ALTERNATIVE THERAPIES BEYOND ANTIBIOTICS: HELICOBACTER PYLORI ERADICATION

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ABSTRACT
Helicobacter pylori is a Gram-negative human gastric pathogen infecting about 30% of children and 60% of adults worldwide and has a possible etiologic role in peptic and gastric ulcers, gastric adenocarcinoma and more rarely, lymphoma of the mucosa-associated lymphoid tissue. Currently, this is managed by the use of a triple therapy, involving the co-administration of two antibiotics (with macrolides, fluoroquinolones, amoxicillin, nitroimidazoles and tetracycline among the most prescribed ones) and ranitidine bismuth and/or a proton pump inhibitor. However, as for other bacteria, during the last decades we have been observing the continuous growth of the number of antibiotic resistant H. pylori strains, especially in countries in which antibiotic prescription is more common. The high prevalence rates worldwide and the real risk of severe gastric diseases development demand the need for good strategies for H. pylori eradication. Still growing antibiotic resistance rate already leads to treatment failure in about 20% of the H. pylori-infected patients, but depending on the therapeutic schema and strain resistance pattern, the failure rate can reach 70%, which has led to the search for alternative therapies. This review focuses on the state of the art of alternative strategies for combating H. pylori infection, not involving the use of antibiotics, currently under investigation, namely vaccines, bacterial photodynamic inactivation, phage therapy, the use of probiotics and natural compounds.

KEYWORDS: Helicobacter pylori; Probiotics; Bacteriocin; Vaccines; Phage therapy.

HELICOBACTER PYLORI INFECTION
In the early 1980s, Barry Marshall and Robin Warren reported the successful isolation and culture of a bacterial species from the human stomach, whose presence was closely
associated with mucosal inflammation.\cite{1} This discovery revolutionized our understanding of upper gastrointestinal tract diseases and triggered a new era in the management of gastritis and peptic ulcer. The bacterium, previously named \textit{Campylobacter pyloridis}, and later on \textit{Helicobacter pylori}, is a gastric Gram negative bacterium belonging to the phylum \textit{Proteobacteria}, \textit{Epsilonproteobacteria} class, \textit{Campylobacterales} order and \textit{Helicobacteraceae} family. \textit{H. pylori} is a microaerophilic, flagellated, spiral bacterium. Currently, the genus \textit{Helicobacter} is composed by more than a dozen gastric species, that have been detected in different animals, such as cats, dogs, pigs and primates, although the zoonotic potential of these species is still unclear.\cite{2}

\textit{H. pylori} is the major human pathogen, affecting over 50\% of the world’s population. It is estimated that \textit{H. pylori}-positive patients have a 10 to 20\% lifetime risk of developing ulcer disease and a 1 to 2\% risk of developing distal gastric malignancy.\cite{3,4} The prevalence of infection is highly dependent on the socioeconomic status of the country, varying from 20 to over 80\% of the population, with higher rates for developing countries, which are characterized by poor sanitary conditions, crowded living conditions and lack of clean water.\cite{5} In developed countries, infection is associated more with lower socioeconomic groups with a prevalence of the infection ranging from 10-60\%.\cite{6} Risk factors include increasing age, large family size and low socioeconomic conditions.\cite{7,10}

\textit{H. pylori} is elegantly well adapted for a lifelong colonization of the gastric mucosa of humans, using complex strategies to maintain an inflammation of the gastric epithelium, while limiting the extent of the immune response in order to prevent its elimination. \textit{H. pylori} has a very strong affinity for epithelial lining of stomach and duodenum, where it attaches and subsequently disrupts microvilli and tight junctions between adjacent cells. Eroded epithelial lining allows acid and bacteria to get through it and establishes pathogen in the mucous layer. Besides the unusual high degree of genetic variability, due to its natural competence for transformation and conjugative transfer of genomic islands and a high recombination rate,\cite{11} some factors have been identified as critical for colonization and persistence of infection such as urease, flagella and adhesins. Then, once having escaped the gastric acidity and reached the gastric mucus layer and epithelium, the bacterium delivers its virulence factors, CagA and VacA, encoded by the \textit{cytotoxin-associated gene A} and the \textit{vacuolating cytotoxin} respectively, the two major toxins which damage the gastric mucosa and cause disease.\cite{12}
Infection by *H. pylori* is usually acquired during childhood.\(^{[13]}\) Most infected individuals remain asymptomatic throughout life. In some cases, this gastric inflammation may evolve toward more severe diseases such as duodenal or gastric ulcers, gastric lymphoma or non-cardia gastric adenocarcinomas.\(^{[14]}\) In 1994, *H. pylori* infection was classified as a type I carcinogen by the International Agency for Research on Cancer, a World Health Organization agency. Studies have also associated *H. pylori* infection to diverse extragastric non-malignant diseases (involving the cardiovascular, hepatobiliary, dermatological, immunological, and haematological systems).

**ANTIBIOTIC THERAPY**

*H. pylori* infection can be difficult to treat using a single antibiotic and requires the use of several antimicrobials. Currently, a triple therapy regimen is considered the most effective treatment for *H. pylori* eradication. Triple therapy, which consists of two antibiotics and a proton pump inhibitor (PPI), administered for 10-14 days\(^{[15-16]}\), is commonly used to eradicate *H. pylori*.\(^{[17]}\) The most commonly used antibiotics are tetracycline, amoxicillin, metronidazole or clarithromycin. A recent phase 3 trial in Europe showed that a quadruple therapy containing two antibiotics, a PPI and bismuth, should be considered for first-line treatment in view of the rising prevalence of clarithromycin-resistant *H. pylori*.\(^{[18]}\) However, the most recent data show that this combination fails in about 20% of the patients.\(^{[19]}\) Alternative regimens using different combinations of the same antibiotics can increase the rate of treatment, such as the sequential treatment which includes a 5-day period with PPI-amoxicillin, followed by a 5 day period with PPI-clarithromycin-metronidazole (or tinidazole)\(^{[20]}\); the three antibiotics given concomitantly together with a PPI\(^{[21]}\); levofloxacin containing triple therapy\(^{[22]}\); and the bismuth-containing quadruple therapy (bismuth salts, metronidazole, and tetracycline plus PPI\(^{[23]}\)) (Table 1).

**Table 1: Recommended treatment regimens for *H. pylori* infection**

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Drug 1</th>
<th>Drug 2</th>
<th>Drug 3</th>
<th>Drug 4</th>
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</thead>
<tbody>
<tr>
<td><strong>First line therapy</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Regimen 1</td>
<td>Omeprazole</td>
<td>Clarithromycin</td>
<td>Amoxicillin</td>
<td>-</td>
</tr>
<tr>
<td>Mechanism of action</td>
<td>Proton-pump inhibitor, neutralize gastric acidity</td>
<td>Inhibits translation i.e. protein synthesis in the bacteria</td>
<td>Inhibits cell wall synthesis</td>
<td></td>
</tr>
<tr>
<td>Regimen 2</td>
<td>Omeprazole</td>
<td>Clarithromycin</td>
<td>Metronidazole</td>
<td>-</td>
</tr>
<tr>
<td>Mechanism of action</td>
<td>Proton-pump inhibitor, neutralize gastric acidity</td>
<td>Inhibits translation i.e. protein synthesis in the bacteria</td>
<td>Inhibits growth of other anaerobic bacteria</td>
<td></td>
</tr>
<tr>
<td><strong>Second line therapy</strong></td>
<td></td>
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<td></td>
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</tbody>
</table>
Regimen 3

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Omeprazole</th>
<th>Bismuth subsalicylate</th>
<th>Tetracycline</th>
<th>Metronidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proton-pump inhibitor, neutralize gastric acidity</td>
<td>Weak anti-acid properties, stimulates absorption of fluid and electrolytes</td>
<td>Inhibits protein synthesis</td>
<td>Inhibits growth of other anaerobic bacteria</td>
<td></td>
</tr>
</tbody>
</table>

EMERGENCE OF DRUG RESISTANCE IN *HELICOBACTER PYLORI*

Various Indian studies highlight the occurrence of drug resistance in *H. pylori* strains isolated from biopsy or stool samples of the patients and as a major obstacle in eradication of this gastro-duodenal pathogen. According to Mukhopadhyay and coworker about 90% of Calcutta strains of *H. pylori* were metronidazole resistant.[24] The drug sensitivity profile of *H. pylori* isolated from different parts of India, namely, Hyderabad, Mumbai and Lucknow was studied at National Institute of Cholera and Enteric Diseases, Kolkata, India.[25] Most of the strains (85%) have shown resistance to metronidazole and 7.5% strains to tetracycline, which is quite high compared to other reports in India. All Kolkata strains are however, highly sensitive to clarithromycin, furazolidone and amoxicillin. Bacterial genotype also has a great influence on the efficiency of proton pump inhibitor-based triple-therapy regimen. The efficiency of a multidrug formulation consisting of a proton pump inhibitor and two antibiotics viz. omeprazole, clarithromycin and amoxicillin was analysed in patients of Eastern India.[26] Bacterial vacA m1 allele was most represented genotype among patients with eradication failures (68%) than in those with successful eradication (39%) (p<0.05). No significant association of vacAs1 (signal sequence allele) or cag pathogenicity island status with persistence was detected. Persistent infection and recurrence after eradication therapy is a great problem in *H. pylori* infection.[27] Thus, current antibiotic-based triple therapies are not practical for global control due to the high cost, genotypic variation in *H. pylori* strains, problems with patients’ compliance and the emergence of antibiotic-resistant strains.[28]

Considering drug resistance mechanisms, increased side effects, low compliance and the high cost of antibiotic therapy, treatment failure rates are increasing and second line treatment strategies need to be developed.[29]

ALTERNATE THERAPIES

The antibiotic era, a period of massive misuse of antibiotics that extends for almost 100 years, since the discovery of penicillin in 1928 by Sir Alexander Fleming,[30] may be reaching an end because of the rapidly increasing emergence of resistance among several species of pathogenic bacteria, including *H. pylori*. This opened doors to an intense research over the last two decades in the search for alternatives to bacterial eradication. Included in the most
exploited alternative approaches to combat *H. pylori* infections are: the development of efficient anti-*H. pylori* vaccines, which has proven to be a hard goal to achieve; the photodynamic inactivation, an old promising approach that was forgotten for decades; the phage therapy, which began to be studied before the discovery of antibiotics, but was forgotten until recently; the use of probiotics, a new fashion in fighting pathogens and promoting health, using other bacteria or their derivates. Additionally, as briefly discussed of effect of natural compounds against *H. pylori* infection.

**VACCINES**

As with other infectious diseases, making a vaccine against *H. pylori* seems to be an obvious priority in the search for alternatives to antibiotics. In the 1990s, it was thought that this task would be easily achieved, not only because of the knowledge on *H. pylori* biology available at the time, but also because of the already vast experience in the vaccines field, accumulated with the development of vaccines against other pathogens. However, all the spent efforts failed so far, which lead us to share with others, the feeling that the prospects for producing an efficient vaccine against *H. pylori* seem further away than they did 15 years ago.\(^3\) Rendering it more difficult is the marked decline on the invested funds from large pharmaceutical companies. Indeed, even considering that a vaccine should be the most cost-effective approach to deal with this microorganism, the decline in the prevalence of *H. pylori* infection observed in the last decade in developed countries\(^3\)\(^2\), is driving the investor’s attention to the research on other human diseases.

In the early 1990’s, as soon as the *Helicobacter felis* mouse model became available\(^3\)\(^3\), several studies were made in order to proof that vaccination could protect against this gastric infection\(^3\)\(^4\)\(^{-}\)\(^3\)\(^6\). The findings of these authors contributed to the early enthusiasm of the field, as mice immunization was considered well succeeded when used prophylactically \(^3\)\(^4\)\(^{-}\)\(^3\)\(^5\), *i.e.*, prior to infection, but also therapeutically after established infection.\(^3\)\(^6\) However, more accurate methods for quantification of *H. felis*, developed later on, revealed that immunization led to significantly reduced colonization levels, but did not achieve complete and sustained eradication of these bacteria in mice.\(^3\)\(^7\) In the late 1990’s, the scientific community’s expectations were again defrauded with the studies using primates. Indeed, although some authors claimed a significant reduction in the number of bacteria colonizing the stomach of rhesus monkeys, therapeutically immunized with some anti-*H. pylori* vaccine candidates\(^3\)\(^8\)\(^{-}\)\(^3\)\(^9\) others, using similar formulations, failed to detect an effect on bacterial load,
only achieving some reduction in the levels of gastritis.\textsuperscript{40} Moreover, when similar experiments were repeated in rhesus monkeys that had never been infected with \textit{H. pylori} before, no effect at all was observed on gastric bacterial colonization, nor on gastric pathological changes.\textsuperscript{41}

Since then, different formulations (including whole cell and recombinant-antigens’ based vaccines), adjuvants and delivery routes have been tested on animal models, offering them a significant (although not complete) protection against experimental infections.\textsuperscript{42} Most of the tested vaccines were based in \textit{H. pylori} key virulence factors, and on abundant and/or surface exposed proteins. These include urease\textsuperscript{38-40; 43-47}, known for its crucial role in regulating the pH during colonization, the well-established virulence factor CagA in conjugation with the VacA toxin and the neutrophil activating protein.\textsuperscript{48} Other examples of tested proteins are: the antioxidant proteins catalase\textsuperscript{49} and thiolperoxidase\textsuperscript{50}; some heat shock proteins (Hsp) (namely HspB)\textsuperscript{51}; the flagellar sheath protein putative Nacetylneuraminylactose- binding hemagglutinin\textsuperscript{52}; and also some adhesins.\textsuperscript{53} In addition, vaccines composed of predicted immunodominant CD4+ T cell epitopes of some of these \textit{H. pylori} proteins have been tested.\textsuperscript{54} Even considering that some have reached clinical trials\textsuperscript{48}, none resulted yet effective for human use.

Rendering difficult the development of effective vaccines is the aforementioned high degree of genetic variability among \textit{H. pylori} strains, required for the full adaptation of the bacterium to the host’s stomach. Thus, acting as a \textit{quasispecies} each individual host harbours a unique \textit{H. pylori} strain\textsuperscript{55-56}, a fact that has been exploited for a variety of studies regarding human population migrations.\textsuperscript{57} Co-infection with multiple closely related clones of \textit{H. pylori} is also a possibility. This fact, underlies the considerable inter-individual variation of the humoral recognition pattern among different infected patients and \textit{H. pylori} isolates, suggesting both variations on the host immune responses and on the antigenic pattern among strains.\textsuperscript{58} Indeed, by studying the immunoproteome of a group of heterogeneous \textit{H. pylori} strains, isolated from Portuguese patients differing in age, gender and \textit{H. pylori}-associated gastric diseases, we have recently presented clear evidence of the variability of the antigenic pattern among \textit{H. pylori} strains. We demonstrated that genetic variability among strains is not reflected in their proteome but, instead, in their immunoproteome.\textsuperscript{59}

Also strongly impairing the effectiveness of the tested vaccines, is the fact that \textit{H. pylori} guarantees its survival and persistence throughout the life of its host, by using a set of
molecular mechanisms to constantly evade the host immune response.\textsuperscript{[60]} Among such successful strategies of bacterial evasion is the relatively anergic lipopolysaccharide of the \textit{H. pylori} cell wall, which mimics the molecular structures of the host cells surface, through the expression of host-related Lewis antigens.\textsuperscript{[61]} Another camouflage used by \textit{H. pylori}, is the expression of proteins at its surface which specifically bind to host-secreted proteins, \textit{e.g.}, bacterial plasminogen-binding proteins (PgbA and PgbB), allowing the bacterium to be coated with host proteins.\textsuperscript{[62]} Moreover, \textit{H. pylori} uses mechanisms of phase variation to switch between different allelic variants of genes encoding its virulence factors, which allows this organism to adapt to the varying conditions in the niche over time.\textsuperscript{[60; 63]} Perhaps the most ingenious evasion mechanism proposed to be used by \textit{H. pylori} is the “altruist autolysis”. Accordingly, in culture some bacteria suffer a programmed autolytic process, releasing their cytoplasmic proteins that are subsequently adsorbed to the external surface of the neighbouring viable cells, ensuring them a range of protective proteins, such as urease.\textsuperscript{[64]} If occurring \textit{in vivo}, such mechanism could also offer a protective effect against host antibodies as these would no longer be able to strongly agglutinate viable \textit{H. pylori}, because the loosely attached surface proteins would detach, removing the antibodies from the bacterial surface.\textsuperscript{[65]} Taking all together, it is our conviction that the development of efficient anti-\textit{H. pylori} vaccines, for both prophylactic and therapeutic purposes, relies on the fully understanding of the interactions between this pathogen and its host immune system.

Antibiotics and vaccines are not the only weapons that can be used against \textit{H. pylori}. The elimination of risk factors such as poverty and poor hygiene, and the improvement in living conditions could also decrease the prevalence of infection.\textsuperscript{[66-67]}

**PHOTODYNAMIC INACTIVATION**

The use of the solar light as a medical tool dates back the ancient Greece, Egypt and India. In 1904, Herman von Tappeiner introduced term “photodynamic action” in his pioneer studies on photobiology. Although it is not clear why he called the process “dynamic”, a term that others tried unsuccessfully to replace\textsuperscript{[68]}, it persisted through the “photodynamic therapy” (PDT), a promising tool in modern cancer treatment. Underlying this clinical method was the discovery of haematoporphyrin ability to accumulate in tumours, together with its phototoxic effect on cancer cells. Currently, PDT is based on the administration of nontoxic, or of low-toxicity in the dark, photosensitive molecules, usually referred to as photosensitizers, that,
once irradiated with light at appropriated wavelength, are activated and, in the presence of O2, trigger a destructive action in biological systems, leading to cell death.[69]

The energy shifts suffered by photosensitizers during PDT is shown in figure. With the absorbing light, the photosensitizer reaches its singlet excited state (1^PS), a highly reactive energetic state from which the photosensitizer may decay by fluorescence to its ground state (PS), or by electronic “intersystem crossing” to its long-lived triplet excited state (3^PS). In this highly reactive triplet state, the photosensitizer reacts with any molecule in its micro environment via energy transfer, two competing pathways called type I and type II reactions, respectively.[70]

Type I reactions are redox reactions, in which the photosensitizer in its excited triplet state exchanges an electron with a neighbouring molecule. Alternatively, in type II reactions, the photosensitizer at its triplet state transfers its energy (not electrons) to the molecular oxygen O2, originating the high-energetic singlet oxygen (1^O2). Due to their short lifetimes and diffusion paths, all the reactive species originated by both types I and II reactions further interact with biomolecules in their immediate vicinity, leading to severe cell damage and, ultimately, death (Fig. 1).

Figure 1: Diagram illustrating the energy shifts of the photosensitizer during PDT. PS, photosensitizer ground state; 1^PS, photosensitizer singlet state; 3^PS, photosensitizer triplet state; ⭐ light at appropriate wavelength.

By now, PDT and various photosensitizers, with porphyrin derivatives among the most widely used[71], have regulatory approval for treatment of several malignant and pre-malignant conditions, including Barrett’s oesophagus and gastric cancer.[72-74] Interestingly,
its application has also been shown to be effective in inactivation of viruses[75], bacteria[76-77], fungi[78] and protozoa[79] In fact, it was already in 1900 that Oscar Raab accidentally discovered that cells of *Paramecium caudatum* were inhibited by the dye acridine orange under illumination, an approach that was immediately put in practice to treat skin infections.[80]

Strongly motivated by the worldwide rise in bacterial resistance to antibiotics[76], the photodynamic inactivation (PDI) of bacteria has gain a new breath and has been widely explored in the past two decades[81], with the studies suggesting that PDI does not induce bacterial resistance[82-83] and its efficiency is independent of the pattern of antibiotic resistance of the strain.[84-85]

Gram-positive bacteria are in general sensitive to PDI.[86] However, its successful application in the eradication of Gram-negative strains has been more challenging. Indeed, with their additional lipid membrane, located externally to the peptidoglycan network, strongly impairing the electrostatic attraction and the access of the photosensitizer to the bacterial cytoplasm, Gram-negative bacteria are not efficiently inactivated with the classical negatively and noncharged photosensitizers.[87] Nevertheless, the use of such photosensitizers in the presence of permeabilization agents (such as CaCl$_2$ and Tris-EDTA) or, alternatively, the use of cationic photosensitizers are considered efficient for *in vitro* PDI eradication of *Escherichia coli*.[88-90] Current research efforts focus on the scrutiny of novel families of photosensitizers and on the study of their structural features that somehow potentiate their antimicrobial effects for *in vivo* use. In fact, physical properties such as the number and type of charge, its distribution over the molecule and the presence of long hydrocarbon chains, have an influence on the hydrophilicity of the photosensitizer and, therefore, on its cellular distribution and PDI effectiveness.[91] Moreover, differences in photochemical properties can lead to differences in $^1$O$_2$ production and decay, when the photosensitizers are taken up by bacterial cells.[90]

Curiously, the nature seems to encourage the use of PDI in eradication of *H. pylori* infection. In fact, this Gram negative bacterium has a natural ability to accumulate photoactive porphyrins, namely protoporphyrin IX and coproporphyrin (by-products of the endogenous heme biosynthesis)[92] Since protoporphyrin IX maximally absorbs light at a wavelength of 410 nm, efficient *H. pylori* killing is possible just by low fluence of violet/blue light (375 - 425 nm)[92-93] or of broad-spectrum conventional white endoscopic light[94], without the need
of any added photosensitizer. Moreover, the localization of the infection in the gastric mucosa facilitates the endoscopic access for light delivery. As shown by Ganz et al.\cite{93}, the delivery of blue light (405 nm, 40 J/cm²) to a 1 cm diameter spot in the gastric antrum, via optical fiber passed through an upper gastrointestinal endoscope, lead to the local death of 90% of the *H. pylori* colony forming units in infected patients. This is a promising procedure that was shown to be safe and feasible for whole-stomach treatment.\cite{95} Recent data have shown that the bactericidal effect of protoporphyrin IX is mediated by cell membrane injury\cite{94} and that of methylene blue is via DNA damage, which is further potentiated by the fact that, in contrast to other Gram-negative bacteria, such as *E. coli*\cite{96}, *H. pylori* expresses fewer genes to repair phototoxicity-induced DNA damage.\cite{97}

Nevertheless, none of the mentioned prospective pilot trials\cite{92-93; 95} achieved complete and sustained eradication of *H. pylori*, stressing the need of further studies for efficient PDI eradication of this bacterium.

**PHAGE THERAPY**

The concept of phage therapy was introduced long before the golden era of antibiotics. However, the discoveries of Sir Alexander Fleming\cite{98} on penicillin effects, led to the ceasing of the research on phage therapy, at least in Western countries. Due to the antibiotic resistance crisis phage therapy is experimenting a renaissance period.\cite{99} Phage therapy consists on the use of all lytic bacteriophages or their lytic proteins, to induce the lysis of the host bacterium cell and thus, eliminating the infecting agent, regardless of the existence of antibiotic resistance.\cite{100} The phage life cycle can be either lytic or lysogenic, with some phages presenting just the lytic cycle (lytic phages), while others having one or the other cycle (temperate phages, *i.e.*, phages that can switch from the lytic to the lysogenic cycle). The first steps of phage infection are similar for both cycles, *i.e.*, adsorption and injection of the phage nucleic acid into the host cell. The next phases in lytic phages are: synthesis of phage nucleic acid and proteins; phage assembly; and phage release of the host cell, usually leading to lysis of the latter. The remaining steps for lysogenic phages consist in the integration of the phage nucleic acid within the host bacterium genome, becoming a prophage, which is replicated with the host cell machinery.\cite{101} An induction event, such as radiation or a SOS response, can produce the switch from the lysogenic to the lytic cycle.\cite{102} Phages control bacterial populations in the wild and can be potentially used as hygiene measures in food production facilities and hospitals, to treat bacterial infections in humans,
animals and crops.\(^{[99]}\) Taking into account the differences between lytic and lysogenic cycles, it is rather easy to understand that lytic phages are more suitable for phage therapy. Indeed, in the case of lysogenic phages, infection does not lead to cell lysis in the majority of times.

Concerning \textit{H. pylori}, the description of phages is still a resumed topic in the literature, although it is growing. The firsts descriptions were made by electron microscopy soon after the discovery of \textit{H. pylori} in the early eighties.\(^{[103-104]}\) In the next decade, there were just two additional publications from the same group\(^{[105-106]}\), describing the spontaneous release of phages from the \textit{Siphoviridae} family. Although these two studies provided a brief description of the genomic map of the phage, the information available at that time did not allow concluding whether it is a lytic phage, or a temperate phage. Fifteen years later, another description of a temperate phage of \textit{H. pylori}, induced by UV, came out.\(^{[107]}\) In the previous two years, there was a burst of information available from genome sequencing projects of \textit{H. pylori} strains, that provided until now the sequences of four complete \(^{[108-109]}\) and three remnant prophages.\(^{[110-111]}\) Although there is no available information on the nature of the life cycle of the described \textit{H. pylori} phages, it is correct to affirm that there is no description of lytic phages. Certainly the screening for lytic phages in this bacterium reservoir could led to their identification, but to our knowledge there are only a couple of studies addressing this issue.\(^{[107; 112]}\)

Taking into account the absence of a currently identified lytic phage in \textit{H. pylori}, the approach that considers the use of phage lytic proteins appears to be more suitable for phage therapy. This approach is applicable for any phage, since the minimum request is the existence of a lysin, the protein responsible for the host bacterial cell wall lysis. The advantage of using lysins resides in the fact that: they are specific for the bacteria species, which means that these proteins would not disturb the gut microbiota; and there is no description of resistance to bacterial lysins.\(^{[113]}\) The most effective way of producing high concentrations of lysins is based on classical molecular biology methods (cloning, expression and purification of the protein).\(^{[114]}\) The main drawback of using lysins against \textit{H. pylori} is that being a Gram-negative, lysins are not capable of crossing its outer membrane from the outside. Indeed, in the nature, lysins hydrolyze the cell wall from the inside, not from the outside as would be the case in a therapeutic scenario. A modification of the lysine, or its encapsulation in a drug delivery system, could help to overcome this limitation. There are some reports of lysins’ modifications that can cross the outer membrane of other Gram-
negative bacteria, such as *Yersinia pestis*.\[115\] Therefore, phage therapy against *H. pylori* is a promising approach that should be further exploited.

**PROBIOTICS**

Research on antimicrobial peptides, with a special interest on bacteriocins of lactic acid bacteria, is entering a new era with novel applications other than food preservation. Many scientists are now focusing on the application of these peptides in medicinal and personal care products. Bacteriocins are small, cationic, amphiphilic peptides produced by some strains of LAB that exhibit structural features typical of members of the eukaryotic channel-forming amphipathic peptides.

Bacteriocins are proteins lethal to bacteria of the same species other than the producing strain. They are a heterogeneous group of ribosomally synthesized peptides or proteins, ranging from small peptides of 19-37 amino acid residues to large peptides with molecular weights of 90000 Da.\[116\] Bacteriocins are considered safer for humans than antibiotics as they have been present in food ever since the origin of humankind. The antimicrobial activity of some of the probiotics may even be based on the production of bacteriocins. In fact, the release of heat-stable, proteinaceous bacteriocins with anti-*H. pylori* activity has been identified in probiotic strains of *Lactobacillus*, *Enterococcus faecium*, *Bacillus subtilis* and *Bifidobacterium*.\[117\] Bacteriocins produced by lactic acid bacteria include nisin A, pediocin PO₂, leucocin K and various types of lacticins.\[117\] Another study demonstrated that bacteriocin action is dependent on *H. pylori* strain and load.\[118\] Also, the gut microbiota may act through the synthesis of bacteriocins. For instance, *Fusobacterium mortiferum*, a phylotype isolated from chicken described in the human stomach,\[119\] synthesizes bacteriocins that inhibit *Salmonella typhimurium*.\[120\] Moreover, the mouth microbiota seems to inhibit *H. pylori* growth through the synthesis of bacteriocins.\[121\] Therefore, in vivo bacteriocin production may be important for the dynamic stability of the gastrointestinal microbiota\[116\] and, consequently, for human health.

The adhesion of *H. pylori* to epithelial cells is important in determining the outcome in *H. pylori*-associated diseases.\[122\] In the gastric mucosa, probiotics either interact with epithelial cells through their secretory components or inhibit colonization and growth of *H. pylori* in the gut or they activate host immune system for eradication of pathogenic bacteria.\[123\] The sharing of glycolipid specificity is a pre-requisite for the *Lactobacillus* strains to have a therapeutic effect on *Helicobacter pylori* eradication through competitive exclusion.\[124\]
MUC5AC glycoprotein has been identified as the primary receptor for H. pylori in the human stomach.\textsuperscript{[125]} There is substantial evidence that probiotics modulate its gastric colonization through competitive exclusion and/or through production of lactic acid, bacteriocins, antibiotics or arginine deiminase (Fig. 2). Different reports support this hypothesis and have proven the efficiency of probiotic microorganism in treatment of H. pylori infection (as given in table 2). In vitro studies showed that Lactobacillus acidophilus, L. johnsonii La1, L. salivarius and Weissella confusa inhibit the attachment of H. pylori to intestinal HT-29 cells\textsuperscript{[126]} or to MKN 45 gastric cell lines.\textsuperscript{[127]} Both in vitro and in vivo mouse model demonstrated that inhibitory effect of Lactobacillus gasseri on H. pylori colonization.\textsuperscript{[128]} In a double-masked, randomized, controlled clinical trial, 326 school children from a low socioeconomic area of Santiago, Chile, with H. pylori infection were treated with both live and heat-killed strains of Lactobacillus johnsonii, L. paracasei and/or carrier once daily for 4 weeks. A $^{13}$C-urea breath test demonstrated a significant decrease in H. pylori colonization in children receiving live L. johnsonii but not the other groups.\textsuperscript{[129]} Both of these studies support the complementary effect of probiotics in the management of H. pylori infection.

Table 2: Probiotic cultures used to treat H. pylori infections\textsuperscript{[130]}

<table>
<thead>
<tr>
<th>Strain</th>
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<td>Bacillus clausii</td>
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<td>Bacillus subtilis</td>
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<td>L. brevis</td>
<td>[135]</td>
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<tr>
<td>L. casei strain Shirota</td>
<td>[136]</td>
</tr>
<tr>
<td>L. casei subsp. DG</td>
<td>[137]</td>
</tr>
<tr>
<td>L. gasseri OLL2716</td>
<td>[138]</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>[124]</td>
</tr>
<tr>
<td>L. salivarius</td>
<td>[139]</td>
</tr>
<tr>
<td>L. salivarius WB1004</td>
<td>[140]</td>
</tr>
<tr>
<td>Lactobacillus GG</td>
<td>[141]</td>
</tr>
<tr>
<td>Weisella confusa</td>
<td>[142]</td>
</tr>
<tr>
<td>L. reuteri and L. paracasei</td>
<td>[143]</td>
</tr>
<tr>
<td>L. rhamnosus (R0011) and L. acidophilus (R0052)</td>
<td>[144]</td>
</tr>
<tr>
<td>L. casei strain Shirota and L. acidophilus</td>
<td>[145]</td>
</tr>
<tr>
<td>Lactobacilli and Bifidobacteria</td>
<td>[127]</td>
</tr>
</tbody>
</table>
Probiotics have been suggested to increase efficacy of *H. pylori* eradication therapy by preventing antibiotic-associated side effects and thus increasing compliance. Cremonini *et al.*[^1] randomized 85 patients with *H. pylori* undergoing eradication with triple therapy to one of four groups: *Lactobacillus casei* subsp. *rhamnosus*, *Saccharomyces boulardii*, *L. acidophilus* plus *Bifidobacterium lactis* or placebo. In all probiotic-supplemented groups, there was a significantly lower incidence of antibiotic-associated diarrhea and taste disturbance relative to placebo. Nevertheless, there was no difference in *H. pylori* eradication or compliance rates between the various groups.

The effects of multi-species probiotic combination on *H. pylori* infection in terms of adhesion, epithelial cell damage, apoptosis and inflammatory responses in Caco-2 cells were evaluated by Myllyluoma *et al.*[^2] All probiotics used in the study inhibited *H. pylori* adhesion. *L. rhamnosus* GG, *L. rhamnosus* LC705, *P. freudenreichii* subsp. *shermanii* JS and the combination inhibited *H. pylori*-induced cell membrane leakage. *L. rhamnosus* GG, *L. rhamnosus* LC705 and the combination initially improved epithelial barrier function but increased the *H. pylori*-induced barrier deterioration after incubation for 24 to 42 h. *L. rhamnosus* GG, *L. rhamnosus* LC705, and *P. freudenreichii* subsp. *shermanii* JS inhibited *H. pylori*-induced IL-8 release, whereas *L. rhamnosus* GG, *L. rhamnosus* LC705 and *B. breve* BB99 suppressed PGE2 release. None of these anti-inflammatory effects persisted when the probiotics were used in combination. The combination thus increased the levels of IL-8, PGE2 and LTB4 released from *H. pylori*-infected epithelial cells. The pro-inflammatory...
actions of the individual components dominated the anti-inflammatory effects when the probiotic bacteria were used in combination. Therapeutic response could be optimized if probiotic strains are characterized before they are used in combination.

In randomized controlled clinical trials on *H. pylori* patients, the effect of fermented milk-based probiotic preparations on *H. pylori* eradication was evaluated on 963 patients at Sitaram Bhartia Institute of Science and Research, New Delhi.\(^{[148]}\) Fermented milk-based probiotic preparations improved *H. pylori* eradication rates by approximately 5-15%, whereas the effect on adverse effects is heterogeneous.

Current antibiotic-based triple therapies are not practical for global eradication due to the genotypic variation in *H. pylori* strains and the emergence of antibiotic-resistant strains.\(^{[28]}\) The drug sensitivity profiles of *H. pylori* isolated from different parts of the World have indicated that the pathogen has acquired resistance to the antibiotics due to point mutations and decreased binding of the antibiotics to the ribosome.\(^{[23-25]}\)

Very few studies have actually focused on role of bacteriocins produced by probiotic bacteria in treating *H. pylori* infection (Table 3). Lacticin A164 of *Lactococcus lactis* subsp. *lactis* A164 and lacticin BH5 of *L. lactis* BH5 are two bacteriocins of lactococcal origin with anti-*Helicobacter* activity that tell pathogen in a dose-dependent manner.\(^{[118]}\) Two more anti-*Helicobacter pylori* bacteriocins namely bulgaricin BB18 produced by *L. bulgaricus* BB18 and enterocin MH3 produced by *E. faecium* MH3 have recently been identified.\(^{[149]}\) These are potential antimicrobial agents and in conjunction with their producers, may have use in applications to contribute a positive effect on the balance of intestinal microflora.

**Table 3: Anti-*Helicobacter pylori* bacteriocins**

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lacticin A164</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em> A164</td>
<td>Kim et al., 2003</td>
</tr>
<tr>
<td>Lacticin BH5</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em> BH5</td>
<td>Kim et al., 2003</td>
</tr>
<tr>
<td>Bulgaricin BB18</td>
<td><em>Lactobacillus delbrueckii</em> sp. <em>bulgaricus</em> BB18</td>
<td>Simova et al., 2009</td>
</tr>
<tr>
<td>Enterocin MH3</td>
<td><em>Enterococcus faecium</em> MH3</td>
<td>Simova et al., 2009</td>
</tr>
</tbody>
</table>

Nisin and lacticins A164 and BH5 inhibited the growth of *Helicobacter pylori in vitro* and may thus be used in the treatment of peptic ulcers.\(^{[150]}\) Nisin (class I) and lacticin (class II) bacteriocins can be considered for treatment of stomach ulcers caused by *H. pylori* and oral mucositis.\(^{[118; 149]}\)
A highly potent Gram positive probiotic lactic acid bacteria *P. acidilactici* BA28 which is a hetero-fermentative and can survive at a pH range from 2.0-9.0, with optimal growth observed between pH 6.5 to 8.5. Microorganism is mesophilic in nature and grows at moderate temperature range (10°C to 42°C). Strain has a good acid and bile tolerance, antibacterial activity and antibiotic sensitivity for its commercial use in various probiotic products. *P. acidilactici* BA28 shows the 69.43% iNOS activity which is comparable to the iNOS activities of the commercially available probiotics such as *Lactobacilli* and *Bifidobacteria*. *P. acidilactici* BA28 is sensitive to a number of antibiotics such as Amoxycillin, Amoxyclav, Ampicillin, Cefazolin, Cefixime, Ciprofloxacin, Co-trimoxazole, Erythromycin, Gentamicin, Gentamycin, Levofloxacain, Linezolid, Norfloxacin, Penicillin-G, Pristhiomycin, Roxithromycin, Streptomycin, Tetracyclin and Tigecycline that supports its GRAS character and usability in various probiotic formulations and as an adjunct with antibiotic therapy. *P. acidilactici* BA28 produces a bacteriocin designated as pediocin BA28 that kills *H. pylori* in a concentration dependent manner. It induces formation of small pores on the cytoplasmic membrane and causes leakage of small inorganic ions, ATP and other molecules from the sensitive cells. Pediocin production phenotype has been linked to genes located on the small cryptic plasmid DNA in case of *P. acidilactici* BA28. *In vivo* studies, carried out in C57BL/6 mice, revealed its potent peptic ulcer eliminating property in *H. pylori* infected animals. This probiotic strain has significantly reduced the attachment of *H. pylori* to gastric tissue thereby reversing the progression of the peptic ulcers to gastric carcinoma stage. Infection was reversed in infected mice receiving probiotic *P. acidilactici* BA28 for a longer duration.

The administration of bacteriocin-producing bacteria, instead of purified products, may be a simpler strategy. Further research is needed as the mechanism of action of bacteriocins is not known, and as for other antimicrobial drugs, the resistance issue should be addressed.

**PHYTOTHERAPY**

Phytotherapy can be defined as the use of plant extracts as medicines or health-promoting agents and is as old as human civilization. Its origins are based on empirical knowledge and scientific validation of these products is still very limited. For example, tea tree oil has broad anti-bacterial activity, including activity against methicillin-resistant *Staphylococcus aureus* or *Candida albicans* and green tea catechins also possess antimicrobial activity. However, the study of herbal medicines to treat *H. pylori* infection is in its early days and few
reports are available. Some authors describe *H. pylori* as susceptible to garlic extract at moderate concentrations in vitro,[156] whereas others report no effect.[157]

The extract of *Pelargonium sidoides* roots (EPs) 7630, a South African herbal remedy currently used to treat acute bronchitis, prevents bacteria from attaching to cell membranes. EPs 7630 inhibit *H. pylori* growth and high potency adhesion to gastric AGS cells and to intact human stomach tissue from patient resections in situ. This suggests that their mode of action is mainly related to their anti-adhesive activity.[158-159]

Similarly, the use of a high molecular mass constituent of cranberry juice inhibits *H. pylori* adhesion to human gastric mucus, suggesting that a combination of antibiotics and a cranberry preparation may improve *H. pylori* eradication.[160-161] A synergistic combination of oregano and cranberry phenolics has been suggested to inhibit *H. pylori* in a laboratory medium. The likely mode of action may be through urease inhibition and disruption of energy production by inhibition of proline dehydrogenase at the plasma membrane.[162] The inhibition of urease, which catalyzes the hydrolysis of urea to carbon dioxide and ammonia, hence increasing the pH, is a potential target for *H. pylori* eradication.[163]

The bark of *Calophyllum brasiliense Camb.* (Clusiaceae), which grows in Brazil and is also known as ‘guanandi’, and its hydroethanolic extract and dichloromethanic fraction have demonstrated anti- *H. pylori* activity in humans.[164] Extracts of the Brazilian *Mouriri elliptica Martius* (Melastomataceae)[165], *Hancornia speciosa Gomez* (Mangaba)[166], *Byronima fagifolia Nied.* (Malpighiaceae)[167] and *Alchornea triplinervia*[168] also appear to have anti- *H. pylori* property. Mexican *Amphipterygium adstringens* (Schltdl.) Standl. (Anacardiaceae), an anacardic acids mixture, exhibits potent dosedependent anti- *H. pylori* activity.[169] Extract of Japanese rice also demonstrated anti- *H. pylori* activity[170] and African Sao Tome plants are also used in traditional therapies for several gastric disorders. These include *Leonotis nepetifolia* (L.) W.T. Ainton var. *nepetifolia* (gastric indisposition), *Solenostenom monastachyus* (P. Beauv.) Briq. ssp. *monostachyus* (stomach pain), *Piper umbellatum* L. (stomach problems), *Bertiera racemosa* (G. Don) K. Shum var. *elephantina* N. Halle´ (stomach pain), *Allophyllus grandifolius* (Baker) Radlk (gastric affection) and *Solanum gilo* Raddi (stomach pain). The anti- *Helicobacter* action of these compounds, either in vitro or in vivo, has not been tested yet.
Isothiocyanate sulforaphane, abundant in broccoli sprouts, may have both a direct antibacterial effect on *H. pylori*, leading to reduced gastritis and an indirect (systemic) effect by increasing the mammalian cytoprotective response.\[^{171}\]

The cold extract, infusion, decoction and simulated digestion of *Larrea divaricata* Cav (jarilla) inhibit clarithromycin- and metronidazole-susceptible and resistant *H. pylori* strains. This plant, usually applied in the treatment of gastric disturbances, may also be useful in peptic ulcer and gastric cancer therapy.\[^{172}\]

*Bacopa monniera* is currently used in Indian medicine to improve intellectual function. A standardized extract of *B. monniera* has anti-*H. pylori* activity in vitro. The antimicrobial effect is due to augmentation of defensive mucosal responses such as mucin secretion, lifespan of mucosal cells and gastric antioxidant effects, rather than offensive acid-pepsin secretion.\[^{173}\]

Propolis is a resinous hive product collected by honeybees from various plant sources usually used in food, beverages and folk medicine for treating various diseases. It shows a broad spectrum biological activity and offers antibacterial activity against *H. pylori*.\[^{174}\] Other kinds of honey also have anti-*H. pylori* activity.\[^{175}\]

Alkyl methyl quinolone alkaloids (AM quinolones) from gosyuyu (Wu-Chu-Yu) and psoralen (extract from *Psoralea corylifolia*)\[^{176}\], a Chinese herbal medicine\[^{177}\], and amu-ru 7, a Mongolian folk medicine\[^{178}\], show antibacterial activity against *H. pylori* in vitro. Luteolin, a component in herbal medicine, can also inhibit *H. pylori*.\[^{179}\] However, the molecular mechanism of action of these herbal extracts is unknown and further investigation is needed. A recent study identified new herbal extracts with antimicrobial activity against *H. pylori*. *Agrimonia eupatoria*, *Hydrastis canadensis*, *Filipendula ulmaria* and *Salvia officinalis* were the most active herbal extracts.\[^{180}\] The same study also tested essential oils and the most effective against *H. pylori*, *Syzygium aromaticum* oil, showed a half maximal inhibitory concentration value 10 times lower than the standard antibiotic ampicillin.

Cytotoxic studies are needed to prove the selective toxicity of these essential oils and to establish the concentrations at which anti-*Helicobacter* activities are not harmful for epithelial cells in the gastrointestinal tract. Finally, clinical trials are necessary to explore the possibility of using herbal medicines as an efficient and low-cost remedy for eradicating *H. pylori*. 
Curcumin (diferuloylmethane), the major yellow pigment present in the rhizome of turmeric (Curcuma longa), has a healing effect in H. pylori infection\textsuperscript{181}, probably by suppressing secretion of metallo-proteinases 3 and 9 by gastric cells, believed to be involved in the development of gastric ulcer and gastric cancer.\textsuperscript{182}

*Nigella sativa* (Ranunculaceae) grows in the Middle East, Eastern Europe and Eastern and Middle Asia and its oil is used as a food additive because of its anti-inflammatory, anti-cancer and antimicrobial activity. *Nigella sativa* seeds contain essential oils such as thymoquinone, dihydrothymoquinone and terpenes that may exercise their antimicrobial activity by disrupting the lipid structure of the cell membrane.\textsuperscript{183} Other herbal extracts also have potential *H. pylori* inhibitory properties, but their mode of action is unknown. These include crude methanol extract of the leaf of *Allium ascalonicum*\textsuperscript{184}; flavonoid constituents of herbal medicines\textsuperscript{185}, such as the ones isolated from the leaves of *Piper carpinia Ruiz & Pav.* (Piperaceae), widely used in folk medicine in tropical and subtropical South American countries and known for their anti-inflammatory and anti-ulcer action\textsuperscript{186}; ‘compound with anti-*Helicobacter* activity’, extracted from celery (*Apium graveolens*) seeds\textsuperscript{187}, and phenolic acid derivatives, acylglycoflavonoids and condensed tannins from *Davilla elliptica* and *Davilla nitida*.\textsuperscript{188} Resveratrol, a polyphenol highly abundant in red grapes\textsuperscript{188}, exhibits anti-inflammatory and anti-cancer activity and has cardioprotective and neuroprotective properties. This protective activity may be due to modulation of inflammatory cytokines such as IL-6, transcription factors such as nuclear factor-kB and regulatory enzymes such as mitogen-activated protein kinases, or to multiple modulatory effects on *H. pylori*-induced IL-8 secretion, reactive oxygen species production and morphological changes.\textsuperscript{188}

Although these substances show promising anti-*H. pylori* properties, a systematic review of Chinese herbal medicines does not support a role as stand-alone therapy\textsuperscript{189} The active ingredients of phytomedicines should be identified to confirm their selective toxicity and determine their mode of action and the dose required to treat infections without harming the patients.

**CONCLUSION**

Nowadays, antibiotic resistance is a significant problem for treatment of diseases caused by virtually known infectious bacteria. The gastric pathogen *H. pylori* is no exception to this rule and during the last years we have observed a continuous and worrying increase of resistant strains. The growing rate of bacterial resistance to antibiotics can soon made humanity enter
in a period, similar to the one we have before the discovery of antibiotics. Thus, we need to focus on finding and exploring alternative anti-\textit{H. pylori} combating strategies with clinical applicability, such as the ones revisited in the review. Probiotics diminish side effects and improve the efficacy of antibiotics, probably because they mimic the function of the human microbiota. Bacteriophage therapy shows potential as an alternative to antibiotics, but it will take some time to verify whether it is feasible as it requires phage isolation and characterization. Phytotherapy is a very promising therapeutic approach that has been used for centuries in traditional medicine. The herbal species used across the globe are highly diverse and only a few studies have identified the nature of the active ingredient or its mechanism of action. Safety must be assessed and each active substance must be characterized. Resistance against these new phyto-therapeutic agents should also be addressed.

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