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EVALUATION OF THE ANTIMICROBIAL POTENTIAL OF NATURAL EXTRACTS ON *HELICOBACTER PYLORI*

Dissertação no âmbito do Mestrado em Bioquímica, orientada pela Professora Doutora Célia Laurinda dos Santos Nogueira e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra.

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List of Acronyms and Abbreviations

^{13}C-urea breath test	UBT
18β-Glycyrrhetinic acid	GRA
1-isothiocyanato-4(R)-methylsulfinylbutane	SF
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
Aadenosine triphosphate	ATP
Acetonic garlic extracts	AGE
Acid-gated urea channel genes	*ureI*
Adherence-associated lipoprotein	Alp
Aliphatic amidase	AmiE
Alkyl hydroperoxide reductase	Ahp
Ammonia	NH_{3+}
Ammonium	NH_{4+}
Apoptosis inhibitor-2 (API2) gene and MALT lymphoma-associated translocation (MALT1) gene	*MALT1- API2*
Aqueous garlic extract	AGE
Arginase enconding gene	*rocF*
ATP-Binding Cassette transporter proteins	ABC
Blood Agar Plate	BAP
Blood group antigen binding adhesin	Bab
Body weight	BW
Bovine lactoferrin	bLf
Cag pathogenicity island	*cag*PAI
Calcium	Ca^{2+}
Campylobacter-like organism	CLO
Carbon dioxide	CO_2
Caudal-type homeobox 2	CDX2
Clinical & Laboratory Standards Institute	CLSI
Colony-forming unit	CFU
Copper resistance determinant	CrdA
Copper(I)-binding protein	CopP
Copper-transporting ATPase	CopA
Cranberry juice/La1	CB/La1
Cytokine	C
Cytotoxin-associated gene A	*cagA*
Dendritic cells	DCs
Deoxyribonucleic acid	DNA
Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol	HyEDA
Dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol	TyEDA
Dithiocarbamates	DTC
DNA-binding proteins	Dps
Endoplasmic reticulum	ER
Enzyme-linked immunosorbent assay	ELISA
Ethanol extract of propolis	EEP
Ethanolic garlic extracts	EGE
European Committee on Antimicrobial Susceptibility Testing	EUCAST

Fe^{3+} dicitrate transport ATP-binding protein	FecE
Fe^{3+} dicitrate transport system permease protein	FecD
Ferric citrate transport protein A	FecA
Ferric iron	Fe^{3+}
Ferritin	Pfr
Ferrous iron	Fe^{2+}
Ferrous iron transport protein B	FeoB
Flagellar Hook Protein	FlgE
Flagellin gene	*fla*
Flagellin subunit protein	Fla
Formamidase	AmiF
Gastric epithelial cell line	AGS
Green tea extract	GTE
Growth-related oncogene	GRO
Guanine	G
H. pylori outer membrane protein	HopZ
H. pylori stool antigen	HpSA
Heat shock protein	Hsp
Helicobacter pylori	*H. pylori* or HP
High-affinity nickel-transport protein	NixA
Histidine-rich proteins	Hpn
Hydrogen	H_2
Hydrogen potential	pH
Hydrogen sulfide	H_2S
Immunoglobulin	Ig
Immunohistochemistry	IHC
Immunoreactive score	IRS
Inner membrane permease	FecD
Interferon-gamma	IFN-γ
Interleukin	IL
International Agency for Research on Cancer	IARC
Iron-cofactored	SodB
Iron-regulated outer membrane gene	*frpB4*
Iron-regulated outer membrane protein B	FrpB
Iron-responsive regulator	Fur
Pathogen-associated molecular patterns	PAMPs
Pepsinogen	PG
Peptic ulcer disease	PUD
Periplasmic binding protein	CeuE
Phosphate-buffered saline	PBS
Polymerase Chain Reaction	PCR
Pronton-gated urea channel	UreI
Propylene glycol extract of propolis	PEP
Prostate stem cell antigen	PSCA
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Refined deep seawater	RDSW
Resistance-nodulation-cell division	RND
Sialic acid-binding adhesin	SabA
Sialyl-Lewis x glyco- sphingolipid	sLe^x
Sulfoxi group	-SO-
Superoxide dismutase B gene	*sodB*
T helper 1	Th1
Temperature sensitive mutant Z filaments	FtsZ

The concentration that inhibits 50% of isolates	MIC_{50}
The concentration that inhibits 90% of isolates	MIC_{90}
The half maximal inhibitory concentration	IC_{50}
Thiol group	-SH
Thiol-peroxidase	Tcp
Toll-like receptor	TLR
TonB-dependent transporter	TBDT
Triterpenoid methylantcinate B	MAB
Tumor necrosis factor alpha	TNF-α
Type IV secretion systems	T4SS
Ultraviolet B	UV-B
United Kingdom	UK
United States of America	USA
Ure gene promotor	P_{ure}
Urease accessory assembly E-H proteins genes	*ureE-H*
Urease accessory protein	Ure
Urease catalytic subunits genes	*ureA*/*B*
Urease subunits	UreA/B
Vacuolating cytotoxin A	VacA
World Gastroenterology Organisation	WGO
World Health Organization	WHO

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Resumo

Helicobacter pylori é uma bactéria Gram-negativa, microaerofílica e patogénica. Coloniza o epitélio gástrico, podendo induzir infeção graças a uma série de mecanismos de adaptação que desenvolveu ao coevoluir com os seres humanos, ou seja, ao evoluir simultaneamente exercendo pressões seletivas. Estima-se que mais de metade da população mundial esteja infetada, com maior incidência nos países em desenvolvimento. A infeção é geralmente adquirida durante a infância e desenvolvida durante a idade adulta, dependendo da relação bactéria- hospedeiro e da patogenicidade dos seus fatores de virulência. Assim, é um fator de risco para o desenvolvimento de patologias gástricas, como a gastrite (atrófica), úlceras pépticas e até mesmo doenças malignas como o linfoma do tipo MALT e o adenocarcinoma gástrico. A infeção é geralmente tratada com um conjunto de antibióticos e medicação supressora de ácido. Contudo, este tipo de terapia tem-se verificado menos eficaz devido ao aumento da resistência bacteriana à ação dos antibióticos. Adicionalmente, tem efeitos secundários adversos associados e nem sempre é acessível, o que cria a necessidade do desenvolvimento de terapias alternativas. Os produtos naturais apresentam-se como uma potencial terapêutica convencional ou adjuvante, devido à sua ampla gama de atividades biológicas e propriedades medicinais. Nas ultimas décadas, têm sido conduzidos vários estudos sobre a ação destes compostos no combate à infeção causada por *Helicobacter pylori*. Estes elementos podem atuar através de diversos mecanismos de ação, incluindo atividades antioxidantes, anti-inflamatórias e anti-adesivas, efeitos antimicrobianos diretos e imunoestimulantes, ou mesmo inibindo fatores de virulência bacteriana. No presente trabalho, são apresentados alguns exemplos de compostos naturais que foram testados em *Helicobacter pylori*, os métodos utilizados e os resultados obtidos. Estão incluídos diversos alimentos como vegetais, frutas, uma especiaria, chá, peixe e azeite, um probiótico, uma proteína animal, plantas medicinais, produtos marinhos, um fungo e um produto resultante da atividade animal. Estes compostos revelaram resultados promissores ao inibir a bactéria e/ou ao melhorar os efeitos que surgem da infeção. Contudo, mais estudos terão de ser realizados para definir a eficácia e segurança dos produtos naturais na generalidade e permitir o seu uso como terapia contra a infeção causada por *Helicobacter pylori.*

Palavras-chaves: *Helicobacter pylori; infeção; terapia; produtos naturais; mecanismos de ação*

Abstract

Helicobacter pylori is a Gram-negative, microaerophilic and pathogenic bacterium. It colonizes the gastric epithelium and can induce infection due to a series of adaptation mechanisms that developed when coevolving with humans, that is, by evolving simultaneously exerting selective pressures. It is estimated that more than half of the world population is infected, with a higher incidence in developing countries. The infection is usually acquired during childhood and developed during adulthood, depending on the bacterial-host relationship and the pathogenicity of its virulence factors. Thus, it is a risk factor for the development of gastric pathologies, such as (atrophic) gastritis, peptic ulcers and even malignant diseases such as MALT lymphoma and gastric adenocarcinoma. The infection is usually treated with a set of antibiotics and acid suppressant medication. However, this type of therapy has been found to be less effective due to increased bacterial resistance to the action of antibiotics. In addition, it has associated adverse side effects and is not always accessible, which creates the need of developing alternative therapies. Natural products are a potential conventional or adjuvant therapeutic, due to their wide range of biological activities and medicinal properties. In the last few decades, various studies have been conducted on the action of these compounds in fighting the infection caused by *Helicobacter pylori*. These elements can act through several mechanisms of action, including antioxidant, anti-inflammatory and anti-adhesive activities, direct antimicrobial and immunostimulating effects, or even inhibiting bacterial virulence factors. In the present work, there are presented some examples of natural compounds that were tested on *Helicobacter pylori*, the used methods and the obtained results. There are included diverse foodstuffs such as vegetables, fruits, a spice, tea, fish and olive oil, a probiotic, an animal protein, medicinal plants, marine products, a fungus and a resulting product from animal activity. These compounds revealed promising results by inhibiting the bacterium and/or by improving the effects that arise from the infection. However, further studies will have to be conducted, to define the effectiveness and safety of natural products in general and to allow their use as a therapy against *Helicobacter pylori* infection.

Keywords: *Helicobacter pylori; infection; therapy; natural products; mechanisms of action*

Chapter I

Introduction

1. Taxonomy of *H. pylori*

Helicobacter pylori (*H. pylori*) was included in the genus *Campylobacter*, firstly as *Campylobacter pyloridis* (Marshall et al., 1984) and after as *Campylobacter pylori* (Marshall et al., 1987). However, there were many different features between *Campylobacter pylori* and other campylobacters, which created the necessity of the genus transition. Today, it is defined as *Helicobacter pylori* due to its morphology: *Helicobacter* are helical organisms *in vivo* and frequently rodlike *in vitro* (Goodwin et al., 1989).

The genus *Helicobacter* comprises microaerophilic bacteria, mostly oxidase and catalase positive and, many times, also urease positive. There are two types of *Helicobacter* species – gastric and nongastric (enterohepatic) – that are highly specific for the organ they colonize (Solnick & Schauer, 2001).

Helicobacter pylori is a cellular organism that belongs to the domain *Bacteria*, to the phylum *Proteobacteria*, to the class *Epsilonproteobacteria*, to the order *Campylobacterales*, to the family *Helicobacteraceae* and to the genus *Helicobacter* (NCBI, 2020).

2. Characteristics of *H. pylori*

Helicobacter pylori (Fig.1) is a Gram-negative bacterium, pathogenic, associated exclusively to gastric mucosa cells. It can have an helical, curved or straight unbranched form and be spiral periodically. It has rounded ends, and due to these characteristics, it is considered a bacillus (Goodwin et al., 1989).

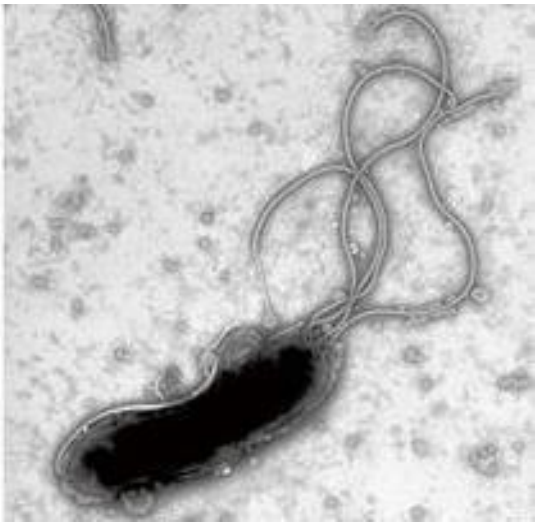

Figure 1 - Transmission electron micrographs of a clinical and type strain of *Helicobacter pylori* (adapted from Bie et al., 2019).

It can also have a coccoid shape, that results of prolonged *in vitro* culture or treatment with antibiotics. This form may represent dead cells (Kusters et al., 1997) or a viable state, nonculturable (Enroth et al., 1999). Due to the prolonged *in vitro* culture, there is a transition to the unculturable cocoid state instead of an increase in colony size. Therefore, when the colonies reach the stationary phase, there is a decline of the growth rate and a morphological change to the coccoid shape (Kusters et al., 1997). In some conditions, this organism can also produce an external glycocalyx (Goodwin et al., 1989).

Visually, the colonies are smooth, translucent and small in size (~1 mm) (Han et al., 1995). Each bacterium has a length of 2 to 4 µm and a width of 0.5 to 1 µm, and two to six unipolar flagella, with terminal bulbs (O'Toole et al., 2000).

3. Genome and strain diversity of *H. pylori*

H. pylori is genetically heterogeneous because of its capacity of colonization and adaptation to different environments – its host. Different hosts differ in gastric conditions and in immune responses to the infection (Kuipers et al., 2000). This genetic differences occur through DNA rearrangement processes, such as introduction and deletion of sequences (Achtman & Suerbaum, 2000; Falush et al., 2003; Suerbauma & Achtman, 2004).

4. Metabolism of *H. pylori*

H. pylori is a highly specialized bacterium; and its specialization comes with a strong adaptation to its environment – the host's stomach. So, metabolic adaptation mechanisms are adjusted to the lifelong infection that results from its activity. There is an absence of many biosynthetic pathways frequently found in bacteria that are less specialized than *H. pylori,* for example, enteric bacteria (Alm et al., 1999; Berg et al., 1997; Doig et al., 1999; Marais et al., 1999; Tomb et al., 1997).

The evolution of molecular techniques, such as genomic comparisons and metabolic studies, allowed scientists to study microorganisms in a detailed way. Due to these type of studies, it was discovered that *H. pylori* has a stripped-down metabolic pathway with hardly any redundancies; and that there are no biosynthetic pathways for some amino acids (Nedenskov, 1994; Reynolds & Penn, 1994). So, this fastidious microorganism requires a complex growth media when it is cultivated, that should have a supplementation with blood or serum. These supplements have a nutritional and a protective function: they are additional sources of nutrients and can possibly protect the bacterium against some toxic effects of metabolism, such as the long-chain fatty acids (Taneera et al., 2002). Once the bacterium lacks some biosynthetic pathways for amino acids, it can only grow in a media with the amino acids arginine, histidine, isoluecine, leucine, methionine, phenylalanine and valine. Some strains also may need a media with alanine and/or serine (Nedenskov, 1994; Reynolds & Penn, 1994).

As said before, this bacterium is positive for some enzymes – urease, oxidase and catalase – that are used in techniques for its identification. Biochemical and genomic studies showed that it can catabolize glucose, but not other sugars (Berg et al., 1997; Doig et al., 1999; Marais et al., 1999; Nedenskov, 1994).

There are some metabolic systems involved in the pathogenicity of the bacterium or connected to it in some way: respiration and oxidative stress defense, nitrogen metabolism and metal metabolism (Kusters et al., 2006).

4.1 Respiration and oxidative stress defense

H. pylori is a microaerophilic bacterium: it only survives in environments with a low oxygen concentration. Despite this, it requires a concentration of at least 2% of oxygen to remain alive (Mendz et al., 1997). It uses oxygen as a terminal electron acceptor, once it cannot utilize alternative electron acceptors, such as formate or nitrate. Nevertheless, it can make use of fumarate to grow anaerobically (Smith & Edwards, 1995). In the human host, the immune cells respond to the infection caused by the bacteria by creating oxidative stress, which generates the necessity for the microorganism to have mechanisms of resistance. Some of these mechanisms are superoxide and peroxide stress defense: the first mentioned takes place via iron-cofactored superoxide dismutase (SodB) (Barnard et al., 2004; Ernst et al., 2005; Seyler et al., 2001) and the second one via catalase (KatA) and alkyl hydroperoxide reductase (AhpC) (Harris et al., 2002; Olczak et al., 2003). Resistance to oxidative and nitrosative stress is simultaneously mediated by a few enzymes: two thioredoxins and its respective reductases and thiol-peroxidase Tcp (Baker et al., 2001; Comtois et al., 2003; Olczak et al., 2002; Olczak et al., 2003; Wang et al., 2005). There are proteins that protect the DNA from the repercussions of reactive oxygen species (ROS), as is the case of the neutrophil activating protein (HP-NAP), a protein that belongs to the Dps family (see also virulence factors, NAP). As this protein is responsible for the activation of neutrophils, it is involved in the production of ROS (Evans et al., 1995). ROS formation is connected with the iron metabolism, once some reactions of this type of metabolism can produce oxygen radicals. So, there are proteins that are both implicated in iron metabolism and oxidative stress resistance, essential for many bacterial functions and for host's colonization: the global iron- responsive regulator Fur, the FeoB iron transporter, the iron-storage protein ferritin and the iron-cofactored SodB (Ernst et al., 2005; Olczak et al., 2003; Seyler et al., 2001; Velayudhan et al., 2000; Waidner et al., 2002). The absence of key components of bacterial oxidative stress resistance (one or more) can result in a lower level of colonization, or even in an incapacity of adaptation to the host, making these components essential to the survival of *H. pylori* (Olczak et al., 2003; Seyler et al., 2001).

4.2 Nitrogen metabolism

In the stomach, the main sources of nitrogen are amino acids and urea. Ammonia has a fundamental role in nitrogen metabolism and acid resistance (Stingl et al., 2002) and,

because of that, *H. pylori* makes use of it through many different metabolic pathways (Bauerfeind et al., 1997; Bury-Moné et al., 2003; McGee et al., 1999; Merrell et al., 2003; Skouloubris et al., 1997; Skouloubris et al., 2001; Van Vliet et al., 2003). Divergent stimuli originate different responses that, in its turn, regulate the alternative pathways that are involved in ammonia synthesis. These pathways can shift to on or off according to the state of the surrounding environment (Fig.2).

Urease is an enzyme with a high activity, that makes part of the principal route of ammonia synthesis. Therefore, *H. pylori* synthesizes massive quantities of this enzyme. Urease activity may be high, but its effective activity depends on the amount of its substrate (the urea) that is available. As urease makes part of the ammonia production, it is present in nitrogen metabolism, contributing to acid resistance and virulence (Bauerfeind et al., 1997) (see virulence factors and pathogenesis, urease). An elevated ammonia production leads to excessive levels of ammonia, that can be removed through the via glutamate synthetase enzyme (Garner et al., 1998). In order to perpetuate in such harsh conditions, the ammonia resulting from enzymatic degradation of urea can be incorporated into amino acid biosynthesis. The fact that there are various alternative pathways for ammonia production (via enzymatic degradation of amides and amino acids) highlights the importance of ammonia in the metabolism of *H. pylori* and its pathogenicity (Fig.2) (Bauerfeind et al., 1997; Bury-Moné et al., 2003; McGee et al., 1999; Merrell et al., 2003: Skouloubris et al., 1997; Skouloubris et al., 2001; Van Vliet et al., 2003).

There are different origins of ammonia when there is a low level of urea, such as amidases – the wide range aliphatic amidase AmiE and the formamidase AmiF – and amino acid deaminases. *H. pylori* has many elements in common with the eukaryotic urea cycle (Mysore et al., 1999) and may have the capacity of producing urea from ammonia. The arginase (RocF) enzyme is a main component of the urea cycle, able to convert L-arginine to L-arnithine and urea; the *rocF* gene has an important role in acid resistance, since it encodes arginase (McGee et al, 1999). The bacterium can control eukaryotic cells through arginase (which controls the nitric oxide production, a free radical that is essential for inflamatory processes and protection against bacteria) (Gobert et al., 2001), and arginine metabolism (Gobert et al., 2002).

4.3 Metal homeostasis

Metals are present in all forms of life, playing a fundamental role in metabolism: they are involved in several biochemical reactions, despite existing in low concentrations

within cells. Metal ions are frequently present in small quantities in the environment, making metal acquisition a fundamental biological process to the organisms. Once they are cofactors of enzymes, they have many different functions: they catalyze elementary functions such as electron transport, redox reactions and energy metabolism. They are also required to control the osmotic pressure. Metal homeostasis is a key element for the good functioning of living beings – both metal limitation and metal overload can decrease growth and even cause cell death due to its toxicity. To assure homeostasis maintenance, bacteria have developed transport systems, a group of mechanisms involved in metal processing: uptake, storage, distribution, incorporation and efflux (Fischer & De Reuse, 2016; Kusters et al., 2006). The role of four metals that are crucial in *H. pylori*'s metabolism – nickel, iron, copper and cobalt – is resumed on the following pages (Kusters et al., 2006).

4.3.1 Nickel

Nickel (Ni^{2+}) is a cofactor of urease and hydrogenase, indispensable components for a successful colonization in the acidic stomach environment. The harsh conditions cause an overexpression of urease and, as a result, the bacterium needs an effective acquisition of this metal to ensure urease optimal activity, which contributes to adapt to this challenging organ, enabling *H. pylori* colonization (Fischer & De Reuse, 2016). Urease activation depends on nickel: this metal enables enzyme activity if present in certain concentrations to allow its maturation (Schauer, 2007). As said before, nickel is a cofactor of another enzyme – hydrogenase, which catalyzes the reversible oxidation of molecular hydrogen. Its activation enables the bacterium to use hydrogen (H_2) as an energy source, which improves bacterial colonization by making another energy source available (Olson & Maier, 2002). Due to the role of this metal in urease and hydrogenase catalytic activities, nickel is considered a virulence determinant, once it permits *H. pylori* to survive and colonize the stomach (Fischer & De Reuse, 2016). Humans obtain nickel through diet and food sources, since there is a low concentration of this metal in the human serum, making it less available (Christensen et al., 1999; Sunderman et al., 1989).

The active transport of some metabolites through the outer membrane in Gram-negative bacteria depends on the TonB machinery and on TonB-dependent transporters (TBDTs). As mentioned before, there is a low concentration of nickel in the human serum, making it less available within the stomach. To guarantee an efficient nickel uptake from the environment, *H. pylori* developed systems that can import sufficient amounts of this metal. The nickel transport system that crosses *H. pylori*'s outer membrane needs FrpB4 TBDT and its energy source is the TonB machinery

including ExbB-ExbDTonB (Schauer et al., 2007; Schauer et al., 2008). FrpB4 is acid-activated and nickel is more soluble at an acidic pH, making this uptake system appropriate for the stomach. Since *frpB4* gene expression is repressed (by NikR) in the presence of nickel, this uptake system may be fully active under nickel-limiting conditions. An alternative nickel uptake system might be FecA3, another *H. pylori*'s TBDT that depends on nickel to be synthetized (Ernst et al., 2006; Romagnoli et al., 2011).

After crossing the outer membrane, nickel binds to NixA. NixA protein (HP1077) is localized in the cytoplasmatic membrane and has a high affinity for nickel, making it a nickel transporter (Fig.2) (Fulkerson et al., 1998; Mobley et al., 1995). This protein belongs to the NiCoT family and its expression is repressed by nickel (Wolfrom et al., 2006; Muller et al., 2011). NixA allows nickel to be taken through the outer membrane at a low capacity (Fulkerson & Mobley, 2000). Inside the cytoplasm, nickel is orientated to its targets, to avoid free nickel ions. Nickel excess has to be avoided due to the potencial cellular damages that it may cause, creating the necessity to store or to export this metal. A mutation in *nixA* gene decreases nickel transport and urease activity, which causes a less efficient colonization (Bauerfeind et al., 1996; Nolan et al., 2002), showing the importance of NixA in nickel transport and the possibility of the existence of alternative ways of transporting this metal through the cytoplasmic membrane (Fischer & De Reuse, 2016). The proton-driven RND-type metal efflux-pump is a nickel export system in *H. pylori*, and is encoded by the *ABC* genes (Stähler et al., 2006).

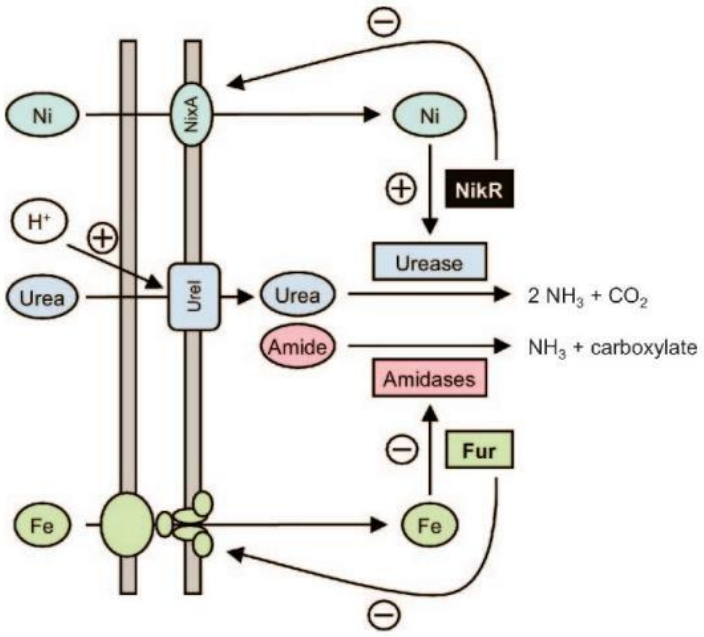

Figure 2 - Schematic representation of the relationships between acid resistance (urease activity and urea transport), nitrogen metabolism (ammonia production), metal metabolism (iron uptake and nickel uptake), and gene regulation (Fur and NikR) in *H. pylori* (reproduced from Kusters et al., 2006).

Metallochaperones (storage proteins) function is to ensure balance in metal concentration, avoiding the toxicity caused by metal overload. These proteins are also in charge of incorporate nickel into urease and hydrogenase (Fischer & De Reuse, 2016). HspA is a homolog of the highly conserved and essential bacterial heat shock protein GroES. This protein exists in high amounts in the bacteria and its role is to bind nickel free ions to store them in the cytoplasm and to contribute to hydrogenase maturation (Schauer et al., 2010). *HspA* gene is upregulated by NikR in the presence of nickel (Muller et al., 2011). There are systems associated with urease and hydrogenase that affect urease activity through its adaptation to the relevant position of nickel, as it is illustrated by hydrogenase accessory proteins (Benoit & Maier, 2003; Mehta et al., 2003; Mehta et al., 2003) and a nickel-binding motif on the HspA chaperone (Kansau et al., 1996).

Along with the urease/hydrogenase accessory proteins and the transport systems, *H. pylori* reveals two histidine-rich proteins (Hpn): Hpn and Hpn-2. These two proteins are small, very rich in His-residues and have a strong binding to nickel (Gilbert et al., 1995; Mobley et al., 1999; Ge et al., 2011; Zeng et al., 2008; Zeng et al., 2011). Transcription of the two genes is also upregulated by NikR in the presence of nickel (Muller et al., 2011). Only Hpn participates in nickel sequestration. The two proteins communicate with each other and both make part of a nickel transfer pathway to urease. Hpn and Hpn-2 were decisive to the capacity of adaptation of *H. pylori* to the stomach during the evolutionary process, as phylogenomic analysis revealed (Vinella et al., 2015).

4.3.2 Iron

In animals (including humans) there is a low concentration of free iron because, depending on the tissue, this metal can be complexed or chelated: complexed into hemoglobin (present in red blood cells) and chelated by transferrin in serum or by lactoferrin at mucosal surfaces, which turns it unavailable to support bacterial development.

There are some iron sources in stomach mucosa: pepsin-degraded food, lactoferrin and heme compounds that damaged tissues release. In gastric environment – acidic, microaerobic conditions – iron has a highly solubility and a low binding affinity to eukaryotic iron-complexing proteins. The principal ionic state of iron present in *H. pylori* is the ferrous iron (Fe^{2+}), that is transported by the bacterium via the FeoB protein system (HP0687). This type of iron acquisition has a major role in colonization (Velayudhan et al., 2000), making it indispensable to adaptation. Another form of iron present in *H. pylori* is the ferric iron (Fe^{3+}), that can be converted to Fe^{2+} by the ferric

reductase (Worst et al., 1998).

There are various types of iron transporters, adaptated to the different forms of iron. Ferric iron is insoluble, so it needs to be expelled to the surrounding environment, through a transport system (TonB system) that can transfer this metal over the outer membrane, which incorporates an iron receptor. A second ferric iron transport system is the ABC transporter, that transfers the iron present in the periplasm to the cytoplasm. *H. pylori* genome encodes some proteins that are involved in iron transport: the putative ferric citrate outer membrane receptor FecA, the FrpB outer membrane receptor (Alm et al., 1999; Berg et al., 1997; Tomb et al., 1997), the periplasmic binding protein CeuE, the inner membrane permease (FecD) and the ATP-binding-protein (FecE) (Velayudhan et al., 2000).

*H. pylori e*xpresses iron storage proteins, such as the Pfr ferritin and the HP-NAP bacterioferritin. The Pfr ferritin works as an intracellular iron reservoir, in wich this metal is released to be used when it exists in limited concentrations. So, this storage protein protects the bacterium against toxicity, free iron-mediated oxidative stress and also supports its growth (Bereswill et al., 1998; Bereswill et al., 2000; Doig et al., 1993; Frazier et al., 1993; Waidner et al., 2002). The HP-NAP bacterioferritin mediates adhesion to mucin (Namavar et al., 1998) and is homologous to bacterioferrins and DNA-binding proteins (Dps family) (Dundon et al., 2001; Tonello et al., 1999).

4.3.3 Copper

Copper works as a cofactor of many proteins – involved in electron transport, oxidases and hydroxylases – and assists in ROS formation (Rensing & Grass, 2003). There are proteins that play a role in copper transport or may behave as copper chaperones, like CopP, a small protein. *H. pylori e*xpresses a copper export system that includes some proteins: CopA (HP1073), CopA2 (HP1503), P-type ATPases (Bayle et al., 1998; Beier et al., 1997; Zhongming and Taylor, 1996) and a copper resistance determinant, CrdA (Waidner et al., 2002).

4.3.4 Cobalt

Cobalt is a cofactor of arginase, an enzyme present in nitrogen metabolism (McGee et al., 1999; Mendz et al., 1998) and part of the immune system response (Gobert et al., 2001; Gobert et al., 2002).

5. Colonization of *H. pylori*

There are four critical steps for a successful colonization of *H. pylori* and consequent infection. First, the bacterium has to enter into the host stomach. Once in there, there is the need to survive in this hostile acidic environment (1). To achieve that, the bacterium neutralizes the acid by activating urease and, as a result, the infection begins. After that, it moves toward host gastric epithelium cells by flagella-mediated motility (2). When *H. pylori* arrives to epithelium cells, bacterial adhesins interact with host cell receptors, enabling an attachment to them (3). So, flagella and adhesins allow a successful colonization and lead to a persistent infection. The next stage of infection consists in a release of toxins by the bacterium, such as cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), causing tissue damage to the host (4). These events induce an inflammatory response by the host, who initiates innate immunity by secreting chemokines through the gastric epithelium layer and activates immune system cells, such as neutrophils. Inevitably, gastric pathologies – ulcer and gastritis – are developed due to this order of events (Fig.3) (Kao et al., 2016).

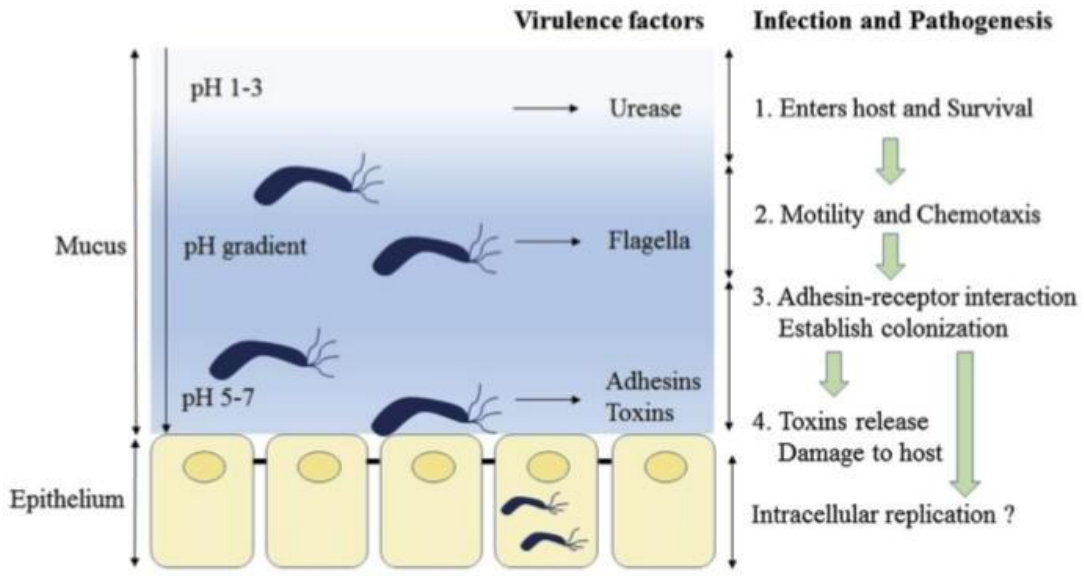

Figure 3 - Schematic diagram of *Helicobacter pylori* infection and pathogenesis (reproduced from Kao et al., 2016).

5.1 Bacterial factors in *H. pylori* pathogenesis

5.1.1 Urease

Regulation of urease activity is an acid acclimation mechanism which consists in adjusting the periplasmic pH in the stomach. This enzyme gene cluster is composed by some genes: catalytic subunits (*ureA*/*B*), an acid-gated urea channel (*ureI*) and accessory assembly proteins (*ureE-H*) (Mobley et al., 1995). The molecular masses of the subunits UreA and UreB are 27 kDa and 62 kDa, respectively. They are encoded by an operon that carries the *ureA* and *ureB* genes (Labigne et al., 1991). There is a second operon, downstream the *ureA*/*B* genes, responsible for encoding UreIEFGH proteins (Cussac et al., 1992; Mobley et al., 1995). There are two promoters in charge of the transcription of the urease gene cluster: P_{ureA} (upstream the *ureA* gene) and P_{ureI} (in the intergenic region between *ureB* and *ureI*) (Akada et al., 2000). The cofactor of urease is nickel, a metal that is inserted into the apoenzyme by four accessory proteins – UreE, UreF, UreG and UreH (UreE is a relevant metallochaperone) –, allowing heterodimer urease activity (Fig.4). However, the mechanism of nickel insertion is not yet well understood (Yang et al., 2014).

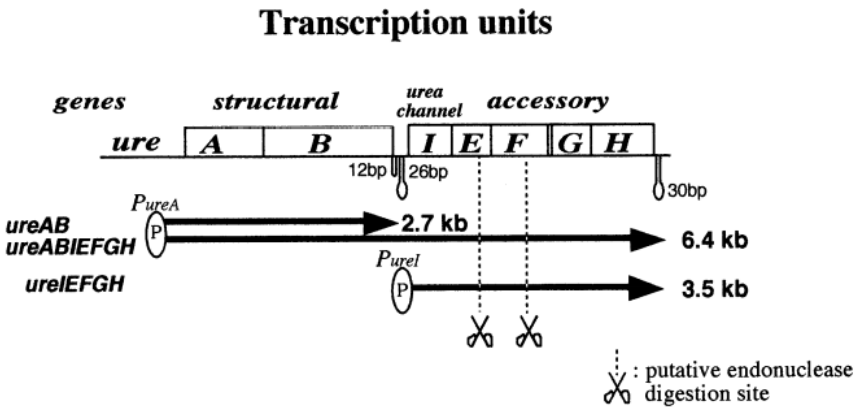

Figure 4 - Transcription units of the *ure* operon of *H. pylori* (adapted from Akada et al., 2000).

Urease can be intracellular or can be found on *H. pylori* surface, and allows the bacterium to be acid resistance. The urease intrabacterial activity is regulated by a channel – the pronton-gated urea channel, UreI. This channel is inserted in the inner membrane and functions to promote homeostasis. It only opens at acidic conditions and fully opens at pH 5.0, which allows a rapid incorporation of urea. So, a lethal alkalization is prevented by this mechanism, inhibiting the entry of urea under neutral conditions, at pH 7.0, by closing the channel at this pH (Weeks et al., 2000). The activity of urease enables the bacterium to produce a big amount of ammonia derived from urea and, because of that, UreI can expel NH_3 and/or NH_4 across the inner membrane to the periplasm (Scott et al., 2010). This type of ammonia

can possibly be assimilated into amino acids, being part of the nitrogen metabolism (Miller & Maier, 2014) (see also nitrogen metabolism).

Urease present on *H. pylori* surface (extracellular urease) breaks urea into carbon dioxide (CO_2) and ammonia (NH_3). When combined with water, amonia forms ammonia hydroxide. Because of this reaction, the bacterium can survive in the harsh acidic gastric juice, once the ammonia hydroxide can neutralize the surrounding microenvironment. This enzyme can also regulate interactions between the bacterium and immune system cells, macrophages, by modulating phagosome pH and megasome formation during phagocytosis. By resisting to this process characteristic of innate immune system, *H. pylori* can survive in the harsh environment present in these type of cells caused by the acidic pH (Schwartz & Allen, 2006).

5.1.2 Flagella

H. pylori flagella enable the bacterium to move from gastric mucosa epithelium layer to the basal layer. The bacterium is composed by two to six unipolar sheated flagella, essential for establish gastric colonization. Flagella are 3 µm in length, approximately, and can carry terminal bulbs (Eaton et al., 1996; Kim et al., 1999). Flagella provide high motility, which is directly related to higher bacterial density. An higher bacterial density increases the host's inflammatory response, resulting in serious gastric pathologies (Kao et al., 2012). For these reasons, flagella can be considered one of the early stage virulence factors (Kao et al., 2016).

Motility is a very complex process that requires many proteins: there are more than 40 proteins involved in flagella's synthesis and functioning (Lertsethtakarn et al., 2011). These organels are constituted by three distinct parts: the basal body (that has many protein structures and is a source of energy), the hook (which is composed by the flagellar hook protein (FlgE) and links the other two parts) and the flagellar filament (which is constituted by the flagellin subunit proteins FlaA and FlaB, encoded by the genes *flaA* and *flaB*, respectively, necessary for total motility) (Josenhans et al., 1995). The flagellar filament needs to be glycosylated to be effective, making glycosylation an essential process for functional flagella (Schoenhofen et al., 2006). Genes involved in flagella encoding can be distributed in three classes, related to its regulation, forming a complex transcriptional network (Niehus et al., 2004). Despite the role of flagella in adherence to mammalian hosts in some bacterial species (specific attachment of flagella to epithelial cells) (Bucior et al., 2012; Merino et al., 2014), there is no evidence of this process in *H. pylori* (Clyne et al., 2000). Flagella do not directly play a role in cell adhesion, but regulators that control flagellar-related genes may affect adhesin expression (Clyne et al., 2000; Kao et al., 2016).

5.1.3 Adhensins

When stomach colonization occurs, *H. pylori* can resist to peristalsis and gastric emptying thanks to interactions between its adhesins and host cellular receptors. Not all *H. pylori* strains express blood-antigen binding protein A (BabA) and sialic acid-binding adhesin (SabA), despite being the best characterized adhesins that have been analized (Ilver et al., 1998; Mahdavi et al., 2002).

There are other adhesins in *H. pylori* that have the capacity of adaption to different tissues: neutrophil-activating protein (NAP) (Teneberg et al., 1997), heat shock protein 60 (Hsp60) (Yamaguchi et al., 1997), adherence-associated proteins (AlpA and AlpB) (Odenbreit et al., 1999), *H. pylori* outer membrane protein (HopZ) (Peck et al., 1999) and lacdiNAc-binding adhesin (LabA) (Rossez et al., 2014).

5.1.3.1 Neutrophil activating protein A (NAP)

NAP belongs to the family of DNA-protecting proteins under starved conditions (Dps). This adhesin stimulates neutrophils to produce high rates of oxygen radicals, which causes tissue damage, assists these cells in the adhesion to endothelial cells (Evans et al., 1995), and induces them to express and release some proteins [interleukin (IL)-8, macrophage inflammatory protein (MIP)-1a, and MIP-1b]. For these reasons, NAP is a positive factor for enhanced bacterial colonization of tissues, since they are more damaged. Owing to the effects caused by NAP in *H. pylori* infection, this protein is considered a hallmark of chronic gastritis, and is responsible for the penetration of some immune system cells into the gastric mucosa, such as neutrophils, lymphocytes and monocytes (Polenghi et al., 2007) (Fig.6). NAP interacts with neutrophils through receptors expressed on their surface, glycosphingolipids (Polenghi et al., 2007), and may favor SabA-mediated binding of host sialylated antigens (Petersson et al., 2006). This protein protects *H. pylori* DNA: it can bind DNA, protecting it from the damage caused by free radicals, or reducing the oxidative stress originated in some reactions (Kottakis et al., 2008; Wang et al., 2006). There is no evidence that NAP is directly associated with gastric inflammation caused by *H. pylori* (Kao et al., 2016).

5.1.3.2 Heat shock protein 60

Heat shock proteins (Hsps) are a well conserved protein family that can be found in prokaryotes and eukaryotes. Their activity is induced by stress events: pH alteration, temperature, lack of oxygen and infection. There are two Hsps that are mostly synthetized by *H. pylori*: GroES-like HspA (Hsp10), and GroEL-like HspB (Hsp60). When the bacterium is exposed to an acidic pH, Hsp60 is highly expressed. So, its

interaction with receptor-like sulfatide may suffer a shift caused by this type of stress, inducing a change in specificity to these receptors (Huesca et al., 1996; Yamaguchi et al., 1997).

Hsps can induce a response from the immune system, which makes them a part of the potential immunogens. They can induce monocytes or gastric epithelial cells to synthesize IL-6, IL-8, tumor necrosis factor alpha (TNF-α) and growth-related oncogene (GRO) (Lin et al., 2010). Anti-Hsp60 antibodies are present in infected patients and their concentration is related with gastritis or gastric cancer evolution (Lin et al., 2010; Tanaka et al., 2009). This type of antibodies can increase the production of IL-8 and TNF-α, modulating *H. pylori* pathogenesis (Fig.6) (Sheu et al., 2003).

5.1.3.3 Blood group antigen binding adhesin (BabA and BabB)

There are three types of *bab* alleles: two *babA* (*$babA_1$ and $babA_2$*) and *babB.* BabA is very important in the initial states of stomach colonization, since the bacterium uses it to bind to fucosylated Lewis B blood-group antigen (Lewis b [Le^b]), that is expressed on the surface of gastric epithelium cells (Ilver et al., 1998). BabA receptor and 0 type blood antigen have similar structures, resulting in a correlation between this type of blood and gastric pathologies (Aspholm-Hurtig et al., 2004).

BabA and BabB are similar in their terminal regions and diverge in the midle region. This region has a relevant role in defining the adhesion capacity of BabB to gastric cells, relating, this way, its expression with an increased risk of peptic ulcer and gastric cancer in western countries, but not in asian countries (Chen et al., 2013; Gerhard et al., 1999; Mizushima et al., 2001). So, the expression of BabB is connected with increased gastric lesions (Sheh et al., 2013). Some *H. pylori* strains do not carry *$babA_2$* or are lacking in BabA due to mutations. Despite this, the bacterium has the ability of recombinate *$babA_1$* and *babB* in some gastric environments, forming a chimeric BabB/A, which can still bind to the Le^b antigen. Hence, the recombination of these two proteins can transform *H. pylori* adherence properties, contributing to its capacity of adaptation and, consequently, an enhanced stomach colonization (Bäckström et al., 2004; Nell et al., 2014; Sheu et al., 2012).

5.1.3.4 Sialic acid-binding adhesin (SabA)

Sialic acid-binding adhesin (SabA) has an important role in *H. pylori* stomach adhesion and colonization of a host with gastritis, since the expression of sialyl-Lewis x glyco-sphingolipid (sLe^x) antigen (which plays a fundamental role in cell-to-cell recognition processes) is higher on gastric epithelium cells surface with

inflammation (Mahdavi et al., 2002; Sheu et al., 2006). *SabB* and *sabA* genes are homologous, but *sabB* may not be included in sLe^x binding, making its role in *H. pylori* adhesion still unclear (Kao et al., 2016; Mahdavi et al., 2002).

There is a high incidence of *sabA* in clinical strains (almost 80%), and there are two types of *sabA* genotypes: type I *sabA* and type II *sabA*, which differ in the regulation of their expression (Alm et al., 1999; Kao et al., 2012; Sheu et al., 2006). There can be formed mixed-genotyped organisms due to the mechanism of regulation of type I *sabA* during infection, resulting in a more effective adaptation to different environments (Kao et al., 2012; Sheu et al., 2006). *H. pylori* density is higher in patients with SabA-positive strains, when compared with patients with SabA-negative strains, sugesting that the interaction of SabA with sLe^x antigen has a major role in stomach colonization. Lewis b (Le^b) blood group antigens are isoforms of Lewis x (Le^x), expressed on *H. pylori* lipopolysaccharides (Heneghan et al., 2000). When there is no Le^b expression, Le^x plays a critical role in colonization (Sheu et al., 2003). This fact is suported by other event: when there is no Le^b expression or the expression is weak, Le^x can ehnance colonization (Sheu et al., 2003; Sheu et al., 2006). Changes in sLe^x-mediated adherence can help *H. pylori* escaping areas with a stronger immune response (Kao et al., 2016).

5.1.4 Toxins and host tissue damage

5.1.4.1 Cytotoxin-associated gene A (CagA)

CagA is present is present in most *H. pylori* strains: the incidence of CagA in western countries is about 60% (Chiurillo et al., 2013; Rezaeifar et al., 2013), while in asian countries it is about 90% (Yamaoka et al., 1999). CagA-positive strains are associated with gastric diseases, such as gastritis, ulcer and gastric cancer (Azuma, 2004; Hatakeyama, 2014; Matos et al., 2013). Were identified two types of CagA: the Western-type CagA and the East Asian-type CagA (Argent et al., 2004; Higashi et al., 2002). The last type mentioned is related with more cytoskeleton changes, which makes it more predictable to be linked with the development of gastric cancer (Argent et al., 2004).

The *cag* pathogenicity island (*cag*PAI) can be found in *H. pylori'*s chromosome and has more than thirty genes (Alm et al., 1999): six genes that are homologous to type IV secretion systems (T4SS), responsible for translocating the protein CagA into the interior (cytoplasm) of the host gastric cells (Odenbreit et al., 2000). β1-integrin is the receptor used by other proteins (CagL, CagY) to translocate CagA into the host cells (Conradi et al., 2012; Tegtmeyer et al., 2014).

CagA can modify host cell signalization through dependent and independent phosphorylations. When phosphorylated, CagA influences cell adhesion, spreading and migration (Higashi et al., 2002; Yamazaki et al., 2003). A studied showed that nonphosphorylated CagA can interact with a host hepatocyte growth factor receptor, leading to cellular proliferation and inflammation, causing tissue damage, which improves *H. pylori'*s capacity of colonization (Suzuki et al., 2009). It can also affect gastric epithelium cell pedestals formation, cytoskeleton's structure and cell proliferation. In addition, due to its activity, CagA can cause a response by gastric epithelium cells, inducing them to release IL-8 (Fig.6) (Boonyanugomol et al., 2011; Boonyanugomol et al., 2013; Kikuchi et al., 2012).

5.1.4.2 Vacuolating cytotoxin A (VacA)

When vacuolating cytotoxin A (VacA) is secreted, it forms an oligomeric structure, composed by two domains. VacA can be inserted into the host cell membrane and is like an anion-selection channel that has the ability to inject bicarbonate and organic anions into the host (Szabò et al., 1999). This way, it assists stomach colonization, once it turns potencial metabolic substrates for *H. pylori* growth available. It can intervene in cellular processes, such as endocytosis. VacA channel can enter into late endossomes, allowing the entrance of anions, which leads to an influx of water, responding to an increase of weak bases, resulting in a larger vacuole (Palframan et al., 2012; Terebiznik et al., 2006). When VacA is applied extracellulary, not entering host cells, it may affect mitochondria due to its activity, since it induces cytocrome C secretion, ER (endoplasmic reticulum) stress and cellular processes (apoptosis) (Akazawa et al., 2013). VacA plays a regulatory role in cell cycle, interfering with genes involved in cell proliferation and death. Due to its nefarious activity to the host, it induces an inflammatory response, as other proteins do, as mentioned above. This induced response is acute, and consists in IL-8 release (Fig.6) (Hisatsune et al., 2008).

All *H. pylori* strains own the *vacA* gene. VacA proteins change in virulence due to distinct genopatterns, that can be present in different genome regions: signal sequence (s1a, s1b, s1c and s2), mid-region (m1, m1T and m2), and the intermediate region (i1, i2 and i3) (Fig.5). These threee regions can combine, originating different subtypes of genotypes, that vary in pathogenicity (Cover et al., 1994; Ferreira et al., 2012). Genotype s1/m1 is known for its facility in damaging cells severely, once it is associated with higher VacA expression (Cover et al., 1994). *VacA* s1 and m1 strains give rise to higher inflammation levels of gastric mucosa, when compared with *vacA* s2 and m2 strains, that are less virulent. The higher inflammation levels can be translated into an increase of the risk for the development of gastric

atrophy and carcinoma, making *vacA* s1 and m1 strains way more virulent. Different subtypes of genotypes can be associated between them and with *cagA*: v*acA* i1 genotype is related with *vacA* s1, *vacA* m1 and *cagA*-positive genotypes, as opposed to v*acA* i2 genotype, that is linked to *vacA* s2, *vacA* m2 and *cagA*-negative genotypes (Ferreira et al., 2012). There can be noticed associations between some gastric diseases and subtypes of genotypes, that diversify according to distinct countries. In patients with gastric cancer, *vacA* s1a and *vacA* s1c subtypes are less expressed, making them less common; while patients with peptic ulcer and chronic gastritis express more *vacA* m1T subtype (Lin et al., 2004).

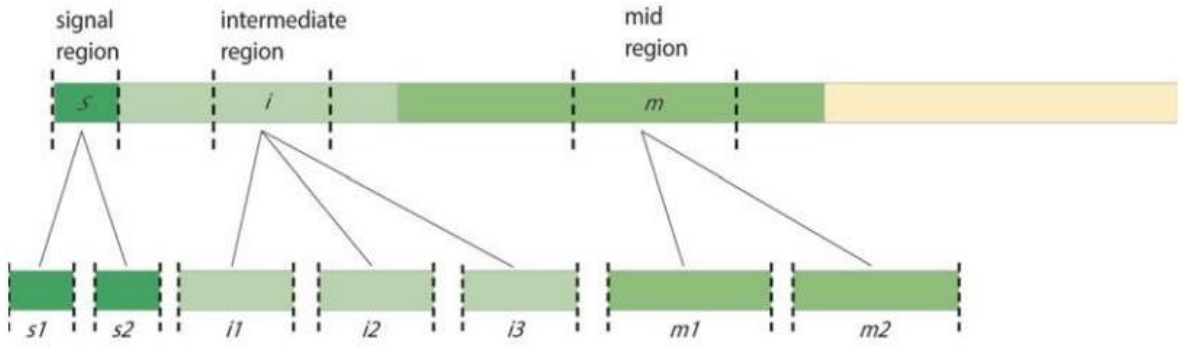

Figure 5 - *vacA* allelic diversity and structure. Significant allelic diversity exists in three regions of the *vacA* gene: the signal region (s1 and s2), the intermediate region (i1, i2 and i3) and the mid-region (m1 and m2) (adapted from Palframan et al., 2012).

H. pylori can occupy host epithelial cells and reproduce inside them - in the plasma membrane or in cytoplasma - within double-layer vesicles. During infection, the bacterium incites autophagic vesicles synthesis, where can occur its replication and also its degradation, after fusing with lysosomes (Chu et al., 2010). *H. pylori* replication inside host cells influences its biological life cycle and constitutes a resistent factor to therapy used against it (Wang et al., 2009). CagA and VacA are fundamental in bacterial virulence: *H. pylori* CagA and VacA wild-types strains obtain higher levels of reproduction inside immune system cells (macrophages), when compared with CagA and VacA mutants (Wang et al., 2009). The dissimilarity in *H. pylori* studies – the use of different host cell lines and strains – generates incoherent results about autophagy effects (Deen et al., 2013). For this reason, further studies are necessary to understand the relevance of autophagy in *H. pylori* infection: its effectiveness as a front line defense and the mechanisms used by *H. pylori* to subsist inside host cells (Kao et al., 2016).

6. Immune system response

Natural immune responses depend on well-conserved invariant structures: the pathogen-associated molecular patterns (PAMPs), which are recognized by different innate sensors, such as toll-like receptors (TLR). Examples of PAMPs are flagellins and LPS (lipopolysaccharide present in the bacterial cell wall), a powerful endotoxin present in Gram-negative bacterial species (Uematsu & Akira, 2006). Several TLRs, such as TLR2, TLR4, TLR5, TLR8 and TLR9 have been linked to *H. pylori* infection. In summary, *H. pylori* triggers a strong immune system response, in which it is common to find an infiltration of inflammatory cells within the gastric mucosa, due to its direct attraction to the different components present in the bacterium, as well as the cytokines expressed by gastric cells in response to *H. pylori*. However, it has been shown that there is a correlation between neutrophil infiltration and the degree of mucosal damage. After exposure to the microorganism, there is a recruitment of immune system cells – neutrophils, macrophages, monocytes, dendritic cells (DCs), natural killer cells (NK) and lymphocytes –, and a release of reactive oxygen species (ROS) and reactive nitrogen species (RNS). *H. pylori* can also promote inflammation by inducing the production of various interleukins, such as IL-1, IL-6 and IL-12, as well as many other proinflammatory factors, such as abnormal cytoskeleton reorganization, proliferation and differentiation, phenotypic cell changes, apoptosis/cytotoxicity, expression of growth factors and defects in connections and polarity, which can lead to a progression to atrophic gastritis (Fig.6) (Conteduca et al., 2012).

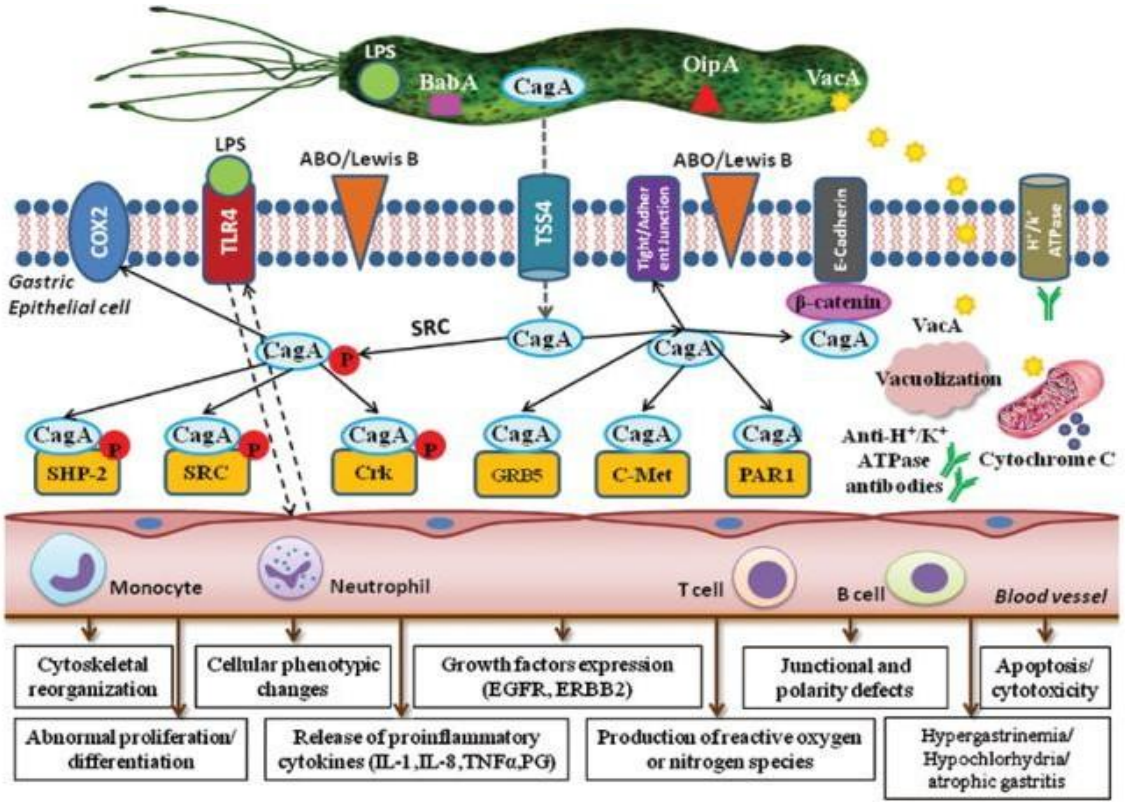

Figure 6 - Pathogenesis of *H. pylori* infection. Several virulence factors, such as CagA and VacA, encoded by *H. pylori* genes, interact with gastric epithelial cells and the immune system, resulting in an inflammatory response, mucosal damage and, eventually, gastric carcinogenesis (reproduced from Conteduca et al., 2012).

7. Infection's acquisition and transmission

The infection caused by this bacterium is the most frequent, with no discrepancy between genders (Atherton et al., 1995). There are two routes of transmission: the iatrogenic route and the person-to-person route. The first one consists of direct contact with infected materials, while the second can be divided in three vias: oral-oral and fecal-oral (characterized by contact with a common source of contamination, such as water or food) and also via gastro-oral, in which direct contact with vomiting occurs, usually present in children. These are the transmission routes since this bacterium can be found in saliva, dental plaque, gastric fluid and feces. *H. pylori* infection is associated to dyspeptic symptoms, usually chronic and capable of causing a negative impact in life quality. Dyspeptic patients commonly suffer from heartburn, epigastric pain, postprandial discomfort, bloating and a heavy feeling in the upper abdominal area (Kim et al., 2004). There are other manifestations that can arise from this infection: nausea, vomiting, loss of appetite, frequent belching, bad breath and weight loss

(Mendall & Northfield, 1995; Dunn et al., 1997; Ribeiro et al., 2004; Momtaz et al., 2012).

Although symptoms usually appear only in adulthood, the acquisition of the bacterium occurs in early stages, during childhood (Ernst & Gold, 1999). The infection is acquired through a vertical transmission from parents to children, as shown in data about families. This route of transmission is the most common way of *H. pylori* colonization (Suerbaum & Josenhans, 2007), as suported by phylogenetic studies of different human populations (Konno et al., 2005; McMillan et al., 2011; Roma-Giannikou et al., 2003). An iranian study about vertical transmission revealed that there is a significant difference of the origin of colonization in children: most cases showed an *H. pylori* strain phylogenetically similar to the strain present in their mothers (Mamishi et al., 2016). Similar animal studies agree with these facts, as evidenced by an investigation about cats, that showed that kittens are passively colonized by the bacterium in an *H. pylori*-infected cat colony (Straubinger et al., 2003).

8. Infection's incidence

H. pylori is present in more than 50% of the world population. Years ago, infection's incidence was about 30% in developed countries and 80% in developing countries (Covacci et al., 1999). Some years later, it still remained the same: >70% still happened in developing countries (Jemal et al., 2011). This bacterium colonizes the stomach and, as a result, is the origin of many human gastric diseases. It can persist in its host during his whole lifetime (Kuipers et al., 2000), causing high rates of morbidity and mortality worldwide. Nowadays, there is a large variation of the prevalence of *H. pylori* infection around the world, with elevated incidence levels reported in South America, sub-Saharan Africa and the Middle East. The lowest incidence levels correspond to Australasia, Switzerland and more generally to North America and Western Europe (Fig.7) (Asfeldt et al., 2008; Ben Mansour et al., 2016; Laszewicz et al., 2014; Luzza et al., 2014; McDonald et al., 2015; Peleteiro et al., 2014; Sanchez Ceballos et al., 2007; Van Blankenstein et al., 2013).

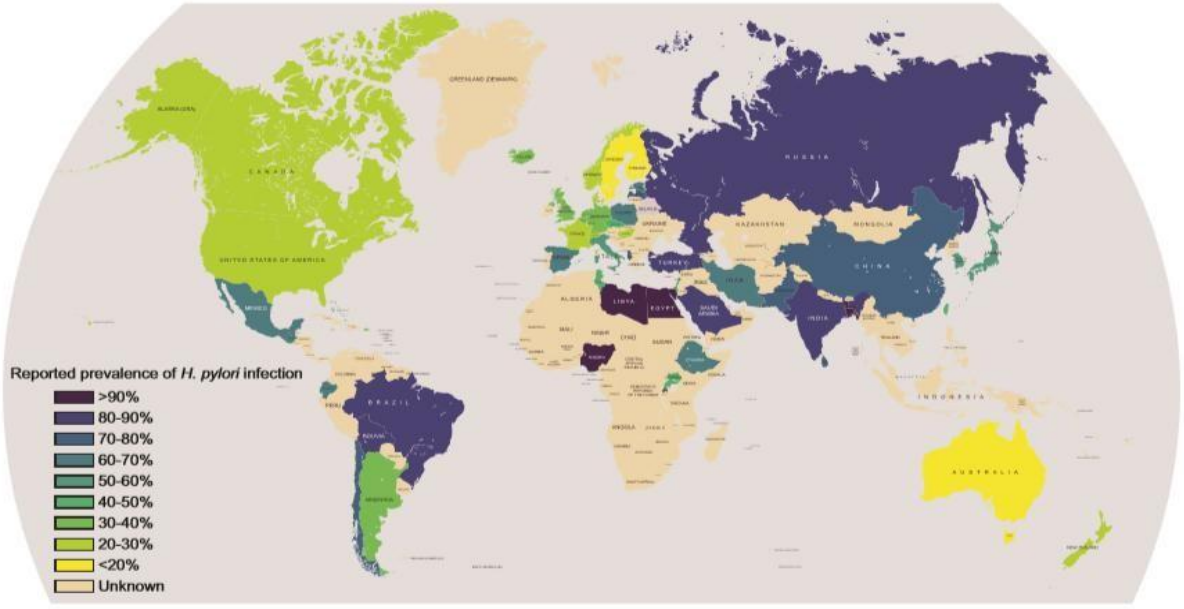

Figure 7 - Worldwide prevalence of *H. pylori* infection (reproduced from Burkitt et al., 2017).

As said before, there are notable differences of infection among distinct regions around the globe. There are some places where the infection occurs at an early age (normally in childhood), which results in a high percentage of infected population by the age of 20 (about 80%). This occurs, for example, in Eastern regions of Africa and in some regions of Latin America. On the other hand, there are countries where the infection rate is low in children under the age of 10, but it increases (about 40%) in adults within 30 to 40 years old. This pattern of infection occurs in some developed countries, such as France, USA, the UK or Australia. When people from high-incidence areas (Japan) migrate to others with a low-risk (USA), the risk of infection decreases, and the descendant generations acquire infection risk levels similar to those intrinsic of the host country (Suerbaum & Michetti, 2002).

In Portugal, despite a steady decline in mortality over the past decades, gastric cancer rates are among the highest in Western Europe (ranking fifth in incidence and mortality when both sexes are considered), especially in the north of the country (Bastos et al., 2013; Morais et al., 2016). In another meta-analysis review, Portugal reveals an estimated infection prevalence of >70% (86.4%), similar of that founded in developing countries (Fig.8) (Hooi et al., 2017).

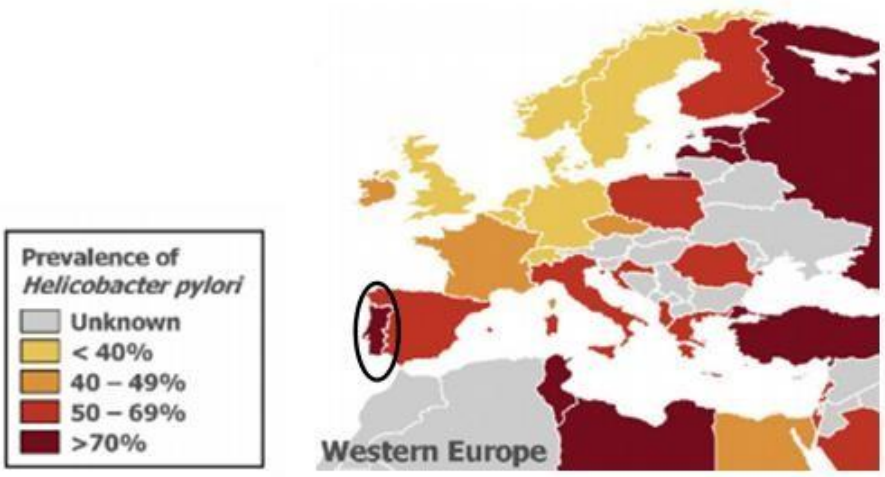

Figure 8 – Western Europe prevalence of *H. pylori* choropleth map. Portugal is surrounded by the black oval line (adapted from Hooi et al., 2017).

9. Social and Economical Impact

9.1 Lethality

H. pylori is the cause of one of the most frequent infections worldwide, which makes it the main cause of infection-induced cancer (Ferlay et al., 2015). The microbial infection and chronic inflamation caused by *H. pylori* is clearly interconnected with the occurrence of gastric cancer, depicting a model of cancer development. Hence, the bacterium has been classified as a class I carcinogen since 1994 by the World Health Organization (WHO) (IARC Working Group, 1994). In 1975, gastric cancer was considered the most common neoplasm; thereafter, has been observed a decrease in mortality from the disease (Bertuccio et al., 2009; Crew & Neugut, 2006). Despite the decrease in mortality, the treatment that was setted to treat the infection (the standard regimen treatment is based on antibiotics – amoxicillin and clarithromycin – and a proton-pump inhibitor) was losing effectiveness and, in 2017, *H. pylori* was considered a high priority bacterium clarithromycin-resistant (priority 2) by the World Health Organization (WHO, 2018).

A decade ago, gastric cancer was the second most lethal type of cancer worldwide: the number of new cases was about 989600 (male-to-female ratio 2:1) and there were 738000 deaths. When its incidence and mortality rates are accounted, gastric cancer exhibits remarkable values: 8% of the total of cancer cases and 10% of total cancer related deaths. The main part of both new cases and gastric cancer-related deaths (about >70%) occured in developing countries, specially in Eastern Asia (Jemal et al., 2011). In 2012, gastric cancer was one of the most lethal cancers (it was the third leading cause of cancer-related death, with more than 720000 deaths worldwide), with an incidence of nearby 1 million cases each year, making it the fifth most diagnosed

cancer (Ferlay et al., 2015). Nowadays, this type of cancer remains one of the most frequent and lethal cancers worldwide (particularly among older males). In 2018, there were over a million diagnosed cases, and it caused about 783000 deaths. Because of these numbers, gastric cancer was the fifth most common neoplasm and the third leading cause of cancer death globally, as it was in 2012 (Bray et al., 2018).

9.2 Social status

Globally, the frequency of the infection varies in accordance with age, race and geographic localization. This wide range of infection rates occurs due to the differences in socioeconomic conditions and, as a consequence, inequalities in public health services (Duque et al., 2012). So, the inequality of access to healthcare systems creates a great discrepancy in gastric health among different countries worldwide (Burkitt et al., 2017). The fact of existing divergence in infection's incidence among ethnic groups of similar socioeconomic conditions may reflect the impact of environmental factors and host genetic variations (Conteduca et al., 2013). However, during the last three decades, it has been observed a decrease of the impact of gastric cancer worldwide (Bray et al., 2018). Incidence and mortality rates are substantially lower, perhaps due to some dietary changes (such as a lower comsumption of salted and preserved food), an evolution in food conservation methods (the accession to refrigeration), the availability of fresh fruits and vegetables and a wider application of *H. pylori* infection eradication therapies (Zullo et al., 2010).

9.3 Economic costs

The economic impact of gastric cancer is expected to increase even further due to the growth of the incidence rate, the costs of treatment after diagnosis and the use of expensive treatments. Thus, this type of cancer manifests as a socioeconomic challenge to governments, health systems, insurers and patients (Nagavarapu, 2015). The main problem in eradicating *H. pylori* is the resistance to antibiotics acquired by this bacterium over the years (Song et al., 2013). Thereby, the development of a strategy that could eradicate the bacterium and prevent gastric cancer simultaneously would help reduce treatment costs for all the identities involved in the process. Additionally, reaching a type of therapy of this kind would ensure quality of life for the patients in risk or with a gastric cancer prognosis.

10. Bacterial-host relation

H. pylori coevolved with humans thousands of years ago. Phylogenetic studies show that *H. pylori* strains are associated with human populations since before 58000 years ago, the time when early humans migrated from Africa (Linz et al., 2007). As previously refered, the bacterium has the ability to interfere in fundamental physiological processes for the cell-activation of growth factors, apoptosis, cell proliferation, angiogenesis and rupture of cell-cell contacts (Hunt, 2004; Malfertheiner et al., 2006).

Although more than 50% of the world's population is infected – even though some infections are asymptomatic –, only less than 2% of infected people develop gastric cancer (Fig.12). These values can be explained by a dynamic balance present in the relationship between the bacterium and the host, that resulted from long-term interactions (Blaser & Atherton, 2004). So, the symptoms that result from infection and, consequently, the carcinogenic effect, depend on the interaction between the virulence of the microorganism and the inflammatory response triggered. Therefore, it can be concluded that the pathogenic characteristics of the bacterium are as relevant as the host response.

The most relevant risk factor for gastric carcinogenesis is the infection caused by *H. pylori*, being a risk factor of at least 80% of cases of this type of cancer (Graham, 2015). Studies revealed that the infection is endemic where the bacterium is endemic (Agha & Graham, 2005) and that its geographical prevalence and predominant form depends on further risk factors, once that gastric carcinogenesis is considered a multifactorial process. These other factors can be divided into main groups: genetic (unalterable, intrinsic) and epigenetic (endogenous or exogenous, potentially modifiable).

The genetic factors rely on the host genotype: cellular processes such as stromal remodelling [specifically the proteins involved, such as matrix metalloproteinases (Tang et al., 2008)], inflammation [pro-inflammatory cytokine profile, that is, polymorphisms at loci encoding cytokines and their receptors (Persson et al., 2011)], prostate stem cell antigen (PSCA) that acts as a tumour suppressor gene (Garcia-Gonzalez et al., 2015; Ichikawa et al., 2015; Mou et al., 2015), apoptosis, proliferation and/or a positive family history.

The epigenetic factors are related with the host's lifestyle (such as diet – dietary salt and nitrosamine intake (Jakszyn et al., 2006; Mendez et al., 2007; Wang et al., 2009), smoking status and alcohol ingestion), environmental causes (such as food preservation methods, nutrition and socioeconomic conditions), bacterial virulence properties (Graham et al., 2009; Kodaman et al., 2014) and non-*Helicobacter* gastric microbiota (Dicksved et al., 2009; Lofgren et al., 2011) (Fig.9). Age is also considered a risk factor, making the elderly being the most vulnerable group (Malfertheiner et al., 2006).

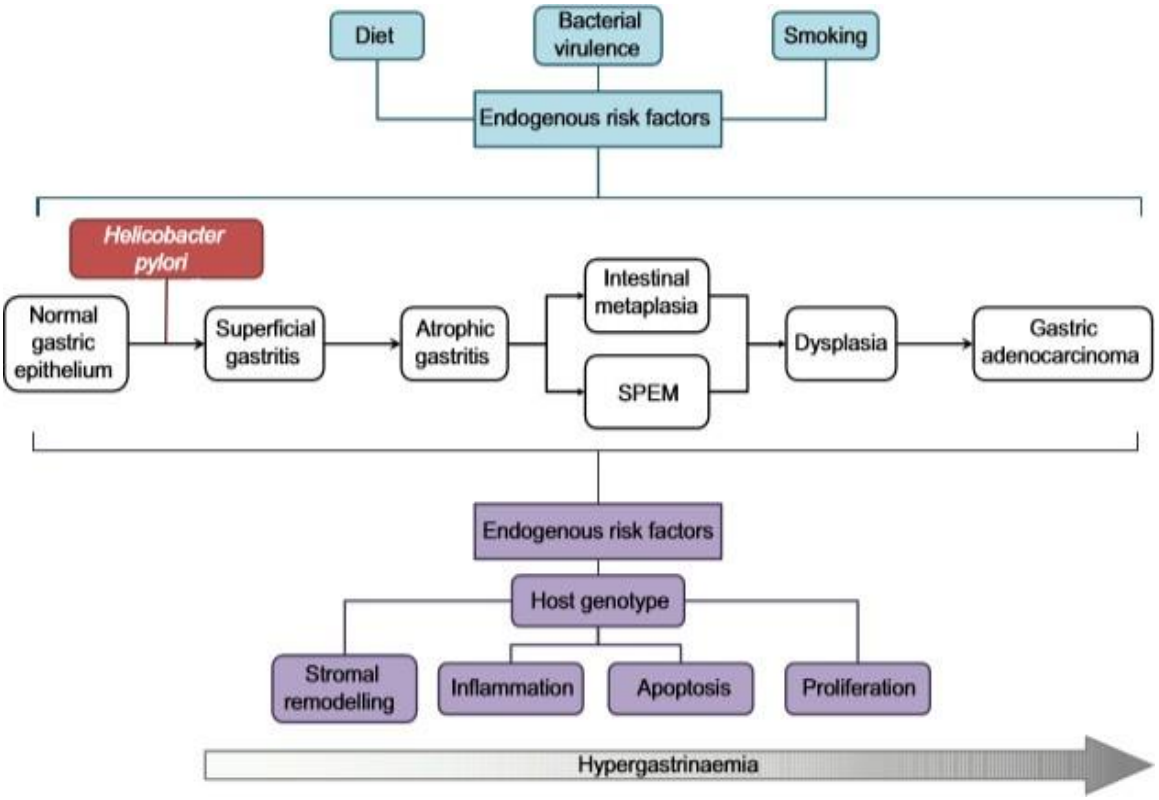

Figure 9 - *Helicobacter pylori* infection and and its progression to gastric cancer. A schematic demonstrating the pathological progression of *H. pylori*-induced gastric pre-neoplasia and highlighting endogenous risk factors for progression towards gastric cancer. SPEM: spasmolytic polypeptide-expressing metaplasia (reproduced from Burkitt et al., 2017).

11. Stomach's anatomy and physiology

The stomach belongs to the digestive tract and is located between the esophagus and the small intestine. It is involved in food digestion, by secreting enzymes and gastric acid. This organ has other functions, such as the secretion of intrinsic factors (as the intrinsic factor required for vitamin B_{12} absorption).

The human stomach is composed by various anatomical parts: oesophagus, cardia, fundus, corpus, antrum and duodenum. There are two types of columnar mucosa that line this organ: the fundus and corpus are lined with oxyntic or corpus glands (profound), and the antrum is lined with antral glands. Oxyntic glands are responsible for acid-secretion and are composed by an epithelial monolayer with an asymmetric proliferative stem cell zone that differentiates into many cell types, such as mucous neck cells, parietal cells, chief cells and enteroendocrine cells. The base of the antral gland is composed by a zone of stem cells that have the capacity to generate the whole gland. When this type of cells divide asymmetrically, there is a migration of daughter cells upwards in the direction of the gastric lumen, where they differentiate into mucous neck, surface mucous or endocrine cells. In the case of corpus glans, the stem cell zone is situated at the isthmus of the gland. Some cells from this zone migrate upwards to differentiate into surface mucous cells; others migrate downwards and differentiate into acid-secreting parietal cells, endocrine cells or zymogen-secreting chief cells. Zymogen consists in the inactive form of a digestive enzyme, such as pepsinogen. In turn, pepsinogen is an inactive form of pepsin, a proteolytic enzyme that is activated by parietal cells when they secrete gastric acid. The mouse stomach shares analogous areas with the human stomach, as in the case of antral and corpus glands. The murine also has a forestomach that is lined with squamous epithelium (Burkitt et al., 2017) (Fig.10).

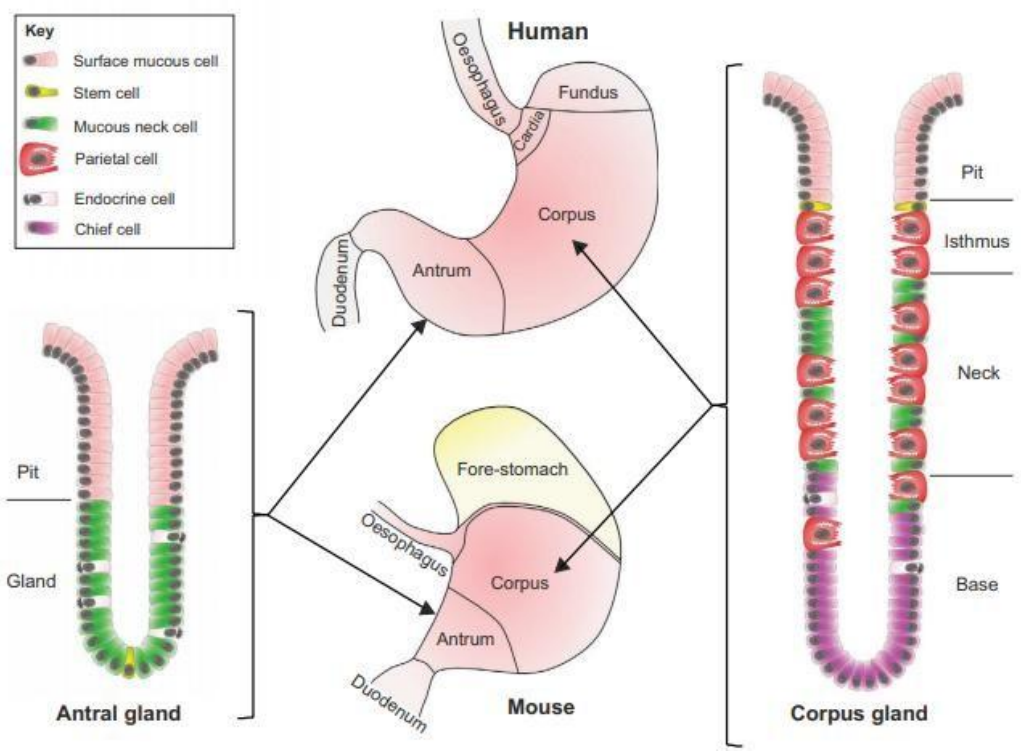

Figure 10 - The anatomy of the human and mouse stomach. A schematic of the anatomy of the human and mouse stomach and the structure of gastric glands (reproduced from Burkitt et al., 2017).

12. Pathologies

12.1 Etiology

The human body has a significant difference between the number of human cells and the number of microbial cells: there are 10 microbial cells for 1 human cell. These microbial cells form the human microbiota, and there are approximately 100 trillion of them. The gut microbiome is composed by a balanced cooperative community, that is extremely complex and a crucial component to the proper functioning of the organism. In healthy individuals, there is a symbiosis interaction, in which the microbiota has many functions, such as protection from pathogens and prevention of tumorigenesis. However, if for any reason this balanced is disrupted, the organism enters into dysbiosis, which can provoke host disease processes (such as cancer) (Candela et al., 2014; Yang et al., 2013).

The infection caused by *H. pylori* can lead to diverse human pathological conditions, such as gastritis (superficial, acute or atrophic) and peptic ulcer disease. In individuals with atrophic gastric mucosa and *H. pylori*-associated gastritis, the emerge of hyperplastic polyps is frequent, but only a few undergo to malignant trasformation.

The most recurrent malignant gastric pathologies originated from *H. pylori*'s colonization are mucosa-associated lymphoid tissue (MALT) lymphoma and adenocarcinoma (nearly 90% of gastric tumors are adenocarcinomas). When associated to gastric carcinoma, the bacterium is mostly involved in the development of intestinal-type and distal forms of the disease. The mechanisms used by the bacterium are clearer for intestinal-type gastric cancer, that slowly evolves from a multifactorial, multi-step inflammatory process, with well-characterized series of histological fases. This type of cancer represents a typical model of gastric carcinogenesis (Correa model, firstly proposed by Correa et al., in 1975), described by pre-neoplastic lesions with high progression risk (atrophic gastritis, intestinal metaplasia and dysplasia)**.** During the evolution of the various stages of gastric preneoplasia, the phenomenon of hypergastrinemia (the levels of gastrin increase in the bloodstream) can be observed (Fig.8) (Burkitt et al., 2009).

As previously mentioned, *H. pylori* infection is necessary, but not sufficient for the development of lesions at the level of the gastric mucosa, which explains why only 10-20% of infected patients with the bacterium develop serious illnesses (Fig.11). These values suggest that the type of immune response to the bacterium – innate and acquired – influences the outcome of the infection for protection, avoidance or pathology (Torres & Torres, 2016).

12.2 Epidemiology

12.2.1 Superficial gastritis

The most common gastric pathology induced by *H. pylori*'s infection is gastritis. Acute gastritis has been described in the context of investigation, either when investigators were exposed to the bacterium accidentally (Sobala et al., 1991) or purposely, to study the induced disease (Marshall et al., 1985).

The initial phase is characterized by a transitional reduction in gastric acid output and by the infiltration of polymorphonuclear leukocyte (including neutrophils, basophils and eosinophils) in the gastric mucosa. The presence of these multilobulated white blood cells (which evidences an active inflammation in the epithelial tissue) along with IgM and IgG seroconversion for the bacterium are typical outcomes of chronic, superficial gastritis (Campbell et al., 2001).

12.2.2 Atrophic gastritis

As mentioned above, atrophic gastritis precedes intestinal metaplasia and gastric cancer. The inflammatory processes that result from immune system's response to the infection destruct the cells that secrete acid into the stomach. So, at this stage, it is common to find fundamental substances impaired, such as hydrochloric acid, pepsin and intrinsic factor. These events promote stomach's neutralization, creating new environmental conditions, which allow the colonization of diverse bacteria species. Consequently, gastric microbiota overgrowth is verified (Lee et al., 2009). The severity of atrophic gastritis can be evaluated according to some clinical parameters: the degree and range of inflammation, the pH of gastric fluids and infection status. A previous study proved that *H. pylori* was absent at the severe stage of atrophic gastritis (characterized by a low acid production) (Fig.11), and that the changes in gastric microbial composition remained the same of those founded in gastric cancer (Engstrand & Lindberg, 2013). Clinical studies have found that there is a bigger chance of developing gastric cancer in cases of atrophic gastritis where *H. pylori*' is not present, suggesting that it plays a crucial role in gastric carcinogenesis. So, there is a correlation between *H. pylori*'s infection and other bacteria present in the stomach, still poorly understood (Cao & Yu, 2015).

12.2.3 Peptic ulcer disease

H. pylori infection increases the risk of peptic ulcer disease (PUD) development (Li et al., 2010). As there has been a reduction in the prevalence of this infection worldwide, the incidence of PUD has also been decreasing (Groenen et al., 2009). Nowadays, this disease is more often associated with non-steroidal anti-inflammatory drug use or to low-dose aspirin (Musumba et al., 2012). When it outcomes from *H. pylori* infection, it results from the condition of preexisting chronic superficial gastritis. In this case, PUD is related with an increased gastric acid secretion levels and a T helper 1 (Th1) polarized immune response (Shimada et al., 2002). Commonly, PUD patients have antral predominant gastritis, that conducts to increased gastrin secretion. Gastrin is an hormone synthesized in the stomach, that stimulates parietal cells to secrete acid; it is also a growth factor and stimulates the proliferation of gastric epithelial stem cell zone, increasing epithelial cell turnover. When this hormone stimulates the parietal cells of the gastric corpus (Fig.10) to increase acid secretion, mucosal ulceration takes place (Fig.11). Excessive gastrin secretion is suppressed with *H. pylori* eradication (Burkitt et al., 2009).

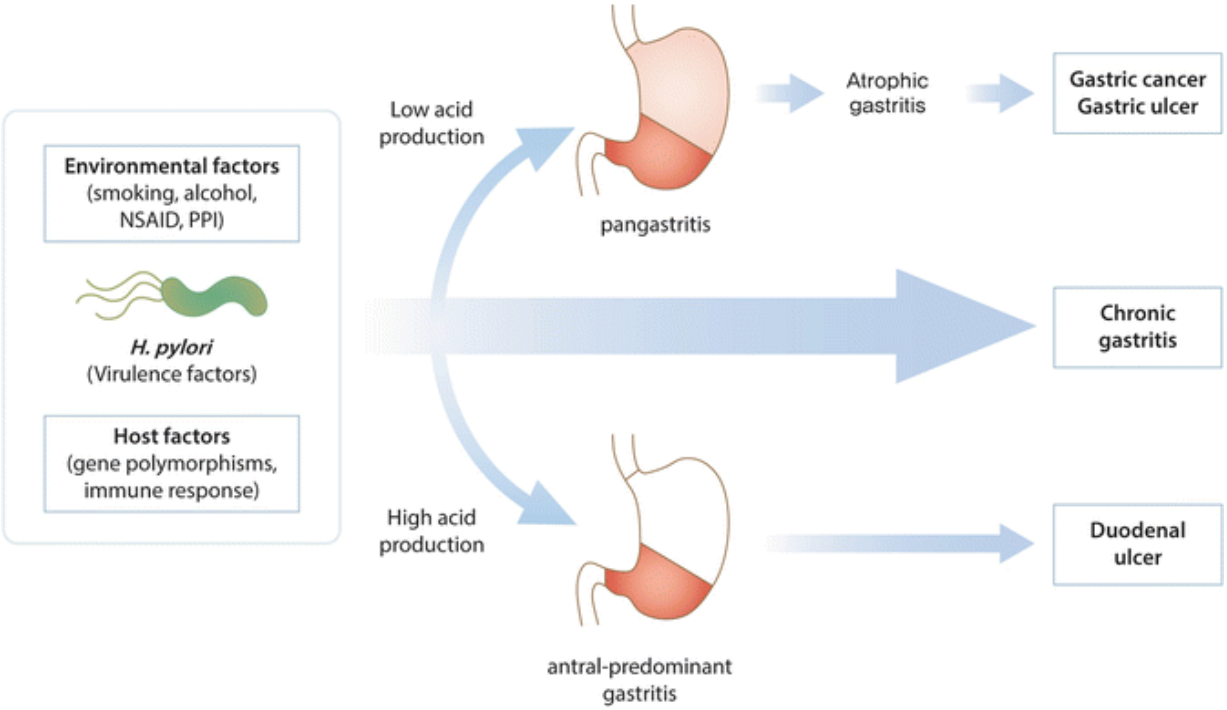

Figure 11 - Schematic representation of the factors contributing to gastric pathology and disease outcome in *H. pylori* infection. NSAID: nonsteroidal anti-inflammatory drug, PPI: proton pump inhibitor (Modified from Kusters et al.) (reproduced from Kim, 2016).

12.2.4 MALT lymphoma

H. pylori is also related with the oncogenic development of mucosa-associated lymphoid tissue (MALT) lymphoma, a distinct subtype of marginal zone B-cell non-Hodgkin's lymphoma. This type of cancer is characterized by the development of B-cell lymphomas within the mucosa-associated lymphoid tissue of the stomach in the gastric extranodal marginal zone. MALT lymphoma incidence rate is low, accounting for only about 3% of all gastric tumors, making it an uncommon consequent of *H. pylori* infection (Khalil et al., 2014), although most reported cases are closely related with this infection (approximately 79%) (Gisbert & Calvet, 2011). One of the most well described typical cytogenetic profile of this tumor is the formation of *MALT1-API2* fusion oncogene, which occurs due to t (11:18) translocation. As a repercussion, *API2* (which encodes the cellular inhibitor of apoptosis 2) is expressed under the control of the *MALT1* promoter (Rosebeck et al., 2011). *MALT1* is responsible for encoding mucosa-associated lymphoid tissue lymphoma translocation protein 1. This protein plays a central function in the T- and B-lymphocytes activation and proliferation, and also in NF-κB (nuclear factor kappa light chain enhancer of activated B cells) activation. The

fusion protein affects the regulation of the alternative pathway NF-κB signalling by enhancing the cleavage of NIK (NF-κB-inducing kinase) (Merga et al., 2016).

12.2.5 Gastric adenocarcinoma and its precursor lesions

It beggins with the infection caused by *H. pylori*, that progresses to chronic active gastritis (present in all infected individuals). Chronic active gastritis may develop into atrophic gastritis and intestinal metaplasia, depending on the influence of environmental and host factors, as refered previously (see bacterial-host relation, Fig.9). This sequence of events happens more frequently in elderly people.

At the stage of atrophic gastritis, there is an unequal loss of parietal cells in the gastric corpus mucosa, which leads to decreasing levels of gastric acid secretion. As a consequence, intraluminal pH increases, with reduction of somatostatin secretion and, consequently, of gastrin secretion (as refered above, gastrin is responsible for stimulating acid secretion and increases epithelial cell turnover) (Burkitt et al., 2009). Over time, some individuals develop intestinal-type metaplasia of the gastric mucosa. Metaplasia is characterized by the substitution of a differentiated epithelial tissue by another, atypical for that anatomical area. Oxyntic glands (see stomach anatomy and physiology, Fig.10) are replaced by CDX2 (caudal-type homeobox 2)-expressing glandular units, that have morphological similarities with the intestinal crypt. After the metaplastic epithelium suffer genomic and phenotypic changes, it may occur an evolution to gastric dysplasia; some patients with intestinal metaplasia (about 20%) develop simultaneous dysplasia. Dysplasia consists in a replacing of normal gastric mucosa by structurally abnormal tissue (and structurally abnormal cells within the tissue, with disarranged disposition) with likely anomalous cell proliferation. Finally, dysplasia can progress to adenocarcinoma (den Hoed et al., 2011) (Fig.12).

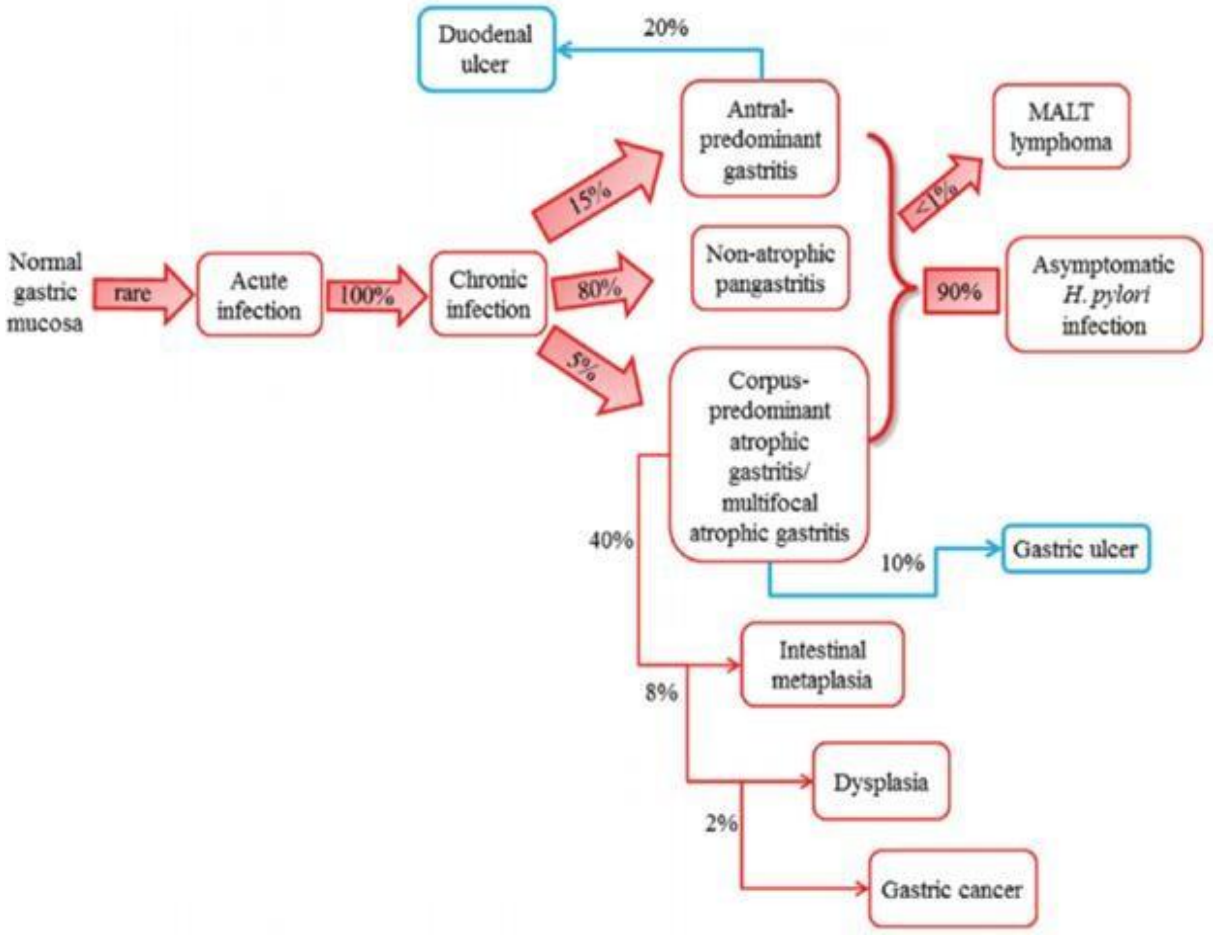

Figure 12 - Natural history of *H. pylori* infection. Chronic gastritis develops in almost all persistently colonized individuals, 90% of whom will remain asymptomatic. Patients with increased acid secretion are more likely to have antral-predominant gastritis, which predisposes to duodenal ulcers. Patients with low acid secretion will more likely develop gastritis in the body of the stomach and are thus more likely to develop gastric ulcer, leading to gastric atrophy, intestinal metaplasia, dysplasia and, finally, in rare cases, gastric carcinoma. *H. pylori* infection induces the formation of mucosa-associated lymphoid tissue (MALT) in the gastric mucosa and MALT lymphoma is another rare complication of *H. pylori* infection (adapted from Conteduca et al., 2012).

13. Infection prevention and treatment

13.1 Diagnosis

The diagnosis for the detection of this bacterium can be made through different tests, divided into two categories: invasive and non-invasive.

Invasives – histology, culture of gastric biopsy and urease test – assume the performance of an endoscopy for collecting gastric biopsies and are the ones with the

greatest specificity and sensitivity. The urease test is a rapid and simple method, that detects the presence of the bacterium in the gastric mucosa through a color change reaction, resulting from urease activity. Although both histology and urease test show whether the bacterium is present or not, being complementary, only histology shows the state of the stomach tissue, providing additional information about possible injuries.

Non-invasive tests – the ^{13}C-urea breath test (UBT), faecal antigen research (with antibodies) and serological detection of an anti-*H. pylori* antibody –, although they do not require a gastric biopsy collection, have high specificity. The ^{13}C-urea breath test, which measures a metabolic function of the bacterium (urease activity), is precise, practical and easily available; it is highly sensitive (94%) and specific (95%). The faecal antigen test has a sensitivity of 91% and a specificity of 93%. The serological test is easily available and is low cost, however, it is not very accurate (low precision: 80-84%).

For more reliable diagnosis, which allows acting accordingly to the strain present in each affected individual, ideally, an urease test should be performed (thus confirming the presence or absence of the bacterium), followed by an histological and cultural examination (the cultural evaluates the sensitivity of the bacterium to antibiotics) (Burkitt et al., 2017; Condecuta et al., 2013; Perri et al., 2002).

As an atempt to extinguish the differences in the classification of gastric dysplasia and cancer between japanese and western doctors, it has been developed the OLGA staging system by gastrointestinal pathologists of an international group: the Operative Link for Gastritis Assessment (OLGA). This system is based on histological phenotypes of gastritis that result from an evolution of stages with increasing risk of developing gastric cancer (Rugge et al., 2011).

As refered before, gastric cancer incidence varies geographically, with highest *H. pylori* infection prevalence in developing countries. In these countries, diagnostic tests are relatively expensive and limited, as well as therapeutic interventions. This fact creates the need to develop screening strategies concordant with the origin country of the infection. The approach differs according to the risk: a high individual risk requires an invasive test (endoscopy), while a more conservative investigation (non-invasive tests) is recommended for individuals in low-risk regions. In these countries, the selection of subjects for screening is based on risk factors for *H. pylori* infection, that are also epidemiological factors: age, country of birth and socioeconomic status (Yeh et al., 2010).

There are different types of gastric polyps – adenomatous polyps, hyperplastic polyps and fundic gland polyps – with distinct origins, morphological features and probability of malignant evolution. Gastric polyps are often asymptomatic and constitute a risk for cancer development (synchronous or metachronous cancer). Because of their frequent association with pre-malignant alterations of the gastric mucosa, this type of polyps are usually analyzed through an esophagogastroduodenoscopy (Goddard et al., 2010).

As previously refered, *H. pylori* infection increases the risk of gastric cancer development as an inital step of carcinogenesis. Due to high prevalence of this infection worldwide, the eradication of the bacterium is the first therapeutical approach to treat chronic gastritis and also to prevent gastric cancer development; either by restoring the inflamed mucosa to a healthy form, or by avoiding the progression of chronic or pre-neoplastic lesions (such as atrophic gastritis and intestinal metaplasia, respectively) (Bornschein et al., 2009).

Atrophic gastritis and intestinal metaplasia progression to gastric cancer can still happen after the eradication of the triggering carcinogen. This statement suggests that there are other factors contributing to carcinogenesis, as may be the case of a no return point of genetic mutations and epigenetic alterations. So, there is a lot to understand about the role of *H. pylori* eradication in gastric pathologies (Fukase et al., 2008).

Accordingo to Conteduca et al., 2013, there is a group of high-risk patients for whose *H. pylori* eradication is advised: "*i) patients with gastric MALT lymphoma; ii) patients with atrophic gastritis; iii) first-degree relatives of patients with gastric intestinal or diffuse type of cancer; iv) patients with unexplained iron-deficiency anemia; v) patients with gastroesophageal reflux disease requiring long-term acid-suppression therapy, due to the potential of proton-pump inhibitors to induce, in the presence of H. pylori, atrophic gastritis, with a subsequent risk of developing gastric cancer; vi) patients with early gastric cancer treated by endoscopic mucosal resection, as it can remove lesions posing a minimal mortality risk, but likely to have higher rates of recurrence and incomplete resections; and vii) patients with partial gastrectomy for gastric cancer*" (Malfertheiner et al., 2007; Malfertheiner et al., 2012).

13.2 Therapy

H. pylori infection is usually treated with a combination of acid suppressant medication (proton pump inhibitors, such as esomeprazole, omeprazole, among others) and antibiotics – amoxicillin, clarithromycin, metronidazole, flouroquinolones (mainly levofloxacin) and tetracycline. However, due to the growing resistance to antibiotics acquired by some strains (mainly clarithromycin, metronidazole and flouroquinolones), this type of treatment has proven to be less effective over time (Aguilera-Correa et al., 2017; Burkitt et al., 2017; Song et al., 2013). Due to this reason, a monotherapy antibiotic treatment is incapable of eradicating the bacterium, and a triple or quadruple therapy is currently required (Gisbert et al., 2007; Hsu et al., 2011). The lack of effectiveness of current therapies triggers the need of developing alternative solutions to treat the infection. A vaccine would be very helpful, but until there is one available, other options need to be considered. Natural products are an appropriate option, due to their particular physiological and physicochemical characteristics and, therefore, their wide range of biological activities and drug-like properties (Cragg & Newman, 2013; Muschietti et al., 2013).

Nevertheless, this type of conventional treatment may not be successful, since *H. pylori* can switch between a replicative state (in which it is susceptible to antibiotics) and a non-replicative state (whereupon it becomes antibiotic-resistant). This oscillation of states/cycles occurs according to the pH of the surrounding microenvironment (lumen's stomach): when the pH is between 4.0 and 6.0, the microorganism can not enter the replicative cycle; while when the pH is between 6.0 and 8.0, the bacterium can enter the replicative cycle. This constitutes a problem because it is more difficult to eradicate bacteria when they are in a phenotypically resistant state (Fig.13) (Kuo et al., 2013; Wu et al., 2012).

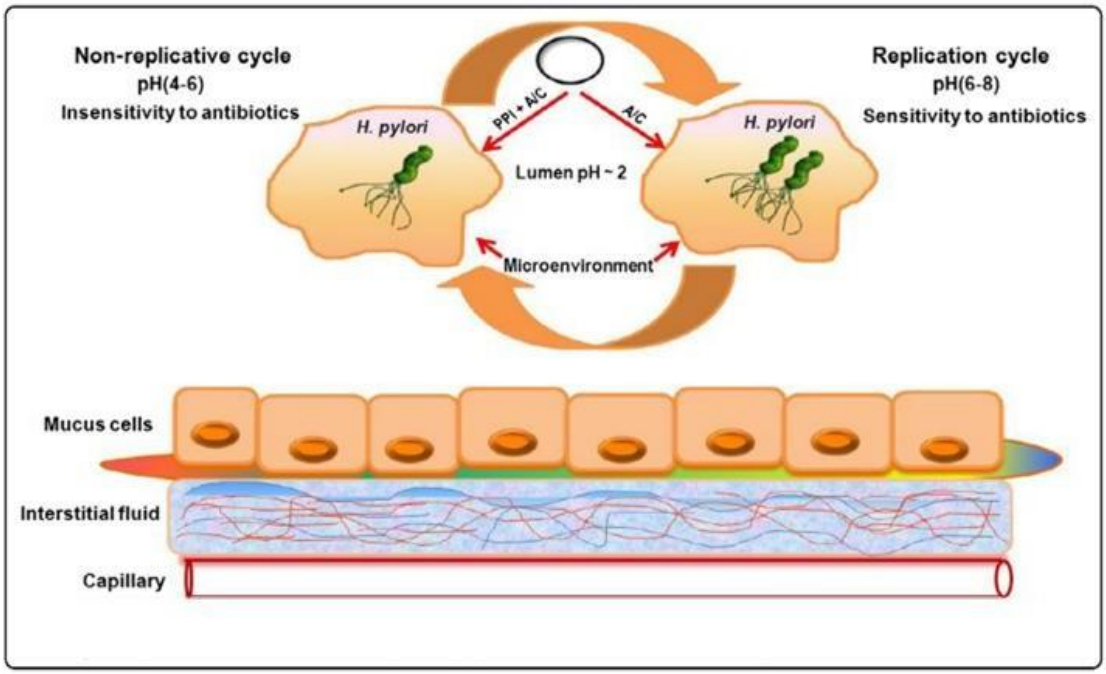

Figure 13 - United of replication of *H. pylori* (antibiotic sensitivity), and a non-replicative state (antibiotics insensitivity). Cycles occurring according to the pH in the microenvironment. PPI: proton pump inhibitor, A: amoxicillin, C: clarithromycin (reproduced from Bonifácio et al., 2014).

13.3 Vaccine

As previously stated, *H. pylori* has acquired antibiotic resistance, increasing the rates of treatment failure. The development of new alternative therapies is a major concern, to improve eradication sucess or, at least, reduce bacterial density. The development of a vaccine (prophylactic or therapeutic) may be a viable solution. It would protect against diseases such as PUD and MALT lymphoma, once it could prevent gastritis by being an immunomodulatory vaccine.

To reach the uninfected patients, a prophylactic vaccine should be administrated in the first years of life. Due to the all factors involved in gastric carcinogenesis, health benefits would only be noticed five decades later. A therapeutic vaccine could be ministered at any time and still (potentially) protect the patient from the development of gastric cancer. However, to maximum prevention, it should be given before 40 years old (Sutton & Boag, 2019). In this type of approach, bacterial constituents can be considered a vaccine target (such as flagella and urease) (Kao et al., 2016).

Due to all the immunosuppressive effects derived from the infection, a prophylactic vaccine would be more efficient than a therapeutic one. However, vaccination studies

in animal models (infected mice with *H. pylori*) have shown a reduction in bacterial colonization (Sutton et al., 2000). Currently, in humans, it still does not exist evidence of the efficiency of vaccination against *H. pylori*. There has been no satisfactory response to vaccine antigens, maybe because *H. pylori* is not accessible to various immune effector mechanisms (since it does not cross the epithelium, only colonizes the gastric mucosa).

Despite the incidence of *H. pylori* infection worldwide, the development of a vaccine against it is not a priority for pharmaceutical companies and, for that reason and due to all costs involved, all advanced vaccines are presently in an early stage of development (in Phase I or preclinical) (Sutton & Boag, 2019).

Many approaches for active immunization have been evaluated: the use of DNA, living vectors, microspheres, new vaccination programs and distinct vias of administration (oral, intranasal, rectal and intramuscular) (Czinn & Blanchard, 2011). Investigations about the roles of bacterial factors have been crucial to understand *H. pylori* infection. Large-scale screening methods will improve the knowledge about *H. pylori* pathogenesis and disease development, which will help vaccine development. New trials of vaccine elaboration may be more efficient using multiple antigens and new strategies to optimize cellular immunity (Kaos et al, 2016; Sutton & Boag, 2019).

13.4 Natural Products

Some natural products (derived from microorganisms, animals, marine organisms and plants) have been used in medicine since prehistoric times as a way to treat and prevent some pathologies, which was essential to the survival and perpetuation of the human specie (Fabricant & Farnsworth, 2001; Shi et al., 2010).

That was how traditional medicine emerged. This is the oldest form of medicine performed, that varies accordingly to the intrinsic culture, by using different approaches and methods to prevent and treat diseases. Nowadays, it has a major role in many countries as a complementary medicine (Abdullahi, 2011; Parasuraman et al., 2014). According to WHO, many people around the world still depend on it (WHO, 2000).

Traditional medicine development was only possible due to the evolution of natural compounds, once there is a vast set of these elements with unic physiological and physicochemical characteristics and, therefore, a wide range of biological activities and drug-like properties. Their diversified chemical structures enforce a modification in

many biological functions, which creates the possibility of developing medicines from them. This process occurs because their are complex and organized elements, with chemical and steric properties that affect the spatial arrengement of atoms, affecting the chemical reactions in which their are involved. The modifications in the molecules make them highly efficient in the selection of targets and, therefore, potencial products for the production of new drugs (Cragg & Newman, 2013; Muschietti et al., 2013). So, natural products are a reliable resource for the production of new remedys, playing a main role in treating ilnesses, especially critical ones (Galm & Shen, 2007).

Since natural products are composed by a large set of constituents – active, invalide and synergistic – there is the need to extract the essence of the product and to separate it from the dross. Some of the active compouns separated from the natural products followed traditional applications while others did not. The emergence of new techniques allowed the onset of synthetic products, which caused concern about the possibility of reduction of the use of natural products in medicine (Joo, 2014). These products have a major role on the rise of new pharmaceutical drugs, thanks to many experiences developed since ancient times. The experiments enabled the acumulation of useful information over years, which included the development of different methods, such as identification, selection, obtainance and preparation of natural products. Consequently, traditional medicine has advanced (Yuan et al., 2016).

As a result of this progress, natural elements and traditional forms of medicine had a strong impact in the evolution of modern medicine, which benefited from the expansion of drugs with similar or different effects of those studied in traditional medicine. It is possible to obtain synergy and high yields by fusing the simple techniques of traditional medicine and complex methods of current technology. Accordingly, if a drug is originated from traditional medicine, there are many benefits, such as cost reductions (Yuan et al., 2016).

There are still some problems in the use of natural products that need to be solved. The synergistic effects of their compounds have unclear mechanisms of action, which create the need of further studies, not only to improve the effectiveness of these products, but also to reduce its adverse effects, in order to generate safer treatments. So, it is necessary to transform these products and to regulate the administered doses, in order to reduce their toxicity and prevent poisoning. For this reason, although natural products are an added value in medicine, people should have some caution in using them.

Traditional medicine has an advantage over modern medicine, by using the synergism of various compounds as a therapy. Current medicine works in an opposite approach, once it is focused on an individual drug. It is well known that some complex diseases are easily treated with a combination of therapies; that is one of the reasons why natural products are so important for the development of new drugs – they are involved in the production of medication for many purposes, such as anticancer, antihypertensive and anti-infective (Joo, 2014; Newman et al., 2003).

Plants are one of the main natural products used in this industry and have been inserted in medicine through its traditional form. Many drugs used to treat cancer derive from plants, making the research for new chemical structures a main goal in current medicine. Thanks to the accessibility of traditional medicine, future research about drugs can be performed, and new plant-based drugs can be originated (Fabricant et al., 2001; Ngo et al., 2013). So, there is a high expectation of new findings in this area, since there are many potencial chemical structures with different bioactivities (Yuan et al., 2016). A plant or a formula contains many constituents, which can function alone or together to produce a pharmacological effect. Since there are so many mechanisms of action, natural products play a significant role in current drug development. Hence, they are much valuable to the pharmaceutical industry and very relevant in the health system (Parasuraman et al., 2014).

Modern technology was ehnanced with screening techniques and combinative chemistry, making some processes available, such as synthesis and fermentation (Li & Vederas, 2009; Winter & Tang, 2012). The revolution in the pharmaceutical industry was based on a high yield synthesis of synthetic drugs, but did not achieve the productivity yields that were expected. So, there was a need to fuse the natural products and modern technology to create new medicines. This insurgence was only possible due to the fusion of the development of new techniques with the diversity of biochemical components and the increasing knowledge about natural products. Hereby, it was possible to generate a library for drug screening, which ehnanced individual therapy (treatment and prevention) by making such valuable data available (Ngo et al., 2013; Zhu et al., 2012).

For the reasons mentioned before, there is an important need to continue to do collaborative research on this topic and to construct networks, once it generates data that allows the discover of new drugs and treatments, by solving the mysteries about the mechanisms involving natural products (Yuan et al., 2016).

Chapter II

Methodologies for testing the antimicrobial potential of natural products on *H. pylori*

1. Background

Various techniques have been developed to detect antibiotic resistance in *H. pylori*. There are some culture-based/phenotipic methods for susceptibility testing of anaerobic bacteria: agar dilution (gold standard), broth microdilution, gradient strips and disc diffusion. Commercial assays of broth microdilution are available, but until further studies are conducted to validate this procedure for testing other bacteria, it should be used only for testing members of the *Bacteroides fragilis* group. Gradient strips are expensive and there are some problems with performance and warnings concerning specific agents. For disc diffusion, EUCAST (The European Committee on Antimicrobial Susceptibility Testing) has no specific breakpoints for *H. pylori* (CLSI, 2012).

The antimicrobial activity of natural extracts should be evaluated according to the methodology described in the standard M45-A2 of *Clinical & Laboratory Standards Institute* (CLSI, 2010). This guideline was written for global application and describes methods for antimicrobial dilution and disc susceptibility testing of infrequently isolated or fastidious bacteria, providing guidance to clinical microbiology laboratories for standardized susceptibility testing for this type of microorganisms. According to CLSI, adequate studies have not been conducted to recommend reproducible disc diffusion breakpoints for many of the organisms. For those, only the broth microdilution test or agar dilution should be performed because disc diffusion results cannot be interpreted reliably. For *H. pylori*, this guideline describes interpretive criteria for agar dilution susceptibility testing.

2. Agar Dilution and Broth Microdilution Methods

Broth or agar dilution methods can be performed to quantitatively measure the *in vitro* activity of an antimicrobial compound against a bacterial isolate. In these assays, MIC (Minimum Inhibitory Concentration) can be determined. According to CLSI, MIC is

defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test. To execute these tests, a series of tubes or plates are prepared with a broth or agar medium, respectively, to which various concentrations of the antimicrobial agents are added. Typically, a series of 5-12 different concentrations of natural extract is tested, derived from 2X serial dilutions [in 0.85% NaCl (sodium chloride)] indexed to base 1 (eg.: 1, 2, 4, 8, 16 µg/mL, etc.), that is, 1:2 (Fig.14).

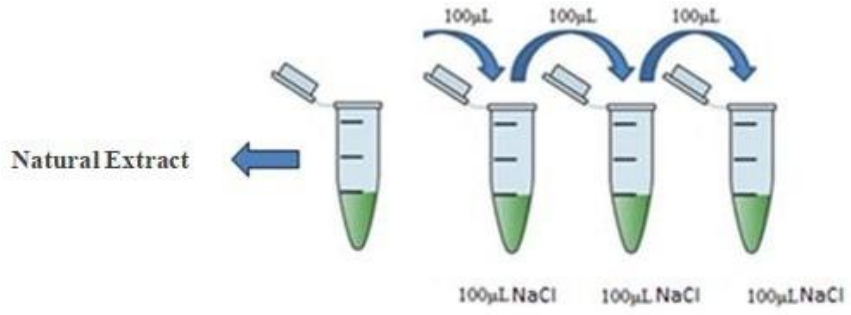

Figure 14 - Representative scheme of the methodology used to obtain the different concentrations of the natural extract to be tested on broth or agar dilution methods. NaCl: sodium chloride.

2.1 Agar Dilution Method

2.1.1 Testing conditions

2.1.1.1 Medium

The medium used for agar dilution is MHA (Muller-Hinton Agar) and aged (2-week-old) sheep blood (5% v/v).

2.1.1.2 Inoculum

It should be prepared a saline suspension equivalent to a 2.0 McFarland standard (containing 1 x 10^7 to 1 x 10^8 CFU/mL) from a 72-hour-old subculture (a pre-incubated culture) from a BAP (Blood Agar Plate). The inoculum (1 to 3 µL per spot) is replicated directly onto the microbial agent-containing agar dilution plates, previously prepared (Fig.15).

2.1.1.2.1 Preparation of the inoculum

The inoculum should be the last thing to be prepared, since the adjusted suspensions must be used in the final inoculation up to 15 minutes after preparation. The preparation of the inoculum is done by the method of direct suspension of the colonies, the most convenient. The suspension is direct, in saline solution (which have an osmotic pressure that does not create instability in the bacteria wall), from isolated colonies selected from the previously incubated 72-hour blood agar plate. The aim is to adjusted the suspension to a 2.0 turbidity on the McFarland scale – a widely used scale in microbiology, which measures the pattern of turbidity caused by bacterial growth – by reading the turbidity/optic density on a suitable device.

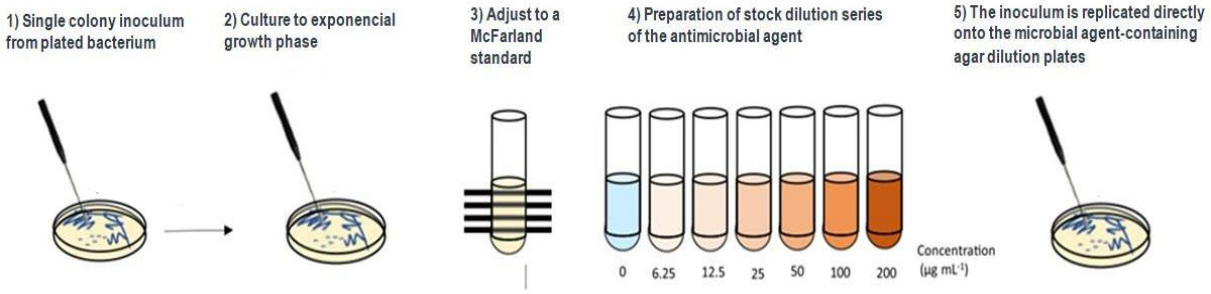

Figure 15 - Flow diagram for undertaking an agar dilution study (adapted from Cotton et al., 2019).

2.1.1.3 Incubation

According to this standard, the incubation is carried out at 35 ± 2ºC for 72 hours in a microaerophilic atmosphere that is achieved using a gas generator system suitable for Campylobacters. An artificial catalyzer is inserted in an hermetic jar, along with cultures. After the jar is sealed, the catalyzer consumes most of the oxygen and releases carbon dioxide into the environment.

2.1.2 Analysis of Results

After the incubation period, colonies growth is observed and MIC is determined (Fig.16). In the case of antibiotics, there are proposed clinical antimicrobial breakpoints for *H. pylori:* CLSI established MIC interpretive standard for clarithromycin, while EUCAST determined values for the other antibiotics used to treat this bacterium infection (CLSI, 2010; EUCAST, 2020).

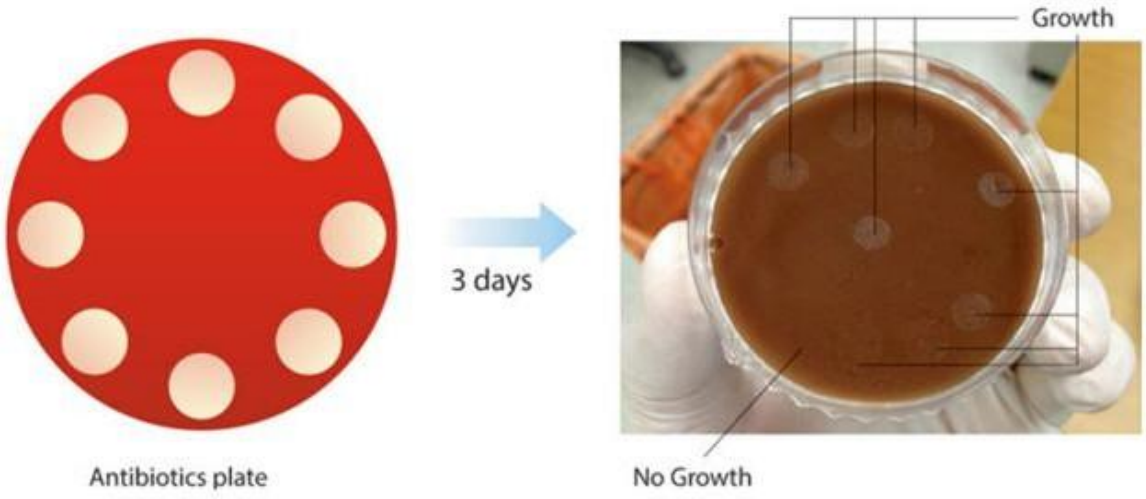

Figure 16 - Representative scheme of the agar dilution method (adapted from Kwon, 2016).

2.2 Broth Microdilution Method

2.2.1 Testing conditions

This method is performed on microplates, and each well should contain: 100 µL of Mueller-Hinton broth supplemented with 10% foetal calf serum inoculated with *H. pylori* (McFarland turbidity standard 2) and 100 µL of the natural extracts to be tested, at different concentrations (Souza, 2011).

2.2.1.1 Controls

Each plate should include a positive, growth control (culture medium and bacteria), a sterility negative control (culture medium only) and an antibiotic control to compare the activity of the antibiotic with that of the natural extract. Clarithromycin is used as the standard drug for growth inhibition (Fig.17).

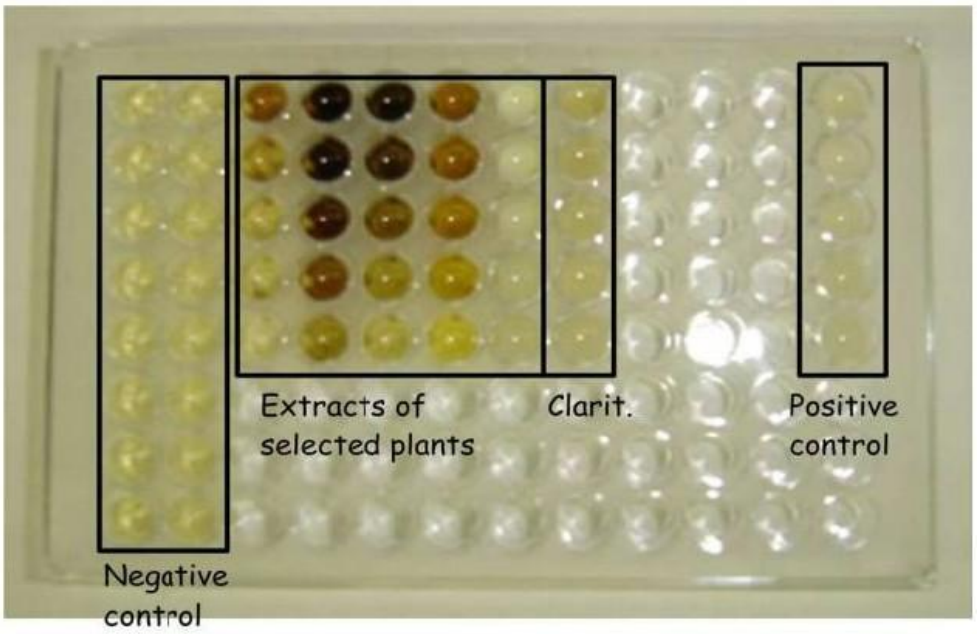

Figure 17 - Photograph of a microdilution plate. Clarit: Clarithromycin (reproduced from Souza, 2011).

2.2.1.2 Incubation

After adding the inoculum, the microdilution plates must be incubated under the same microaerophilic conditions mentioned above, for 3-5 days.

2.2.2 Analysis of Results

After the incubation, plates are visually examined and MIC is determined by comparing the turbidity of the wells with the extracts with the turbidity of the positive growth control. So, MIC corresponds to the first well with less turbidity than the growth control. The content of each well that presents a minor turbidity than the positive control is replicated on solid medium Mueller-Hinton agar with 5% sheep blood, to ascertain whether growth has occurred or not. Therefore, MBC (minimum bactericidal concentration) – defined as the lowest concentration that kills the bacteria – is determined (Souza, 2011).

3. Disc Diffusion Method

The disc diffusion is a simple and cheap method to assess antimicrobial susceptibility. This technique consists in placing impregnated discs with the antimicrobial agent on an agar plate with replicated bacteria to check if there is any antimicrobial activity. After incubation, the antimicrobial susceptibility can be visualized by a growth inhibition zone and determined by measuring the size of the radius or diameter of the inhibition halo (Fig.18) (Souza, 2011).

As this assay is not suitable for mucoid strains or fastidious organisms (Kwon, 2016), it can only be done a comparision between the size of the growth inhibition halo of the evaluated extract with antibiotics or other extracts, to ensure the tested agent is more, less or just as efficient.

3.1 Testing conditions

3.1.1 Inoculation

The discs can be prepared using serial dilutions of the extracts, by being imbibed in 25 µL of each dose of extract and fraction. They should be deposited on the surface of the plate inoculated with *H. pylori* (a suspension of McFarland turbidity standard 2) and positioned at a distance of 30 mm apart and no closer than 24 mm apart when measured center to center, to minimize overlap of inhibition zones. Clarithromycin, the standard drug, can be used as control.

3.1.2 Incubation

The plates should be incubated under the same conditions described before, for 3-5 days.

3.2 Analysis of Results

After the incubation period, the growth inhibition halos should be quantified with a digital pachymeter, in duplicate. Mean values ≥10 mm are considered active (Souza, 2011).

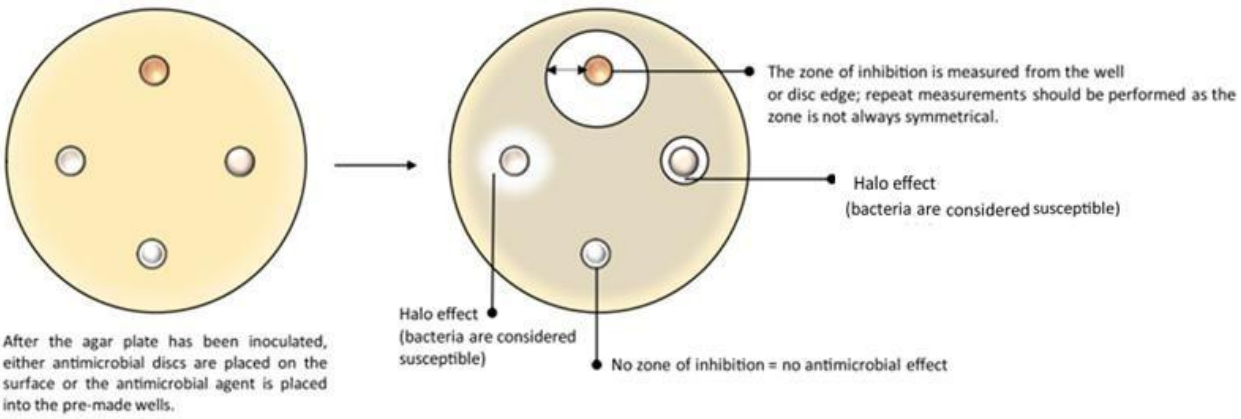

Figure 18 - Schematic representation of the disc diffusion assay (adapted from Cotton et al., 2019).

4. Gradient strips (Etest)

Etest is a quantitative variant of the disc diffusion test. It complements disc diffusion method and others currently used for bacterial susceptibility testing. Since Etest is based on the use of a stable preformed gradient, it can be used for testing a wide range of fastidious and/or slow growing organisms (Kwon, 2016). However, there are commercially available predefined strips for antibiotics, not for natural extratcs.

After the same conditions of inoculation and incubation mentioned on disc diffusion method, the antimicrobial activity is assessed by the visualization of the growth inhibition halo. As the strip contains the MIC predefined gradient, the MIC value can be determined at the point where the growth/inhibition margin of the organism intersects the edge of the calibrated strip (Fig.19) (Schwalbe et al., 2007).

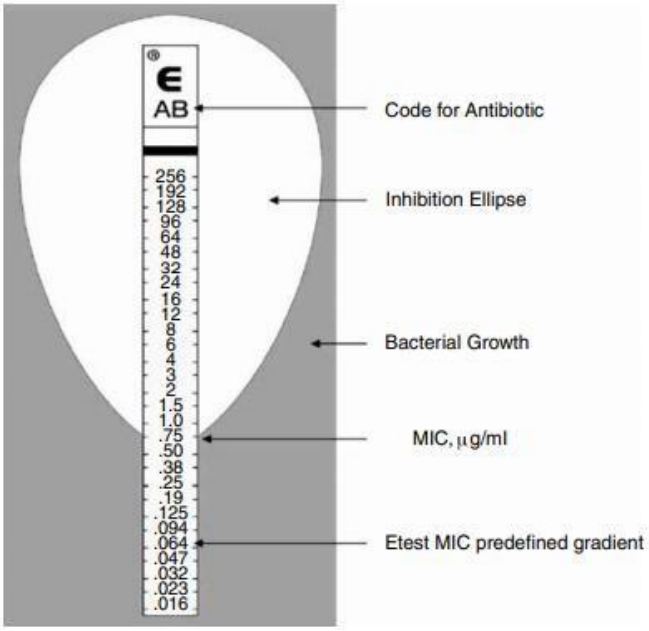

Figure 19 - Schematic diagram of the Etest inhibition ellipse showing the MIC at the intersection of the growth of the organism and the calibrated strip (reproduced from Schwalbe et al., 2007).

Chapter III

Natural products on *H. pylori*

1. Background

An elevated number of natural products has been studied for their capacity of inhibiting *H. pylori* growth *in vitro*. Some compounds can even decrease bacterial colonization or virulence *in vivo*, both in animal models or human clinical trials (more limited). The reduction in bacterial load and pathogenicity can attenuate infection symptoms and evolution by mitigating gastritis, inhibiting the progression of atrophic gastritis and perhaps delaying or preventing gastric cancer development (Fahey et al., 2015).

Many foodstuffs have exhibited anti-*H. pylori* activity, showing prominent and promising effects against *H. pylori*-induced gastrointestinal diseases. Generally, nature-derived compounds can provide great benefits to human health, and even ameliorate the adverse effects and the development of many diseases. An alternative therapy based on a diet treatment, even without completely eradicating the bacterium in treated patients, would have a positive impact on many factors, such as in cost reduction, treatment tolerability and social acceptance (Fahey et al., 2015; Takeuchi et al., 2014).

A considerable number of potential effective alternatives and adjuvant treatments have been reported, in which diverse natural compounds are included: water, dairy products, vegetables, fruits, oils, spices, medicinal plants, essential oils, pure phytochemicals and products resulting from animal activity (like propolis). Other compounds like probiotics (such as *Lactobacillus* species and *Saccharomyces boulardii*) and vitamins also demonstrated therapeutical potential (Fahey et al., 2015; Takeuchi et al., 2014).

Various plants, such as *Cinnamomum verumm*, *Pistacia lentiscus*, *Punica granatum* and *Terminalia chebula,* have been used over centuries by native and traditional healers as a medicine for gastrotintestinal syndromes, probably involving *H. pylori* infection. This vast set of living beings has been extensively studied for constituting potential antimicrobial products for prevention and/or treatment of many infections. In recent years, plant preparations and their isolated components have received special attention for their ability in supressing *H. pylori* infection (Fahey et al., 2015; Savafi et al., 2015).

Essential oils are defined as the mixture of low molecular weight compounds, usually rich in organic compounds like aldehydes, ketones, phenols or terpenes. These oils are commonly acquired from plants through steam extraction and distillation. A wide variety of these extracts has been described as a result of their diverse biological effetcs against *H. pylori*: anti-inflammatory, antimicrobial, antioxidant and immune stimulatory activity (Bergonzelli et al., 2003).

In a general way, daily-consumed natural products are safe and helpful for human welfare. When multiple types of food are conjugated, their effect on colonization and inflammation can show enhanced activity or a significative synergy against *H. pylori* (Keenan et al., 2010; Keenan et al., 2012).

Natural products can act by many mechanisms of action, including: antioxidant, anti-inflammatory and anti-adhesive activities, direct antimicrobial and immune stimulatory effects, or even by inhibiting bacterial virulence factors, such as the enzyme urease (Fig. 20) (Fahey et al., 2015).

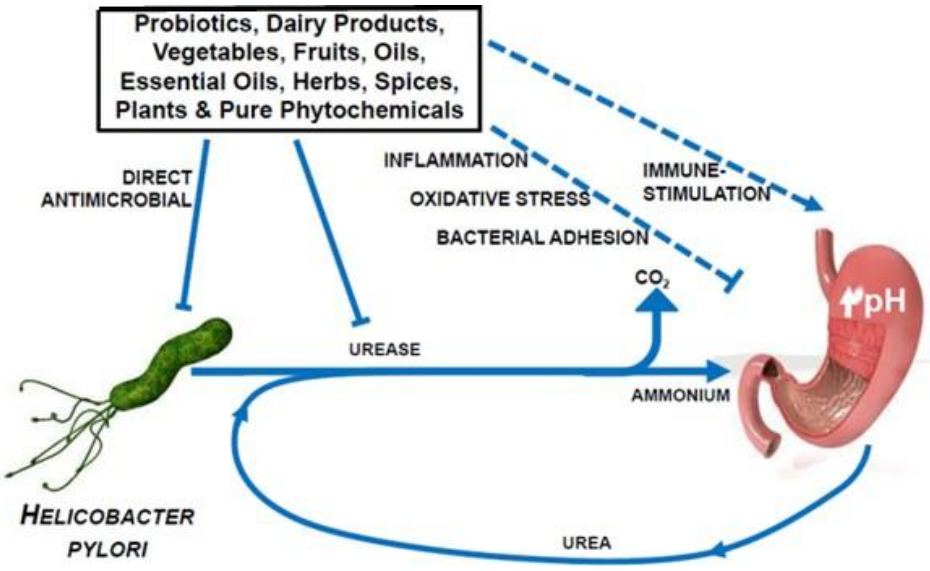

Figure 20 - Representative scheme of the mechanisms of action of natural products against *H. pylori* (adapted from Fahey et al., 2015).

In the present work, some examples of sundry natural compounds that were tested on *H. pylori* in the last decades, the methods that were used and the obtained results are presented. There are included varied foodstuffs like vegetables (broccoli and garlic), fruits (almond skins and cranberry juice), a spice (curcumin), tea (green tea), one fish (snakehead fish), oil (virgin olive oil), a probiotic (*Lactobacillus johnsonii)*, an animal protein (bovine

lactoferrin), medicinal plants (licorice and *Oenothera biennis*), marine products (refined deep seawater and the seaweed *Laminaria japonica*), a fungus (*Antrodia camphorata*) and a product that results from animal activity (propolis). Some products were evaluated together: cranberry juice was conjugated with the probiotic *Lactobacillus johnsonii* and *Laminaria japonica* with *Oenothera biennis*. There are examples of *ex vivo*, *in vitro* and *in vivo* studies, both on animal models and humans. The characteristics of each product are summarized in Table 1, while their mechanisms of action (sometimes not achieved) are in Table 2.

2. Garlic (*Allium sativum*)

Garlic (*Allium sativum*) has been widely consumed worlwide because of its medicinal properties: it regulates blood pressure, decreases blood sugar and cholesterol levels, displays a preventive role in cardiovascular diseases, is effective against pathogens (bacterial, fungal, viral and parasitic infections), has antitumor and antioxidant effects and enhances the immune system (Ayaz & Alpsoy, 2007). Many components and forms of garlic have been tested throughout the years: garlic oil, garlic powder and allicin (allyl 2-propene thiosulfinate and their diallyl constituents) (O'Gara et al., 2000).

In 1996, Cellini et al. studied the antibacterial effect of aqueous garlic extract (AGE) against *H. pylori in vitro* using two different varieties of garlic (red pellicle or white pellicle). Fresh cloves of garlic suffered a preparation to become AGE. They were peeled, minced and ground in a blender. Then, garlic pulp was shook with a phosphate buffer and refrigerated. After that, the larger particles were removed by squeezing the product through a gauze cloth. The supernate of garlic extract (AGE) was then filtered, sterilized through a filter and lyophilized.

Through agar dilution method, MIC was calculated. AGE inhibited the bacterial growth in a concentration between 2-5 mg/ml. For both AGE types, the concentration needed to inhibit 90% (MIC_{90}) of isolates was 5 mg/ml. The MBC values were the same or two-fold higher than those for MIC. The extracts were used either fresh or boiled. Heat treatment reduced the inhibitory activity of extracts, since the boiled AGE were less efficient in MIC and MBC values, from two- to four-fold, when compared with the fresh AGE. A proton-inhibitor (omeprazole) was combined with AGE in a ratio of 1:250 to evaluate the effect of these two components toghether. In 47% of strains, a synergistic effect (the effect of the cooperative action of agents, greater than its isolated effect) was observed. The bacteriostatic and bactericidal activity of AGE against *H.*

pylori showed the importance of further *in vivo* studies, since these garlic extracts could have reliable treatment aplications.

Some years later, in 2004, Canizares et al. isolated allicin (natural allicin is more stable than synthetic allicin) and allyl-methyl plus methyl-allyl thiosulfinate from acetonic garlic extracts (AGE) by high-performance liquid chromatography. The chromatographic method was optimized, allowing the isolation of natural thiosulfinates extracted from garlic by organic solvents and avoiding complex synthesis and purification procedures, which turned the methodology easier and cheaper, providing the maximum yield of oil and optimum bacteriostatic properties against *H. pylori*.

The organosulfur compounds tested inhibited the *in vitro* growth of *H. pylori*. The investigators tested the capacity and effectiveness of isolated natural thiosulfinates, enabling the recognition of the main compounds responsible for the bacteriostatic activity shown by AGE along with the ethanolic garlic extracts (EGE). The concentrations of the active compounds in the microbiological test corresponded to the MIC 16 mg/L for allicin and 24 mg/L for the allylmethyl plus methyl-allyl thiosulfinate. These MIC values are lower than those for synthetic allicin and allyl methyl thiosulfinate (20 and 30 mg/L, respectively), which indicates that natural thiosulfinates have better stability when compared to synthetic compounds and, consequently, a higher bacteriostatic action (Yu & Wu, 1989).

An additional microbiological analysis was performed to highlight the possibility of a synergic effect against *H. pylori* between the two organosulfur compounds. MIC values were compared with the ones for the individual components and, as opposed to the expected (18 mm), the inhibition halo obtained was 21 mm. So, when combined, the action of the two thiosulfinates was more efficient than when they were tested individually, which presupposes the existence of synergy.

In the thiosulfinates' isolated form, only compounds with allyl radicals linked to the sulfoxi group (-SO-) exhibited bacteriostatic properties, as they only react with the sulfhydryl groups in amino acids. These properties can imply the obstruction of the -SH groups of essential proteins and the destruction of the sulfhydryl groups of membrane proteins, which would stop bacterial proliferation (Cavallito & John, 1944). Effets of this kind were not evident in other nonallylsubstituted thiosulfinates, therefore, these garlic derivatives are potencial compounds that can be used in the treatment of *H. pylori* infection.

Other *in vitro* studies that show anti-*H. pylori* activity have been conducted, but they differ in efficacy. Diallyl tetrasulfide and allicin exhibited lower MICs than garlic powder, oil and diallyl disulfide (O'Gara et al., 2000). A garlic derivative, allitridi (diallyl trisulfide) inhibited *H. pylori* growth in a dose-dependent manner (Liu et al., 2010). An *ex vivo* study using garlic oil determined that allyl/methyl sulfides have a preventive effect against *H. pylori*-induced gastritis and thus gastric cancer (O'Gara et al., 2008). An *in vivo* study concluded that selenium-enriched garlic ingestion inhibited the development and progression of chronic gastritis induced by *H. pylori* in mongolian gerbils (Gu et al., 2007). In a clinical trial, a long-term administration of garlic supplements has not decreased *H. pylori* infection (Gail et al., 2007), being in accordance with a previous study (Graham et al., 1999). However, in a recent clinical trial, it was concluded that raw garlic has anti-bacterial effects against *H. pylori* (Zardast et al., 2016). *H. pylori* treatment (amoxicillin and omeprazole) for two weeks and vitamin or garlic supplementation (extract and oil) for seven years were associated with a statistically significant reduced risk of death due to gastric cancer (Li et al., 2019). In a review and meta-analysis, it was concluded that allicin (as an add-on therapy) improves *H. pylori* eradication, healing of ulcers and remission of symptoms (Si et al., 2019). Despite the initial results of clinical trials, it can be concluded that garlic is a good agent to be used in infection's treatment.

3. Almond (*Prunus dulcis*)

The almond is a fruit enriched in nutrients and phytochemicals, such as vitamin E, monounsatured and polyunsatured fatty acids, that ensure a wide range of biological activities: anticancer, antimicrobial, antiviral, anti-inflammatory and antioxidant (Amico et al., 2006; Mandalari et al., 2010). Almond skins also contain polyphenols, that still remain bioaccessible during simulated digestion in the gut (Mandalari et al., 2010). Flavonoids (polyphenolic secondary metabolites) are known for their biological activities: anticancer, antimicrobial, antiviral, antimutagenic and anti-inflammatory (Marino et al., 2010; Miceli et al., 2011).

Bisignano et al., 2013 analyzed the inhibitory effect of natural almond skins against *H. pylori* before and after a simulation of the human digestion. Pure flavonoid compounds such as epicatechin, naringenin and protocatechuic acid were also evaluated. The authors had previously proven that polyphenols from almond skins are active against a range of food-borne pathogens (Mandalari et al., 2010).

The antibacterial activity of almond skins was tested on *cagA*-positive and -negative clinical isolates, whose *cagA* and *vacA* genes were identified by PCR (Polymerase

Chain Reaction) a molecular technique that amplifies a copy of a gene). Natural almond skins (NS) were prepared from almonds by treatment with liquid nitrogen. For *in vitro* digestion studies, two types of digestion of natural almond skins were simulated: under gastric and duodenal conditions. To obtain the almond skin extracts, polyphenol-rich extracts from NS, NS post *in vitro* gastric digestion (NS G) and NS post *in vitro* gastric plus duodenal digestion (NS G + D) were prepared. Powder prepared from blanched skins was obtained by hot water extraction. This product suffered sequential extraction with methanol to obtain the extracted blanched skin powder, removing phenolic compounds. Both digestions (gastric and gastric plus duodenal digestion) were simulated using proper solutions and enzymes, in a shaking incubator (Bisignano et al., 2013).

The susceptibility of strains was assessed through the standard agar dilution method. The most effective antibacterial product was the natural almond skin (MIC_{50} of 64 μg/ml and MIC_{90} of 128 μg/ml), the second was natural skin post gastric digestion (MIC_{50} of 128 μg/ml and MIC_{90} of 256 μg/ml), followed by natural almond skin post gastric plus duodenal digestion (MIC_{50} of 256 μg/ml and MIC_{90} of 512 μg/ml). Between the pure flavonoid compounds, the one who showed the greatest efficacy against *H. pylori* was protocatechuic acid (MIC of 128 μg/mL against 50% of strains and a MIC of 256 μg/mL against 90% of strains); whereas the less efective was epicathechin (MIC of 512 μg/mL against 50% of strains and a MIC of 1024 μg/mL against 90% of strains) (Bisignano et al., 2013).

NS was the most active compound agains *H. pylori* maybe because it has the highest polyphenols concentrations, compared to the total phenolic content present in both simulated digestions. The main polyphenols identified in NS were catechin, epicatechin, kaempferol and isorhamnetin (Mandalari et al., 2010), and their combination may be responsible for these promising results. There are several reported mechanisms of action of flavonoids agains *H. pylori*: isoflavones and chalcones inhibit urease (Ansari et al., 2005; Xiao et al., 2007); others may neutralize vacA via interference of the toll-like receptor 4 signaling induced by the bacterium (Lee et al., 2005; Tombola et al., 2003). As a matter of fact, polyphenols that were found in almond skins are effective *in vitro* against *H. pylori* (independently of genotype status) and should be studied *in vivo*, conjugated with antibiotics (Bisignano et al., 2013).

4. Propolis

Propolis is defined as a resinous mixture that honeybees collect from botanical sources, such as tree buds and sap flows. It contains a variety of compounds, such as

chalcones, many distinct acids and their derivatives, hydrocarbons, amino acids and some minerals (Walker & Crane, 1987), that provide a big range of biological effects: anti-angiogenic, antiapoptotic, anti-enzymatic and anti-inflammatory (Shapla et al., 2018).

Bonvehí & Gutiérrez, 2012, tested the antimicrobial activity of 19 propolis extracts collected in different regions in the Basque Country against some fungal and bacterial isolates, including *Helicobacter pylori.* The active compounds were extracted from finely ground propolis with two types of solvents – ethanol extract of propolis (EEP) and propylene glycol extract of propolis (PEP) –, with intermittent manual shaking. Then, the insoluble fraction was filtered. To evaluate the inhibitory activity of propolis, the agar diffusion method was performed. The extracts were considered active against both bacteria and fungi if the zone of inhibition was bigger than 6 mm (based on the criteria setted by EUCAST). *H. pylori* showed the largest inhibition zones (from 13 to 20 mm) and a MIC (MIC was defined as the lowest solution concentration that inhibited at least 90% of the microorganism growth after incubation) from 6 to 14 mg/ml, the second lowest MIC value of the three Gram-negative bacteria tested.

Basque propolis extracts had an effective and dose dependent activity, regardless of their geographical origin and the solvent used for the extraction. The phenolic substances present in the extracts were responsible for the inhibiton of the microorganisms, since the samples with highest total phenolic content demonstrated the best antimicrobial activity. Phytochemical compounds, mainly flavonoids (a group of secondary metabolites of the polyphenol class) – flavone, flavonol, flavanone and dihydroflavonol – are predominant in the phenolic fraction and have distinct biological properties, making them the best candidates to assess the quality of Basque propolis. The antimicrobial activity of propolis had a positive correlation with flavonoids compounds' content, making it a promising product to apply for the treatment of *H. pylori* infection.

Propolis carries several types of flavonoids that are associated with antioxidative effects in many different ways, such as hydrogen donors, reducing agents and chelating metal ions (Cao et al., 1997; Rice-Evans et al., 1996). Consequently, it protects cellular molecules and keeps regular cellular structure and function (Valente et al., 2011). A following *in vitro* study showed that propolis ethanolic extracts constituted by distinct phenolic compounds strongly inhibited *H. pylori* growth and urease production (Baltas et al., 2016), which agree with the results of Bonvehí & Gutiérrez. However, in a clinical trial, Brazilian green propolis had minimal therapeutic effects (Coelho et al., 2007). More clinical trials could be performed using different types of propolis, in order to understand if the therapeutic effect remains minimal.

5. Curcumin (*Curcuma longa*)

Curcumin (diferuloylmethane) is the most active compound of the perennial herb *Curcuma longa,* commonly known as turmeric. Turmeric powder is extracted from the rhizomes of the plant and is usually used as a spice (Sharma et al., 2005). Several studies have confirmed the biological effects of curcumin: antimutagen, antioxidant, anti-inflammatory and anti-infectious (Bengmark, 2006; Duvoix et al., 2005; Holt et al., 2005).

De et al., 2009, studied the effect of curcumin against *H. pylori* isolates from India and in infected mice. The shikimate pathway is indispensable for the synthesis of important metabolites, such as aromatic amino acids. There are many enzymes involved in this pathway that have getting attention for being potential targets in nontoxic drug development. One of those enzymes is shikimate dehydrogenase (SDH), encoded by the *aroE* gene of *H. pylori*. A previous study has proved that curcumin is a noncompetitive inhibitor of SDH (Han et al., 2006), making it a promising compound for therapy progress.

The antibacterial activity of curcumin was analyzed *in vitro* through agar dilution method. The MIC of curcumin (defined as the lowest concentration of the compound at which there was no visible growth) ranged between 5 µg/ml to 50 µg/ml, clarifying its effectiveness against this bacterium, irrespective of the genetic composition of strains.

Sequence analysis of the *aroE* gene of *H. pylori* revealed that curcumin-mediated growth inhibition may not always depend on the shikimate pathway, seeing that strains that have identical sequences also owned differential curcumin MICs. The explanation may come from the strain-to-strain variations in the rate of curcumin uptake/efflux or maybe from the fact that curcumin may have other antimicrobial effects that do not only involve the shikimate pathway. A studied suggested that curcumin may prevent bacterial cell proliferation by inhibiting the assembly dynamics of FtsZ, a protofilament involved in cell division (Rai et al., 2008).

To understand curcumin activity *in vivo*, De et al., 2009 performed a study: a group of mice were inoculated with *H. pylori* and sacrificed, while other group was firstly infected and then orally fed with curcumin for 7 days. After the sacrifice, gastric tissues of the mice were checked for *H. pylori* colonization (by an urease test, which in the case of the group of mice that were orally fed with curcumin allowed the evaluation of the effect of the compound) and histology. The infection was still confirmed by PCR (using the *H. pylori*-specific gene *vacA*) and quantitative culture in blood agar plates. From quantitive culture, it was noted that

curcumin eradicated the bacterium (regardless of the strain). With the aim of confirming the therapeutic potential of curcumin, *H. pylori*-specific gene *vacA* was amplified by PCR with DNA from the two groups of mice, through comparing them. The results showed that, after treatment, the bacterium was completely eliminated. In histology assays (microscopic observations), it was observed that this natural product is highly active in healing the damaged gastric tissues. Thereby, *in vitro* and *in vivo* data are in concordance. In conclusion, curcumin manifested great effectiveness both in *H. pylori* eradication and in damaged gastric tissue restoration, evidencing its therapeutic potential.

In an *ex vivo* study, curcumin rhizome suppressed *H. pylori*-induced cytidine deaminase in gastric epithelial cells and showed an inhibitory effect, most likely by chemopreventive mechanisms (Zaidi et al., 2009). Sintara et al., 2010 conducted an *in vivo* investigation, in which curcumin supplementation reduced the NF-κB activation and macromolecular leakage (increased in gastric infammation) in *H. pylori*-induced infection in rats. Administration of curcumin-based therapy to infected patients has not showed effective results in *H. pylori* eradication. Despite that, when added to the standard regimen (a proton pump inhibitor plus amoxicillin and clarithromycin), it improves dyspepsia symptoms (Khonche et al., 2016) and ameliorates oxidative stress and histopathologic changes in chronic gastritis associated to *H. pylori* infection (Judaki et al., 2017). In another triple therapy (non-antibiotic), it reduced the serologic signs of gastric inflammation (Di Mario et al., 2007). So, curcumin can be used as an adjuvant to improve symptoms and inflammation that arise from the infection.

6. Broccoli (*Brassica oleracea*)

Yanaka et al., 2009, studied the effect of broccoli sprouts in *H. pylori* infection, in infected mice and humans. Broccoli sprouts are abundant in isothiocyanate sulforaphane [SF; 1-isothiocyanato-4(R)-methylsulfinylbutane] in the form of glucoraphanin, the inert glucosinolate precursor of sulforaphane (the primary isothiocyanate from broccoli). Isothiocyanate has biological activity in cruciferous plants (in which broccoli is included), making it relevant for its bactericidal potential. For this reason, foods enriched in this type of compounds are implyed in diets to prevent cancer.

Fresh broccoli and broccoli sprouts possess myrosinase, a membrane-associated enzyme that hydrolyzes glucoraphanin (present in the vacuole). This enzyme is also a

part of the microbiota of the lower intestine of animals and humans. So, glucoraphanin becomes bioavailable when uncooked broccoli is chewed, but there is a significant fraction that only is hydrolyzed when it passes through the gastrointestinal system. In mammals, sulforaphane induces protective (phase 2) enzymes, exhibiting cellular antioxidative, anti-inflammatory and antiangiogenic outcomes mostly via the transcription factor Nrf2 (nuclear factor erythroid 2–related factor 2) (Fahey et al., 1997). Previous studies had shown the anti-*H. pylori* activity of sulforaphane both *in vitro* and *in vivo* (in mice) (Fahey et al., 2002; Haristoy et al., 2003; Haristoy et al., 2005) and even in a pilot study, in which there was an eradication of infection in three of nine patients (Galan et al., 2004).

To study the effect of SF, mice were orally treated with SF-rich broccoli sprouts and a high-salt diet (because it has been proved that a high-salt treatment exaggerates *H. pylori*–induced gastritis in mice). The level of inflammation and atrophy of the gastric mucosa was measured, and the apoptosis was assessed immunohistochemically. The oxidative DNA injury and the phase 2 detoxification enzyme activity in gastric mucosal membranes were quantified by specific ELISA (enzyme-linked immunosorbent assay) kits. *H. pylori* colonization of mice gastric mucosa was numerically determined through viable counts of bacteria. The results evidenced that this kind of diet reduced bacterial colonization, impaired mucosal expression of tumor necrosis factor-α and interleukin-1β, attenuated corpus inflammation and averted gastric corpus atrophy induced by high salt-diet. In mice that suffered a knock out in *nrf2* gene, the healing effect described was not detected, highlighting the crucial role of proteins that depend on this transcription factor, involved in SF-dependent protection.

In the human study, only volunteers with gastritis that did not take medication (antibiotics, proton pump inhibitors or antiulcer drugs) were accepted. A control group was set by consuming a placebo, alfalfa sprouts, which do not contain SF. Urine samples were collected for measurement of dithiocarbamates (DTC), a group of metabolites of sulforaphane. Stool samples for *H. pylori* stool antigen (HpSA) were collected to analyze HpSA values with an ELISA kit, and the intensity of colonization was assessed by the UBT (both tests are biomarkers of *H. pylori* colonization). Blood samples were collected to measure serum pepsinogens I and II (PGI and PGII), biomarkers of gastric inflammation.

The tests showed that the consumption of broccoli sprouts decreased the levels of urease (determined by the ^{13}C-urea breath test), *H. pylori* stool antigen and serum pepsinogens I and II. After the treatment, values returned to their original quantity in

two months. In conclusion, the ingestion of fresh broccoli sprouts boosts the chemoprotection of the gastric mucosa against the oxidative stress caused by *H. pylori* infection, in both infected mice and humans, due to antibacterial activity of the bioactive metabolite, sulforaphane, that induces systemic cytoprotective enzymes.

7. Bovine lactoferrin (bLf)

Di Mario et al., 2006 studied the effect of bovine lactoferrin (bLf) in *H. pylori* eradication through a randomized, multicentred study. Bovine lactoferrin is a glycoprotein constituted by one polypeptide chain that belongs to the transferrin family. This glycoprotein plays the function of transporting iron (two atoms) with high affinity in different environments, including acidic conditions (Baker et al., 1991). It can be found in milk, saliva, tears, bile, blood plasma and mucosal and genital secretions (Britigan et al., 1994). BLf shows antibiotic activity *in vitro* and *in vivo* against *H. pylori* (and many other infectious agents) due to its capacity of binding iron with high affinity, making it impossible for the bacteria to use this metal, compromising its development and activity (Levay & Viljoen, 1995). It also has the ability to inhibit the attachment of the bacterium to gastric epithelial cells, which reduces the bacterial load and, as a consequence, the inflammation (Dial & Lichtenberger, 2002).

The antimicrobial efficacy of bLf was analyzed in addition to a standard triple therapy – esomeprazole 20 mg, clarithromycin 500 mg and tinidazole 500 mg – for 7 days, as a first-line treatment for *H. pylori*-positive patients (with various exclusion criteria). Patients were divided into three groups: group A, that only received the triple therapy refered; group B received a daily dosage of 200 mg of bLf for 1 week followed by the same treatment of group A; and group C, that also received a daily dosage of 200 mg of bLf for 1 week, administered at the same time as the standard triple therapy. The colonization of the bacterium was evaluated by histology and the C^{13}-urea breath test or by histology and the *H. pylori* stool antigen-test (HpSA).

Group A showed an eradication rate of 77%, group B exhibited 73% and group C revealed the highest rate, 90%. When the prevalence of side effects – fatigue, dizziness, headache, diarrhoea, tiredness, bitter taste and skin rash – was assessed, group A showed the highest one (9.5%), followed by group B (9%) and group C (8.2%). In this study, it can be observed that when bLf is combined with the standard 1-week triple therapy, there is an increase in infection's eradication rate, which results due to the many points of attack against *H. pylori*. This may occur due to various factors, like

the ones mentioned above (being an iron chelator and having the ability of inhibit the attachment of the bacterium to gastric epithelial cells) and others, such as: immune-modulatory activity on the gastric mucosa (Levay & Viljoen, 1995; Sanchez et al., 1992; Singh et al., 2002), a suggested antioxidant action and a non-iron dependent activity (Baldwin et al., 1984; Gutteridge et al., 1981).

The combination of the antibiotics (clarithromycin and tinidazole) with bLf may manifest a synergic action against the bacterium, leading to its destruction. So, an adjuvant treatment with bovine lactoferrin along with these specific medicines causes a high *H. pylori* eradication rate (89.5%) associated with low side effects, making it a reliable therapy for this infection.

8. Green tea (*Camellia sinensis*)

Camellia sinensis is a plant species whose leaves and leaf buds are a source of tea, the most common beverage in the world (Shoae Hassani et al., 2009). In a variety of animal models, the administration of green tea extracts or polyphenols inhibits tumorigenesis in several organs, including the stomach (Yang et al., 1993; Yang et al., 1997). Green-tea polyphenols have various anticarcinogenic effects, such as strong antioxidant activity, inhibition of nitrosation and cell proliferation, and induction of apoptosis among carcinoma cells (Yang et al., 1997; Ahmad et al., 1997).

Matsubara et al., 2003 studied the effect of foodstuffs (including green tea) in the inhibition of urease activity, which is essential for *H. pylori* colonization. Urease plays a fundamental role in *H. pylori* colonization, protecting the bacterium against the harsh stomach environment (Marshall et al., 1990). Previous studies have already proved that without this enzyme, the bacterium cannot induce infection in animal models – mice, gnotobiotic piglets and mongolian gerbils – pointing its importance in disease development *in vivo* (Eaton & Krakowka, 1994; Tsuda et al., 1994; Wirth et al., 1998).

Tested plant-derived samples *in vitro*, of which some tea (black tea, jasmine tea and oolong tea) and rosemary extracts evidenced inhibithory urease activity. Samples of plant-derived foodstuff were extracted with 50% methanol, while tea catechin (phenolic compounds) derivatives were dissolved in the same solvent and then serially diluted. To estimate urease inhibition, an assay was performed using jack bean and *H. pylori* enzymes. The aim was to measure ammonia production (urease catalyzes the hydrolysis of urea into ammonia and carbon dioxide) determining its concentration through coloration with the indophenol reaction, readed in a colorimeter.

Green tea extract (GTE) stood out of the analyzed products, showing the strongest IC_{50} value (the half maximal inhibitory concentration (IC_{50}) measured the potency of the products in inhibiting urease). *Camellia sinensis* is the source of green tea, one of the most consumed drinks worldwide. Tea leaves carry diverse types of catechins. This fact caused the need to analyze the urease inhibitory effect of the different forms separately. Epigallocatechin gallate, gallocatechin gallate, gallocatechin and epigallocatechin were the catechins that showed the strongest results (lower IC_{50} values). These types of phenolic compounds differ from the ones examined (that presented a weaker inhibitory effect) due to a specific feature: they possess a hydroxyl group at the 5'-position in the B-ring.

Since GTE presented the best potential in inhibiting urease activity *in vitro*, its effect *in vivo* was assessed on mongolian gerbils with *H. pylori*-induced gastritis. The animals were inoculated with *H. pylori;* it was given sterilized broth to the control group, while the experimental one received water supplemented with GTE. Mongolian gerbils were then sacrificed and their stomachs were resected. Gastric lesions (edema and hemorrhage) were observed macroscopically, stomach weight was measured and the level of gastritits was scored microscopically. *H. pylori* colonization was counted resorting to inoculation (onto agar plates) of scraped mucosa samples.

Gastric lesions were suppressed by GTE (at concentrations of 500–2000 ppm), that also reduced the microscopic scores of gastritis in a dose-dependent manner and the average stomach weights. From this data, it can be concluded that urease inhibition may be one of the factors that contributes to *H. pylori* infection eradication in the present animal model. Other potential mechanisms of catechins that may be involved are their antibacterial (Mabe et al., 1999) and antioxidant potential (Nanjo et al., 1996; Rice-Evans et al., 1996). The relation between the ihnibition of the studied enzyme and the 5'-hydroxyl group of the active components was not acknowledged. In conclusion, tea and tea catechins have a strong capability to inhibit *H. pylori* urease activity and to exhibit gastritis supression in mongolian gerbils. Due to these reasons, they can be considered a safe natural product to enforce gastric diseases therapy caused by this bacterium.

Other *in vitro* and *in vivo* studies corroborate this data about green tea. Catechin compounds (epigallocatechin-3-gallate) present in leaves and buds inhibited *H. pylori* growth *in vitro* (Yanagawa et al., 2003), as well as extracts of black and green tea (Diker & Hascelik, 1994). Non-fermented and semi-fermented methanol:water mixture extracts of young shoots of *C. sinensis* had inhibitory effects against *H. pylori* and urease function and production (Shoae Hassani et al., 2009). When combinated with sucralfate (an anti-ulcer drug), green tea catechins showed a significant reduction of

bacterial load in mongolian gerbils (Takabayashi et al., 2004). Green tea concentrates influenced gastric colonization/pathology in infected mice (Ruggiero et al., 2007). However, other studies about the effects of green tea consumption show controversial data. It is certain that green tea consumption decreases the risk of chronic atrophic gastritis development (Kuwahara et al., 2000; Shibata et al., 2000), but while some investigations indicate that it reduces the risk of stomach cancer (Inoue et al., 2014; Kono et al., 1988; Setiawan et al., 2001), others stipulate that there is no beneficial effect in consuming it (Nagano et al., 2001; Tsubono et al., 2001). Even though the information about green tea is not unanimous, it is a drink that may benefit health.

9. Virgin Olive Oil (*Olea europaea*)

Olive oil is an intrinsic food of the Mediterranean diet, used in many meals. This vegetable oil has many biological properties: anti-inflammatory, antioxidant, anti-cancer, among others. It is composed by an elevated percentage of oleic acid and is also enriched in other minor components, such as squalene, tocopherols, aliphatic alcohols, triterpenic and phenolic compounds. Virgin olive oil is obtained from the olive fruit without the need to go through a purification process (García-González et al., 2008).

Olive leaf extract is very active against *H. pylori* and other microorganisms (Sudjana et al., 2009). Phenolic compounds have revealed *in vitro* antimicrobial activity against *H. pylori* (Medina et al., 2009; Romero et al., 2007). Romero et al., 2007 noticed that a low concentration of pure dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (TyEDA) was able to eradicate the bacterium. Later, Medina et al., 2009 also perceived that some phenolic compounds composed by dialdehydic structures like TyEDA and HyEDA (dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol) were the reason why virgin olive oil showed a strong antibacterial effect against *H. pylori.*

Thanks to these findings from previous studies, Castro et al., 2012 studied the effect of virgin olive oil on *H. pylori*-infected patients with dyspeptic symptoms (with some exclusion criteria) and confirmed its efficiency *in vitro*. Castro et al., 2012 started by performing chemical analyses of phenolic compounds – HyEDA; TyEDA; HyEA, oleuropein aglycon; TyEA, ligustroside aglycon – fatty acids, sterols, squalene, tocopherols and aliphatic alcohols.

Then, the *in vitro* effect of both olive oil extracts on *H. pylori* was evaluated. The bacterial suspension in PBS (phosphate-buffered saline) was mixed with the oils

(extracts washed seven times with PBS were also tested) during 5 minutes, incubated and then the surviving CFU were counted. As expected, both extracts from olive oils A and B eradicated the bacterium, even when they were washed. Both wash and unwashed oils contained sufficient concentrations of TyEDA and HyEDA, that were capable of inducing the antibacterial outcome. After these conclusions, Castro et al. carried on with the clinical trials.

Two types of pilot studies were conducted: in the clinical study I, patients took 30 g of washed virgin olive oil A (obtained from the fruit of the Manzanilla variety) during two weeks. The oil was mixed with distilled water and separated (eight times); lastly, it was filtered. After 1 month, individuals who tested positive for the infection received 30 g of unwashed virgin olive oil for another two weeks. In the clinical study II, patients ingested the same amount of a distinct virgin olive oil (B type, obtained from the fruit of the Picual variety) with a different chemical composition in fatty acids and phenolic compounds. Infection status was accessed by the ^{13}C-urea breath test (24–72 hour and 4–6 weeks of the last dose taken).

The clinical trial I manifested an eradication rate of 27% with intention to treat (this type of analysis considers all patients) and 40% per protocol (only patients who completed the entire study); the second one exhibited an eradication rate of 10 and 11%, respectively. The oil caused some drawbacks, like taste dislike and nausea, experienced by some individuals (participants fasted for 30–60 minute after the intake). For this reason, other methods of oil administration should be considered. Oils A and B had a similar composition in fatty acids, sterols, squalene, aliphatic acids and tocopherols, but differed in phenolic compounds percentage, mainly in TyEDA and HyEDA, which was higher in oil A than in oil B. This chemical variation may justify the contrast in eradication rates between the studies I and II.

The results of this clinical trial were significant and should be supported by a new trial, without the first step of using washed oil (firstly intended as a placebo). Many variables such as olive diversity, processing methods and storage circumstances affect the olive oil's constitution (García et al., 2003). It would be relevant to understand the role of the many variables on *H. pylori* eradication *in vivo*.

10. Refined Deep Seawater

Deep seawater is collected in many facilities in Japan. After going through a purification process, this water can be consumed as refined deep seawater (RDSW). RDSW is a mineral-rich, safe and clean drinking water, that not only supports life, but also has

properties for human health improvement: it can relieve atopic dermatitis, prevent arteriosclerosis and can enhance hyperlipidemia, constipation and blood pressure, etc. This type of water is widely ingested and does not cause any kind of side effects on long-time consumers or medical complications on sick people. Thanks to these particularities, RDSW has various applications in the fishing industry, engineering and medicine (Hataguchi et al., 2005; Katsuda et al., 2008; Miyamura et al., 2004). Despite this, the efficiency of RDSW in infections has not been analyzed until 2012.

Kawada et al., 2012 studied the inhibitory effects of RDSW on *H. pylori* infection through *in vitro* and *in vivo* (animal and human) assays. The deep seawater was collected offshore, desalinated and conditioned to increase mineral concentration, originating distinct stages of hardness and magnesium:calcium (Mg:Ca) ratios.

The *in vitro* experiments were performed using 20 types of RDSW with combinations of 5 Mg/Ca ratios and 4 degrees of hardness. Bacterial growth and motility were assessed to evaluate the inhibitory effects of RDSW on *H. pylori*. Both parameters were inhibited depending on hardness. When the level of hardness was unaltered, the variation of the Mg/Ca ratio was responsible for the anti-*H. pylori* activities. All strains were inhibited for one of the 5 Mg/Ca ratios (at least) at a harness of 1000.

The results of the *in vitro* assay were then transposed to *in vivo* tests in *H. pylori*-inoculated mongolian gerbils. The animals received commercial mineral water (hardness around 30) and RDSW with no mineral content as a control, and RDSW with a hardness of 1000, with 5 different Mg/Ca ratios for 2 weeks. After the stipulated experimental time, mongolian gerbils were sacrificed, their stomachs were removed and the CFU were counted after the incubation using treated gastric tissue. Stomach colonization was significantly reduced by 2 types of RDSW ((Mg:Ca ratio of 3:1 and only Mg), in contrast with the commercial water.

Finally, RDSW was examined in asymptomatic *H. pylori*-infected individuals in a clinical trial. Patients drank 1L of the same 5 types of RDSW examined in the mongolian gerbils, every day during 10 days. Infection status was achieved by an UBT, done before and after the experimental period ($\Delta^{13}C$). To estimate the effect of the tested waters, the value of $\Delta^{13}C$ was considered a reflection of *H. pylori* colonization. There was a reduction of $\Delta^{13}C$ values in the experimental group when compared to the control. The antibacterial activity was observed in 91% of patients, for at least one of the 5 types of RDSW, with a significantly decrease in types with Mg:Ca ratio of 1:2 and 3:1.

In the *in vitro* tests, the reduction rate of CFU was an index of growth inhibition, while the reducted migration of colonies on the soft agar medium was an indicator of motility inhibition. CFU reduction increased proportionally to higher hardness and magnesium content, while the decreased motility raised accordingly to bigger hardness and calcium percentage. So, it can be concluded that growth is mostly influenced by magnesium and motility by calcium. The two minerals are either adsorbed on the surface or taken into the bacterium, and their inhibitory role occurs via distinct mechanisms. Motility is an essential factor for a successful gastric colonization, and a high bacterial load contributes to a persistent infection (Eaton et al., 1992; Eaton et al., 1996). The inhibition of these factors can lower the risk of infection, mitigate gastric damaged tissue and alleviate the consequent symptoms. The mechanisms by which these two minerals suppress the biological activities of *H. pylori* and their particular composition need to be achieved for future therapeutic application. A genetic analysis should be performed to understand how the minerals can influence *H. pylori* gene regulation and expression. This way, it would be possible to develop a RDSW appropriated for each person. The types of RSDW that contained mostly magnesium (Mg:Ca ratio of 3:1 and only Mg) showed the best inhibitory effect in mongolian gerbils assay. So, this outcome is in accordance with the *in vitro* tests, highlighting the great importance of magnesium and calcium in *in vivo* inhibition of *H. pylori*. As remarked before, *H. pylori* adsorbs and/or absorbs the two minerals, and this process may be influenced by a certain Mg:Ca ratio, as it can be observed in Mg:Ca ratio of 3:1. It is possible that magnesium and calcium exhibit a superior inhibitory activity when used in a balanced ratio (promoting intake), comparing to the single effect of each one. Hereupon, these two minerals may generate a synergistic inhibitory effect, reducing *H. pylori* gastric colonization.

RDSW has many beneficial aspects with regard to human health, such as preventing mineral deficits. This study presented promising results, but it would be necessary to evaluate the combined effect of RDSW and antibiotics, to ensure a safe and effective treatment. Concluding, refined deep seawater is a propitious product to prevent and treat *H. pylori* infection as an adjuvant therapy.

11. Cranberry juice (*Vaccinium macrocarpon*) and *Lactobacillus johnsonii* La1

Gotteland et al., 2008 studied the effect of cranberry juice and *Lactobacillus johnsonii* La1 in children. Cranberry (*Vaccinium macrocarpon*) is a native fruit from North America, known for its potential in preventing and treating urinary tract infections (Stothers, 2002). Studies about cranberry extracts (and other berries, such as blueberries and blackberries) have verified anti-*H. pylori* activity, which probably results from their high content of proanthocyanidins, a high-molecular-weight class of polyphenols (Burger et al., 2000; Chatterjee et al., 2004; Lengsfeld et al., 2004). *Vaccinium* species may exhibit anti-inflammatory and antioxidant activity, helpful to mitigate infections caused by pathogens (Määttä-Riihinen et al., 2005). Cranberry juice can also prevent bacterium adhesion to human cells. As related in *in vitro* and *in vivo* studies, it avoids *Escherichia coli* from adhering to the uroepithelium (Howell, 2002) and *H. pylori* to the gastric mucosa (Burger et al., 2000; Chatterjee et al., 2004). In an animal study, there was an eradication rate of 20% in infected mice (Xiao & Shi, 2003). A clinical trial demonstrated that cranberry juice was capable of eradicating *H. pylori* in 14.4% of patients (Zhang et al., 2005). Additionally, a mix of berries extracts enhanced *H. pylori* strains susceptibility to clarithromycin (Chatterjee et al., 2004).

Lactobacillus johnsonii La1 (La1) is a probiotic strain that has the capacity to persist in the human gastrointestinal tract, adjusting the microbiota (Garrido et al., 2005). *In vitro* studies have reported many beneficial effects of La1: it restores the local and systemic immune responses (Haller et al., 2000; Haller et al., 2000) and displays antimicrobial activity against gastrointestinal pathogenic microorganisms (Bernet et al., 1994; Bernet-Camard et al., 1997), in which *H. pylori* is included (Michetti et al., 1999). Clinical trials have claimed that some types of probiotic strains may influence *H. pylori* colonization and infection and/or reduce the unpleasant effects that arise from the standard treatment (Gotteland et al., 2006; Pantoflickova et al., 2003). A study has suggested that this positive effect in *H. pylori* inhibition may occur due to the synthesis of organic acids and/or bacteriocins (toxins generated by bacteria to inhibit the growth of related bacteria), which prevents bacterium development and its attachment to the epithelial cells of the stomach (Gotteland et al., 2006). Clinical trials using La1 have indicated that this probiotic decreases *H. pylori* colonization, despite of not inhibiting it (Cruchet et al., 2003), even when the intake is more frequent (Gotteland & Cruchet, 2003).

The high potential of both foodstuffs on *H. pylori* inhibition and their probable distinct mechanisms of action conducted this clinical trial, on which an additive or synergistic outcome was expected. Gotteland et al., 2008 previously checked that the juice can not ihnibit the activity of the probiotic, through the agar diffusion method. Asymptomatic children infected with *H. pylori* ingested 200 mL of cranberry juice (cranberry concentrate diluted in potable tap water) and 80 mL of La1 every day, during 3 weeks. To assess the infection status, an ^{13}C-urea breath test was taken before and after the experimental period. Patiens who tested positive in the second test also took a third one after one month. Children were divided into four groups: cranberry juice/La1 (CB/La1), placebo juice/La1 (La1), cranberry juice/heat-killed La1 (CB), and placebo juice/heat-killed La1 (control).

There was an eradication rate of 1.5% in the control group, 14.9% in the La1, 16.9% in the CB and 22.9% in the CB/La1. The group that included both products showed a higher eradication, but not significant, when compared with the single effect of each tested element: CB or La1, 6% and 8%, respectively. However, *H. pylori* was detected in 80% of children who took the third ^{13}C-urea breath test.

The results of the present study confirm that regular consume of cranberry juice and/or La1 influences *H. pylori* eradication in a positive way in asymptomatic children. The expected synergistic effect was not noticed, but it was observed an irrelevant additive effect. The high percentage of positive tests in the third ^{13}C-urea breath test taken suggests that the bacterium had been temporary inhibited but not totally eradicated. Despite this, it may be concluded that both products are functional and can be safely consumed – alone or simultaneously – for extended periods of time.

12. FEMY-R7 (*Laminaria japonica* and *Oenothera biennis*)

Kim et al., 2015 studied the effect of FEMY-R7, composed by *Laminaria japonica* and *Oenothera biennis* extracts, in mice and humans. Extracts of the seaweeds *Laminaria japonica* and *Cladosiphon okamuranus* are extensively utilized in Oriental medicine because of their content of fucoidan. This sulfate polysaccharide compound acts as an active component, due to a range of biological activities: anti-inflammatory, antioxidant, anti-tumor and anti-coagulative (Feldman et al., 1999; Wang et al., 2010).

Consequently, it is efficient in improving the imune system, ischemia and inflammation (Bojakowski et al., 2001; Li et al., 2005). Fucoidan also has the capability of intervening in the adhesion of *H. pylori* to the gastric epithelium *in vivo* (mongolian gerbils and humans) (Shibata et al., 1999; Shibata et al., 2003). *Oenothera biennis* (commonly known as evening primrose) is a medicinal plant whose seeds have shown activity against *H. pylori*, which comes from its tenor in tanins (polyphenols of vegetable origin). A study corroborated that *Oenothera biennis* extract inhibits *H. pylori* spread *in vitro* and prevents this bacterium from adhering and colonizing the gastric tissue (Funatogawa et al., 2004). Cai et al., 2014 previously proved that *Laminaria japonica* extract (LJE) and *Oenothera biennis* seed extract (OBE) eradicated the bacterium *in vitro* and *in vivo*, from mice stomachs. FEMY-R7 has also demonstrated its anti-*H. pylori* potential in humans (Kim et al., 2014).

Laminaria japonica was washed, dried and grinded. After these procedures, the extract was obtained with hot water and then filtered. *Oenothera biennis* seeds were extracted with 60% ethanol. The mixture of LJE and OBE (1:1), FEMYR7, was then performed.

Mice were orally inoculated with the pathogen 3 times at 2-day breaks and orally administrated with FEMY-R7 (total 20, 64 or 200 mg/kg/day), at the same time during 2 weeks. In the clinical trial, infected patients received FEMY-R7 capsules (total 1.62, 5.2 or 16.2 mg/kg/day - mice matching doses) twice a day, before a meal, during 8 weeks.

In bacterial identification assays, mice were sacrificed after the final administration and a biopsy of their gastric mucosa was realized. Mice and human feces were also collected. Samples were applied to CLO (Campylobacter-like organism) kits to assess urease activity, through a color changing reaction. To identify the bacterium from the CLO-positive stools, fecal samples were inoculated and tested through oxidase and catalase reaction, nitrate reduction and H_2S formation.

In mice, (CLO)-detection tests on gastric mucosa and feces revealed that FEMY-R7 decreased the urease-positive reactivity in a dose-dependent manner: to 70, 20, and 10% for gastric mucosa and to 80, 50, and 20% for feces. In humans, FEMY-R7 reducted the positivity ratios to 70, 40, and 30% in feces, respectively.

Dose dependency of CLO reaction and bacterial identification ratios presented a satisfactory correlation between mice and human feces. As refered before, *H. pylori* was eradicated in mice in a dose-dependent manner (200 mg/kg/day displayed a great efficacy). Despite this, fecal and gastric mucosa CLO tests did not match. In CLO tests, fecal samples manifesting a partially-positive reaction were not identified in bacterial

culture. This can be explained by the fact that dead bacterium may still release urease, that remains in stools. The positive results of FEMY-R7 in *H. pylori* eradication propose the administration of this extract as an adjunct product in infection treatment.

13. Stout camphor fungus (*Antrodia camphorata*)

Antrodia camphorata (Polyporaceae) is a medicinal mushroom commonly known as stout camphor fungus or ‘‘jang-jy” and ‘‘niu-chang-chih” in Taiwan, that only grows within the heart-wood wall of an endemic tree. This fungus is widely consumed in many asian and european countries as a food dietary supplement. It has many health benefits: prevents cancer and helps in the treatment of various conditions, such as diarrhea, abdominal pain, food and drug intoxication, skin itches, hypertension and liver cancer (Tzeng & Geethangili, 2011). There have been a lot of identified secondary metabolites of this mushroom. One of them of special importance, the triterpenoid methylantcinate B (MAB), is capable of displaying cytotoxicity against diverse cancer cell lines (Yeh et al., 2009). Geethangili et al., 2010 concluded that MAB reduces *H. pylori* inflammation in gastric epithelial cell line (AGS cells), although they did not fully understand the associated molecular mechanisms.

The bacterium interacts with AGS cells directly, translocating CagA protein into them via type IV secretion system (T4SS). CagA is then tyrosine phosphorylated, inducing inflammatory processess, such as the activation of IL-8 via nuclear factor (NF)- B signaling pathway (nuclear factor kappa-light-chain-enhancer of activated B cells) (Odenbreit et al., 2000; Brandt et al., 2005). CagA mimics a host cell factor to activate or inactivate some intracellular signaling pathways (Odenbreit et al., 2000). CagA translocation and phosphorylation are mediated by microdomains enriched in cholesterol, located in the plasma membrane (lipid rafts) (Lai et al., 2008; Lai et al., 2011; Murata-Kamiya et al., 2010). Hereupon, cholesterol depletion (by competition of the binding sites) can be a potential therapeutic target to reduce the CagA-induced pathogenesis (Lai et al., 2008). Chemical structures of MAB are identical to those of cholesterol. Because of that, it can be of major interest to understand the binding process of MAB to CagA, responsible for the anti-*H. pylori* activity. In the present study, Lin et al., 2013 studied the mechanisms of competition between MAB and CagA to bind to cholesterol.

Dried powder of *A. camphorata* fruiting bodies was extracted with chloroform and hexane in an extractor. The various constituents were then separated following specific

extraction and isolation methods. To assess the cytotoxicity of MAB in AGS cells, an MTT assay was performed (cell metabolic activity was measured by a colorimetric reaction). A Western Blot analysis of AGS cells was realized to quantify specific proteins and the hummingbird phenotype (a phenotype that is the result of two events: the induction of early motility and elongation) was determined. Luminescence was measured in a luciferase activity assay, in which AGS cells were seeded with transfect cells with either IL-8-Luc or NF- B-Luc plasmids. IL-8 secretion was setted by ELISA. The location of *H. pylori* and CagA was visualized by immunofluorescence (to quantify fluorescence intensity) and confocal (laser scanning) microscopy. Structural protein comparisons and their models were explored and performed using bioinformatic programs. To ascertain the binding ihnibition of cholesterol and CagA by MAB, a dot blot analysis was executed (dot blot is a simplified version of the western blot method).

Lin et al., 2013 found that MAB restrained the translocation and phosphorylation of CagA in a dose-dependent manner, reducing the hummingbird phenotype in infected AGS cells. In this phenotype, the early cell scattering relies on a CagA-dependent T4SS factor, while the cell elongation depends on CagA phosphorylation. This events cause an impact on the immune reaction, enhancing pathogenesis (Backert & Selbach, 2008; Hatakeyama, 2008). MAB suppressed the activation of CagA induced via NF- B and blocked p65 (a member of the NF- B transcription complex) nuclear translocation, evidencing MAB anti-cancer activity. MAB also repressed the activity of IL-8 luciferase and its secretion, which means that the immune stimulation caused by the bacterium diminished with this triterpenoid. Previous studies reported that triterpenoids reduce serum cholesterol levels, which indicates that they modulate cholesterol synthesis, boosting anti-hyperlipidemic potential (Melo et al., 2009; Tzeng & Geethangili, 2011). MAB did not change cellular cholesterol level, which points that CagA functional inhibition did not happen by cholesterol reduction in the membrane rafts. As previously stated, CagA may interact with membrane cholesterol (Lai et al., 2008; Murata-Kamiya et al., 2010). The present investigation reported that MAB and cholesterol interact with CagA in a similar mode.

All together, these findings denote that MAB attenuates *H. pylori* infection inhibiting the binding of cholesterol with CagA by competing with it. So, this compound has a great potential in modulating CagA-induced pathogenesis. However, *in vivo* studies should be fulfilled to discern if there is a correlation with the *ex vivo* outcomes.

14. Snakehead fish (*Channa striata*)

H. pylori infection causes an innate immune response, which leads to the inflammation of the gastric mucosa and recruitment of distinct cells and diverse innate immune system components through the release of chemokines and cytokines (Pacheco-Fernández et al., 2019). One of the cytokines is the macrophage migration inhibitory factor (MIF), that can also be induced by endotoxins, exotoxins, stress and other bacterial infections (Yu et al., 2015). This factor regulates the immune response (innate and adaptive), the inflammatory processes progress and diseases mediated by the immune system, including gastrointestinal illnesses: gastritis, ulcer, gastric malignancy and colon cancer (Xia et al., 2009). MIF evokes other proinflammatory cytokines –- interleukin-1 beta (IL-1β), IL-8, IL-6, tumor necrosis factor-α (TNF-α) and interferon-gamma (IFN-γ) – which increase the inflammation by recruiting neutrophils, macrophages and T-cells into the gastric mucosa (Schindler et al., 2018). A previously study has confirmed that *H. pylori* infection raised MIF levels, which notably declined after a firstline eradication treatment (Kebapcilar et al., 2010).

Snakehead fish (*Channa striata*), also known as chevron snakehead, stripped snakehead and “ikan gabus” in Indonesian, is a widely consumed fish in Asia-Pacific, particularly in Indonesia. This animal has medicinal value: it accelarates the recovery process of a disease, heals wounds, reduces pain and fever, assists in many skin disorders cure and has anti-inflammatory properties (Wahab et al., 2015). Snakehead fish extract has been studied due to its potential in decreasing inflammation (Dwijayanti et al., 2015) and its antibacterial and antifungal effects *in vitro* (Andini & Prayekti, 2019; Zulaikha et al., 2020). Moreover, there was a great reduction of cytokine levels in pulmonary tuberculosis patients administered with capsules of this extract and antituberculosis drugs (Paliliewu et al., 2013).

Yulizal et al., 2020 studied the effect of snakehead fish extract supplementation to first-line eradication regimen on MIF expression in albino rats (*Rattus norvegicus*) induced by *H. pylori* infection. The pure extract powder was comprised in 500 mg capsules, which were then diluted in 0.5% carboxymethyl cellulose and administered orally to rats by intragastric gavage during one week.

Mice were divided into four groups: groups 1 and 2 as negative (without *H. pylori* inoculation), and positive (with *H. pylori* inoculation) control, respectively, and treatment groups, both with *H. pylori* inoculation, group 3 as first-line eradication regimen group (aqueous solution of amoxicillin 50 mg/kgBW + clarithromycin 25 mg/kgBW +

omeprazole 20 mg/kgBW) and group 4 as supplementation group (the same eradication treatment as group 3 and snakehead fish extract with a dosage of 300 mg/kgBW). Groups 2, 3 and 4 also received an aqueous solution of streptomycin (an antibiotic used to treat tuberculosis) (5 mg/ml) before the inoculation, and omeprazole (a proton pump inhibitor) with a dose of 400 µmol/kgBW, before the inoculation and during the following 6 days.

To determine the infection status, the rats were sacrificed, subject to a surgical procedure and an urease test was performed, before (a rat from each group) and after the inoculation. An immunohistochemistry (IHC) assay was performed with the remaining gastric tissue. IHC is a technique of identification of antigens in tissues, using specific antibodies conjugated with an enzyme that catalyzes a colorful reaction when the antigen-antibody binding occurs. MIF expression was examined using immunoreactive score (IRS). IRS was setted as the outcome of multiplication between the percentage score of immunoreactive cells and color intensity scores of these cells at IHC. Eradication testing procedure was accomplished using a section of gastric mucosal that was putted in a saline solution, then macerated and homogenized. *H. pylori* colonies were identified morphologically and biochemically. Colonies were calculated using a colony counter.

Streptomycin and omeprazole pretreatment inhibited other bacteria growth and decreased gastric acidity, respectively. Inoculated groups showed a higher MIF expression compared to group 1 (negative control), which was correlated with a stronger bacterial density, as concluded by group 2 (positive control) results. In groups 3 and 4 (treatment groups), *H. pylori* was eradicated and MIF expression decreased, indicating that the first-line eradication regimen was very effective, as another study suggests (Kebapcilar et al., 2010). Supplementation group (group 4) manifested a significant reduction of MIF expression relatively to group 3, evidencing the therapeutic potential of the snakehead fish extract when compared to the single administration therapy used in this study.

Snakehead fish contains therapeutic potential due to its content in certain compounds: amino acids, fatty acids, albumin and minerals (Haniffa et al., 2014). Albumin can inhibit biofilm formation (Smith et al., 2017), has antibacterial properties and antioxidant effects as some minerals, such as zincum, cuprum and ferrum, by protecting cells against oxidative stress (Hidayati et al., 2018). Anti-*H. pylori* activity of some amino acids has been confirmed *in vitro* (Siebert et al., 2018). An anti-inflammatory and antioxidant synergistic activity between amino acids (lysine, arginine, aspartic and

glutamic acid) and fatty acids (linoleic, arachidonic, stearic and oleic acid) has been reported (Galla et al., 2012). An *in vivo* study using snakehead fish based cream acknowleged that linoleic and arachidonic acids affected proinflammatory cytokines, while leukocyte activity and its adhesion molecules expression were reduced by stearic and oleic acids, respectively (Abedi et al., 2012). Lining up all these facts, snakehead fish is a great food to fight *H. pylori* infection and can be consumed as an adjuvant medicine. However, a clinical trial should be performed in order to understand its effectiveness in humans.

15. Licorice (*Glycyrrhiza glabra*)

Various natural products have been used in traditional chinese medicine, including the licorice root (*Glycyrrhiza glabra*). 18β-Glycyrrhetinic acid (GRA) constitutes the main integrant purified from this plant. Licorice herb has many known therapeutic properties, widely used in clinic: antitumor, anti-UV-B irradiation, antioxidative, antimicrobial, antiprotozoal, antiviral, anti-inflammatory and even hepatoprotective (Hosseinzadeh & Nassiri-Asl, 2015). Root extract of *Glycyrrhiza glabra* favors the gastrointestinal system by acting as an anti-ulcer, protecting gastric epithelial cells and regulating gastrointestinal motility (Aly et al., 2005; Chen et al., 2009; Oh et al., 2009). Licorice can repair PUD by protecting the mucosa through secretin secretion (secretin is a digestive hormone that has many functions such as gastric acid regulation and osmoregulation), and by enzyme inhibition, resulting in antibacterial and anti-adhesive effects on *H. pylori* (Chatterji et al., 2001). This anti-adhesive property was also supported in another study about the effect of this herb in several bacteria (Asha et al., 2013).

Wittschier et al., 2009 conducted an *in vitro* study in which the polysaccharide released from the root of licorice inhibited *H. pylori* adhesion to human gastric mucosa. *Glycyrrhiza glabra*'s rhizomes and leaves can prevent *H. pylori* multiplication *in vitro*, even in clarithromycin-resistant strains (Fukai et al., 2002). Cao et al., 2016 indicated that GRA has protective effects *in vivo*, once it notably attenuated *H. pylori*-infected gastritis in mongolian gerbils. Yoon et al., 2019 conducted a clinical trial administrating fermented milk containing *Lactobacillus paracasei* (a probiotic) and *Glycyrrhiza glabra*; together, these two products reduced bacterial density and improved histologic inflammation in *H. pylori*-infected patients.

Hajiaghamohammadi et al., 2016 evaluated the effect of adding licorice to the standard treatment regimen of *H. pylori.* Patients suffering from dyspepsia [either with PUD or non-ulcer dyspepsia) and many exclusion criteria] who tested positive for rapid urease test were selected. Two treatment regimens were applied during two weeks: triple therapy including clarithromycin (500 mg twice a day), amoxicillin (1 g every day) and omeprazole (20 mg twice a day) (control group) or the same therapy supplemented with licorice [D-Reglis, a dry extract (380 mg twice a day)]. Both groups received 20 mg of omeprazole after the testing period, once a day, for four weeks. *H. pylori* eradication was measured six weeks after the treatment, using *H. pylori* stool antigen (HpSA) test.

The difference in eradication was significant between groups – treatment group achieved an eradication of 83.3% and the control group of 62.5% – and the treatment was more effective in PUD patients, which was an outcome in line with another report (Rahnama et al., 2013). The addition of licorice clearly enhanced bacterial eradication, compared to the standard clarithromycin-based triple therapy, as confirmed in a previous *in vitro* study (Wittschier et al., 2009). Two clinical trials have compared the effectiveness of quadruple therapy including licorice or bismuth (a metal). In both studies, the regiment using licorice showed a higher eradication rate, which indicates that D-Reglis has the potential to replace bismuth (Ali et al., 2014; Rahnama et al., 2013).

In dyspeptic patients, licorice not only produces an antibacterial effect against *H. pylori*, but also has an anti-inflammatory action (Puram et al., 2013; Raveendra et al., 2012). This outcome proposes a higher dose of licorice prescription and a longer treatment duration. In conclusion, adding licorice to this type of therapy would be an adequated first line treatment, particularlly in PUD patients living in clarithromycin and/or amoxicillin-resistant areas. It could also benefit infected individuals by reducing adverse effects caused by drugs and their high prices.

Table 1. Natural products with anti-*Helicobacter pylori* potential.

Natural Product	Type of Natural Product	Family	Part / Extract	Active Compound	Reference
Stout camphor fungus (*Antrodia camphorata*)	Fungus (mush-room)	Polypora-ceae	Fruiting body (dried powder extracted with chloroform)	Triterpenoid methylantcinate B	Lin et al., 2013
Almond (*Prunus dulcis*)	Foodstuff (fruit)	Rosaceae	Skins (hot water and methanol extraction)	Polyphenols (catechin, epicatechin, kaempferol and isorhamnetin)	Bisignano et al., 2013
Garlic (*Allium sativum*)	Foodstuff (vegetable)	Amaryllida-ceae	1. Clove: aqueous garlic extract 2. Aceto nic garlic extracts	1. Unknown 2. Thiosulfinates: allicin and allylmethyl plus methyl-allyl thiosulfinate	1. Cellini et al., 1996 2. Canizares et al., 2004
Propolis	Resulting animal activity	-	Ethanoli c and propyle ne glycol extract	Phenolic compounds: flavonoids (flavone, flavonol, flavanone and dihydroflavonol)	Bonvehí & Gutiérre z, 2012
Turmeric (*Curcuma longa*)	Foodstuff (spice)	Zingibera-ceae	Rhizome	Curcumin (Diferuloylmetha ne)	De et al., 2009
Gree n tea (*Cam ellia sine nsis*)	Foodstuff (plant)	Theaceae	Leaves and leaf buds (extracted with metanol)	Phenolic compounds: catechins (epigallocatechin gallate, gallocatechin gallate, gallocatechin and epigallocatechin)	Matsubara et al., 2003
Snakehead fish (*Channa striata*)	Foodstuff (fish)	Channi-dae	Pure extract powder (diluted in 0.5% carboxymet hyl)	Unknown (amino acids, fatty acids, albumin and minerals)	Yulizal et al., 2020
Refined deep seawater	Foodstuff (water)	-	Desalination and refination to increase the mineral concentratio n	Magnesium and calcium	Kawada et al., 2012
Broccoli (*Brassica oleracea*)	Foodstuff (vegetable)	Brassica-ceae	Sprout (diluted in distilled water)	Isothiocyanate sulforaphane	Yanaka et al., 2009

FEMY-R7 (*Laminaria japonica* and *Oenothera biennis*)	Seaweed and Plant	Laminariaceae and Onagraceae	*L .japonica* plant (hot water extract) and *O. biennis* seeds (ethanol extract) 1:1	Fucoidan	Kim et al., 2015
Bovine lactoferrin (bLf)	Animal glycoprotein	Transferrin	-	-	Di Mario et al., 2006
Virgin Olive Oil (*Olea europaea*)	Foodstuff (plant)	Oleaceae	Oil (washed oil: mixed with water 1:1)	Phenolic compounds with dialdehydic structure (TyEDA and HyEDA)	Castro et al., 2012
Licorice (*Glycyrrhiza glabra*)	Foodstuff (plant)	Fabaceae	Roots	18β-Glycyrrhetinic acid (GRA)	Hajiagh amohammadi et al., 2016
Cranberry (*Vaccinium macrocarpon*) and *Lactobacillus johnsonii* La1	Foodstuff (fruit and probiotic strain)	Ericaceae and Lactobacillaceae	Fruit (cranberry concentrate diluted in water)	Polyphenols (proanthocyanidins)	Gotteland et al., 2008

Table 2. Studies of the anti-*Helicobacter pylori* effects of natural products.

Natural Product	Type of Study	Dose and Duration	MIC / Eradication Rate	Mechanisms of Action	Referen-ce
Stout camphor fungus (*Antrodia camphorata*)	*Ex vivo* (gastric epithelial cells)	10, 20 and 50 M for 6h	IC_{50}: 50 µm	Ameliorating effect on *H. pylori*-induced modifications; Inhibition of CagA translocation and phosphorylation	Lin et al., 2013
Almond (*Prunus dulcis*)	*In vitro*	-	MIC_{50}: 64 µg/ml and MIC_{90}: 128 µg/ml	Unknown (anti-bacterial activity)	Bisignano et al., 2013
Garlic (*Allium sativum*)	*1. In vitro* *2. In vitro*	- -	1. MIC_{90}: 5 mg/ml 2. allicin MIC: 16 mg/L allylmethyl plus methyl-allyl thiosulfinate MIC: 24 mg/L	1. Unknown (bacteriostatic and bactericidal activity; synergistic effect with omeprazole, ratio of 1:250) 2. Synergic effect between the two organosulfur compounds; Inhibition of bacterial proliferation	1. Cellini et al., 1996 2. Canizares et al., 2004
Propolis	*In vitro*	-	MIC: 6–14 mg/ml	Unknow (anti-*H. pylori* activity)	Bonvehí & Gutiérrez, 2012
Turmeric (*Curcuma longa*)	*In vitro* *In vivo* (mice)	- 25 mg/kg once daily for 7 days	MIC: 5–50 mg/ml 100%	Strong anti-bacterial activity; Ameliorating effect on *H. pylori*-induced gastric damage	De et al., 2009
Green tea (*Camellia sinensis*)	*In vitro* *In vivo* (mongolian gerbils)	- 500, 1000 and 2000 ppm for 6 weeks	IC_{50}: 13 lg/ml 32%, 36%, and 16%	Inhibithory urease activity	Matsubara et al., 2003
Snakehead fish (*Channa striata*)	*In vivo* (rats)	amoxicillin 50 mg/kgBW + CAM 25 mg/kgBW + omeprazole 20 mg/kgBW + fish extract 300 mg/kgBW for 7 days	-	Significant reduction of MIF (macrophage migration inhibitory factor) expression	Yulizal et al., 2020

Table 2. Continued

Natural Product	Type of Study	Dose and Duration	MIC / Eradication Rate	Mechanisms of Action	Referen-ce
Refined deep seawater (RDSW)	*In vitro* *In vivo* (mongolian gerbils) Clinical trial	5 Mg/Ca ratios and 4 degrees of hardness 5 RDSW types (hardness of 1000, 5 different Mg/Ca ratios) for 2 weeks 1L of the same 5 RDSW types for 10 days	-44% for Mg 240 mg/L, hardnes s of 1000 -High CFU decrease with Mg:Ca of 3:1 and only Mg -91%	Bacterial growth and motility inhibition, may produce synergistic inhibitory effects; Reduced stomach colonization; Anti-bacterial activity	Kawada et al., 2012
FEMY-R7 (*Laminaria japonica* and *Oenothera biennis*)	*In vivo* (mice) Clinical trial	20, 60, 200 mg/body weight/day for 2 weeks 100, 320 and 1000 mg/person/day for 8 weeks (1.62, 5.2 and 16.2 mg/kg/day)	60, 20 and 0%, respectively 70, 20 and 0%, respectively	Anti-bacterial effect and *H. pylori* eradicating activity	Yanaka et al., 2009
Broccoli (*Brassica oleracea*)	*In vivo* (mice) Clinical trial	3 μmol/mouse/day of glucoraphanin for 2 months 70 g/day of glucoraphanin (420 μmol/70 g dose) for 8 weeks	- Reduced gastric bacterial colonization and inflammation	Anti-bacterial activity (systemic cytoprotective enzymes induction)	Kim et al., 2015
Bovine lactoferrin (bLf)	Clinical trial	200 mg bLf for 7 days + 20 mg esomeprazole+ 500 mg CAM+500 mg tinidazole for 7 days	bLF followed by triple therapy: 73% triple therapy followed by bLf: 90%	Inhibition of bacterial activity and development by iron binding; Inhibition of bacterial attachment to gastric epithelial cells	Di Mario et al., 2006
Virgin Olive Oil (*Olea europaea*)	Clinical trial	1.30 g of washed and unwashed virgin olive oil A for 14 days with an interval of 1 month 2. 30 g of a virgin olive oil B for 14 days	1. ITT 27% and PP 40% 2. ITT 10% and PP 11%	High anti-bacterial activity; *H. pylori* increased eradication	Castro et al., 2012
Licorice (*Glycyrrhiza glabra*)	Clinical trial	500 mg CAM + 1 g amoxicillin + 20 mg omeprazole + 380 mg D- Reglis (a dry extract) for 2 weeks	83.3%	Anti-bacterial effect (enhanced bacterial eradication)	Hajia-gham-oham-madi et al., 2016

Cranberry (*Vaccinium macrocarpon*) and *Lactobacillus johnsonii* La1	Clinical trial	200 mL of cranberry juice and 80 mL of La1 every day for 3 weeks	22.90%	Inhibitory effect on *H. pylori* adhesion to gastric mucosa; Reduced *H. pylori* colonization; Additive effect, not relevant	Gotteland et al., 2008

MIC: Minimum Inhibitory Concentration; MIC_{50}: the concentration that inhibits 50% of isolates; MIC_{90}: the concentration that inhibits 90% of isolates; IC_{50}: half maximal inhibitory concentration.
BW: body weight.
CAM: clarithromycin.
ITT: Intention-to-treat analysis.

Chapter IV

Conclusions

The human body possesses a varied microbiota, that harbors different species of microorganisms living in dynamic ecological communities (Turnbaugh et al., 2007). The gastric microenvironment is characteryzed by a low partial oxygen pressure, a high acid concentration and the presence of digestive enzymes. Because of these particularities, this organ is hostil to commensal bacteria. Therefore, bacterial factors of the intrinsic community play a significant impact on their adaptation and virulence. As a consequence, they contribute to the emergence of gastric pathologies and even to cancer development (Conteduca et al., 2012; Crew & Neugut, 2006). Due to these statements, it is of major interest to understand the mechanisms associated with gastric carcinogenesis.

H. pylori is a bacterium capable of inducing gastric infection, strongly influencing the evolution of malignant outcomes and its precursor lesions. Despite not being an intracellular pathogen, bacterial-host cellular interactions may benefit this pathogenic agent. This bacterium has an enhanced capacity of surviving in the stomach's harsh conditions, which enables its replication and consequent microcolonies formation. Through diverse mechanisms of action, it contributes to the development of a chronic infection (Hatakeyama, 2009).

H. pylori stimulates innate and acquired immune systems, through DNA arrangements, such as point mutations and recombinations. These cellular mechanisms provide the capability to reduce identification by generating disctinct epitopes. This pathogen also uses host plasma membranes by sequestering itself into the gut lumen. This establishment causes an evasion and manipulation of the immune system, less efficient in this gastric area. *H. pylori* not only downregulates the activation of immune cells, but it also mimicks host antigens (WGO, 2011). Bacterial adhesion to gastric epithelial cells provokes an inflammatory response, in which many immune cells are recruited: neutrophils, B and T lymphocytes, macrophages and plasma cells. As a consequence, high quantities of reactive oxygen and nitrogen species are released, causing epithelial cell damage and carcinogenesis (Correa & Houghton, 2007). The infection is generally acquired during infancy, inducing a life-long chronic gastritis and other pathologies, like PUD and MALT lymphoma. As it has an elevated incidence worldwide, many efforts have been invested for treating this infection.

There are well-defined *in vivo* models to study *H. pylori*-induced gastric pathology. The mongolian gerbil is the standard animal model to evaluate the effects of this infection because they rarely develop gastritis without being infected with *H. pylori* (Bhattamisra et al., 2018; Jeung et al., 2012). There are other animals models, like mice, who need to be colonized with a higher number of CFU than mongolian gerbils. Besides this, when infected, these animals develop histological changes that resemble those observed in humans. Due to these reasons, this animal has been a model to study gastritis (Bhattamisra et al., 2018), peptic ulcers (Werawatganon, 2014) and gastric carcinogenesis (Jeung et al., 2012). However, as there is a wide range of potential pathological outcomes, a single animal can not replicate every stage and/or disease. So, other animal models have been used, like (transgenic) mice, gnotobiotic piglets and rhesus macaques. Due to animal welfare, to the associated costs and to the complexity of *in vivo* models, there was a need to develop *in vitro* and *ex vivo* models. Over the past decade, there has been an effort to expand this field, giving origin to gastric organoids (three-dimensional, primary gastric gland cultures) like *ex vivo* gastric mucosal culture models, such as long-lived epithelial cultures derived from primary gastric tissue or from induced pluripotent stem cells (McCracken et al., 2014; VanDussen et al., 2015). *Ex vivo* models enable a systematic approach, but further studies need to be done in order to not be reductionist. These models should include host epithelial, mesenchymal and immune compartments, as well as a diverse microbiota. By developing models that mimic gastric epithelial pathologies in culture, *ex vivo* and *in vivo* outcomes can be compared, and new therapeutical strategies can be design for infected individuals (Burkitt et al., 2017).

There is a large number of factors involved in *H. pylori*-induced infection's prevention and treatment. On a global level, countries should take measures to help eradicate poverty, by improving the acess to safe drinking water and food, appropriate sanitary conditions and a health system that would garantee the welfare (by providing medical care, affordable diagnostic tests and medication) of all population.

On an individual level, people should be cautious with their lifestyle, since it as a high impact on disease development. Having a balanced diet (rich in vegetables, avoiding processed foods), practicing physical exercise and avoiding risky behaviours (smoking, alcoholism, drugs intake) are conducts that can help avoid the infection (and its progress).

In clinical, there has been a collective effort to find the adequate treatment to fight this infection. A vaccine against this bacterium would be very helpful, but until there is one available, alternative options that function either as a main or an adjuvant (combined with antibiotics) therapy need to be explored.

As *H. pylori* constitutes a risk factor for gastrointestinal pathologies development, guidelines recommend medicating all infected patients (irrespective of the clinical presentation), acting on an individual level and preventing infection's spread between the community (Bennett et al., 1980).

The main interest is to find new therapies to eradicate *H. pylori*, due to the increasing bacterial resistance to antibiotics (resulting in conventional therapy failure) and the adverse effects that result from this approach. Standard eradication regimens also have compliance problems, and their efficiency relies on varied factors, such as age, patient compromise, local antibiotic policies and cultural habits on diet, food conservation and hygiene practises (Calvet et al., 2011). Additionally, there is a high cost associated with this type of treatment and it may be controversial to apply this approach in areas where the infection is endemic. Over the past decades, natural products have received special attention in *H. pylori* infection treatment, either as a main-stream therapy or as an adjuvant, owing to their biological particularities and drug-like properties. Furthermore, they are safe, non-toxic, have a better cost-effectiveness relation when compared to antibiotics and provoke minimal side effects (Gotteland et al., 2008). Natural products (like medicinal plants) that have been traditionally used for healing gastrointestinal ilnesses are a potential target for discovering selective and strong anti-*H. pylori* therapies.

It has been proposed that new therapies should be compared with a current effective treatment (Graham & Fischbach, 2010). Guidelines also suggest that a therapy for *H. pylori* should have an eradication rate above 80% of intention-to-treat-basis (in which the data from all initial subjects is considered in the primary analysis) to be considered sucessful (Malfertheiner et al., 2007), which may be difficult to achieve.

There is a wide range of promising studies about natural compounds – *ex vivo*, *in vitro*, *in vivo* and clinical trials – which involve their identification, characterization and extraction. The antimicrobial potential of these products is confirmed several times; however, further investigations are required in order to develop nutraceutical drugs. It is necessary to determine the pharmacokinetics (assesses the effects that the body has on the drug) and pharmacodynamics (studies the mechanisms of action of drugs) involved, the therapeutic safety window, toxicity and likely synergistic activities between the elements (Shapla et al., 2018).

A high number of bacterial colonies is significantly associated with a worst clinical condition. By reducing *H. pylori* load, natural products can lead to the improvement of the gastric mucosa microenvironment, decreasing the risk of gastric diseases development, even in asymptomatic patients (Morimoto et al., 2007).

Many studies have shown that some natural products like foodstuffs (such as tea, garlic, cranberries, broccoli) and plant extracts inhibit *H. pylori* growth *in vitro*. The anti-*H. pylori* bioactivity of many tested products has been mainly assigned to phenolic compounds, despite not having been fully demonstrated (Castro et al. 2012). However, when tested *in vivo*, most of them reveal less effectiveness, perhaps because the gastric mucosal microenvironment protects the bacterium from these substances (Coelho et al., 2007; Menezes et al., 2006; Wang et al., 2011).

If the active compounds exhibit less antibacterial activity *in vivo* than *in vitro*, the administration of natural extracts or foodstuffs should not represent a threat for human health. Nevertheless, there must be some caution in extrapolating data from *in vitro* to *in vivo* studies. The effectiveness of a product needs to be verified in *in vivo* assays, particularly in clinical trials. When intaking foods or food-based components, it is essential to consider individual personal conditions such as taste, allergies and other medical questions (Castro et al. 2012). By following this experimental course, natural products can get to the point of translational medicine (Takeuchi et al., 2014).

Even so, clinical trials are fraught with difficulties that result in treatment failure. Many endpoints or biomarkers are available, which involve diagnosis tests, like endoscopy and/or gastric biopsy. However, medical, economic, ethical and logistical limitations can influence the flow of the study, by restricting the number of evaluated subjects, for example. The majority of the studies studies focus on symptomatic patients, but studying infected asymptomatic individuals could be more profitable, since this population can be used as a target for preventive strategies (Fahey & Kensler, 2013). Another problem related to clinical assays is the existence of many guidelines for *H. pylori* treatment that differ between countries. Therefore, a suitable, universal guideline for standardize the application of *H. pylori* treatment should be created, based on the evaluation of the *in vivo* effects (Takeuchi et al., 2014).

^{13}C-urea breath test (UBT) results should be cautiously interpreted due to the existence of a “gray zone”, in which they are inconclusive. Executing UBT after treatment reduces its sensitivity, maybe because there are remaining bacteria with inhibited urease activity. In fact, if *H. pylori* is not totally eradicated, a low intragastric bacterial load that

the UBT can not detect may remain in the organism (Gisbert & Pajares, 2004; Sheu et al., 2000).

There is a lack of compliance among non-clinical assays, as well. As stated before, CLSI only stipulates a standard culture-based method for susceptibility testing of *H. pylori*, the agar dilution method. To ensure a greater coherence among the techniques, testing parameters for the remaining methods (broth microdilution, disc diffusion and Etest) should be established. When agar dilution, disc diffusion and Etest are compared, the results are not always consistent (Mégraud et al., 1999; van der Wouden et al., 1999). Both broth or agar dilution methods results are significantly influenced by methodology, which must be meticulously controlled in order to obtain reproducible outcomes (intralaboratory and interlaboratory). Moreover, all these techniques are prolonged and tend to fail in about 10% of cases, mainly due to samples contamination or *H. pylori* growth failure (Gerrits et al., 2006).

More *in vitro* assays can be conducted, using products that have not yet been tested. However, it would be more interesting and useful to understand the mechanisms of action of these elements and their effects in *in vivo* and in clinical trials, as it can differ a lot from the *in vitro* outcomes. More associations between natural products and antibiotics could be studied, since it is possible that they can exert a synergistic effect against the bacteria.

The scientific community has been putting so much effort on the study of natural products and their bioactive constituents as potential targets for the prevention and treatment of *H. pylori* infection, as well as other disorders. This type of therapy (usually diet-based), commonly as an adjuvant medication, can work as a solution for conventional antibiotic therapy failure and/or inaccessibility. Effectiveness and safety of natural products have yet to be defined, so that they may be extrapolated to acceptable medication and be used as a current therapy. In this way, it would be a great contribution to the improvement of public health and to a better life quality.

Chapter V

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