

# *Helicobacter pylori* Biofilm-Related Drug Resistance and New Developments in Its Anti-Biofilm Agents

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**Abstract:** *Helicobacter pylori* is one of the most common pathogenic bacterium worldwide, infecting about 50% of the world's population. It is a major cause of several upper gastrointestinal diseases, including peptic ulcers and gastric cancer. The emergence of *H. pylori* resistance to antibiotics has been a major clinical challenge in the field of gastroenterology. In the course of *H. pylori* infection, some bacteria invade the gastric epithelium and are encapsulated into a self-produced matrix to form biofilms that protect the bacteria from external threats. Bacteria with biofilm structures can be up to 1000 times more resistant to antibiotics than planktonic bacteria. This implies that targeting biofilms might be an effective strategy to alleviate *H. pylori* drug resistance. Therefore, it is important to develop drugs that can eliminate or disperse biofilms. In recent years, anti-biofilm agents have been investigated as alternative or complementary therapies to antibiotics to reduce the rate of drug resistance. This article discusses the formation of *H. pylori* biofilms, the relationship between biofilms and drug resistance in *H. pylori*, and the recent developments in the research of anti-biofilm agents targeting *H. pylori* drug resistance.

**Keywords:** *Helicobacter pylori*, biofilm, biofilm formation, anti-biofilm molecules, antibiotic resistance, resistance mechanism

## Introduction

*Helicobacter pylori* is a Gram-negative microaerobic spiral rod-shaped bacterium that was first cultured and identified by Professors Marshall and Dr. Warren.<sup>1</sup> This bacterium primarily colonizes the gastric mucosal surface and has been linked to chronic gastritis, peptic ulcer, gastric mucosa-associated tissue lymphoma (MALT), gastric cancer, and other upper gastrointestinal disorders.<sup>2</sup> Standard triple therapy (proton pump inhibitor + two antibiotics) and bismuth quadruple therapy (proton pump inhibitor + bismuth + two antibiotics) are the most commonly used during eradication therapies for *H. pylori*.<sup>3</sup>

*H. pylori* secrete proteins, polysaccharides, extracellular DNA (eDNA), and other molecules to create extracellular polymeric substances (EPS) after colonizing the gastric mucosa, which are wrapped and adhered to each other by bacteria to form biofilms.<sup>4</sup> Unlike planktonic bacteria, colonies that form biofilm structures are highly resistant to the harsh external environment, including antibiotic exposure.<sup>5</sup> It is well established that when bacteria develop biofilms, their resistance to antibiotics increases by up to 10–1000 times.<sup>6</sup> Therefore, the formation of *H. pylori* biofilms is most likely the primary cause of long-term chronic infection, multiple drug resistance, and treatment failure.

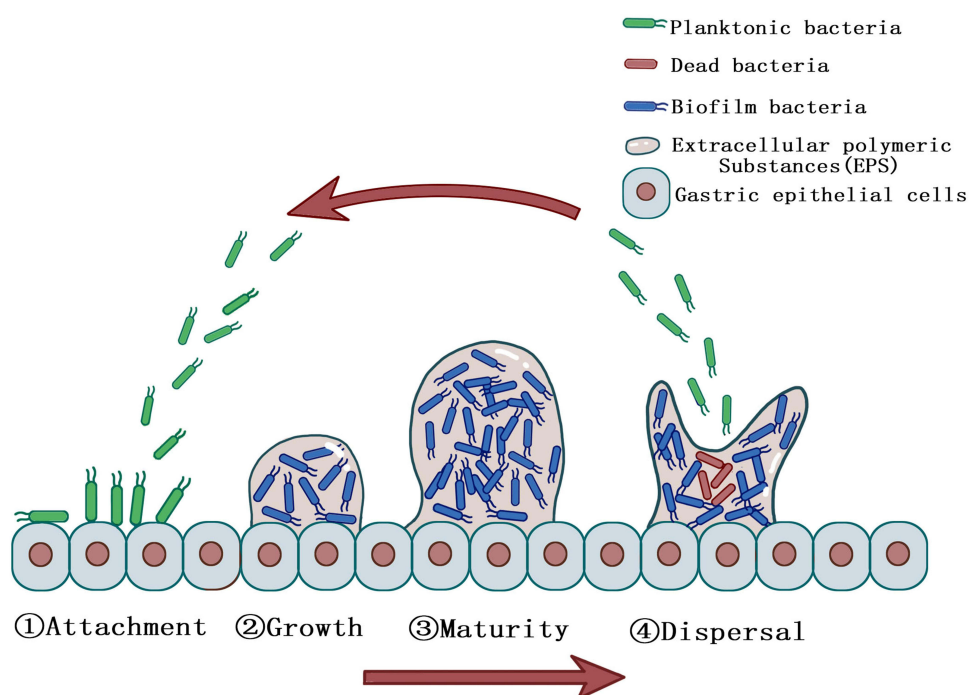
Consequently, strategies targeting biofilms can be applied to alleviate *H. pylori* drug resistance. Research should be directed at developing anti-biofilm agents/molecules and determine their minimum effective concentration that can completely eradicate biofilm with maximum potency without causing unwanted side effects to the host.

## ***Helicobacter pylori* Biofilm**

The formation of *H. pylori* biofilms decreases efficacy of conventional eradication treatments.<sup>7</sup> Individual planktonic bacteria which grow on agar plates or broths are often used as platforms for antibiotic susceptibility testing. However, these bacteria do not exist as independent individuals, and the majority do not live as a single species.<sup>8</sup> About 80% of the world's bacteria are known to exist as biofilms,<sup>9</sup> and this has been their major means of survival for billions of years.<sup>10</sup> Bacteria that form biofilms adhere to one another using extracellular polymeric substances (EPS) composed of polysaccharides, proteins, extracellular DNA (eDNA), and share information using quorum sensing (QS) system, allowing them to live in an organized manner similar to that of animal populations.<sup>4</sup> In the event of a threat, such as drastic changes in temperature and pH, nutrient and oxygen deficiency, antibiotic exposure, or other similar events, they are able to respond immediately.

### **Steps of *H. pylori* Biofilm Formation**

Majority of *H. pylori* strains are capable of forming biofilms in vivo and in vitro depending on the strain.<sup>11,12</sup> Clinical strains isolated from the gastric mucosa of patients have been reported to have higher capacity to form biofilm than other strains.<sup>13</sup> The formation of biofilms by *H. pylori*, like other bacteria, is divided into four steps: (1) attachment, (2) growth, (3) maturity, and (4) dispersal (Figure 1).<sup>14</sup> *H. pylori* adheres to the gastric epithelial cells in the gastric sinus. Co-adhesion is the term used to describe adhesion that occurs between the bacterial cytosol and the gastric epithelial cells.<sup>15</sup> This process is driven by bacterial structures such as flagella, pili, and lipopolysaccharides, and it is also involved in the initial step of *H. pylori* pathogenicity. Bacterial adhesion to a surface can upregulate the secretion of intercellular signaling molecules via a QS mechanism within minutes and co-produce EPS with surrounding bacteria to establish firm and irreversible microcolonies.<sup>16,17</sup> Bacteria in microcolonies continue to proliferate and produce EPS, which promotes bacterial coaggregation and results in the formation of an early biofilm structure.<sup>15</sup> The biofilm matures after 2–4 days after initial adhesion and is maintained for some time.<sup>17</sup> When nutrients are depleted in the biofilm and waste metabolites accumulate to a specific concentration threshold, the biofilm disintegrates into the dispersal stage. This process is mediated by several mechanisms, including termination of the synthesis of biofilm matrix compounds, degradation of



**Figure 1** Steps of *H. pylori* biofilm formation. Planktonic *H. pylori* (green) adhere to the surface of gastric epithelial cells and secrete molecules to create extracellular polymeric substances (EPS) to form an early biofilm structure and biofilm *H. pylori* (blue) are protected from the adverse external environment. After the biofilm matures, dead *H. pylori* (red) gradually appear due to the lack of nutrients in the biofilm and the biofilm dispersal into the next cycle.

the matrix, and disruption of non-covalent interactions between matrix components.<sup>18</sup> After dispersal, the bacteria undergo the next stage of expanded infection and biofilm formation in a new ecological niche. In this regard, if the concentration or dose of antimicrobial agents is insufficient, or if only anti-biofilm agents without antimicrobial activity are used during the eradication of *H. pylori* with biofilm formation, it will not be possible to effectively eradicate all bacteria, but rather flush out only the biofilm matrix, and the dispersed flora may re-adhere to the gastric epithelium, further expanding the size of the biofilm and the scope of infection. This might be the primary reason why, in some studies, the size of the biofilm increased rather than decreased following the application of sub-inhibitory doses of antibiotics.<sup>19</sup> This also implies that some anti-biofilm agents that lack antimicrobial activity may require to be supplemented with antibiotics to improve the therapeutic effect, as they may otherwise make the infection more severe.

## Regulatory Pathways Affecting Biofilm Formation of *Helicobacter pylori*

Research on regulatory pathways affecting *H. pylori* biofilm formation is still in its infancy. The most extensively pathway is the intercellular communication mechanism known as the quorum sensing (QS) system.<sup>20</sup> Bacteria are capable of autonomous growth, division, sensing, and adaptation to environmental signals. The process of biofilm formation is a community behavior in which bacteria interact with one another and regulate gene expression in response to population density changes, allowing bacteria to adapt to changes in the external environment.<sup>21</sup> The transition between these two states is governed by QS signaling molecules such as N-acyl-homoserine lactones (AHL), autoinducing peptide (AIP), autoinducer-2 (AI-2), and diffusion signaling factor (DSF).<sup>22,23</sup> Cole et al found that specific mutations in the *cagE* type IV secretion gene and quorum-sensing gene *luxS* may be associated with enhancing the ability of *H. pylori* biofilm formation.<sup>24</sup> Elsewhere, Wong et al used comparative genomics to sequence the entire genomes of 32 biofilm-forming clinical strains and found that genes involved in *H. pylori* biofilm formation included alpha (1,3)-fucosyltransferase, flagellar protein, 3 hypothetical proteins, outer membrane protein, and a *cag* pathogenicity island protein.<sup>25</sup> These genes play a role in bacterial motility, lipopolysaccharide (LPS) synthesis, Lewis antigen synthesis, adhesion, and/or the type-IV secretion system (T4SS). The outer membrane protein AlpB plays a critical role in the formation of strong biofilms by the TK1402 strain.<sup>26</sup> In ArsRS mutant strains, the outer membrane protein HomB is required for hyperbiofilm formation and aberrant regulation of this gene is sufficient to induce a hyperbiofilm phenotype.<sup>27</sup> SpoT has been implicated in biofilm formation in multi-drug resistant bacteria by upregulating the efflux pump HP1174 and the neutrophil-activating protein (NapA; HP0243).<sup>28,29</sup> The transporter proteins HP0939, HP0497, and HP0471 are implicated in *H. pylori* biofilm formation.<sup>30</sup>

## Methods for Detecting Biofilms

The most frequently used staining method for the quantitative determination of in vitro biofilms grown attached in micro-titer polystyrene well plates is crystalline violet (CV).<sup>31,32</sup> After staining, the scanning electron microscope is used to observe the three-dimensional structure of biofilms.<sup>11</sup> In contrast, the crystal violet staining method has its limitations, since it requires repeated washing, which invariably decreases the number of biofilm cells. Other methods for detecting biofilm formation include the tissue culture plate method,<sup>33</sup> bioluminescence analysis,<sup>34</sup> transmission percentage (%T) method,<sup>35</sup> and some other biofilm imaging techniques such as fluorescence microscopy examination, confocal laser scanning microscopy (CLSM), infrared spectroscopy, and optical fluorimetry.<sup>36,37</sup>

## The Mechanism of Drug Resistance in *Helicobacter pylori* Biofilm

At present, the mechanisms of *H. pylori* biofilm resistance are not fully elucidated, and there is considerable room for doubt and evidence. However, according to current research, they are mostly related to the resistance mechanisms described below.

### EPS Barrier Protection

EPS plays a primary role in *H. pylori* biofilm resistance. Because the target of antibiotics is typically situated within the bacterial cell and EPS is located in the outermost layer of the biofilm, EPS wraps around the bacterium, avoiding direct interaction of the body's immune cells with the bacteria and decreasing antibiotic penetration.<sup>38</sup> Furthermore, because

EPS is generally negatively charged and some of antibiotics are positively charged, the EPS component of the biofilm also forms a natural charge barrier, limiting antimicrobial agents transport.<sup>39</sup>

## *H. pylori* Coccoid Formation

There are two forms of viable *H. pylori*, the spiral form, which is highly culturable and colonizable, and the viable but non-culturable (VBNC) coccoid form, also known as the persistent form, which is a dormant state of the bacterium.<sup>40,41</sup> When the external environment is unfavorable for *H. pylori* growth and reproduction, such as lack of nutrients, changes in oxygen concentration or pH, and antimicrobial drug intervention, *H. pylori* form biofilms and undergoes transformation from spiral to coccoid forms.<sup>42</sup> In general, antimicrobial drugs have excellent inhibitory and bactericidal effects only on bacteria in their active phase, while dormant bacteria located deep inside the biofilm are difficult to kill. Lewis observed that while most cells in the biofilm are sensitive to antibiotics, a small proportion of persistent cells survive, independent of the antibiotic concentration.<sup>43</sup> Biofilm-protected cells can withstand large dosages of antibiotics as well as immunological defense mechanisms. When antibiotic concentrations are reduced, coccoid *H. pylori* transform back into a reproducible spiral and repopulate the biofilm or disperse out of it to form a new biofilm.<sup>43,44</sup>

## Involvement of Efflux Pumps

The efflux pump is a multidrug transporter protein that is found on the bacterial cell membrane. The pump transports antimicrobial drugs out of the bacterium, thereby decreasing the intracellular concentration of antimicrobial drugs which promotes drug resistance. It is the primary cause of multi-drug resistance in *H. pylori*. A previous study showed that when biofilms were exposed to clarithromycin, they developed substantial levels of resistance compared to planktonic cells, and significant expression of efflux pump genes was detected in these biofilm cells.<sup>45</sup> Other studies have revealed that the expression of efflux pump genes Hp605, Hp971, Hp1327, Hp1489, Hp118, and Hp1174 is remarkably higher in bacteria that form biofilms than in planktonic bacteria,<sup>5,28</sup> implying that efflux pumps and biofilms can work synergistically to increase drug resistance.

## Other Drug Resistance Mechanisms

Research has revealed that increased propagation of antibiotic resistance genes in biofilms through horizontal gene transfer, integration of conjugative elements, and natural transformation leads to drug resistance.<sup>46–48</sup> Point mutations at positions 2142 or 2143 in the V structural domain of 23S rRNA in biofilms results in development of drug resistance.<sup>45</sup> Hathroubi et al found that biofilm formation causes changes in outer membrane proteins associated with antibiotic resistance and that increasing proteinase K levels can alleviate clarithromycin resistance.<sup>49</sup> Furthermore, eDNA in biofilms promotes microbial adhesion, inhibits antibiotic diffusion, and chelates cations.<sup>50</sup> Some extracellular enzymes in the biofilm may have hydrolytic effects on antibiotics.<sup>51</sup>

## Anti-Biofilm Agents Against *H. pylori*

### Natural Products

As indicated in Table 1, most antibiofilm agents are mainly isolated from natural products, many of which are “secondary” metabolites and can be produced by microorganisms,<sup>52</sup> such as phytochemicals, biosurfactants, antimicrobial peptides, and microbial enzymes, etc.<sup>53</sup> In addition, several quorum sensing inhibitors and probiotics have been found to show anti-biofilm activity.<sup>54,55</sup> It is interesting to note that all natural products in Table 1 have anti-*H. pylori* biofilm activity, and nearly all of them also have antibacterial ability. These natural products have good anti-biofilm and antibacterial abilities in vitro, whereas some of the natural products such as *Pistacia vera* L. oleoresin, Dihydrotanshinone I (DHT), Amu-ru 7, and *Casearia sylvestris* leaf derivatives are effective against *H. pylori* both in vitro and in vivo.<sup>56–59</sup> It is noteworthy that some of the natural products tested for anti-biofilm and antibacterial ability were carried out using *H. pylori* strains that were resistant to one or more drugs.<sup>12,56,60</sup> This suggests that some natural products have the potential to alleviate *H. pylori* multidrug resistance. Evidence from prior studies has indicated that *H. pylori* eradication therapy requires a combination of different antibiotics such as clarithromycin (CLR), levofloxacin

**Table I** Natural Anti-Biofilm Agents Targeting *H. pylori* Infection

Natural Anti-Biofilm Agents	<i>H. pylori</i> Strains	In vivo/in vitro	Antibacterial Activity	Synergistic Antibiotics	References
<i>Chelidonium majus</i> and <i>Corydalis cheilanthifolia</i> Extracts	<i>H. pylori</i> 8064	in vitro	√	AMX	[88]
<i>Atractylodes lancea</i> volatile oils	NCTC 11637	in vitro	√		[89]
<i>Pistacia vera</i> L. oleoresin	clinical strains	in vitro and in vivo	√	LVX	[56]
Dihydrotanshinone I	G27 ATCC 43504 26,695 NSH57 clinical strains BHKS159	in vitro and in vivo	√	MTZ	[57]
Armeniaspirol A	G27 Hp159 clinical strains BHKS159	in vitro and in vivo	√		[12]
curcumin	ATCC 43504	in vitro			[90]
3-Bromopyruvate or Sertraline	ATCC 51932 <i>H. pylori</i> 8064	in vitro	√		[91]
Antimicrobial Peptide Cathelicidin	SSI <i>H. pylori</i> 10,783	in vitro	√		[92]
Alginate Lyase	ATCC 43629	in vitro		CLR	[93]
<i>Aloe vera</i> inner gel	<i>H. pylori</i> 3/2013/A <i>H. pylori</i> 21/2011/A <i>H. pylori</i> 16/2012/A ATCC 43629	in vitro	√		[60]
Amu-ru 7	ATCC 43503 NCTC 11638 clinical strains	in vitro and in vivo	√		[58]
<i>Hibiscus rosa sinensis</i> L. Flower	ATCC 43504 ATCC 51932 clinical strains	in vitro	√		[94]
Rhamnolipid	SSI	in vitro	√	CLR, AMX	[95]
Sodium Lauryl Sulfate	SSI	in vitro	√		[96]
<i>Lactobacillus salivarius</i> LN12	SSI ATCC43504 clinical strains	in vitro	√	CLR, AMX	[64]
Resveratrol	ATCC 43629 clinical strains	in vitro	√	LVX	[97]
Myricetin	ATCC 700824 ATCC 51932	in vitro	√	CLR, AMX LVX, MTZ, TET	[65]

(Continued)

Table 1 (Continued).

Natural Anti-Biofilm Agents	<i>H. pylori</i> Strains	In vivo/in vitro	Antibacterial Activity	Synergistic Antibiotics	References
<i>Casearia sylvestris</i> leaf derivatives	ATCC 43504	in vitro and in vivo	√		[59]
<i>Lactobacillus plantarum</i> LN66	ATCC 43504	in vitro		CLR, LVX	[98]
Nimbolide	G27 26,695 HPAG I SSI J99 7.13 USUI01 USUI03UA1182 (ATCC 700684)	in vitro	√		[99]
Carvacrol and Thymol	NCTC 11637 (ATCC 43504)	in vitro			[100]
Dioscin	<i>H. pylori</i> J99	in vitro	√	CLR, MTZ, LVX	[101]

(LVX), amoxicillin (AMX), metronidazole (MTZ), and tetracycline (TET).<sup>61–63</sup> A combination of classically used antibiotics with natural products can synergistically fight against *H. pylori*. *Pistacia vera* L. oleoresin synergizes with levofloxacin to suppress drug-resistance in *H. pylori* strains.<sup>56</sup> When *Lactobacillus salivarius* LN12 cell-free supernatant (CFS) was used in combination with AMX and CLR, they disrupted the biofilm structure of some strains much more effectively than when each agent was applied alone.<sup>64</sup> Myricetin was the only natural product that synergized with all five traditional anti-*H. pylori* antibiotics to disrupt the transition of *H. pylori* from spiral to coccoid forms.<sup>65</sup> In comparison, several anti-biofilm agents appear to be more effective in eradicating *H. pylori* than a combination of some antibiotics. Armeniaspirol A (ARM1) exerted potent antibacterial activity against *H. pylori* (including multidrug-resistant strains). Moreover, a combination of ARM1 and omeprazole more effectively killed *H. pylori* in vivo compared to standard triple therapy in a mouse model of multidrug-resistant *H. pylori* infection.<sup>12</sup> The combination of DHT and omeprazole also showed superior *H. pylori*-killing effect than standard triple therapy, suggesting that DHT may be suitable anti-*H. pylori* drug when combined with a proton pump inhibitor.<sup>57</sup> From the above, it follows that natural products have great potential to combat *H. pylori* biofilms and to address the problem of drug resistance in *H. pylori*.

## Nanoparticles

In recent years, nanomaterials have also been used to eradicate *H. pylori* biofilms and minimize drug resistance.<sup>66–68</sup> New Synthesized Silver Ultra-NanoClusters (SUNCs) alone or in combination with metronidazole exhibit good anti-biofilm and antibacterial activity.<sup>69</sup> Nanodrugs made of berberine derivatives and rhamnolipids (RHL) penetrated the mucus layer and effectively cleared *H. pylori* biofilms in vitro and in vivo.<sup>70</sup>

## Acetylcysteine

N-acetylcysteine is the only molecule in clinical trials that has been found to be effective against *H. pylori* biofilms.<sup>71</sup> It is an antioxidant that breaks down of mucus and is most frequently used to treat chronic respiratory tract infections.<sup>72</sup> Numerous studies have demonstrated that NAC inhibits bacterial adhesion, decreases the viability of sequestered cells, disrupts mature biofilms of a variety of bacteria, and inhibits the production of extracellular polysaccharide substrates.<sup>73–75</sup> NAC pretreatment improves the outcome of patients with refractory *Helicobacter pylori* infection before initiating triple therapy.<sup>71</sup>



## Other

A previous study showed that Extremely Low-Frequency Electromagnetic Fields (ELFs) can reduce *H. pylori* biofilm adhesion and formation.<sup>76</sup> A combination of curcumin and blue light irradiation for more than 6 minutes disrupted *H. pylori* mature biofilms by more than 50% and enhanced the antimicrobial effect.<sup>77,78</sup> An Electrolyzed Superoxidized Solution exerted antibacterial and anti-biofilm effects against *H. pylori*.<sup>79</sup>

## Shortcomings of the Current Study

First, researchers have made significant progress in understanding biofilms for opportunistic pathogenic bacteria, particularly those found in hospitals. Many of these bacteria are typically sequestered on surfaces of indwelling medical devices.<sup>80</sup> For example, *Pseudomonas aeruginosa*, which can form biofilms on medical equipments such as catheters, implants, and contact lenses,<sup>81</sup> *Escherichia coli*, which can also form biofilms on surfaces of indwelling catheters,<sup>82</sup> *Acinetobacter baumannii*, which has been reported to cause ventilator-associated and catheter-associated biofilm infections.<sup>83</sup> Staphylococcus, which can result in mechanical heart valves-associated and central venous catheter-associated biofilm infections,<sup>84</sup> and *Candida*, which can form biofilms on medical devices such as vascular catheters, joint prostheses, and dialysis catheters,<sup>85</sup> etc. To date, there are few studies on *H. pylori* biofilms, and research is still at the preliminary stage. Given that biofilms may be an important cause of *H. pylori* drug-resistant and long-term infections, significant research attention should be directed at *H. pylori* biofilms. Second, most anti-biofilm agents targeting *H. pylori* have been mainly tested in vitro using only standard strains such as SS1, ACTC43503, NCTC11639, and G27, which are not geographic- or strain-specific.<sup>7,86</sup> Even when clinical strains were used, the majority of them were isolated from infected patients and cultured in vitro to form biofilms. This does not perfectly reflect the biofilm formation process in the in vivo environment.<sup>87</sup> Currently, there is no clinical guideline and therapeutic agent for biofilm infections. In vivo and clinical trials must be improved and practiced. Finally, rapid and accurate diagnostic tools for *H. pylori* biofilms should be developed for effective treatment and prevention of long-term chronic infection. In addition, conventional microbial culture, molecular biology, and other tests, should be carried out in the early stages of biofilm infection, for accurate diagnosis and subsequent anti-biofilm treatment.

## Conclusion and Perspectives

The emergence of drug resistance in *H. pylori* has become a unique clinical challenge. The formation of *H. pylori* biofilms is considered an important factor contributing to antibiotic resistance in humans. Thus, anti-biofilm agents should be developed because they have strong antagonistic effect against bacterial biofilms. This will decrease drug resistance hence increase the eradication rate of *H. pylori* and providing us with a new approach to address the antibiotic resistance problem. Unconfirmed speculation suggests that anti-biofilm agents will most likely become the new treatment approach in addressing the failure of *H. pylori* eradication therapy and multi-drug resistance in the near future.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare that they have no conflicts of interest.

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