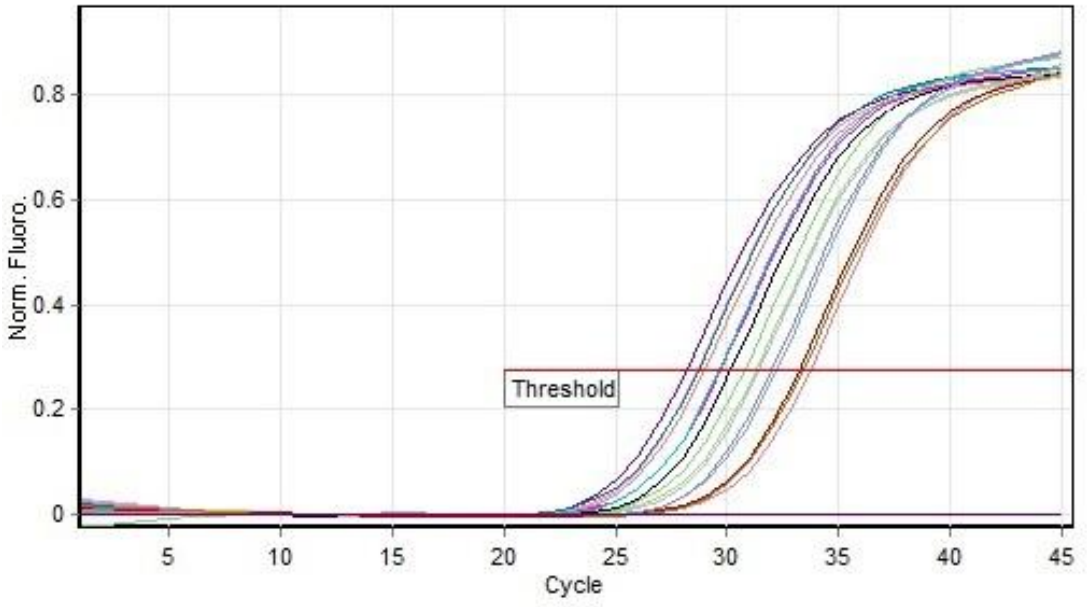


Figure 4: Amplification plot of cycle number against fluorescence for *DEFB4*. The threshold is shown, as calculated via the corresponding efficiency plot (Figure 3).

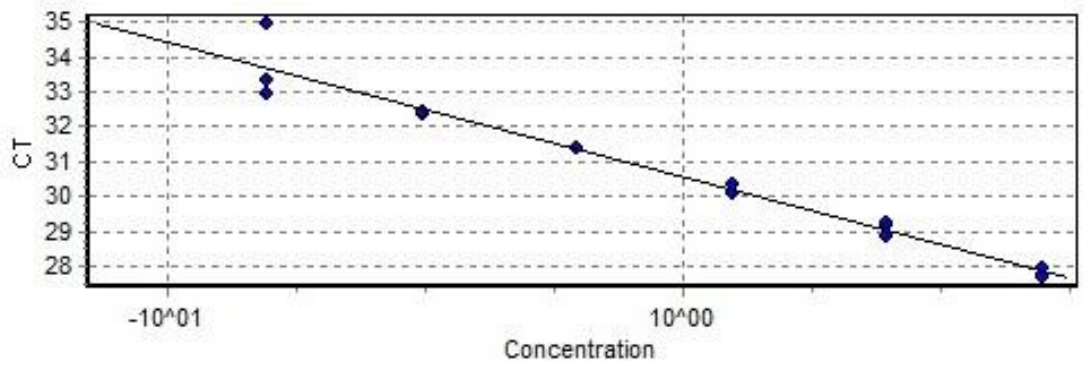


Figure 5: Efficiency plot for *DEFA5*. A four-fold dilution series was carried out in triplicate using *DEFA5* primers and positive control cDNA.

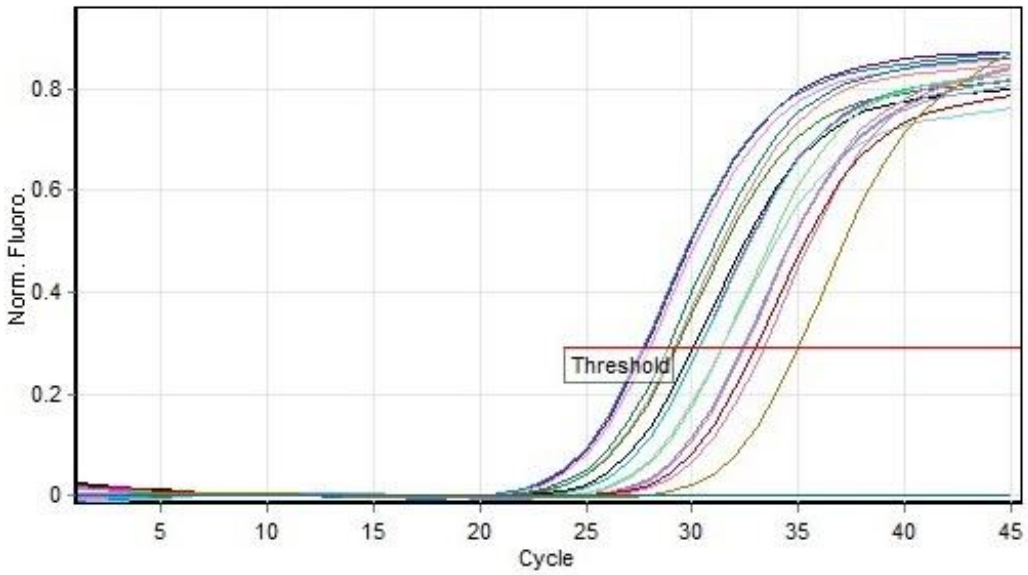


Figure 6: Amplification plot of cycle number against fluorescence for *DEFA5*. The threshold is shown, as calculated via the corresponding efficiency plot (Figure 5).

8.2 : Appendix 2

Appendix 2: Consistency between antibiotic susceptibility phenotype (E-test) and genotype-based predictions using CARD for all 36 *H. pylori* isolates.

H. pylori clinical isolates	Amoxicillin		Clarithromycin		Metronidazole		Levofloxacin	
	E-test results	CARD results	E-test results	CARD results	E-test results	CARD results	E-test results	CARD results
11637	S	R	S	R	R	S	S	S
45a	S	R	S	R	S	R	S	S
45c	S	R	S	R	S	R	S	S
77a	S	R	S	R	R	R	S	S
77c	S	R	S	R	R	R	S	S
120a	R	R	S	R	S	R	S	S
120c	R	R	S	R	S	R	S	S
194a	S	R	R	R	R	S	S	S
194c	S	R	R	R	R	S	S	S
249c	S	R	R	R	R	S	S	S
265a	S	R	S	R	S	R	S	S
265c	S	R	S	R	R	R	S	S
295a	S	R	S	R	R	R	S	S
295c	S	R	S	R	R	R	S	S
308a	R	R	R	R	S	R	R	R
308c	R	R	R	R	S	R	R	R
322a	S	R	S	R	S	R	S	S
322c	S	R	S	R	R	R	S	S
326a	S	R	S	R	R	R	S	S

326c	S	R	S	R	R	R	S	S
335a	S	R	S	R	R	S	S	S
335c	R	R	R	R	R	S	S	S
350a	R	R	R	R	R	S	S	S
350c	R	R	R	R	R	S	S	S
391c	R	R	R	R	R	R	S	S
439a	S	R	S	R	R	R	S	S
439c	S	R	S	R	R	R	S	S
444a	S	R	S	R	S	R	S	S
444c	S	R	S	R	S	R	S	S
495a	S	R	S	R	R	R	S	S
495c	S	R	S	R	R	S	S	S
537a	S	R	S	R	S	R	S	S
565a	S	R	S	R	S	R	S	S
565c	S	R	S	R	R	R	S	S
732a	S	R	S	R	S	R	S	S
732c	S	R	S	R	R	R	S	S

*R=resistant, S=susceptible, a=gastric antrum biopsy and c=gastric corpus biopsy.