Inhibition of *Helicobacter pylori* growth by *Actinomyces oris* and the implications for gastric carcinogenesis

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Abstract

H. pylori infection is the causative agent of intestinal-type gastric adenocarcinoma. The emergence of multidrug resistant *H. pylori* strains increases the burden of *H. pylori* infection and decreases the effectiveness of the current antimicrobial treatments. *H. pylori* abundance in the stomach decreases with the progression to gastric adenocarcinoma and is replaced by other members of the gastrointestinal tract and oral microbiome, including *Actinomyces oris*. The aim of this project was to understand the interaction between *H. pylori* and *A. oris in vitro* using co-culture systems. An oral *A. oris* isolate completely inhibited *H. pylori* in a co-culture assay and significantly decreased the growth of the related bacterium, *Campylobacter jejuni.* A gastric *A. oris* isolate and *Actinomyces viscosus* also inhibited *H. pylori* growth. The inhibition was shown to be mediated by two or more secreted factors of molecular weights both lower and higher than 5 kDa. These inhibitors are heat-resistant, acid-independent, sensitive to proteolytic activity and secreted in the stationary phase of growth. *A. oris* culture supernatant did not affect the growth of a panel of Gram-positive and Gram-negative bacterial species. Mass spectrometry analysis of *A. oris* culture supernatant revealed that it contains a 34 kDa protein of an uncharacterised function that has a lysozyme domain and a G5 domain. Analysis of the genomes of the *Actinomyces* strains used in the study with AntiSMASH and BAGEL4 databases indicates that they harbour putative biosynthetic gene clusters of three bacteriocins; Linocin_M18, Lanthipeptide and Sactipeptide. In conclusion, *Actinomyces* species produce inhibitors of *H. pylori* growth. Further work should focus on conclusive identification of the inhibitor and exploiting its potential as a novel antimicrobial against *H. pylori* infection.

DEDICATION

I would like to dedicate this thesis to my amazing uncle Turki, who empowered me, provided me with love and an endless support throughout the ups and downs of my educational journey. Without you, none of this would be possible.

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CHAPTER 1:

INTRODUCTION

1. Gastric cancer

1.2. Epidemiology of gastric cancer

Gastric cancer is one of the most common cancer types worldwide accounting for 5.7% of cancer cases and 8.2% of all cancer deaths in 2018 (Bray *et al.*, 2018). There are around 1 million new GC cases reported annually. The latest estimation of gastric cancer global incidence and mortality rates by the International Agency for Research on Cancer was reported in 2018. The number of new GC cases and deaths were 1,033,700 and 782,685, respectively (Bray *et al.,* 2018) The average number of new GC cases in the United Kingdom is 6,500 between 2016 and 2018, accounting for 2% of all cancer cases in the UK reported in the same period (CANCER RESEARCH UK, 2023). In the United States, 27,600 GC new cases and 11,010 GC-related deaths were reported in 2020 (Siegel *et al.*, 2020).

Although GC was the fifth most common cancer type reported in 2018, it was the second cause of cancer-related deaths in the same year given that the disease typically becomes symptomatic only in the advanced stages. Detection of the disease usually occurs after malignancy has invaded the epithelial lining and reached the muscularis propria, the muscle layer of the stomach. The survival rate of these patients is 5 years or even less (Correa, 2004) (Figure 1.1).

The global incidence and mortality rates vary between genders. Incidence of GC is higher in males than females, whereby GC is the third most common cancer after lung and prostate cancers among males. The global number of new GC cases represented 5.7% of all new cancer cases reported in 2018. The global number of deaths caused by GC represented 8.2% of cancer deaths in the same year. GC incidence and mortality rates also vary depending on geographical region, with more than 50% of the new cases reported in developing countries (Bray *et al.*, 2018). The Age-standard

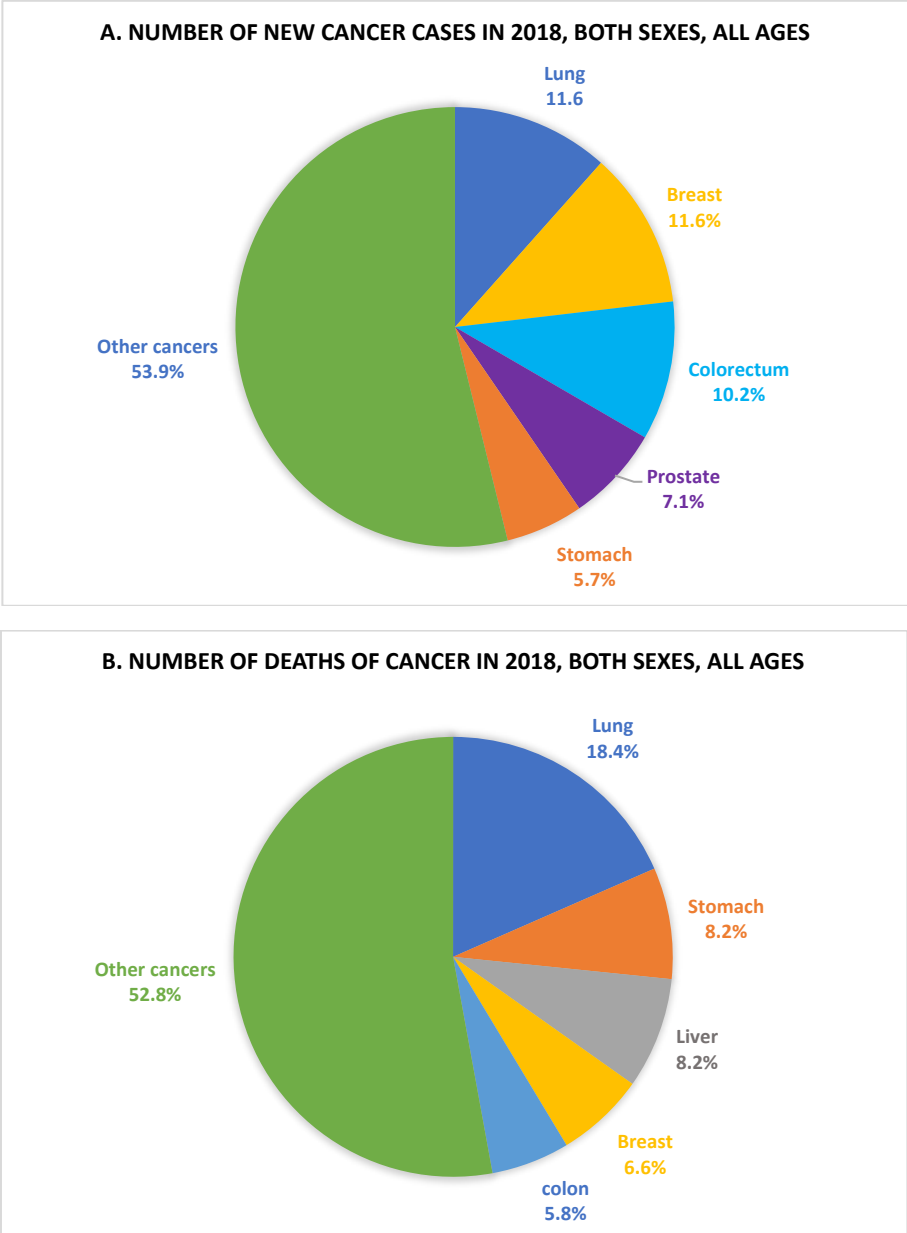

Figure 1. 1 The global number of cancer new cases and the number of global cancer related deaths in 2018. Gastric cancer was the fifth common cancer type representing 5.7% of new cancer cases in 2018 **(A).** The number of global cancer related deaths, GC was the second cause of cancer related deaths representing 8.2% of all cancer related deaths in the 2018 **(B).** Data are from the International Agency for Research on Cancer (IARC) (Siegel *et al.*, 2020).

Incidence rate (ASR) is usually used to determine the cancer risk within a region/group depending on the ASR value. A low-risk region is at ASR of <10 per 100,000, intermediate risk region is at ASR value of 10-20 per 100,000 and a high-risk region is at ASR value of >20 per 100,000. High ASR values are found in Asia, in which male ASR values were double the ASR rate observed in females. Low ASR values are found in Western countries. There is a 10-fold increase in GC incidence rate in Asia, including South Korea and Japan, than in western countries (Bray *et al.,* 2018). This variation can be attributed to predisposing factors such as lifestyle, diet and genetics. Although GC morbidity rates in Japan and South Korea are high, the mortality rates are lower due to improved surveillance and screening methods, which allow for early detection (Quach *et al.*, 2019).

The global incidence rate of GC has declined steadily in the past years due to several factors. One of the main factors is the discovery of the link between gastric ulcers, GC and *Helicobacter pylori* infection in the 1980s, by Drs Barry Marshall and Robin Warren (Marshall and Warren, 1984). Treatment of *H. pylori* infection with antibiotics reduced the risk of developing GC in many patients. However, *H. pylori* infection is still a problem in low income countries, where GC cases remains high. Other factors such as improved hygiene and food preservation methods have played a role in reducing the incidence of GC (Strong, 2018).

1.2. Gastric cancer types

There are different systems that classify GC based on histopathological and molecular characteristics. For example, Lauren's classification of GC divides GC histologically into two main subtypes: diffusible type adenocarcinoma (D-TAC); intestinal-type gastric adenocarcinoma (I- TGAC) (Lauren, 1965). A third subtype, indeterminate type, was introduced later that describes rare histology (Leocata *et al.*, 1998). Diffusible adenocarcinoma affects mainly young people and originates from a

normal gastric epithelium and does not result from gradual histological changes (Brawner *et al.*, 2014). Intestinal-type gastric adenocarcinoma is common amongst the older population, and it is characterised by a series of gastric mucosal histological changes, predominantly in the distal stomach. This carcinogenic cascade is known as the 'Correa's cascade' and details the stepwise progression from superficial gastritis (mainly caused by *H. pylori* infection) to chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and finally adenocarcinoma (Figure 1. 2) (Correa, 2004) Although Lauren's classification classifies GC into 3 broad histological subtypes, it was the first classification that links GC to *H. pylori* infection.

The World Health Organization (WHO) has classified gastric cancer histologically into more detailed subgroups. The WHO classification of GC includes tumours of less frequency that are absent in the Lauren's classification. GC is divided into several subgroups: tubular; papillary; mucinous; signet-ring cell carcinoma; mixed carcinomas (equivalent to the indeterminate type of the Lauren's classification) (Fléjou, 2011).

The cancer genome atlas (TCGA) has classified GC into 4 molecular subtypes. They have evaluated 295 gastric adenocarcinoma cases using different platforms such as DNA sequencing, RNA sequencing genome/exom/methylome and protein arrays. These subtypes are: Epstein-Barr virus (EBV) positive tumours (8.8%); microsatellite instable tumours (21.7%); tumours with chromosomal instability (49.8%); genomically stable tumours (19.7%) (Cancer Genome Atlas Research, 2014). The TCGA system classifies GC based on molecular findings from gastric biopsies. Although it classified one subtype based on EBV status, it does not include a subtype based on *H. pylori* status, most likely due to the decrease in *H. pylori* abundance with the progression to GC.

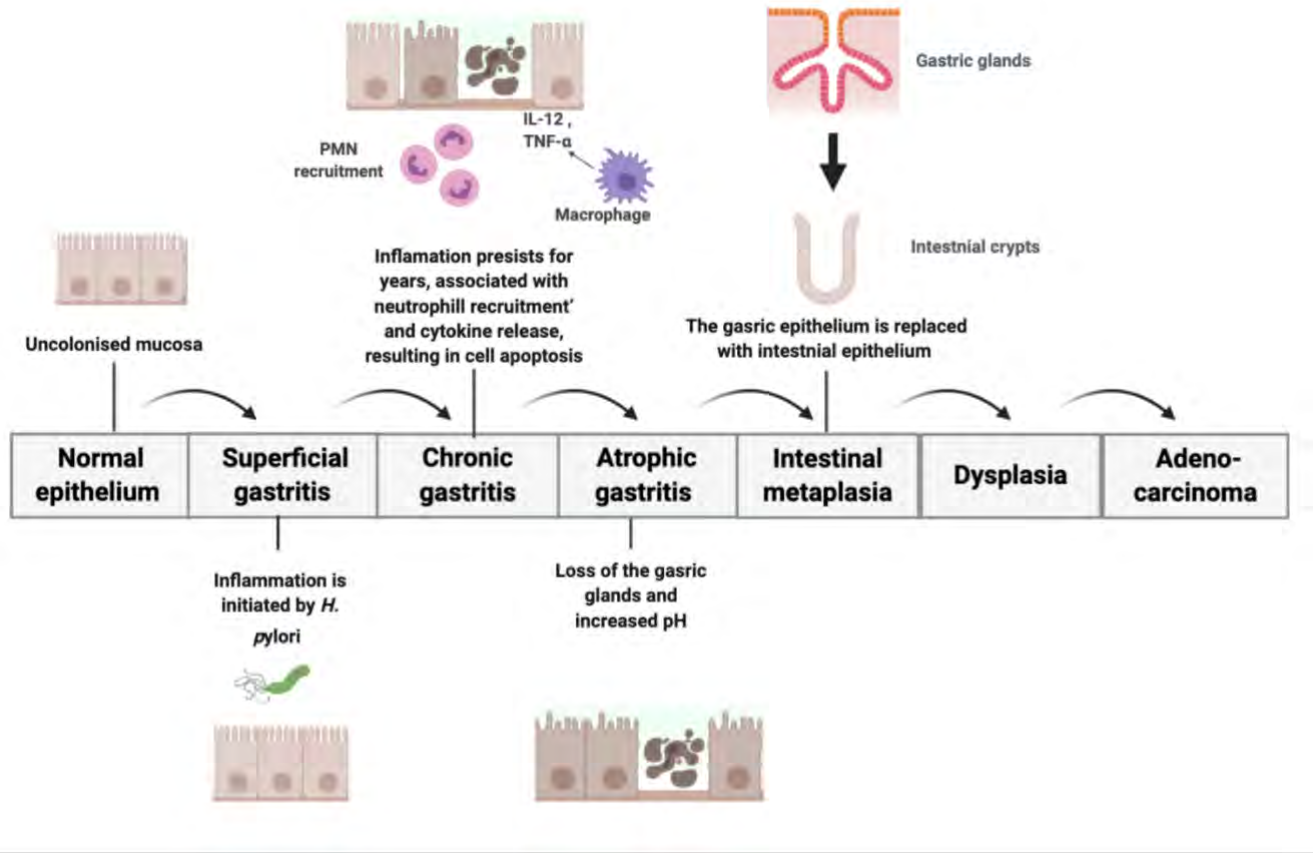

Figure 1. 2 Correa's model of gastric carcinogenesis. *H. pylori* colonises the stomach lining, resulting in superficial gastritis. The inflammation of the gastric mucosa can proceed to chronic gastritis and subsequently atrophic gastritis. Atrophic gastritis is followed by intestinal metaplasia, dysplasia and gastric adenocarcinoma. Figure was created using Biorender (https://biorender.com/).

1.3. Gastric cancer risk factors

Cancer can result from both genetic and environmental factors. The biggest risk factor for GC development is *H. pylori* infection, which is discussed in detail in the next section. However, there are other factors such as high-salt diet, obesity, alcoholism and smoking that are involved in the development of GC.

1.3.1. Dietary and lifestyle factors

Studies have shown that healthy dietary habits, which include a high intake of fresh vegetables (rich in β carotene, vitamin C, E and foliate) and fruits, low sodium diet and high red meat consumption decreases the risk of developing GC (Nomura *et al.*, 2003, Buckland *et al.*, 2015). High intake of salted food, smoked meat and pickled vegetables are considered to be a predisposing factor to cancer (Lin *et al.*, 2014). Higher GC incidence rates are reported in regions such as Eastern Europe and East Asia where most people consume highly salted and preserved food. Furthermore, lower GC incidence rates are reported in western countries, which can be attributed to an improved diet, which is low in salt and rich in fresh vegetables and fruits.

Alcoholism increases the risk of developing GC. A metanalysis study, which included over 334,557 GC cases, showed a positive correlation between excessive alcohol consumption and the risk of developing GC (Nomura et al., 2003, Lagergren *et al.*, 2000). The international Agency for Research on Cancer (IARC) has classified acetaldehyde which is derived from ethanol in alcoholic drinks as a group 1 carcinogen. (Baan *et al.*, 2007, Lachenmeier and Salaspuro, 2017, Seitz and Stickel, 2007). The exact mechanism underpinning how alcohol plays a role in carcinogenesis is not yet fully understood.

Tobacco smoking increases the risk of developing GC. This finding was supported by multiple case-control and prospective studies. The risk of developing gastric cancer is higher in chronic smokers (Batty *et al.*, 2008, Chao *et al.*, 2002). The risk of developing GC is also higher in occasional smokers than in chronic smokers. Male smokers are at a 60% increased risk of developing GC, whilst female smokers are at a 20% increased risk of developing GC (Ladeiras-Lopes *et al.*, 2008). The mechanism by which smoking can increase GC risk is still not fully understood.

1.3.2. Genetic mutations

Cancer can result from an inherited genetic mutation such as single nucleotide polymorphisms (SNPs). Many studies have investigated the role of genetics in the development of GC. The risk of developing D-TGAC is approximately 3-fold higher in individuals with a family history (La Vecchia *et al.*, 1992). In 1998, Dr Parry Guilford linked a truncating mutation in the tumour suppressor gene, *CDH1,* to an increased risk of developing the diffusible type of GC (Guilford *et al.*, 1998). However, there has not been any conclusive data about specific mutations that can explain the variation of GC incidence rate between different countries.

Gastric carcinogenesis involves multiple cellular events that drive carcinogenesis, such as mutations in the tumour suppressor genes, cell adhesion molecules, DNA repair genes and telomerase activation and SNPs (Yokozaki *et al.,* 2001). For example, SNPs in genes involved in cell cycle regulation, DNA methylation and metabolism result in increased risk of cancer development (Deng *et al.*, 2017). *DNMT1* encodes for DNA methyltransferase 1, involved in DNA methylation. SNP rs16999593 occurs on the C-allele of *DNMT1* resulting in a histidine substitution to arginine at position 97 of the resulting enzyme, disrupting the function of the gene. Overexpression of *DNMT1* results in aberrant DNA methylation of the tumour suppressor genes, thereby increasing the risk of developing GC

(Tian *et al.*, 2019). A study by Ranzani *et al.* (1995) indicated that mutations in p53 gene plays an important role early in gastric carcinogenesis of intestinal type and facilitate the transition between metaplasia and dysplasia (Ranzani *et al.*, 1995). Infection with *H. pylori* triggers hyperplasia of the gastric stem cells. This event is followed by DNA hypermethylation at the D17S5 locus, p53 mutation loss of both RAR beta and pS2 CD44 abnormal transcripts and replication error. These mutations were detected in around 30% of people with intestinal metaplasia (Tahara, 2004).

1.3.3. Epstein-Barr virus infection

Epstein-Barr virus (EBV) is a type of human herpes virus, which induces overexpression of B cell lymphoma 2 gene (bcl-2); an anti-apoptotic protein, that ultimately leads to GC. This finding was confirmed by a study that included analysis of 14 EBV-positive gastric carcinoma biopsies using RNA *in-situ* hybridisation to confirm the presence of EBV in the tumours, followed by immunohistochemistry to detect the expression of bcl-2 protein. Expression of bcl-2 protein was found in 71% of the tumours tested. The same study suggests that EBV-tumours can result from the proliferation of a single EBV infected cell (Czopek *et al.*, 2003). Detection of EBV and *H. pylori* in GC specimens have been reported in multiple studies. However, these studies vary in their detection methods and sample sizes. The prevalence of co-infection with EBV and *H. pylori* was 34% in patients with gastritis, gastric ulcers and dysplasia (Dávila-Collado *et al.*, 2020).

1.3.4. *H. pylori* infection

H. pylori infection is the causative agent in the development of 95% of the intestinal type of gastric adenocarcinoma cases (Zhang *et al.*, 2014). Patients infected with *H. pylori* (CagA+) strains are at 5.8-fold higher risk of developing gastric adenocarcinoma than non-infected subjects (95% confidence interval) (Parsonnet *et al.*, 1997). The correlation between *H. pylori* infection and gastric ulcers and ultimately GC has been studied extensively. Shibata *et al.* (2002) also investigated the

association of *H. pylori* infection and GC tumours and concluded that *H. pylori* (CagA+) strains are strongly associated with abnormalities in p53 protein function and overexpression of bcl-2 protein (Shibata *et al.*, 2002).

H. pylori and EBV produce a life-long persistent infection that induce chronic inflammation of the gastric mucosa which might proceed to gastric cancer. Rihane *et al.*, (2021) investigated the effect of co-infection of *H. pylori* and EBV in 100 tissue samples of gastric cancer. The study reported that co-infection with *H. pylori* and EBV was detected in 16% of the cases. There was also a significant correlation between increased patient age and the co-infection (Rihane *et al.*, 2021).

Studies have shown that *H. pylori* infection can play a protective role against some diseases such as allergies, asthma, inflammatory bowel disease and oesophageal acid reflux. For example, it has been proposed that the immune response to *H. pylori*-infection, which includes the tolerance to dendritic cells, the production of protective cytokines and the preferential differentiation of T-cells play a role in the protection from autoimmune diseases (Arnold and Müller, 2016). *H. pylori* infection has been associated with a lower risk of developing oesophageal acid reflux due to the decreased acidity of the stomach associated with *H. pylori* infection (Scida *et al.*, 2018).

1.4. *Helicobacter pylori*

1.4.1. Microbiology

The *Helicobacter* genus belongs to the Campylobacterota phylum (Oren and Garrity, 2021). The genus comprises 18 identified species that typically colonise the gastrointestinal tract of both humans and animals. *Helicobacters* are non-invasive, non-spore forming, micro-aerophilic Gram-negative bacteria. They are spiral in shape and measure around 3.5 x 0.5 μm.

Helicobacter pylori was first described by Dr. Marshall and Dr. Warren in the early 1980s. They reported the presence of an S-shaped curved bacilli with up to 5 polar flagella that are used for motility in 135 gastric biopsies (Marshall and Warren, 1984). They were awarded the Nobel prize for their discovery of *H. pylori* and its role in causing gastric ulcers. Following their discovery, many studies have focused on the role of *H. pylori* in gastric-related diseases and the development of gastric adenocarcinoma (Linz *et al.*, 2007).

1.4.2. *H. pylori* prevalence

H. pylori colonises 50% of the world's population (Brawner et al., 2014). Generally, the prevalence of *H. pylori* infection varies depending on factors such as race, age, geographical region and socioeconomical status. Most of the data on *H. pylori* infection rates come from seroprevalence studies. One disadvantage of these studies is that it does not distinguish between current and previous infection.

A systematic review of the global *H. pylori* prevalence reported variation in *H. pylori* infection prevalence between countries whereby the highest *H. pylori* infection prevalence was recorded in Mexico (90%) and the lowest *H. pylori* infection prevalence was recorded from Finland (13%) (Peleteiro *et al.*, 2014). Another systematic review of the global *H. pylori* infection prevalence that included 62 countries reported a significant variation in *H. pylori* infection prevalence across different geographical regions. The lowest *H. pylori* prevalence was 24% reported from Oceania and the highest *H. pylori* infection prevalence was 70% reported from Africa. On an individual country level, the highest *H. pylori* infection prevalence levels were reported from Nigeria (85%) and the lowest *H. pylori* infection prevalence levels were reported from Switzerland (18.9%). The same systematic

review reported that *H. pylori* infection prevalence in the United States and the United Kingdom were of 35.6% and 35.5%, respectively (Hooi *et al.*, 2017).

H. pylori infection prevalence also varies between socioeconomic levels whereby high *H. pylori* infection prevalence is associated with low socioeconomic status and low hygiene and crowded living conditions (Lane *et al.*, 2002, Cheng *et al.*, 2009, Mentis *et al.*, 2015). *H. pylori* infection prevalence of the adult population in developing countries can be greater than 70% compared with 40% or less in developed countries. For example, it is estimated that *H. pylori* infection prevalence is 90% in Bangladesh (Ahmad *et al.*, 2007), 80% in India (Ahmed *et al.*, 2007), 65% in Brazil (Zaterka *et al.*, 2007) and between 60% - 70% in China (Zhang *et al.*, 2008). However, *H. pylori* infection prevalence is 22.1% in Denmark, 26.2% in Sweden and 24% in Australia and New Zealand (Hooi et al., 2017).

There are limitations to the assays commonly used to detect *H. pylori* infection in epidemiological studies. For example, serological assays fail to differentiate between the current and the past infections which affects the accuracy of the infection prevalence results. Many studies have compared the accuracy and the sensitivity of the common tests used for the detection of *H. pylori*. The rapid urease test (RUT) is a measure of the of gastric urease produced by *H. pylori* and is widely used to diagnose *H. pylori* infection. Based on multiple epidemiological studies, the sensitivity range of the RUT falls between 83.3% and 86.9%. The specificity of the RUT ranges between 95.1 and 97%. The same studies reported that while the sensitivity of serological assays is high, their specificity is relatively low and therefore it is not ideal for the detection of *H. pylori* infection (Miftahussurur and Yamaoka, 2016). Kazemi *et al.,* (2011) compared the sensitivity and the specificity of the assays used to detect *H. pylori* infection. The sensitivity and the specificity of RUT was of 93%,75% respectively and the sensitivity and the specificity of serology was of 50%, 54% respectively (Kazemi *et al.*, 2011).

A meta-analysis study by Best *et al.,* (2018) reviewed the accuracy of the non-invasive assays for the diagnosis of *H. pylori* infection. They concluded that the RUT had higher odd ratio than serology. The sensitivity of the RUT was higher than serology at a fixed specificity of 0.90. The gold standard for the detection of *H. pylori* infection is immunohistochemistry of gastric biopsies. However, the method is invasive and therefore not suitable for large-scale epidemiological studies (Best *et al.*, 2018).

1.4.3. *H. pylori* virulence factors

The outcome of *H. pylori* infection depends mainly on the strain type that colonised the stomach. The *H. pylori* genome is genetically heterogeneous which means that different genes produce the same or similar phenotype. Thus, different strains of *H. pylori* vary in expression of virulence factors.

The cytotoxin-associated gene A protein (CagA) is *H. pylori'*s most studied virulence factor. It is encoded by the *cag* PAI, a 40-kb DNA insertion element containing up to 31 genes that are flanked by 31-bp repeats (Wroblewski *et al.*, 2010). *H. pylori* is commonly classified into a CagA positive or a CagA negative strain depending on the presence and absence of *cagA* gene. *H. pylori* delivers the CagA protein (around 140-kDa) to targeted cells via a type 4 secretion system, which is also encoded on the *cag*PAI. Once injected to the targeted cells, CagA is phosphorylated resulting in aberrant morphological changes of the epithelial cells. CagA also results in mitogenic and pro-inflammatory cell responses and loss of cell polarity by dysregulating mitotic spindle formation (Saadat *et al.*, 2007, Umeda *et al.*, 2009). The cytotoxic activity of CagA was confirmed with different studies on different cell line types and animal models (Wroblewski et al., 2010). Studies on transgenic mice expressing CagA confirmed the link between CagA and gastric epithelial cell proliferation and carcinoma. In particular, translocation of CagA to the nucleus activates genes involved in gastric cancer, such as

caudal type homeobox 1 (CDX1). This gene encodes intestinal transcription factor required in intestinal metaplasia seen in Correa's model (Murata-Kamiya *et al.*, 2007). Based on these findings, CagA was classified as a type 1 oncoprotein.

H. pylori also injects peptidoglycan into host epithelial cells via *cag* PAI. Peptidoglycan interacts with the nucleotide binding oligomerization domain containing 1 gene (NOD1), a cellular sensor of peptidoglycan in epithelial cells. This interaction results in production of the pro-inflammatory cytokine IL-8 and is a hallmark of *H. pylori* infection. Peptidoglycan also activates other signalling pathways such as the phosphatidylinositol 3-kinase (PI3K-AKT), leading to increased cell migration and decreased cell apoptosis. CagA affects mitochondrial function by decreasing mitochondrial transmembrane potential (Nagy *et al.*, 2009).

H. pylori also interferes with the cell signalling functions using the virulence factor vacuolating cytotoxin A (VacA). VacA is an 87 kDa protein and is present in all *H. pylori* strains. However, the number of *vacA* alleles and forms within the genome vary between different *H. pylori* strains, resulting in differences in *vacA* expression and variations in the degrees of disease severity and cell toxicity (Wroblewski *et al.,* 2010). VacA is a potent cytotoxin that disrupts gastric epithelial barrier functions. It disturbs the late endosomal compartments resulting in vacuole formation *in vitro* and disruption of the tight junctions. It also induces epithelial cell apoptosis by activating caspase-8 and caspase-9. Caspases are cysteine proteases that play an important role in cell apoptosis (Manente *et al.*, 2008, Wroblewski *et al.*, 2010)

VacA plays a role in ulcerogenesis by interfering with the gastric epithelial cell receptor, Receptor-type Protein Tyrosine Phosphatase β (RPTPβ). This receptor modulates proliferation, adhesion and

differentiation of epithelial cells. Administration of neutralised VacA orally to wild type RPTPβ$^{+/+}$ mice induced the formation of gastric ulcers and gastric atrophy after two days of administration. In contrast, RPTPβ$^{-/-}$ mice did not show a sign of gastric lesions after administration of VacA (Fujikawa *et al.*, 2003). Moreover, VacA binds to T-cells and once internalised within the cell, it interferes with the transcription factors required for activation of T-cells by preventing phosphorylation. Therefore, it inhibits the production of IL-2 and the activation of the IL-2 receptor and ultimately inhibits the proliferation of T-cells (Gebert *et al.*, 2003). *H. pylori* uses VacA to evade the immune system by inducing dendritic cells to secrete IL-10 and IL-18. These anti-inflammatory cytokines promote Treg differentiation (Brawner *et al.,* 2014). Treg cells play an important role in maintaining peripheral tolerance, preventing inflammation and autoimmune diseases (Vignali *et al.*, 2008).

Infection with *cag*PAI positive *H. pylori* strains increases the risk of developing gastric ulcers and GC. However, infection with *cag*PAI negative *H. pylori* strains can also induce gastric ulcer and GC (Yuan *et al.*, 2017). The ability of the infecting *H. pylori* strain to induce gastric diseases depends on the combination of the virulence factors that it has. For example, *vacA* gene is polymorphic having three polymorphic regions; the signal (s1/s2), the intermediate (i1/i2) and the mid (m1/m2) regions. Different forms of the *vacA* alleles results in different levels of cytotoxicity and activity, s1/m1/i1 variants are the most active and toxic forms (Amilon *et al.*, 2015). Several studies have linked *vacA* i1 type with the development of peptic ulcers, gastric atrophy and GC (Yordanov *et al.*, 2012, Basso *et al.*, 2008, Winter *et al.*, 2014). Studies have shown association between *cagA* and *vacA* genotypes and gastric disease development. Most *cag*PAI negative *H. pylori* strains are *vac*A s2. In contrast, most *cag*PAI positive *H. pylori* stains are *Vac*A s1 (Rhead *et al.*, 2007, Atherton, 2006).

H. pylori is also linked to the production of the novel cytokine A proliferation-inducing ligand (Xu *et al.*) by macrophages that plays a role in B-cells proliferation during gastric lymphomagenesis (Munari *et al.*, 2011). On a molecular level, *H. pylori* infection results in genetic alterations that transform normal B cells to malignant ones. These genetic alterations are responsible for the activation of nuclear factor kappa B (NF-κB) and subsequently changes in the immunity inflammation and apoptosis (Isaacson, 2005).

H. pylori has other virulence factors such as outer membrane proteins, adhesion proteins and the production of hydroxyl ion that increases the oxidative stress resulting in DNA damage (Wroblewski *et al.,* 2010) (Figure 1.3).

1.4.4. The role of *H. pylori* in gastric adenocarcinoma

The World Health Organization have classified *H. pylori* as a type 1 carcinogen based on several epidemiological and histological studies (Correa *et al.*, 1990, Cho and Blaser, 2012). Multiple serological sensitivity studies have shown a correlation between *H. pylori* seropositivity and higher risk of developing GC, up to 16-fold more risk than seronegative individuals (Hansson *et al.*, 1993, Miehlke *et al.*, 1997, Barreto-Zuñiga *et al.*, 1997).

A prospective study on 1,526 Japanese indicated that patients with severe gastric atrophy, corpus-predominant gastritis and intestinal metaplasia are at higher risk of developing GC and that eradication of *H. pylori* can prevent the progression of I-TGAC (Uemura *et al.*, 2001). The finding that *H. pylori* eradication can affect the development of GC was also shown in multiple antimicrobial therapy studies. Antimicrobial therapy of *H. pylori* in a trial study done by Correa *et al.* (2000) showed an elevated regression rate of gastric atrophy and intestinal metaplasia in comparison to the

control group (Correa *et al.*, 2000). Another Japanese study on 132 patients with early stages of I-TGAC and who had *H. pylori* infection showed that antibiotic therapy to eradicate *H. pylori* significantly decreased the recurrence of new carcinomas (Uemura *et al.*, 1997).

The association between *H. pylori* infection and the progression to the intestinal type of GC also was shown in multiple animal studies. *H. pylori* infection induced gastric ulcers, duodenal ulcers and intestinal metaplasia in Mongolian gerbil model (Hirayama *et al.*, 1996, Ohkusa *et al.*, 2003). Subsequent studies have shown that long term *H. pylori* infection results in the development of Gastric adenocarcinoma in the same animal model (Watanabe *et al.*, 1998, Honda *et al.*, 1998, Ogura *et al.*, 2000, Zheng *et al.*, 2004)

1.4.5. *H. pylori* induced mucosa-associated lymphoid tissue lymphoma

Lymphoma is a type of blood malignancy that arises from white blood cells. It usually involves the lymph nodes, bone marrow and spleen. However, it can also be detected in other sites of the human body and is referred to as non-Hodgkin lymphoma or extranodal lymphomas (Zucca *et al.*, 1997). Gastric extranodal lymphomas represent around 45% of all extranodal lymphomas. It accounts for 2-8% of all gastric tumours (Zucca and Cavalli, 2000). The annual average number of cases of MALT lymphoma in the United Kingdom is 14,176 cases per year (Cancer Research, 2022,July). The mucosa associated lymphoid tissue lymphoma (MALT-lymphoma) is a slow growing non-Hodgkin lymphoma that arises from B-cells, which forms a dense lymphoid infiltrate that attack and destroy the gastric glands resulting in a lymphoepthelial lesion (Zucca et al., 1997). *H. pylori*-induced gastritis can lead to MALT onset in the gastric mucosa and therefore, it has been postulated that the development of MALT in the gastric mucosa is an indication of *H. pylori* infection (Isaacson, 2005).

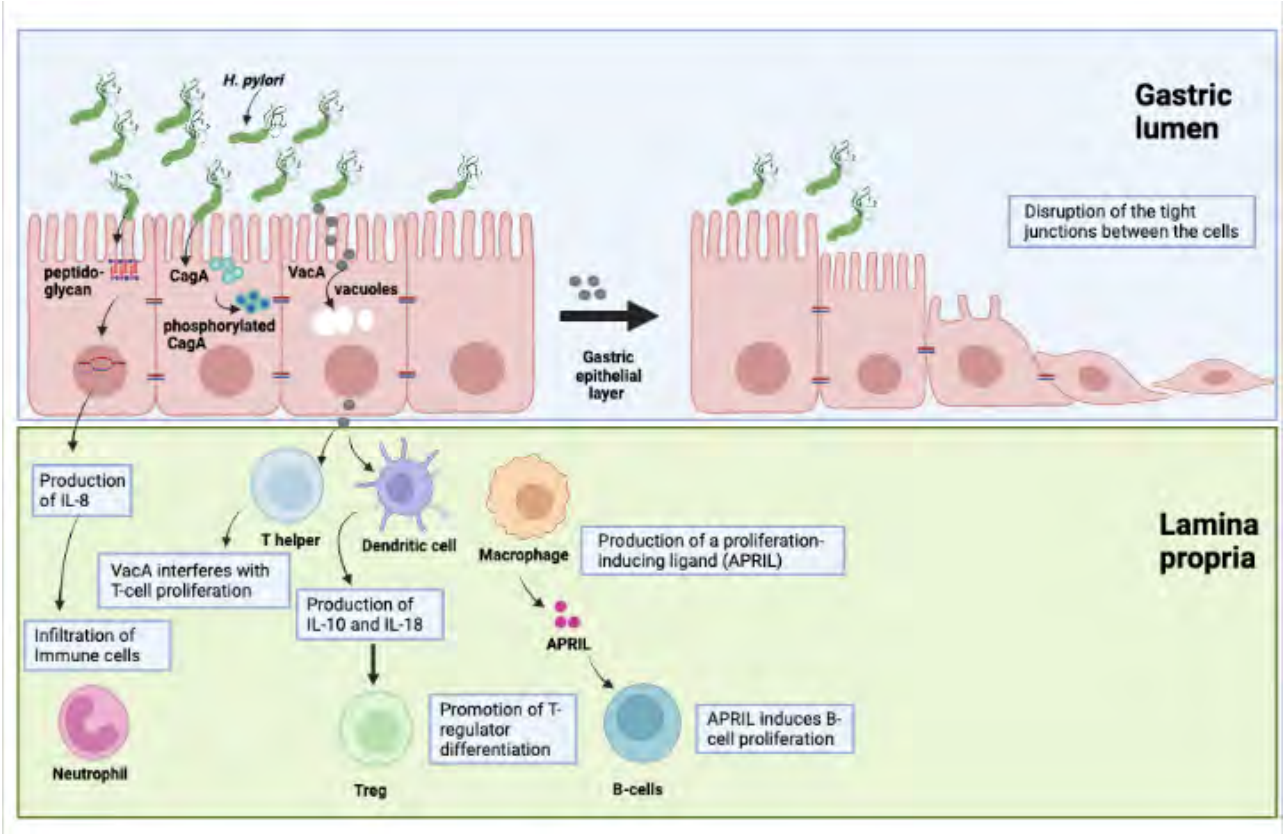

Figure 1. 3. *H. pylori* pathogenesis. The major virulence factors produced by *H. pylori* are shown here as either secreted or injected effector molecules. *H. pylori* injects peptidoglycan into the epithelial cells via *cag*PAI and stimulating the production of IL-8 and subsequent recruitment of immune cells to the lamina propia. CagA is an effector protein injected into epithelial cells also via *cag*PAI. Upon injection into host cells, CagA is phosphorylated by host cell kinases inducing transcription of pro-inflammatory genes. CagA also disrupts the tight junctions between epithelial cells. Vacuolating cytotoxin (VacA) induces vacuoles inside the epithelial cells, interferes with the production of IL-2, inhibits T cell proliferation and stimulates dendritic cells to produce IL-10 and IL18, which subsequently promote T-reg differentiation. *H. pylori* also stimulates the production of APRIL, which induces B-cells proliferation. Image created using BioRender **(https://www.biorender.com/).**

1.5. Antimicrobial resistance of *H. pylori*

Treatment of *H. pylori* infection includes a combination of multiple antibiotics. Mono antibiotic therapy failed to reach sufficient efficiency (>80% efficiency level) (Graham, 1994). Several antibiotics are effectively able to eradicate *H. pylori* infection, including clarithromycin, metronidazole, amoxicillin, rifabutin, levofloxacin and tetracycline. *H. pylori* eradication therapy includes a combination of two or three of these antibiotics and a proton pump inhibitor (PPI). The Public Health England and the National Institute for Heath and Care Excellence guidelines for the first line of treatment for *H. pylori* infection includes PPI, amoxicillin and either clarithromycin or metronidazole. The second line of treatment is used if the symptoms persist, which includes a PPI, amoxicillin and either tetracycline or levofloxacin (National Institute and Care Excellence, 2022)

Challenges with *H. pylori* eradication therapy are the limited options of effective antibiotics and the over prescriptions of certain antibiotics (Thung *et al.*, 2016, Kasahun *et al.*, 2020). In recent years, global reports of increasing rates of drug resistance in *H. pylori* was coupled with a decrease in the success of the eradication therapies (Malfertheiner *et al.*, 2017, Savoldi *et al.*, 2018). All the recommended *H. pylori* eradication therapies worldwide failed in around 10-30% of the patients (Li *et al.*, 2015). Therefore, the World Health Organization (WHO) have classified *H. pylori* in 2017 as one the 20 most threatening pathogens to humans due to their antimicrobial resistance (Tacconelli *et al.*, 2018).

1.5.1. Mechanism of drug resistance of *H. pylori*

Most of the drug resistance mutations are encoded with the chromosome (insertion, deletion, frameshift, nonsense and missense) rather than acquiring it from plasmids (Tuan *et al.*, 2019, Lauener *et al.*, 2019). These mutations occur at random in one bacterial cell and administration of

antimicrobial therapy acts as a selective force that selects for the growth of that mutant (Binh *et al.*, 2014, Gerrits *et al.*, 2002b).

H. pylori resistance to β-lactams such as amoxicillin includes alteration of the PBP1A binding site, which affects drug penetration (Nakamura *et al.*, 2003, Gerrits *et al.*, 2006, Okamoto *et al.*, 2002). *H. pylori* also alters the membrane permeability to β-lactams through mutations of *hofH*, *hefC* and *hopC* genes, which code for efflux pumps and porins (Dore *et al.*, 1999).

Moreover, *H. pylori* develops resistance to tetracycline through base-pair substitution mutations in the 16S RNA binding site of tetracycline (Dailidiene *et al.*, 2002, Gerrits *et al.*, 2002a). Another study has suggested that *H. pylori* uses efflux pump to decrease the drug concentration in the cytoplasm (Wu *et al.*, 2005). Fluoroquinolones such as levofloxacin and delafloxacin are also used to treat *H. pylori* infections. *H. pylori* acquire resistance to Fluoroquinolones by three mechanisms; altering the drug intake mediated by efflux systems, mutations in the DNA gyrase or topoisomerase IV (*topoIV*) and secreting enzymes that decrease drug activity (Aldred *et al.*, 2014). *H. pylori* resistance to clarithromycin is caused by a point mutation in the 23S rRNA gene (Gong and Yuan, 2018, Hu *et al.*, 2016, Versalovic *et al.*, 1996). Furthermore, *H. pylori* resist metronidazole through reducing the drug uptake and increasing drug efflux (Dingsdag and Hunter, 2018). Mutations in genes encoding the RecA protein that is involved in DNA repair are also reported in *H. pylori*. *H. pylori* also uses efflux pumps to decrease metronidazole concentrations in the cell (Albert *et al.*, 2005, Moore *et al.*, 1995).

There are global reports of emergence of multidrug resistant (MDR) *H. pylori* strains, which is a serious threat to the current available treatment options. MDR *H. pylori* strains have developed

resistance two or three drug families at the same time (Boyanova *et al.*, 2019). The mechanism of drug resistance is discussed in the previous section. However, the ability of *H. pylori* to form biofilms also contribute to the development of MDR *H. pylori* strains. Biofilms are a complex structure of bacterial communities adherent to a surface that forms a matrix filled with proteins and polysaccharides and act as a shield of protection. Biofilms are a method of bacterial survival as it prevents the penetration of drugs and at the same time allows horizontal gene transfer of drug resistance genes and overexpression of efflux pumps (Burmølle *et al.*, 2006, Madsen *et al.*, 2012).

1.5.2. Implications of *H. pylori* antimicrobial resistance

H. pylori capability to acquire exogenous DNA through conjugation and transformation results in a highly diverse population in a single colonised individual. *H. pylori* remarkable diversity in the genotype and phenotype allows it to develop antibiotic resistance rapidly (Sepulveda, 2013, Mégraud, 2004). The emergence of SDR and MDR *H. pylori* decreases *H. pylori* eradication efficiency and subsequently complicates the clinical outcomes of gastric diseases. Meta-analysis studies on *H. pylori* resistant strains to clarithromycin reported reductions in the efficacy of all regimens containing clarithromycin with a reduction of 66% in the treatment success of a clarithromycin triple treatment course (Dore *et al.*, 2000, Zou *et al.*, 2020). Regimens containing metronidazole also decreased in efficiency by 38% (Fischbach and Evans, 2007).

The drop in success of the antimicrobial therapies to eradicate *H. pylori* was also reported from regimens containing amoxicillin, levofloxacin, tetracycline and rifabutin (Nishizawa *et al.*, 2011). *H. pylori* eradication therapy also select for drug resistance among other microbial species of the gastrointestinal tract (Adamsson *et al.*, 2000). Studies have suggested that *H. pylori* eradication therapy induces gastrointestinal dysbiosis (Chen *et al.*, 2018, Guo *et al.*, 2020). Failure of these treatment therapies

increase the chances of developing MDR and SDR mechanisms in gastrointestinal microbiota (Guo *et al*., 2020).

1.6. The development of a vaccine against *H. pylori* infection

Antibiotic resistance limits the availability of effective antibiotic treatment to eradicate *H. pylori* as discussed in the previous section. Extensive research is currently conducted to find strategies to develop a vaccine against *H. pylori* infection. However, all the reported vaccine strategies were unsuccessful including vaccines based on the whole cell extract (Raghavan *et al.*, 2002), the adhesion antigens (Talebi Bezmin Abadi, 2016) and the flagellar antigens (Skene *et al.*, 2007). The failure of these strategies can be attributed to the complexity of the immune response to *H. pylori* infection and the genetic diversity of *H. pylori*. Although multiple studies are currently focused on developing an effective vaccine against *H. pylori* infection, many of them are still in preclinical phase (Dos Santos Viana *et al.*, 2021).

1.7. Potential alternative therapeutic approaches to treat *H. pylori* infection

The complications of *H. pylori* eradication therapy are a serious threat that faces human health. With the limitation in the treatment options and the absence of an effective vaccine against *H. pylori*, development of alternative therapeutic approaches is the only promising option to tackle the global burden.

1.7.1 The use of probiotics to treat *H. pylori* infection

Probiotics are a group of bacteria that colonise the gastrointestinal tract and have benefits for human health (Sazawal *et al.*, 2010). Studies have shown that the use of probiotics with antibiotics treatment can be helpful especially in diseases such as diarrhoea, inflammatory bowel diseases cardiovascular diseases and allergic reactions (Liu *et al.*, 2018, Saadat et al., 2007). It has been shown that probiotics inhibit *H. pylori* growth through competing over adhesion and subsequently inhibits *H. pylori*

colonisation. For example, *Saccharomyces boulardii* prevent the adhesion of *H. pylori* to the gastric epithelial layer through binding to sialic acid receptor (Sakarya and Gunay, 2014). *Lactobacillus reuteri* compete with *H. pylori* over binding to the gangliotetraosylceramide (asialo-GMI) and the sulfatide receptors of the epithelial cells *in vitro*. *L. reuteri* shares glycolipid specificity with *H. pylori* and therefore it was suggested that the use of *L. reuteri* as probiotic to prevent early colonisation of *H. pylori* (Mukai *et al.*, 2002). Several probiotics are also known to secrete antimicrobial compounds, such as bacteriocins that inhibit *H. pylori* growth. For example, *Bacillus subtilis* have been reported to produce a bacteriocin (Amicoumacin A) that inhibits *H. pylori* growth *in vitro* (Pinchuk *et al.*, 2001). Multiple strains of *Enterococcus faecium* also produce a bacteriocin and lactic acid that inhibit the growth of *H. pylori in vitro* (Kang and Lee, 2005, Tsai *et al.*, 2004). *H. pylori* interferes with the expression of mucus genes, such as MUC1 and MUC5 (Van den Brink *et al.*, 2000). It has been shown that probiotics such as *Lactobacillus rhamnosus* triggers the expression of MUC2 and MUC3 genes (Mack *et al.*, 1999), which can interfere with *H. pylori* colonisation by increasing mucus secretions.

Keikha *et al.* (2021) reported improved efficiency of *H. pylori* eradication therapy in a mouse model when combined with a probiotic (*Lactobacillus casei, Lactobacillus reuteri, L. rhamnosus GG,* and *S. boulardii)* (Keikha and Karbalaei, 2021). Sgouras *et al.* (2004) investigated the effect of probiotic adminisitration on *H. pylori* induced gastritis. The study used the sydney strain of *H. pylori* along with nine *H. pylori* clinical isolates. The inhibition effect of *L. casei* was tested using *in vitro* co-culture assays and an *in vivo* mouse model. *L. casei* inhibited all the tested *H. pylori* strains only in co-cultures. However, the main observation of the study was a significant reduction in *H. pylori* colonization in the C57BL/6 mice model after administration of *L. casei* (Sgouras *et al.*, 2004). Lactobacilli are one of the main members of the human gastrointestinal microbiota. Several *Lactobacillus* species are known to inhibit *H. pylori* growth and reduce *H. pylori* induced-inflammation (Chen *et al.*, 2019). Other animal

studies have also shown that probiotics can reduce *H. pylori* colonisation and *H. pylori*-induced gastritis by modulating IL-8, IL-12 and TNF-α (Sgouras *et al.*, 2005, Ushiyama *et al.*, 2003). Clinical trials of combined antibiotic therapy with probiotics in both adults and children showed improved *H. pylori* eradication efficiency (Keikha and Karbalaei, 2021).

Despite the promising potential of using probiotics in treating *H. pylori* infection, there are challenges with developing a therapeutic approach using probiotics. Studies on treating *H. pylori* infection with probiotics differ in the experimental design, treatment duration and the type of probiotics used in the clinical trials, which affects the interpretation of the results. Moreover, one of the side effects of probiotics supplements is serum elevated histamine levels and digestive disorders (Gao *et al.*, 2015). Therefore, more research is needed using larger sample size to validate these studies and determine a universal recommendation of including probiotics in *H. pylori* treatment.

The observation that a member of the gastrointestinal microbiota can inhibit *H. Pylori* growth in an *in vitro* model and reduce *H. pylori* colonization in an animal model might explain the difference in the stomach microbial composition between *H. pylori* infected individuals and non-infected individuals. Therefore, understanding *H. pylori* interaction with members of the oral and gastric microbiota can help in finding alternative therapeutic options and preventative measures for gastric diseases.

1.7.2 Novel compounds against *H. pylori* infection

The need to develop an effective treatment to eradicate *H. pylori* infection is crucial. Therefore, many studies were focused on finding novel compounds to treat *H. pylori* infection. For example, Jia *et al.* (2022) reported a novel compound produced by *Streptomyces armeniacus* that exhibits anti-*H. pylori* effect. Armeniaspirol A (ARM1) exhibits a potent effect against *H. pylori* and MDR *H. pylori* strains, interferes with the biofilm formation and eliminates *H. pylori* biofilms by a dose dependent manner. The

inhibition activity of ARM1 was assessed in an animal model in combination with omeprazole and induced effective elimination of *H. pylori* infection (Jia *et al.*, 2022). González *et al*. (2019) tested the inhibitory effect of four natural flavonoids on *H. pylori* growth *in vitro*; apigenin, chrysin, kaempferol and hesperetin, all had a bactericidal activity against *H. pylori* (González *et al.*, 2019). Although these compounds hold potential in *H. pylori* treatment, the development of drugs from a natural compound is complicated and requires extensive toxicity and safety assessments followed by clinical trials to approve the use of it in clinical applications.

1.8. The role of the gastric microbiome in gastric cancer

1.8.1. The history of microbiome studies

The human microbiome is a group of microorganisms that colonise different parts of the body such as the gut, the skin, the oral cavity. Microbiome members have a mutualistic or parasitic relationship with the host and play an important role in homeostasis, immunity, digestion, vitamin production and endocrine regulation and other important body functions (Montoliu *et al.*, 2013). For many of years, microbial research was limited by the tools available to investigate microbial infections, such as light microscope and traditional lab culturing methods. However, most of the microbiome members cannot be grown in traditional laboratory methods and require additional unique growth conditions, media and atmosphere (Ley *et al.*, 2006). The advances in sequencing techniques in the early 2000s allowed the identification and the ecological characterisation of these bacterial communities. These culture-independent techniques led to a rapid growth in studies aimed at understanding the role of these bacteria in human physiology and disease (Cantarel *et al.*, 2012, Turnbaugh *et al.*, 2006).

1.8.2. Tools to study the microbiome

One of the major issues with microbiome studies is the accurate identification and characterisation of the microbial communities. Microbiome studies often use the microbial gene amplicon sequencing technique to identify the hypervariable regions of small subunit ribosomal RNA gene (16S). The method involves the amplification of the 16S gene that is conserved within each bacterial species using polymerase chain reaction (PCR) amplification (Caporaso *et al.*, 2011, Sanschagrin and Yergeau, 2014). The main advantage of using 16S rRNA sequencing technique is that it provides species-level identification of bacterial species within the screened microbial community without the need for culturing. However, the disadvantages of using 16S rRNA sequencing technique is that it is subjected to variation in PCR amplification frequencies and incomplete online databases. Moreover, the results are of the relative abundance of the screened sequence rather than a quantitative number of the bacterial species. The method does not provide functional information about the amplified sequences (Jo *et al.*, 2016).

In order to avoid the limitations of using 16S rRNA sequences, many microbiome studies have instead used high-throughput shotgun metagenomics sequencing. The method involves amplifying the whole bacterial genome with random primers to sequence overlapping regions of the genome. This is followed by mapping the amplified sequence to a reference database or generate a *de novo* assembly of the sequence reads. Shotgun metagenomic sequencing is a more sensitive technique and provides an enhanced detection of the bacterial species and strain level, increased recognition of bacterial diversity and functional predictions of the genes (Ranjan *et al.*, 2016). However, shotgun metagenomics is more expensive and requires meticulous data analysis (Kuczynski *et al.*, 2011, Sims *et al.*, 2014). One of the drawbacks of using metagenomics in microbiome studies is the variation the sample size, inappropriate data analysis. Dubourg *et al.* (2013) showed that the number of bacterial species isolated by culture from

stool samples exceeded the results of pyrosequencing (Dubourg *et al.*, 2013). Some studies that compared culturing and sequencing techniques have shown differences in the proportions of Gram-positive and Gram-negative bacteria (Gill *et al.*, 2006, Turnbaugh *et al.*, 2007).

Culturomics involves culturing bacteria from body samples. Microbiome studies have used culturomics to fill the gaps of sequencing techniques (Patel and Lebwohl, 2011). Culturomics is used to optimise the traditional culturing techniques by showing that every microorganism is culturable under the right conditions and using the right tools. (Lagier *et al.*, 2015). However, culturomics is subjected to the risk of contamination and variation in technical skills (Bilen *et al.*, 2018).

1.8.3. Gastric microbiome

The stomach was long believed to be hostile to bacterial growth. However, the discovery of the gastric microbiome shifted the understanding of the gastric microbial composition (Sgambato *et al.*, 2017). It is estimated that the microbial density of the stomach is between 10^1-10^3 colony forming units/g (Sheh and Fox, 2013, Wroblewski *et al.*, 2010). However, most of these bacteria cannot survive the stomach defence mechanisms.

There is a great discrepancy in the results of gastric microbiome studies because of differences in the sample size, population ethnicity and patient diet, all of which influence the composition of the gastric microbiome (David *et al.*, 2014). Nonetheless, these studies have limitations as they are based on very sensitive sequencing techniques. They are also subject to factors such as drug intake and diet that can vary between tested subjects. One disadvantage of using sequencing data in microbiome studies is that results are based on the distribution or relative abundance of bacteria and therefore dose not quantify the actual number of a particular bacteria in the samples tested. Understanding polymicrobial interactions

and how they can shape immunity and play a role in carcinogenesis requires methods beyond sequencing techniques.

These methods only detect the presence or absence of certain bacteria. Sample collection can affect the accuracy of the sequenced DNA. For example, endoscopy is used in stomach biopsies and there is a possibility of oral bacteria contamination during the insertion and removal of the endoscope (Spiegelhauer *et al.*, 2020). Therefore, these methods do not provide differentiation between the resident bacteria and the transient/contaminant bacteria nor determine the cause-and-effect relationship.

1.8.4. *H. pylori* influence on the composition of the gastric microbiome

Although there are inconsistencies regarding the most prominent members of the gastric microbial communities, there is some consensus that oral bacteria dominate the gastric niche during the intermediate to late stages of gastric cancer. Studies have reported that the main genera that dominate the gastric mucosa other than *Helicobacer* were *Streptococcus, Rothia, Lactobacillus, Veillonella, Prevotella, Neisseria* and *Hemophilus* (Alarcón *et al.*, 2017, Yu *et al.*, 2017). Studies using molecular sequencing techniques have reported that *Streptococcus* was the most dominant genus in the stomach regardless of *H. pylori* infection, however, *Prevotella, Neisseria, Veillonella* and *Rothia* were also high in abundance in the gastric tissue (Schulz *et al.*, 2016, Bik *et al.*, 2006). Studies on the bacterial composition of *H. pylori* infected individuals describe conflicting results (Liatsos *et al.*, 2022). For example, Li *et al*. (2017) reported that the gastric microbial diversity is greatly influenced by *H. pylori* infection. *H. pylori* is the most dominant bacterial species in the stomach of *H. pylori*-infected individuals while non-*H. pylori* Proteobacteria such as *Haemophilus, Neisseria, Stenotrophomonas*, and *Serratia* were the major bacterial genera reported from the stomach of *H. pylori* negative individuals (Li *et al.*, 2017). However, Bik *et al*. (2006) used a 16S rDNA clone library method to show that there was

no significant difference in gastric microbiota between *H. pylori* infected individuals and *H. pylori* non-infected individuals (Bik *et al.,* 2006). Andersson *et al.* (2008) and Li *et al.* (2017) showed a significant decrease in gastric microbial diversity with *H. pylori* infection using 454 pyrosequencing technology and 16S rDNA sequencing (Andersson *et al.*, 2008, Li et al., 2017), implying that *H. pylori* dominates the stomach of *H. pylori* infected individuals. The main studies that described the gastric microbial profile are listed in Table 1.1.

1.8.5. The role of *H. pylori* and the gastric microbiome in GC

There is also a discrepancy between microbiome studies on the exact bacteria that are enriched during a certain stage of the gastric carcinogenic cascade. Several studies aimed at identifying the dominant bacteria during the different stages of the gastric carcinogenic cascade reported low abundance of *H. pylori* in the later stages of carcinogenesis and an enrichment of other bacterial genera such as *Lactobacillus*, *Streptococcus*, *Escherichia, Shigella, Veillonella, Actinomyces* and *Prevotella* (Figure 1.5). The dysbiosis in the stomach microbiome composition might result from the changes in the stomach epithelium lining during the progression to GC. The loss of the parietal glands, where *H. pylori* colonises, results in reduced acidity of the stomach allowing other bacteria to colonise the stomach. Another possible explanation to this observation would be that some of these bacteria outcompete or inhibit *H. pylori* growth. However, there is still no conclusive scientific evidence that explains the dysbiosis of gastric microbiota and the decrease in *H. pylori* abundance with the progression to GC (Yang *et al.*, 2021, Cho and Blaser, 2012, Dicksved *et al.*, 2009).

Coker *et al.* (2019) investigated the shifts in microbial diversity associated with the gastric carcinogenic cascade. The study used 16S rRNA gene analysis of gastric biopsies from cases of superficial gastritis, atrophic gastritis intestinal metaplasia and gastric cancer. A significant shift in gastric microbial

composition was observed in samples of intestinal metaplasia and GC, with a significant enrichment of oral bacteria in samples of GC ($p < 0.05$) (Coker *et al.*, 2018).

The degree of *H. pylori*-induced inflammation is affected by pre-infection with gastric microbiota (Lofgren *et al.*, 2011). Altering microbiota composition with antibiotics resulted in a marked decrease in $CD4^+$ T-helper cells and IFN-γ transcript levels in gastric tissue of C57BL/6N mice model (Rolig *et al.*, 2013). A transgenic insulin-gastrin (INS-GAS) mice infected with *H. pylori* and a complex microbiota developed gastric adenocarcinoma of a higher rate with more severe pathology than (INS-GAS) mice infected only with *H. pylori* (Lertpiriyapong *et al.*, 2014). Their finding can suggest that some of these bacteria outcompete or inhibit *H. pylori* growth. However, there is still no conclusive scientific evidence that explains the dysbiosis of gastric microbiota and the decrease in *H. pylori* abundance with the progression to GC (Yang *et al.*, 2021, Cho and Blaser, 2012, Dicksved *et al.*, 2009). These findings might suggest that the atrophy caused by *H. pylori*-induced inflammation alters the gastric microbiota composition. This alteration can lead to colonization of other commensal bacteria that might escalate the progression to gastric adenocarcinoma. Therefore, this has an implication on the use of these bacteria as probiotics.

The interaction of *H. pylori* with members of the oral and gastric microbiota is an area of interest aimed at understanding how these interactions influence and shape the gastric microbiota composition and trigger gastric diseases. For example, Khosravi *et al*. (2014) investigated the interaction of *H. pylori* with *Streptococcus mitis* and *Lactobacillus fermentum*, which are commonly found in healthy stomach and gastric disease, using *in vitro* co-culture assays. *S. mitis* inhibited *H. pylori* growth and induced conversion of *H. pylori* from spiral-shaped to coccoid as a result of oxidative stress. The study suggested that *S. mitis* secrete a compound that mediate the inhibition of *H. pylori* growth. Metabolic analysis of

S. mitis culture supernatant resulted in a candidate compound that shares homology with an anti-cancer compound (Tenovin-6) (Khosravi *et al.*, 2014a). Their findings led to the suggestion that members of the oral and gastric microbiota modulate *H. pylori* physiology and possibly gastric diseases. Nonetheless, there is still no conclusive evidence that confirms whether the alteration in the gastric microbial composition drives the carcinogenic cascade or whether it is a result of precancerous lesions that was initiated by *H. pylori* infection, host and environmental factors or a combination of these factors.

1.9. *Actinomyces*

Actinomyces are Gram-positive, filamentous, facultative or strict anaerobic bacteria that belongs to the *Actinomycetaceae* family of the Actinobacteria phylum. The genus inhabitant the oral cavity, gut and female genital tract of humans. There are more than 20 described species of this genus, most of members are members of the human microbiota and do not normally cause disease (Könönen and Wade, 2015). *Actinomyces* spp. abundance in the oral cavity is relatively moderate representing 0.1% to >2.0% of the oral microbiota reported from a geographically diverse group (Segata *et al.*, 2012). *Actinomyces* spp colonise the oral cavity of humans during the first year of life along with other members of the oral microbiota such as *Streptococcus, Staphylococcus, Neisseria, Lactobacillius* and *Veillonella* (Nelson-Filho *et al.*, 2013). Könönen *et al.* (1991) investigated the anaerobic bacterial composition of the oral cavity using samples of 44 infants, *Actinomyces* spp were detected in 32% of the samples of the 2 months old, 80% of the samples of the 6 months old and 91% of the samples of the one-year-old (Könönen *et al.*, 1999). However, there are no studies that illustrate the mechanism by which *Actinomyces* spp establish colonisation of the human oral cavity.

Actinomyces spp. are opportunistic pathogens that only invade tissues when the host-microbial balance is disturbed and the mechanism by which these bacteria establish the infection is yet to be investigated.

Table 1. 1 The main studies that described the gastric microbial profile.

Sample size	Method	Main findings	Reference
160 biopsies from GC patients	16S rRNA sequencing	*H. pylori* is the dominant bacterial species in the non-malignant gastric tissue followed by members of the following genera: *Enterococcus; Lactobacillus* and *Streptococcus.*	(Yu *et al.,* 2017).
215 gastric biopsies (130 samples were from *H. pylori*-positive and 84 samples were *H. pylori*-negative)	16S rRNA sequencing	No significant difference in the gastric microbial profile was detected between *H. pylori* positive samples and *H. pylori* negative samples. The main bacterial species detected were: *Streptococcus parasanguinis; Streptococcus mitis; Streptococcus salivarius; Streptococcus anginosus; Neisseria flavescens; Neisseria perflava; Rothia mucilaginosa; Lactobacillus fermentum; E. coli; Klebsiella pneumonia, Burkholderia pseudomallei, Bacillus cereus; and Acinetobacter baumannii.*	(Khosravi *et al.*, 2014b)
Gastric biopsies and gastric juices of 12 persons	16S rDNA amplicons pyrosequenced	The main bacterial genera that colonise the stomach were *Propionibacterium, Lactobacillus, Streptococcus and Staphylococcus.*	(Dicksved *et al.,* 2009)
6 gastric biopsies (3 samples were from *H. pylori*-positive and 3 samples were *H. pylori*-negative)	16S rRNA sequencing	A significant decrease in the gastric microbial diversity was observed in the *H. pylori* positive samples. The main bacterial genera detected from *H. pylori*-negative samples were *Streptococcus; Actinomyces; Prevotella; and Gemella*	(Andersson *et al.,* 2008)

23 gastric biopsies (12 *H. pylori* positive samples)	16S rDNA clone library approach	No significant difference was observed between *H. pylori*-positive samples and *H. pylori*-negative samples. The main genera that dominate the stomach are *Streptococcus, Prevotella, Rothia, Fusobacterium, veillonella.*	(Bik *et al.,* 2006)
33 individuals	Illumina MiSeq platform targeting the 16S rDNA.	The main genera detected from the samples were *Helicobacter (H. pylori), Haemophilus, Serratia, Neisseria and Stenotrophomonas.* A significant decrease in microbial diversity was observed in *H. pylori*-positive samples.	(Li *et al.,* 2017)

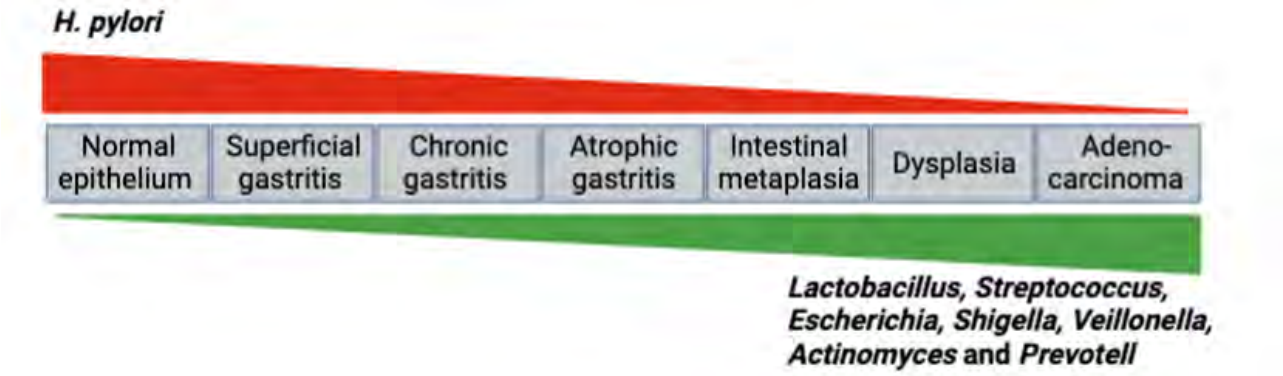

Figure 1. 4 The gastric microbiota changes during the progression to gastric adenocarcinoma. *H. pylori* abundance in the stomach decreases with the progression to gastric cancer and other bacteria such as *Lactobacillus, Streptococcus, Escherichia, Shigella, Veillonella, Actinomyces* and *Prevotella* are enriched in the gastric mucosa in the later stages of the carcinogenic cascade (Yang et al., 2021, Cho and Blaser, 2012, Dicksved et al., 2009, Coker et al., 2018).

A. israelii is the causative agent of actinomycosis, a rare chronic bacterial infection that affects immunocompromised patients and low hygienic individuals. Actinomycosis can be detected in various body sites however, pelvic actinomycosis (in women with contraceptive, devices such as intrauterine devices) and pulmonary actinomycosis (affecting smokers with poor oral hygiene) are the main common actinomycosis types. The treatment includes prolonged antimicrobial therapy that ranges between three to six months to facilitate the drug penetration into the infected tissue (Valour *et al.*, 2014). In a study that compared the microbial composition of the stomach microbiota, *A. oris* was detected in biopsies from the stomach of *H. pylori*-negative individuals and was absent in biopsies from the stomach of *H. Pylori*-positive individuals (Schulz *et al.*, 2018). *A. oris* is a member of the oral and gut microbiota that plays an important role in biofilm formation in the mouth. Biofilm of the teeth surface is a polymicrobial layer that protects from oral infections. *A. viscosus* colonises the human oral cavity and lives in a dynamic relationship with the host. However, it can cause dental plaques and actinomycosis on some occasions such as poor oral hygiene or smoking (Chalmers Natalia *et al.*, 2008, Palmer *et al.*, 2003).

Studies on the interaction of *Actinomyces* spp with oral and gastric microbiota are limited. Nonetheless, it has been reported that culture supernatants of oral strains of *A. viscosus, A. naeslundii and A. odontolyticus* significantly inhibit the proliferation, metabolic enzymatic activity, hyphae formation, adhesion and biofilm formation of *Candida albicans*. *C. albicans* is a serious oral pathogen that causes *C. albicans*-associated stomatitis and thrush (Guo *et al.*, 2015). Another study on the role of oral bacteria on *H. pylori* growth using the stab culture method for inhibition assessment showed that *A. viscosus* inhibits *H. pylori* growth. However, the study did not include any quantitative or mechanistic analyses (Ishihara *et al.*, 1997).

1.10. Wider importance of this study and preliminary work

The interaction of *H. pylori* with members of the oral and the gastric microbiota is not fully understood. Most of the modern gastric microbiota studies are based on new generation sequencing techniques that show correlation rather than causation. Data from these studies does not indicate the mechanism of bacterial dysbiosis observed in gastric diseases. Understanding the interaction between *H. pylori* and resident and transitional bacteria is crucial to understanding bacterial interactions and their role in driving gastric carcinogenesis. Moreover, this avenue of research could provide the rationale for alternative therapeutic strategies to treat *H. pylori* infections especially with the emergence of MDR *H. pylori* strains and the lack of an effective *H. pylori* vaccine.

To study interactions of *H. pylori* with members of the gastric cancer associated microbiome, a MSc student in the Rossiter laboratory, Paida Katsande, first investigated the impact of certain bacterial species on *H. pylori* growth (MSc thesis, University of Birmingham). Katsande reported a negative effect of *Prevotella intermedia* on *H. pylori* growth. Her study also showed that *Actinomyces oris* inhibited *H. pylori* growth in a co-culture and that the inhibition was mediated by a factor secreted into the medium. *Actinomyces viscosus* C505 also inhibited *H. pylori* growth in a co-culture medium. **This led us to hypothesise that *Actinomyces* spp. produce one or multiple bacteriocins that inhibit *H. pylori* growth. Understanding the regulation and biochemical characteristics of these inhibitors could further our understanding of why *H. pylori* abundance decreases during gastric cancer and identify a novel compound for the treatment *H. pylori* infections.**

1.12. Aims

There are 2 main aims of this study.

1. Determine the conditions by which *A. oris* secretes the inhibitors of *H. pylori* growth.
2. Identify the potential inhibitors produced by *A. oris* using biochemical and bioinformatics techniques.

CHAPTER 2:

MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 2.1. Materials are listed in Table 2.2 *H. pylori* is routinely grown from a glycerol stock onto horse blood agar plate with Skirrow's supplement (HBS plates) for 2 days at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2 or in a candle jar with CampyGen sachets (Oxoid) at 37°C (passage number 0, P0). *H. pylori* was then passaged 2-3 times before using it for experimental work. To prepare *H. pylori* cells for experiments, 2-3 full loops from *H. pylori* plate (P2 -P4) were inoculated in brain heart infusion broth with 10% fetal bovine serum (FBS) supplemented with Skirrow's supplement (vancomycin (6.2 mg/ml), polymyxin B (0.155 mg/ml) trimethoprim (3.125 mg/ml)) and incubated statically under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2 for 1-2 days. Liquid cultures were grown vertically in a vented T25 tissue culture flasks (Corning®). *H. pylori* plates of passages of 3,4 and 5 were strictly used for experimental work. The same growth protocol was followed to grow *C. jejuni*.

A. oris, *V. parvula* and *P. intermedia* were grown from glycerol stocks onto blood agar and incubated in a candle jar with AnaeroGen sachet (Oxoid) at 37°C for 2 days (P0). This is followed by passaging the bacteria on blood agar and incubating it for another day. For liquid culture of *A. oris*, the bacterium was initially grown in BHI medium supplemented with 10% FBS media, (FBS was removed from the growth medium in the later experiments). Liquid cultures were grown at the same anaerobic conditions, unless otherwise stated.

All other bacterial strains used in this study were grown onto horse blood agar. Bacteria used for inhibition screening assays were quality control strains provided by the Microbiology Diagnostic Laboratory at the National Guard hospital, king Fahad Medical City, Riyadh Saudi Arabia. Bacterial strains were regularly grown from glycerol stock on Horse blood agar at 37°C for a day. An overnight

culture was prepared by inoculating a single colony into liquid BHI media and incubated at 37°C. Glycerol stocks of bacterial strains were made by adding 700 µl of an overnight liquid culture to 700 µl of BHI with 30% glycerol and stored at – 80°C.

2.2. Co-culture assay

Overnight cultures of the tested bacteria were strictly used in this assay. Measurements of the optical density at 600 nm of the bacterial cultures were normalised to an OD_{600nm} of 1. Bacteria tested were inoculated at a starting inoculum of OD_{600nm} of 0.01 into BHI media supplemented with 10% FBS, as either a single culture or co-culture and incubated for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was assessed by enumerating viable cells by serially diluting cultures and plating the viable cells for colony forming unit analysis. *H. pylori* was incubated for 5-7 days onto HBS plates, *V. parvula, P. intermedia* and *A. oris* for 2-3 days onto blood agar plate under the standard growth conditions for the isolation of single colonies.

2.3. *Actinomyces oris* culture supernatant harvesting

The whole growth of an *A. oris* plate of passage number 1 was inoculated into 200 ml of BHI medium for 24 h (the incubation period was increased to 48 h in later experiments to reach an OD_{600nm} of 2.2 or more). *A. oris* culture was divided in 50 ml aliquots in 50 ml Falcon tubes, (Merck), followed by centrifugation at 3300 x g for 3 h. Supernatant of the cultures were collected, and filter sterilized using 0.22 µm Vacuum Filtration system (Coring) in the cold room or on ice. Filtered supernatant were aliquoted in 7 ml aliquots and snap frozen with liquid nitrogen before storing at – 80°C. The protocol initially included growing *A. oris* in BHI with 10% FBS, FBS was removed from the medium in later experiments.

Table 2. 1. Bacterial strains used in this study

Bacteria	**American Type Culture Collection (ATCC) number**	**Source**
Veillonella parvula	n/a	This study
***Prevotella intermedia* (oral isolate)**	n/a	This study
Actinomyces oris	n/a	This study
***Actinomyces oris* (gastric isolate)**	n/a	This study
Actinomyces viscosus	C505	ATCC
Campylobacter jejuni	33560	ATCC
Klebsiella pneumoniae	1705	ATCC
Escherichia coli	25922	ATCC
Escherichia coli	BW3115	This study
***Salmonella* Typhimurium**	14028	ATCC
Pseudomonas aeruginosa	1744	ATCC
Staphylococcus aureus	25923	ATCC
Staphylococcus epidermidis	1228	ATCC
Enterococcus faecalis	29212	ATCC
Streptococcus mitis	n/a	This study
Staphylococcus epidermidis	14990	ATCC

Table 2. 2. Materials used for bacterial growth

Material	Manufacturer	Catalogue number
Blood agar	OXOID	CM0055
Defibrinated Horse blood	EO labs	DHB100
Brain heart infusion	OXOID	CM1135
fetal bovine serum	Thermo fisher	A38401
Campygen	OXOID	CN0025
Vancomycin	VWR	1205950010
Polymyxin B	VWR	A08900001
Trimethoprim	VWR	J6305306
Amphotericin B	VWR	12350000

2.4. Inhibition screening assay

An overnight culture of the tested bacteria was inoculated into fresh BHI-10% FBS medium as well as in a medium supplemented with 50% of the *A. oris* culture supernatant. The optical density at 600 nm reading of the overnight cultures were normalised to an OD_{600} of 1. Bacteria tested were inoculated at a starting inoculum of OD_{600nm} of 0.01. Flasks were incubated statically for 48 h at 37°C in an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Growth of bacteria was assessed using optical density measurements at 600 nm.

2.5. The time-course of *A. oris* inhibition on *H. pylori* growth

Overnight cultures of the indicated bacteria were inoculated into fresh BHI-FBS medium at a starting inoculum OD_{600nm} of 0.01, as either a single culture or co-culture. Cultures were grown statically at 37°C in an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured at hours 0, 14, 24 and 37. One flask was used for each time point and discarded after measurements were taken to eliminate variation in O_2 levels during sampling. Bacterial growth was measured using viable colony count enumeration and measurement of the optical density at 600 nm. *H. pylori* was incubated for 5-7 days, and *A. oris* for 2-3 days under the standard growth conditions for the isolation of single colonies.

2.6. Dialysis of *A. oris* culture supernatant

A. oris culture supernatant (SN) was thawed on ice and was transferred to a SnakeSkin™ dialysis tubing, 3.5 K MW CO and the sample was placed in a 1 L beaker filled with 500 ml of BHI medium and stirred for 24 h in the cold room. The media was changed 3 times during dialysis. The dialysed SN was filter sterilized using 0.22 μm filter and tested for inhibition activity at 50% concentration following the same protocol described in section 2.4.

2.7. Size exclusion of *A. oris* culture supernatant using 5 kDa viva spin column

|Active *A. oris* SN was thawed on ice and 15 ml aliquots of the SN were transferred to a viva-spin concentrator with a 5 kDa MW cut-off (Sartorius). Columns were centrifuged at 3300 x g at 4°C for 4 – 5 h until the volume went down to 1.5 ml. The retentate was routinely dialysed against BHI prior to use in experiments. Both the retentate and the filtrate were processed on ice and used for inhibition assays at 50% concentration. The same inhibition assay protocol described in section 2.4 was followed to test the inhibition activity of the retentate and the filtrate.

2.8. Heat treatment of *A. oris* culture supernatant

A. oris SN was thawed on ice, and 1 ml aliquots were transferred to 1.5 ml tubes (Eppendorf). The tubes were then placed on a heat block at 95°C for 5 minutes. Heat treated SN was tested for inhibition activity at 50% concentration using the inhibition protocol described in section 2.4.

2.9. Proteinase K effect on *H. pylori* growth

The indicated concentrations of proteinase K (PK) were tested for their effect on *H. pylori* growth by adding the enzyme to the liquid growth medium. Parallel cultures of the same PK concentrations were included in the experiment with the addition of phenylmethylsulfonyl fluoride (PMSF) at 1µg/ml concentration to inhibit the activity of PK enzyme. This was followed by addition of overnight culture of *H. pylori* at a starting inoculum of OD_{600nm} of 0.01. Flasks were incubated according to the standard growth conditions for *H. pylori*, growth was measured using optical density at 600 nm.

2.10. TrypLE treatment of *H. pylori*

The indicated concentrations of TrypLE were tested on *H. pylori* growth and were added to the liquid growth medium followed by addition of an overnight culture of *H. pylori* at a starting inoculum of

OD_{600} of 0.01. Flasks were incubated according to the standard growth conditions for *H. pylori*, growth was measured using optical density at 600 nm.

2.11. TrypLE digestion of *A. oris* culture supernatant

TrypLE (Sigma) was added to the indicated samples at a volume of 100 µl/ml for 0 and 30 minutes. TrypLE was inactivated by addition of the liquid growth medium (containing 10% FBS). The liquid growth medium was added to the treated SN, retentate and filtrate at 50% concentration. Overnight culture of *H. pylori* was added at a starting inoculum of OD_{600} of 0.01. Flasks were incubated according to the standard growth conditions for *H. pylori*, growth was measured using optical density at 600 nm.

2.12. Lyophilisation of *A. oris* culture supernatant

A. oris culture supernatant (SN) was aliquoted into glass round flasks and lyophilized for 48 h using a lyophiliser. Deionised distilled water was added to the lyophilized *A. oris* SN to make up a volume of 10 x concentrated *A. oris* SN. The lyophilized *A. oris* SN was filter sterilized using 0.22 µm filter and tested for inhibition activity at 5% concentration using the inhibition protocol described in section 2.4.

2.13. Protein analysis

2.13.1. SDS-PAGE

Proteins were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (tris-glycine SDS-PAGE). Gels were poured using 1 mm thickness Mini-PROTEAN® Tetra Handcast Systems (Bio-Rad). Gels were made with a resolving gel (10% acrylamide) and a stacking gel (4% acrylamide). The resolving gel was prepared by adding 1.5 M Tris-HCL, pH 8.8 buffer, 20% (w/v)

SDS and 10% (w/v) ammonium persulphate (APS) and 1 x TEMED, deionized water was added to make up the final volumes required. The stacking gel was prepared by adding 0.5 M Tris-HCL pH 6.8 buffer to 20% (w/v) SDS, 10% (w/v) ammonium persulphate (APS) and 1 x TEMED, deionized water was added to make up the final volumes required. The running buffer (25 mM Tris-HCL, 200mM glycine, 0.1% SDS) was used for electrophoresis. A broad range ladder (10-250 kDa) was used for protein size estimation. A voltage of 120 V was applied to the gels for the first 15 minutes and increased afterwards to 150 V for 30 minutes to yield a better separation of the proteins.

2.12.2. Coomassie Brilliant Blue staining

Gels were routinely stained with the Fast Coomassie stain (0.05% Coomassie Brilliant Blue, 0.4 M Citric acid, 20% Propan-2- ol). De-staining was achieved by microwaving gels in water at full power for 60 seconds. Gels were left to cool at room temperature for 5 minutes before pouring the Fast Coomassie and washing the gels with hot water and microwaving it again at full power for 60 seconds. This step was repeated 7-10 times to achieve the desired intensity of the stain to visualise the proteins.

3.2.17. Mass spectrophotometry

The desired bands were cut from the SDS PAGE gel of *A. oris* SN with a razor blade, placed in an Eppendorf 1.5 ml tube and sent to the Mass spectrophotometry (MS) department at the Collage of Biosciences, University of Birmingham, Birmingham. The detected spectra were analysed using Proteome Discoverer version 2.2 and *A. oris* database obtained from UniProt (https://www.uniprot.org/), accessed in December 2021.

2.12.4. Bioinformatic analysis of the potential inhibitors

The amino acid sequence of the two candidate proteins were detected within the retentate sample of *A. oris* culture supernatant is presented in Table 2.4. The amino acid sequence of the two proteins in FASTA format was analysed using Blastp alignment search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The amnio acid sequence of the resulted homologous protein hits in FASTA format was obtained from the National Library of Medicine (https://www.ncbi.nlm.nih.gov/). The candidate proteins detected from *A. oris* culture supernatant were aligned with their homologous proteins using Clustal Omega multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). The protein domains were determined using InterPro database (https://www.ebi.ac.uk/interpro/). Analysis of the putative biosynthetic gene clusters of *A. oris* was preformed using AntiSMASH database (https://antismash.secondarymetabolites.org/).

2.13. Genomic sequencing

Bacterial strains were sent to the Microbes NG, University of Birmingham, for whole genome sequencing, The complete genome sequences were determined using Miseq 46 llumine platform, (2x250bp) 30x coverage. The N50 values and the genome assembly sizes were provided by MicrobesNG. The Average Nucleotide Identify value (ANI) and the GC% of the clinical *A. oris* isolates used in this study were calculated using the ANI calculator tool by EZ Biocloud (https://www.ezbiocloud.net/tools/ani).

2.14. Statistical analysis

Results were evaluated using Student *t* test and one-way ANOVA followed by Dunnett's multiple comparisons test, GraphPad Prism version 8.0.0 for MacBook, GraphPad Software, San Diego, California USA, (www.graphpad.com). *P* values of <0.001 (***), <0.01 (**), and <0.05 (*) were considered statistically significant**.**

Table 2. 3. Amino acid sequence of the proteins used in this study

Protein	UniPro/NCBI accession number	Amino acid sequence (FASTA format)
A0A1Q8XG29_9ACTO by *A. oris*	A0A1Q8XG29_9ACTO	MGRHSQTSSLSTTLAGLGSLASKKRAASASHGRRRAEGPAKTS LTPMLFKAGGAAAAFSLAVSGAAYAAISAGDDEGRSSSGGSFG LIGGESDGQAKAAATPKAGAGKIASAQTSTTTVDEPQVHSTVK KETDSLPKGETKVETAGVDGLVRTTYEVTTQDGKEVSRTPVA QVVVTKKVDEVVLVGTGEQQDQQAQQAQQQAAQQAQSAGD GQAAQANGGSEGSNGSTPAPAANPGAGTDPDSAKAIARSMMA SHGWGDSEFSCLESLWTRESSWNYQAENASSGAYGIPQALPGT KMSEVADDWATNPTTQITWGLNYISGRYGTPCSAWAHSESVG WY
A0A1Q8WLI2 by *A. oris*	A0A1Q8WLI2	MSSRTTARHRKATRALTPLDDFAPTARRGLAVAASSGLALTM MASGANAAGHTEVTSSGTIEASGVTPGVGIFAANAREALAAR QQVTMSQVNWVTEAAPEVEAVAPAPAAPQAPAAPEAPAAQA EQTSSEAAAPAAADQGTDSAAADTQAAQATPAAAPASTSNSS VVAIAMQYVGAPYVWGASGPSAFDCSGFTQYVYAQVGISLPR TSSEQAVSGTPVSAAEAQPGDLVTWPGHVGIYAGDGKVIDAG SEDTGVVYRDLWDSPSFVRVG
Phospholipase produced by *M. sorbitolivorans* and	WP_162785780.1	MNQHETATKSLLLKSHRERTARRSKRTAIITGSVAAALVIAGA VATGTTAIAAESGPRNAVTVELATGRVADQAQAAIDAAQSVM ERAADDNIDTPHLEDRVAELAEYAATPSTIVEARTARVIELTET VTEKATAYEKQ VAEEEAKAEEEAAAAAVAAAEAQAEANTPEGAKATARSIMSS TYGWGDDQFACLDSLWTKESGWNYQASNASSGAYGIPQSLPG SKMSTVADDWETNATTQVTWGLDYISRAYGTPCSAWAHSQS VNWY
Lytic transglycosylase produced by *R. agropyri*	WP_246226640.1	MVNVVDPSSGAVASPYYQAPARFNGDAAQRLVVAGGIERTVE RDSFAAQAKPTPTPTPTPVAPPAQAATSETEEAAPAAAAPVVR AATPDPGSAKAIAYDMIVQRGWADSEYTCLVSLWNRESGWN VYAENKSSGAYGIPQALPGSKMATVGSDWASNPSTQITWGLG YISGRYGSPCGAWAHSESVGWY
C40 peptidase produced by *A. populi*	WP_109093344.1	MKVSSKQIIAPGVSGLVVGAMLGGLVPAAQADDDAAANQTA AFTAAHATPEVTATKAVTATEKIEKADKVSFETLDATVTVTEP EPEPVVEEAQAETAAATDTAATDTTASDSESAATESETATAAP STAGGVVGIARQY VGAPYVWGAAGPTAFDCSGFTSYVYAQMGINLPRSSGAQQSA GTPVSASEAQPGDLVWWPGHVGIYTGNGNHIAARNPSTGVQE GPVYGSPTYIRVG
Glycoside hydrolyse produced by *Terrabacter* spp	NUS41473.1	MSARTNHGRHRAARRPIALAPTGVAGRRVAVAAAAGGLLVST FASAGAAQAAPVDTDAAKKLSTVDLGALTDQAREALEAAPV VTVDAKAKVDVEKVTAKIAAEAEITPAPEPEPEPVVEQVADTS STDDASRSSETASR SDEREAVEAPASANGSAIVSIAMRYVGVPYVSGGSSPSGFDCS GLTQYVYAQVGISLPRTSSAQRYAGTVVSASEAKPGDLVWTP GHVAIYAGDGMQIDAPKPGDVVKYRAIWQSNPTFIRVG

CHAPTER 3:

Investigation of the inhibition of *Helicobacter pylori* growth by *Actinomyces oris*

3.1. Introduction

The review of the literature in chapter one established that the gastric microbial composition differs depending on *H. pylori* infection status. Sequencing studies suggest that the stomach of *H. pylori* non-infected individuals is more diverse in microbial composition than the stomach of *H. pylori* infected individuals in which it is dominated by *H. pylori* (Cho and Blaser, 2012, Maldonado-Contreras *et al.*, 2011, Andersson et al., 2008). However, as discussed in chapter 1, sequencing techniques are a sensitive method that determine relative bacterial levels in the samples but does not indicate if the bacteria were transient, contaminant or resident bacteria within the gastric mucosa.

Although *H. pylori* causes 95% of gastric adenocarcinoma cases (Shiotani *et al.*, 2013), the abundance of *H. pylori* in the gastric mucosa decreases with progression to gastric adenocarcinoma, whereas there is an enrichment of other bacterial species such as *Lactobacillus* spp., *Streptococcus* spp., *Veillonella* spp. and *Prevotella* spp. (Yang et al., 2021). The mechanism driving this dysbiosis is unknown. Multiple studies have reported that several species of *Lactobacillus* and *Streptococcus* inhibit *H. pylori* growth *in vitro* (Khosravi et al., 2014a, Sgouras et al., 2004, Chen et al., 2019). Ishihara *et al.* investigated the interaction of oral bacteria such as *Streptococcus mutans, Streptococcus oralis and Actinomyces viscosus* with *H. pylori,* using a qualitative growth assay. All of the oral bacteria tested had a negative effect on the growth of *H. pylori,* yet the extent and the factor driving this inhibition was not characterised (Ishihara et al., 1997). The decrease in the abundance of *H. pylori* in the later stages of gastric cancer might imply that these bacteria outcompete or inhibit *H. pylori* growth *in vivo*. Therefore, understanding *H. pylori* interactions with members of the gastric and oral microbiota is essential to determine their effect on *H. pylori* growth and subsequent role in gastric carcinogenesis.

Paida Katsande showed that an oral *A. oris* isolate and *A. viscosus* C505 strain inhibited *H. pylori* growth in a co-culture assay. *A. oris* inhibition of *H. pylori* growth was mediated by a factor secreted into the medium (MSc thesis, University of Birmingham). The first aim of work presented in this chapter was to confirm the preliminary results of Katsande. Subsequent experiments were designed to determine whether the ability of *A. oris* to inhibit growth of *H. pylori* was specific to an oral isolate of *A. oris* used in previous work or whether it extended to other clinical isolates. It was also important to confirm whether *A. oris* inhibits growth of bacteria closely related to *H. pylori*, and to extend the investigations to other Gram-positive and Gram-negative bacteria. The aim of the current work was to design experiments to further characterise any inhibitor secreted by *A. oris* biochemically.

3.2. Results

3.2.1. The interaction of *H. pylori* with members of the gastrointestinal tract microbiome

In order to assess the interaction of *H. pylori* with members of the GI tract microbiome, species enriched in the later stages of GC or in the stomach of *H. pylori* negative individuals were chosen for this assay. These include *Veillonella parvula*, *Prevotella intermedia* (Noto and Peek, 2017) and a member of the oral microbiota, *Actinomyces oris*, one of the most prominent Actinobacteria in the oral cavity (Siegel *et al.*, 2016). To investigate whether any of these three species directly inhibit the growth of *H. pylori*, cultures of *H. pylori* and the strain to be tested were grown in BHI-FBS medium for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2 either alone or in a co-culture. Growth was measured by serially diluting cultures and plating the cells for colony forming unit analysis.

H. pylori grew equally well in the presence or absence of *V. parvula* to 1.5 x10^8 CFU/ml. Conversely, the cell density of *V. parvula* was 2.1 x 10^9 both in the presence or absence of *H. pylori* (Figure 3. 1

A). It was concluded that *V. parvula* did not inhibit *H. pylori* growth. Similar results were obtained with the co-cultures of *H. pylori* and *P. intermedia*, *H. pylori* grew to a final density of 1x 10^8 CFU/ml in both the single and the co-culture flasks (Figure 3.1 B). However, no *H. pylori* colonies were recovered from the co-culture in the presence of *A. oris* (Figure 3.1 C). It was concluded that *A. oris* inhibited the growth of *H. pylori* under the conditions tested.

3.2.2. Growth of *H. pylori* in a co-culture with a gastric *A. oris* isolate

The inhibitor of *H. pylori* growth reported in the previous experiment was produced by an oral *A. oris* isolate from a patient at the University of Birmingham Dental Hospital. However, it remained to be investigated whether a gastric *A. oris* strain (g*A. Oris*) will have the same effect on *H. pylori* growth. A member of the Rossiter laboratory isolated *A. oris* from a gastric biopsy provided by a collaborator at the Queen Elizabeth Hospital, Birmingham. The strain was co-cultured with *H. pylori* following the same protocol used in the previous experiment.

H. pylori grew to a density of 1.3 x 10^8 CFU/ml in the single culture. g*A. Oris* inhibited *H. pylori* growth in the co-culture (below the detection limit of 100 CFU/ml). g*A. Oris* grew to 3 x 10^8 both independently and when co-cultured with *H. pylori*. (Figure 3.2 A). It was concluded that both the oral and the gastric isolates of *A. oris* inhibited the growth of *H. pylori* under the conditions tested.

3.2.3. Growth of *H. pylori* in a co-culture with *Actinomyces viscosus* C505

In order to determine whether other *Actinomyces* species also inhibit *H. pylori* growth, *H. pylori* was co-cultured with *A. viscosus* C505, which is a reference strain obtained from the American Type

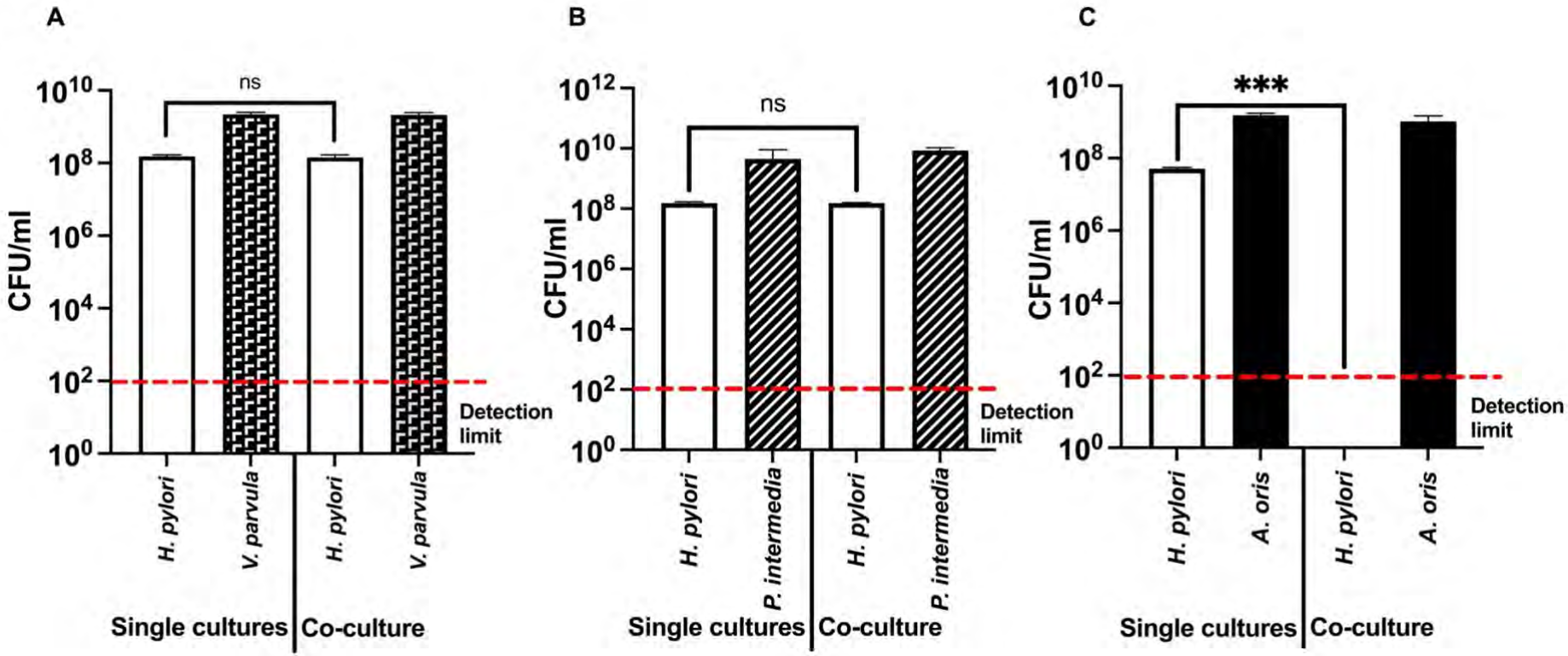

Figure 3. 1. Co-culture assay of *H. pylori* with *Veillonella parvula, Prevotella intermedia* and *Actinomyces oris*

An overnight culture of the indicated bacteria was inoculated into fresh BHI-FBS medium at a starting inoculum OD_{600nm} of 0.01, as either a single culture or co-culture. Cultures were grown statically for 48 h at 37°C in an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. *H. pylori* was plated onto horse blood agar with Skirrow's selective supplement and placed in a candle jar with a CampyGen gas pack at 37° C for 5-7 days. *V. parvula, P. intermedia* and *A. oris* were plated onto horse blood agar, without selection, and placed in a candle jar with an AnaeroGen sachet at 37° C for 2-3 days. The detection limit of 100 *H. pylori* CFUs is represented as a dashed line. Results shown are the mean from three independent experiments, +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

Culture Collection. The same co-culture protocol of the previous experiments was followed. *H. pylori* grew to 2 x10^7 CFU/ml when on its own. No *H. pylori* cells were detected from the co-culture medium. *A. viscosus* C505 grew equally well both in the single culture and in the co-culture to 3.8 x10^8 CFU/ml (Figure 3.2 B). This result indicates that the inhibition of *H. pylori* growth is not limited to *A. oris*.

3.2.4. The effect of *Actinomyces* species on the growth of *Campylobacter jejuni*

Campylobacter and *Helicobacter* are two genera of microaerophilic Gram-negative bacteria belonging to the epsilonproteobacteria class of the phylum Proteobacteria (On, 2001). In order to determine whether *Actinomyces* species also inhibit bacteria related to *H. pylori*, *Campylobacter jejuni* was co-cultured with *A. oris* or *A. viscosus* C505 using the same protocol followed in the previous experiments (Figure 3.3 A, B).

Although both *Actinomyces* species tested significantly reduced the growth of *C. jejuni* (Figure 3.3), this was a much milder reduction in growth than observed with *H. pylori* (Figure 3.2). *A. oris* and *A. viscosus* C505 grew equally well in the single and the co-culture medium. It was concluded that *Actinomyces* species had a minor effect on *C. jejuni* growth.

3.2.5. The effect of *A. oris* and *A. viscosus* C505 culture supernatants on *H. pylori* growth

An oral isolate of *A. oris* and a reference strain of *A. viscosus* inhibited *H. pylori* growth in a co-culture assay. In order to test whether this inhibition phenomenon is produced by a factor secreted into the medium, the supernatants from cultures of *A. oris* and *A. viscosus* C505 were tested for

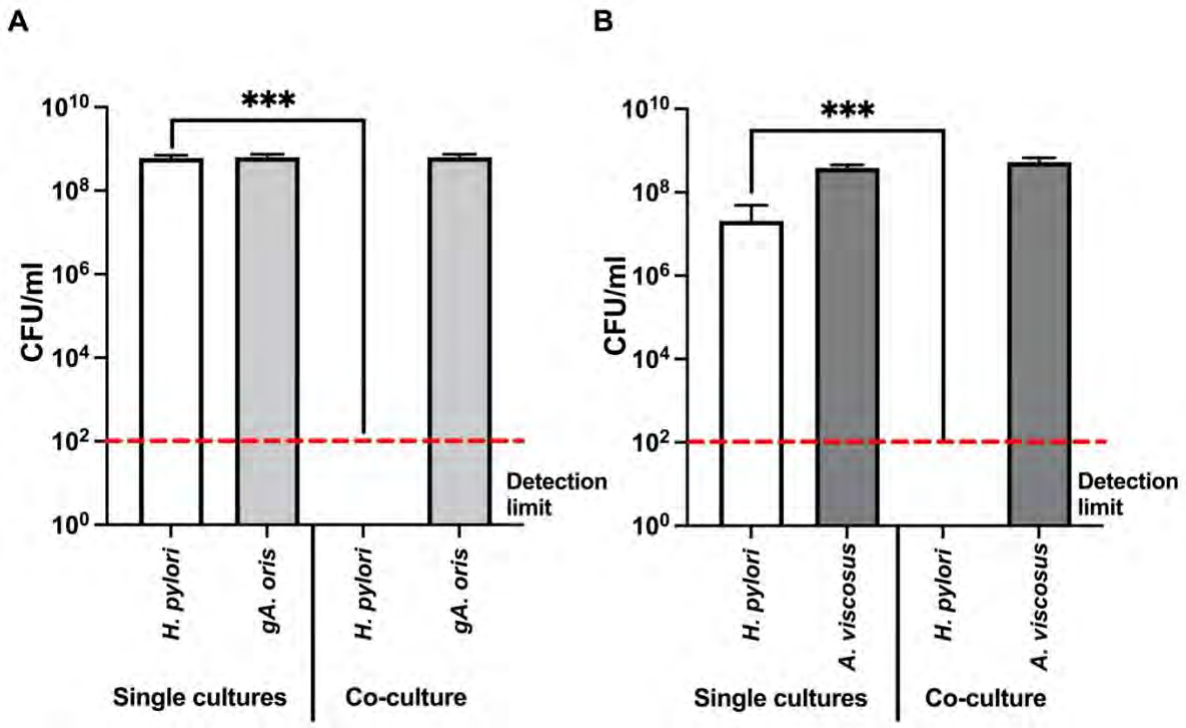

Figure 3. 2. Growth of *H. pylori* in a co-culture with a gastric *A. oris* isolate or with *A. viscosus* C505

An overnight culture of *H. pylori* with either *gA. Oris* **(A)** or *A. viscosus* **(B)** were inoculated into fresh BHI-FBS medium at a starting inoculum of an OD_{600nm} of 0.01, as either single culture or co-culture in which both bacteria were inoculated into the same flask. Cultures were then grown statically for 48 h at 37° C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. *H. pylori* was plated onto horse blood agar with Skirrow's selective supplement and placed in a candle jar with a CampyGen gas pack at 37° C for 5-7 days. *G A. oris* and *A. viscosus* C505 were plated onto horse blood agar, without selection, and placed into a candle jar with an AnaeroGen sachet at 37° C for 2-3 days. The detection limit (DL) of 100 *H. pylori* CFU's is represented as a dashed line. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

inhibition activity. *A. oris* and *A. viscosus* C505 were grown anaerobically in BHI medium for 24 h to an OD_{600nm} of 2.3. The culture supernatant was harvested by centrifugation followed by filter sterilization to remove any cell debris.

The inhibition activity of *A. oris* and *A. viscosus* C505 culture supernatants (SN) was tested at 50% (v/v) concentration. *E. coli* BW25113 supernatant was used in the experiment as a control (E. SN).

H. pylori grew to an OD_{600nm} of 1.6 in the positive control. Addition of 50% of *A. oris* SN significantly reduced *H. pylori* growth to an OD_{600nm} of 0.08. E. SN did not have any effect on *H. pylori* growth (Figure 3.4 A). Addition of 50% of *A. viscosus* C505 SN decreased *H. pylori* to a lesser extent than *A. oris* SN reaching an OD_{600nm} of 0.54 compared with an OD_{600nm} of 1.4 of the positive control This result suggests that the *A. oris* and *A. viscosus* C505-mediated inhibition of *H. pylori* growth is driven by a factor that is secreted into the medium and that *A. viscosus* C505 culture supernatant inhibits *H. pylori* growth to a lesser extent than *A. oris* SN (Figure 3.4 B).

3.2.6. Effect of the removal of foetal bovine serum from *A. oris* growth medium on the production of the *H. pylori* growth inhibitor

A. oris was initially grown in a BHI medium supplemented with 10% foetal bovine serum (FBS) for 24 h. FBS is rich in immunoglobulins and other proteins that can complicate the identification and purification of the inhibitor produced by *A. oris* (Figure 3.5 A). In order to determine whether FBS is essential for the production of the inhibitor of *H. pylori* growth, two supernatants from *A. oris* cultures that had been grown in the presence or absence of FBS were tested for the inhibition activity

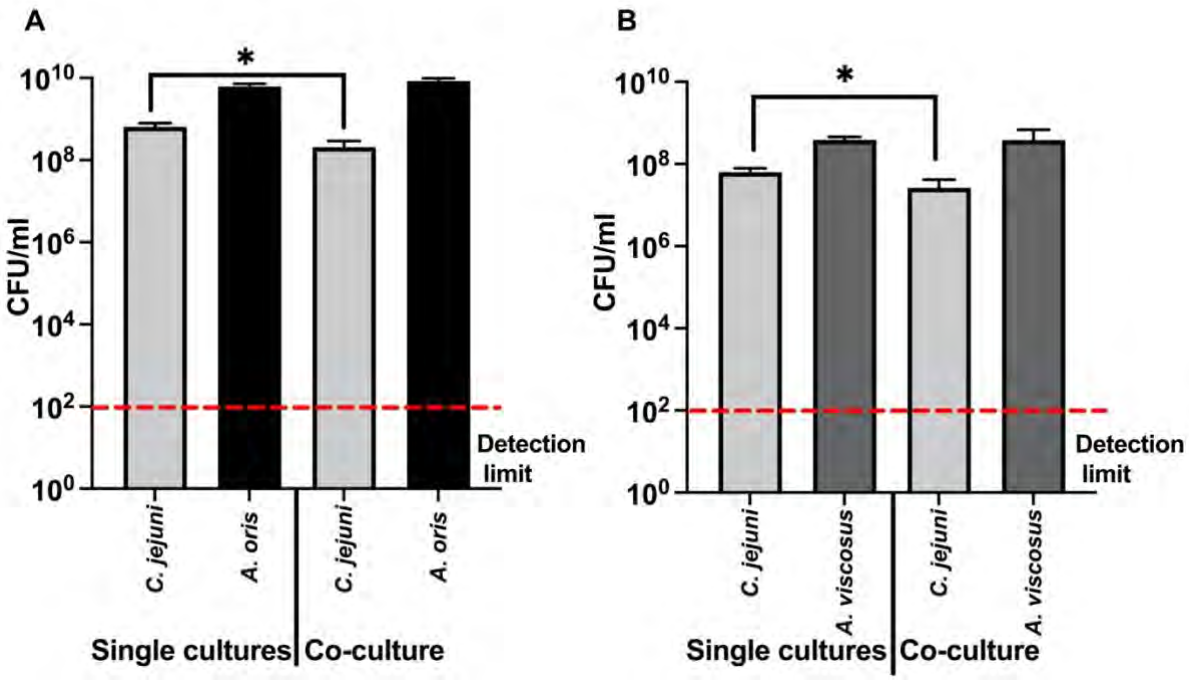

Figure 3. 3. Co-culture assay of *C. jejuni* with *A. oris* and *A. viscosus* C505
An overnight culture of *C. jejuni* with either *A. oris* **(A)** or *A. viscosus* C505 **(B)** were inoculated into fresh BHI-FBS medium at a starting inoculum of OD_{600nm} of 0.01, as either a single culture or co-culture in which both bacteria were inoculated into the same flask. Cultures were then grown statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. *C. jejuni* was plated onto horse blood agar with Skirrow's selective supplement and placed in a candle jar with a CampyGen gas pack at 37° C for 5-7 days. *A. oris* and *A. viscosus* C505 **(B)** were plated onto horse blood agar, without selection, and placed into a candle jar with an AnaeroGen sachet at 37° C for 2-3 days. The detection limit (DL) of 100 *C. jejuni* CFU's is represented as a dashed line. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, * $p \leq 0.05$.

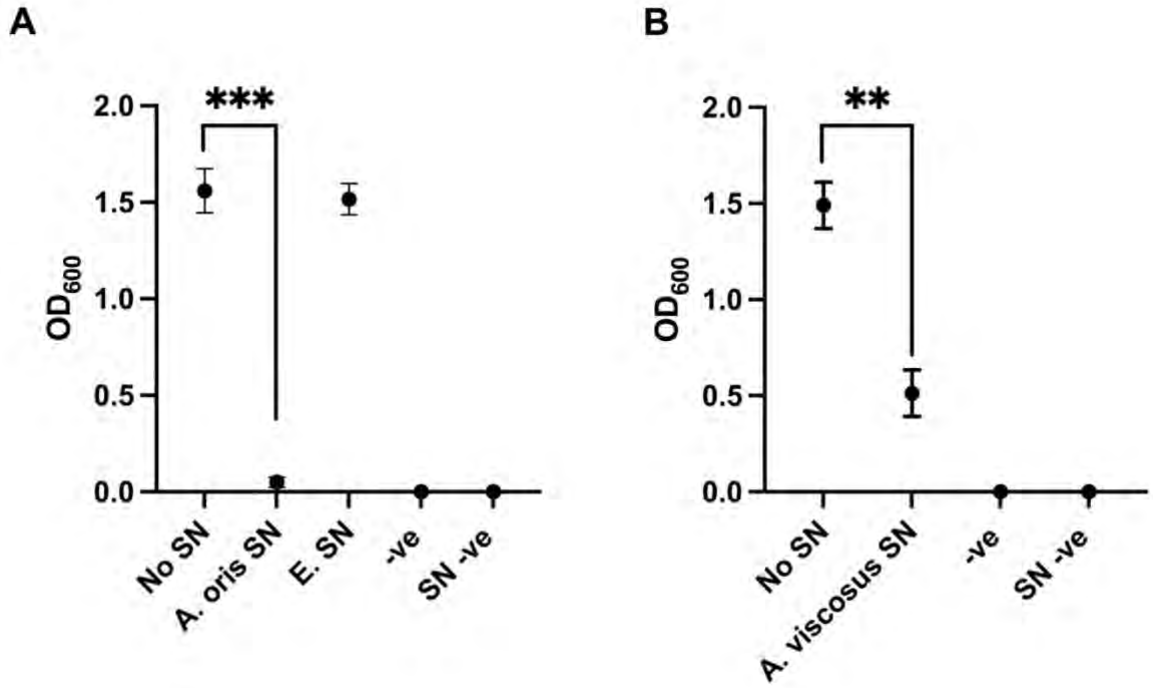

Figure 3. 4. The effect of *A. oris* culture supernatant or *A. viscosus* C505 culture supernatant on *H. pylori* growth

An overnight culture of *H. pylori* was inoculated into fresh BHI-FBS medium at a starting inoculum of an OD_{600nm} of 0.01, as either a single culture or a culture supplemented with 50% of *A. oris* supernatant (*A. oris* SN) or *E. coli* supernatant (E. SN) **(A)**. Cultures were then grown statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. The ability of *Actinomyces viscosus* C505 **(B)** culture supernatant to inhibit *H. pylori* growth was determined using the same protocol as in Figure A. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$, ** $p \leq 0.01$.

of *H. pylori* growth following the same protocol used in the previous experiments.

Both supernatants tested significantly inhibited *H. pylori* growth at a similar level. *H. pylori* grew to an OD_{600nm} of 1.4 in the single culture (Figure 3.5 B). This result confirmed that the addition of FBS to the growth medium of *A. oris* is not required for the production of the inhibitor of *H. pylori* growth.

3.2.7. Effect of the *A. oris* cell density on the production of the inhibitor

In order to determine the growth phase at which the *A. oris* inhibitor is produced, *A. oris* was grown anaerobically at 37°C in BHI cultures for 8 h, 24 h and 48 h. The time points were chosen to reflect the early, mid and late stages of growth (OD_{600} of 1, 2 and 2.8, respectively). The culture supernatants, designated SN B, C and D, were harvested and tested for inhibition activity of *H. pylori* growth following the same protocol used in the previous experiments, Table 3.1 shows the OD_{600nm} measurements of each of the cultures. SN A is an active supernatant that was previously shown to inhibit *H. pylori* growth that was included as a positive control.

SN B failed to inhibit *H. pylori* growth while the rest of the supernatants tested showed a significant inhibition and SN D showed the strongest inhibition (Figure 3.6). This result suggests that the inhibitor is either produced at later stages of *A. oris* growth or that its production is proportional to the density of growth.

3.2.8. Comparison of the effects of *A. oris* culture supernatant prepared under anaerobic or microaerobic conditions on *H. pylori* growth

Oxidative stress plays a major role in the production of many bacteriocins produced by Gram-positive bacteria (Jack *et al.*, 1995). In order to determine whether oxygen is required for the

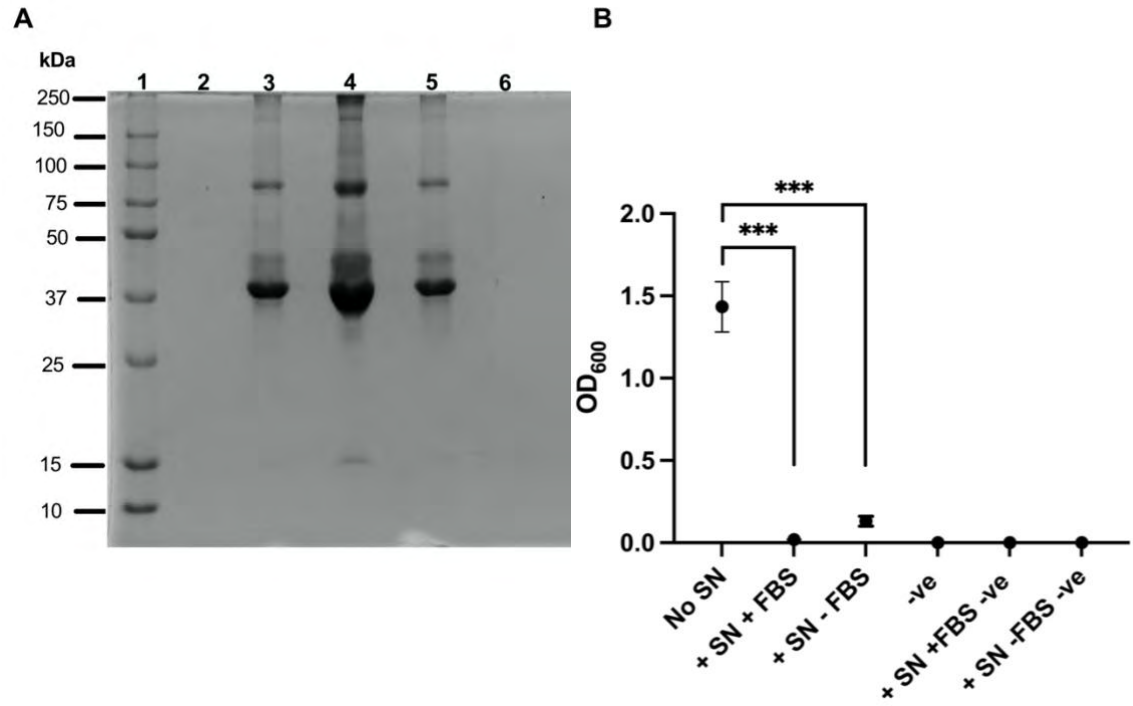

Figure 3. 5. Effect of the removal of FBS from the growth medium on growth and production of the *H. pylori* inhibitor

A. oris culture supernatants grown with and without foetal bovine serum (FBS) were separated using SDS-PAGE **(A)**. Lane 1 shows the Broad range ladder (Biorad) used for protein size estimation. Lanes 2-6 contain the following samples; 2: BHI only, 3: BHI with 10% FBS, 4: FBS only, 5: SN grown with 10% FBS and 6: SN grown without FBS. **(B)** The activity of the indicated supernatant fractions of *A. oris* against *H. pylori.* An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as in a medium supplemented with 50% of the indicated *A. oris* culture supernatants. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Data shown are the mean from three independent repeats +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

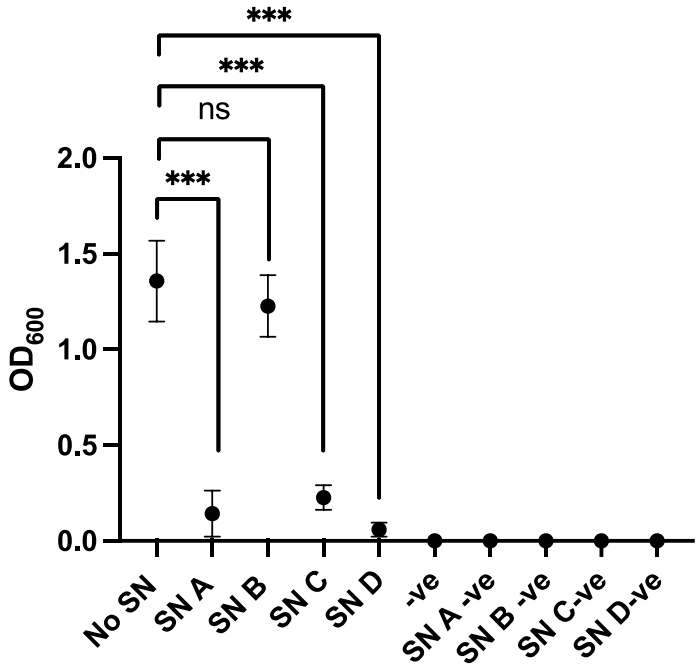

Figure 3. 6. Effect of *A. oris* cell density on the production of the inhibitor
An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as in a medium supplemented with 50% of *A. oris* cultures supernatants (SN). SNA is a control from an active *A. oris* culture supernatant. SN B, C and D are supernatant fractions prepared from *A. oris* cultures grown to an OD_{600nm} of 1, 2.1 and 2.8, respectively. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600nm. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

conditions to an equal OD_{600nm} of 2.2. Supernatants from both cultures were harvested and tested for inhibition activity against *H. pylori* following the same protocol described in the previous experiments.

The mean value of *H. Pylori* growth in the positive control was an OD_{600nm} of 1.4. The supernatant from the *A. oris* anaerobic culture (anae SN) significantly inhibited *H. pylori* growth almost completely to an OD_{600nm} of 0.07. The supernatant from the microaerobic *A. oris* culture (m SN) did not significantly affect *H. pylori* growth (Figure 3.7). Data from this experiment indicates that *A. oris* produces the inhibitor of *H. pylori* growth when grown as a pure culture under anaerobic conditions and that *A. oris* produces the inhibitor under microaerobic conditions only in the presence of *H. pylori* and *C. jejuni.*

3.2.9. Effect of freezing and thawing on the activity of the inhibitor produced by *A. oris*

In order to assess the effect of freezing and thawing on the inhibition activity of *A. oris* SN for storage purposes, two freezing techniques were tested for maintaining the inhibition activity of the SN. Snap freezing with liquid nitrogen is a technique by which samples are frozen rapidly to temperatures of lower than -80 °C. The rapid freezing helps in maintaining sample integrity by forming ice crystals and therefore reducing water in the sample. This method of freezing is commonly used for storing samples with unstable proteins and lipids. *A. oris* SN was snap frozen with liquid nitrogen and stored at -80°C. The supernatant was also directly frozen slowly in a- 20°C freezer. After storage for a week, the supernatants were thawed and tested for inhibition activity following the same protocol described in the previous experiments.

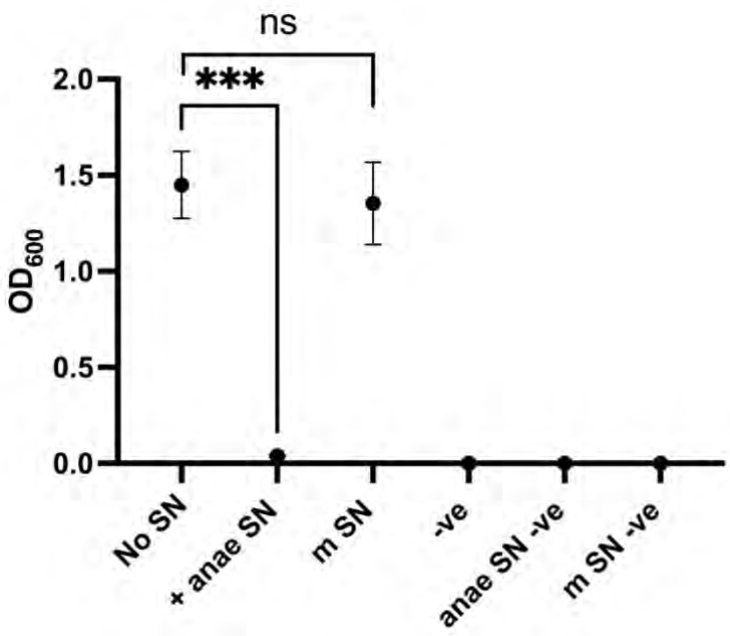

Figure 3. 7. Comparison of the effects of *A. oris* culture supernatant prepared under anaerobic or microaerobic conditions on *H. pylori* growth

An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as into a medium supplemented with 50% of the two *A. oris* culture supernatants tested. The supernatant from *A. oris* cultures that were grown anaerobically or microaerobically are designated anae SN and m SN, respectively. Microaerobic cultures were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Data shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

Both supernatants had retained the inhibition activity of *H. pylori* growth after freezing. No difference in the level of inhibition of *A. oris* SN was observed between the two freezing techniques used (Figure 3.8 A, B).

3.2.10. The effect of *A. oris* culture supernatant on the growth of Gram- negative bacteria

To determine the effect of *A. oris* culture SN on the growth of other Gram-negative bacteria, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli* were grown in a medium supplemented with 50% *A. oris* (SN). Bacterial strains used for *A. oris* (SN) inhibition screening were reference strains of common pathogens of the respiratory and gastrointestinal tract of humans. The same protocol for the inhibition assay used in testing the effect of *A. oris* SN on *H. pylori* growth (section 3.2.5) was followed in this experiment. *H. pylori* was included in the experiment as a positive control of *A. oris* SN inhibition activity.

S. Typhimurium grew equally well in the presence and the absence of *A. oris* SN (Figure 3.9 A). The mean value of the growth of *P. aeruginosa* was an OD_{600nm} of 10.2 in the single culture compared with an OD_{600nm} of 9.4 in a medium supplemented with 50% *A. oris* SN (Figure 3.9 B). *K. pneumoniae* reached similar levels of growth of OD_{600nm} of 10 in both conditions tested (Figure 3.9 C). No difference was observed between the growth levels of *E. coli* with and without the addition of *A. oris* SN (Figure 3.9 D). *H. pylori* growth was significantly inhibited in all of the screening assays. Data from this experiment indicate that *A. oris* SN does not inhibit the growth of any of the Gram-negative bacteria tested.

3.2.11. The effect of *A. oris* supernatant on the growth of Gram-positive bacteria

In order to determine whether *A. oris* SN affects the growth of Gram-positive bacteria,

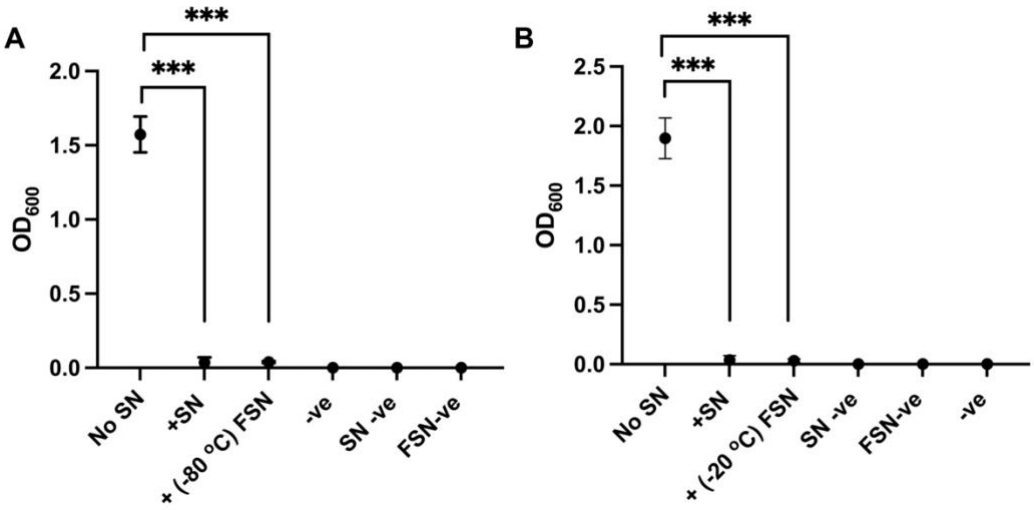

Figure 3. 8. Effect of freezing and thawing on the activity of the inhibitor produced by *A. oris*
An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as into a medium supplemented with 50% of the frozen *A. oris* culture supernatants to be tested. **(A)** *A. oris* culture supernatant that was snap frozen with liquid nitrogen and stored at -80°C. **(B)** *A. oris* culture supernatant that was slowly frozen at – 20°C. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p< 0.001$.

Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis and Streptococcus mitis were grown in a medium supplemented with *A. oris* culture supernatant. The same protocol used when testing the effect of *A. oris* supernatant (SN) on *H. pylori* growth (section 3.5) was followed in this experiment. *H. pylori* was included in the experiment as a positive control of *A. oris* SN inhibition activity. The growth of both *Staphylococcus* species tested increased slightly with the addition of 50% *A. oris* SN in the medium (Figure 3.10 A, B). *A. oris* SN did not affect *E. faecalis* growth as both cultures tested had an OD_{600nm} of 3.6 (Figure 3.10 C). The same result was observed with *S. mitis* (Figure 3.10 D). *H. pylori* growth was significantly inhibited in all the screening assays. These results indicate that *A. oris* SN does not inhibit the growth of any of the Gram-positive bacteria tested.

3.2.12. The time-course of *A. oris* inhibition on *H. pylori* growth

To determine the time-course of *A. oris* inhibition of *H. pylori* in a co-culture, both bacteria were co-cultured in BHI-FBS medium and bacterial growth was assessed at the time points 0, 14, 24 and 37 h. Overnight cultures of both bacteria were inoculated at a starting inoculum of OD_{600nm} of 0.01 as either a single or a co-culture. Cultures were incubated at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2.Growth was measured by serially diluting samples and plating for colony forming unit analysis.

A. oris grew in the single culture to a level of 1.7 $x10^9$ CFU/ml at 14 h and had decreased slightly to 7.5 $x10^8$ CFU/ml after 24 h. After 37 h, the density had increased to 6 $x10^9$ CFU/ml. *A. oris* growth in the co-culture reached similar levels of the single culture except for at 14 h which was of 2.1 $x10^7$ CFU/ml. *H. pylori* growth of the single culture increased over the time-course tested (1.5 $x10^6$ CFU/ml to 1.9 $x10^8$ CFU/ml). The number of *H. pylori* cells recovered from the co-culture at 14 h

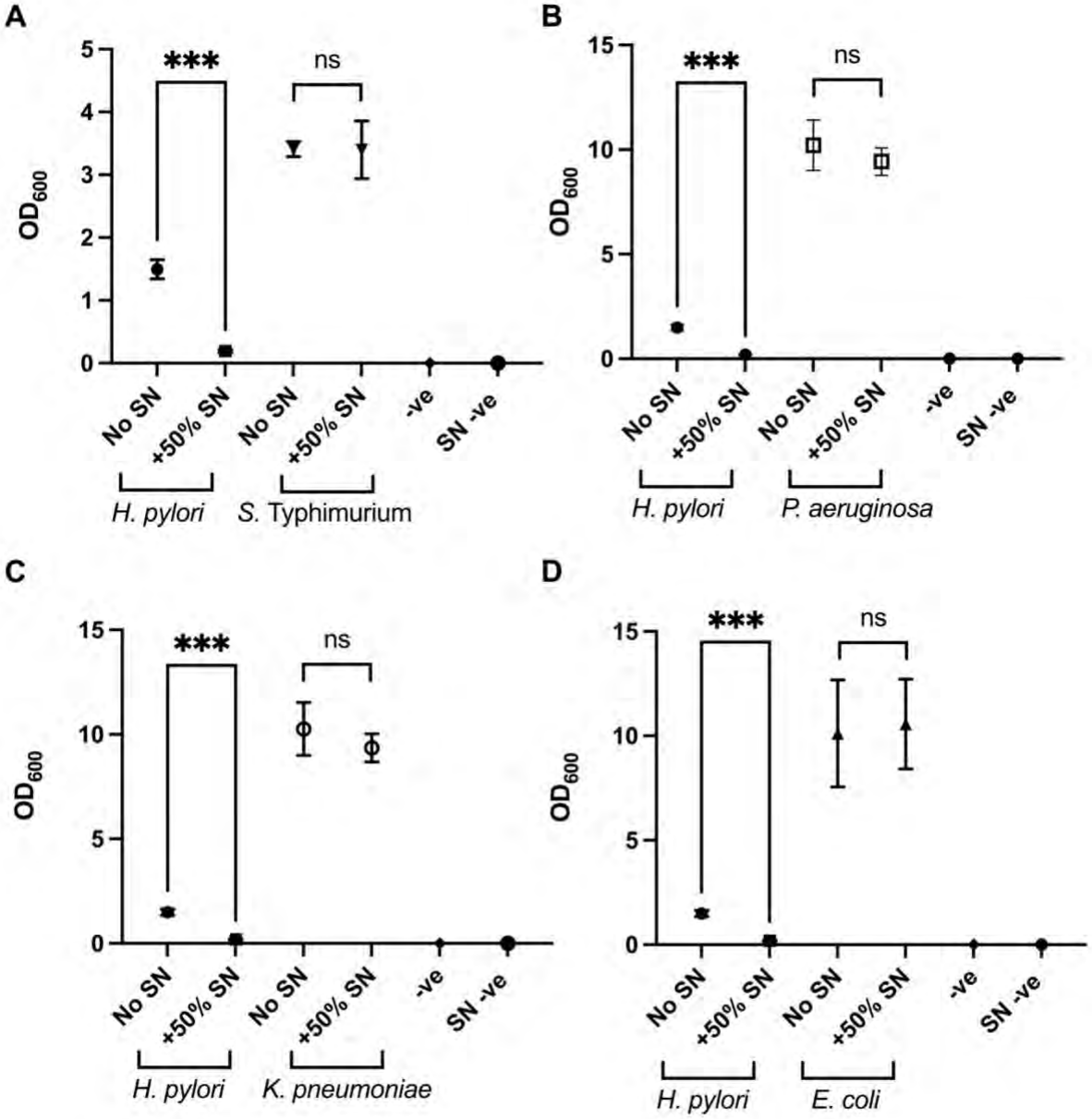

Figure 3. 9. The effect of *A. oris* culture supernatant on the growth of Gram-negative bacteria
An overnight cultures of *S. enterica* serovar Typhimurium **(A)**, *P. aeruginosa* **(B)**, *K. pneumoniae* **(C)**, *E. coli* **(D)** and *H. pylori* were inoculated at a starting OD_{600nm} of 0.01 in BHI-FBS medium as well as in a medium supplemented with 50% of *A. oris* culture supernatant (SN). Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

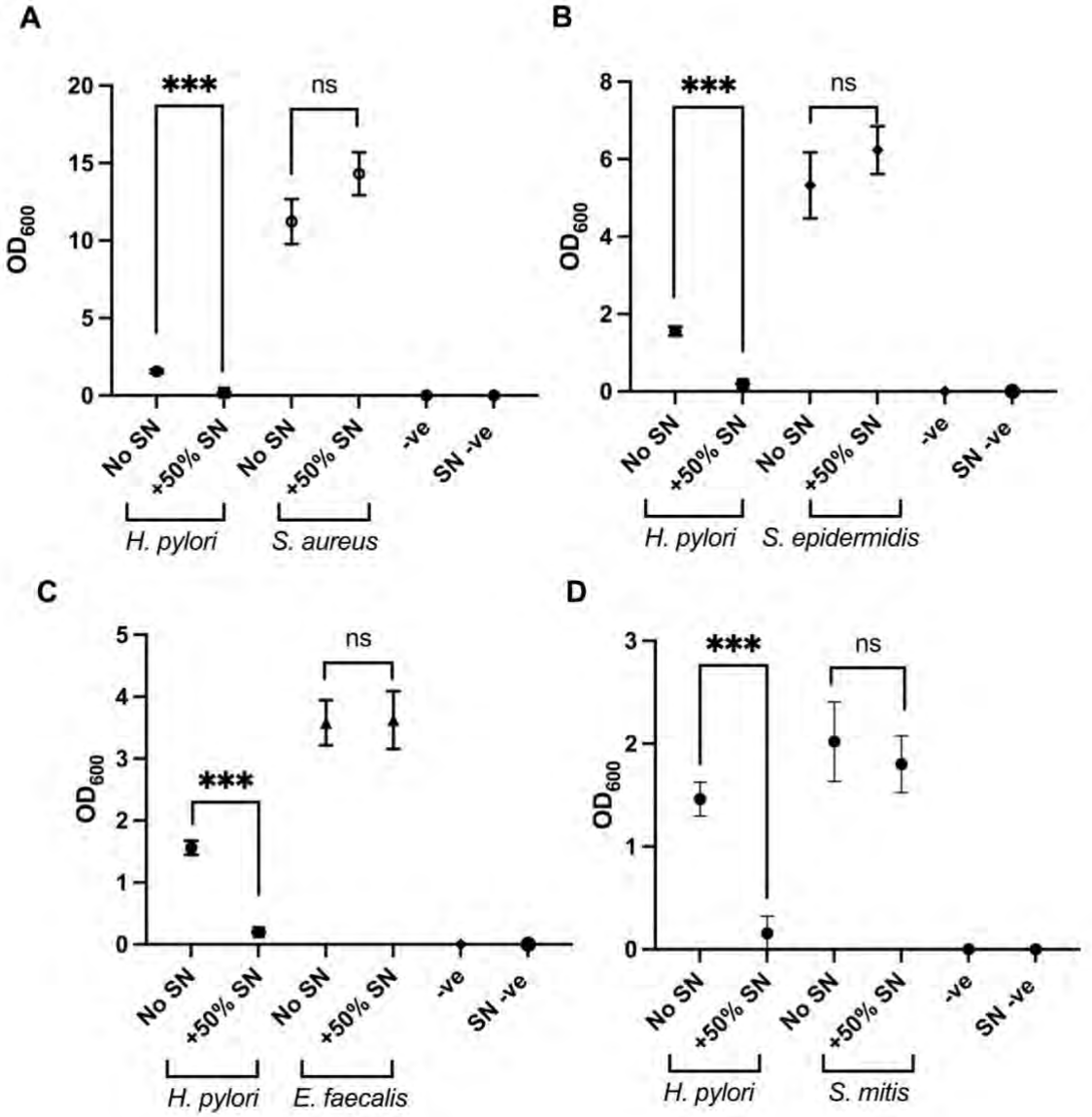

Figure 3. 10. The effect of *A. oris* supernatant on the growth of Gram-positive bacteria
An overnight cultures of *Staphylococcus aureus* **(A)**, *Staphylococcus epidermidis* **(B)**, *Enterococcus faecalis* **(C)**, *Streptococcus mitis* **(D)** and *H. pylori* were inoculated at a starting OD_{600nm} of 0.01 in BHI-FBS medium as well as in a medium supplemented with 50% of *A. oris* culture supernatant (SN). Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

and 24 h were of 5 x10^5 CFU/ml and 1.1 x10^5 CFU/ml, respectively. No *H. Pylori* cells were detected at 37 h (Figure 3.11 A). Data from this experiment suggest that *A. oris* might produce the inhibitor at the stationary phase of growth (Figure 3.11 B).

3.2.13. Effect of dialysis on the inhibition activity of *A. oris* culture supernatant

The pH of the *A. oris* culture supernatant (SN) is 5.0. To test whether acidity contributes to the inhibition effect of *A. oris* SN on *H. pylori* growth, *A. oris* SN was concentrated using a concentrator with a 5 kDa MW cut-off (viva-spin column, Sartorius). The SN, retentate and filtrate were dialysed against BHI media using a 3.5 kDa molecular wight cut-off (MWCO) dialysis tubing for 24 h at 4°C. The medium was changed 3 times during dialysis. The pH of the SN, retentate and filtrate was 6.5 post-dialysis. Dialysed SN, retentate and filtrate were tested for inhibition activity following the same protocol used when testing the effect of *A. oris* SN on *H. pylori* growth (section 3.2.5).

A. oris SN and retentate maintained the inhibition activity. However, the filtrate lost the inhibition activity post dialysis whereby *H. pylori* growth was of an OD_{600nm} of 1.6 compared to an OD_{600} of 1.4 of the positive control (Figure 3.12). Results from this experiment suggest that the acidity of *A. oris* SN does not play a role in the inhibition activity of the SN nor the retentate. The loss of the inhibition activity of the filtrate is an indication of the small molecular size of the inhibitor as it escapes the pores of the 3.5 kDa MWCO dialysis tubing.

3.2.14. Size exclusion of *A. oris* culture supernatant using a 5 kDa viva spin column

Actinomyces species are known to secrete different types of antimicrobials or bacteriocins that range in molecular weight (Sugrue *et al.*, 2020). Each type has a distinct inhibitor molecular size range that

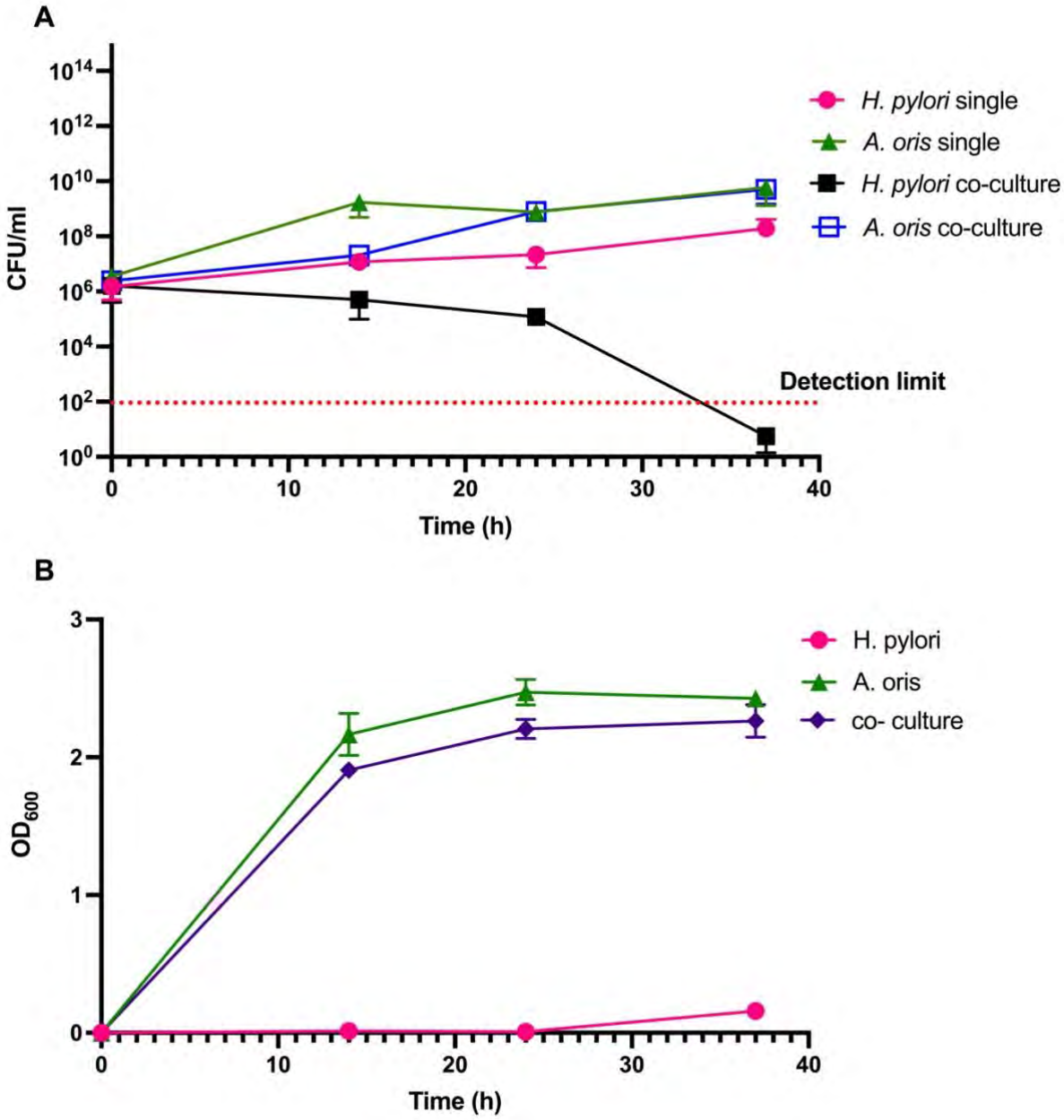

Figure 3. 11. The time-course of *A. oris* inhibition on *H. pylori* growth
An overnight culture of the indicated bacteria was inoculated into fresh BHI-FBS medium at a starting inoculum OD_{600nm} of 0.01, as either a single culture or co-culture. Cultures were grown statically at 37°C in an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured at the time points 0, 14, 24 and 37. *H. pylori* was plated onto horse blood agar with Skirrow's selective supplement and placed in a candle jar with a CampyGen gas pack at 37°C for 5-7 days. *A. oris* was plated onto horse blood agar, without selection, and placed in a candle jar with an AnaeroGen sachet at 37°C for 2-3 days. The detection limit of 100 *H. pylori* CFUs is represented as a dashed line **(A)**. OD_{600nm} measurements of each of the conditions tested at the time points 0, 14, 24 and 37 **(B)**. Results shown are from of three independent experiments +/- the standard deviation.

distinguishes one from another (Simons *et al.*, 2020). To determine the size of the *A. oris* inhibitor, *A. oris* SN was concentrated using a concentrator with a 5 kDa MW cut-off (viva-spin column, Sartorius). The SN and the retentate were routinely dialysed to ensure the pH was consistent throughout the experiments. The retentate (molecules >5 kDa) and the filtrate (molecules <5 kDa) were tested for inhibition activity following the same protocol used when testing the effect of *A. oris* SN on *H. pylori* growth (section 3.2.5). Growth was assessed using optical density at 600 nm.

Both the retentate and filtrate inhibited *H. pylori* growth at the same level as the supernatant (Figure 3.13). This result suggests that *A. oris* might be producing multiple inhibitors of different molecular sizes. Data from this experiment also indicated that the inhibitors are stable throughout the separation process of centrifugation at 3300 x g for 3- 4 h at 4°C.

3.2.15. Effect of heat treatment at 95°C on the inhibition activity of the *A. oris* culture supernatant

In order to determine the effect of denaturation on the activity of the inhibitor of *H. pylori* growth. An *A. oris* culture supernatant was heat treated for 5 minutes at 95°C. The inhibition activity of the heat-treated *A. oris* SN (no FBS) on *H. pylori* growth was assessed following the same protocol used when testing the effect of *A. oris* supernatant (SN) on *H. pylori* growth (section 3.2.5).

A. oris culture supernatant maintained the inhibition activity after heat treatment at 95°C for 5 min. The mean of *H. pylori* growth of the positive control was equal to an OD_{600} of 1.4 (Figure 3.14). This result confirms that the inhibitor of *H. pylori* growth is stable when heated under the conditions tested.

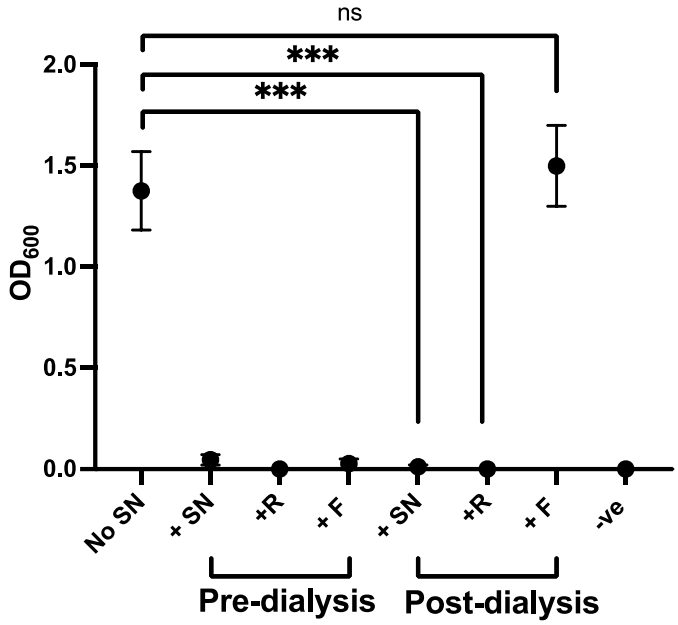

Figure 3. 12. Effect of dialysis on the inhibition activity of *A. oris* culture supernatant
A. oris culture supernatant (SN), retentate (R) and filtrate (F) were dialyzed using SnakeSkin™ dialysis tubing with a 3.5 K MWCO against BHI media for 24 h at 4°C. An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as into a medium supplemented with 50% of *A. oris* culture supernatant (SN), retentate (R) and Filtrate (F), pre and post dialysis. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Data shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p< 0.001$.

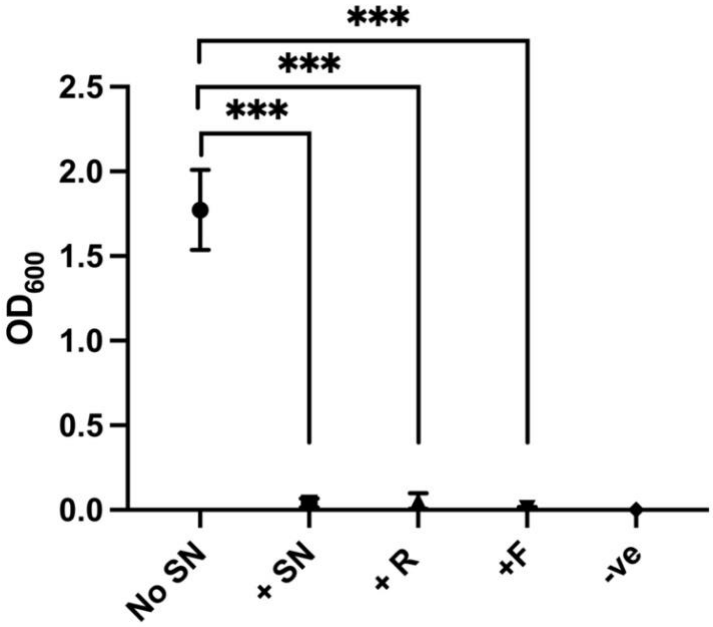

Figure 3. 13. Size exclusion of *A. oris* culture supernatant using a 5 kDa molecular wight cut-off concentrr

A. oris culture supernatant was concentrated using a 5 kDa molecular wight cut-off concentrator (Sartorius). An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as into a medium supplemented with 50% of *A. oris* culture supernatant (SN), retentate or concen®te (R) and Filtrate (F). The SN and the R were dialysed before the addition to the BHI-FBS medium. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Data are from 3 independent repeats. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

3.2.16. The effect of proteolytic enzymes on the growth of *H. pylori*

Two proteolytic enzymes were chosen for the digestion of *A. oris* culture supernatant to determine whether the inhibitor produced by *A. oris* is a protein. Proteinase K is a serine protease of a broad range that cleaves the peptide bonds adjacent to the carboxyl group of aliphatic and aromatic amino acids. Trypsin is another serine protease that is commonly used when digesting gastric biopsies prior to isolation of *H. pylori* (Peretz *et al.*, 2015) and therefore it should not affect the growth of *H. pylori*. The subsequent experiments were designed to test the effect of these enzymes on the growth of *H. pylori* in order to determine the enzyme type that will be used to digest *A. oris* culture supernatant.

3.2.16.1. Effect of proteinase K on *H. pylori* growth

The effect of different concentrations (0.1 mg/ml; 0.5 mg/ml; and 1 mg/ml) of proteinase K on *H. pylori* growth were tested. PMSF at 1 μg/ml concentration was used to stop the activity of proteinase K. PMSF was added to the control flasks after the addition of the proteinase K to stop the enzymatic activity before the addition of *H. pylori*. This was followed by the addition of an overnight culture of *H. pylori* at a starting OD_{600nm} of 0.01. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm.

All of the tested PK concentrations inhibited *H. pylori* growth. *H. pylori* grew to an OD_{600} of 1.4 in the positive control (NO PK). Addition of PMSF neither prevented the inhibition of *H. pylori* growth caused by addition of proteinase K, nor did it inhibit *H. pylori* growth (Figure 3.15). It was concluded that proteinase K inhibits *H. pylori* growth and is therefore not suitable for digesting proteins in the *A. oris* culture supernatant.

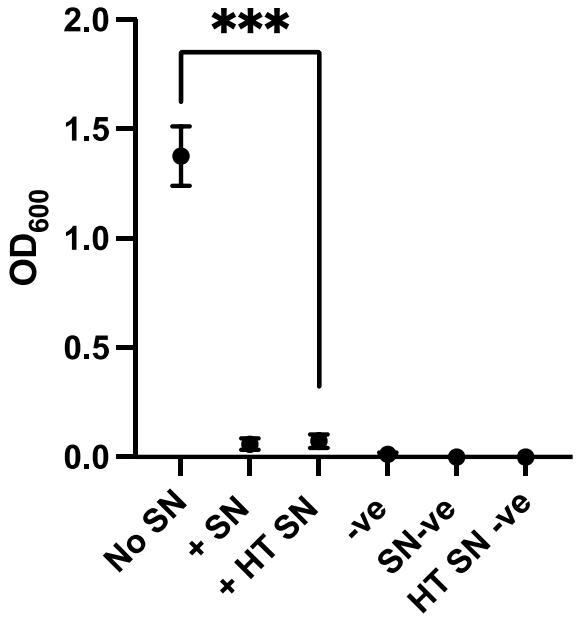

Figure 3. 14. Effect of heat treatment at 95°C on the inhibition activity of *A. oris* culture supernatant

An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as into a medium supplemented with 50% of *A. oris* culture supernatant (SN) and heat-treated SN (HT SN). Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test.

3.2.16.2. The effect of the protease TrypLE on *H. pylori* growth

TrypLE (ThermoFisher Sci) is a derivative of Trypsin in which the enzyme cleaves the peptide bonds on the C-terminal sides of arginine and lysine residues in proteins with greater specificity. The enzyme is widely used in cell culture experiments and has proven to gently and more efficiently detach the proteins on the surface of the cell lines making it an excellent candidate for testing on *H. pylori* growth (Chetty *et al.*, 2013). Information on the composition and the concentration of TrypLE are kept confidential by the manufacturer. The effect of different volumes of TrypLE as supplied by the manufacturer (1x) were added to the *H. pylori* growth medium as follows: 1 µl/ml; 10 µl/ml; and 100 µl/ml. An overnight culture of *H. pylori* was then added at a starting inoculum OD_{600nm} of 0.01. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2.

Bacterial growth was measured using optical density at 600 nm. None of the TrypLE concentrations tested inhibited *H. pylori* growth (Figure 3.16). This result indicated that TrypLE is suitable for digesting proteins in the *A. oris* culture fluid without inhibiting subsequent *H. pylori* growth.

3.2.17. Effect of TrypLE enzyme on the inhibition activity of *A. oris* culture supernatant

In order to determine whether the inhibitors of *H. pylori* growth produced by *A. oris* are proteins, *A. oris* culture supernatant (SN) was separated using a viva-spin column with a 5 kDa MW cut-off size. The culture supernatant (SN), the retentate and the filtrate were treated with 100 µl/ml TrypLE enzyme (x1) for the time points 0 (T0) and 30 min (T30). TrypLE was inactivated by the addition of FBS at a final concentration of 10% (v/v). The TrypLE-treated SN, retentate and filtrate samples were then added to BHI medium at a 50% (v/v) followed by addition of an overnight *H. pylori* culture at

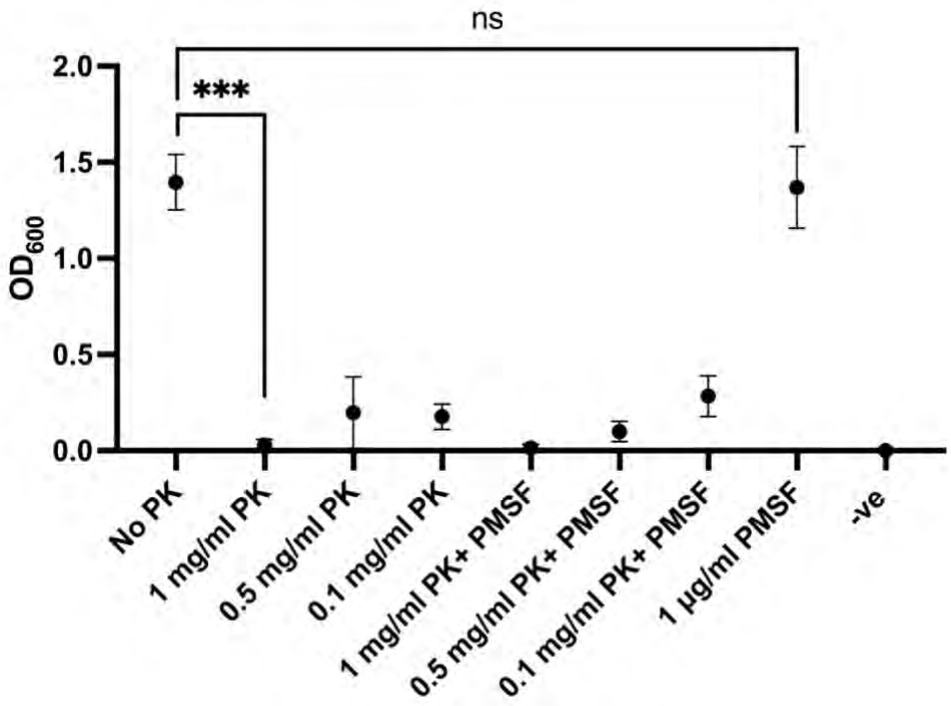

Figure 3. 15. Effect of proteinase K on *H. pylori* growth

An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium containing the different concentrations of proteinase K enzyme; 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml. PMSF was used to stop the activity of PK enzyme at 1 µg/ml concentration. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Results shown are the mean from three independent experiments +/- the standard deviation Statistics were calculated using an unpaired *t*-test, *** $p <$ 0.001.

starting inoculum of an OD_{600} of 0.01. Two positive controls were used in the experiment: single *H. pylori* culture (No SN) and *H. pylori* grown with 100 µl/ml TrypLE (+TE). *A. oris* SN partially lost inhibition activity after TrypLE treatment for 30 min. (Figure 3.17 A). TrypLE-treated retentate showed partial inhibition as *H. pylori* grew to an OD_{600} of 0.65 (Figure 3.17 B). TrypLE-treated filtrate lost the inhibition activity at T0 and T30 as *H. pylori* grew to an OD_{600} of 1.7, a similar level of the positive control (Figure 3.17 C). It was concluded that the inhibitors of *H. pylori* growth produced by *A. oris* are proteins.

3.2.18. The effect of lyophilisation on the inhibition activity of *A. oris* culture supernatant

Lyophilisation or dry freezing is a common way of concentrating large volumes of proteins. The first step of purifying the potential inhibitors requires generating a large volume of an active *A. oris* culture supernatant (SN) followed by concentrating the sample. *A. oris* SN (100 ml) was lyophilised for 48 h until the SN was completely dried. The lyophilised SN was suspended in deionised distal water to make up a 10 x concentrated sample and was tested for the inhibition activity on *H. pylori* growth at 5% concentration. The lyophilised SN lost the inhibition activity, *H. pylori* grew to an OD_{600nm} of 1.75 (Figure 3.18).

3.3 Discussion

The results presented in this chapter indicate that the tested *V. parvula* and *P. intermedia* strains do not inhibit *H. pylori* growth and that multiple members of *Actinomyces* produce one or more inhibitors of *H. pylori* growth. These results are consistent with the findings of Paida Katsande showing that an *A. oris* isolate is able to inhibit *H. pylori* growth in co-culture. The inhibitors of *H. pylori* growth produced by an oral *A. oris* strain is mediated by secreted factors that are heat-resistant, acid

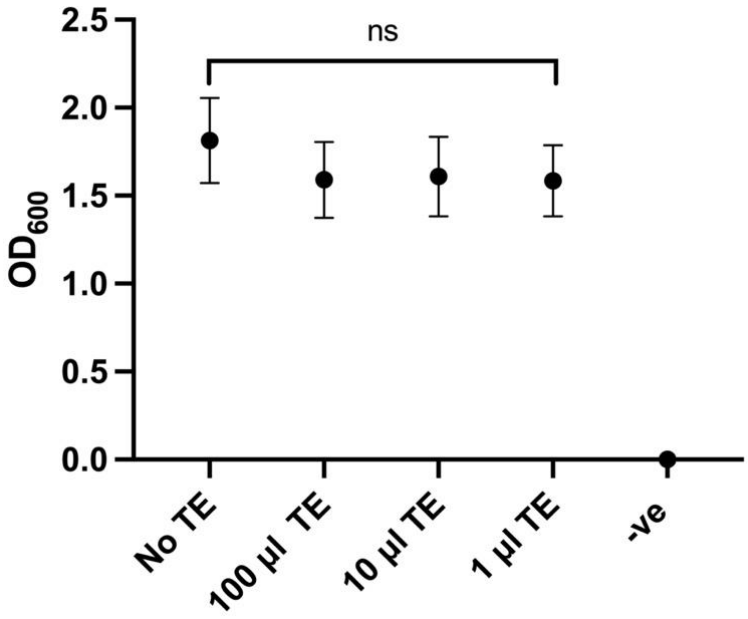

Figure 3. 16. The effect of the TrypLE enzyme on *H. pylori* growth.
An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium containing the following volumes of TrypLE enzyme (x1); 1 µl/ml; 10 µl/ml and 100 µl/ml. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using a one-way ANOVA test followed by Dunnett test.

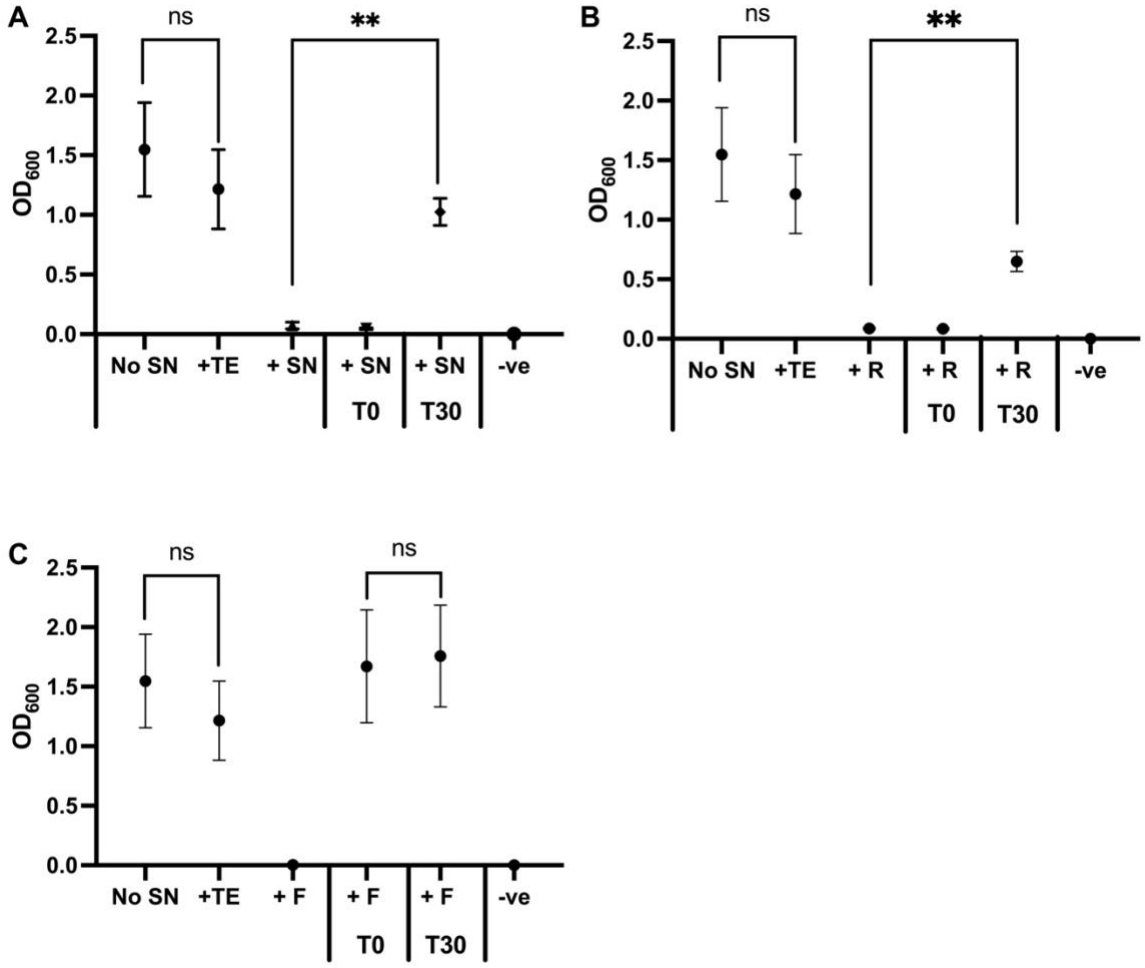

Figure 3. 17. Effect of TrypLE enzyme treatment on the inhibition activity of *A. oris* culture supernatant and retentate

A. oris culture supernatant (SN) **(A)** retentate (R) **(B)** and filtrate (F) **(C)** were treated with 100 µl/ml TrypLE enzyme (x1) for 0 and 30 min (T0 and T30, respectively). No SN indicates *H. pylori* culture grown without *A. oris* culture supernatant and +TE indicates *H. pylori* culture grown with 100 µl/ml TrypLE enzyme only. An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as into a medium supplemented with and without 50% of *A. oris* TrypLE-treated SN and retentate. Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using a one-way ANOVA test followed by Dunnett test, ** $p \leq 0.01$.

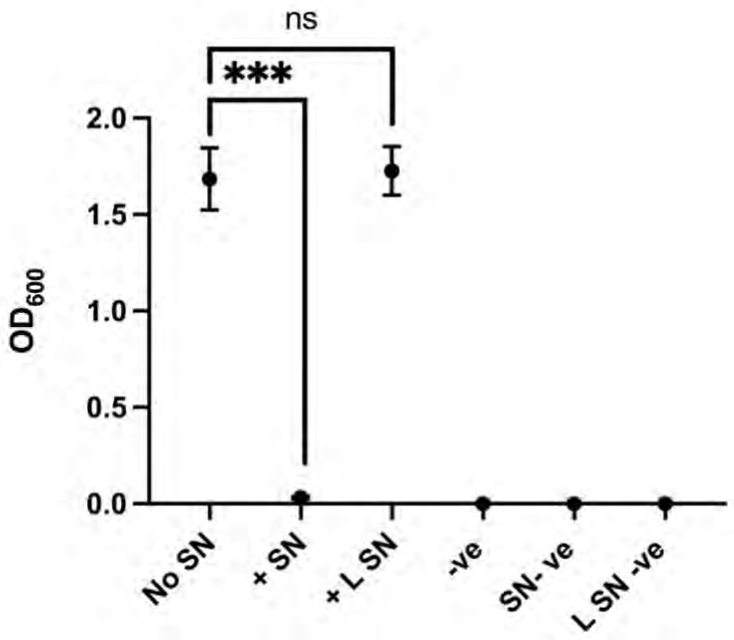

Figure 3. 18. The effect of lyophilisation on the inhibition activity of *A. oris* culture supernatant
An overnight culture of *H. pylori* was inoculated at a starting OD_{600nm} of 0.01 into BHI-FBS medium as well as into a medium supplemented with 50% of *A. oris* culture supernatant (SN) and with 5% of lyophilised *A. oris* SN (L SN). Flasks were incubated statically for 48 h at 37°C under an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Bacterial growth was measured using optical density at 600 nm. Results shown are the mean from three independent experiments +/- the standard deviation. Statistics were calculated using an unpaired *t*-test, *** $p < 0.001$.

independent, stable when stored at -20°C and –80°C, sensitive to proteolytic treatment and of sizes lower and higher than 5 kDa. The inhibition range is narrow as none of the tested Gram-positive and Gram-negative bacteria were inhibited by *A. oris* culture supernatant with the exception of *C. jejuni*. The inhibitors produced by *A. oris* are secreted at the stationary phase of growth, under anaerobic conditions and in the presence of *H. pylori* and *C. jejuni* in the same medium.

3.3.1 The biochemical characteristics of *A. oris* inhibitors

Bacteriocins are biologically active peptides or proteins produced by bacteria that function as antimicrobials. Bacteriocins are diverse in range of inhibition, mechanisms of action, structure and molecular sizes (Simons et al., 2020). For example, heating *A. oris* culture supernatant to 95°C for 5 minutes did not affect the inhibition activity on *H. pylori* growth. Bacteriocins of Gram-positive bacteria are of 4 types all of which are heat-resistant except some of type III bacteriocins (Sugrue et al., 2020). *A. oris* SN contain two or more inhibitors of different molecular sizes and therefore denaturing the filtrate and the retentate of *A. oris* SN fractions independently will give more conclusive evidence on the nature of these inhibitors. Small peptides found in the filtrate might are more stable than large proteins when heated at 95°C.

Ultrafiltration of the *A. oris* culture supernatant using a 5 kDa concentrator did not inactivate the inhibitors of *H. pylori* growth. The retentate and the filtrate inhibited *H. pylori* growth to the same extent as the supernatant fraction indicating that *A. oris* produces multiple inhibitors of sizes smaller and larger than 5 kDa. Type I bacteriocins (lantibiotics) are ribosomally-synthesized and post-translationally modified antimicrobial peptides that are heat-resistant and have a molecular size of <5 kDa (Nishie *et al.*, 2012). The classical example of lantibiotics is Nisin A produced by *Lactococcus lactis,* which has a wide application in food preservation due to the broad spectrum of its antimicrobial

activity (Okuda *et al.*, 2013). Type II bacteriocins are of a molecular size of < 10 kDa and type III bacteriocins are large molecules of 30 kDa or more (Simons et al., 2020). However, the retentate sample is 10 x more concentrated than *A. oris* culture supernatant and the filtrate. Therefore, it remains to be investigated whether diluting the retentate sample 10 x would inhibit *H. pylori* growth.

The data from the TrypLE experiment indicate that *A. oris* culture supernatant and the retentate lose inhibition activity after digestion with the enzyme, suggesting that it might be of protein nature. This characteristic is consistent with the fact that all types of bacteriocins are sensitive to proteolytic activity. Digestion of the filtrate resulted in loss of the inhibition activity at T0 which might imply that the enzyme concentration used in the experiment was higher than the concentration required to digest small peptides. The loss of activity of the SN after TE treatment for 30 min rules out that the inhibitor of *H. pylori* growth present within the filtrate sample is a small molecule but rather is a small protein or a peptide. Future experiments will be aimed at testing a range of TrypLE concentrations (0.5 x and 0.1 x) to confirm the nature of the inhibitor of the filtrate. It remains to be investigated whether increasing the incubation period of TE treatment to 60 min and 120 min would completely degrade the inhibitor. Additionally, most proteolytic digestions of bacteriocins reported in the literature used a purified form of the bacteriocins, which yields a more conclusive result (Valdés-Stauber and Scherer, 1994, Maisnier-patin *et al.*, 1996, Pantev *et al.*, 2002). Purifying the potential inhibitors and digesting the purified proteins would be a better approach to confirm these results.

3.3.2 The inhibition spectrum of the inhibitors of *H. pylori* growth

A. oris culture supernatant inhibited *H. pylori* growth completely and significantly reduced *C. jejuni* growth. However, *A. oris* culture supernatant did not affect the growth of any of the Gram-positive and Gram-negative bacteria tested, suggesting that the inhibition range is narrow. The specificity of bacteriocins activity varies, in which some have been shown to have a narrow target range, affecting

closely related bacteria or competitor bacteria (Simons *et al.,* 2020), whilst some have a broad target range, for example, Lysostaphin produced by *S. aureus* is a type III bacteriocin that is active only against bacteria within the same genus (Bastos *et al.*, 2010). According to Klaenhammer, the target of the majority of bacteriocins produced by lactic acid bacteria is narrow (Klaenhammer, 1993), with few exceptions such as plantaricin, produced by *Lactobacillus plantarum* which has a broad range of activity (Messi *et al.*, 2001).

The type of the inhibition assay used in this study was chosen following Papagianni *et al.'s* recommendation of using a liquid assay to yield a more reilable result (Papagianni *et al.*, 2006). The inhibition assay protocol was modified to support the growth of *H. pylori* where bacteria were grown in a liquid medium that contains FBS for 48 h. However, one of the issues of this protocol was that the 48 h incubation period is double the incubation period required to grow the screened indicator bacteria and therefore might enable the surviving population of the weakly inhibited bacteria to grow. The screened indicator bacteria do not require FBS for growth and so the addition of a rich source of nutrients to the medium might also help the surviving population of the weakly inhibited bacteria to divide during the last 24 h of incubation leading to false screening results. This can be explained by the bacterial persistence phenomenon that describes a subpopulation of a homogenous bacterial community that have a non-inherited antibiotic resistance. Persister cells appear in the exponential phase and have low protein synthesis levels and slow growth rate. Studies have shown that these cells have higher levels of expression of genes involved in stress responses that enable them to resist killing by inhibitors (Khlebodarova and Likhoshvai, 2018).

Moreover, the concentration of the inhibitor might affect the weak inhibition effect. For example, antibiotic concentration-dependent response is a phenomenon whereby bacterial exposure to a lower

antimicrobial concentration of the one needed to inhibit bacteria induces tolerance. Subinhibitory antibiotic concentration induces mechanism of antibiotics resistance by the receiver bacteria (Bernier and Surette, 2013). Moreover, bacterial communities are heterogenous and a subpopulation can poses resistance genes that enable antibiotic tolerance which might shift the whole population response to an inhibitor or a toxin (Bernier and Surette, 2013). Therefore, decreasing the incubation period and using a concentrated sample of the inhibitors would be a better approach to detect weakly inhibited bacteria.

3.3.3 The difference in the inhibition activity between *A. oris* and *A. viscosus*

A. oris culture supernatant inhibits *H. pylori* growth completely at a 50% concentration. In contrast to this finding, the same concentration of *A. viscosus* C505 culture supernatant inhibits *H. pylori* growth to a lesser extent. The difference in the inhibition activity might be attributed to the fact that *Actinomyces* species can harbour one or multiple bacteriocin operons within their genome. A study by Sugrue *et al.* (2020) screened *Actinomyces* genus for potential bacteriocins operons using the BAGEL 4 database. The study reported that the genus is rich in potential bacteriocin genes. The study also showed that *A. viscosus* C505 genome harbours only Type III bacteriocins while the majority of the screened *A. oris* strains harbour multiple bacteriocins operons (Sugrue *et al.,* 2020).

Additional studies have also suggested that the distribution of certain bacteriocins can vary between bacteria within the same genus or species. For example, Salivaricin A (SalA) is a bacteriocin that belongs to class I of bacteriocins produced by *Streptococcus salivarius*, a predominant member of the oral flora. A number of variants of *salA* gene have been identified within the genome of 90% of *S. pyogenes* strains, however, only strains belonging to M serotype and T pattern 4 (M proteins and T-antigens, from pili) produce the active peptide.(Wescombe *et al.*, 2006). Future experiments will

be aimed at ultra-filtrating *A. viscosus* C505 supernatant and the gastric *A. oris* isolate culture supernatants using a 5 kDa concentrator and testing the activity of both the filtrate and the retentate in order to confirm which of the inhibitors of *H. pylori* growth are expressed by these strains.

3.3.4 Inhibition of *C. jejuni* growth by *A. oris* culture supernatant

The retentate and the filtrate of *A. oris* culture supernatant inhibited *H. pylori* growth, indicating that the oral strain used in this study is able to produce multiple inhibitors of different sizes. This might explain the difference in the inhibition activity on *C. jejuni* growth in which one of the inhibitors might affect *C. jejuni* growth resulting in a weak inhibition activity. In the context of bacteriocins, the target of bacteriocins varies, some bacteriocins have enzymatic activity such as endopeptidases in which it degrades and disrupts the cell wall (type III; lysostaphin, zoocin A and helvetici) (Joerger and Klaenhammer, 1986). Others target the lipid II part of the cell membrane, an essential molecule for peptidoglycan synthesis, inducing pores in the cell wall and increasing the permeability leading to cell lysis and death. Examples of these bacteriocins are type 1 bacteriocins (Nisin and epidermin) (McAuliffe *et al.*, 2001). However, the current evidence is not enough to determine why *H. pylori* is more sensitive to the inhibition activity by *A. oris* than *C. jejuni* and further characterisation of these inhibitors will give more information about their targets in the bacterial cell wall. For example, testing the retentate and filtrate of *A. oris* culture supernatant on *C. jejuni* growth will determine which of inhibitors is active against *C. jejuni*. The determination of the size range of the active inhibitor against *C. jejuni* will give an insight into the potential inhibitor size, which will be useful for the purification process.

3.3.5 Factors influencing the production of inhibitory molecules by *A. oris*

Cell density and bacterial growth phase are two important factors in the production of the potential inhibitors of *H. pylori* growth. *A. oris* culture supernatant fractions from cultures grown to an OD_{600nm} of 1 failed to inhibit *H. pylori* growth, implying that the inhibitor is produced at the stationary phase of growth. Further evidence to support this observation is that *H. pylori* struggles to replicate in a co-culture medium with *A. oris* for the first 24 h of the incubation and its growth dramatically drops between 24 h and 37 h. This reduction in viable cell counts was observed when *A. oris* reached an OD_{600nm} of ~2.2. This finding is consistent with previous reports showing that most bacteriocins are secreted in the stationary phase of growth (Meade *et al.*, 2020, Mortvedt-Abildgaa *et al.*, 1995, Jiménez-Díaz *et al.*, 1993).

The effect of the growth medium composition on the activity of antimicrobials have been shown in many studies on improving the antibiotics and the bacteriocins production and activity. Al Farraj *et al* (2020) investigated the difference in antibiotic production by *Streptomyces* spp. AS4 when grown in media supplimented with different nutrient sources. Addition of yeast extract and $CaCl_2$ to the growth medium significanlty increased the production of the antibiotic (Al Farraj *et al.*, 2020). The influence of pH, incubation period and the medium composition on antibiotics production was shown in several studies (Ibrahim and Elkhidir, 2011, Poulikakos and Falagas, 2013, Wang and Liu, 2008, Quinn *et al.*, 2020).

Todorov and Dicks investigated the effect of growth medium composition of the activity of two bacteriocins; ST28MS and ST26MS produced by *Lactobacillus plantarum*. The activity of both bacteriocins was low when *L. plantarum* was grown in BHI medium (800 AU/ml) and the production of the bacteriocins reached levels of 12,800 arbitrary units/ml (AU/ml), 6400 AU/ml respectively,

when *L. plantarum* was grown in MRS medium supplemented with 20 g/l glucose. BHI and MRS media contain only 2 g/l of glucose, however, bacteriocin production is increased when glucose added to the growth medium as bacteria use it as a carbon source. Their findings suggest that specific nutrients are required to enhance bacteriocin activity and production. The effect of cell density on the production of the bacteriocin was also observed in this study, the 12,800 AU/ml activity level of ST28MS was recorded from a culture of an OD_{600nm} of 9.65 and the 6400 AU/ml activity level of ST26MS was recorded from a culture of an OD_{600nm} 8.81 (Todorov and Dicks, 2005). This observation was also reported from studies that investigated the effect of specific nutrients and growth conditions on increasing the activity of bacteriocins produced by *Lactobacillu pentosus* (Todorov and Dicks, 2004), *Lactobacillus curvatus* (Mataragas *et al.*, 2003), *Leuconostoc mesenteroides* (Krier *et al.*, 1998), *Pediococcus damnosus* (Nel *et al.*, 2001), and *Enterococcus spp.* (Abriouel *et al.*, 2001).

It remains to be investigated whether addition of glucose or other carbon sources to the growth medium of *A. oris* will enhance the production of the inhibitor of *H. pylori* growth. The initial protocol of harvesting *A. oris* culture supernatant included growing the bacteria in a medium supplemented with 10% FBS. Removal of the FBS was an essential step to minimise the amount of the proteins present in the sample prior to the purification of the potential inhibitors. The inhibition activity of *A. oris* culture supernatant was not affected by removing FBS from the medium, suggesting that FBS is not required for the production of the inhibitors.

Our data shows that *A. oris* produces the potential inhibitors of *H. pylori* growth under anaerobic conditions but not under microaerobic condition when it is grown as a pure culture. However, *A. oris* produces the inhibitor when grown under microaerobic conditions in a co-culture with *H. pylori* and *C. jejuni*. Grander *et al.* proposed a theory suggesting that bacteriocin production increases when the

producer lineages are at intermediate frequencies with a competitor (Gardner *et al.*, 2004). Bacteria sense changes in their environment and respond to these changes through various mechanisms to gain a fitness advantage and protection from competitor microbes (Kümmerli *et al.*, 2009). For example, quorum sensing is a mechanism that the majority of bacteria use in biofilms for communication. (Rumbaugh *et al.*, 2009). In the context of bacteriocin production, a proposed "competition sensing hypothesis" suggests that bacteria evolved to sense and respond to competition in their environment. The hypothesis was based on a metanalysis of bacteriocin production pathways in which competition related stressors (nutrient competition and cell damage) triggers the stress response pathways and leads to upregulation of bacteriocin production (Cornforth and Foster, 2013). Colicin is a bacteriocin produced by *E. coli* and it has been shown that colicin is produced spontaneously by cells with low fitness. However, the production of colicin is significantly increased when responding to attacks from competitors via a competition sensing (Mavridou *et al.*, 2018). *A. oris* is a facultative anaerobic bacterium that grows better in absence of oxygen, implying that oxidative stress affects the production of the inhibitor. However, the current results do not provide enough evidence to determine the mechanism by which *H. pylori* and *C. jejuni* trigger the production of the inhibitor. Competition over oxygen is overruled as this variable is removed because the inhibition assay is carried out in a hypoxic incubator where the level of oxygen is maintained at 5% throughout the incubation period. Therefore, it remains to be investigated whether the production of the inhibitors by *A. oris* is a result of nutrient competition or a currently uncharacterised response to the presence of *H. pylori* and *C. jejuni* in the same medium. Future experiments will be aimed at testing the effect of adding *H. pylori* culture supernatant to *A. oris* microaerobic cultures in order to determine whether it triggers the production of the inhibitor. Future experiments may include comparing concentrated samples of *A. oris* supernatants from different cultures (aerobic, microaerobic and anaerobic cultures) on SDS-PAGE

gel. The aim of this experiment is to test whether the bands produced in the anaerobic conditions are also produced in other growth conditions.

Microbiota are known to produce bacteriocins to help them persist in their ecological niches and for protection against pathogenic bacteria that compete with them over limited resources in their environment. The colonisation of bacteriocin-producing bacteria of the oral cavity, inhibit the adhesion of pathogens to the oral cavity cells (Heilbronner *et al.*, 2021). It has been shown that multiple members of the oral microbiota including *A. viscosus* inhibit *H. pylori* growth in *vitro* experiments. However, the mechanism of the inhibition of *H. pylori* growth was not investigated in the study (Ishihara *et al.,* 1997).

3.3.6 Purification of *A. oris* inhibitors

Most of the bacteriocins purification protocols are developed for lactic acid bacteria. The first characterised bacteriocin from *Actinomyces* was reported by Sugrue *et al.,* (2020), a type II bacteriocin produced by *A. rumincula* called actifensin. Their purification protocol involved purifying the bacteriocin from culture free supernatant using Amberlite XAD beads C_{18}. (Sugrue *et al.,* 2020). Amberlite XAD beads are a type of aqueous impregnated resin system (AIRS), a purification method used for the separation, purification and concentration of samples. One of the advantages of using AIRS is that it is a one-step method that is far less complicated than using traditional purification methods such as ammonium sulphate method or fractionating it using High Performance Liquid chromatography. The AIRS method is commonly used to purify enzymes and yields high purity of the sample (Tan *et al.*, 2018). However, the reliability of using this technique in the purification of bacteriocins was assessed by Abdulaziz *et al* 2020 who showed that it was suitable to purify BLIS bacteriocin from *Lactobacillus bulgaricus* (Abdul Aziz *et al.*, 2020). The future purification of *A. oris* inhibitors of *H. pylori* growth will follow Sugrue *et al.,* (2020) protocol.

One of the aims of the project was to purify candidate inhibitors and develop a high-throughput screening method to test the purified inhibitors. However, the challenges of the detection of novel antimicrobial compounds and bacteriocins include determining the type of assay that yields an accurate measurement of the inhibitory effect of the purified inhibitor. Most detection assays used in the literature were well diffusion assays or a spotting method. Therefore, attempts were made to develop the disk diffusion assay in order to test the inhibition activity of the purified inhibitors. However, spotting 10 µl of *A. oris* culture supernatant onto blood agar inoculated with *H. pylori* did not produce detectable zones of inhibition. The method will require further optimisation to produce a reliable result and due to the time limitation, it was not possible to optimise the disk diffusion assay.

The well diffusion assay was described by Tagg and McGiven and the assay depends on the rapid diffusion of the bacteriocin from the well into the solid medium which is subsequently inoculated with the target bacterium (Tagg and McGiven, 1971). The well diffusion technique was afterwards considered to be a standard screening assay used for the detection of many bacteriocins (Schillinger and Lücke, 1989, Batdorj *et al.*, 2006, Wang *et al.*, 2018, Sugrue et al., 2020). The spotting method was described by Kekessy and Piguet in 1970 in which the screened bacteria were spread at a standard low inoculum onto solid medium that is low in nutrients, such as LB or Muller-Hinton plates followed by spotting 10 µl of bacterial culture supernatant. The inhibition effect was reported as low, medium or strong based on the zone of inhibition produced by the culture supernatant (Valdés-Stauber and Scherer, 1994, Oscáriz *et al.*, 1999, Choyam *et al.*, 2015, Al-Madboly *et al.*, 2020). These methods allow for the detection of weakly inhibited bacteria and are therefore better at determining the range of inhibition among the screened bacteria.

Lai *et al* compared the difference between methods for bacteriocin screening; the spotting method and the cross-streaking method. The cross-streaking method was found to be more reproducible and allowed the detection of 50 out of 80 screened strains where the spotting method enabled the detection of 30 bacteria out of 80 (Lai *et al.*, 1983). Their findings suggest that factors such as the type of media used for inhibition screening and incubation period can affect the results of the screening assay.

CHAPTER 4:

Identification of the potential inhibitors of *H. pylori* growth

4.1. Introduction

Actinomyces belong to the phylum Actinobacteria, a ubiquitously distributed type of bacteria in humans, soil, plants and insects. Members of this phylum are known to produce a wide range of antimicrobial secondary metabolites and bacteriocins (Das *et al.*, 2018). Data from the previous chapter suggest that the *A. oris* inhibitors of *H. pylori* growth might be bacteriocins. Bacteriocins are ribosomally synthesized peptides produced by bacteria for defence and survival mechanisms. They vary in their inhibition range and molecular size with most of them being produced by Gram-positive bacteria. Bacteriocin biosynthesis genes can be found on the chromosome, plasmids or transposons. The gene clusters often have minimum genetic machinery that include immunity genes to prevent self-toxicity (Benítez-Chao *et al.*, 2021).

Results from the previous chapter indicate that *A. oris* secretes two or more inhibitors of *H. pylori* growth, which are low and high molecular weight, heat-resistant, active in low pH, secreted in the stationary phase of growth and have a narrow range of inhibition with the high molecular weight inhibitor being sensitive to proteolytic enzymes. These properties all match the characteristics of some type 1 and type III bacteriocins (Benítez-Chao et al., 2021, Dicks *et al.*, 2018).

Many *in silico* tools are used for the identification of biosynthetic gene clusters (BGCs) of secondary metabolites and bacteriocins. AntiSMASH and BAGEL4 are online servers used to screen bacterial and fungal genomes for potential antimicrobial gene clusters (https://antismash.secondarymetabolites.org/) (http://bagel5.molgenrug.nl/) (Medema *et al.*, 2011, Pohl *et al.*, 2019).

Therefore, the aims of the work described in this chapter were to

1. Identify candidate inhibitors present within the retentate sample of *A. oris* SN, using mass spectrophotometry.
2. Identify candidate inhibitors present within the genomes of the *Actinomyces* strains used in this study and the publicly available *A. oris* genomes, using AntiSMASH and BAGEL4 databases (Medema *et al.*, 2011, Pohl *et al.,* 2019).

4.2. Results

4.2.1. Separation of proteins within the *A. oris* culture supernatant

In order to identify the potential inhibitors of *H. pylori* growth, *A. oris* SN was concentrated 10x using a 5 kDa viva spin column to generate two fractions: the retentate containing proteins over 5 kDa and the filtrate containing proteins and metabolites under 5 kDa. Proteins in the filtrate and retentate were separated using SDS-PAGE and visualised by Coomassie Brilliant Blue staining. As expected, no bands were detected from the culture supernatant or in the filtrate, most likely due to a low concentration of proteins in the unconcentrated culture supernatant and the limitations of detecting low molecular weight proteins using a 12% SDS PAGE gel, respectively (Figure 4.1). However, four bands were detected in the retentate fraction of the SN. The band sizes were 34 kDa, ~36 kDa, 55 kDa and ~60 kDa. The two smaller size bands were cut from the gel and sent for mass spectrophotometry analysis.

4.2.2. Mass spectrophotometry analysis of the 34 kDa band

The criteria followed when analysing protein hits from the mass spectrophotometry mainly included selecting proteins with (1) high confidence level, (2) a score sequest value of greater than 15, and a high coverage percentage that reflects the percentage of the detected peptides that belong to a specific protein present in the sample. The confidence level is determined by

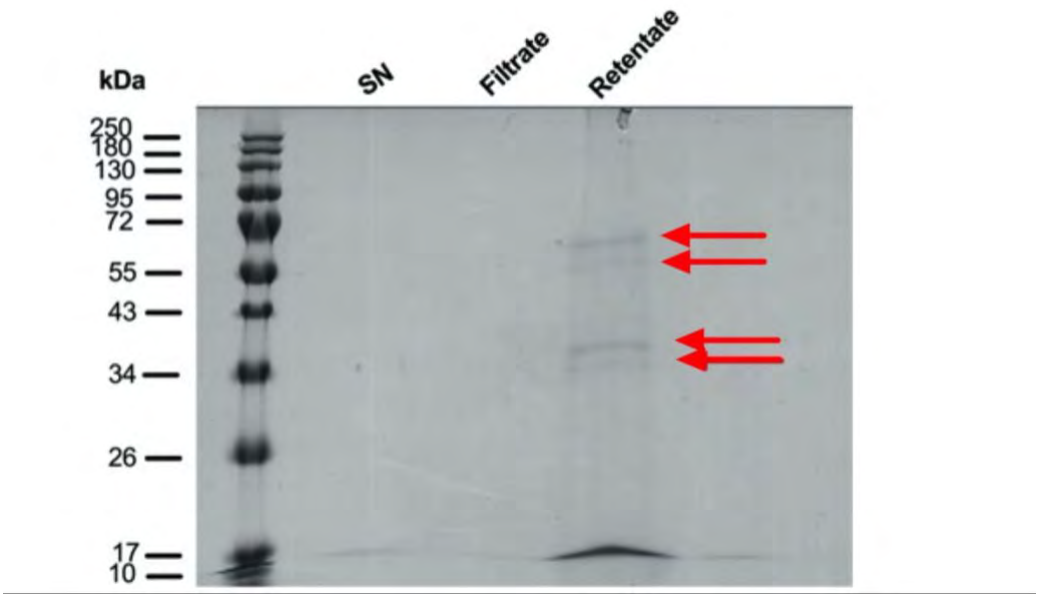

Figure 4. 1**. SDS-PAGE gel of *A. oris* culture supernatant, the filtrate and the retentate** *A. oris* culture supernatant (SN) was separated using a 5 kDa MW cutoff size viva spin column. 10 µl of the SN, the filtrate and the retentate were loaded to the wells, proteins were separated using SDS-PAGE gel with 12% acrylamide for 3 h at 120 V. Broad range ladder was used for protein size estimation.

the false discovery rate (FDR) where the high confidence level has an FDR of 1% meaning that the hit is 99% accurate. Medium confidence level indicates 95% accuracy and low confidence levels indicates 90% accuracy. The score sequest value represents how well the peptide spectrum matches to the theoretical mass spectrum of the fragmented peptides: the higher the score, the more significant is the match (Martiánez-Bartolomé *et al.*, 2008).

The 34 kDa band contained 84 protein hits with most of the hits belonging to fimbrial proteins or outer membrane proteins, such as ABC transporter proteins. However, the score sequest values, the confidence level and the FDR score of most of the proteins were very low, and therefore, these hits were considered insignificant. The most prevalent significant protein hits detected in the 34 kDa band was an uncharacterized protein with a G5 domain, UniProt #A0A1Q8XG29, a score sequest value of 69.14 and a coverage percentage of 47%. The A0A1Q8XG29 protein molecular mass is 34,608 kDa. The second most prevalent protein detected in the sample was an uncharacterised 27,117 kDa protein that belonged to the NlpC/P60 family protein, UniPro #A0A1Q8WLI2 with a score sequest value of 17.1 and a coverage percentage of 20% of the sample.

4.2.3. Mass spectrophotometry analysis of the ~37 kDa band

The same criteria used in the previous section for the mass spectrophotometry data analysis was followed to analyse the ~37 kDa band. The 37 kDa band did not contain candidate proteins with a significant score sequest, FDR rate and a coverage percentage. The highest score sequest value was 29.12, detected from a fimbrial protein with a coverage percentage of 32%. The A0A1Q8XG29 protein was also detected in the band with a score sequest value of 26.1 and a coverage percentage of 19% of the band. Detection of the A0A1Q8XG29 protein in the 37 kDa band might be a result of cross-contamination when cutting the band, as the 2 bands were close together. The sequest score

values and the coverage percentages of the other proteins detected within the 37 kDa band were very low.

4.2.4. Analysis of proteins detected within the oral *A. oris* retentate sample

The amino acid sequences of the A0A1Q8XG29 protein and the A0A1Q8WLI2 protein in FASTA format were analysed using the InterPro database in order to determine the protein domains and to give an indication of their potential function. The two proteins were also screened for homologous proteins using the BLAST alignment search tool. The A0A1Q8XG29 protein and the A0A1Q8WLI2 protein were aligned with their homologous protein hits from the blast alignment search, using the Clustal omega server.

4.2.4.1. The A0A1Q8XG29 protein

MALDI-TOF MS detected 16 peptide fragments that belong to the A0A1Q8XG29 protein (Figure 4.2.). The A0A1Q8XG29 protein is 340 amino acids in length, has a mass of 34,604 kDa and has no known function. Two domains were predicted using the InterPro database: the G5 domain that covers the region between amino acids 109-189 and a lysozyme-like domain that covers the region between amino acid 262-332 (Figure 4.3.). The BLAST search of the A0A1Q8XG29 protein resulted in more than 100 hits of hypothetical proteins of unknown functions, phospholipases and proteins containing lytic transglycosylase domains from different bacteria. The Expect values (E. value) is a parameter used in bioinformatics to describe the number of hits expected to result from a chance. The lower the E value, the lower the likelihood that the alignment of the two sequences resulted from a chance (National Library of Medicine, 2022, June). The most significant values of the E values and percentage identity belonged to a phospholipase enzyme produced by *Microbacterium*

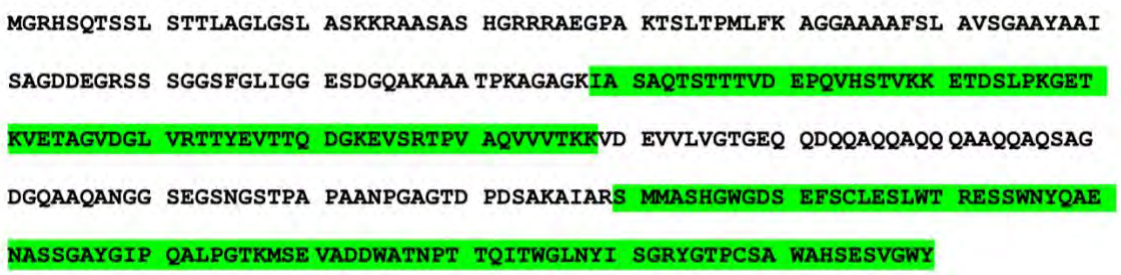

Figure 4. 2. The peptide fragments of the A0A1Q8XG29 protein detected by MALDI-TOF MS
The 16 detected peptides are highlighted in green, alignment of the detected spectra to *A. oris* database, obtained from UniProt (https://www.uniprot.org/) was done using Proteome Discoverer version 2.2. Figure was created using Bioender (https://biorender.com/).

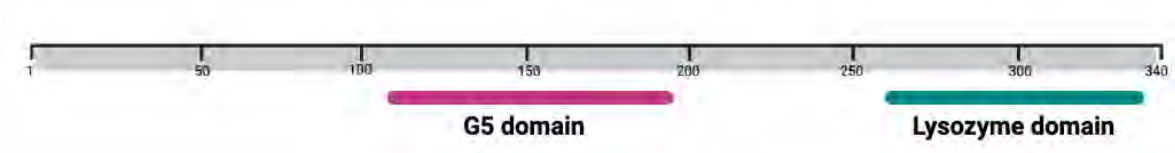

Figure 4. 3 Predicted protein domains within the A0A1Q8XG29 protein Schematic representation of the predicted domains within the A0A1Q8XG29 protein using the InterPro database. G5 domain is located between amino acids 109-189 and the homologous domain to lysozyme is located between amino acids 262-332. Figure was created using Biorender (https://biorender.com/).

sorbitolivorans with an identity percentage of 76.53%, E value of 7e-46 and query coverage of 28%. The second most significant hit belonged to a lytic transglycosylase domain containing protein produced by *Rathayibacter agropyri* with an identity percentage of 74.49%, E value of 1e-44 and query coverage of 28%. The BLAST search of the A0A1Q8XG29 protein also resulted in several hits of other uncharacterised proteins that contain the lysozyme-like domain or proteins containing lytic transglycosylase domains. The two most significant homologous proteins were used for multiple sequence alignment using Clustal omega. Alignment of the A0A1Q8XG29 protein with the 2 most significant protein hits shows that they align at the lysozyme-like domain site (Figure 4.4).

4.2.4.2. The A0A1Q8WLI2 protein

The function of the A0A1Q8WLI2 protein is unknown, the size of the protein is 27 kDa and it consists of ~ 270 amino acids (Figure 4.5). The A0A1Q8WLI2 has a NlpC/P60 domain that is usually found in papaine-like peptidases, which are a type of cysteine peptidase (Figure 4.5). The BLAST search of the A0A1Q8WLI2 protein resulted in more than 100 hits that belong to the C40 family peptidases and glycoside hydrolases from different bacteria. Proteins with the lowest E values and percentage identity were used for the protein alignment using Clustal Omega. The A0A1Q8WLI2 protein is homologous to a C40 peptidase produced by the Gram-positive anaerobic bacterium, *Ancrocorticia populi.* The percentage identity of the C40 protein peptidase hits was 67.62%, the E value was of 2e-39 and the query coverage was of 39%. The C40 peptidases are involved in bacterial cell wall degradation (Faheem *et al.*, 2016). The A0A1Q8WLI2 protein is also homologous with a glycoside hydrolyse produced by *Terrabacter* spp, a Gram-positive aerobic bacterium. The percentage identity of the hit was 67.5%, the E value was of 4e-37 and the query coverage was of 39%. The rest of the

Figure 4. 4. Multiple sequence alignment of the A0A1Q8XG29 containing protein produced by *A. oris* with phospholipase produced by *M. sorbitolivorans* and lytic transglycosylase produced by *R. agropyri*

BLAST search tool was used to find homologous proteins of the A0A1Q8XG29 protein produced by *A. oris.* The sequence alignments were performed using Clustal-omega (EMBL-EBI). Perfect matches residues were displayed with *, amino acids substitutions of groups with strongly similar properties is represented by (Bass *et al.*) and amino acids substitutions of groups with weakly similar properties is represented by (.) , cov represents the percentage coverage and pid represents the percentage identity of the proteins to the A0A1Q8XG29 protein. The lysozyme domain is outlined by a red box. Protein sequences were obtained from blastp database (WP_162785780.1, WP_246226640.1). The A0A1Q8XG29 protein sequence was obtained from UniPro. Figure was created using Mview (https://www.ebi.ac.uk/Tools/msa/mview/).

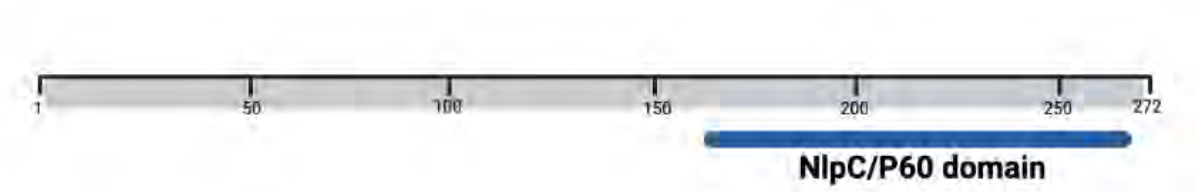

Figure 4. 5. Predicted protein domains within the A0A1Q8WLI2 protein produced by *A. oris*. Schematic representation of the predicted domain within A0A1Q8WLI2 protein using InterPro NlpC/P60 domain is located between amino acid 162-272. Figure was created using Biorender (https://biorender.com/).

C40 peptidase and the glycoside hydrolyses hits had similar homology values. Therefore, the two most significant homologous proteins were used for multiple sequence alignment using Clustal omega. Alignment of the A0A1Q8WLI2 protein produced by *A. oris* with the C40 peptidase produced by *Ancrocorticia populi* and glycoside hydrolyse produced by *Terrabacter* spp. shows that the 3 proteins align best towards the NlpC/P60 domain (Figure 4.6).

4.2.5. Identification of the potential antimicrobial biosynthetic gene clusters present within the genomes of *Actinomyces* strains used in this study and the publicly available genomes of *A. oris*.

As shown in Chapter 3, *A. oris* filtrate fractions also inhibited *H. pylori* growth, suggesting that an inhibitor below 5 kDa is also being produced. However, no bands were detected from the filtrate sample on an SDS PAGE gel. Detection of small size proteins (<5 kDa) using SDS PAGE gel is limited, and it requires high concentrations of the SDS PAGE gel, followed by Silver staining to increase the sensitivity of the detection. Therefore, in order to predict the inhibitor of the filtrate sample of *A. oris* SN, the oral and gastric *A. oris* isolates used in this study were whole genome sequenced, using Illumina sequencing (2x250bp) with 30x coverage.

The Orthologous Average Nucleotide Identity (OrthoANIu) value is a measure of similarity between two genome sequences and it is used for the classification of bacteria (Lee *et al.*, 2016). *A. oris* strain FDAARGOS_1051 (RefSeq GCF_016127955.1) was used as a reference genome. The OrthoANIu values of the genomes of the two *A. oris* isolates used in this study are higher than 95%, confirming that the two isolates are *A. oris* (Table 4.1.) The resulting sequences, the *A. viscosus* C505 genome (NZ_LR134477.1) and 34 published *A. oris* genomes were analysed bioinformatically, using the AntiSMASH database (https://antismash.secondarymetabolites.org/) (Medema *et al.,* 2011) and BAGEL 4

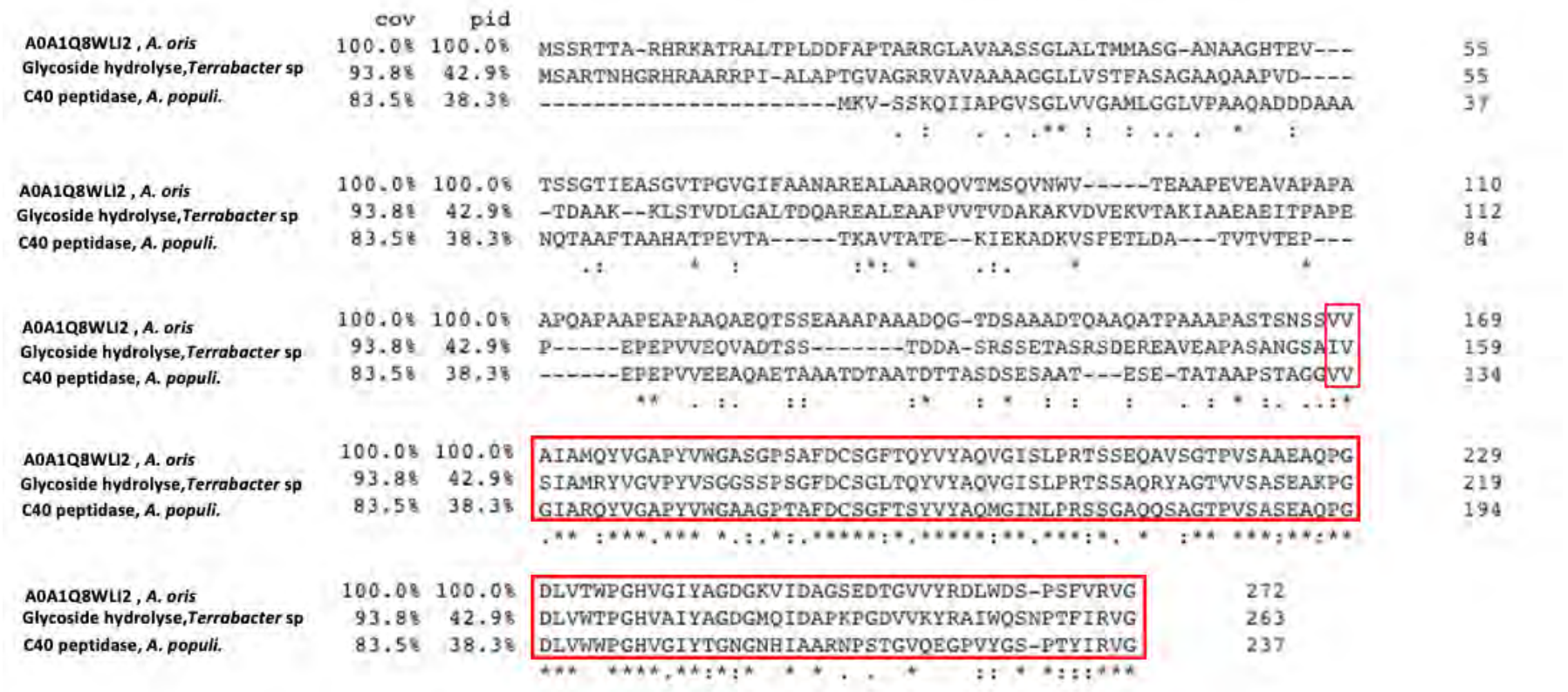

Figure 4. 6 Multiple sequence alignment of A0A1Q8WLI2 protein produced by *A. oris* with C40 peptidase produced by *A. populi* and glycoside hydrolyse produced by *Terrabacter* spp BLAST search tool was used to find homologous proteins of the A0A1Q8WLI2 protein produced by *A. oris*. The sequence alignments were performed using Clustal-omega (EMBL-EBI). Perfect matches residues were displayed with *, amino acids substitutions of groups with strongly similar properties is represented by (Bass et al.) and amino acids substitutions of groups with weakly similar properties is represented by (.), cov represents the percentage coverage and pid represents the percentage identity of the proteins to the A0A1Q8WLI2 protein. The NlpC/P60 domain is outlined in a red box. Protein sequences were obtained from blastp database (WP_109093344.1, NUS41473.1). The A0A1Q8WLI2 protein sequence was obtained from UniPro (A0A1Q8WLI2). Figure was created using Mview (https://www.ebi.ac.uk/Tools/msa/mview/).

Table 4. 1. *A. oris* clinical isolates genomes assembly sequence information using *A. oris* strain FDAARGOS_1051 (RefSeq GCF_016127955.1) as a reference genome

Bacteria	Assembly size	GC content (%)	OrthoANIu value (%)	N50
Oral *A. oris* isolate	3,094,965	68.56	96.44	35082
Gastric *A. oris* isolate	3,276,940	67.12	96.38	31231

(http://bagel5.molgenrug.nl/) (Pohl *et al.,* 2019) to search for putative biosynthetic gene clusters encoding bacteriocins and antimicrobial peptides present within the *A. oris* genomes.

Two potential bacteriocins were detected with AntiSMASH screening among the three *Actinomyces* strains used in the study. A significant putative BGC of Lanthipeptide (less than 5 kDa) was detected within the oral and the gastric *A. oris* isolates and *A. viscosus* genomes. The operon consists of Lanthipeptide class III gene (*ctg1_749*); ABhydrolase gene (*ctg1__745*); aldehyde dehydrogenase gene (*ctg1_757*) and other transport and regulatory genes. These genes are involved in the function and secretion of Lanthipeptides class III. The operon included significant putative homologous genes for ABC transporter, suggesting that *A. oris* secretes the lanthipeptide through an ABC transporter channel. BAGEL4 search detected Lanthipeptide BGC in the gastric *A. oris* and *A. viscosus* genomes and not in the oral *A. oris* genome. Lanthipeptide BGC was detected in 6 genomes of the 34 publicly available *A. oris* genomes using AntiSMASH and only in 3 *A. oris* genomes of the 34 publicly available *A. oris* genomes using BAGEL4 (Table 4.2., Table.4.3.).

A significant putative BGC hit of a homologous gene to Linocin_M18 (31 kDa) was identified among the genomes of both isolates of *A. oris* and *A. viscosus*. In detail, AntiSMASH and BAGEL4 analysis of *A. oris* genome detected a putative BGC, the Linocin_M18-like operon, of the ribosomally synthesised and post-translationally modified peptide product gene (RiPP, Linocin_M18-like protein). The linocin_M18 operon consists of the *ctg1_321* gene that encodes the DyP-type encapsulating protein, DyP type peroxidase (*ctg1_320*), a tRNA methyltransferase complex GCD14 (*ctg1_322*) and other regulatory genes. Linocin_M18 BGC was detected in 30 genomes of the

publicly available *A. oris* genomes using AntiSMASH and in 28 genomes using BAGEL4 (Table 4.2., Table.4.3.).

A BGC of Sactipeptides was detected in all the *Actinomyces* strains used in this study using BAGEL4 database only. Sactipeptides are ribosomally synthesised and post translationally modified peptides that are known to have antimicrobial activity. Sactipeptides BGC was detected in 27 genomes of the publicly available *A. oris* genomes. However, Sactipeptides BGC was not detected when *A. oris* genomes were screened using AntiSMASH tool.

4.3 Discussion

4.3.1 Potential functions of the A0A1Q8XG29 protein

Mass spectrophotometric analysis of the two bands detected from the SDS-PAGE gel of *A. oris* culture supernatant indicated that it contained a protein of unknown function, the A0A1Q8XG29 protein. The G5 domain consists of 80 residues that contain conserved glycine residues. The exact function of the G5 domain is still not fully known. However, the domain is associated with a range of enzymes such as glycosyl hydrolases and streptococcal IgA peptidases in bacteria (Bateman *et al.*, 2004). Several suggested functions of the domain have been proposed that include co-factor binding, regulation of the enzymatic domain and an acetylglucosamine binding site that is a common feature of proteins containing G5 domains (Bateman *et al.*, 2005, Paukovich *et al.*, 2019, Pluvinage *et al.*, 2013). N-acetylglucosamine (GlcNAc) is a key component of the peptidoglycan layer and plays a major role in the integrity of the bacterial cell wall (Konopka, 2012).

The A0A1Q8XG29 protein also has a lysozyme domain. The lysozyme superfamily is a group of enzymes that catalyse the hydrolysis of the glycosidic bond between carbohydrates (Henrissat *et al.,* 1995). Lysozymes are found in a variety of organisms as a defence mechanism to protect against bacteria and secreted by bacteriophages to infect bacteria. Lysozymes hydrolyse the β-1, 4 glycosidic bonds between MurNAc and GlcNAc of the peptidoglycan cell wall (Pei and Grishin, 2005). The bacterial cell wall consists of alternating sugar residues; β (1,4) N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). A peptide chain of 5 amino acids is attached to the MurNAc that cross links with another peptide chain forming the peptidoglycan layer. Disruption of the peptidoglycan layer results in immediate cell lysis
and death. The UniProt analysis of the A0A1Q8XG29 protein indicates that it has a signal sequence and is therefore likely to be secreted by *A. oris*.

Alignment of the A0A1Q8XG29 protein with the homologous proteins detected by BLAST search; the phospholipase produced by *M. sorbitolivorans* and lytic transglycosylase domain-containing protein produced by *R. agropyri* indicates that they align best towards the lysozyme domain, suggesting that the shared function between the three proteins is the lysozyme effect of degrading peptidoglycan. Further functional characterisation of the G5 domain containing protein is required to confirm whether the A0A1Q8XG29 protein is an inhibitor of *H. pylori* growth. The first step of confirming the activity of the A0A1Q8XG29 protein is to purify the protein followed by testing the activity of it on *H. pylori* growth ot both.

Type III bacteriocins are known to have enzymatic functions that degrade the peptidoglycan of the targeted cells. Bacteriocins of this type are large molecules of >30 kDa and vary in their heat sensitivities and lytic activities (Simons et al., 2020). For example, zoocin A produced by

Streptococcus equi subsp. zooepidemicus 4881 belongs to type III bacteriocins and function as a bacteriolytic enzyme that binds to the penicillin binding receptors of the peptidoglycan layer of the targeted cell and hydrolyse it (Beatson *et al.*, 1998). Other examples of type III bacteriocins with enzymatic functions are lysostaphin produced by *Staphylococcu*s spp. (Bastos et al., 2010), and helviticin J produced by *Lactobacillus helveticus*(Joerger and Klaenhammer, 1986). Most Gram-negative bacteria are resistant to bacteriocin activity because the outer membrane protects the peptidoglycan layer. However, pesticin produced by *Yersinia pestis* is an example of a bacteriocin that can penetrate the outer membrane of Gram-negative bacteria such as *H. pylori* . Pesticin has a lysozyme domain that hydrolyses the peptidoglycan layer of the targeted cells, a first report of a bacteriocin with lysozyme-like activity (Patzer *et al.*, 2012). *A. oris* produces a protein of an unknown function (the A0A1Q8XG29 protein) that has a lysozyme domain. Therefore, purifying the A0A1Q8XG29 protein followed by testing the activity of it on *H. pylori* growth is required to confirm that it is responsible of *A. oris* inhibition of *H. pylori* growth.

4.3.2. The A0A1Q8WLI2 protein

The mass spectrophotometry analysis of the 34 kDa band indicated that the band contained a significant hit of an uncharacterised protein of 27 kDa in size, the A0A1Q8WLI2 protein. The detection of a 27 kDa size protein in the 34 kDa band might be due to technical issues during the separation of *A. oris* culture supernatant using SDS PAGE gel electrophoresis such as the quality of the gel and the buffer used, the duration of the separation of proteins and the electrical current used. In SDS-PAGE gel electrophoresis, proteins migrate with the electrical current through the mesh-like matrix. Proteins with lower molecular weight migrate quicker than larger proteins. However, the structure of the protein affects the mobility of the proteins. Therefore, samples are routinely boiled for 5 min prior to separation. Globular proteins such as outer membrane proteins tend to aggregate

Table 4. 2. Antimicrobial biosynthetic gene clusters (BGC) detected within the genomes of *Actinomyces* spp. used in this study, using AntiSMASH database

Bacterial strain/ GenBank reference number	Sample type	Linocin_M 18	Lanthipeptide	Type-1 polyketide synthase	Thiopeptide	Ladderane
***A. oris* (this study)**	oral	+	+	+	+	+
***A. oris* (this study)**	gastric	+	+	+	-	+
***A. viscosus* C505**	upper respiratory tract	+	+	-	+	+
GCA_016127955.1	unspecified clinical isolate	+	-	-	+	-
GCA_001553935.1	oral	+	-	-	+	-
GCA_001682715.1	oral	+	-	-	-	-
GCA_001929365.1	cerebral abscess	+	-	-	+	-
GCA_001929375.1	oral	-	-	-	+	-
GCA_001937365.1	cerebral abscess	+	+	-	+	-
GCA_001937385.1	Soft lesion	+	-	-	+	-
GCA_001937415.1	oral	+	+	-	+	-
GCA_001937425.1	oral	+	+	-	+	-

GCA_001937445.1	oral	+	-	-	-	-
GCA_001937485.1	oral	+	-	-	+	-
GCA_001937505.1	intrauterine contraceptive device	+	+	-	+	-
GCA_001937535.1	blood culture	+	+	-	-	-
GCA_001937545.1	oral	+	-	-	+	-
GCA_001937555.1	oral	+	-	-	+	-
GCA_001937655.1	oral	-	-	-	+	-
GCA_001937665.1	oral	-	-	-	+	-
GCA_001937675.1	oral	-	-	-	+	-
GCA_001937715.1	leathery lesion	+	-	-	+	-
GCA_001937725.1	leathery lesion	+	-	-	+	-
GCA_002847555.1	urine	+	-	-	+	-
GCA_006546825.1	septum	+	-	-	-	-
GCA_027945475.1	oral	+	-	-	-	-
GCA_905373015.1	oral	+	-	-	-	-
GCA_905373285.1	oral	+	-	-	+	-
GCA_905373335.1	oral	+	-	-	-	-

GCA_916048155.1	oral	+	-	-	+	-
GCA_916050205.1	oral	+	-	-	+	-
GCA_916438615.1	oral	+	-	-	+	-
GCA_937981545.1	digestive system	+	-	-	+	-
GCA_937982005.1	digestive system	+	-	-	-	-
GCA_946221355.1	skin	+	-	-	-	-
GCA_000180155.1	oral	+	-	-	-	-
GCA_023169925.1	oral	+	-	-	+	-

Table 4. 3. Antimicrobial biosynthetic gene clusters (BGC) detected within the genomes of *Actinomyces* spp. used in this study and the 34 publicly available *A. oris* genomes using BAGEL4 database

Bacterial strain/ GenBank reference number	Sample type	Linocin_M18	Lanthipeptide	Thiopeptide	Sactipeptide
Oral *A. oris*	oral	+	-	-	+
Gastric *A. oris*	gastric	+	+	-	+
***A. viscosus* C505**	upper respiratory tract	+	+	-	+
GCA_016127955.1	unspecified clinical isolate	+	-	+	-
GCA_001553935.1	oral	+	-	+	-
GCA_001682715.1	oral	+	-	-	+
GCA_001929365.1	cerebral abscess	+	-	+	+
GCA_001929375.1	oral	-	-	-	+
GCA_001937365.1	cerebral abscess	+	-	-	+
GCA_001937385.1	Soft lesion	+	-	-	+

GCA_001937415.1	oral	+	+	-	+
GCA_001937425.1	oral	+	+	-	+
GCA_001937445.1	oral	+	-	-	+
GCA_001937485.1	oral	+	-	-	+
GCA_001937505.1	intrauterine contraceptive device	+	-	-	+
GCA_001937535.1	blood culture	+	+	-	+
GCA_001937545.1	oral	+	-	-	+
GCA_001937555.1	oral	+	-	-	+
GCA_001937655.1	oral	-	-	-	+
GCA_001937665.1	oral	-	-	-	+
GCA_001937675.1	oral	-	-	-	+
GCA_001937715.1	leathery lesion	+	-	-	+
GCA_001937725.1	leathery lesion	+	-	-	+
GCA_002847555.1	urine	+	-	-	+

GCA_006546825.1	septum	+	-	-	+
GCA_027945475.1	oral	+	-	-	+
GCA_905373015.1	oral	+	-	-	+
GCA_905373285.1	oral	+	-	-	+
GCA_905373335.1	oral	+	-	-	-
GCA_916048155.1	oral	+	-	-	+
GCA_916050205.1	oral	+	-	-	+
GCA_916438615.1	oral	-	-	-	-
GCA_937981545.1	digestive system	+	-	-	+
GCA_937982005.1	digestive system	-	-	-	+
GCA_946221355.1	skin	+	-	-	+
GCA_000180155.1	oral	+	-	+	-
GCA_023169925.1	oral	+	-	+	-

when boiling at 98°C which affects the mobility of these protein across the gel leading to a slower migration. Other factors such as the acidity of the sample buffer and the use of organic solvent also cause in protein aggregation (Sagné *et al.*, 1996). This phenomenon can explain the detection of a 27 kDa protein in the 34 kDa band.

The exact function of the NlpC/P60 domain has not been identified (Xu *et al.,* 2009). The NlpC/P60 domain is often associated with peptidase enzymes that are involved in the bacterial cell wall degradation and modification. The BLAST search of the A0A1Q8XG29 protein resulted in significant homology hits with C40 peptidase enzyme which functions as a cell wall modifier. The C40 peptidases have a conserved catalytic residue of; cysteine, histidine, histidine (Aramini *et al.*, 2008) that is absent from the A0A1Q8XG29 protein. However, these preliminary data need further characterisation to confirm whether the NlpC/P60 protein is a cell wall modification enzyme that maintain *A. oris* physiology or is an inhibitor of *H. pylori* growth.

4.3.3. Potential antimicrobial operons detected within the genome of the *Actinomyces* strains used in this study and the publicly available *A. oris* genomes

Two online databases were used to screen for potential antimicrobial BGCs among the genomes of the *Actinomyces* strains used in the study and the publicly available *A. oris* genomes. AntiSMASH detected Lanthipeptide and Linocin_M18 BGCs in more screened genomes than BAGEL4 did. Both databases are routinely used to screen for potential antimicrobial BGCs by comparing a previously identified antimicrobial BGC to target gene clusters.

AntiSMASH database uses validated rules that define the biosynthetic clusters present within a genome in order to form a BGC. AntiSMASH uses profile hidden Markov (pHMMs) models to

identify these BGCs. These includes PFAM, TIGRFAMs, SMART, BAGEL, Yadav *et al*. 2009 (Yadav *et al.*, 2009), and therefore, increases the detection of the potential BGCs. The developers of AntiSMASH have improved the annotation and detection of RiPP such as Lanthipeptide by detecting the prepeptide of the cluster and commonly associated tailoring enzymes (Blin *et al.*, 2021).

BAGEL 4 uses a combination of direct mining of precursor peptides and indirect rule-based approach to identify potential bacteriocins BGCS and can accurately predict the mature bacteriocin sequence (Zhong *et al.*, 2020, Russell and Truman, 2020).

Analysis of the whole genomes of *Actinomyces* strains used in this study using AntiSMASH indicates that Lanthipeptide and Linocin_M18 homologue genes were conserved among the three bacteria used in this study. However, BAGEL4 screening detected Lanthipeptides BGC in the gastric *A. oris* and *A. visocusu* genomes only. Lanthipeptides are classified as type I bacteriocins, a ribosomally synthesised and post-translationally modified peptide product (RiPP). They have a small molecular mass of less than 5 kDa, are heat-resistant and known to have antimicrobial activity (Lagedroste *et al.*, 2020).

BAGEL4 screening resulted in the detection of Sactipeptides BGC in all the three strains used in the study and in 27 genomes of the publicly available *A. oris* genomes. Sactipeptides are small peptides that exhibit antimicrobial activities. For example, Thuricin CD is a Sactipeptides produced by *Bacillus thuringiensis* that has a narrow range of inhibition (Rea *et al.*, 2010).

A. oris secretes an inhibitor of less than 5 kDa into the medium as shown when testing the filtrate of *A. oris* SN on *H. pylori* growth. Further investigations are required to confirm if the inhibitor of *H.*

pylori growth detected in the filtrate of *A. oris* SN belongs to the lanthipeptide class or Sactipeptides class of bacteriocins.

Linocin_M18 is a type III bacteriocin produced by *Brevibacterium linens.* The protein is produced in the logarithmic phase of growth, has antimicrobial activity against some of the Gram-positive bacteria used in the study. Linocin-M18 did not affect *S. aureus* and *E. faecalis* and 3 different strains of *Streptococcus salivarius* growth. This correlates with the effect of *A. oris* SN on *S. aureus*, *E. faecalis* and *Streptococcus* growth shown in chapter 3. However, Linocin_M18 inhibition activity was not tested on *H. pylori* growth in that study. Linocin-M18 has a molecular mass of 31 kDa, is sensitive to proteolytic enzymes, heat labile and stable under low pH (Valdés-Stauber and Scherer, 1994). The analysis of Linocin_M18 protein structure revealed that these proteins are merely capsids that encapsulate DyP peroxidases and are involved in oxidative stress adaptation. The antimicrobial activity of the encapsulin proteins such as Linocin_M18 is instead a result of the cargo protein, indicating that both proteins are required for Linocin_M18 function. The capsid protein (Linocin_M18) is required for the stability and the delivery of the cargo protein (DyP peroxidase) The genome of the three *Actinomyces* strains used in this study harbours the DyP peroxidase and the DyP-type encapsulating protein (Liocin_M18-like protein) (Putri *et al.*, 2017). Further investigation is required to determine whether the *Actinomyces* strains use Linocin_M18-like bacteriocin to inhibit *H. pylori* growth. Future experiments would include the purification of Linocin_M18 from *A. oris* SN and testing the inhibition effect on *H. pylori* growth.

Ladderanes are unique lipids commonly found in the membranes of anammox bacteria. They consist of C18 and C20 fatty acids that contain either 3 or 5 concatenated cyclobutene rings. The exact role of these lipid is still not fully understood, however they are proposed to be involved in ammonium

oxidizing process under anoxic conditions (Moss Frank *et al.*, 2018). The antimicrobial activity of Ladderanes was not investigated before and therefore it remains to be investigated whether ladderane is responsible for the inhibition activity on *H. pylori* growth by *A. oris.* Ladderanes BGC was only detected in the AntiSMASH screen and was not detected when screening *A. oris* genomes using BAGEL4.

The accuracy of the detection of putative antimicrobial BGC vary between the two tools used in this study. This variation depends on the type of the detected BGCs and the algorithm that these databases use. Predication of BGCs depends on specific sequences which may not be present in all bacteriocins. It also depends on sequence similarities between the database gene sequences and the target gene sequence, which can be limited to the previously identified bacteriocins sequences leading to false negative results (Russell and Truman, 2020).

There was no correlation between the sample isolation site and the presence of a certain bacteriocin BGCs. The *A. oris* strains that harbour putative antimicrobial BCGs were isolated from different human body sites and not limited to strains isolated from the oral and gastric samples where *A. oris* might interact with *H. pylori.*

4.3.4. Limitations of the detection of proteins from bacterial culture supernatant using SDS PAGE.

Results presented in chapter 3 show that the retentate of *A. oris* SN inhibits *H. pylori* growth. However, the SDS-PAGE gel of the retentate (10 x concentrated) sample of *A. oris* SN stained with Coomassie Brilliant Blue contained a band of 34 kDa, which was excised and analysed by mass spectrometry. Further analysis indicated that this is an uncharacterised protein with a G5 domain.

Linocin_M18 has a molecular weight of 31 kDa but was not detected in the mass spectrometry analysis. Furthermore, proteins of lower than 5 kDa in size could not be detected using this method. However, Coomassie Brilliant Blue staining sensitivity is relatively low and does not detect all the proteins present within a sample. Silver staining is a stain that can be used as an alternative to Coomassie brilliant blue, which offers higher sensitivity for protein detection in which the silver binds to the sidechain of the amnio acid i.e., carboxyl group (Kurien and Scofield, 2012). Valdés-Stauber *et al*., (1994) biochemically characterised linocin_M18 and used sliver staining to detect linocin_M18 from *B. linens* culture supernatant SDS-PAGE gel (Valdés-Stauber and Scherer, 1994). Halami *et al* (2011) investigated the biochemical properties of a bacteriocin produced by *Pediococcus pentosaceus*. Their protocol included Silver staining in order to detect the small size bacteriocin of 5 kDa from a SDS-PAGE (16%) gel of *P. pentosaceus* culture supernatant (Halami *et al.*, 2011). Silver staining of the SDS-PAGE gel was employed by several researchers to detect low concentration or small size bacteriocin from culture supernatant samples (Aktypis *et al.*, 1998, Whitford *et al.*, 2001). Future experiments will be aimed at optimising the production of *A. oris* inhibitors using different nutrient resources as discussed in chapter 3 to yield a better concentration of the inhibitors from a culture supernatant. This will be followed by separating the samples using 16% SDS PAGE gel electrophoresis to detect lower molecular weight proteins from the filtrate followed by silver staining to detect the potential bacteriocins present within the sample.

CHAPTER 5:

FINAL DISCUSSION

5. Final discussion

The effectiveness of *H. pylori* infection treatment regimen decreased in the past years especially with the emergence of resistant *H. pylori* strains. The need to develop alternative antimicrobial agents is crucial to treat *H. pylori* infection and to prevent the progression to GC. One disadvantage of using broad spectrum antibiotics is that it does not select for the pathogenic bacteria and affects the human microbiome, leading to the development of antimicrobial resistance among microbiome members (Konstantinidis *et al.*, 2020). Therefore, developing an antimicrobial therapy to treat a single bacterial infection would be a promising approach to treat the targeted bacterial infection and preserve the microbiome diversity and integrity.

Results presented in this thesis indicate that members of *Actinomyces* produce inhibitors of *H. pylori* growth. The investigation of the inhibition phenomenon presented in chapter 3 show that an oral *A. oris* isolate produce two or more inhibitors of molecular weights of lower and higher than 5 kDa in size, pH independent, heat resistant, sensitive to proteolytic enzymes and produced during the stationary phase of growth. Mass spectrometry analysis of *A. oris* culture supernatant indicated that it contains an uncharacterised protein with a potential inhibition activity (the A0A1Q8XG29 protein). Results from chapter 4 indicate that the three *Actinomyces* strains used in this study harbours biosynthetic gene clusters of lanthipeptide and linocin M_18 bacteriocins.

5.1. Hypothesised mechanism of action of the A0A1Q8XG29 protein

Results from chapter 4 indicated that a concentrated sample of *A. oris* SN contained a protein of an unknown function of a 34 kDa size. Analysis of the protein functional domains indicated that the protein has a G5 domain that recognises the N-acetylglucosamine part of peptidoglycan and a lysozyme domain that hydrolyses the glycosidic bonds of peptidoglycan.

Lysozyme enzyme is part of the innate human immunity protecting from both Gram-positive and Gram-negative bacteria despite the difference in their call wall components. The innate immune cells produce other factors such as defensins (Hancock and Scott, 2000) and lactoferrin (Ellison and Giehl, 1991) to permeabilise the outer membrane of Gram-negative bacteria and enable the lysozyme enzyme to access the peptidoglycan layer. Sugrue *et al.* reported a defensin-like bacteriocin of 4,091 Da in size produced by *A. rumincula* that shares more than 50% identity with eukaryotic defensins. The study reported that actifinsin was common among *Actinomyces* genus including multiple *A. oris* strains (Sugrue *et al.,* 2020). The gene encoding for actifinsin (*afa*A) was not detected with the *Actinomyces* strains used in the study, therefore, it remains to be established whether *Actinomyces* secrete other compounds to permeabilise *H. pylori* outer membrane and facilitate the action of the G5 domain containing protein.

The inhibitors produced by *A. oris* did not affect the growth of any of the Gram-positive bacteria tested. Gram-positive bacteria are known to confer lysozyme resistance through peptidoglycan O-acetylation, a modification of the glycan strands of the peptidoglycan layer. It has been reported that this mechanism is employed primarily by pathogenic bacteria to escape innate immunity. For example, pathogenic species of Staphylococci O-acetylate their peptidoglycan layer, conferring resistance to lysozyme activity. In contrast, the non-pathogenic species of *Staphylococcus* are sensitive to lysozyme activity because they do not O-acetylate their peptidoglycan layer (Bera *et al.*, 2006). All of the Gram-positive bacteria used in this study are pathogenic bacteria that are reported to use peptidoglycan O-acetylation to protect them from the lysozyme activity (Sychantha *et al.*, 2018). This might explain the negative effect of *A. oris* SN on the growth of the tested Gram-positive bacteria.

The genes responsible for peptidoglycan modification in *H. pylori* are *patA* and *pdgA*. A study by Wang *et al.,* (2012) showed that mutants of any of the previous genes were more susceptible to lysozyme effect than the wild type (WT) *H. pylori*. Lysozyme treatment (30 mg/ml) for 6 h resulted in a lower survival of the *patA* mutant (10^4 cells/ ml) and the WT *H. pylori* (~ 10^6 cells/ ml) than the untreated *H. pylori* (10^9 cells/ ml). This finding suggests that *patA* plays a role in protecting from lysozyme and that *H. pylori* strains that lack the *patA* gene are more susceptible to lysozymes. The same study screened the whole genomes of 30 *H. pylori* strains using a BLAST search for the putative *patA* gene, only four strains out of the screened genomes harboured a putative *patA* gene, indicating that *patA* is not well distributed among the screened *H. pylori* genomes (Wang *et al.*, 2012). Analysis of the whole genome of the *H. pylori* strain used in this study showed that it only harbours the *pdgA* gene and it lacks the *patA* gene.

C. jejuni is intrinsically resistant to lysozyme activity through peptidoglycan O-acetylation. The genes encoding the enzyme peptidoglycan acetyltransferase *patA* and *patB* are well distributed in *C. jejuni*. Iwta *et al.,* (2016) reported that *patA* and *patB* genes were detected in all of the 96 *C. jejuni* whole genome sequences used in the study using BLAST alignment (Iwata *et al.*, 2016). This might explain the difference in the inhibition activity of *A. oris* culture supernatant on *H. pylori* and *C. jejuni* as *A. oris* culture supernatant completely inhibits *H. pylori* and partially inhibits *C. jejuni.*

5.2. Challenges with characterisation of the inhibitor

Commensal bacteria have evolved to preserve their microenvironment and maintain their ecological niche through multiple mechanisms including antimicrobial production. The specificity of the targets

depends merely on the interaction between these bacteria and whether they threaten the fitness of the antimicrobial producer in their ecological niche (Abt and Pamer, 2014).

Despite that bacteriocins are extensively studied over the years, their utilization and medical application are challenging (Stote *et al.*, 2017). There is no report of a universal approach to purify and characterise all types of bacteriocins. This is because bacteriocins vary in their structure, chemical properties and their targets. Therefore, bacteriocin purification method can vary and maybe developed extensively for a certain bacteriocin (Stote et al., 2017).

A. oris inhibitors activity assessment was limited to two types of fastidious bacteria that require specific growth conditions and medium. Therefore, characterising these inhibitors required longer periods and multiple optimisation steps that affected the progress of the project. Different purification approaches, such as lyophilisation was employed as mentioned in chapter 3, however, this method was not successful, which also contributed to the delay in the purification of *A. oris* inhibitors.

5.3. Future work

The evidence presented in this thesis indicates that *A. oris* produce two or more inhibitors of *H. pylori* growth. One of the inhibitors is below 5 kDa in size and analysis of the whole genome of the *Actinomyces* strains used in this study indicates that lanthipeptide gene operon is conserved among the three strains. The retentate sample of *A. oris* SN inhibits *H. pylori* growth, suggesting that *A. oris* produce another inhibitor of a size higher than 5 kDa that inhibits *H. pylori* growth.

H. pylori stains are diverse genetically because of their high intraspecies recombination and mutation rates (Cover, 2016). Individuals are infected with *H. pylori* with a highly diverse population of the

bacterium. *H. pylori* diversity is observed between different regions of the human stomach (Wilkinson *et al.*, 2022). Results from chapter 3 indicate that *A. oris* SN inhibits the growth of SS1 strain of *H. pylori*. Future experiments may include testing the effect of *A. oris* SN on different strains of *H. pylori* which would represent a better model that reflects *H. pylori* population in the stomach.

The method used in this thesis to identify the candidate inhibitors of *H. pylori* growth was running *A. oris* SN on an SDS-PAGE followed by cutting out the resulted bands. Mass spectrometry was used afterwards to identify the proteins present within the resulted bands. However, a better approach to profile the proteome of *A. oris* would be to use Liquid Chromatography with tandem mass spectrometry LC-MS/MS. The method is widely used technique in proteomics to identity and quantify proteins and peptides in samples with complex mixtures. The technique is high in sensitivity and specificity because it separates and analyse peptides based on their biochemical properties such as charge, mass and hydrophobicity. Therefore, it allows the detection of more proteins and peptides than other techniques. One of the advantages of LC-MS/MS is that it identifies the post-transitional modifications of peptides which makes it a well-suited approach to analyse bacteriocins (Karpievitch *et al.*, 2010, Pérez-Llarena and Bou, 2016). Future experiments would include proteomic analysis of *A. oris* SN using LC-MS/MS technique to identify and analyse the potential inhibitors produced by *A. oris*.

Following the purification and the identification of the inhibitors of *H. pylori* growth, *in vitro* assays will be employed to assess if these inhibitors are able to inhibit *H. pylori* growth in a cell line model such as AGS cells.

Bacteriocin applications in industry and medicine depends on the stability of the bacteriocin. For example, lysostaphin is a staphylococcal bacteriolysin that has a promising clinical application to treat staphylococcal infections. Several *in vitro* and *in vivo* studies showed its potentials when used solely or in combination with antibiotic treatment (Bastos *et al.,* 2010). Sigma-Aldrich commercially sell a recombinant lysostaphin expressed in *E. coli* used in DNA extraction kits (Klesius and Schuhardt, 1968). Studies on lysostaphin clinical applications show that lysostaphin kills the pathogenic Staphylococcal strains while having no toxicity on the cells or affecting the gut microbiome. The bacteriocin is digested by the intestinal proteases, maintains activity in the serum when combined with polyethylene glycol (Williamson *et al.*, 1994, Walsh *et al.*, 2003, Schaffner *et al.*, 1967).

The work presented in this thesis has provided a new insight into the interaction between members of *Actinomyces* and *H. pylori.* The evidence provided in this thesis can be used as a foundation for new investigations aimed at finding alternative approaches to tackle *H. pylori* infection.

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