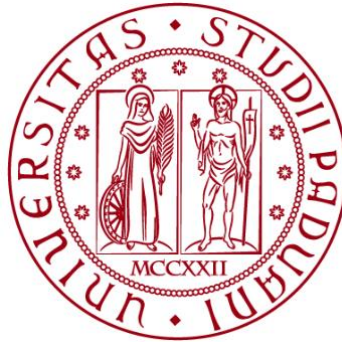


UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea magistrale in Molecular Biology



TESI DI LAUREA

Do intraspecies interactions affect *Helicobacter pylori* community composition?

Relatore: Prof. Gil G. Rosenthal
Dipartimento di Biologia

Correlatore*: Assoc. Prof. Sandra B. Andersen
University of Copenhagen

Laureando/a: Tulay Ugurlu

ANNO ACCADEMICO 2023/2024

TABLE OF CONTENTS

ABSTRACT	3
INTRODUCTION	4
1. SOCIAL EVOLUTION THEORY FOR MICROORGANISMS	6
2. Helicobacter Pylori	9
2.1. H. pylori and humans: an ancient relationship	9
2.2. History	10
2.3. Morphology	10
2.4. Persistence and adaptability	11
2.5. H. pylori and disease	12
2.6. Virulence factors of H. pylori	12
2.6.1. Vacuolating cytotoxin A	13
2.6.2. VacA factor in the immune response of the host	14
2.7. H.pylori and benefits of its infection	16
METHODOLOGY	19
1. Infection of C57BL/6 neonatal mice with PMSS1/PMSS1-VacA H. pylori	19
2. H. pylori quantification in vivo	21
3. H. Pylori PMSS1/PMSS1-vacA in vitro growth assay	23
4. H. pylori (PMSS1-PMSS1/vacA) quantification in vitro	25
5. Statistical analysis and Plots	27
RESULTS	28
1. Quantification of H.pylori in vivo samples	28
1.1. Spot plating of the initial concentration of H.pylori used to infect mice	28
1.2. qPCR with glmm primers	29
1.3. qPCR with aphA3 primers	30
2. Quantification of H.pylori bacterium in vitro	31
2.1. Quantification of H.pylori bacterium using Optical Density Spectrometer	32
2.2. Quantification of H.pylori bacterium with qPCR and Qubit assays	34
2.3. Quantification of H.pylori bacterium using qPCR with glmm and aphA3 primers	37
2.3.1. Quantification of H.pylori bacterium using qPCR with aphA3 primers	38
2.3.2. Quantification of H.pylori bacterium using qPCR with glmm primers	39
2.4. Measurement of the ratio between the knock-out and wild-type H.pylori in mix-culture samples	40
DISCUSSION	41
Future importance	43
REFERENCES	45

ABSTRACT

Helicobacter pylori is a bacterium thought to inhabit the human stomach for at least 300,000 years. This ancient association between the host and the bacteria is involved in pathogenesis of gastric diseases. Most of the bacterial strains produce factors that influence the host on a large scale. Since the discovery of the *H. pylori*, the majority of the research that has been done focused on the pathology of diseases associated with these bacteria. However, recent research demonstrates potential benefits of its infection on modern diseases, which developed after discovering new hygiene practices and antibiotic usage. Therefore it is crucial to understand the factors that are affecting how these bacteria behave.

The emphasis on the host environment and medical treatment in order to eradicate the infection, is to some degree neglecting the actual microbial interactions. Bacteria are known to have social lives, communicating via various pathways, even affecting the behavior of the host, protecting against diseases, and helping digest our food. In this paper we focus on the microbial social evolution theory, both in vivo and in vitro environments, to observe the host effects on microbes and microbial interactions alone such as competition, cooperation and cheating, respectively. The research in this project addresses the impact of *H. pylori* sociality on the host as well as on each other. Because in the end, social behaviors can be the traits that matter for host colonization and persistence. Therefore, affecting the progression of the infection.

Keywords: H.pylori, Social evolution, Vacuolating toxin(vacA), Cooperation, Cheating

INTRODUCTION

Research in the medical field is expanding, with a growing focus on preventing disease outbreaks. The development of resistance and harmful mutations, which can render previously effective medicine and treatments useless, is particularly concerning. Drug-resistant bacteria are a group of bacteria that were initially susceptible to the effects of an antimicrobial drug, but later developed resistance to it. The issue of antibiotic resistance, particularly in pathogenic bacteria, is a pressing concern within the medical domain. The previously manageable bacterial diseases have now become problematic as a result of the emergence of antibiotic-resistant bacteria. Moreover, when used for disease treatment, antibiotics not only target the causative bacteria, but also impact numerous other bacteria in the body that exhibit antibiotic sensitivity[1]. It turned out that this destruction was not without its drawbacks. As infection rates caused by some microorganisms decreased, the occurrence of asthma and allergies, also known as "modern diseases," increased. In this research, we focus on a notable example of such a microorganism: *Helicobacter pylori*.

Microorganisms are known to inhabit a wide variety of mammals, both internally and externally. Diverse species and strains of bacteria have colonized humans throughout their existence, a phenomenon that we now understand impacts their overall well-being. The composition of the human microbiome exhibits variability across individuals due to fluctuations in diets, environmental influences, and inheritance. Our understanding of the microbiome remains limited, particularly regarding the mechanisms of communication among microbial populations and their coevolutionary dynamics with humans[2].

A working knowledge of antibiotics is not the only prerequisite for preventing disease development. While antibiotics may work in normal treatment circumstances, the urging issue with antibiotic-resistant bacteria forces us to look more deeply into the interaction between the microbes that cause these illnesses. These interactions are reported to be "social" as bacteria exhibits behaviours such as cooperation, cheating, etc. Furthermore, social behaviors are considered one of a population's primary dynamics, and in the case of microorganisms, it's possible that "sociality" could

influence the course of a specific infection they cause. In order to target social behaviors as an innovative method for managing the transmission and severity of infectious diseases, it is imperative to conduct additional research on the host-symbiont relationship between humans and their microorganisms[3].

In this paper, we focused on a bacteria that has an intriguing social life in the gastric mucosa. *Helicobacter pylori* interacts with the host's immune system, potentially impacting metabolism and other microbiomes in the stomach and gut[4]. It is possible to manipulate these interactions with mutant bacteria, which will exploit the wild-type bacteria. As a result, we applied this theory to *Helicobacter pylori* to see how it would shape the social interactions between these two different strains of bacteria. Consequently: This has brought us to our primary question:

To what extent does intraspecies interaction influence the composition of *H. pylori* communities?

1. SOCIAL EVOLUTION THEORY FOR MICROORGANISMS

Biologists have been fascinated by the study of cooperation for centuries, but the recognition of social behavior in microbes has only occurred much later. In the past, there was a prevailing assumption that bacteria and other microorganisms led solitary lives, lacking the cooperative behaviors that have attracted significant attention in the study of animals like mammals, birds, and insects. In the process of creating the social evolution theory, scientists focused on these animals, not giving much thought to microorganisms. Even though microorganisms, including bacteria, are responsible for the vast majority of biodiversity observed on our planet, only recently researchers reported microbes engaging in a diverse range of social behaviors. The wide spectrum of these behaviors allows the microorganisms to communicate, cooperate, and coordinate to perform behaviors such as dispersal, nutrient acquisition, biofilm formation, and quorum sensing[3]. Hence, the examination of their social behaviors presents a valuable prospect for assessing the social evolution theory across diverse groups of organisms.

When an action has effects on both the organism executing it and the recipient, it is considered social. When the recipient gains from a behavior that raises the actor's direct fitness, it is mutually beneficial; however, when the recipient loses, it is selfish. The word "cooperation" describes an action that improves the recipient's fitness; as a result, depending on how it affects the actor, cooperation may be altruistic or mutually beneficial[5]. Microorganisms' social interactions rely on a range of external actions generated by individual cells, which can impact the reproductive effectiveness of neighboring cells. Individuals share extracellular products and resources known as "public goods," such as enzymes, which benefit both the producer and the group as a whole. However, there are certain cells that have the tendency to not produce these public goods for the group. These cells, often referred to as 'cheaters', take advantage of the rewards of social actions without bearing any cost[6].

As previously pointed out, the theory of social evolution developed by drawing upon the initial observations made on animals. However, the cooperative behavior of organisms posed a challenge to the evolution theory based on the 'survival of the

'fittest' principle, as it could be susceptible to manipulation by non-cooperative individuals. Evolutionary experiments have demonstrated that a significant level of genetic similarity among cells typically leads to the observation of cooperative behavior.

The primary factor contributing to the genetic similarity between two individuals is their shared ancestry. Scientists use kin selection theory to explain cooperative behaviors within a species. In microbial contexts, beneficial molecules produced by bacteria can be used to illustrate the problem of cooperation in preventing cheaters from gaining the benefit of public goods. In bacteria, the phenomenon of clonal growth leads to the coexistence of closely related organisms, while limited diffusion ensures that public goods remain in close proximity to producers. As a result, the advantages of cooperation are typically distributed among cells that are related and possess the gene for cooperation. Therefore, cooperation is favored through kin selection [7].

Cooperation is a common practice among microorganisms, and the acknowledgment of the social aspect has led to an increased focus on studying microbial cooperation. Microbes have emerged as a highly suitable model system due to their rapid evolutionary processes, easy laboratory manipulation, ability to make mutants, and potential for molecular and genetic research. 'cheaters' that exploit the cooperation of others have the potential to undergo rapid evolution if they are selectively favored[8]. Consequently, using microorganisms to comprehend social evolution theory is more convenient and fast, and it has the potential to offer an alternative perspective to the theory that scientists overlooked due to their reliance on animal systems [3].

In this research, we focus on a notable example of such a microorganism: *Helicobacter pylori*. Despite research on other bacterial species, the social evolution of *H. pylori* has remained unexplored. Research indicates that these bacteria exhibit cooperative behavior by using signaling and binding to influence the host environment [9]. Therefore, we propose that *H. pylori* participates in social behaviors like cooperation and cheating within the host's stomach tissue, with a specific focus on the "virulence factor vacuolating cytotoxin A (VacA)" as a public

good. The first step in figuring out the social side of making a possible good is to compare the fitness of the wild type, which is thought to have social behavior, and the mutant, which doesn't, when grown separately in monocultures or in a mixture[3]

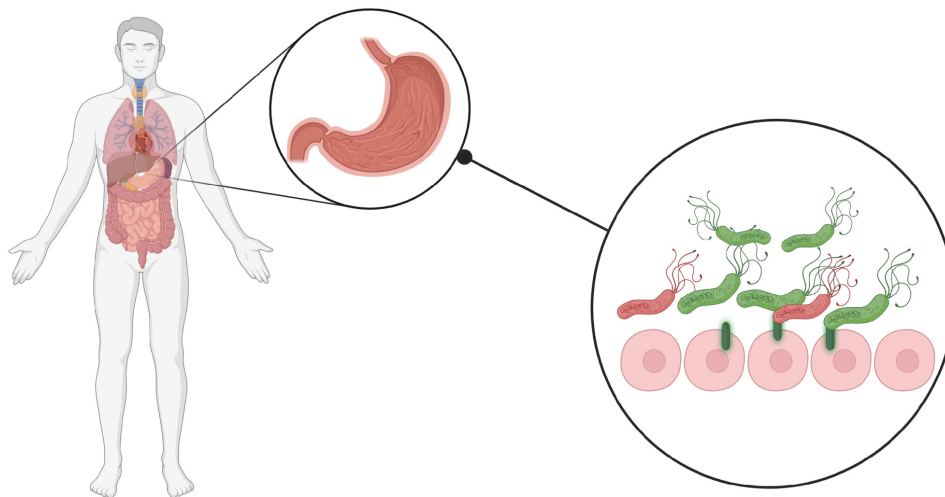


Fig.1. Representation of a social behavior in H. pylori as an example organism: cheaters (red) exploiting public goods produced by cooperators (green) inside the stomach.

This study aims to determine the relative advantage of non-producers when producers are present. This will be done by comparing wild-type and knock-out strains of certain genes in mice that were gavaged with mono- and mixed cultures. Furthermore, clinical implementation is still required. We expect the potential health consequences of microbial sociality, given that public goods produced by wild-type

organisms can benefit the entire group. As a result, *Helicobacter pylori*'s social interactions are critical to research because they can alter the microorganism's ability to infect individuals and persist throughout their lifetime, affecting host health[10].

2. HELICOBACTER PYLORI

2.1. H. pylori and humans: an ancient relationship

Helicobacter pylori is a gram-negative bacteria found in human stomachs. It is very common that they are the dominant microorganisms compared to other existing bacteria inside the stomach. They owe their persistent colonization to the genetic modification and evolution they went through over the course of years. These characteristics gave them an opportunity to survive the high acidity and innate adaptive immune system[2].

H. pylori is a prevalent bacterium that colonizes the stomachs of only humans naturally. It is a frequent occurrence for these microorganisms to exhibit dominance over the other coexisting bacterial species inside the gastric environment. The persistent colonization can be attributed to the genetic modifications and evolutionary changes they underwent over an extended period of time. The acquired traits enabled them to adapt and thrive in environments characterized by low acidity. Evidence gathered from experimental infections of mice shows *H. pylori* has undergone evolutionary changes that have resulted in a modification of the adaptive immune response of individuals towards the promotion of immunological tolerance[11]. *Helicobacter pylori* can either exist in humans as a transitory or persistent infection. Without antibiotic medication, early-life stomach colonization is extremely likely to last a lifetime. Research has demonstrated that *Helicobacter* specifically targets the immune system throughout the early stages of development and possesses the ability to modify certain pathways in its own favor. Researchers later discovered that this mechanism partially contributes to the prevention of asthma and allergic disorders[12].

2.2.History

H. pylori was first cultured from gastric tissue by Warren and Marshall in 1982. First, scientists were curious about the survival conditions inside the stomach. Because of its acidity, it was an unlikely place to be inhabited by microorganisms. To that end, Barry Marshall infected himself with the bacteria to prove his hypothesis was true, as well as the bacteria involved in the process of developing acute gastric diseases. Marshall later cured the infection with antibiotics and fulfilled Koch's postulates, initiating research into *H. pylori* as a pathogen[13].

The correlation between *H. pylori* and gastric cancer has garnered significant attention globally due to the classification of *H. pylori* as a "group 1 (definite carcinogen)" by the International Agency for Research on Cancer (IARC), a subsidiary body of the World Health Organization (WHO), in 1994[14]. Following the discovery of *Helicobacter*, the persistent colonization by the bacteria of animals was unsuccessful. Scientists have used these animal models to infer the effects of bacteria on humans, but they have not succeeded in mimicking the key features of the infection. Therefore, using mice would be economically and scientifically logical, considering its similarity with human immunological responses to *Helicobacter*. Another challenge was developing a bacterial strain capable of effectively colonizing the mouse. In 1997, Lee et al. developed the mouse-adapted Sydney strain (SS1) of *H. pylori*, which has the ability to express some of the key factors of the bacteria, such as *VacA* toxin. This strain showed promising results in terms of colonization persistence. Moreover, the C57BL/6 mouse had higher colony-forming units (10⁶–10⁷ CFU/g) in the stomach tissue. However, the pre-mouse Sydney strain (PMSS1) can also infect mice and express more virulence factors than SS1, such as *cagA*, and this was the strain we used in our experiments[15].

2.3.Morphology

H.pylori is primarily characterized by a curved to spiral morphological form, although certain members exhibit either a short or tapered rod shape. Most *Helicobacter* exhibit a fundamental morphology characterized by an S shape and polar, sheathed

flagella. However, there are variations in the size and number of spirals and turns observed. The primary components of *H. Pylori* flagella are the basal body, hook, and flagellar filament. The flagellar filament comprises two flagellins, namely FlaA and FlaB, which are encoded by the genes *flaA* and *flaB*. FlgE forms the hook structure, connecting the basal body and flagellar filament. The basal body consists of multiple protein structures and serves as a crucial energy source for facilitating motility [16]. The organism's spiral morphology and flagella facilitate its ability to navigate through the gastric mucus gel. Its length ranges from 0.5 to 5 μm , and it possesses a cluster of 5 to 7 polar sheathed flagella. The observed morphology has been found to be associated with the highest level of in vitro motility. The morphology of organisms also exerts influence on various physiological processes, such as their ability to move and spread, their capacity to form microbial aggregates or biofilms, their ability to withstand environmental stressors, and their interactions with other organisms [17].

2.4.Persistence and adaptability

H. pylori establishes itself in our gastrointestinal tracts during childhood and likely remains present throughout our lifespan. This suggests a high level of adaptation to the specific niche and the capacity to elude the innate immune response of the host. The spiral shape and flagella of the organism enable it to move in a corkscrew motion through the gastric mucus gel. Additionally, it has multiple adhesins that allow it to selectively adhere to the epithelium. *Helicobacter pylori* possesses various mechanisms to defend against gastric acid, one of which is the acid acclimation mechanism. Significantly, 15% of bacteria's protein composition is comprised of preformed cytoplasmic urease[2]. When the pH of the external environment falls below 6.5, a distinct channel is activated in the cytoplasmic membrane of bacteria, facilitating the entry of urea. The neutralization of the periplasm by ammonia generated through urea hydrolysis facilitates the preservation of the cytoplasmic membrane potential. *H. pylori*, similar to other human commensal bacteria, has developed distinct mechanisms to evade activating the immune response[18].

H. pylori moves through the gastric mucosa epithelium layer to the basal layer, where the pH value is close to 7.0, by the action of polar sheathed flagella. Previous studies

showed that flagella-mediated motility is essential for the *H. pylori* colonization of the gnotobiotic piglet and mouse gastric mucosa. Mutagenesis of just about any gene in the motility and chemotaxis systems abolishes the ability of *H. pylori* to infect the stomach and establish colonization[6].

2.5. *H. pylori* and disease

H.pylori can colonize the stomach for decades without causing harm, but its presence is linked to an increased risk of diseases such as peptic ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma. *H.pylori* is a bacterium that mainly resides in the gastric mucus layer and does not directly engage with host cells. Nevertheless, certain organisms adhere to gastric epithelial cells and sporadically become intracellular. This adherence activates multiple signaling pathways and can result in the release of toxins or effector molecules. *H.pylori* triggers an immune response in humans, leading to the infiltration of leukocytes into the affected tissues in individuals who are consistently colonized by the bacteria. The host's inflammatory response to *H. pylori* is relatively mild, but it is more significant and intricate compared to the response to other bacteria in the intestinal lumen. Some strains of this bacterium produce factors that help them attach to the host epithelium; however, the majority of the bacteria (non-producers) present in the mucus do not produce the tools to help them contact the tissue [19].

In this study, by adapting social evolution theory to microorganisms, we try to explain whether or not an *H. pylori* non-producer of a key virulence factor could exploit wild type producer *H.pylori* in the host environment.

2.6. Virulence factors of *H. pylori*

Colonization is linked to the infiltration of inflammatory cells into the gastric mucosa, which is referred to as gastritis. Various strains of *H. pylori* elicit different levels of gastric inflammation, which can be attributed to their unique capacities to engage with the host [20]. Certain strains exhibit "host interaction" factors, which are also

referred to as virulence factors. The mechanism by which these factors contribute to disease is increasingly being comprehended, although the advantage for *H. pylori* in possessing them remains uncertain. One possible explanation is that the alterations in epithelial cells caused by *H. pylori*, either directly or potentially through inflammation, facilitate enhanced nutrient transportation to the bacterium. Furthermore, these interactions with hosts can lead to changes in the ecological niche, either creating more favorable conditions for the survival of *H. pylori* or creating less favorable conditions for the survival of competing bacteria [18].

There are several known toxins in *H. pylori* that utilize colonization and persistence, including *vacA* and *cagA*. In this research paper, we are focusing on *VacA* toxin.

Our main research question concerning this toxin is:

Is the *vacA* factor a prerequisite for *Helicobacter pylori* to grow in the host's stomach tissue? And if the production of *vacA* is cooperative or not?

We aim to answer this question by comparing the growth and quantity of bacteria in monoculture and mixed cultures in vitro and in vivo (mice stomach). Using *vacA* to knock out *H. pylori* and wild-type *H. pylori*, who produce this factor.

2.6.1. Vacuolating cytotoxin A

There are many bacterial toxins known to enter the plasma membrane, disrupting the host's metabolism. Unlike most of them, the *vacA* toxin has no enzymatic activity. By forming anion channels on the host membrane, it has been classified by scientists as a pore-forming toxin[21].

The gene *VacA* of *H. pylori* encodes a secreted protein, *VacA*, which was first discovered due to its capacity to cause vacuolation in cultured epithelial cells. All strains possess the *vacA* gene; however, there is significant variation in the *vacA* sequences among different strains. The regions with the highest diversity are found

at the 5' signal terminus, the mid-region, and the intermediate region. VacA sequence diversity is associated with variations in vacuolating activity. The transcriptional start site of the *H. pylori* vacA gene is situated approximately 120 nucleotides from the start codon. The presence of a stem-loop structure in the 5' untranslated region (UTR) of the vacA transcript enhances the stability of the vacA mRNA, especially in situations of environmental stress. The expression of vacA is controlled in accordance with the growth phase, with the most significant levels of transcription taking place during the late logarithmic phase. Some research studies suggest vacA transcription is controlled in response to factors such as low pH, iron concentration, salt concentration, and bacterial interaction with host cells [21], [22].

The vacuolating cytotoxin A gene is thought to produce a protoxin with a mass of 140 kDa. The released vacA toxin consists of the p33 and p55 domains, which are vacA functional domains, later forming an oligomeric structure. The exposure of VacA oligomers to either acidic or alkaline pH leads to the breakdown of VacA oligomers into individual monomers. It has been suggested that VacA initially binds to the plasma membrane of host cells as a single molecule, where it then forms a group of molecules and inserts itself to create a functional membrane channel [23]. This oligomeric complex has the ability to integrate into the host cell membrane and functions as an anion-selective channel. This channel has the ability to secrete bicarbonate and organic anions into the cytoplasm of the host. This channel is thought to have an effect on the colonization of *H. pylori* as it allows the efflux of molecules that could be important for growth [16].

2.6.2. VacA factor in the immune response of the host

VacA is considered a multifunctional toxin and is suggested to affect the host system in multiple possible ways:

Endosomes are intracellular organelles enclosed by a membrane and primarily responsible for the organization and transportation of lipid vesicles and their contents to and from the cell's outer membrane. Endocytosis is a cellular process in which a substance is enclosed by a cell membrane, forming a small sac internally that

contains the ingested substances. Before the VacA toxin can do anything, it must first undergo endocytosis to be internalized. Both the p33 and p55 subunits of VacA are necessary for the process of internalization, and the formation of vacuoles relies on the acid-activation of VacA. Although VacA primarily binds as a single molecule, its ability to create vacuoles depends on the formation of oligomerized VacA. The VacA channel, which is taken up by endocytosis, permits the entry of anions into late endosomes. This results in the buildup of weak bases and subsequently leads to the formation of large vacuoles due to the influx of water[24].

Mitochondria, the energy-producing organelles, are essential for a wide range of cellular processes in humans. Their main responsibilities include carrying out aerobic respiration, producing adenosine triphosphate (ATP), creating fatty acids, and aiding in the formation of iron-sulfur clusters. Previous research also suggests that VacA, when applied externally, specifically targets mitochondria[25].

Moreover, mitochondria play a crucial role in the intrinsic apoptotic pathway, regulating cellular self-destruction when needed: Leading to release of cytochrome c from mitochondria into the cytosol, triggering the activation of pro-caspases and ultimately resulting in cell death [24].

This is evidenced by its ability to trigger the release of cytochrome C, endoplasmic reticulum stress, and apoptosis. VacA perturbs the equilibrium between cell proliferation and apoptosis by influencing genes that govern the cell cycle. Moreover, VacA not only induces vacuolation but also stimulates apoptosis in gastric epithelial cells [9].

During apoptosis, cytochrome c is released from the mitochondrial intermembrane space into the cytoplasm through an unknown mechanism. This triggers downstream executioner caspases, leading to cell death. It is hypothesized that VacA can form membrane-embedded pores at the inner mitochondrial membrane, causing the mitochondrial electrochemical membrane potential (ψ) to dissipate. VacA-mediated reduction in ψ has been linked to the release of cytochrome c [26]. Research has demonstrated that the inhibition of vacA channel formation hinders the release of cytochrome c, indicating that the formation of pores is a prerequisite for cellular apoptosis [25].

There are some hypothesis about how vacA reaches the mitochondria such as; vacA forming ionic channels within vacuoles and release vacA monomers into the cytoplasm or osmotic swelling leading to destruction and release of vacA factor into the cytoplasm[27].

While the exact impact of VacA on the overall harmful effects of *H. pylori* is not yet fully comprehended, this toxin seems to be involved in the initial stage of the infection. Additionally, by affecting the immune response of cells, it may contribute to the long-term presence of the infection[11]. VacA disrupts T-cell activation and proliferation by manipulating the T-cell receptor pathway and the cell cycle. VacA disrupts T cell activation pathways, impeding cell division and the release of IL-2(Interleukin-2), which is a cytokine that is synthesized by mainly T cells, as a reaction to antigen stimulation, potentially by causing cell cycle arrest. Additionally, it interferes with the process of calcium signaling, thereby inhibiting the transcription of IL-2. The interaction between VacA and T cell components, as well as other immune molecules, indicates that VacA has a broad influence on the immune response[28]. Moreover, CD4+T cells, along with CD8+T cells, make up the majority of T lymphocytes. CD4 t cells mediate the immune response, and VacA specifically affects human CD4 t cells. It does this by blocking the activity of a transcription factor called the nuclear factor of activated T cells, which in turn inhibits the proliferation of T cells. VacA not only affects CD4 T cells, but it also hinders the presentation of antigens by B cells and disrupts the normal activities of CD8 T cells, macrophages, and mast cells[18].

2.7. H.pylori and benefits of its infection

The recent disappearance of *H. pylori* from human populations marks a significant shift from our long-standing microbial companionship. As *H.pylori* considered to be part of humans normal microbiota, our physiological and immunological systems have evolved with *H. pylori*'s persistent colonization of the stomach. Therefore, this absence disrupts these finely tuned systems, which can be the cause of modern diseases. From an evolutionary standpoint, the presence or absence of *H. pylori* could have been relatively neutral. However, from a 21st-century perspective, its

absence appears to have costs, especially later in life. This emerging understanding suggests early-life exposure to *H. pylori* may be beneficial, while its absence later in life may contribute to disease susceptibility. For example, the lack of *H. pylori* is believed to have wider implications for current health issues, such as obesity and allergic disorders[12].

As new evidence emerged researchers have become interested in the complex connection between the colonization of *Helicobacter pylori* (*H. pylori*) and allergic disorders. This has provided insight into the possible immunological consequences of its absence in modern populations. The core of this investigation centers around the hygiene hypothesis, which suggests that being exposed to infections during early childhood influences immune responses, possibly protecting against allergies and autoimmune diseases. Furthermore, this hypothesis is supported by the "disappearing microbiota hypothesis"; the decreasing presence of certain well-established microbial companions, such as *H. pylori*, may be a factor in the increasing occurrence of allergic disorders[12].

Studies have suggested a negative correlation between *H. pylori* and allergic diseases, although the presence of factors such as crowded living conditions and childhood hygiene levels make it difficult to interpret the results. However, there have been proposed mechanisms focusing on the role of regulatory T regulator cells (Tregs) in controlling immune responses. Research indicates that there is a connection between the presence of *H. pylori* and increased levels of Tregs, and these cells display immunosuppressive characteristics, which may play a role in preventing allergic diseases. Studies conducted on mice have provided additional insight into this phenomenon, revealing that *H. pylori*-induced regulatory T cells (Tregs) inhibit other immune responses, thereby promoting the colonization of the bacterium. These findings suggest that there may be a direct connection between *H. pylori*, Tregs, and the decreased likelihood of developing allergic and autoimmune diseases[18].

Further evidence supporting this hypothesis is that the strains of *H. pylori* that have a higher level of interaction with the host, as compared to other strains, are more likely to be linked to the prevention of modern diseases. However, in order to completely

clarify this hypothesis, it is essential to conduct additional interventional and mechanistic studies using appropriate animal models and human populations.

As research progresses in understanding the complex relationship between *H. pylori* colonization and immune regulation, we may gain a better understanding of its role in allergic disorders. And it would be more rational to study this association in developed countries, where *H. pylori* infections are becoming less common as a result of antibiotic use and adapting hygiene standards in daily life. This knowledge could potentially lead to the development of new therapeutic strategies and interventions[2].

METHODOLOGY

1. Infection of C57BL/6 neonatal mice with PMSS1/PMSS1-VacA *H. pylori*

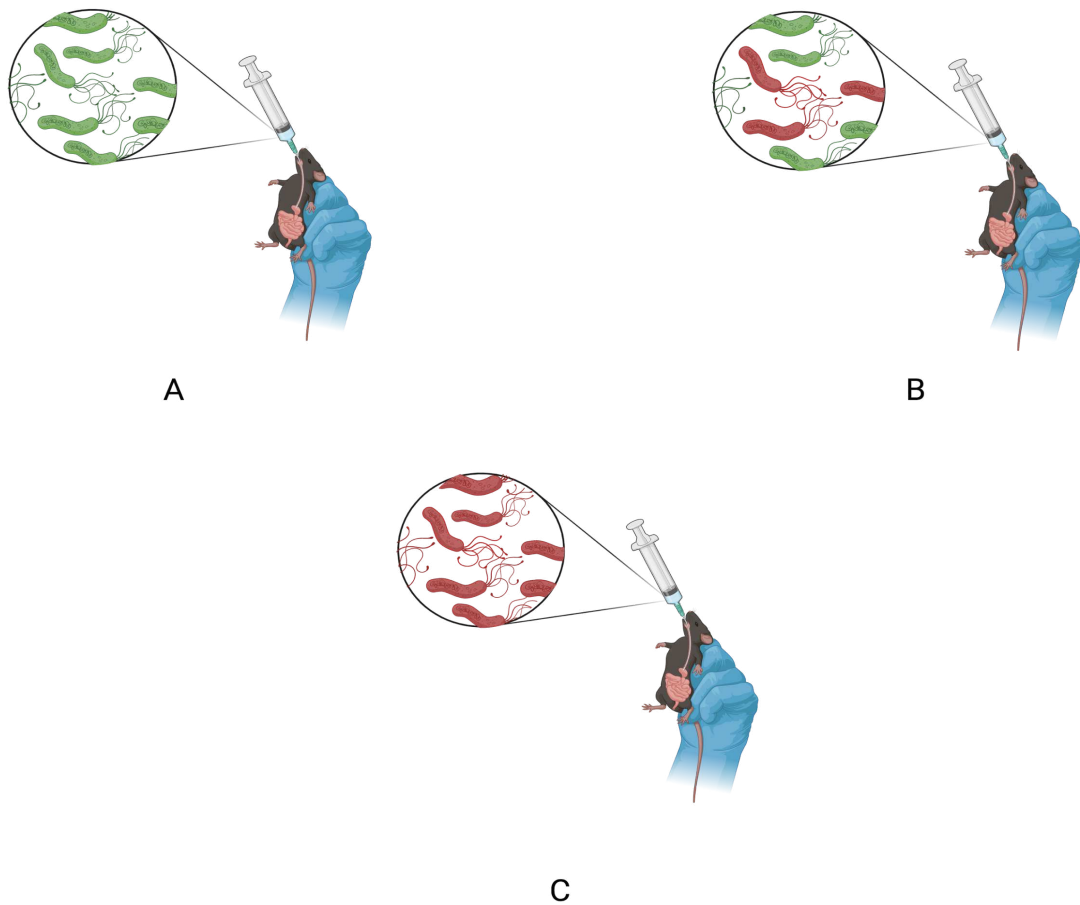


Fig.2. Mice are infected with Helicobacter pylori solution via oral gavage. A) C57BL/6 mouse infected with PMSS1. B) C57BL/6 mouse infected with PMSS1-vacA. C) C57BL/6 mouse infected with PMSS1 and PMSS1-vacA.

We used the standard C57BL/6 mouse model in these experiments, which we purchased from Janvier aged 6 weeks. Mice were split into three groups of five mice

each. The pre-mouse Sydney strain (PMSS1) can also infect mice and express more virulence factors than SS1, such as *cagA*, and this is the one we use. These groups were then given a 200µl solution of *H. pylori* PMSS1, PMSS1-*vacA*, or a mix of the two by mouth. The first group was infected with PMSS1-*vacA H. pylori*, the second group with PMSS1 *H. pylori*, and the third group with a mix culture of these two strains, respectively; 1:10 *vacA* to wild type[29].

The solution of *H. pylori* was grown on TSA sheep blood plates (Thermo Fisher Scientific) to count the initial bacteria amount with the spot plating method. The method is executed as follows: A blood agar plate is used as a growth medium for *H. pylori* solution. We prepared serial dilutions of the original solution and using a pipette, 10 ul of the solution are spotted onto separate sections of the agar in a way that prevents the bacteria from overlapping inside solutions that are diluted differently. The blood agar was incubated, allowing bacteria to form colonies. After incubation, colonies were counted in the 10⁻⁵ diluted solution by using the following formula[13]:

$$\text{CFU/mL} = (\text{Volume plated in mL} \times \text{Colonies}) \times \text{Dilution factor}$$

2. *H. pylori* quantification in vivo

After five weeks, mice were killed by cervical dislocation and tissue samples were collected from the stomach. The antral part of the stomach was cut into four almost equal parts, and the tissues were sealed inside a sterile 1.5 mL tube. DNA extraction from ¼ of the tissue samples was carried out using the Qiagen DNA purification kit.

For the DNA extraction, 180µl of ATL Buffer and 20µL of Proteinase K are added into a 1.5µL tube containing the mouse sample. Tubes were vortexed. Then, samples were placed in the 56°C incubator for 3 hours. After incubation, samples were vortexed, added 200µl of AL buffer, and vortexed again. 200µl of absolute ethanol was added, and the whole volume was transferred on to the spin column. Samples were centrifuged at 8000 rpm for 1 minute, and the flow-through was discarded. After adding 500 µl of Buffer AW1 and centrifuging at 8000 rpm for 1 minute, the flow-through was discarded. Followed by the addition of 500µL of Buffer AW2 and centrifugation at 13000 rpm for 3 minutes, discarding flow-through. The spin column was placed on a new sterile 1.5-mL tube, and the DNA was eluted in 100 µL elution buffer AE. Samples were left at room temperature for 1 minute. Tubes were centrifuged at 8000 rpm for 1 minute, and this step was repeated one more time. Followed by a measurement of the DNA content of the samples with the Qubit assay. Later, the DNA solution was kept in the freezer for storage.

Firstly, to detect bacterial infection, conventional PCR was performed with OipA primers targeting for amplification a region upstream of the OipA gene (SB Andersen, unpublished).

OipA F: CGCCGATACTACCTTGTCCCT

OipA R: GAAGTATCCAGGCGCTCCAT

Next, *H. pylori* in samples were quantified using the quantitative PCR technique (qPCR). The qPCR was conducted using a 20-µL reaction mixture comprising

10 μ L of 2x qPCR SyGreen Mix, 0.8 μ L of forward primer and 0.8 μ L of reverse primer, and 2 μ L of template.

Primers used in this study were glmM F/R and AphA3 F/R. Two different experiments were set, one with glmM targeting a sequence present in both strains of *H. pylori* and one with AphA3 primers targeting the kanamycin resistance gene, used for creating the vacA knock-out *Helicobacter pylori* (PMSS1-vacA), and therefore only present in this strain.

The Mx3005P qPCR machine was used to perform duplicate runs of the samples and standards. The qPCR protocol involved an initial denaturation step at 95 °C for 3 minutes, followed by 40 repeated cycles of 95 °C for 5 seconds and 60 °C for 30 seconds, and 1 cycle of 95 °C for 1 minute, 60 °C for 30 seconds, and 95 °C for 30 seconds.

3. *H. Pylori* PMSS1/PMSS1-vacA in vitro growth assay

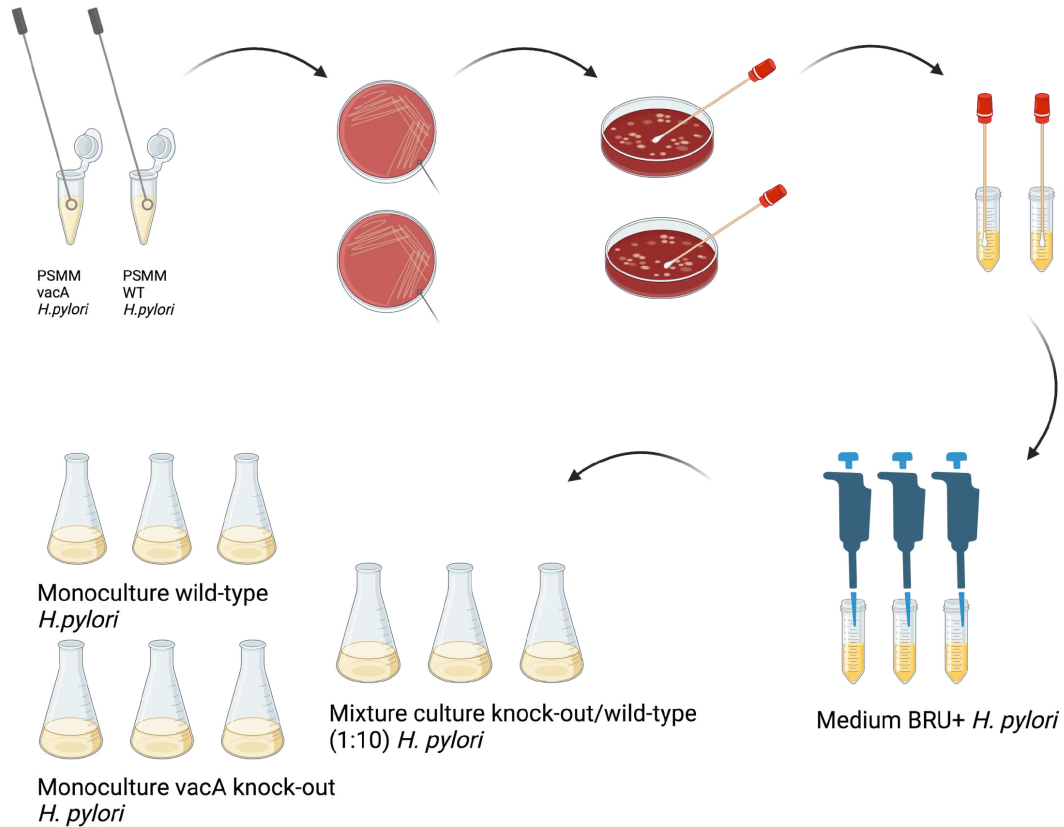


Fig. 3. Presentation of work flow for *H. pylori* growth in vitro.

H. Pylori strains were grown on TSA sheep blood plates (ThermoFisher Scientific). Firstly, it was plated on a blood agar with the streaking method. With a sterile loop, bacteria are collected from a glycerol stock and are successively streaked over the agar medium in different patterns. As the streaking proceeds, bacterial cells are spread over the blood agar and kept in an incubator at 37 °C, 10% CO₂, and 6% O₂ over three nights.

After growth on these plates, bacteria colonies are collected by scraping the surface of the agar with a sterile tool, and cells are re-suspended in 1.5 mL of Brucella broth. The bacterial solution was inoculated into liquid Brucella broth media with 10% heat-inactivated fetal bovine serum (FBS) and vancomycin. Cultures were incubated overnight with shaking at 100 rpm. The shaking helps to distribute nutrients evenly

and prevents the clumping of bacterial cells. The following day, the motility of bacteria is checked with a microscope. Later cultures were centrifuged at 2000 rpm at room temperature for 10 minutes, yielding culture supernatants and bacterial pellets. The supernatant is discarded, and the pellet is saved. The pellets composed of bacteria are re-suspended with 500 µl of BrucElle media.

Two replicates are made for each monoculture of knock-out and wild-type *H. pylori*, and a mixture culture is made using 100µl from previous cultures. 2 replicates for WT bacterial cultures (300µl), 2 replicates for KO bacterial cultures (300µl), 2 replicates for the 1:10 KO-WT mixture (270µl WT + 30µl KO, 90µl PMSS1 strain, and 10µl PMSS1-VacA strain per culture), and the control culture without the bacteria. In total, nine flasks are used, each containing the relevant strain or strains (100 µl) and Brucella media (18 ml) with FBS (400 l) and 0.06 mg/ml Vancomycin. The bacteria are standardized and added at the same initial density in all liquid cultures (OD600 of 0,006), ensuring consistency in the starting conditions for the experiment and allowing for accurate comparisons between different cultures or experimental conditions. The cultures are left to incubate for 72 hours.

The OD600 spectrophotometer is used due to its working principle, which measures the scattered light by the existing microorganisms inside the solution to give an idea about the density of the bacteria in a sample. Therefore, to assess the microbial growth in solutions, an aliquot of 100µL was taken and processed by an OD600 spectrophotometer for the time points at which bacteria were checked. Another 100µL aliquot was taken at serial time points (12, 24, 48, and 72 hours post-inoculation), for a total of 4 samples for each culture.

4. *H. pylori*(PMSS1-PMSS1/vacA) quantification in vitro

DNA extraction on longitudinal samples was performed using the QIAGEN DNeasy Blood and Tissue Kit. For the DNA extraction, cells were harvested by centrifuging the tubes of the previously stored culture for 10 minutes at 5000 x *g* (7500 rpm). Supernatant discarded. The remaining pellet was resuspended in 180µl of ATL Buffer. DNA was extracted using the following modifications to the protocol: The solution was incubated at 56°C for 1 hour. At the end, DNA was eluted in 100 µl of elution buffer AE.

A real-time/ quantitative PCR (qPCR) assay is used to amplify the targeted DNA sequence of *H. pylori* and detect the amount of genetic material in our given samples. For this essay, two different experiments were run with different sets of primers, both for in vivo and in vitro samples. Primers were chosen according to their binding ability to targeted sequences. A master mix solution was prepared, including SyGreen Mix Lo-ROX (PCRBIO SYSTEMS), forward and reverse primers, ddH₂O, and the template DNA. A positive and a negative control were added, as well as six standards, while running the qPCR assay.

Standards were made by amplifying 600 bp region surrounding the target site by PCR, purifying the PCR product, quantifying the concentration by Qubit, and making serial dilutions for building a standard curve, included in all qPCR assays.

Real-time PCR with glmM primers: A primer pair was developed for detecting the glmM gene in *H. pylori* by the utilization of polymerase chain reaction (PCR). The glmM gene plays a crucial role in bacterial cell wall formation and microorganism growth, and it can be found in any *H. pylori* strain. Therefore, this primer is used to detect *H. pylori* genetic material present in the given sample.

glmM R: 5'-GCATTCACAACTTATCCCCAATC-3'

glmM F: 5'-GGATAAGCTTTTAGGGGTGTTAGGGG-3'

Real-time PCR aphA3 primers: In the process of generating a knock-out strain of *Helicobacter*(PMSS1-VacA) for the vacA factor, the vacA gene was excised and

subsequently replaced with a kanamycin-resistant gene within the organism's genome.

Positive selection involves the introduction of a kanamycin-resistant gene, specifically a neo cassette that is designed to be compact and short in order to minimize the likelihood of random insertions. Subsequently, the growth medium is supplemented with the antibiotic kanamycin, which selectively inhibits growth of cells lacking the mentioned gene. As a result, only the knock-out bacteria are obtained. The *aphA3* primers amplify the kanamycin gene area in the knock-out strain, whereas they are unable to amplify the wild-type strain (PMSS1) due to the absence of the corresponding resistance gene.

aphA3 F: ATCGAGCTGTATGCGGAGTG

aphA3 R: CGCGCGGATCTTTAAATGGA

5. Statistical analysis and Plots

Data were analyzed in R (R.4.2.2.) using the packages tidyverse, ggplot2, and dplyr.

Our data from the OD spectrometer were grouped by different variables such as "Strain" and "Time". Our "Time" points indicate the beginning, 12h-, 24h-, 48h-, and 72h- post-incubation and "Strain" for WT, KO, and MIX *H. pylori*. Sample size, average OD600, and 95% confidence limits around the mean among the replicates were calculated using R. ggplot2 was used to plot the mean OD600 values over a period of time for each strain.

For statistical testing of in vivo and in vitro samples, a one-way ANOVA was conducted. Tukey's Honest Significant Difference (HSD) test was conducted for post-hoc comparisons to identify specific differences between groups when the ANOVA indicated significant differences. Additionally, the Kruskal-Wallis test was conducted to compare the medians of more than two groups.

RESULTS

1. Quantification of *H.pylori* in vivo samples

The second experiment set up was in vivo environment. The experiment started by introducing knock-out *H.pylori* and wild-type *H.pylori* to neonatal mice.

The mouse in vivo experiment was conducted infecting 3 separate groups of 5 mice. The aim was to determine if *vacA* knock-out (KO) *H.pylori* would exhibit improved outcomes when coexisting with wild-type (WT) *H.pylori* or when existing independently. In order to conduct an experiment, I analyzed the stomach tissues of mice that have been infected for 5 weeks with three different bacterial inocula: KO, WT, and a mixture of KO and WT with a ratio of 1:10.

1.1. Spot plating of the initial concentration of *H.pylori* used to infect mice

The colony-forming units of *H. pylori* were counted for 10–5 dilutions and plated with 10µl for three different solutions. We used the formula provided in the methods section of this paper.

We count 44 and 39 colonies of wild-type *H. pylori* in replicate plates for the solution that infects the first group of mice. The estimated bacterial count inside the 200 µl of solution was 83 million.

We count 43 and 45 colonies of knock-out *H. pylori* in replicate plates for the solution that infects the second group of mice. The calculated amount of bacteria inside the 200 µl solution was 88 million.

We count 35 and 29 colonies of the mixed culture of KO and WT *H. Pylori* in replicate plates for the solution that infects the third group of mice. The amount of mixed bacteria inside the 200µl solution was 64 million.

1.2. qPCR with glmm primers

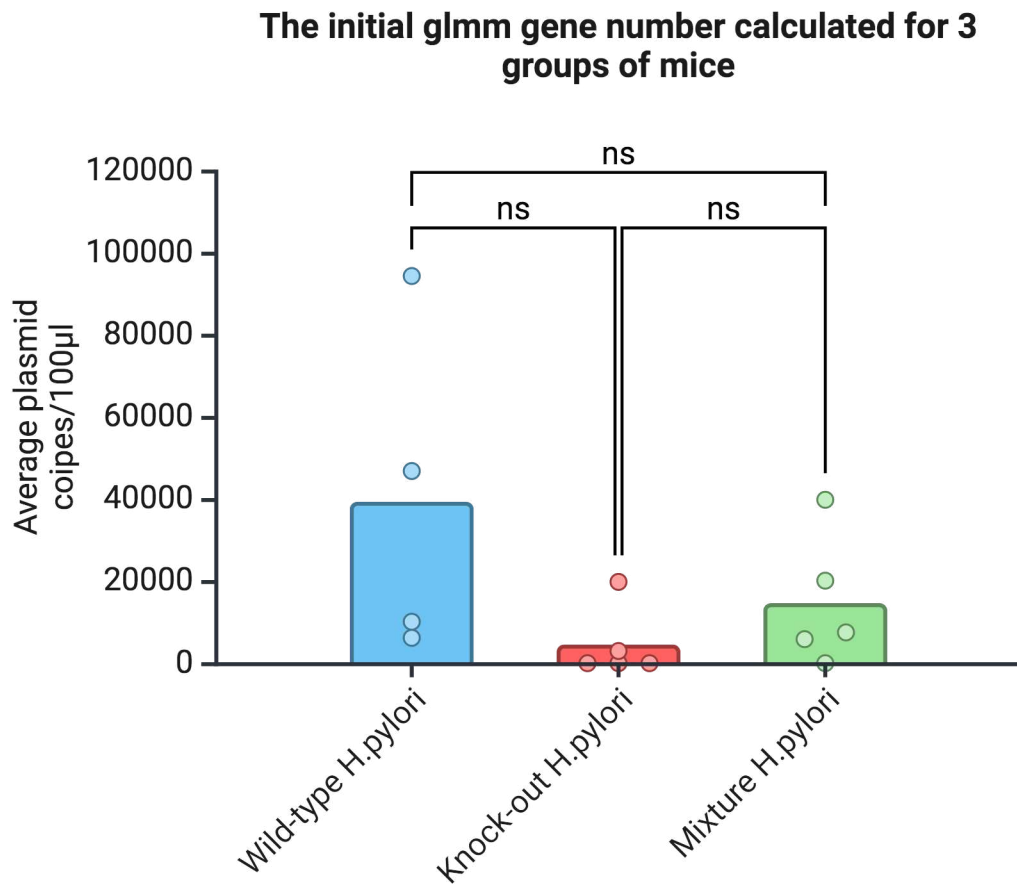


Fig.4. Bar graph representation of mice groups infected with KO, WT, and a mixture of KO and WT with a ratio of 1:10.

We found that the quantity of DNA in the mixed environment is lower than that in the wild-type environment (Fig.4.). The final quantification of knock-out bacteria is lower than that of wild-type bacteria. Even though the comparison between groups in the bar graph shows the WT *H. pylori* had a better chance of establishing itself inside the stomach compared to the mixture and KO *H. pylori*, there were large variations between samples and the amount of bacteria was not significantly different between these groups determined by one-way ANOVA ($F(2,11) = 2.418$, $p = .135$).

1.3. qPCR with *aphA3* primers

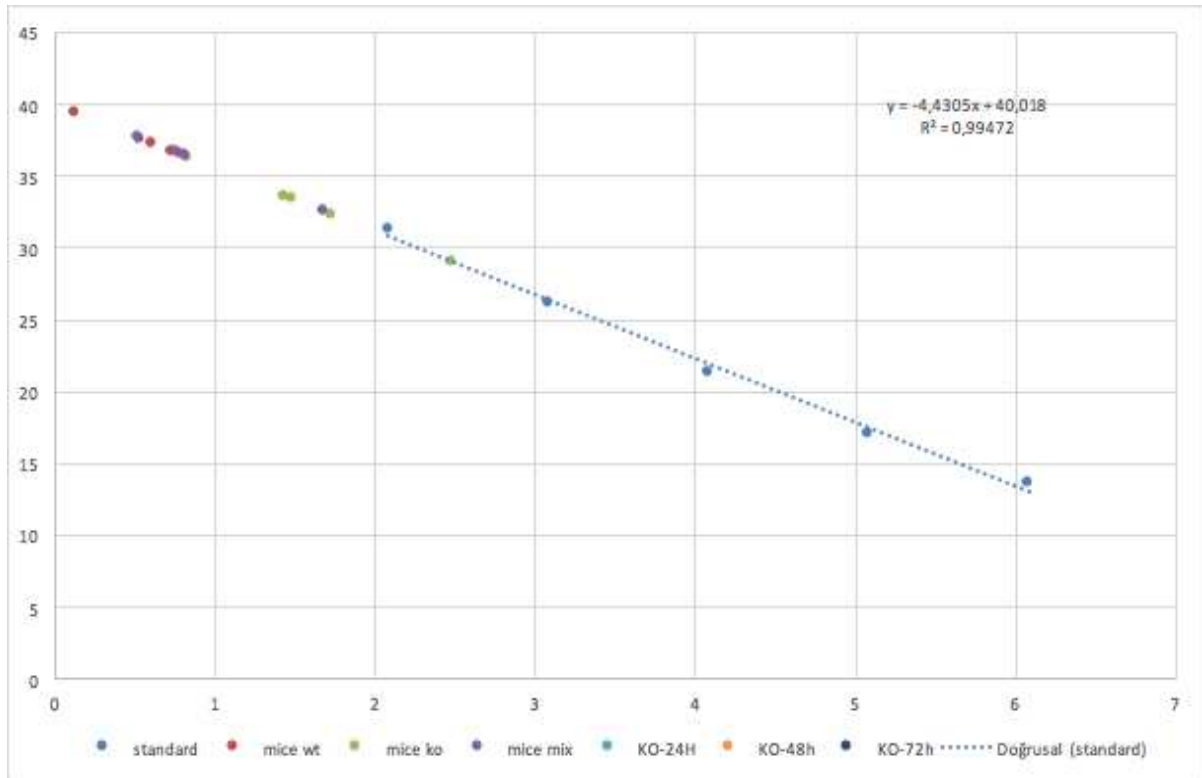


Fig.5. Graph illustrates the standard curve derived from quantitative polymerase chain reaction (qPCR). Data obtained from DNA samples collected from the stomach tissue of mice (y-axis representing the Ct values and x-axis representing the log(gene copy) number).

The cycle threshold (Ct) values observed in the in vivo samples were rather high, indicating a low abundance of gene copies compared to the standards. This is what we anticipated since *aphA3* primers are useful when used on knock-out bacteria because they can only recognize and bind a particular area present in these bacteria. In the context of in vivo samples, it was hypothesized that the knock-out samples would exhibit reduced infection rates due to the absence of a crucial genomic component that facilitates bacterial survival within the host. Therefore, it is expected that we detected low number of gene copies which are under the detection limit with

qPCR suggesting that KO strains indeed were not successful colonizing on their own.

The issue with the low detection of these copy numbers was that we could not detect the number of KO in the mixture samples, which could ultimately determine whether our hypothesis could be true or not. Additionally, we could have determined if the KO/WT ratio fluctuated over time and whether the KO benefited from the WT bacteria. We expected this to happen since the KO is a cheater and can use the product-vacA toxin that the WT bacteria (which is a cooperator) produced to colonize itself better in the stomach. As a result, we expected an increase in the KO/WT ratio, but because the qPCR was not specific enough, we could not detect the number of KO bacteria and, consequently, the WT bacteria in the mixture samples.

2. Quantification of *H.pylori* bacterium in vitro

The purpose of my research project is to investigate the impact of vacA on the total infection rate of *H. pylori*.

We conducted two separate experiments. The first one took place in an in vitro setting. We performed an in vitro experiment where we individually and collectively transferred distinct strains of bacteria into a liquid culture to observe their growth within a defined time frame. As vacA is a virulence factor that exploits the host's tissue to colonize more rapidly, it is not beneficial for the bacteria in vitro since there is no live tissue available for exploitation. Hence, based on the in vitro experiment, we anticipated observing a negligible disparity in the bacterial growth across various experimental configurations, or a slight cost to the wild-type strain if it produces vacA in these conditions.

2.1. Quantification of *H. pylori* bacterium using Optical Density Spectrometer

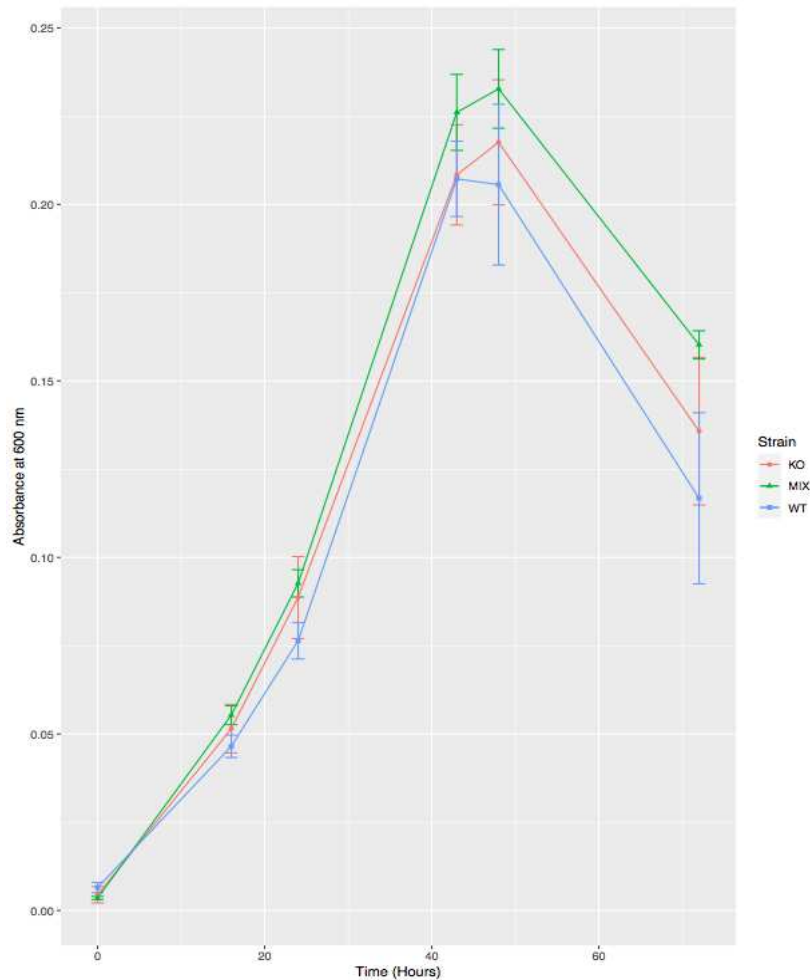


Fig.6. OD measurements were taken longitudinally for 72h from 100 μ l taken from the bacterial cultures.

The optical density measurement exhibited minor variations in bacterial growth over the time period, which was subsequently determined to be statistically insignificant. This outcome was anticipated due to the consistent conditions of the environment, including temperature, shaking rate, and compounds present in the cultures. The sole distinguishing factor was the *H. pylori* strains used in this experiment.

To ensure an accurate comparison between the growth rates of monocultures and mix-cultures, the experiment started with an identical concentration of bacteria in each flask, thereby eliminating any potential misinterpretation.

The graph shows consistent growth for both mono-cultural and mixed-cultural groups, up until the 48-hour incubation period.

However, the OD spectrometer recorded a drop in density during the 48–72-hour period. We anticipated this, given that the bacteria had entered the phase of decline or death in their growth cycle. This can be attributed to insufficient nutrition for an over-growing population and a lack of available space. The OD measure does not distinguish between dead and alive cells but the decrease in OD may be due to clumping.

Consequently, this graph suggests there are no significant changes when it comes to growth rate between different strains of *H.pylori* in the beginning, 12 hour period and 48.

- At the 0 and 12 hours time points, the p-values (0.452 and 0.541 respectively) suggest that there are no significant statistical differences in the OD600 values between the strains throughout these initial time periods.
- At the 24-hour mark, there is a significant difference between the strains (p-value = 0.002), indicating that the strains exhibit dissimilar growth patterns at this point.
- At the 48-hour mark, the p-values of 0.059 exceed the standard significance level of 0.05, indicating that there are no significant changes.
- After 72 hours, there are substantial differences in growth between the strains (p-values= 0.031 and 0.020 respectively), indicating that the strains exhibit noticeable variances in growth over time.

We only saw a difference in growth rate between the strains during measurements in 24 hour and 72 hour compared to other time points which they show no significance.

2.2. Quantification of *H.pylori* bacterium with qPCR and Qubit assays

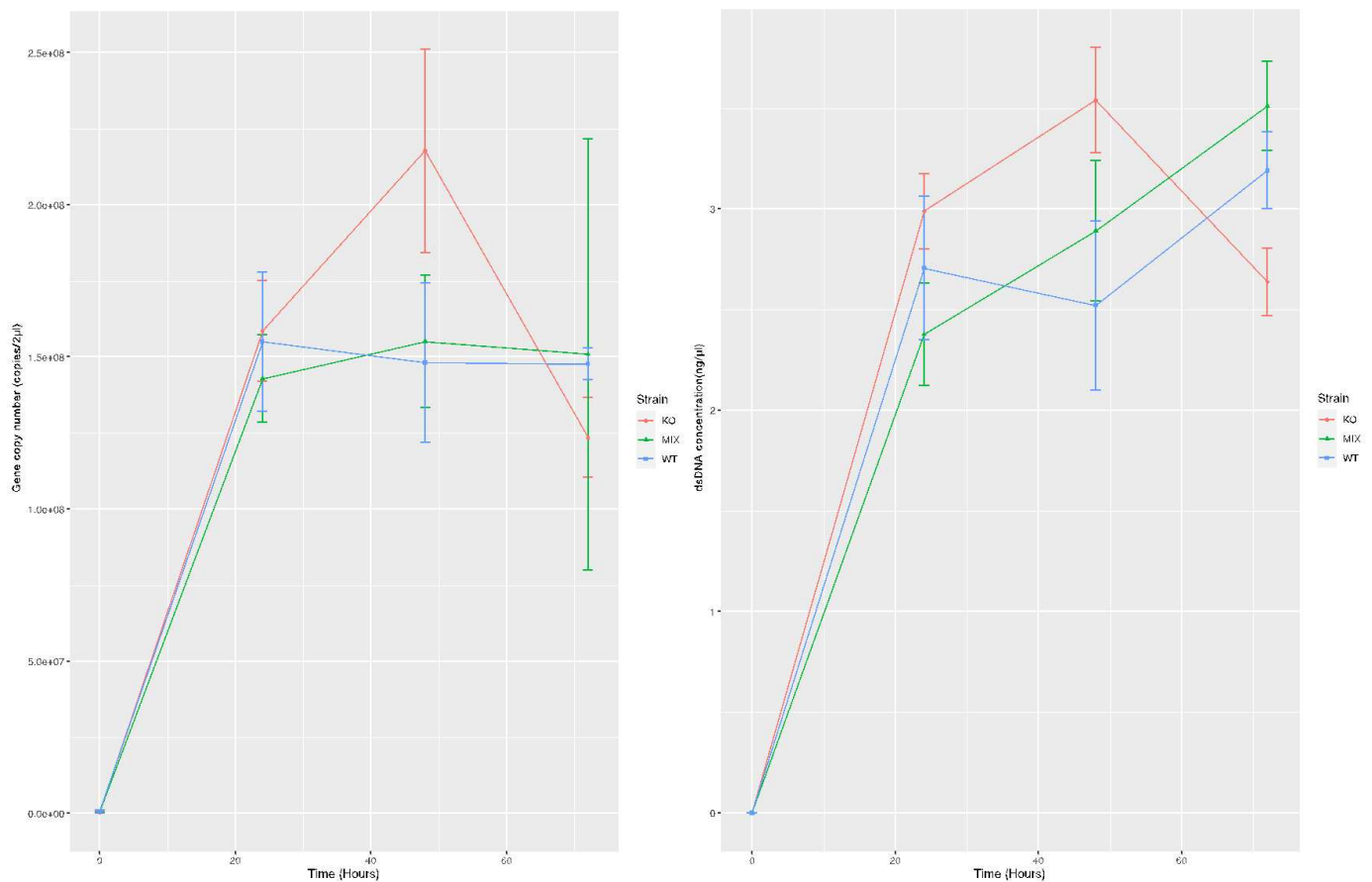


Fig.7. R plots are generated to visualize: a. The measurements obtained using the Qubit assay and b. the quantification performed using q-PCR. These plots are based on longitudinal samples collected during an in vivo experiment.

We extracted DNA from the samples taken after incubation for 24 hours, 48 hours, and 72 hours. We first measured the DNA content in these samples using the Qubit assay, followed by qPCR. Figure 7.a and 7.b display the change in DNA content over the specified time period. The bacterial content in the samples increased until the 24 hour, where the WT and MIX groups stabilized while the KO growth is seen higher in the graph. But we could not find any significant differences between these 3 groups.

Time(hours)	F-statistic	p-value
Initial(before starting incubation)	0.209	0.8199
24th hour post-incubation	0.196	0.8295
48th hour post-incubation	1.674	0.2963
72nd hour post-incubation	0.207	0.8215

Table.1. Presentation of a statistical test using one-way ANOVA for measurements with a qPCR assay. The analysis was done using gene copy numbers in each sample.

Time(hours)	F-statistic	p-value
Initial(before starting incubation)	NaN	NaN
24th hour post-incubation	1.52	0.323
48th hour post-incubation	2.64	0.186
72nd hour post-incubation	5.78	0.066

Table.2. Presentation of a statistical test using one-way ANOVA for measurements with the Qubit assay. Analysis was done by detecting the double-stranded DNA numbers in each sample after DNA extraction.

Even though the KO *H.pylori* lacks the ability to produce VacA toxin, they showed similar growth as the wild-type *H.pylori* in monocultures. Again, when we compare mix-culture to monocultures of KO and WT, we see no significant difference (Table.1, Table.2). This is what we would expect when the vacA toxin is not relevant outside of the host environment, and would suggest that the WT does not produce much vacA in the host tissues absence.

Despite measuring the DNA content using different parameters—qubit uses ng/ml, while qPCR uses gene copy number calculated from the Ct values—the results in both graphs remain similar.

All of the samples had a similar concentration of bacteria before starting the incubation process, however this was likely too low to estimate precisely.

The stationary phase that the populations of wild-type and mixture bacteria observed in both the 48-hour time frame and the optical density (OD) graph indicates that results from Qubit and QPCR assays were similar with OD measurements (Fig.1).

2.3. Quantification of *H.pylori* bacterium using qPCR with glmm and apha3 primers

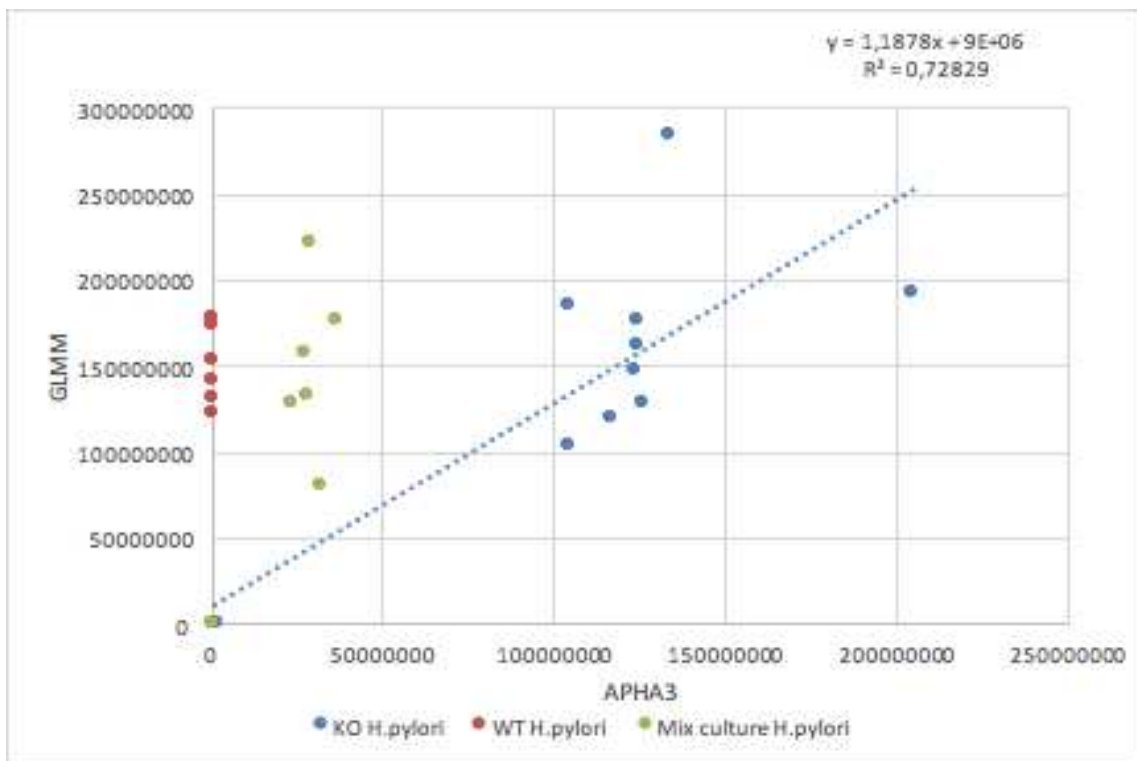


Fig.8. Correlation graph between the gene copy numbers measured with qPCR using two different sets of primers; y-axis representing the log gene copy numbers detected from qPCR done with GLMM and x-axis represents the qPCR done with APHA3 primers.

The graph above compares the bacterial gene copy numbers estimated using apha3 primers and copy numbers estimated with glmm primers with qPCR. As the absolute quantification of gene copy numbers with qPCR is not entirely comparable between primer pairs, we use this as a way to standardize gene count between primer pairs. The glmm primers amplifies both the WT and the KO and the apha3 primers only the knock-out *H. pylori*. Ideally, we would therefore expect to see a linear correlation between the bacterial counts using both primers for the KO, and use the slope of the fit to quantify the number of knockout bacteria within the mixture samples. A linear regression $R^2 = 0.72$ shows a reasonable fit but with outliers.

2.3.1. Quantification of *H.pylori* bacterium using qPCR with *aphA3* primers

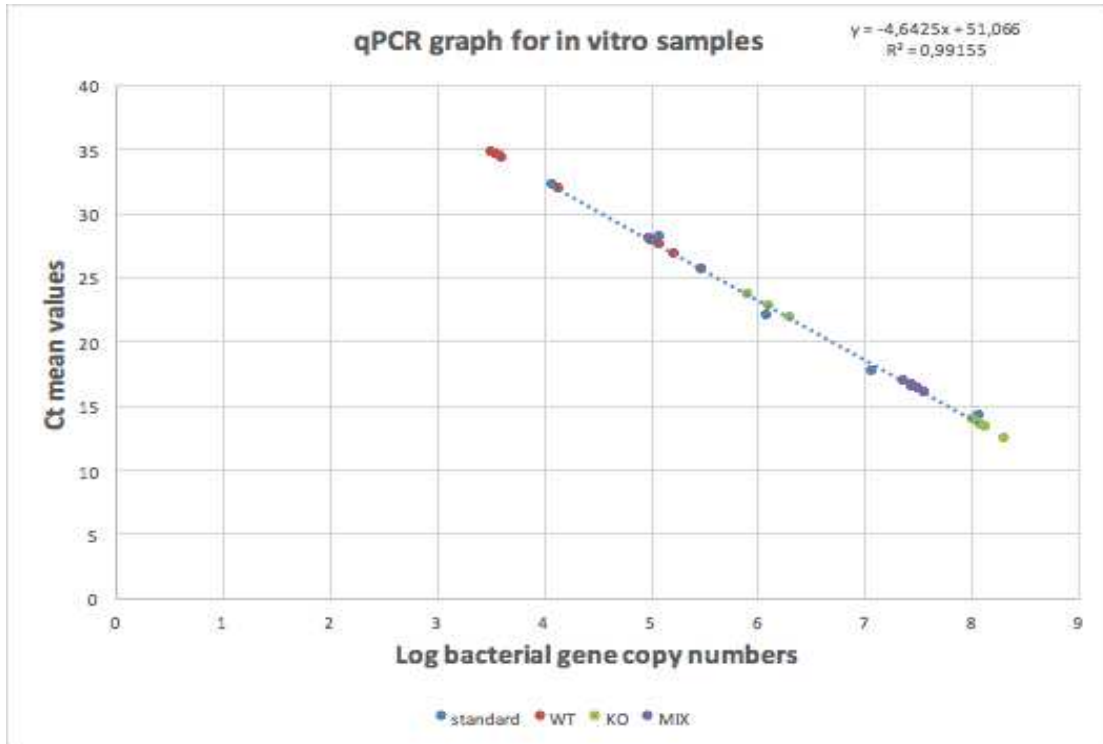


Fig.9. Standard curve of Ct values for *H.pylori* gene copies from longitudinal in vitro samples.

Ct values measured from the in vitro samples allowed us to interpret the amount of *H. pylori* DNA in the samples. A standard curve amplifying known quantities of *H. pylori* DNA is used to estimate the amount of *H. pylori* DNA in the samples. There is a negative correlation between the gene copy numbers and the Ct value. As the Ct value gets higher, the original DNA content of the sample is considered low.

As we mentioned earlier, *aphA3* primers amplify knock-out PMSS1 *H.pylori*, and the results from the standard curve matched our expectations: For wild-type Ct, values are high and the gene number is low, confirming the *aphA3* primers did not bind the wild-type bacteria as they lack the target region for these primers. Therefore, amplification did not take place, and we did not detect wild-type *H.pylori* in this QPCR assay. As we see in the figure, gene copy numbers are high when we look at the knock-out bacteria, as these primers specifically amplify these bacteria. Moreover,

the mix sample of wild-type and knock-out bacteria clearly shows higher gene copy numbers than the wild-type samples, confirming the presence of knock-out *H.pylori* in our mixture samples.

2.3.2. Quantification of *H.pylori* bacterium using qPCR with glmm primers

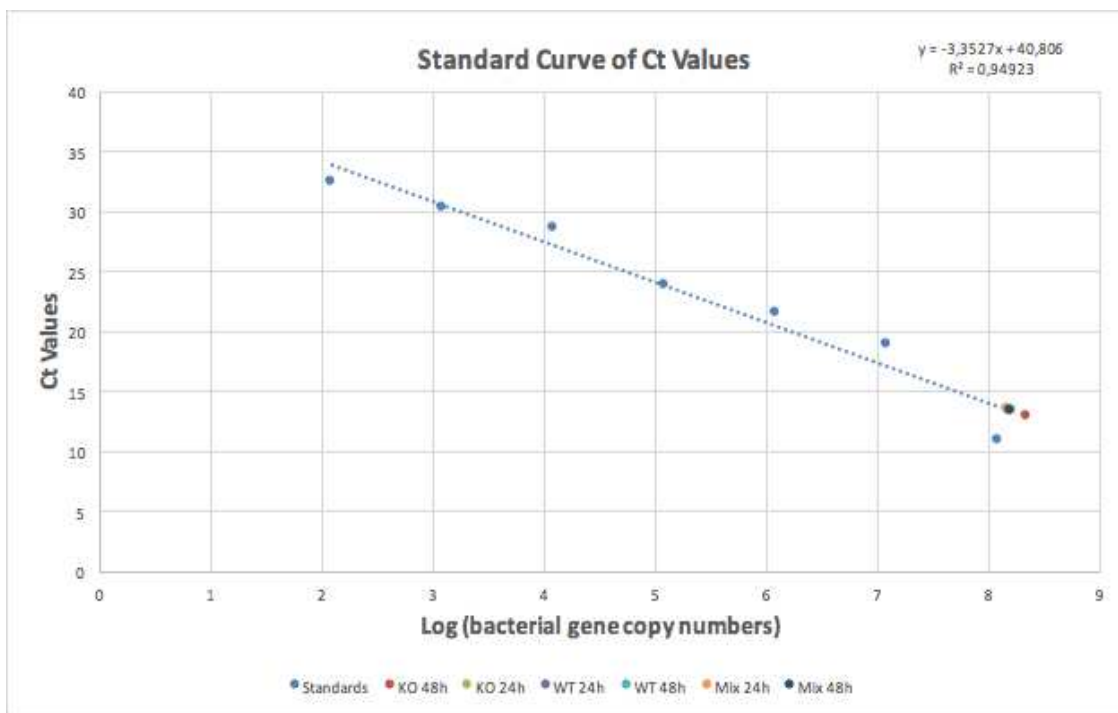


Fig.10. Standard curve generated from the qPCR results performed with glmm primers. For this graph samples taken from 24h and 48h post-incubation cultures used.

In the standard curve demonstrating ct values, both monocultures and mix culture samples show high log gene copy numbers. The samples were chosen to represent the log phase of the growth phase of the bacteria as their numbers are the highest during this period. As seen in the figure, the log gene copy numbers differ little and as we mentioned before this might suggest these bacteria (wild-type and knock-out bacteria) shows similar fitness. And the *vacA* might matter in the presence of hosts' tissue.

2.4. Measurement of the ratio between the knock-out and wild-type *H.pylori* in mix-culture samples

Time(hours)	Mixture Culture 1	Mixture Culture 2
	KO / WT ratio	KO / WT ratio
Initial(before starting incubation	0,032193557	0,010485886
24th hour post-incubation	0,65995638	0,63630474
48th hour post-incubation	0,663468283	0,697349115
72nd hour post-incubation	0,665551512	0,679307418

Table.3. The ratio between the knock-out *H.pylori* and wild-type *H.pylori* in mixture cultures. The samples were collected longitudinally. Results belong to the samples taken from the beginning, 24 hour, 48 hour, 72 hour post-incubation.

We measured the initial ratio between the knock-out and wild-type *H.pylori* that are cultured and incubated for 72 hours. The initial rates show a rise within the following 24 hours, however, this outcome may be related to the usage of a low bacteria concentration at the onset of the experiment. As a result, detecting the initial genomic content in these samples may not be reliable. However, in the following days, the ratio between these bacteria remained relatively the same, with no significant changes seen. This is in line with our expectations from in vitro experiments, as the *vacA* toxin does not play a significant role in this situation, the knock-out bacteria cannot cheat, and the ratio between them remains constant ($p=0.112$). Therefore, we suggest wild-type and knock-out *H.pylori* might have similar fitness and the same growth rates in vitro.

DISCUSSION

Our hypothesis focuses on the effect of microbial interactions on the host and the social behaviors that microbes exhibit toward one another. My research questions are based on the social evolution theory for microbes explained in the introduction part of this paper, which provides a framework to better understand interactions such as competition, cooperation, and cheating. The aim of this project was to analyze bacterial social interactions by quantifying *H. pylori* populations in vivo and in vitro. We wanted to test whether during infection, *H. pylori* can exhibit cooperative behavior that non-cooperative "cheater" bacteria can exploit. Specifically, the research question for this project was whether production of the vacA toxin production is cooperative or not. As previously mentioned, experts believe that vacA toxin plays a crucial role in the gastric environment, enhancing *H. pylori* colonization and leveraging the bacteria's persistence in humans[22]. We anticipated that a wild-type *H. pylori* producing vacA would do best in monoculture infections, but in mixed-culture infections, the KO would be more able to invade and benefit from the presence of the cooperative WT-*H. pylori* within the mice's stomachs.

We first tested the effect of vacA production in vitro, where we expect to see no benefit of vacA production as it is only important in the host environment should have similar growth rates for monocultures (KO and WT *H. pylori*) and mix-cultures (KO:WT *H. pylori*, 1:10, respectively). Moreover, the ratio between the KO and WT should remain steady, as the VacA has no additional benefit to both of these bacteria, so there is no possibility for the KO to cheat. Optical density measurements showed results as expected, after quantification by Qubit and qPCR assays, we also found there was no difference between the quantity of genomic content in the samples. In the mixed culture the ratio 0.6 remained steady during incubation.

This result may be explained by the vacA toxin not affecting how the bacteria behaves in vitro and its importance remains significant in the gastric environment.

During optical density measurements in the 24- and 72-hour periods, there were significant differences between groups' growth rates. This result may be explained by the fact that, although there is a difference, it's likely too small to be biologically meaningful for measurements taken at 24 hours. The data from 72-hour incubated cultures may not be as relevant as data from other hours due to the bacteria's tendency to clump together during this decline/death phase, which in turn affects OD measurements. OD spectrometers assume a homogeneous bacterial suspension, therefore the presence of clumps causes an uneven distribution, resulting in inconsistent data. Furthermore, bacterial clumps can scatter light differently in contrast to individual cells causing varying optical density (OD) results.

The experiment in mice explores the idea of these bacteria coexisting inside the stomach and affecting each other's behavior. We expected the KO *H.pylori* would be able to colonize better with wild-type bacteria as they could use the *vacA* virulence factor produced by the wild-type bacteria. It could be inferred that cheaters can take up goods produced by other bacteria, the cooperators, which have metabolic costs. Thus, the cheater bacteria have a fitness advantage over the cooperators. Although in the long term, as the WT *H.pylori* pays the cost of these interactions, the KO *H.pylori* would proliferate faster than WT *H.pylori*, and eventually the overall bacteria population would decrease since the *vacA* per bacteria would not be enough to sustain all of the population.

In contrast, for in vitro experiments, in tissues infected with WT *H. pylori*, the quantity of bacteria was higher than in tissues infected with KO alone, so here there was a potential for the KO to cheat on the WT. The bacterial load was also lower than the WT in mixture cultures. However, overall bacterial loads were low in the mice, which makes quantification with qPCR challenging. The ANOVA test done on these data demonstrated that the p value is higher than 0.005, which means there are no significant differences between these groups.

We could unfortunately not get meaningful qualifications of the KO in the mix culture, to test if it increased in frequency compared to the starting point. We performed two separate qPCR assays with *aphA3* and *glmm* primers, as mentioned in the methodology part of this paper. We intended to use *aphA3* primers to determine the

number of KO *H. pylori* present in the mouse infection mixture. However, detecting KO in vivo samples proved challenging. The problem with the low detection of these copy numbers was that it prevented us from determining the number of KO present in the mixture samples, a crucial step in verifying the validity of our hypothesis. Additionally, we could have determined if the KO/WT ratio fluctuated over time and whether the KO benefited from the WT bacteria. We tested if the KO is a cheater and can use the product-*vacA* toxin that the WT bacteria (which is a cooperator) produced to colonize itself better in the stomach. As a result, if KO is indeed a cheater, then we expected an increase in the KO/WT ratio, but because the qPCR was not sensitive enough to detect low copy numbers, we could not detect the number of KO bacteria and, consequently, the ratio of KO to WT bacteria in the mixture samples. For future reference, it may be better to use more specific quantification methods, such as the TaqMan qPCR assay. Another challenge was the small sample size which is a total of 15 mice, each group containing 5 individuals. Therefore, further investigation with a larger sample size has a chance to prove this theory. We infected mice as adults, which results in lower bacterial load than infection early in life[30].

Future importance

Antibiotics have gained importance since their discovery, helping to save many lives from harmful bacteria. They have proven to be a useful type of medicine that many people today rely on for survival[1]. Due to the nature of antibiotic treatment, the emergence of resistance in bacteria is not surprising news.

Despite the usefulness of antibiotics, these treatments have been hindered by misuse and abuse by patients globally, with little knowledge on how the medicine should be used. This could possibly be due to the somewhat high reputation that comes from antibiotics being looked at as a miracle cure, and people might have been unaware of their limitations. However, due to their nature, bacteria inevitably develop resistance, which means that the economic influence of society is of importance[31]. This development is both an evolutionary response and an adaptation to antibiotics, resulting in various mutations that render antibiotics ineffective in treating bacterial infections. Several articles have highlighted the

importance of addressing the rise in resistant bacteria and investing more to explore alternative antibiotic treatment methods in the future. Antibiotics currently treat *H. pylori*, but as the bacteria become resistant to the drugs, they are more likely to persist throughout life. These bacteria are known to have social lives, sharing genes and products among themselves. Therefore, it is not wrong to say that when these bacteria gain resistance to drugs, the wild-type could suppress in the environment, eventually resulting in a strong infection.

In addition to what we said, there is also another aspect of using antibiotics, which is the eradication of these bacteria, that could be beneficial to us. Several papers suggest that *H. pylori*-infected humans show less asthma and related disorders compared to those who don't have this bacteria. The potential benefits shown in these studies mostly come from the *H. pylori* strains that interact extensively with the host. Therefore, it is also important to consider the host-symbiont relationship that can lead to these outcomes. Therefore, in our research, we considered both the microbe-microbe interactions in vitro and the host-microbe(*H. Pylori*) interactions in vivo. Even though we have data from the in vitro environment, the interactions in the gastric tissue are beyond our comprehension at the moment, so it is important to add these aspects to future studies[20].

Furthermore, because bacterial pathogens cause diseases through communication and cooperation, the idea of using cheats to weaken these behaviors has emerged. Exploiting the cooperative behavior of wild-type bacteria through cheat therapies has the potential to effectively reduce the severity of disease pathogens[32].

We can easily create cheater bacteria due to their ease of manipulation, which we can then incorporate into clinical studies in the future. Given the ease of manipulating these bacteria, it is possible to create invaders that take advantage of cooperative traits, which are often linked to virulence[3]. Therefore, these behaviors may offer a promising novel approach to reducing the severity of *H. pylori*-induced diseases without completely eliminating the bacteria from the stomach. Therefore, it's possible that these bacteria could still serve a beneficial purpose without causing pathogenic outcomes.

REFERENCES

- [1] Y. A. Helmy *et al.*, “Antimicrobial Resistance and Recent Alternatives to Antibiotics for the Control of Bacterial Pathogens with an Emphasis on Foodborne Pathogens,” *Antibiotics*, vol. 12, no. 2, 2023, doi: 10.3390/antibiotics12020274.
- [2] J. C. Atherton and M. J. Blaser, “Coadaptation of *Helicobacter pylori* and humans: Ancient history, modern implications,” *Journal of Clinical Investigation*, vol. 119, no. 9, pp. 2475–2487, 2009, doi: 10.1172/JCI38605.
- [3] S. A. West, S. P. Diggle, A. Buckling, A. Gardner, and A. S. Griffin, “The social lives of microbes,” *Annu Rev Ecol Evol Syst*, vol. 38, pp. 53–77, 2007, doi: 10.1146/annurev.ecolsys.38.091206.095740.
- [4] N. Kodaman *et al.*, “Human and *Helicobacter pylori* coevolution shapes the risk of gastric disease,” *Proc Natl Acad Sci U S A*, vol. 111, no. 4, pp. 1455–1460, 2014, doi: 10.1073/pnas.1318093111.
- [5] S. A. West, A. S. Griffin, A. Gardner, and S. P. Diggle, “Social evolution theory for microorganisms,” *Nat Rev Microbiol*, vol. 4, no. 8, pp. 597–607, 2006, doi: 10.1038/nrmicro1461.
- [6] C. A. Molina and S. Vilchez, “Cooperation and bacterial pathogenicity: An approach to social evolution,” *Revista Chilena de Historia Natural*, vol. 87, no. 1, pp. 1–9, 2014, doi: 10.1186/s40693-014-0014-2.
- [7] L. J. Belcher, A. E. Dewar, C. Hao, M. Ghoul, and S. A. West, “Signatures of kin selection in a natural population of the bacteria *Bacillus subtilis*,” *Evol Lett*, vol. 7, no. 5, pp. 315–330, 2023, doi: 10.1093/evlett/grad029.
- [8] P. Smith and M. Schuster, “Public goods and cheating in microbes,” *Current Biology*, vol. 29, no. 11, pp. R442–R447, 2019, doi: <https://doi.org/10.1016/j.cub.2019.03.001>.
- [9] D. B. Polk and R. M. P. Jr, “*Helicobacter Pylori*: Gastric Cancer. NIH Public Access,” vol. 10, no. 6, pp. 403–414, 2010, doi: 10.1038/nrc2857.Helicobacter.
- [10] H. C. Leggett, S. P. Brown, and S. E. Reece, “War and peace: Social interactions in infections,” *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 369, no. 1642, 2014, doi: 10.1098/rstb.2013.0365.
- [11] W. Fischer, S. Prassl, and R. Haas, “Virulence mechanisms and persistence strategies of the human gastric pathogen *Helicobacter pylori*,” *Curr Top Microbiol Immunol*, vol. 337, pp. 129–171, 2009, doi: 10.1007/978-3-642-01846-6_5.
- [12] I. C. Arnold, I. Hitzler, and A. Müller, “The immunomodulatory properties of *Helicobacter pylori* confer protection against allergic and chronic inflammatory disorders,” *Front Cell Infect Microbiol*, vol. 2, no. February, p. 10, 2012, doi: 10.3389/fcimb.2012.00010.
- [13] M. K. Patel, G. N. Ryan, A. M. Cerny, and E. A. Kurt-Jones, *Methods for In Vivo and In Vitro Analysis of Innate Immune Responses to Helicobacter pylori Infection*. 2012. doi: 10.1007/978-1-62703-005-2_24.
- [14] H. J. Ahn and D. S. Lee, “*Helicobacter pylori* in gastric carcinogenesis,” *World J Gastrointest Oncol*, vol. 7, no. 12, pp. 455–465, 2015, doi: 10.4251/wjgo.v7.i12.455.
- [15] A. Lee, J. O’Rourke, M. C. De Ungria, B. Robertson, G. Daskalopoulos, and M. F. Dixon, “A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain,” *Gastroenterology*, vol. 112, no. 4, pp. 1386–1397, Apr. 1997, doi: 10.1016/s0016-5085(97)70155-0.

- [16] C. Y. Kao, B. S. Sheu, and J. J. Wu, "Helicobacter pylori infection: An overview of bacterial virulence factors and pathogenesis," *Biomed J*, vol. 39, no. 1, pp. 14–23, 2016, doi: 10.1016/j.bj.2015.06.002.
- [17] J. O'Rourke and G. Bode, "Morphology and Ultrastructure.," H. L. T. Mobley, G. L. Mendz, and S. L. Hazell, Eds., Washington (DC), 2001.
- [18] T. L. Cover and M. J. Blaser, "Helicobacter pylori in Health and Disease," *Gastroenterology*, vol. 136, no. 6, pp. 1863–1873, 2009, doi: 10.1053/j.gastro.2009.01.073.
- [19] R. FitzGerald and S. M. Smith, *An Overview of Helicobacter pylori Infection*, vol. 2283. 2021. doi: 10.1007/978-1-0716-1302-3_1.
- [20] S. Ansari and Y. Yamaoka, "Survival of Helicobacter pylori in gastric acidic territory," *Helicobacter*, vol. 22, no. 4, pp. 1–13, 2017, doi: 10.1111/hel.12386.
- [21] N. J. Foegeding, R. R. Caston, M. S. McClain, M. D. Ohi, and T. L. Cover, "An overview of Helicobacter pylori VacA toxin biology," *Toxins (Basel)*, vol. 8, no. 6, pp. 1–21, 2016, doi: 10.3390/toxins8060173.
- [22] P. Boquet and V. Ricci, "Intoxication strategy of Helicobacter pylori VacA toxin," *Trends Microbiol*, vol. 20, no. 4, pp. 165–174, 2012, doi: 10.1016/j.tim.2012.01.008.
- [23] T. L. Cover, P. I. Hanson, and J. E. Heuser, "Acid-induced dissociation of VacA, the Helicobacter pylori vacuolating cytotoxin, reveals its pattern of assembly," *Journal of Cell Biology*, vol. 138, no. 4, pp. 759–769, 1997, doi: 10.1083/jcb.138.4.759.
- [24] S. L. Palframan, T. Kwok, and K. Gabriel, "Vacuolating cytotoxin A (VacA), a key toxin for Helicobacter pylori pathogenesis.," *Front Cell Infect Microbiol*, vol. 2, no. July, p. 92, 2012, doi: 10.3389/fcimb.2012.00092.
- [25] D. C. Willhite and S. R. Blanke, "Helicobacter pylori vacuolating cytotoxin enters cells, localizes to the mitochondria, and induces mitochondrial membrane permeability changes correlated to toxin channel activity," *Cell Microbiol*, vol. 6, no. 2, pp. 143–154, 2004, doi: 10.1046/j.1462-5822.2003.00347.x.
- [26] K. R. Jones, J. M. Whitmire, and D. S. Merrell, "A Tale of Two Toxins: Helicobacter Pylori CagA and VacA Modulate Host Pathways that Impact Disease.," *Front Microbiol*, vol. 1, p. 115, 2010, doi: 10.3389/fmicb.2010.00115.
- [27] S. L. Palframan, T. Kwok, and K. Gabriel, "Vacuolating cytotoxin A (VacA), a key toxin for Helicobacter pylori pathogenesis.," *Front Cell Infect Microbiol*, vol. 2, no. July, p. 92, 2012, doi: 10.3389/fcimb.2012.00092.
- [28] C. Utsch and R. Haas, "Vaca's induction of vaca-containing vacuoles (VCVs) and their immunomodulatory activities on human T cells," *Toxins (Basel)*, vol. 8, no. 6, 2016, doi: 10.3390/toxins8060190.
- [29] A. Müller and J. V Solnick, "Inflammation, immunity, and vaccine development for Helicobacter pylori.," *Helicobacter*, vol. 16 Suppl 1, pp. 26–32, Sep. 2011, doi: 10.1111/j.1523-5378.2011.00877.x.
- [30] I. C. Arnold *et al.*, "Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia.," *Gastroenterology*, vol. 140, no. 1, pp. 199–209, Jan. 2011, doi: 10.1053/j.gastro.2010.06.047.
- [31] C. A. Hart and S. Kariuki, "Antimicrobial resistance in developing countries.," *BMJ*, vol. 317, no. 7159, pp. 647–650, Sep. 1998, doi: 10.1136/bmj.317.7159.647.
- [32] E. Yurtsev, H. Xiao Chao, M. Datta, T. Artemova, and J. Gore, "Bacterial Cheating Limits the Evolution of Antibiotic Resistance," in *APS March Meeting Abstracts*, in *APS Meeting Abstracts*, vol. 2012. Feb. 2012, p. Q42.008