Joana Rita de Sousa Bernardino

Bachelor's Degree in Biotechnology

Haem uptake study in *Helicobacter pylori* clinical strains isolated from children with iron deficiency anaemia

Dissertation for obtaining the Master's Degree in Biochemistry for Health

Supervisor: Jordi Zamarreño Beas, Postdoctoral Researcher at Molecular Mechanisms of Pathogen Resistance Laboratory, Instituto de Tecnologia Química e Biológica António Xavier – NOVA University of Lisbon (ITQB/UNL)

Co-supervisor: Mónica Oleastro, Researcher at Department of Infectious Diseases, Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA)

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Abstract

Anaemia is a disease that results of reduced red blood cells or haemoglobin levels, limiting blood's oxygen transport throughout the body, usually caused by the lack of nutrients. Iron deficiency anaemia (IDA) is a type of anaemia caused by the lack of iron, affecting 1.2 billion world's population, according to a Global Burden of Disease Study (2016). Patients with IDA present paleness, fatigue, dyspnoea, and headache.

Helicobacter pylori is a Gram-negative human pathogen that colonises the gastric mucosa in 50% world's population, being the major cause of peptic ulcer disease. Recent reports indicate that *H. pylori* infection is correlated with haematological disorders like IDA. However, how *H. pylori* triggers iron deficiency in the host is still unknown. One hypothesis is that *H. pylori* infection causes gastrointestinal damage and stomach acidity, which can lead to iron malabsorption. A second hypothesis is that the infection causes iron scavenging through their iron acquisition systems reducing host's iron availability.

H. pylori possess different iron/haem uptake systems; however, these are poorly described and their role in infection is still not well assessed. In this study, we explored the possible correlation between *H. pylori* and IDA through these uptake systems. Overall, we compared gene expression of genes involved in iron/haem acquisition in non-anaemic (NA) and IDA strains and the growth rate and viability of these strains in different conditions: rich, low-iron and haemin supplemented media.

Our results did not show striking differences in target genes' expression between IDA and NA strains. Moreover, growth and viability assays did not reveal a significant phenotype of IDA strains compared to NA strains. Although, our first approach did not bring significant explanations on the relation between *H. pylori* infection and IDA, this work serves as a first exploratory study to understand the importance of iron/haem metabolism in *H. pylori* infection.

Keywords: *Helicobacter pylori*, iron deficiency anaemia, haem uptake, clinical strains, quantitative reverse transcription PCR.

Resumo

A anemia é uma doença resultante da redução dos níveis de glóbulos vermelhos ou hemoglobina, limitando o transporte de oxigénio do sangue pelo corpo, causada pela falta de nutrientes. A anemia ferropénica (AF) é causada pela falta de ferro, afetando 1,2 biliões da população mundial de acordo com um Estudo Global de Carga de Doenças (2016). Pacientes com AF apresentam palidez, fadiga, dispneia e cefaleia.

Helicobacter pylori é um patógeno humano de Gram-negativo colonizador da mucosa gástrica em 50% da população mundial, sendo a principal causa de úlcera péptica. Estudos recentes indicam que a infeção por *H. pylori* está correlacionada com distúrbios hematológicos como a AF. No entanto, não se sabe como se desencadeia a deficiência de ferro no hospedeiro. Uma hipótese é a infeção causar danos gastrointestinais e acidez estomacal levando à má absorção de ferro. Outra, é provocar a eliminação de ferro através de sistemas de aquisição deste, reduzindo a sua disponibilidade no hospedeiro.

H. pylori possui diferentes sistemas de absorção de ferro/heme, estando pouco descritos e o seu papel na infeção não está bem avaliado. Neste estudo, exploramos a possível relação entre *H. pylori* e AF através desses sistemas. Comparámos a expressão de genes envolvidos na aquisição de ferro/heme em estirpes não anémicas (NA) e AF, e a taxa de crescimento e viabilidade dessas estirpes em diferentes condições: meios rico, com baixo teor de ferro e suplementado com hemina.

Os resultados não mostraram diferenças relevantes na expressão dos genes alvo entre as estirpes NA e AF. Os ensaios de crescimento e viabilidade também não revelaram um fenótipo significativo de estirpes AF comparando às NA. Embora esta abordagem não tenha revelado explicações significativas sobre essa relação, este estudo serve como um primeiro trabalho exploratório para entender a importância do metabolismo do ferro/heme na infeção por *H. pylori*.

Palavras-chave: *Helicobacter pylori*, anemia ferropénica, absorção de heme, estirpes clínicas, Transcrição reversa quantitativa seguida de PCR

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Abbreviations

A	Anaemic
BHI	Brain heart infusion
cagA	Cytotoxin-associated gene A
cDNA	Complementary DNA
Ct	Threshold cycle
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal bovine serum
G+C	Total sum of the quantity of guanines with cytosines
gDNA	Genomic DNA
IARC	International Agency for Cancer Research
IDA	Iron deficiency anaemia/anaemic
IgA1	Immunoglobulin A1
IgG	Immunoglobulin G
NA	Non-anaemic
NCBI	National Center for Biotechnology Information
NIH	National Institutes of Health
OMPs	Outer membrane proteins
PCR	Polymerase chain reaction
rRNA	ribosomal Ribonucleic Acid
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SYBR	Synergy Brands Incorporated
TAE	Tris-Acetate-EDTA
TSB	Tryptic Soy Broth
WHO	World Health Organization

Chapter I. Introduction

1. *Helicobacter pylori*

1.1 History

Helicobacter pylori (*H. pylori*) is a Gram-negative spiral bacterium that colonises the human gastric mucosa [1,2,3], early called as *Campylobacter pyloridis* due to a few similarities with the genus *Campylobacter* [3]. It was firstly described in 1982 by the pathologist Robin Warren, and an internal medicine's trainee, Barry Marshall [1,4]. Barry Marshall, to prove that this bacterium colonises and infects the stomach, attempted to do the Koch's postulates for bacteria, which consists in growing an *in vitro* pure culture of the bacteria obtained from infected individuals sampled from their gastric mucosa, to be then introduced in non-infected individuals and followed to observe if they developed the same disease[5]. For this experiment, Marshall himself and another 3 individuals drank a 3-day isolated culture resuspended in alkaline peptone water. Previously biopsy specimens of antrum and duodenal cap of all subjects were observed to make sure that bacteria were not present in their gastrointestinal mucosa. After one week, they showed symptoms as vomiting, headaches, mushy faeces, ill-tempered and bad breath. It was a Gram-stained biopsy observed by microscope which finally confirmed the cause of peptic ulcers that was in fact due to *H. pylori* infection[5]. This was ratified by the successful treatment with antibiotics which fully suppressed all the symptoms and healed all the infected patients[5].

After the discovery that this Gram-negative bacterial infection could be linked to gastritis and stomach or duodenum ulceration, Barry Marshall and Robin Warren won a Nobel Prize in Physiology or Medicine in 2005 [6].

A couple years after the discovery of *H. pylori*, the associated infection was described as causing an inflammation in the gastric mucus leading to chronic superficial gastritis and with polymorphonuclear cell infiltration, known as chronic active gastritis. In 1994, in a conference organized by the National Institutes of Health (NIH), it was determined that despite of causing stomach ulceration, *H. pylori* infection can also lead to duodenal ulceration, and was determined as the major cause of peptic ulcer disease [1,3]. It is known to be related also with Menetrier disease (hypertrophic protein-losing gastritis) in adults, and also, many studies report its association with haematological disorders like iron deficiency anaemia and, iron and vitamin B12 deficiency[7–9]. Osteoporosis and cardiovascular diseases are also another diseases that are associated with *H. pylori* infection [10].

In children, *H. pylori* infection can be manifested by symptoms like chronic diarrhoea and growth retardation [11,12].

H. pylori infection can cause gastrointestinal atrophy which can promote the development of gastric cancers [1,3,11]. For this reason, in 1994, the International Agency for Cancer Research (IARC), a branch of the World Health Organization (WHO) described *H. pylori* infection as a Group I human carcinogenic [3], which is the second leading cause responsible for cancer deaths in the

whole world [1,3]. Among these cancers are, for example, gastric non-Hodgkin's lymphomas, another lymphoproliferative disorder, gastric mucosa-associated lymphoid tissue lymphoma (MALToma) and adenocarcinoma of the antrum (the distal part of the stomach)[1].

1.2 Morphology

These bacteria are 2.5 to 5.0 µm long and 0.5 to 1.0 µm wide, displaying four to six unipolar flagella with 30 µm long and 2.5 nm thick that confer high motility, especially in the gastric mucosa [1,3]. Its cell wall can be coated with glycocalyx up to 40 nm of thickness covered with circular subunits with 12 to 15 nm of diameter [3]. It has a spiral shape that is very important for bacteria motility[13].

1.3 Genome and plasmids

In 2017, there were already almost 700 *H. pylori* whole genome sequences in NCBI, 86 were complete genomes[14]. Its genome size averages 1.63 megabases (mb) (NCBI) within a range from around 1.4 to 2.6 mb[15], and holds a minimum of two copies of each 16S and 23S rRNA genes[1]. Specific regions of its genome are known to help in bacteria colonisation in the host individual being potential important for *H. pylori* infection. *H. pylori* 26695 strain (frequently used in laboratorial experiments) complete sequence was determined in 1997 [15]. And it has around 1.730 protein coding sequences[15].

Guanine plus cytosine (G+C) percentage averages 38.9 % in *H. pylori* strains (NCBI). Each strain has different plasticity zones of G+C and these zones' percentage also differs within the different zones, so they might be transferred horizontally by transduction[16], or conjugation (DNase-resistant conjugation-like mechanism[17]), or natural transformation (type IV secretion system-dependent[18]). Around 40% of *H. pylori* strains isolates carry plasmids with sizes between 1.5 to 23.3 kb[1,3]. These are cryptic plasmids[19] that do not transport virulence factors[1], and there are already described a few that are mobilisable participating in conjugative transfer functions within bacterial strains, like DNA-transfer[19]. These factors explain the major multiplicity in *H. pylori* strains' genome[16].

1.4 Transmission

H. pylori transmission route is by person-to-person transmission and can be vertical, if it occurs between individuals like family members, or horizontal, if it occurs between couples or by environment contamination[20,21]. That being said, it can also be divided by two ways of transmission: oral-oral transmission and faecal-oral transmission[1,3,22]. Faecal-oral transmission is the most important route of transmission, that can occur between individuals, but also by the ingestion of contaminated food, e.g., raw vegetables fertilized with contaminated faeces, or consumption of contaminated water [1,3]. Patients usually remain asymptomatic for many years and as a chronic infection it persists for many years, decades or even the whole life[23].

1.5 Epidemiology of infection

This bacterium is often acquired in childhood (before 10-year-old) based on serological tests on random people. Therefore, *H. pylori* infection affects more frequently children than adults, and the risk of being infected increases throughout life. Consequently rates of infection earlier in life have been decreasing, showing a cohort effect[2,3,24]. This infection is more abundant in developing countries, around 80% of infected population[15], and in developed countries the prevalence of this infection is usually much lower but still very high among low-income communities[3]. Also, studies of periodontal disease in *H. pylori* infected individuals and also, water, food contamination and socioeconomic factors show their correlation with *H. pylori* infection[25]. Although the percentage of incidence of this infection of children from families with socioeconomic difficulties might be decreasing[26].

H. pylori infection affects approximately 50% of people all over the world[15,27,28]. In Portugal, according to a study of 2011, *H. pylori* infection's prevalence observed was 31.6 % in children aged of 0 to 15 years with a crescent increasing between those ages [3].

1.5.1 Detection

Gastric biopsy specimens are usually collected from the antrum and the corpus with standard forceps into saline or transport medium and cultured to be analysed no longer than up to 24 hours. One way to detect *H. pylori* infection is a rapid urease test, since urease breaks down urea into carbon dioxide and ammonia, and ammonia raises the pH, which can be read on a pH indicator. It can also be detected by histological examination, bacteriological tests (Gram-staining, antibiotics susceptibility but the best and precise method for detection is culturing samples in selective and non-selective media with blood under microaerophilic environment at 37 °C. Polymerase chain reaction (PCR) is also used for its detection, and the primers used are the ones corresponding to genes that encode urease, 16S ribosomal RNA, a specific 26-kDa protein and an uncharacterized 1,9-kilobase-pair fragment of chromosomal DNA; or also targeting virulence genes such as *cag*A [3].

1.5.1 Treatment

There are many types of therapies that can be administrated for this infection disease, in order to eradicate it and reach regression of the ulcers or tumours caused by this bacterium, those treatments normally require antibiotics (more than one) and an acid suppressive agent, like proton pump inhibitors (PPI) [1,3,29].

1.5.1.1 Triple and Quadruple therapies

The first-line therapy used to treat *H. pylori* infection is the triple therapy with proton-pump inhibitor (PPI) with amoxicillin and clarithromycin (PPI-AC). Due to resistance of clarithromycin, in some patients it can be used the non-bismuth quadruple concomitant therapy with PPI, amoxicillin, clarithromycin and nitroimidazole[30,31].

Bismuth therapy can help to overcome bacterial resistance, and it is usually applied in patients allergic to penicillin or those that PPI-AC therapy fails. It is normally used as a quadruple therapy combined with PPI, tetracycline and metronidazole[30].

The duration of these therapies is normally 14-day for non-bismuth quadruple concomitant therapy and standard triple plus bismuth, and 10-day for bismuth quadruple therapy[30].

Overall, quadruple therapies should be always preferred as these are the only ones that have better eradication rate due to patients' resistance to clarithromycin[30].

Still, newer therapies alternatives are awaited as, for example, a designed vaccine[31].

1.5.1.2 Treatment resistance

One of the most common reasons of treatments failure in bacterial infections is antibiotic resistance. In the case of *H. pylori,* it presents resistance to several antibiotics used to treat the associated infection, such as, clarithromycin, levofloxacin and metronidazole, especially in patients where these drugs were previously used in treatment of this or other infectious diseases[29,32].

The second main cause of resistance is patient's non adherence to therapy due to eradication treatments complexity, excessive number of pills that the patient take per day, drugs side effects, lack of information of therapy administration, treatment interruption or high cost[32].

Finally, there are many other secondary reasons for treatment failure, as patient's genetics and lifestyle (age, smoker, obesity, and diabetes), microbial mechanisms (gastric environment variations and abundant bacteria) or virulence factors (e.g., *cagA*, *vacA*)[32].

1.6 Virulence and infection

H. pylori infection induces an inflammatory response by secreting chemokines, by a bacterial type IV secretion system (T4SS, that is also found in other bacteria like, *Legionella pneumophila* and *Brucella*)[33], that will trigger inflammatory cells, like neutrophils, in the mucosa, or by local and systemic humoral immune responses, like local and systemic immunoglobulin (Ig) A response (mainly by IgA1 subclass), and systemic IgG response [3,27].

It colonises the gastric mucosa of infected people and in acidic environments. Occasionally, they can also be found in the oesophagus, duodenum, Meckel's diverticulum or in the rectum. Many virulence factors contribute to the colonisation/infection process, helping in avoiding acidic contact and neutralize it, and in moving along the epithelium cells. Those virulence factors are *H. pylori*'s motility, adherence to the gastric epithelium, extracellular matrix proteins and cellular antigens, and the production of enzymes such as urease [3]. Most of these strains are also naturally competent for uptake DNA[1,34], so they are currently suffering transformation and because of this it is easy to manipulate genetic material of these bacteria[34]. *H. pylori* also

possesses flagellin genes and the cytotoxic virulence factors genes, vacuolating cytotoxin A gene (*vac*A) and cytotoxin-associated gene A (*cag*A) [1,3].

H. pylori invades the gastric mucosa and adheres to host cells passing through mucins that are protecting the gastric mucosa, with the help of adhesins[15,28]. Those adhesins are outer membrane proteins (OMPs), like for example, sialic acid-binding adhesin SabA, that binds to sialyl-Lewis x (sLex) antigen, and blood group antigen-binding adhesin BabA, that binds to Lewis b (Leb) antigen[35–37]. Virulence factors and injected into the host cells also with the help of these two adhesins[38] (Figure 1). SabA rises the inflammatory response and helps in host nutrients utilization. BabA rises the inflammation of the mucosa and stimulates sialyl-Lewis x/a expression. Both adhesins expression is regulated by the bacteria according with necessity[38].

H. pylori have two to six flagella that helps in this bacterium motility through the gastric epithelium mucosa[39]. Flagellin genes, *fla*A and *fla*B, are the major subunits belonging to the filament are assembled to nearly the end of each flagellum and are essential for full motility of this pathogen. The hook is composed by *flgE* (structural protein) and *fliD* (hook-associated protein), located at the beginning and the tip of the flagellum, respectively. And there are another proteins belonging to each flagella being a total of approximately 20 proteins[39].

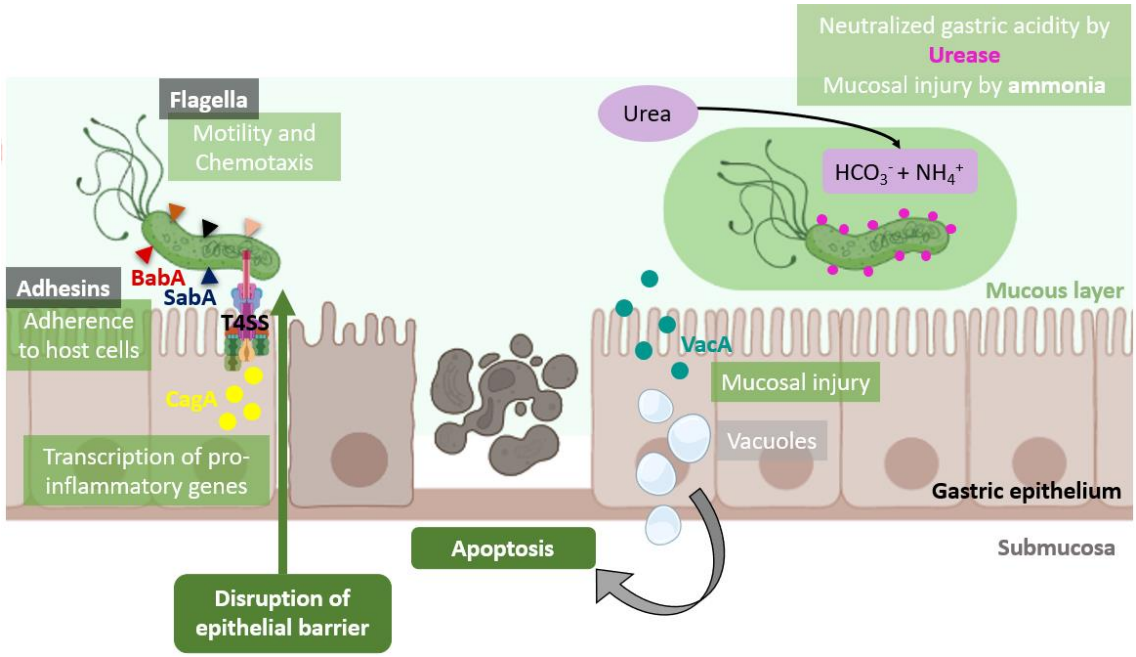

Figure 1 – *Helicobacter pylori* infection pathogenesis with its virulence factors CagA and PAI, VacA and adhesins, BabA and SabA (Based on the paper: Wen and Moss (2009), Cancer Letters[31]).

Helicobacter pylori strains adhere to the gastric mucosa cells by adhesins, BabA, that binds to Le^b, and SabA, that binds to Le^a and sLe^x, helping in the delivery of other virulence factors into the host cells. CagA is injected to host epithelial cells by the *H. pylori* T4SS. Inside the cell CagA can affect signalling pathways by mechanisms dependent or not of phosphorylation process. This may provoke cytoskeletal reorganization, increase proliferation as well as motility; induce inflammation, apoptosis and mitogenic gene expression; and junctional and polarity defects. VacA will induce cytoplasmic vacuolation, mitochondrial injury, the release of cytochrome c and lead to apoptosis.

There is a nickel-containing hexameric molecule, which is an urease gene cluster with seven genes, ureABIEFGH (Figure 2), that encodes urease structural (UreA and UreB) and accessory (UreIEFGH) proteins for urease synthesis with the presence of nickel ions (Ni^{2+})[40]. Urease catalyses hydrolysis of urea transforming it in ammonia and carbon dioxide, neutralising the pH of the acidic stomach environment helping bacterium survival in this environment[15,28].

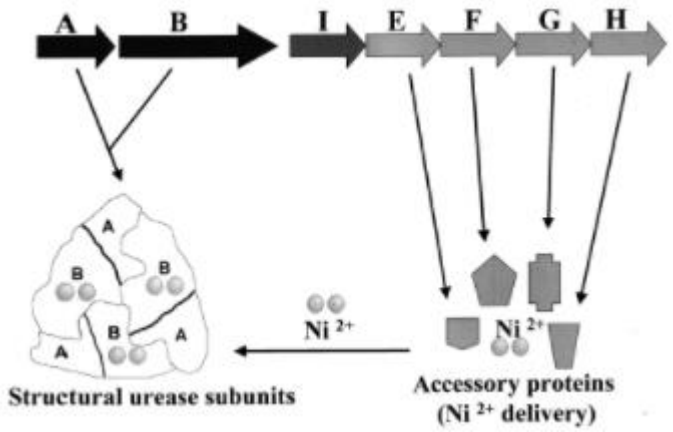

Figure 2 – Urease gene cluster that encodes structural and acessory urease genes for urease synthesis by nickel ions delivery (Mobley (2001) ASM Press[28]).

Gamma-glutamyl transpeptidase (GGT) is also another important virulence factor that helps *H. pylori* colonisation, once that it activate the bacterial defence against the immune response that is initiated by the host[41], generating reactive oxygen species and also converting host's glutamine into glutamate and ammonia and host's glutathione into glutamate and cysteinylglycine[42].

Also, after invasion by this bacterium, the two major virulence factors mentioned above contribute to the development of the disease[15,28].

Virulence factor VacA is produced by all *H. pylori* strains[38]. This toxin is released to the extracellular surface or form aggregates in the surface of bacterium to be injected into host cell. Inside the host, VacA induces cytoplasmic vacuolation, cell apoptosis, autophagy, and T-cell proliferation inhibition (Figure 1)[38,43].

Some *H. pylori* strains have the capability of producing CagA protein[33], a toxin that is injected to the host gastric epithelial cells via a type IV secretory system (T4SS) and changes cytokine signalling and cell cycle control [38](Figure 1). This is a 40 kb DNA region known as Cag-pathogenicity island (PAI)[15,28] and its production boosts inflammation and appearance of ulcers or gastrointestinal carcinoma[35]. CagL is the adhesin that mediate CagA delivery into host cells[28]. Inside the host CagA is phosphorylated inducing cell changes (CagA phosphorylation-dependent cell signalling), then it will act together with intracellular effectors and induces cell elongation. In CagA phosphorylation-independent host cell signaling, CagA being only translocated inducing junctional and polarity defects in the cell, proinflammatory and mitogenic gene expressions[44] (Figure 1). Although most of T4SSs structures are known, Cag T4SS is wider and complex but

remain still not fully clear[33]. A study with a co-culture of these type of strains (with the capacity of expressing *cagA*) with AGS cells demonstrated that these toxins are essential for *H. pylori* survival in stomach [45].

2. *Helicobacter pylori* and iron deficiency anaemia

Anaemia is a result of reduced red blood cells (RBCs) or haemoglobin levels, which have physiological consequences, such as, diminishment the blood capacity to transport oxygen[46–48]. The lack of oxygen transported throughout the body may cause a broad range of symptoms like fatigue, weakness, dizziness[49]. Anaemia can occur due to a nutritional deficiency, in this case, of iron (iron deficiency anaemia - IDA) but there is another lack of nutrients that can provoke anaemia, like iron (iron deficiency anaemia), folate, B12 vitamin or A vitamin. Although, infectious diseases can also be associated with anaemia like malaria, tuberculosis, HIV, parasitic infections and *H. pylori* infection[7,46,50]. In adults, anaemia is diagnosed when haemoglobin levels are lower than 13.0 g/dL (male) and 12.0 g/L (female). In children with 5, 12 and 15 years old, the levels of haemoglobin are lower than 11, 11.5, 12 g/dL, respectively, and in pregnant women haemoglobin levels are below 11.0 g/dL[47].

Anaemia affects one third worldwide and iron deficiency anaemia is the most common among all types of anaemia according to WHO[51,52]. In young children (aged 6 to 59 months) and women of reproductive age (15 to 49 years old) diagnosed with anaemia, it was estimated, in 2019, a prevalence of 39.8% (269 million children) and 29.9% (half a billion women within these ages), respectively[46,52–54].

Iron deficiency can be absolute, if iron levels from total body are at a low or depleted level, or it can be functional if iron levels from total body are normal or increased but it is not well provided to the bone marrow[52].

Iron deficiency anaemia is a clinical condition that affects more than 1.2 billion of the world's population, data of a Global Burden of Disease Study from 2016[55]. It affects mostly women and individuals with other clinical conditions, especially with chronic diseases, and the older age group of people[51]. This disease can be caused by higher iron requirements, mostly in children and women. The most frequent symptoms of an iron deficiency anaemia condition are very similar to all types of anaemia, being the most frequent symptoms, paleness, fatigue, dyspnoea and headache[56].

Blood loss (by e.g. gastrointestinal carcinomas, benign gastric ulceration and angiodysplasia), iron malabsorption (by e.g. bypass gastric surgery, gastrectomy or *H. pylori* infection), chronic diseases (by e.g. chronic heart failure, chronic kidney disease, cancer, rheumatoid arthritis, obesity or inflammatory bowel diseases), and a vast group of rare genetic mutations, are some of the reasons of an iron deficiency anaemia diagnosis[56]. Haem iron (Fe^{2+} - ferrous iron, found in e.g. meat, poultry and seafood) is more easily absorbed than non-haem iron (Fe^{3+} - ferric iron, found e.g. in black tea, cacao, cereals and dried fruit), being the percentage of total absorption of iron in the first one more than 40% [57].

As referred before, *H. pylori* infection is associated with anaemia[7,50], IDA)[58] and iron deficiency because, it is thought that iron levels decrease from IDA in some cases, might be due to the gastrointestinal damage or stomach acidity (leading to malabsorption) caused by *H. pylori*. Another cause of this decrease can also be both the host (human) and *H. pylori* trying to scavenge iron simultaneously leading to a drastically decrease of host iron levels[36,59].

Bacteria normally have siderophores, proteins that can scavenge iron, and uptake iron by specific OMPs with the auxiliary energy-transducing system (TonB-ExbB-ExbD)[60]. Although for *H. pylori* there is still no described siderophore, there are many proteins described to participate in iron biosynthesis[36].

Apart from elemental iron uptake, there are other molecules that bacteria can exploit to obtain iron. This is the case of haemoglobin and haem, two molecules present in the human body that can be uptaken by bacteria and be used as iron source. Indeed, the main source of iron for bacterial pathogens is haem[61].

3. Haem

Haem is an iron containing porphyrin, a protein cofactor that plays an essential role in several biological processes in humans and most living organisms[62]. Examples of those biological processes are oxygen transport and storage, gas sensing, electron transport, catalysis of enzymatic reactions, drug metabolism and cellular respiration[62,63]. Haem can be found in macromolecules, such as, haemoglobin (Hb), around 67% of haem found in Hb, and Hb is present in erythrocytes, haemopexin, cytochromes and myoglobin[61,63].

3.1 Bacterial haem uptake

Bacteria can acquire haem by uptake from the extracellular medium or by endogenously synthesising it[64]. Here, I will describe the haem uptake systems that have been previously described in bacteria.

3.1.1 Gram-positive bacteria

In Gram-positive bacteria, there are surface-exposed receptors, NEAT-containing surface proteins, that bind to extracellular haem and transfer it through peptidoglycan cell wall to then be transported through the inner membrane into the cytoplasm by a dedicated ABC transporter Shuttle protein [64](Figure 3). This type of haem uptake system is described for, for example, *Staphylococcus aureus* (iron-regulated surface determinant system – Isd). Some Gram-positive bacteria possess additional proteins, called haemophore, that have high affinity for haem and are secreted to the extracellular environment to scavenge haem and direct it to the haem receptors[65,66]. This is the case of *Bacillus anthracis*, which encodes for IsdX1 (capable of removing haem from haemoglobin and passing it to other proteins) and IsdX2 (not well defined function), two characterised haemophores[65](Figure 3). Also, transpeptidases linked to the inner membrane, sortases, in Gram-positive strains, may also have a important role in pathogenesis virulence process helping, for example, in haem transport, nutrients uptake, sporulation and cell

attachment[67]. SrtA and SrtB are the two transpeptidases proteins involved in haem uptake in *S. aureus*[68].

In many organisms, haem can be utilised as source of iron. Indeed, in Gram-positive bacteria, haem can be degraded by a haem oxygenase (HO) protein to obtain free iron, like in *S. aureus*, *Bacillus anthracis* and *Listeria monocytogenes*[69].

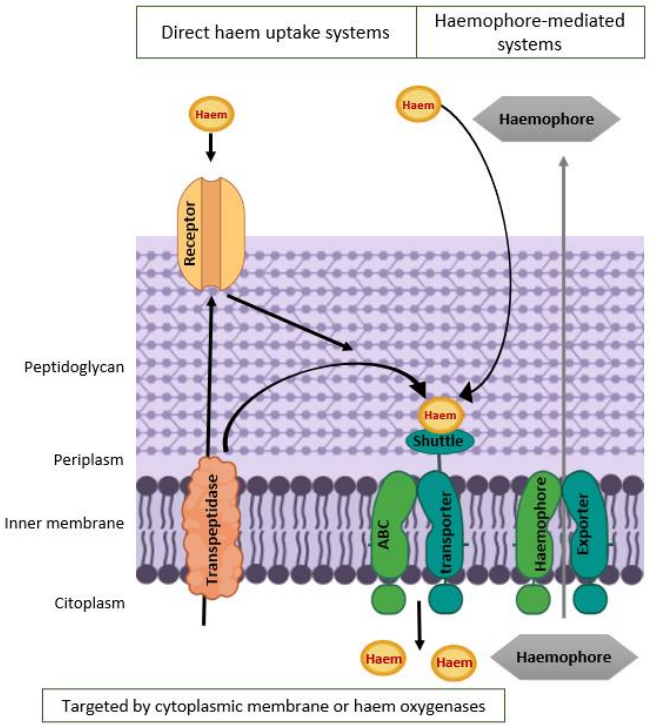

Figure 3 – A schema of the two different haem uptake systems of Gram-positive bacteria (Own authorship – based on the scheme represented in Anzaldi and Skaar, 2010 [49]).

Those two haem uptake systems are the direct haem uptake systems (on the left) and haemophore-mediated systems (on the right).

3.1.2 Gram-negative bacteria

Gram-negative bacteria encode for similar haem uptake systems. According to the different proteins involved in haem uptake, three different types of haem uptake systems have been identified in Gram-negative bacteria: direct haem uptake systems, bipartite haem receptors and haemophore-mediated haem uptake systems[65](Figure 4).

Direct haem uptake systems start with haem recognisation by a TonB-dependent outer membrane transporter (TBDT), and it is transported through the outer membrane and the periplasm in an active process, using the energy from a specific group of inner membrane proteins complex, TonB-ExbB-ExbD complex. After that, haem is then internalised in the cell by the action of an inner membrane ATP-binding cassette (ABC) transporter, and the haem dedicated ABC transporter works as a haem shuttler, sending haem molecules to the cytoplasm. binds to a shuttle protein that shuttles it to the ABC inner membrane transporter that transports it to the cytoplasm[28,71,30]. *Pseudomonas aeruginosa, Campylobacter jejuni* and *Escherichia coli* are some of the Gram-negative bacteria that utilizes this type of haem uptake system[65].

In the case of bipartite haem receptors, the difference from direct haem uptake systems is that it starts with haem being recognised by a TBDT and also another outer membrane protein instead of just one outer membrane protein, as it happens in *Neisseria gonorrhoeae*[65].

Haemophore-mediated haem uptake systems, differs from the other two systems once that starts by haem being uptaken by an haemophore that is secreted by the bacteria by an haemophore receptor[61], like in *Serratia marcescens*[65,72].

As in Gram-positive, in Gram-negative haem can also be degraded by a haem oxygenase (HO) protein to obtain free iron. In *C. jejuni* and *H. pylori* different haem oxygenase enzymes have been identified, ChuZ[73,74] and HugZ[70,75], respectively. The majority of haem uptake systems are regulated by Fur protein (ferric uptake repressor) in Gram-negative bacteria[70], such as in *H. pylori*[76], *C. jejuni* and *P. aeruginosa*[77].

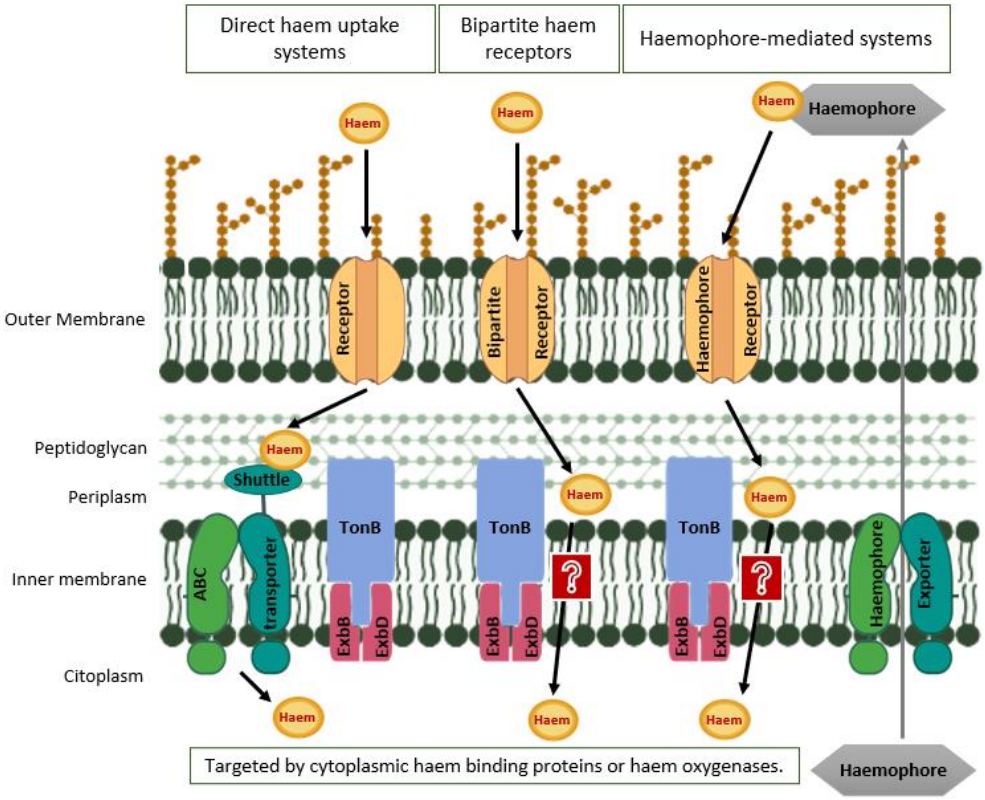

Figure 4 – A schema of the three different haem uptake systems for Gram-negative bacteria (Own authorship – based on the scheme represented in Anzaldi and Skaar, 2010[47]).

Those three systems are the direct haem uptake systems (at the left), bipartite haem receptors (at the middle) and haemophore-mediated systems (at the right). These bacteria need the help of the energy of TonB-ExbBD complex to help haem go through the outer membrane into the periplasm.

3.1.3 Haem uptake in *Helicobacter pylori*

In *H. pylori* there is not a complete haem uptake system described in the literature. However, there are some proteins that participate in haem uptake that have been described and studied[70,78–80].

Starting with GroEL (HP0010), it is a chaperonin described to be a siderophore, and its ability to possibly bind haem and to be a haemophore is still unknown[80]. Interestingly, GroEL, which is known in most bacteria to be a chaperon system to ensure good folding of proteins by blocking certain interactions among polypeptides that could lead to aggregation[81]. In *H. pylori* it has been proposed that these proteins are haemophores secreted outside the cell to facilitate haem uptake[80]. So, it is thought that *H. pylori* haem uptake system is a haemophore-mediated system (Figure 5).

It is also described that this bacterium has three iron-regulated outer membrane proteins (IROMPs), that target haem and/or haemoglobin. These belong to ferric regulated protein family (FrpB), which is a family of IROMPs: FrpB1 (HP0876) is described to bind both haem and haemoglobin iron, and to probably participate in other processes once that it is possible to observe an expression in an iron-repressed medium[78], and also Kato S. paper from 2017 showed that FrpB1 is more expressed in non-anaemic than iron deficiency anaemic clinical strains[36]; FrpB2 (HP0915) can acquire iron but only from binding to haemoglobin and it is not expressed without iron resource[82]; and FrpB3 (HP1512) that is described as capable of binding to haem[79].

Also, a different type of haem oxygenase was identified in *H. pylori*. This haem oxygenase was denominated as HugZ (HP0318 in *H. pylori* strain ATCC 26695). In *H. pylori*, haem oxygenase HugZ degrades haem to biliverdin and carbon monoxide (CO) releasing free iron, so this can be utilized by bacteria. HugZ is also induced by iron starvation[70,75].

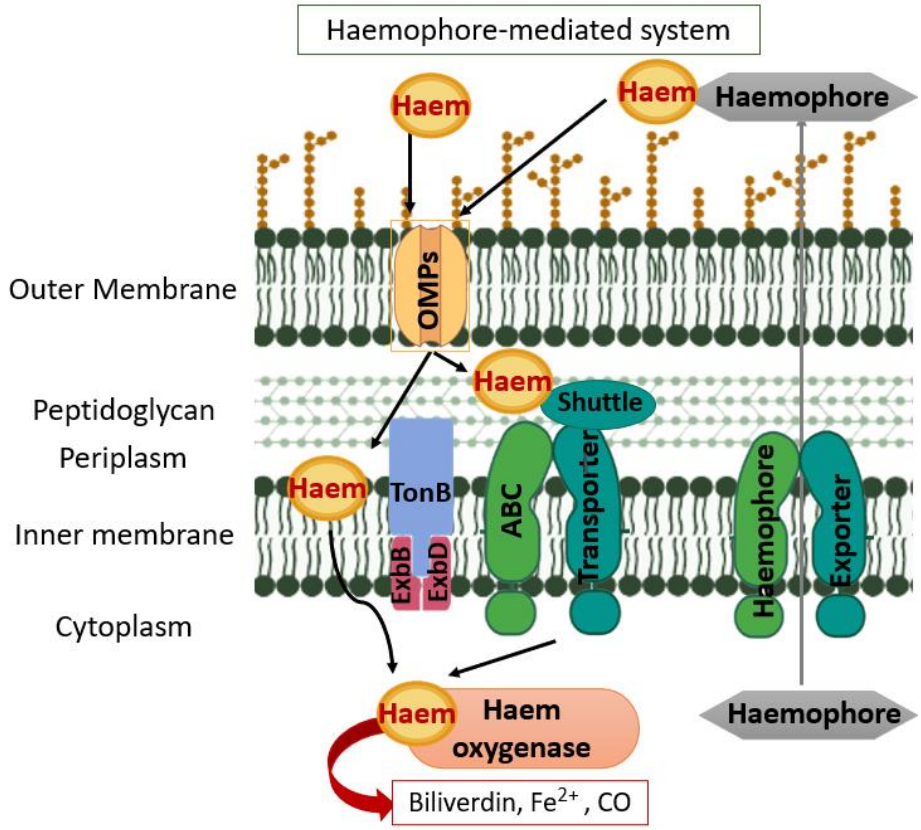

Figure 5 – *Helicobacter pylori* putative haem uptake systems, with FrpB2 IROMP exclusion for haem uptake and a possible haemophore (GroEL), also with another known bindings to IROMPs (Own authorship).

4. Goal

With this study, we wanted to observe if haem uptake is related with the presence of the clinical condition of iron deficiency anaemia.

For this, we designed a scientifical approach to evaluate whether haem uptake and iron deficiency anaemia might be related.

The points are: 1 – Evaluate the gene expression levels of haem uptake involved genes in iron deficiency anaemic and non-anaemic *H. pylori* strains; 2 – Determine the ability of anaemic vs. non-anaemic to grow in rich medium with or without supplementation of haem and with iron starvation.

This is an exploratory project to give insights for future experiments and studies targeting haem uptake in *H. pylori*.

Chapter II. Materials and methods

1. Strains selection and growth conditions

H. pylori 26695 reference strain and anaemic, iron deficiency anaemic and non-anaemic *H. pylori* clinical strains were selected from the bacterial collection of the Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA). Clinical strains selection was done anonymously and, from a total of around 160 strains paediatric patients that tested positive for *H. pylori* infection from 2017 to 2020, a pre-selection of 33 strains isolated from patients with a clinical diagnosis of anaemia was made. From these, a total of 6 strains were selected based on the absence of other risk factors for the anaemia/iron deficiency anaemia, besides *H. pylori* infection. Also, 4 of *H. pylori* strains from infected paediatric patients without anaemia were selected randomly to work as a control for this study (Table 1). The paediatric group was chosen since, contrary to adults, the development of disease in children occurs over a shorter period, suggesting a stronger link between infection and disease.

Table 1 – Clinical information of the clinical strains isolated from the non-anaemic and iron-deficiency anaemic patients.

Strain lab number	Clinical conditions	Patient's age	Year of strain isolation
9971	Iron deficiency anaemia	15	2017
11456	Iron deficiency anaemia	10	2019
11546	Iron deficiency anaemia	8	2019
11686	Iron deficiency anaemia refractory to *H. pylori* treatment	14	2020
11695	Anaemia	16	2020
11801	Iron deficiency anaemia, Coeliac disease	15	2020
12037	Non-anaemic	12	2020
12038	Non-anaemic	14	2020
12208	Non-anaemic	15	2021
12211	Non-anaemic	12	2021

Ethical approval for the study was obtained from the Health Ethics Commission from INSA.

H. pylori strains, previously stored in cryogenic tubes (AHN Biotechnologie GmbH) with tryptone soya broth (TSB) and 20% (vol/vol) glycerol at -80 °C, were grown in Wilkins Chalgren Anaerobe agar (Biogerm®) and Brain Heart infusion broth with 10% of Fetal Bovine Serum (FBS, Gibco®) when grown in solid and liquid media, respectively. Liquid cultures and plates were grown inside a jar where the atmosphere was previously exchanged to microaerophilic conditions (6% O_2, 7% CO_2, 3.5% H_2, and 83.5% N_2) using Axonomat® III.

1.1 Strains culture

The stocks at -80ºC were streaked out in Wilkins Chalgren Anaerobe agar plates and were incubated at 37 °C for 2 to 3 days. Each strain was subsequently restreaked into fresh plates and incubated around 24 hours in the same conditions. Pre-inoculums were generated from this plate by inoculating a portion of the plate using a swab, in a 50 mL falcon with 10 mL of Brain Heart Infusion (BHI) (Oxoid) broth supplemented with 10% of Fetal Bovine Serum (FBS) (Gibco®) and with 20 µL of antibiotics and antifungals mix (Ab/Af mix, Table 2). Then, strains were incubated overnight at 37 °C, with shaking at 150 rotations per minute (rpm) with the falcon cap unscrewed (Figure 6).

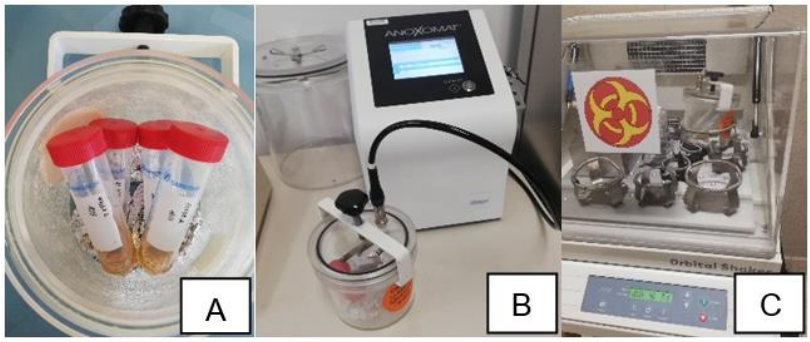

Figure 6 – Pre-inoculums in a Axonomat jar (A), inserting microaerophilic conditions into the jar (B) and the jar inside the orbital shaker incubating (C) (Own authorship).

Table 2 - Antibiotics and antifungal mix components and respective concentrations.

Name	Type	Concentration	Brand
Polymyxin B sulphate	Antibiotic	0.3 mg/L	Carl Roth GmbH + Co. KG
Trimethoprim	Antibiotic	6 mg/L	Sigma-Aldrich – Merck KGaA
Amphotericin B	Antifungal	5 mg/L	Carl Roth GmbH + Co. KG
Vancomycin hydrochloride	Antibiotic	12.5 mg/L	Carl Roth GmbH + Co. KG

On the next day, OD_{600} was measured, in an Ultraviolet-Visible spectrophotometer (UV-1603, Shimadzu). All pre-inoculums were diluted to an OD_{600} ~0.1 in fresh BHI broth with 10% of FBS in 10 mL volume in a 50 mL unscrewed falcon. Cultures were incubated at 37 °C with 150 rpm until achieving an OD_{600} 0.4-0.7 (~7-9 hours of growth). After this, 6 to 8 mL of culture ($\sim 3.2 \times 10^9$ bacterial cells/mL) were collected, and cells were pelleted by centrifuging at ~7000 G for 5 minutes. Cell pellet was resuspended in 400 µL of BHI broth, and then 600 µL of RNAlater™ were added to stabilise and protect RNA with immediate RNase inactivation. RNAlater resuspended pellets were incubated at room temperature (RT) for 1 hour, and then stored at 4 °C for a maximum of one month or snap-frozen in liquid nitrogen and stored at -80 °C until performing RNA extraction.

1.2 Supplemented growth

Reviving of the plates and pre-inoculums were done the exact same way as a normal growth explained in the previous topic. After measuring the OD_{600} on the next day, pre-inoculums were diluted to OD_{600} ~0.1 in fresh BHI broth with 10% of FBS in 11 mL total volume in a 50 mL Erlenmeyer flask, then 1 mL was used to measure the OD_{600} before adding the supplement, in this case 10, 20 µM of haemin and 250 µM of 2,2'-dipyridyl. Cultures were prepared always in triplicates and were incubated at 37 °C with 150 rpm for 3, 6 and/or 8 hours. After this, some of the cultures of haemin supplemented media were collected (6 to 8 mL), and cells were pelleted the same way as previous ones. Other cultures were used for a viability assay during its growth, which is explained in the last topic of this chapter (Chapter II.5.1.1); for this assay the initial total volume was 11 mL and 100 µL (1 mL to measure the OD_{600} and 100 µL to add it to a 96-well plate, both before adding the supplement).

2. Genomic DNA extraction

Genomic DNA was needed to test the primers and it was extracted from strain 26695. An overnight (~16h) culture of 1 mL of an overnight (grown in 10 mL BHI with 10% of FBS and 20 µL of Ab/Af mix) was pelleted and gDNA extraction was performed by following NZY Tissue gDNA Isolation Kit protocol (NZYTech), according to the manufacturer's instructions. Then gDNA was stored at 4 or -20 °C.

3. RNA extraction

RNA extraction was performed according to Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories) Spin Protocol to obtain a high-quality DNA-free total RNA from the quantity of $\sim 3.2 \times 10^9$ bacterial cells/mL already collected in INSA. At the end, RNA sample was obtained, and it can be used right away or stored at -20 or -80 °C.

The amount of total RNA contained in samples was measured in Nanodrop by microvolume spectrophotometer (Nanodrop™ 2000c Spectrophotometer, Thermo Scientific™) to proceed to the cleaning protocol.

DNA contaminations were removed from the RNA extracted performing a protocol using TURBO DNase™ enzyme and TURBO DNA-*free*™ Kit (Invitrogen, Thermo Fisher). A water bath at 37 °C or a thermocycler was also necessary for the incubations. The amount of purified RNA was measured in Nanodrop as before.

A conventional PCR assay was performed to verify that DNA was removed from RNA samples. Housekeeping gene primers for the strain 26695, were used in all PCR assays. For PCR assay, Taq DNA Polymerase with ThermoPol® Buffer protocol (New England Biolabs, Inc.) was followed adding 50 ng/µL of gDNA (positive control), water (negative control) and RNA samples, to the respective tubes. PCR reactions' conditions for thermal cycler (T100™ Thermal Cycler, Bio-Rad Laboratories) were, an initial denaturation at 95 °C for 30 seconds, followed by 30 cycles with 95 °C for 30 seconds, 55 °C for 30 seconds and 68 °C for 25 seconds (1 min per

kb) with a final extension at 68 °C for 5 minutes, and ending with 4 °C infinite hold, until taking out samples and placing them on ice. If no amplification was observed, the sample was DNA-free.

Electrophoresis assays were performed to verify the integrity of RNA samples and to check the presence or not of DNA contamination in RNA samples. RNA samples and PCR products were observed in a 1% and 2% agarose gel respectively, containing TAE running buffer and stained with SYBR™ Safe gel stain (Invitrogen, Thermo Fisher Scientific). Electrophoresis for RNA samples were performed at 80 V for 30 min and for PCR products at 100 V for 30 minutes. The gel was then observed with UV illumination.

4. PCR primers design

For a Reverse transcription quantitative PCR assay (qRT-PCR), PCR primers are needed so, the first thing was to design the target genes' primers. Target genes were chosen using some databases resources such as, Uniprot (Universal Protein Resource), KEGG (Kyoto Encyclopaedia of Genes and Genomes), and Microbes Online. PCR forward (Fw) and reverse (Rv) primers were then designed using both Primer BLAST (Basic Local Alignment Search Tool) from NCBI website (U. S. National Library of Medicine – National Centre for Biotechnology Information), and Editseq and Primer Select programs (DNASTAR, Inc.). The parameters used for primers' designing is described in Table 3. All primer pairs and the housekeeping gene 16S rRNA chosen, represented in Table 4, were tested following the Taq DNA Polymerase with ThermoPol® Buffer protocol (New England Biolabs, Inc.) with 50 ng/µL of genomic (gDNA) *H. pylori* strain 26695, using the same Thermal Cycler and same conditions mentioned before in the previous topic (RNA extraction). PCR products were analysed in an 2% agarose gel electrophoresis, to confirm if they correspond to the expected amplicon length.

Table 3 – Primer designing parameters.

Primer length	18-24 bps			
PCR product size	**Min** 100		**Max** 170	
Tm (primer melting temperatures in °C)	**Min** 59	**Opt** 60	**Max** 61	**Max Tm difference** 1
Database	Refseq RNA (refseq_rna)			
Organism	*Helicobacter pylori* 26695 (taxid:85962)			

Table 4 - Primer pairs' sequences of chosen target genes for haem uptake in *Helicobacter pylori* 26695 strain.

Gene	Sequence	Length (nts)	Tm (° C)	Amplification product (nts)
16S rRNA				
Fw primer	CGACCTGCTGGAACATTACTGACG	24	59.6	111
Rv primer	GCCCTCCAACAACTAGCATCCATC	24	59.7	
frpB1				
Fw primer	AAAGAGGGAAAGCGAACGGGAATA	24	60.1	149
Rv primer	AAAGCCTTGAGTGTAGCGGTGGTT	24	59.2	
frpB2				
Fw primer	GCCTGGAGGTTTGGTGTGGATG	22	60.0	129
Rv primer	CCGGCGGCTCTAAAATCAAAATAC	24	59.0	
frpB3				
Fw primer	CAGCCCTCTCTAAAGCCAATGACC	24	59.2	129
Rv primer	GGCCGCCTTCATGCTTGTATTT	22	59.0	
groEL				
Fw primer	AAAGCCGGAAGGATTGTGATTGAC	24	59.3	129
Rv primer	GTCATAATCGCTTGTCGTGCTTGC	24	59.7	
hugZ				
Fw primer	GAGCGAAGTGGCTGAGCATTTTG	23	59.9	169
Rv primer	AAACGCTTTGTCAAACTCCGCC	22	59.2	
fecD				
Fw primer	GGCGATGGCGATAGCGGTAGT	21	59.9	136
Rv primer	CACCCCTGAAAGCACCAACGA	21	59.4	
COG-Dlte				
Fw primer	GAAGACACGCCCATTGAAGAGGTT	24	59.4	147
Rv primer	GCTCACACGCCCTGCTATGGA	21	59.7	
tonB				
Fw primer	GCTGAGTCGGCTAAACCCAAAGAA	24	59.7	143
Rv primer	TCAGGCTTAGGTTTTGGTTTTGGC	24	59.5	
modA				
Fw primer	GGCGTGTTGGTTTTATGGAGTGAA	24	58.9	120
Rv primer	GCTGGCTTTTCCATAAGGGGCTA	23	59.5	
omp11				
Fw primer	ATGGTTTGGGGCTAGGGTGTATGG	24	60.8	114
Rv primer	AGATTGACAATCAAATCGCCACCG	24	60.8	

5. Reverse transcription quantitative PCR

Reverse Transcription quantitative Polymerase Chain Reaction (qRT-PCR) was performed to study the genes' expression. For this experiment, RNA samples were reverse transcribe into cDNA (complementary DNA) and for this synthesis it was used the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics). It was followed the protocol of Standard Procedure for Qualitative qRT-PCR: Reverse transcription was performed, adding to a microcentrifuge tube, 1 ng of total RNA in a total volume of 20 µL reaction, using both anchored-oligo (dT)18 priming and random hexamer primers and incubating for 10 min. at 65 °C, to denature RNA secondary structures. Then, to this template-primer mix it was added a master mix that contains a reverse transcriptase enzyme as well as a specific buffer for this enzyme and other components necessary to transcribe the RNA. Lastly, cDNA was transcribed after incubations of 30 min. at 50 °C, followed by 5 min. 85 °C, and ending with infinite hold of 4 °C. It was needed a conventional thermocycler to perform the incubations.

qRT-PCR assays were performed using the LightCycler® 480 SYBR Green I Master kit, in a 96-well plate using a LightCycler® 96 System (Roche Diagnostics). Each well contained 2 µL of a 1:2 dilution of the synthesised cDNA and 13 µL of the PCR mix provided by the kit, having a total volume reaction of 15 µL. All qRT-PCR samples were amplified following the same cycle steps described in Table 5.

Table 5 – qRT-PCR cycle steps conditions.

Step		Target (°C)	Hold (min/sec)	Rate (°C/s)
Pre-incubation		95	5'	4.4
Amplification (45 cycles)		95	10"	4.4
	Annealing	55 (primer dependent)	10"	2.2
	Extension	72 (mode: single)	10" (amplicon length/25)	4.4
Melting Curve		95	5"	4.4
		65	1'	2.2
		97 (mode: continuous)	5 acquisitions/°C	0.1
Cooling		40	10'	2.0

5.1 qRT-PCR data analysis

qRT-PCR fold change was calculated based on the comparative C_T method ($2^{-\Delta\Delta C_T}$) [83,84] and based on that method, calculations were made following the equation below:

$$Fold\ change = 2^{-\Delta\Delta C_T}$$

$$\Delta C_T = C_{T_{NA\ 12038\ strain\ of\ a\ certain\ gene}} - C_{T_{Other\ clinical\ strain\ of\ the\ same\ gene}}$$

Gene higher and lower expressions were observed designing graphics with fold changes values in a log10 scale, by using GraphPad Prism® 5 software.

5.1.1 Statistics analysis to qRT-PCR data

Statistical analysis was determined using Wilcoxon-Mann-Whitney test, using IBM® SPSS® Statistics software. This test was performed to evaluate if there is a difference between the mean expression[85] of two independent groups (in this case: non-anaemic 12038 strain versus the other non-anaemic and iron-deficiency anaemic strains) in a dependent variable (in this case: each target gene).

Statistical analysis was performed using 2^{-Ct} values, once that it is a linear form of Ct values and Ct raw values should never be used on statistical analysis because they do not correctly represent the variation[84]. In qRT-PCRs for only normal growth of strains, statistical data was calculated between 2^{-Ct} data of non-anaemic (NA) 12038 strain (N=3) against 2^{-Ct} data each other strains (N=3 per each strain, N_{total}=6). In qRT-PCRs for normal and haemin supplemented growth of NA 12038 strain and IDA 11801 strain, statistical data was calculated between 2^{-Ct} values of the NA 12038 strain growth in normal media against all the other strains' conditions (IDA 11801 strain growth in normal media and, IDA 11801 strain and NA 12038 strain growth in haemin supplemented media).

It is important to observe if the difference of mean ranks in each gene is significantly different between the two strains that are being compared. From that, we can conclude if there is a significant difference in each gene expression between the two strains that will be compared. For this, we must look to the p-values (Asymptotic Significance (2-tailed)), if p-value<0.05, we can conclude that the data is significant and correlate. On the contrary, if the p-value>0.05 we can conclude that there is no significance and relation in these data. This last, will be our null hypothesis.

6. Viability growth assay

The preparation of the strains' cultures was made as explained in the topic Supplemented growth of this Chapter. Before adding 20 µM of haemin to the cultures and put them to grow, 100 µL of each pure culture's flask was added to half of the row A of a 96-well plate (column 1-3 control flasks, column 4-6 haemin supplemented flasks), leaving the other 6 wells of the rest of that row for the other 100 µL of pure culture after the growth of 8 hours. To the remaining 84 wells were added 180 µL of sterile PBS 1X, like exemplified in Figure 7 – Empty 96-well plate with a scheme to what to add in half of the plate at 0 hours of growth (A) and half of the plate ready to perform the viability assay (B) (Own authorship).A, to dilute the pure culture successively 7 times (dilutions 10^{-1} to 10^{-7}).

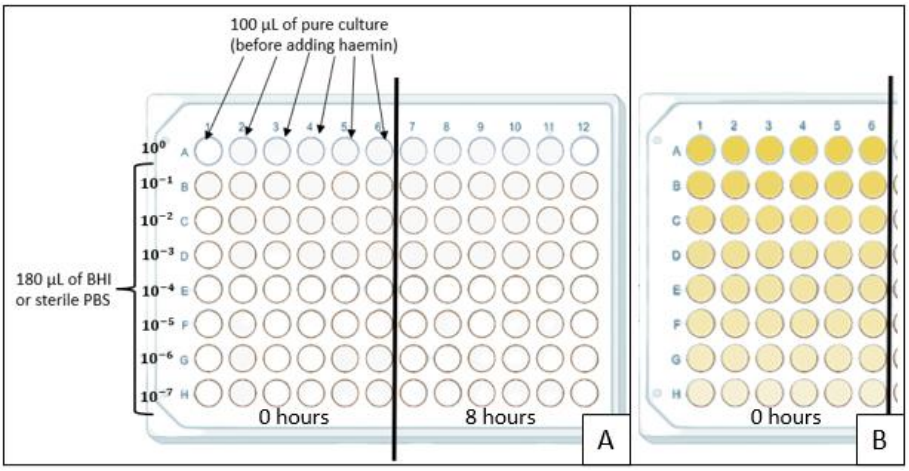

Figure 7 – Empty 96-well plate with a scheme to what to add in half of the plate at 0 hours of growth (A) and half of the plate ready to perform the viability assay (B) (Own authorship).

Flasks' cultures were left to grow in same conditions as explained in the topic Supplemented growth as well.

After having the 96-well plate with the dilutions, with a multichannel pipette, 20 µL of each well of pure culture (row A, column 1-6) were pipetted to the row below (row B, column 1-6), doing up and down mix in each well before pipetting. This procedure was successively repeated throughout the rest of the rows, until having all half of the plate loaded, as exemplified in Figure 7 – Empty 96-well plate with a scheme to what to add in half of the plate at 0 hours of growth (A) and half of the plate ready to perform the viability assay (B) (Own authorship).B.

After this, 5 µL of each column's well (A1 to H1) was pipetted to Wilkins Chalgren Anaerobe agar (Biogerm®) plates until having all A1-6 to H1-6 wells pipetted into plates, as exemplified in Figure 8.

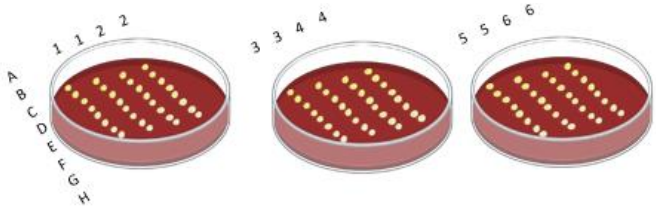

Figure 8 – Wilkins Chalgren Anaerobe agar plates exemplification of viability assay, for counting colonies (Own authorship).

Then, all plates were incubated in an Axonomat jar with microaerophilic conditions at 37 °C, until colonies are visible enough for counting by eye vision.

After 8 hours of incubation of the Erlenmeyer flasks of cultures with haemin supplementation and respective flasks of controls, the procedure mentioned before was repeated in the other half of the 96-well plate.

All the procedure was repeated for DIP supplementation and respective controls. After all plates having countable colonies, they were counted. The numbers of all counted cells per each

dilution, 10^{0} to 10^{-7} (if countable), were then inserted in a table in Excel (Microsoft Office) and CFU's/mL were calculated from 10^{-4} dilution, since it was the one first dilution higher countable dilution.

After viability assays, all cells were also collected, RNA was extracted, and for those strains with haemin supplemented growth RNA extracted samples were transformed in cDNA to further perform a RT--qPCR to observe the influence of 20 μM haemin addition in the gene's expression. .

Chapter III. Results and discussion

In this work, we want to evaluate the expression levels of different genes involved in haem homeostasis in order to reveal a possible correlation with iron deficiency anaemia. I will describe the process, by which we selected the target genes and analyse their expression. The results obtained by qRT-PCR and the growth/viability assays were performed in presence and absence of haemin and 2,2'-dipyridyl.

1. Describing the target genes

H. pylori possess haem uptake systems (see Chapter I.3.1.3), however, these systems have not been completely described and also, we cannot exclude the possibility of the existence of alternative haem uptake systems that have not been discovered due to the low number of studies in this topic. Bearing that in mind, we searched within *H. pylori* strain 26695 genome for homologs of proteins involved in haem, iron and haemoglobin uptake in other bacteria. The results obtained are listed in Table 6.

Table 6 – Proteins involved in haem uptake in other bacterial pathogens, and its homology with *Helicobacter pylori* 26695 strain proteins

Protein name and function	Bacterial species	Identity	Cover	E-value	*Helicobacter pylori* 26695 homologous protein (respective KEGG ID number)
IsdF – Heme transporter	*Staphylococcus aureus*	30%	84%	7e-29	FecD (HP0889) Iron (III) dicitrate ABC transporter, permease protein
HmuU – Haemin transport system permease protein	*Corynebacterium diphtheriae*	48%	65%	4e-11	
PhuU – ABC transporter protein	*Pseudomonas aeruginosa*	36%	92%	1e-42	
PhuV – ABC transporter protein		32%	76%	9e-22	ModD (HP0475) sulfate/molybdate ABC transporter ATP-binding protein
HmuV – Haemin import ATP-binding protein	*Corynebacterium diphtheriae*	31%	82%	1e-24	FecE (HP0888) iron (III) dicitrate transport ATP-binding protein
FrpB3 – Outer membrane protein	*Helicobacter pylori* J99	97%	100%	0.0	FrpB iron-regulated outer membrane protein (HP1512)

FrpB1 – Outer membrane protein	*Helicobacter pylori* J99	98%	100%	0.0	FrpB iron-regulated outer membrane protein (HP0876)
FrpB2 – Outer membrane protein	*Helicobacter pylori* J99	96.81%	69%	0.0	FrpB iron-regulated outer membrane protein (HP0915)

Other proteins involved in haem and iron uptake systems were also used as templates for homology search in *H. pylori* 26695 but did not exhibit any similarities with *H. pylori* enzymes (see Appendix A).

Our first approach revealed that the genes, *fecD*, *modD* and *fecE* from *H. pylori* 26695 might be involved in haem uptake. Some of these genes were found in operon with other genes (see Appendix B) which we thought could be interesting targets.

Together with haem uptake components already identified in *H. pylori* (*frpB1, frpB2, frpB3, groEL*, *hugZ* and *tonB*), we obtained our final list of target genes to analyse their expression levels (Table 7).

Table 7 – Final target genes chosen with a putative role in haem uptake in *Helicobacter pylori*.

Gene	KEGG ID	Expected/known function	Length (bps/nts)
16S rRNA	HPr04	Housekeeping gene	1499
frpB1	HP0876	OM receptor	2376
frpB2	HP0915	OM receptor	750
frpB3	HP1512	OM receptor	2634
groEL	HP0010	Haemophore	1641
hugZ	HP0318	Haem oxygenase	756
fecD	HP0889	IM transporter	981
COG-Dlte	HP0890	IM transporter	771
tonB	HP1341	IM transporter	858
modA	HP0473	IM transporter	741
omp11	HP0472	IM transporter	561

OM: Outer membrane; IM: Inner membrane

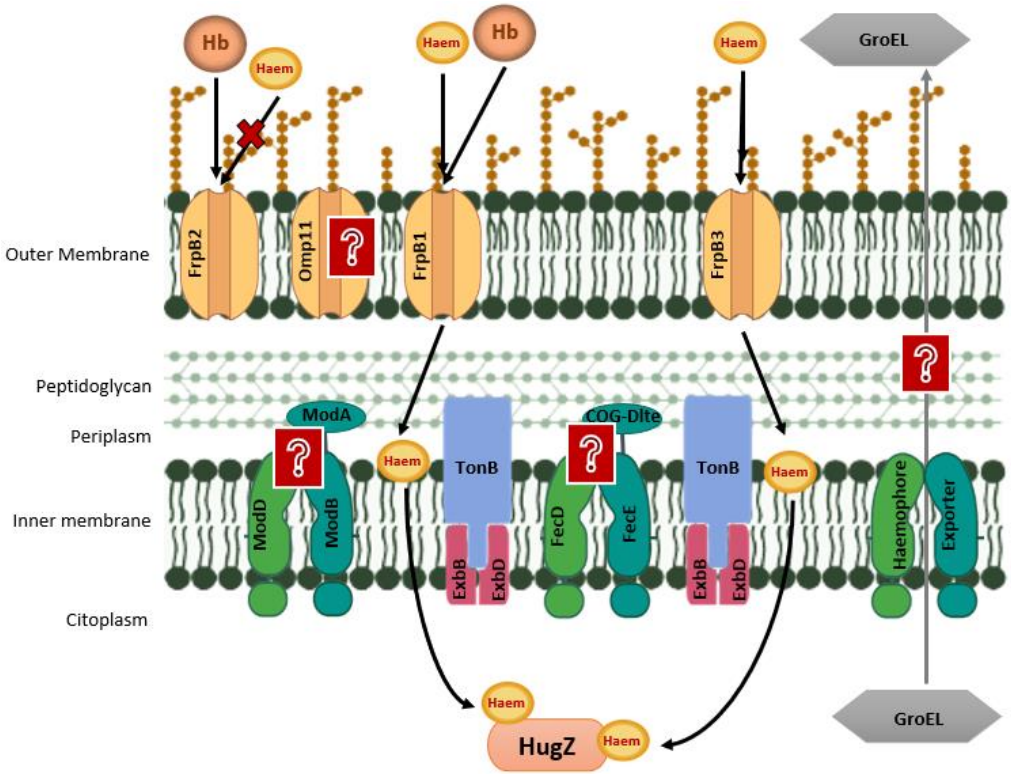

Figure 9 – Haem uptake in *Helicobacter pylori* strain 26695.

Haemophores are proteins secreted to scavenge extracellular haem, they can be recognised by outer membrane receptors to actively uptake haem. Haem can also be recognised by TonB-ExBD-dependent outer membrane receptor protein FrpB1 or FrpB3) or a secreted haemophore and transported to the periplasm. Haem is then internalised by the action of dedicated ABC transporters located in the inner membrane. Finally cytoplasmic haem can be degraded by the action of haem oxygenase HugZ to obtain free iron.
In this figure we represent the putative components of *H. pylori* haem uptake systems. Question marks indicate that there is no experimental evidence, and our assumption is based on protein homology with other haem uptake systems.
Represented in the schema there is also an outer membrane protein described to acquire haemoglobin (Hb) and to not acquire haem (represented with an X) - FrpB2, and other putative haem uptake proteins with a question mark (?): Omp11 which is an outer membrane protein, ModA that belongs to ModADB ABC transporter, COG-Dlte and FecD that belongs to FecDE-COG-Dlte ABC transporter, and chaperonin GroEL that might have the function of haemophore.

2. Gene expression analysis

2.1 Gene expression analysis in rich medium

qRT-PCR gene expression analysis was performed in RNA extracted from cultures grown in liquid BHI media supplemented with 10% FBS under microaerobic conditions until an OD_{600} of 0.4-0.7.

The results obtained are represented as fold change using a non-anaemic strain as reference (Strain 12038), as explained in Chapter II.5.1. To facilitate comparisons, we chose a non-anaemic strain to analyse fold change expression, thus, if we had a tendency between non-anaemic strains, we should observe a fold change value near 1, whereas, if we see a difference with anaemic strains, their fold change values should be higher or lower than 1.

Expression levels of the 4 outer membrane receptors are shown in Figure 10. Fold change values of *frpB1* in NA strains ranged from 11.10 to 94.02 (11.10, 60.50, 94.02). In relation to the NA reference strain, this high fold change values show that 12038 strain has lower basal expression of *frpB1* gene compared to the other NA strains. IDA strains showed more heterogeneity. These results could be dispatched within two different groups, strains 11456, 11546 and 11801 exhibiting a fold change~1 (2, 2.1, 2.3 respectively) or strains 11686 exhibiting a fold change>1 (7.3). Except for strain 9971 that exhibited a very high fold change (72.5) according to the IDA strains. Also, the A 11695 strain showed very low fold change values comparing to all strains. These results indicate that *frpB1* is higher expressed in most of NA strains we have tested, compared to the other strains. However, this is not the case of the reference NA strain (12038) and IDA 9971 strain which showed a very low and high fold-change, respectively. These results are in agreement with previous observations by Kato, S. and colleagues where they observed a trend of *frpB1* gene expression, where NA strains have higher expression of this gene than IDA strains[36].

frpB2 gene expression in NA strains ranged from fold changes of 0.36 to 1.31 (0.36, 0.44, 1.31) and in IDA strains from 0.18 to 2.99 (0.18, 0.69, 0.73, 1.39, 1.74,2.99). This means that in relation to the NA reference strain, this gene has a similar gene expression in all the strains, not existing a tendency in neither NA nor in A/IDA strains.

Fold change values for *frpB3* gene in NA strains were 1.8 and 8 for strains 12037 and 12208, respectively and 0.2 for 12211, having this last strain a much lower expression compared to all the other NA strains, including the reference strain, which means that in general NA strains have a higher expression of *frpB3* gene. IDA strains fold changes can be divided into a higher and a lower expression groups. The IDA strains with fold change values ~3 were 11456, 11546 and 11686 (1.4, 3.1, 3.2, respectively) and IDA strains with fold change values <1 were 11801 and 9971 (0.1 and 0.5, respectively). Both NA and IDA strains have different gene expression comparing each one of them, therefore we conclude that *frpB3* has a different gene expression in each strain. Anaemic 11695 strain Ct values were too high to be considered for *frpB3* gene expression analysis.

In *omp11*, fold change values in NA strains ranged from 0.86 to 3.68, being more expressed in 12208 and 12211 strains (2.4 and 3.7, respectively) compared to the NA strain of reference and less expressed in NA strain 12037 (0.9), so 2 strains had higher expressions and other 2 lower expressions which showed that NA strains did not have the same gene expression levels. In IDA strains, the fold change values ranged from 0.4 to 20.1 (1.4 for 11456, 0.4 for 11546, 3.2 for 11686, 20.1 for 11801 and 9 for 9971), not existing a tendency among these values. Anaemic 11695 strain's fold change was ~4. All strains have as well different levels of gene expression and there was no tendency in each group of strains.

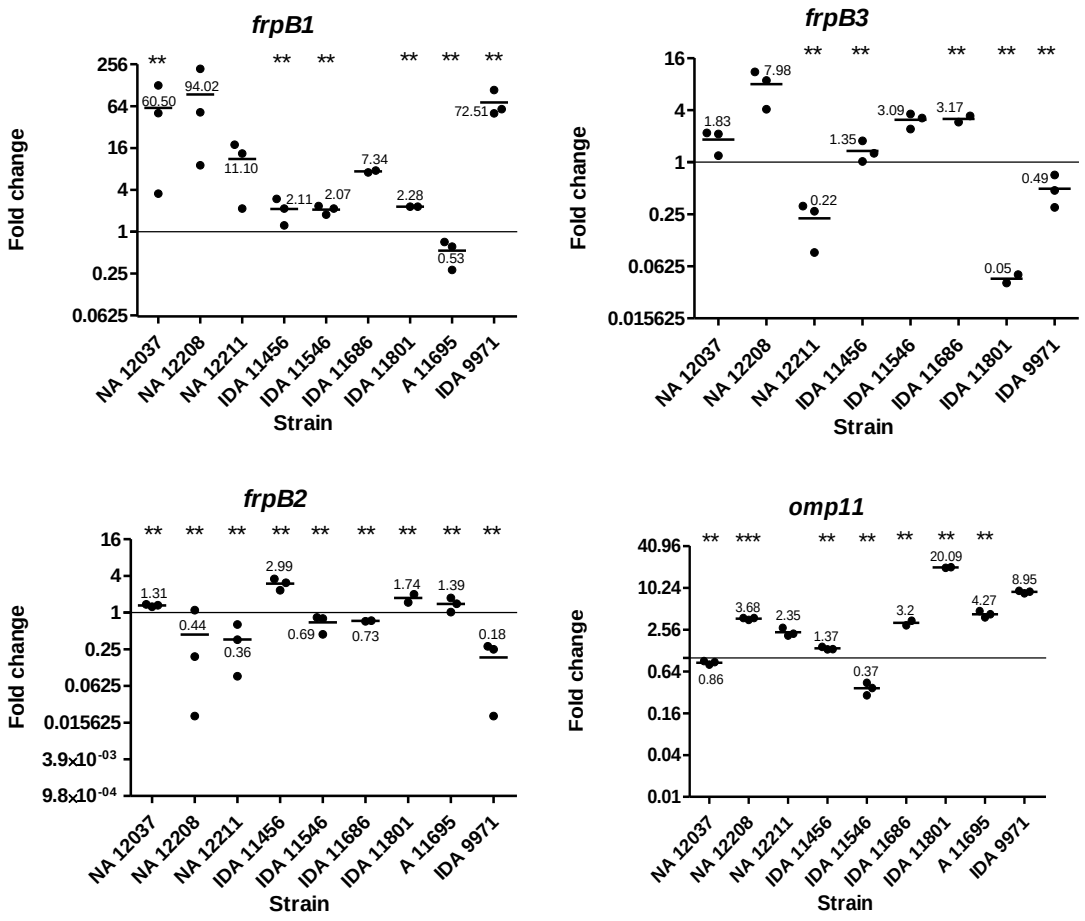

Figure 10 – Outer membrane *Helicobacter pylori* 26695 target genes expression analysis from a normal growth of all NA and IDA clinical strains in study, with each strain's triplicates mean, represented by the "line" and its values.

NA and IDA clinical strains were grown in liquid BHI medium at 37 °C under microaerobic conditions until OD~0.4-0.7. At this point RNA was extracted and gene expression was analysed by qRT-PCR. Results are expressed as fold change with the reference strain (NA 12038). Each sample was analysed in triplicate. Each dot represents one replicate, and the line represents the mean of the triplicate.

The statistical significance was established using Wilcoxon-Mann-Whitney test (******* p-value<0.05; ****** p-value<0.1). NA – Non-anaemic; IDA – Iron-deficiency anaemic; A – Anaemic

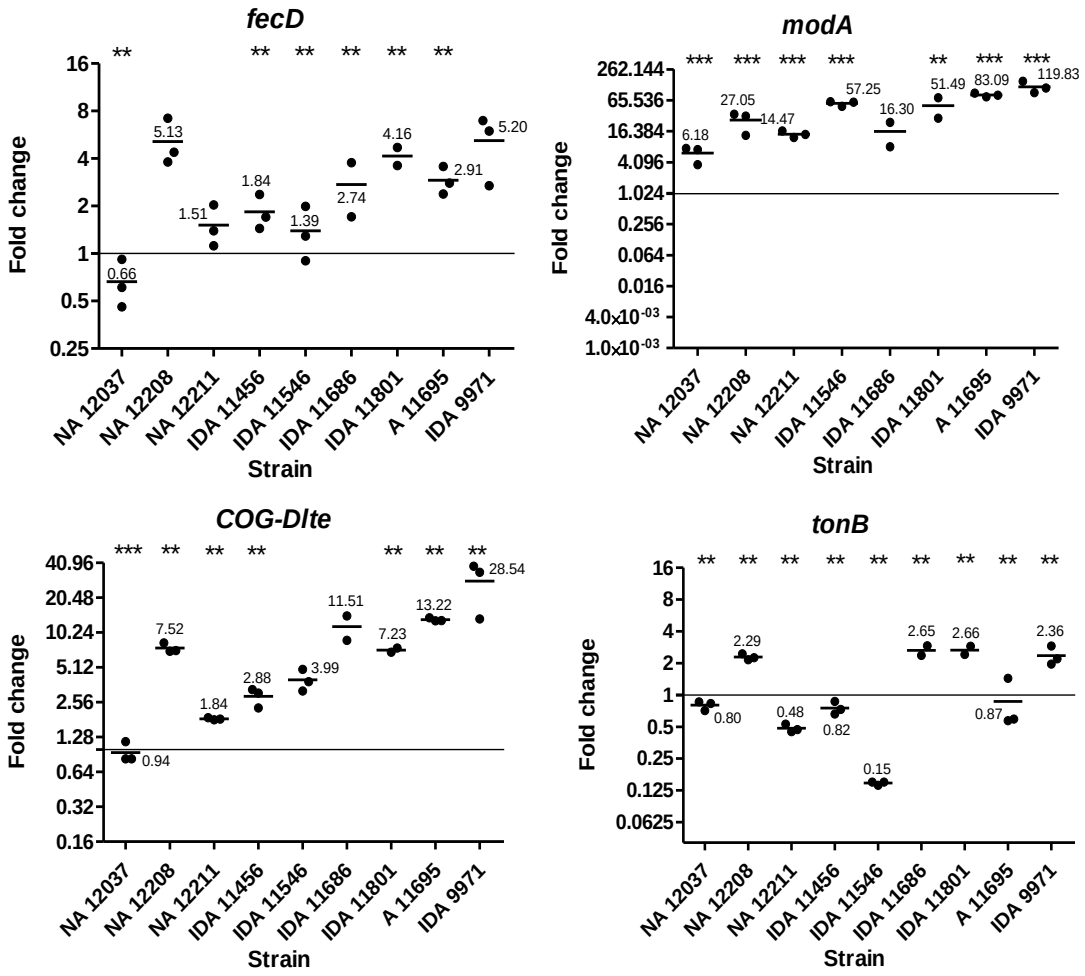

Figure 11 - Inner membrane proteins (ABC transporters and *tonB*) *Helicobacter pylori* 26695 target genes expression analysis from a normal growth of all NA and IDA clinical strains in study, with each strain's triplicates mean, represented by the "line" and its values.

NA and IDA clinical strains were grown in liquid BHI medium at 37 °C under microaerobic conditions until OD~0.4-0.7. At this point RNA was extracted and gene expression was analysed by qRT-PCR. Results are expressed as fold change with the reference strain (NA 12038). Each sample was analysed in triplicate. Each dot represents one replicate, and the line represents the mean of the triplicate.

The statistical significance was established using Wilcoxon-Mann-Whitney test (******* p-value<0.05; ****** p-value<0.1). NA – Non-anaemic; IDA – Iron-deficiency anaemic; A – Anaemic

In Figure 11, there are represented the fold change values of genes belonging to ABC transporters and a gene from TonB-ExBD complex.

In *fecD* fold change are 0.7, 1.5, 5.1 for 12037, 12211 and 12208, respectively, having 2 NA strains more expressed, being the and the reference NA strains less expressed, having no tendency among them. IDA strains have fold change values regarding *fecD* gene, around 3 (1.4 for 11546, 1.8 for 11456, 2.7 for 11686, 4.2 for 11801 and 5.2 for 9971, being this gene in all IDA strains as well as A 11695 strain (fold change of 2.9) more expressed than in the reference strain. However, there is no clear difference between NA and IDA strains.

In *COG-DIte* fold change in NA strains 12037, 12208 and 12211 are 0.9, 1.8 and 7.5, respectively, which are similar to *fecD* gene that belongs also to the same inner membrane complex. In IDA strains fold changes for *COG-DIte* ranges from 2.9 to 28.5 (11456, 11546, 11686, 11801, 9971, fold changes are 2.9, 4, 7.2, 11.5, 13.2, 28.5, respectively). So, every IDA strains have different gene expressions.

The gene expression in *modA* have a range of 6.18 to 27.05 in NA strains 12037, 12211 and 12038 (6.18, 14.47, 27.05, respectively), so 3 out of 4 NA strains have higher expression of this gene. IDA strains fold changes range from 16.3 to 119.8 (16.3 for 11686, 51.5 for 11801, 57.3 for 11546 and 119.8 for 9971), so all IDA strains are more expressed than the NA strain of reference, also A strain have a fold change of 83.1 which is also similar. From this data, all strains are more expressed and, in average, IDA strains show a greater level of expression comparing to NA group. Here, IDA 11456 strain had high Ct values, so it was not possible to analyse that data.

Lastly, in *tonB* gene expression analysis, NA strains have fold changes for NA strains 12211, 12037 and 12208 of 0.5, 0.8, 2.3, meaning that 3 out of 4 NA strains are less expressed. In IDA strains fold change values for this gene are around 2.5 for 3 of them, and other 2 are less expressed as well as the anaemic strain. This gene might be expressed similarly among all the NA strains, although IDA strains have different gene expressions.

In Figure 12 – Haem oxygenase, *hugZ*, and putative haemophore, *groEL*, *Helicobacter pylori* 26695 target genes expression , there are represented the fold change values of genes belonging to a haem oxygenase gene and a putative haemophore. In *hugZ* gene expression levels for NA strains go from fold changes <1 (0.12, 0.26, 1.02 for strains 12211, 12037 and 12208, respectively), meaning that for NA strains this gene is not much expressed compared to the control. For IDA strains, this gene expression levels are also <1 (0.01 for 11546, 0.2 for 11456, 0.5 for 9971, 1.1 for 11801 and 1.5 for 11686) being all IDA strains with lower gene expression for *hugZ*. Anaemic strain shows higher expression levels with a fold change of 5.76. So, this gene is lower expressed in all NA and IDA strains comparing to the NA strain of reference, except for the anaemic strain.

In *groEL*, gene expression levels go from fold change range of 4.3 to 14.1 (4.3 for 12037, 8.9 for 12211, 14.1 for 12208) in NA strains, showing that this gene is higher express in 3 out of 4 NA strains. In IDA strains fold change values are 1.8 for 11546, 1.9 for 11546, 2.9 for 9971, 5.7 for 11801 and 6.3 for 11686, so for IDA strains this gene is also higher expressed comparing to the reference strain. Anaemic strain has a fold change of 3.2, also higher expressed. Meaning that this gene is higher expressed in all strain compared to the reference strain.

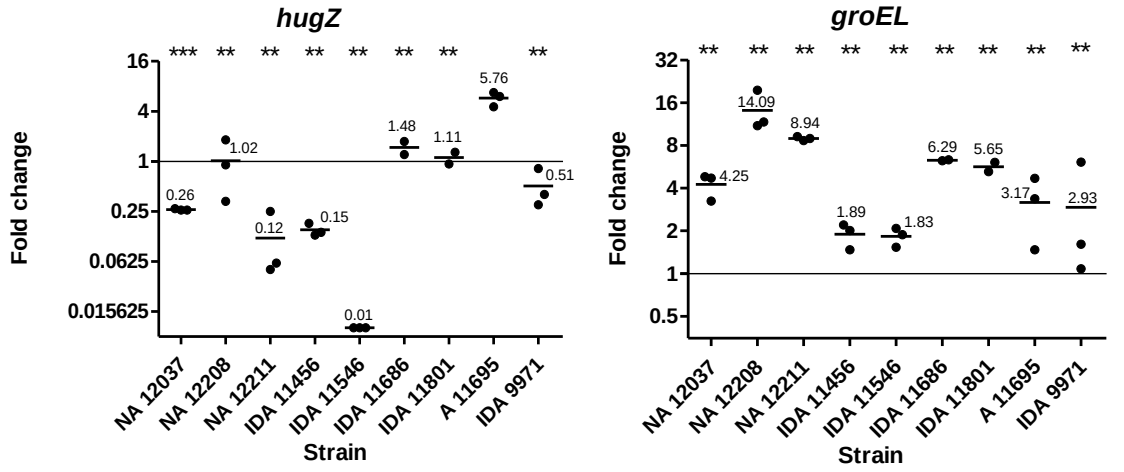

Figure 12 – Haem oxygenase, *hugZ*, and putative haemophore, *groEL*, *Helicobacter pylori* 26695 target genes expression analysis from a normal growth of all NA and IDA clinical strains in study, with each strain's triplicates mean, represented by the "line" and its values.

NA and IDA clinical strains were grown in liquid BHI medium at 37 °C under microaerobic conditions until OD~0.4-0.7. At this point RNA was extracted and gene expression was analysed by qRT-PCR. Results are expressed as fold change with the reference strain (NA 12038). Each sample was analysed in triplicate. Each dot represents one replicate, and the line represents the mean of the triplicate.

The statistical significance was established using Wilcoxon-Mann-Whitney test (******* p-value<0.05; ****** p-value<0.1). NA – Non-anaemic; IDA – Iron-deficiency anaemic; A – Anaemic

Unfortunately, it was not possible to finish our purpose, that was to repeat qRT-PCR for three biological replicates, so the calculations were performed by using the three replicates' values, that resulted from the same biological sample, for each strain (except for 11686 and 11801 strains), that we only had two replicates because there was not enough cDNA to do a third replicate.

Overall, we did not observe a clear difference between the NA and the IDA strains that could point to a putative role of a gene or genes products in the disease process. However that can mean that all strains behaviour differently from each other, not having a comparable gene expression, and therefore it is not possible to conclude something from these genes' expression assays.

2.2 Gene expression analysis in haemin supplemented growth

We wanted to see if the addition of haemin to the media, impacted differently the expression levels of the target genes in NA and iron-deficiency anaemic IDA strains, thus we performed qRT-PCR experiments in NA 12038 and IDA 11801 strains, grown in BHI liquid medium with 10% FBS supplemented with 20 µM of haemin.

Fold change values were calculated using NA 12038 strain grown in the absence of haemin as reference.

In Figure 13, are represented the gene expression levels of the 4 outer membrane receptors. Fold change values in *frpB1,* showed that in NA strain there is 4-fold induction in the presence of haemin, and that IDA strain has no effect in *frpB1* expression in the presence of haemin.

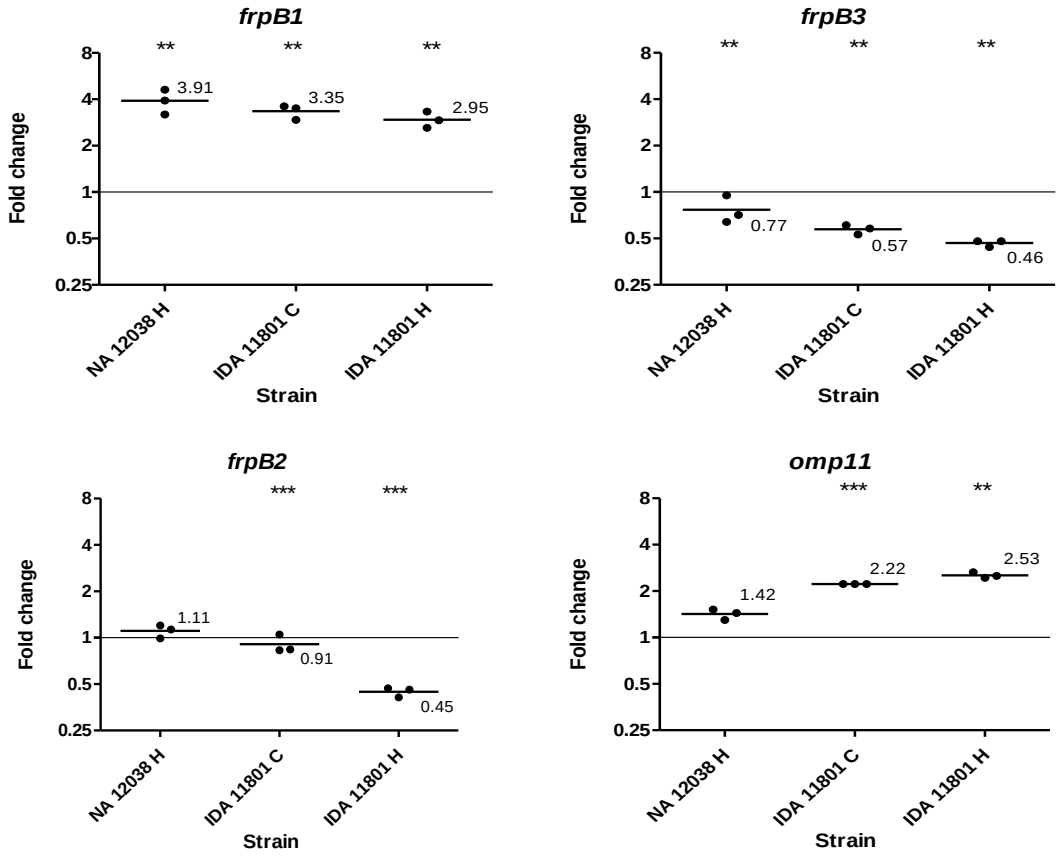

Figure 13 – Outer membrane *Helicobacter pylori* 26695 target genes expression analysis from a normal and haemin supplemented growth of NA 12038 and IDA 11801 strains, with each strain's triplicates mean, represented by the "line" and its values.

NA and IDA clinical strains were grown in liquid BHI medium (C) and liquid BHI medium supplemented with 20 µM of haemin (H), at 37 °C under microaerobic conditions until OD~0.4-0.7. At this point RNA was extracted and gene expression was analysed by qRT-PCR. Results are expressed as fold change with the reference strain (NA 12038) grown in liquid BHI medium (C). Each sample was analysed in triplicate. Each dot represents one replicate, and the line represents the mean of the triplicate.

The statistical significance was established using Wilcoxon-Mann-Whitney test (******* p-value<0.05; ****** p-value<0.1). NA – Non-anaemic; IDA – Iron-deficiency anaemic.

In *frpB2* fold change values in the NA strain shows that this do not respond to haemin addition and that IDA strain decrease to a fold change of 0.5.

In *frpB3*, there are fold changes around 0.5 in both the IDA and NA strains, and haemin addition seemed to not affect much the gene expression.

In *omp11* fold change values shows that in both NA and IDA strains haemin addition does not affect much this gene expression, although it shows higher basal levels in IDA strain.

In Figure 14, there are represented the graphs for inner membrane genes from ABC transporters and a gene belonging to TonB complex.

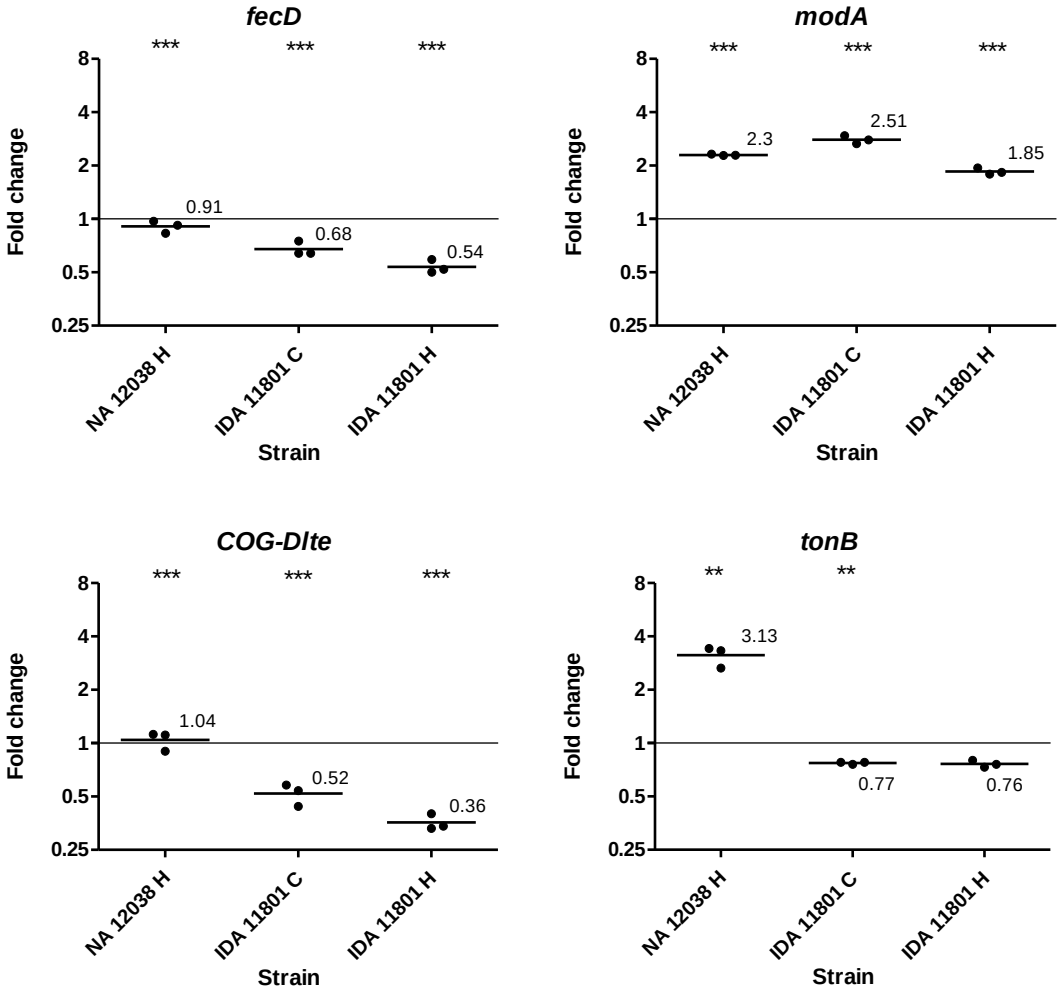

Figure 14 - Inner membrane proteins (ABC transporters and *tonB*) *Helicobacter pylori* 26695 target genes expression analysis from a normal and haemin supplemented growth of NA 12038 and IDA 11801 strains, with each strain's triplicates mean, represented by the "line" and its values.

NA and IDA clinical strains were grown in liquid BHI medium (C) and liquid BHI medium supplemented with 20 µM of haemin (H), at 37 °C under microaerobic conditions until OD~0.4-0.7. At this point RNA was extracted and gene expression was analysed by qRT-PCR. Results are expressed as fold change with the reference strain (NA 12038) grown in liquid BHI medium (C). Each sample was analysed in triplicate. Each dot represents one replicate, and the line represents the mean of the triplicate.

The statistical significance was established using Wilcoxon-Mann-Whitney test (******* p-value<0.05; ****** p-value<0.1). NA – Non-anaemic; IDA – Iron-deficiency anaemic.

In *fecD* fold change values shows that are no striking differences between NA and IDA strains, meaning that haemin does not affect its gene expression.

The gene expression in *modA* gene is represented with higher fold values in NA strains in the presence of haemin, although in IDA strains haemin does not affect its expression.

In *COG-Dlte* fold change values shows a lower basal level of expression in IDA, although haemin might repress its expression. In NA strains this gene expression seem to not be affected by haemin addition.

In *tonB*, there is a clear higher expression in this gene for the NA strain, and in IDA strain haemin addition seems to not affect its expression.

In Figure 15 – Haem oxygenase, *hugZ*, and putative haemophore, *groEL*, *Helicobacter pylori* 26695 target genes expression analysis , there are the graphs regarding the putative haemophore *groEL* and the haem oxygenase *hugZ*. In *groEL* gene expression analysis, haemin addition seems to not affect its expression in both NA and IDA strains. And in *hugZ*, there is a lower basal level for this gene expression in IDA strain, however the presence of haemin does

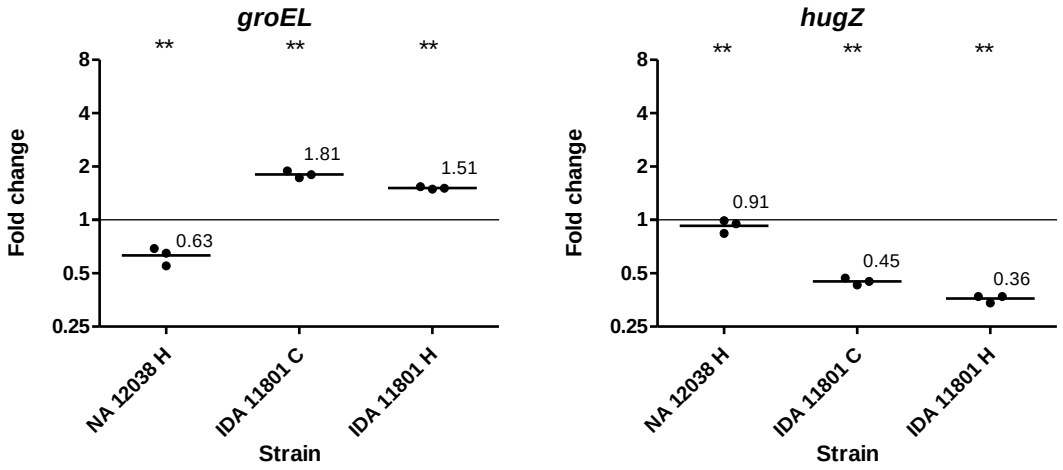

Figure 15 – Haem oxygenase, *hugZ*, and putative haemophore, *groEL*, *Helicobacter pylori* 26695 target genes expression analysis from a normal and haemin supplemented growth of NA 12038 and IDA 11801 strains, with each strain's triplicates mean, represented by the "line" and its values.

NA and IDA clinical strains were grown in liquid BHI medium (C) and liquid BHI medium supplemented with 20 μM of haemin (H), at 37 °C under microaerobic conditions until OD~0.4-0.7. At this point RNA was extracted and gene expression was analysed by qRT-PCR. Results are expressed as fold change with the reference strain (NA 12038) grown in liquid BHI medium (C). Each sample was analysed in triplicate. Each dot represents one replicate, and the line represents the mean of the triplicate.

The statistical significance was established using Wilcoxon-Mann-Whitney test (******* p-value<0.05; ****** p-value<0.1). NA – Non-anaemic; IDA – Iron-deficiency anaemic.

not affect the expression in both NA and IDA strains.

Our results showed that haemin addition significantly increased the expression of *frpB1*, *tonB* and *modA* genes and might decrease the expression levels in *COG-Dlte* gene in the NA strain, also in IDA strains the presence of haemin seemed to decrease the expression of *frpB2*

gene. In the other genes, haemin addition did not affect their expression in both NA and IDA strains.

In previous results published by Carrizo-Chávez and colleagues, they observed an increase of *frpB1* gene expression upon addition of an external source of haem, although, in their study, they grown *H. pylori* strain in M63 medium supplemented with ferric citrate because they refer that in previous studies describe that medium with peptone and tryptone could block uptake of haem[78]. In our case *frpB1* expression only increased in NA strain and not in IDA strain[78]. In the other hand, our results are not in agreement with previous results published by González-López and colleagues showed that *frpB1* expression levels was increased with haemoglobin but not with haem, also that *frpB2* increased with haem but not with haemoglobin, and that *frpB3* was increased with haem[79].

Also, haemin addition did not affect groEL and hugZ gene expression, which is interesting since *groEL* is thought to be an haemophore and *hugZ* is described as a haem oxygenase. González-López and colleagues also describes in previous studies that *groEL* has affinity for haem[80] and Guo, Y. and colleagues described that *hugZ* has an haem oxygenase activity and binds haemin *in vitro*, and also shows that it synthesise haem[86].

Finally, we are using clinical strains, so that might explain these different results from what is published already, although for further studies it could be interesting to repeat these experiments and also to test more NA and IDA strains to see if there is a trend in these gene expressions among them and see if there are differences between the two group of strains.

3. Viability assays

We also wanted to do a viability study to understand if the addition of haemin and an iron chelate agent would influence the growth of NA and IDA strains. Strains were grown in a rich liquid media (BHI with 10% FBS) with and without 10 µM, 20 µM of haemin and 250 µM of DIP (iron chelate agent) supplementation. OD_{600} values were taken at time 0, 3, 6 hours for 10 µM of haemin supplementation growths and at 0 and 8 hours for 20 µM of haemin and 250 µM of DIP.

3.1 Growth in haemin supplemented medium

In Figure 16 – Growth of *Helicobacter pylori* strain 26695 supplemented with haemin., it's represented the graph of growth data after a normal and 10 µM haemin supplemented growth to serve as a control.

Our results showed that addition of haemin did not affect the growth rate of *H. pylori* 26695 strain. OD_{600} values measured after 3 hours and 6 hours of growth in the presence or absence of haemin were similar in both cases (Figure 16).

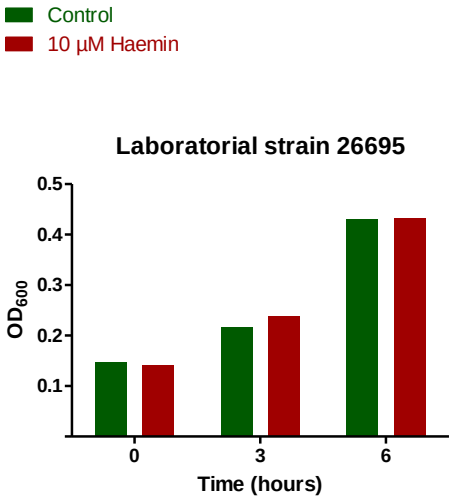

Figure 16 – Growth of *Helicobacter pylori* strain 26695 supplemented with haemin.

H. pylori 26695 strain was grown in BHI media supplemented or not with 10 µM of haemin.

Similar results were obtained in NA and IDA strains grown in liquid media supplemented with 10 µM of haemin (Figure 17 and Figure 18).

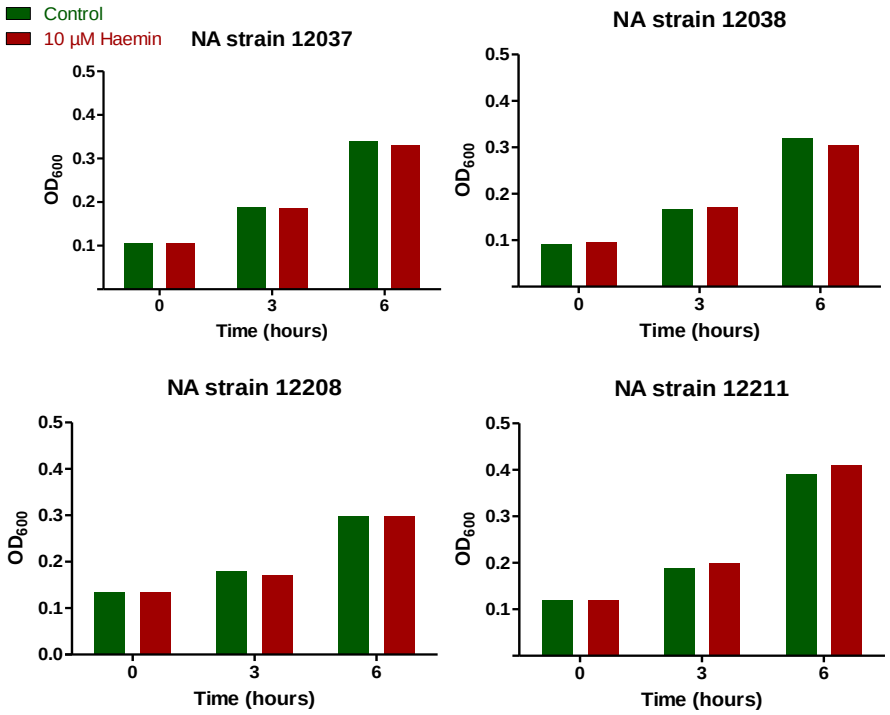

Figure 17 – Growth of *Helicobacter pylori* non-anaemic strains supplemented with haemin.

H. pylori non-anaemic strains were grown in BHI media supplemented or not with 10 µM of haemin.

NA – Non-anaemic

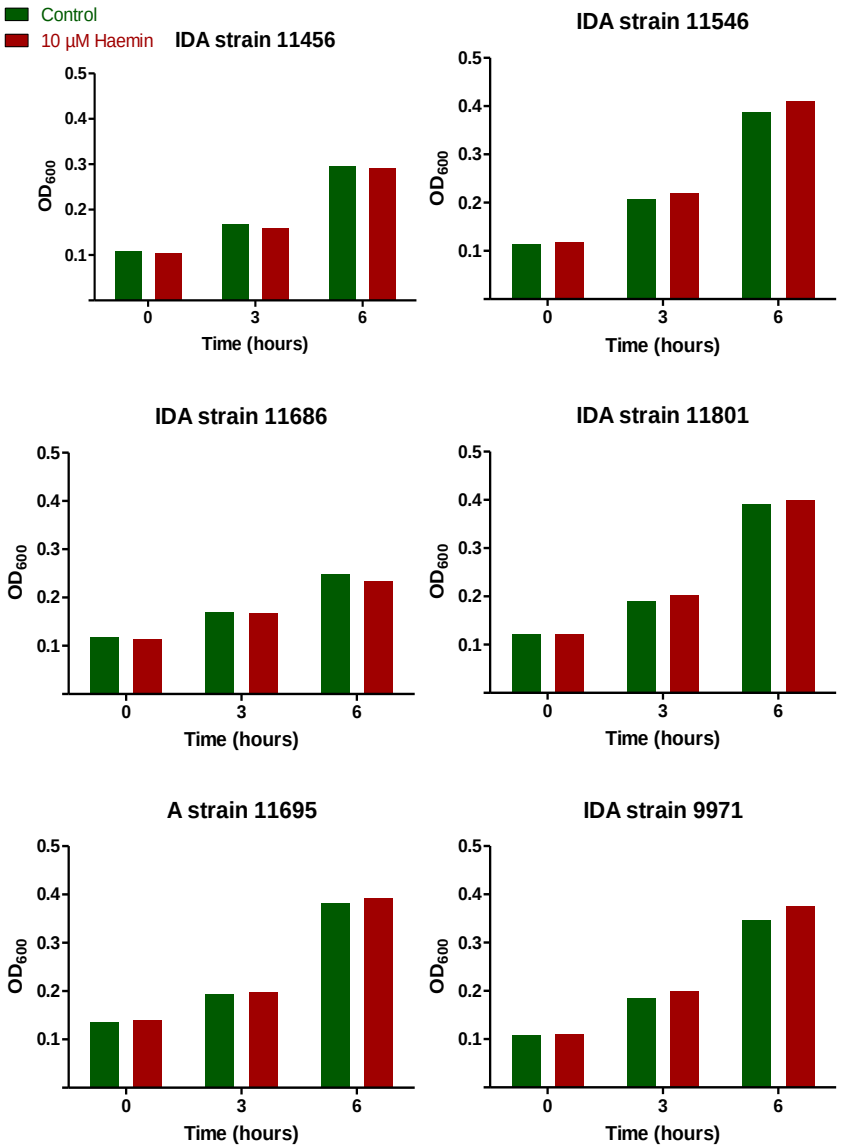

Figure 18 – Growth of *Helicobacter pylori* iron deficiency anaemic and anaemic strains supplemented with haemin.

H. pylori iron deficiency anaemic and anaemic strains were grown in BHI media supplemented or not with 10 µM of haemin.

IDA – Iron-deficiency anaemic; A – Anaemic

Also, with 20 µM of haemin supplemented media the same results were obtained for NA 12038 and IDA 11801 strains (Figure 19).

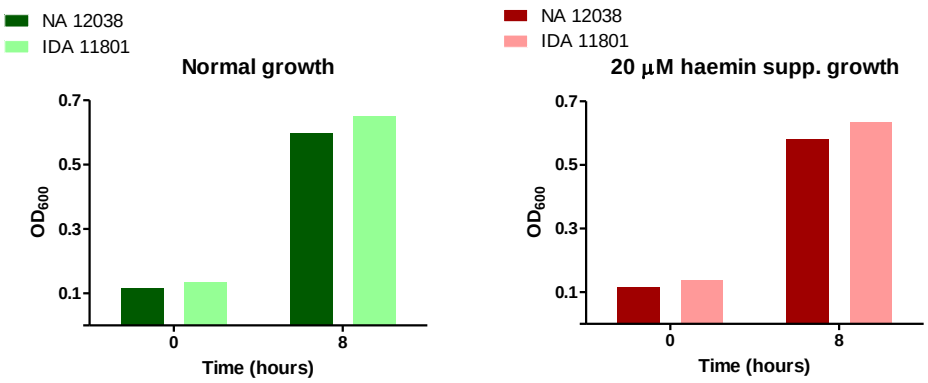

Figure 19 – Growth of *Helicobacter pylori* non-anaemic 12038 and iron deficiency anaemic 11801 strains supplemented with haemin.

H. pylori non-anaemic 12038 and iron deficiency anaemic 11801 strains were grown in BHI media supplemented or not with 20 µM of haemin.

NA – Non-anaemic; IDA – Iron-deficiency anaemic

Thus, our results indicate that addition of haemin do not affect the growth rate of *H. pylori* strains. However, this assay was done with a single concentration of haemin and would be interesting to see if different concentration would show significant results, maybe starting by trying one non-anaemic and another iron deficiency anaemic strains with different concentrations of haemin (40 µM/50 µM/100 µM), to see if any of these concentrations changes the growth of these strains. Moreover, after evaluating the growth rate of both strains, we also wanted to evaluate the viability of these strains under haemin supplementation.

3.2 CFU/mL in haemin supplemented growth

To measure the viability of *H. pylori* growing with the addition of extracellular source of haem, NA 12038 and IDA 11801 strains' colonies were counted after the growth with and without 20 µM haemin supplementation and CFU/mL was calculated.

In both Figure 21 – CFU/mL from a normal and 20 µM haemin supplemented growth of non-anaemic (NA) strain 12038. and Figure 20 – CFU/mL from a normal and 20 µM haemin supplemented growth of iron-deficiency anaemic (IDA) strain 11801., we can observe that haemin did not significantly change the CFU's/mL.

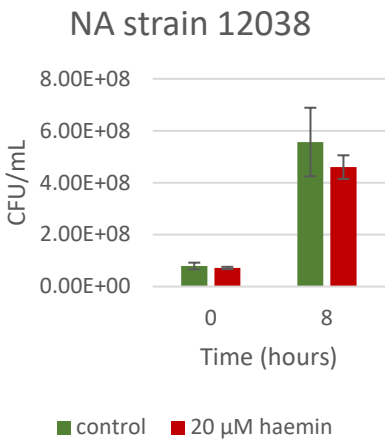

Figure 21 – CFU/mL from a normal and 20 µM haemin supplemented growth of non-anaemic (NA) strain 12038.

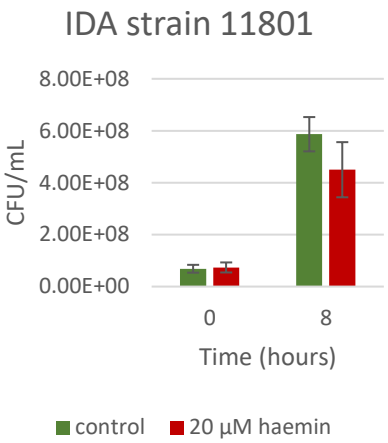

Figure 20 – CFU/mL from a normal and 20 µM haemin supplemented growth of iron-deficiency anaemic (IDA) strain 11801.

3.3 Growth in iron restricted medium

In Figure 22, there are represented the graphs of growth data with 250 µM DIP supplementation of NA 12038 and IDA 11801 strains. The addition of 250 µM of DIP decreased the growth of both non-anaemic and iron-deficiency strain, being more decreased in the IDA strain, as a result of iron depletion. However, we could not see a clear distinction between growth effect in NA strain compared to IDA strain.

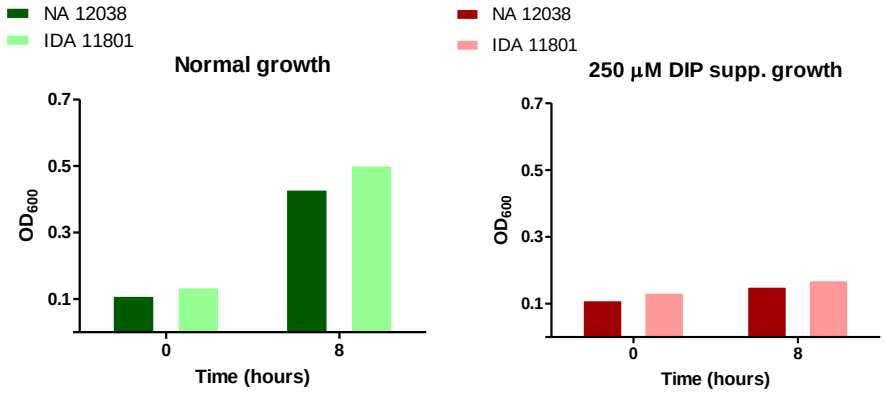

Figure 22 – Growth of *Helicobacter pylori* non-anaemic 12038 and iron deficiency anaemic 11801 strains supplemented with DIP.

H. pylori non-anaemic 12038 and iron deficiency anaemic 11801 strains were grown in BHI media supplemented or not with 250 µM of DIP.

NA – Non-anaemic; IDA – Iron-deficiency anaemic

Our results did not reveal a growth phenotype in IDA compared to NA strain. Nevertheless, it might be interesting to test a different range of DIP concentrations, between 100 and 250 µM that might be more informative to disclose differences in the phenotype. Moreover,

after evaluating the growth rate of both strains, we also wanted to evaluate the viability of these strains under iron starvation.

3.4 CFU/mL in DIP supplemented growth

Again, the measure of viability for the same strains was done but this time after the normal and 250 µM DIP supplemented growth and then calculated CFU/mL.

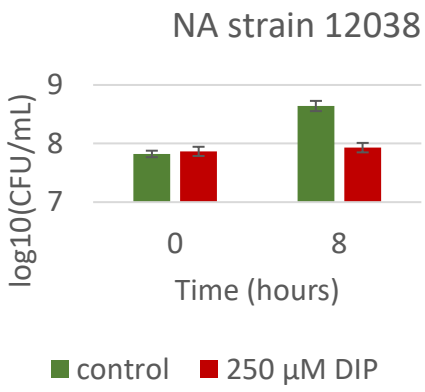

Figure 23 – CFU/mL from a normal and 250 µM DIP supplemented growth of non-anaemic (NA) strain 12038.

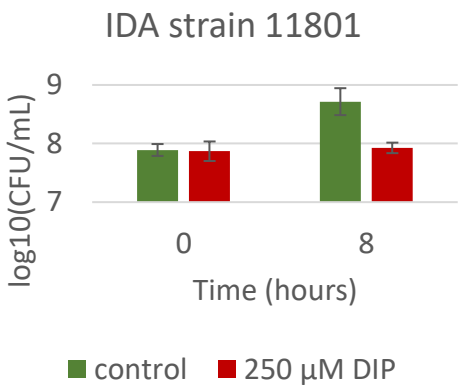

Figure 24 – CFU/mL from a normal and 250 µM DIP supplemented growth of iron-deficiency anaemic (IDA) strain 11801.

Our results showed that there are no major differences in both NA and IDA strains.

Chapter IV. Conclusions and Future work

For this study, we performed several experiments to observe NA and IDA strains target genes for haem uptake expressions by qRT-PCR, then study the growth rate of each strain and its viability with and without different concentrations of haemin and DIP supplementation to rich media.

In qRT-PCR experiments a final optimised protocol for this experiment in *H. pylori* strains was defined. Overall, our results showed that there is no evidence of target genes to be more expressed in iron-deficiency anaemic strains comparing to non-anaemic strains and vice versa, when grown in rich media. However, haemin supplementation showed that gene expression of *frpB1*, *tonB* and *modA* genes significantly increased and might decrease the expression levels in *COG-Dlte* gene in the NA strain, and in IDA strains the presence of haemin seemed to decrease the expression of *frpB2* gene.

In viability assays results we have seen no effect on growth viability of haemin or iron starvation. Thus, our results do not seem to indicate a phenotype correlation between IDA strains and haemin/iron deficiency.

To conclude, it is important to refer that this is a first exploratory project and results might be taken as a first approach. Also, more biological samples should be analysed to confirm our results.

Previous results by Kato, S. and colleagues described a trend of *frpB1* gene expression in rich media, where NA strains have higher expression of this gene than IDA strains[36] which is in agreement with our results. Also other studies, regarding the presence of haemin in the media, published by Carrizo-Chávez and colleagues demonstrate an increase of *frpB1* gene expression in haemin supplemented different media[78], although in our case it only increased its expression in NA strain and not in IDA strain. Also, González-López and colleagues showed that *frpB1* expression levels was increased with haemoglobin but not with haem, *frpB2* increased with haem but not with haemoglobin, and that *frpB3* was increased with haem[79], but our results showed no striking differences. Also, haemin addition did not affect *groEL* and *hugZ* gene expression, which is interesting since González-López and colleagues also describes in previous studies that *groEL* has affinity for haem[80] and Guo, Y. and colleagues described that *hugZ* has an haem oxygenase activity and binds haemin *in vitro*, and also shows that it synthesise haem[86].

I think it would be nice to pursue this study and to grow all the NA and IDA strains with supplementation of haemin and in an iron-depleted media (increasing both concentrations) and perform qRT-PCR as well, to have more gene expression data for getting more clear conclusions. It would be also important to repeat all of that with much more clinical strains, non-anaemic as well as iron-deficiency anaemic, to critically evaluate if there is or not a trend towards a specific group of strains.

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Appendixes

Appendix A Other genes related with iron/haem uptake that were search for homology with *H. pylori* 26695 strain

Protein name and function	**Specie**	**Identity**	**Cover**	**E-value**	***H. pylori* 26695 homologous protein (respective KEGG ID number)**
HPB23 – Peroxiredoxin-1 (haem-binding 23 kDa)	*Rattus norvegicus*	45%	63%	6e-56	TsaA (HP1563) alkyl hydroperoxide reductase
Peroxide-responsive transcriptional repressor PerR	*Bacillus subtilis*	25%	91%	1e-18	Chain A, Ferric Uptake Regulation Protein
Anr transcriptional regulator	*Pseudomonas aeruginosa*	No homology			
NarL putative nitrate/nitrite response regulator	*Pseudomonas aeruginosa*	No homology			
HemO haem oxygenase	*Pseudomonas aeruginosa*	No homology			
PhuT haem-transporter	*Pseudomonas aeruginosa*	No homology			
HasR haem uptake outer membrane receptor	*Pseudomonas aeruginosa*	No homology			
Haem-responsive two componente system response HrrA	*Corynebacterium diphtheria*	No homology			
HtaA cell-surface haemin receptor	*Corynebacterium diphtheria*	No homology			
HtaB cell-surface haemin receptor	*Corynebacterium diphtheria*	No homology			

HtaC cell-surface haemin receptor	*Corynebacterium diphtheria*	No homology
HmuT haemin-binding periplasmic protein	*Corynebacterium diphtheria*	No homology
OxyR regulator responsible for induction of oxygen-dependent HemF expression	*Escherichia coli*	No homology
IsdB NEAT domain protein	*Staphylococcus aureus*	No homology
IsdH NEAT domain protein	*Staphylococcus aureus*	No homology
IsdA NEAT domain protein	*Staphylococcus aureus*	No homology
IsdG staphylobilin-forming haem oxygenase	*Staphylococcus aureus*	No homology
IsdI staphylobilin-forming haem oxygenase	*Staphylococcus aureus*	No homology
HasA haemophore	*Serratia marcescens*	No homology

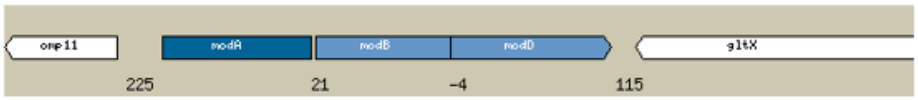
Appendix B Omp11, ModA and FecD positioning in *H. pylori* 26695 genome

VIMSS18892: **HP0472 omp11 outer membrane protein (omp11) (NCBI ptt file), 186 a.a.** [Helicobacter pylori 26695]

VIMSS Predicted Operon

VIMSS18893: **HP0473 modA molybdenum ABC transporter, periplasmic molybdate-binding protein (modA) (NCBI ptt file), 246 a.a.** [Helicobacter pylori 26695]

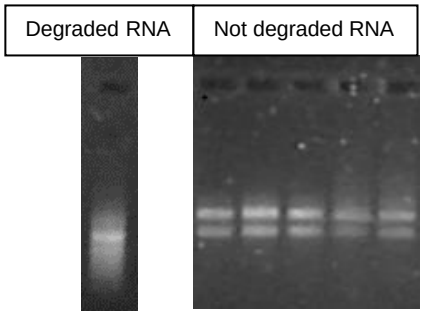
VIMSS Predicted Operon



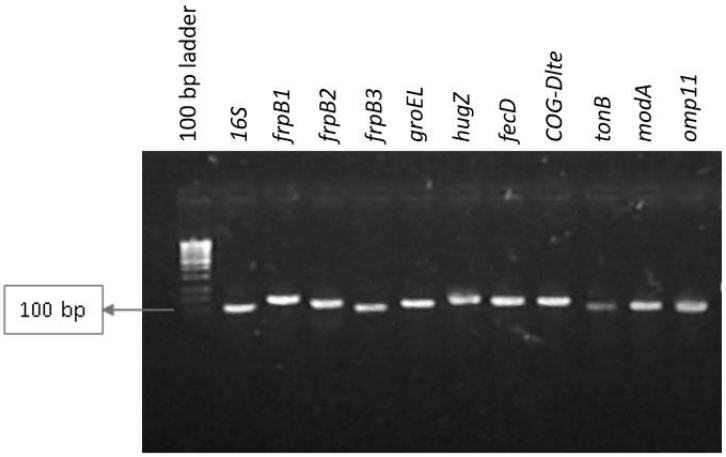
VIMSS19303: **HP0889 fecD iron(III) dicitrate ABC transporter, permease protein (fecD) (NCBI ptt file), 326 a.a.** [Helicobacter pylori 26695]

VIMSS Predicted Operon

Appendix C Electrophoresis agarose gel: extracted RNA



Appendix D Electrophoresis 2% agarose gel saw by UV light of PCR products of primers testing by a conventional PCR



Observing Electrophoresis 2% agarose gel saw by UV light of PCR products of primers testing by a conventional PCR figure, it was possible to confirm that all primer pairs work well and have the expected amplicon length, except *tonB* gene that had a faint band. New primers for tonB gene were designed and tested again and showed better results (electrophoresis gel not shown).