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THE PRODUCTION OF N-NITROSAMINES BY GUT BACTERIA

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A dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfillment for the degree of Master of Science in Medicine.

Johannesburg, 2020

Declaration

I **Madaliso Phakathi** declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Masters of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

M.Phakathi

(Signature of candidate)

On the 13th day of July 2020

Dedication

I would like to dedicate this body of work to my support structures; to my parents who support everything I do even if it seems out of reach. To my friends, there are a lot of you but mainly to Mukundi Masakona, Nkazimulo Mnguni and Rixile Mahlaule; a special place in my heart hates you for annoying me to keep writing when all I wanted was a holiday. To my sister who was forced to stay up with me and listen to my venting, and to my colleagues who were always willing to help when I was in need.

Lastly, I would like to dedicate this to the dreamers. To those who keep pushing even when it seems bleak. Your dreams are valid!

Abstract

Cancer is one of the leading causes of morbidity and mortality worldwide. Gastrointestinal cancers have increased in prevalence. Studies have shown that cancer risk increases with exposure to carcinogenic compounds which can be found in the environment and in food. Nitrosamines are an example of such carcinogens, with *in vivo* studies in animal models demonstrating exposure to nitrosamines results in the development of particular cancers and the associations between nitrosamine exposure and cancers in humans. Nitrosamines are formed from nitrites, a common food preservative, and amines. It is now realized that micro-organisms may facilitate the production of nitrosamine compounds, with the gut microbiome playing a significant role.

We therefore investigated whether or not the gastric inhabiting bacterium associated with gastric cancer, *Helicobacter pylori* alone, or *Bacillus cereus* and the combination of these, were able to produce nitrosamines. Strains of *H. pylori* and a reference strain of *B. cereus* were cultured in the presence of precursors to nitrosamine compound formation, nitrites, nitrates, a secondary amine alone, or in combination, in a standard reaction assay. Samples were extracted and analyzed for nitrosamine formation using gas chromatography with detection and identification by mass spectrometry.

Although particular nitrosamine compounds were only produced by the individual bacteria, the important finding was that many more nitrosamines were also produced, and some at significantly elevated concentrations, by culturing *H. pylori* in supernatant of *B. cereus*. Many of these nitrosamines have been implicated in cancer formation.

Cancer formation has been attributed to many different factors and the production of nitrosamines through microbial actions, specifically the combination of *H. pylori* and *B. cereus*, may be of relevance in gastrointestinal cancers. Further studies, including testing supernatants in animal models are required to confirm the findings of this study.

Acknowledgements

I would like to acknowledge and thank my supervisor, Prof Geoffrey Candy, for his support and patience throughout the duration of this project. Your guidance and wisdom will always be highly appreciated. I would also like to thank Professor Karl Rumbold for his advice concerning this project. A special thanks to my colleagues; Anza Thiba, Sarhana Dinat and Hannah Mullah for their advice and support when I was completely lost. But most importantly for the laughs, you guys made this journey a breeze. To James Ayojedi, thank you for imparting all your *Helicobacter* knowledge on me. Lastly, to Eric Morifi, Refilwe Moepa and the Wits university department of Chemistry, thank you for your assistance, and for allowing us to use your facilities for sample analysis, this project would not have come to completion without you guys.

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List of abbreviations

B. cereus	*Bacillus cereus*
BC	*Bacillus cereus*
BHI	Brain Heart Infusion
Cag	Cytotoxin associated gene
CBA	Colombia Blood Agar
DPA	Diphenylamine
E. coli	*Escherichia coli*
FBS	Fetal Bovine Serum
FDA	Food and drug administration
GC-MS	Gas Chromatography Mass spectrometry
GIT	Gastrointestinal tract
H. pylori	*Helicobacter pylori*
HP	*Helicobacter pylori*
m/z	mass to charge ratio
MS	Mass Spectrometry
NICD	National Institute for Communicable Diseases
NO	Nitric Oxide
NOS	Nitric Oxide synthase
P. aeruginosa	*Pseudornonas aeruginosa*
P. denitrificans	*Paracoccus denitrificans*
P. morganii	*Proteus morganii*

PDAC	Pancreatic Ductal Adenocarcinoma
Ppb	Parts per billion
Ppm	Parts per million
S. aureus	*Staphylococcus aureus*
Spp	Species
NO_2^-	Nitrite
NO_3^-	Nitrate

Chapter 1

Introduction

1 Literature review

1.1 Cancer

Cancer has been ranked as one of the leading causes of mortality globally, especially in people under the age of 71. Cancer incidence continues to be on the rise. In 2018 alone, it was estimated that globally; there were 17.1 million new cases of cancer reported (Bray *et al*., 2018). Additionally, the rise in cancer incidence has been attributed, like many other diseases, to the growth of the population and improved diagnosis.

Cancer incidence increases based on genetics, life style choices which include smoking and drinking, as well as environmental factors or the interaction of these (Khan *et al.,* 2010). With genetics playing a role in cancer formation; there is a higher prevalence of cancer related deaths in men compared to women (Bray *et al*., 2018).

Substances that cause or exacerbate the formation of cancer are known as carcinogens (Alberts *et al*., 2002). Well known carcinogens are found in cigarettes, the environment and in foods such as processed meats, and from industrial chemicals and processes (Jarvholm *et al*., 1986). As such, cancer risk increases with exposure to carcinogenic agents. Nitrosamines are an example of such carcinogens. These are compounds that are mostly found in foods and can be formed through microbial reactions in the gut. Such production requires the presence of a nitric oxide (NO) precursor and an amine (IARC, 1998).

Gastrointestinal cancers appear to be increasingly common, as such, gastric cancer has been ranked the second leading cause of cancer related deaths (Parkin *et al*., 1999; Wroblewski *et al*., 2010; Van Cutsem *et al*., 2016). Gastric cancer is the leading cause of morbidity and mortality in especially in the Southern African region. It was estimated that gastric cancer accounted for approximately 12% of all cancer deaths in 2003 (Kelley and Duggan, 2003). The incidence has since increased. In 2018, gastric cancer was found to be responsible for over 100 000 new cases, and was estimated to be responsible for 783 000 deaths globally (Bray *et al*., 2018).

Prognosis of gastric cancer is poor with only 1 in 5 patients being able to survive more than 5 years after diagnosis (Hooi *et al*., 2017). Additionally, patients with early stage gastric cancer can be asymptomatic, making it more difficult to diagnose in the early stages (Van Cutsem *et al*.,

2016). Treatment of gastric cancer requires surgical resection, which is determined by the size, location and tumor stage (Van Cutsem *et al*., 2016).

Western Asia has the highest number of gastric cancer cases and the highest mortality rate due to the cancer (Bray *et al*., 2018). Gastric cancer incidence rates differ in migrants, as rates among first generation Japanese migrants in Hawaii are seen to be lower than those among Japanese people living in Japan (Bray *et al*., 2018). This highlights the role that a change in lifestyle, diet and geography may have on cancer incidence.

Gastric cancer is an example of a cancer that is caused by bacterial infection (Schwabe and Jobin, 2013). The presence of bacteria along with secondary amines and nitrite or nitrate has been found to result in nitrosamine formation (Hill *et al*., 1973)

1.2 Nitrosamines

The risk of getting cancer increases with exposure to carcinogenic agents. Studies have identified several carcinogens in foods such as pasta, milk, meat, and fish (Park *et al*., 2015). Nitrosamines are, as such, toxic compounds found in food and have been proposed to have a carcinogenic role in gastric cancer. They are a group of N-nitroso compounds which are formed by chemical or microbial reactions between secondary amines and naturally occurring NO precursors such as nitrates and nitrites (Figure 1.1), known as nitrosating agents (IARC, 1998). Nitrosamines may be found in the environment and water (Grebel *et al*., 2006), other food products such as meat (De Mey *et al*., 2017) or in foods preserved with nitrites (Wolff and Wasserman, 1972) Alternatively, these nitrosating agents can be produced endogenously in saliva, in gastric juice and by gut bacteria (Hwang *et al*., 1994). Nitrosamines consist of an amine ($-NR_2$) as well as an N-nitroso functional group (-N-N=O), giving rise to various N-nitroso compounds that may either be volatile or non-volatile. As such, nitrosamines have a molecular formula of $(R)_2N-N=O$, where R is any aryl or an alkyl group (Figure 1.2). Nitrosamines are considered to be powerful mutagens as prolonged exposure can lead to the formation of tumors (De Mey *et al*., 2017). There are five main type of nitrosamines, namely; N-nitrosodiethylamine, N-nitrosodibutylamine, N-nitrosopiperidine, N-nitrosopyrrolidine and N-nitrosomorpholine (Figure 1.3). All of which have been identified as possible carcinogens for humans.

Nitrosamine compounds have been associated with different types of cancers. These include; esophageal, stomach, pancreatic and colon cancers (Liteplo *et al*., 2002; McCreery and Balmin,2017). Eighty percent of all the N-nitroso compounds that were known in 1976, were tested in many animal species and were shown to induce tumors (Neurath *et al*., 1976). However, many other nitrosamine compounds that have since been discovered have not been investigated.

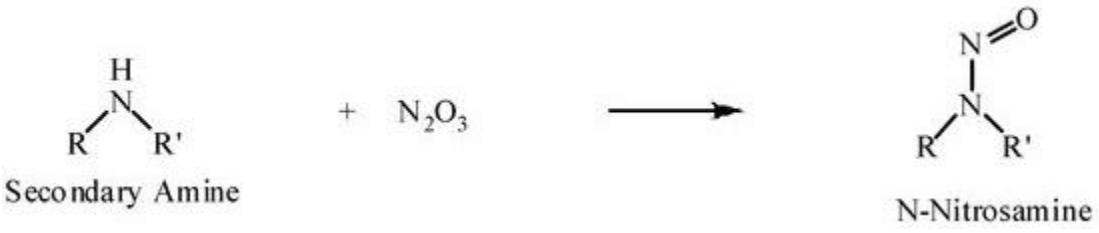


Figure 1.1: The reaction of a secondary amine with dinitrogen trioxide to form a nitrosamine (Lewis et al., 2013)

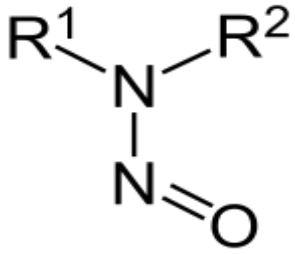
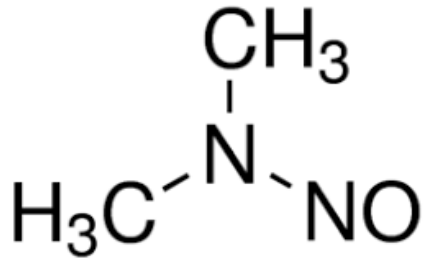
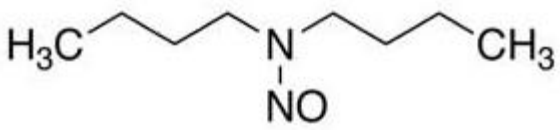
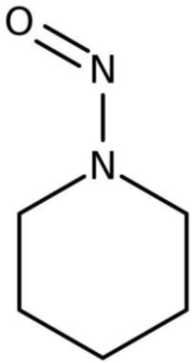
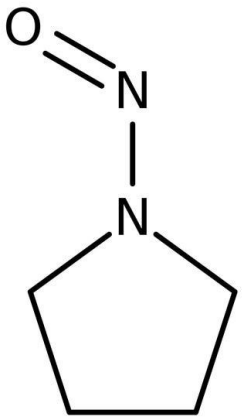


Figure 1.2: The molecular formula of nitrosamines. (Barrington, 2011)

1. 

2. 

3. 

4. 

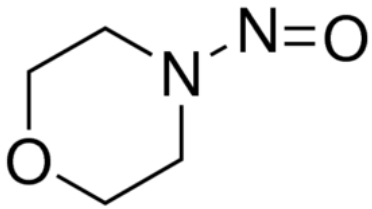
5. 

Figure 1.3: Chemical structure of the 5 main nitrosamines; 1) N-nitrosodiethylamine, 2) N-nitrosodibutylamine, 3) N-nitrosopiperidine, 4) N-nitrosopyrrolidine and 5) N-nitrosomorpholine (Kadmi *et al*., 2014)

1.2.1 Amines and nitrites in the natural environment

Primary amines have the chemical formula R-NH2 while secondary amines have the formula R-2NH. Secondary amines are considered to be weaker acids when comparing to alcohols. Amines have been found in plants and wine and the most prominent amines found in nature are dimethylamine and diethylamide (Neurath *et al*, 1976). Additionally, piperidine and pyrrolidine have been found on plant derived materials. N-nitroso pyrrolidine occurs in many food products however the composition of amines in the human environment is not fully known (Neurath *et al*., 1976).

Neurath and colleagues (1976) set out to find amines in different food products. Their study detected 40 primary and secondary amines. These amines contained different chromatographic properties in samples from fresh vegetables, pickles, and fish along with bread, cheese, and water. Twenty-one amines were identified by mass spectrometry to be secondary amines which as we know, are precursors for the production of carcinogenic nitrosamines (IARC, 1998). The most prominent secondary amines identified were pyrrolidine, piperidine, N-methylbenzylamine, N-methylaniline and N-methylphenethylamine. Moreover, the highest number of amines found were in red radishes and spinach (Neurath *et al*., 1976)

Dimethylamine is a naturally occurring amine with the chemical formula $(CH_3)_2NH$. It is found in animals, plants and in food. Dimethylamine is a precursor to industry compounds and reacts with carbon disulphate to produce dimethyl dithiocarbamate (Fay *et al*., 1997). Some studies have shown that most of N-nitroso compounds found in the body are produced from *in vivo* conversion (Lin, 1986), as such, dimethylamine has been found to be present in human urine following the ingestion of fish (Fay *et al*., 1997).

Nitrates are natural compounds and are found in plant substances such as fruit and vegetables (Wolff and Wasserman, 1972). This is mainly because fertilizers used to grow such plants are rich in nitrates. Another common source of nitrates is water. Nitrates can be reduced to nitrites under different conditions such as the storage of foods, which can be toxic. The biggest concern is the interaction of amines with nitrites which may be chemically or metabolically activated (Wolff and Wasserman, 1972). Such reactions yield N-nitroso compounds, which are known to be carcinogenic.

1.2.2 Spectroscopic properties of amines

Ultraviolet absorptions of saturated amines occur at short wavelengths (220nm) and have been considered to be useful for identification.

1.2.3 Tumorigenesis by nitrosamines and cytochrome P450

Nitrosamines play a pivotal role in cancer formation as they have been linked to tumorigenesis by the activation by the P450 cytochrome enzyme. Cytochrome P450 hydroxylates the carbon adjacent to the N-nitroso group to yield an α-hydroxynitrosamine (Tricker and Preussmann, 1991). Thereafter an alkyldiazohydroxide is formed by the cleavage of the carbon-nitrogen bond resulting in the spontaneous elimination of an aldehyde. This then leads to the production of alkyl diazonium ions by the decomposition of alkyldiazohydroxide. These ions are the ultimate carcinogens that bind to and react at the nucleophilic sites of numerous cellular compounds. The cancer is thus initiated as a consequence of DNA alkylation by dialkylnitrosamines resulting in a range of modified bases, of which the most important is the formation of O^6-alkylguanine and O^4-alkylthymine. These particular modified bases lead to the inclusion of non-complementary bases during polyribonucleotide or polydeoxyribonucleotide synthesis (Tricker and Preussmann, 1991).

Nitrosamines have been frequently used to induce cancers in animal models (McCreery and Balmin 2017) (Table 1.1), as they have been identified as strong mutagens (Lijinsky, 1999). Alternatively, nitrosamine compounds can also be formed in the gut by a reaction of a nitrate with a secondary amine. This has been demonstrated *in vivo* in rats (Alan *et al*., 1971) whose poor gastric acid production permits a flora similar to that in achlorhydric man (Forsythe *et al*., 1988). Nitrosamine formation has been demonstrated by different bacteria (Hawksworth and Hill, 1971; Fong and Chan, 1973; Calmels *et al*., 1988), however, we are unaware of studies that have found nitrosamine production by specifically *H. pylori* and *B. cereus*.

Table 1.1: Common nitrosamines and the cancers they have been found to induce in animal models. Compiled from Act *et al*. (2011)

Name	Method of administration	Cancer caused	Organism of study
N-Methyl-N'-nitro-N-itrosoguanidine (MNNG)	Added to drinking water, stomach tube or intraperitoneal injection or intrarectal instillation	Forestomach and glandular stomach Large intestine Small intestine Liver	Rats
	Drinking water	Forestomach	Male hamsters
	Drinking water	Forestomach and glandular stomach	Dogs
N-Nitrosodi-n-butylmine	Oral exposure Or subcutaneous injection	Urinary bladder tumors (papilloma or squamous carcinoma)	Mice Rats Hamsters Guinea pigs Rabbits
	Oral or prenatal exposure	Tumor in respiratory tract	Hamsters
	Oral exposure	Tumor of the upper digestive tract (pharynx, esophagus, forestomach)	Rats Hamsters

N-Nitrosodiethanolamine (NDELA)	Oral (drinking water)	Liver cancer (hepatocellular carcinoma) Kidney tumors(adenoma)	Rats
	Subcutaneous injection	Nasal cavity(adenocarcinoma) Trachea (papilloma Liver (hepatocellular adenoma))	Hamsters
N-Nitrosodiethylamine	Intraperitoneal injection	Liver	Mice Rats Hamsters
	Intramuscular injection	Liver tumor	Birds
	Oral administration	Kidney Upper digestive tract	Mice Rats Hamsters Dogs
	Oral administration Intravenous Prenatal administration	Kidney tumors	Rats

N-Nitrosodimethylamine	Oral administration	Liver Bile duct	Rats Mice Hamsters Rabbits Guinea pigs Fish
	Inhalation	Respiratory tract Lung tumor	Mice Rats
	Oral administration Inhalation Intraperitoneal injection	Kidney tumor	Rats
N-Nitrosodi-n-propylamine	Oral (drinking water)	Liver cancer Malignant and benign tumors of the esophagus	Rats
	Subcutaneous injection	Lung tumors	Rats Hamsters
	Intraperitoneal injection	Liver tumor	Monkeys
N-Nitroso-n-ethylurea	Oral administration	Tumors of the nervous system (Brain, spinal cord, peripheral nerves)	Rats Mice

			Hamsters Rabbits
	Intraperitoneal injection	Brain tumors	Mice
	Intravenous injection	Tumors of the brain and peripheral nerves	Rats
	Prenatal exposure	Benign tumors Leukemia Liver Endocrine glands	Mice
	Oral administration	Leukemia	Rats
	Intravenous injection	Kidney Ovary Uterus Vagina	Rats
		Ovary Uterus Bone Skin Blood vessels	Monkeys

4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1- butanone	Subcutaneous injection	Benign/Malignant nasal cavity tumor Lung cancer Liver Blood vessels	Rats
N-Nitroso-N-methylurea	Prenatal exposure	Tumors of the nervous system Kidney tumors Benign tumors of the lungs Liver	Rats
	Injection exposure	Digestive tract tumors	Rats Hamsters
N-Nitrosomethylvinylamine	Oral exposure (drinking water)	Tongue Pharynx Benign and malignant tumors of the esophagus	Rats
N-Nitrosonornicotine	Oral exposure (drinking water)	Cancer of the nasal cavity Esophageal tumors	Rats
	Subcutaneous injection	Tracheal tumors	Hamsters
	Intraperitoneal injection	Benign lung tumors	Mice

	Stomach tube Oral exposure (drinking water) Intraperitoneal injection	Nasal tumors	Hamsters
N-Nitrosopiperidine	Oral exposure (drinking water) Intraperitoneal injection	Benign lung tumor	Mice
	Oral administration	Upper digestive tract	Mice
	Oral exposure (drinking water)	Benign/malignant upper respiratory tract tumors	Hamsters
	Oral exposure (drinking water) Subcutaneous injection Intravenous injection	Benign /malignant liver tumor	Mice
	Intraperitoneal injection	Liver cancer	Monkeys
N-Nitrosopyrrolidine	Oral administration	Liver cancer	Rats
	Oral exposure (drinking water)	Liver cancer	Hamsters
N-Nitrososarcosine	Oral administration	Cancer of the nasal cavity	Mice
	Oral exposure (drinking water)	Benign/Malignant tumors of the esophagus	Rats
	Intraperitoneal injection	Liver cancer	Newborn mice

1.3 Gut Microbiota

The bacteria of the human gastrointestinal tract (GIT) are important for the healthy maintenance of the gut and ensuring correct physiological functioning of the human body (Zhang *et al*., 2015). It is estimated that the human gut is home to more than 10^4 species, and a total number of 10^{14} microorganisms, this number exceeds that of human cells. There are six main phyla found in the gut and they are: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobio* and *Fusobacteria* (Zhang *et al*., 2015). *Bacteroidetes* and *Firmicutes* contribute to over 90% of the overall gut microbiota (Qin *et al*., 2010). Bacteria can be commensal and play different beneficial roles; from aiding in digestion, to promoting immune functions. Additionally, commensal bacteria can aid in barrier protection and may play a role in preventing pathogen entry into the mucosal tissues (Zhang *et al*., 2015).

Commensal bacteria colonize the human gut from birth and have been estimated to exceed 1000 species (Qin *et al*., 2010; Zhang *et al*., 2015). The microbial makeup of the gut changes with age as the microbiome make up of infants is different to that of adults. This change occurs rapidly and the gut makeup of an adult is established by the time a child is a year old (Qin *et al*., 2010; Zhang *et al*., 2015). However, not all bacteria can survive in the human gut, therefore, the community of bacteria in the human gut is determined by diet, the gastric environment including the pH, temperature as well as the overall health of the human, taking into account the use of certain medications such as antibiotics (Rowland *et al*., 2018).

Not all gut bacteria are beneficial for the human body as others are pathogenic; causing chronic diseases including inflammatory bowel disease, obesity and most importantly, in the context of this research, cancer. An imbalance (dysbiosis) in bacteria composition can also cause intestinal symptoms including bloating, abdominal pain and diarrhea. Evidently, the microbiome make-up differs among healthy people when compared to cancer patients (Zhang *et al*., 2015). Cancer can also be associated with gut bacteria, these cancers include gastrointestinal cancer, prostate cancer, pancreatic cancer and gastric cancer. It is suggested that gut bacteria may activate macrophages to produce chromosome breaking factors which further lead to cancer formation (Zhang *et al*., 2015). One such bacteria is *Helicobacter pylori* (*H. pylori*), this is a human pathogen that has been identified to play a role in cancer formation (Kelley and Duggan, 2003; Bornschein and Malfertheiner, 2014).

1.4 The microbiome and cancer

Clinical evidence has shown that the microbiota is likely to play a role in cancer formation. The proposed method of action is through inflammation and epithelial damage (Schwabe and Jobin, 2013), also termed barrier failure. The most common example of barrier failure is ulcerative colitis, this defect in the intestinal barrier may cause disease development and then increase the risk of cancer formation (Schwabe and Jobin, 2013). It is estimated that 99% of microbiota are located in the gastrointestinal tract (Schwabe and Jobin, 2013) which is one of the areas most affected by cancer. Additionally, cancer can be formed through the release of carcinogenic molecules by these microbial organisms, through the production of tumor promoting metabolites (Schwabe and Jobin, 2013).

Cancer caused by microorganisms is formed when mechanisms that regulate human physiological pathways are disturbed (Schwabe and Jobin, 2013). It has been seen that eradication of bacteria in mice species decreases the development of cancer, on the other hand, it has also been seen that bacteria have tumor promoting effects (Dapito *et al*., 2012). This would then highlight the role that the microbiome may play on cancer formation.

Gastric cancer is the most common example of a cancer that is caused by bacterial infection (Schwabe and Jobin, 2013). The bacterium *H. pylori* has been identified to be the main risk factor for stomach cancer and is responsible for almost 90% of all new stomach cancer cases (Kelley and Duggan, 2003; Bray *et al*., 2018). It is however not very clear how the bacteria may cause or influence the cancer formation, but we believe that the bacteria may play a role in mediating the formation of carcinogenic compounds.

1.5 *Helicobacter Pylori*

H. pylori is a Gram-negative, microaerophilic, gastric pathogen that has been found to be a major cause of chronic inflammatory gastritis and peptic ulcer (Taylor and Blaser, 1991; Wroblewski *et al*., 2010). It was first identified as a cause of peptic ulcer in 1983, and chronic infection has been associated with the increased risk of gastric adenocarcinoma (Wroblewski *et al*., 2010). The bacterium has the ability to inhabit and infect the gastrointestinal system, more specifically the epithelial lining of the stomach (Hooi *et al*., 2017).

H. pylori has also been identified as a bacterium that is the strongest risk factor for pancreatic and gall cancers and as a result, *H. pylori* has been classified as a Group I carcinogen and it is considered a necessary but insufficient cause of gastric cancer. This has earned *H. pylori* some growing attention over the years (Wroblewski and Peek, 2013).

1.5.1 Prevalence of *H. pylori* infection

Globally, *H. pylori* infections have become less prevalent. This is due to improved sanitation, standards of living and methods of treatments (Hooi *et al*., 2017). This, however, only applies to developed countries (Hooi *et al*., 2017). As such, developing countries with decreased socioeconomic statuses bear the burden of *H. pylori* infection (Salih, 2009; Hooi *et al*., 2017). Even with the decreased infection rates, *H. pylori* has been found to colonize more than 50% of the world population (Parssonnet *et al*.,1991; Wang *et al*., 2017), however, infection differs geographically with developing countries having higher incidences in both cancer and bacterial infection (Taylor and Blaser, 1991; Salih, 2009).

H. pylori infection has always been a public health problem. In 2015, it was estimated that globally, 4.4 billion people were infected with the bacterium (Hooi *et al*., 2017). A study by Hooi and colleagues (2017) which analyzed a total of 184 papers found that from the period of 1 January 1970 to 1 January 2016, Africa had the highest estimate of *H. pylori* infections with a prevalence of 79.1%. The country with the highest incidence was Nigeria with approximately 87.7%. The United states on the other hand only had a prevalence of 35.6%.

There are differences in *H. pylori* prevalence within the same country. These differences may be attributed to differences in race. In a study by Everhart *et al*. (2000), they looked at the Hispanic population in the Unites States. They found that *H. pylori* infection was higher in Hispanic (61.6%) and non-Hispanic blacks (52.7%). Infection in non-Hispanic white individuals ranged from 18.4%-26.2% (Everhart *et al*., 2000). It is however unclear to what extent the differences in prevalence are ascribed to socioeconomic factors, or other factors such as genetics and race.

The geographical difference in *H. pylori* infection has been attributed to the rates of acquisition of the infection with improved hygiene playing a role in decreasing the rates of infection. In this

regard, the burden that *H. pylori* infections have on the health system is highlighted, and eradication strategies need to be improved.

1.5.2 Pathogenesis of *H. pylori*

H. pylori infection usually occurs in early childhood and if not detected, can continue throughout an individual's adulthood. Most people infected with *H. pylori* show no clinical symptoms or peptic ulcerations during the early stages of infection, and infection can thus go unnoticed (Salih, 2009). The differences in symptom manifestation among individuals may be explained by the variety of *H. pylori* species which are constantly evolving.

H. pylori has various pathogenic factors. It produces urease; which hydrolyzes urea into carbon dioxide and ammonia. This decreases the pH in the stomach and allows the bacteria to thrive in the acidic gastric environment. Additionally, *H. pylori* have flagella; tail-like structures that have been adapted for mobility and to assist in the colonization (Suerbaum and Michetti, 2002). Among the range of *H. pylori* strains, the majority of the strains express the 95-kD vacuolating cytotoxin (Vac); VacA, which is a secreted exotoxin (Suerbaum and Michetti, 2002). The toxin inserts itself into the human epithelial-cell membrane and forms a hexameric anion-selective, voltage dependent channel. The bacteria use this channel to release bicarbonate and other organic anions. It has been suggested that this may provide some nutrients for the bacteria (Suerbaum and Michetti, 2002). Having multiple mechanisms of action, the VacA cytotoxin also targets the mitochondrial membrane and causes the release of cytochrome-c and induces apoptosis.

Some *H. pylori* strains contain the cytotoxin associated gene (Cag) pathogenicity island. CagA possessing strains are associated with cancer (Yamaoka *et al*., 2008), and the Cag pathogenicity island is a marker for infection associated with inflammation caused by *H. pylori* (Graham and Yamaoka, 1998). It is suggested that the CagA pathogenicity island may cause cancer by increasing the levels of inflammation. Increased inflammation results in the mucosa being more susceptible to acid and thus resulting in duodenal ulcers and the development of atrophic gastritis. Furthermore, the risk of developing gastric cancer also increases with the development of multifocal atrophic gastritis and intestinal metaplasia (Graham and Yamaoka, 1998).

H. pylori has been adapted to the gastric mucosal environment and is thus able to enter the mucosal barrier, swim and attach to epithelial cells. It is able to evade the human immune

response and thus thrive in the GIT. Furthermore, the *H. pylori* genome has been found to change during chronic infection (Suerbaum and Michetti, 2002). This is done by introducing pieces of foreign DNA from other *H. pylori* strains during infection, allowing the bacterium to thrive in infecting the host.

H. pylori causes chronic gastric inflammation in the host (Suerbaum and Michetti, 2002). The infection by the bacterium recruits' neutrophils and then T and B lymphocytes and macrophages, and causes epithelial cell damage. *H. pylori* binds to class II major-histocompatibility-complex that are on the surface of gastric epithelial cells. This results in the apoptotic activity of the cells (Suerbaum and Michetti, 2002). People who have been infected by *H. pylori* show increased levels of interleukin-1B, interleukin-2, interleikin-6, interleukin-8 and tumor necrosis factor, these are pro inflammatory cytokines responsible for mediating immune responses. This further leads to host tissue damage.

In a study that reviewed the epidemiology of *H. pylori* infection, it was found that early childhood is the most common period of infection and transmission (Goh *et al*., 2011). There are several methods of transmission as far as *H. pylori* is concerned, the first being direct transmission. An individual can ingest the bacterium via oral-oral, gastro-oral or fecal-oral pathways (Khalifa *et al*., 2010). It has been suggested that *H. pylori* can also be an oral inhabitor, as a result, it has been suggested that *H. pylori* can be transmitted orally from one person to another. An example would be the premastication of food by mothers before feeding an infant (Khalifa *et al*., 2010). Another method of transmission is the gastro-oral route. It is suggested that the bacteria may be able to move from the gut to the mouth through gastric reflux of stomach contents. Lastly; fecal-oral transmission, this has been suggested as *H. pylori* successfully been identified in human fecal matter. *H. pylori* can also be transmitted indirectly through unclean drinking water, contaminated food that is not adequately prepared or dairy products such as milk (Go, 2002).

1.5.3 *H. pylori* infection and cancer

H. pylori strains all differ due to mutations, this causes differences in cancer risk depending on the *H. pylori* strain that is present (Peek and Blaser, 2002). Geographical differences of *H. pylori* related cancer incidence have been found to be dependent on the strain of *H. pylori* prevalent in that specific area (Yamaoka *et al*., 2008) with the CagA positive strain being the most cancer associated strain. These strains contain the Cag pathogenicity island. This a gene that encodes an immunodominant protein, it however has an unknown function (Akopyants *et al*., 1998). The presence of the Cag pathogenicity island has been correlated with the severity of *H. pylori* associated pathologies such as peptic ulcer and gastric cancer (Nilsson, *et al.*, 2003). Cag positive strains of *H. pylori* are suggested to provoke an inflammatory response that could potentially be damaging to the host tissue. This inflammation results in the synthesis of the pro inflammatory cytokine IL-8 and this has been seen on gastric biopsies and cultured cells (Crabtree *et al*., 1994). Additionally, *H. pylori* induces a signaling cascade involving calcium-calmodulin and extracellular signal regulated kinase that leads to the activation of the transcriptional regulate NF-kb which activates interleukin-8 production (Nozawa *et al.*, 2002; Tegtmeyer *et al.*, 2011).

The Cag pathogenicity island facilitates the injection of bacterial proteins into eukaryotic cells. *H. pylori* attaches to cells and delivers the pathogenicity island encoded proteins into the host cells (Guillemin *et al*., 2002). CagA has been identified to alter the actin cytoskeleton of cells, however, molecular events that lead to this alteration of the actin cytoskeleton are not known (Guillemin *et al*., 2002). It is proposed that *H. pylori* adheres to cells in the proximity of the apical-junction complex. Therefore, CagA interferes with the assembly of functional tight junctions and alters the composition of junction protein complexes (Amieva *et al*., 2003).

South African strains of *H. pylori* do not have CagA (Hooi *et al*., 2017). This is contradicting to results by Idowu *et al* (2019) who found that found that 62% of their study population had the CagA virulence gene. The inconsistency in CagA information on a South African context may explain why even though Africa has the largest prevalence of *H. pylori* infection, the gastric cancer incidence is not as high as other countries. It has been identified that CagA positive

strains have certain genes upstream of CagA that are absent in CagA negative strains. These genes have been found to be essential in the induction of interleukin 8 (Akopyants *et al*., 1998).

H. pylori's mechanism of action is also believed to include the increased susceptibility to bacterial overgrowth, which then results in the increased bacterial conversion of dietary nitrates into carcinogens (Schwabe and Jobin, 2013). According to Wei and colleagues (2019), *H. pylori* cannot be detected in pancreatic juice by PCR, therefore, this suggests that *H. pylori* cannot trigger pancreatic carcinogenesis directly, instead, it indirectly acts by causing inflammation and immune escape (Wei *et al*., 2019)

A study by Uemura and colleagues (2001) looked at individuals with a range of ulcer conditions to identify if the ulcers were caused by *H. pylori*. They used endoscopy for identification. The results showed that out of 1526 patients, 1246 patients were positive for *H. pylori* infection, which is 82%. In follow up procedures, they found that gastric cancer developed in 36 of the 1246 patients and in none of the *H. pylori* negative patients. Similar results were found by Parssonnet and colleagues (1991) who followed a cohort of 128992 people between the mid-1960s. Eighty-four percent of these had previously been infected with *H. pylori*. From this study, only 186 cases of gastric carcinoma were reported.

It has been proposed that in the stomach, *H. pylori's* ability to activate the macrophage system through the L-arginine/NO pathway, may lead to increased endogenous NO formation. Oxidation of NO forms nitrosating compounds and these species are converted to the nitrosating agents; dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4), which may interact with amines and nitrates to produce nitrosamines or may directly damage DNA.
Co-infection of *H. pylori* with other bacteria can cause tumor development (Goh *et al*., 2011). *H. pylori* produces ammonia through its urease-mediated activity by degrading urea (van Vliet *et al*., 2003), and because not much is known about *H. pylori'*s ability to directly synthesize NO, this project hypothesized that a symbiotic relationship with other bacteria such as *Bacillus cereus (B. cereus)* would yield the production of NO, and subsequently produce nitrosamine compounds.

1.6 *Bacillus cereus*

B. cereus is a Gram-positive, spore forming bacteria that is able to grow in both aerobic and anaerobic environments (Granum and Lund, 1997; Majed *et al*., 2016). It can be found in air, soil and water (Carey *et al*., 2017). It has been associated with food poisoning, especially caused by rice (Granum and Lund, 1997; Carey *et al*., 2017). Additionally, it has been found to play a role in human infections of the eyes, central nervous system and respiratory tract (Bottone, 2010)

Food poisoning caused by *B. cereus* is induced in two ways: one being the diarrheal type which is caused by enterotoxins and this is caused in the vegetative growth of *B. cereus*, and two-the emetic type which is caused when the emetic toxin is produced by growing cells in the food. *B. cere*us grows after the heating and cooling of food as heat causes spore germination. *B. cereus* produces an emetic toxin and three different enterotoxins (Granum and Lund, 1997). The emetic toxin is a ring-shaped structure of three repeats of four amino acids and they play a role in food poisoning.

Food poisoning caused by *B. cereus* is considered mild as it usually lasts for less than 24 hours. Additionally, *B. cereus* caused diseases have also been found to differ due to geographical regions, where Japan has been found to have the emetic type while Europe and North America have been reported to have the diarrheal type of infections (Granum and Lund, 1997). *B. cereus* has been found to be infectious if doses range from $5x10^4$ to 10^{11} colony forming units.

1.6.1 Prevalence of *B. cereus* infection

B. cereus is known to predominantly be found in rice. In this regard, Kim *et al*. (2009) found a 95% prevalence of *B. cereus* in rice samples. Other studies have found that *B. cereus* prevalence in samples such as soil, feces and vegetables ranges from 10-88% (Hassan and Nabbut, 1995). In a study that examined 100 normal and 50 diarrheal feces samples, results showed that 6% of the diarrheal feces and 7% of the normal human feces were positive for *B. cereus* (Hassan and Nabbut, 1995). The same study found 100% *B. cereus* in soil samples tested, 60% in dehydrated milk and 68% in rice. *B. cereus* is therefore widely available in the everyday food we consume.

1.6.2 Pathogenesis of *B. cereus*

B. cereus is an opportunistic pathogen, it mostly targets individuals who are immunocompromised. It has also been associated with several diseases such as metabolic disorders and malignant diseases. The natural environmental reservoir for *B. cereus* includes fresh and marine waters as well as vegetables (Bottone, 2010) which aids in the colonization of the human GIT when these products are consumed (Bottone, 2010). *B. cereus* spores germinate once they have come into contact with organic matter or are within a host. However, *B. cereus* also has a saprophytic life cycle, this means that the bacterial spores germinate in soil and are able to survive in this environment until the bacteria is taken up by another host. When growing in soil, *B. cereus* switches from a single cell to a multicellular phenotype which allows it to translocate through the soil (Bottone, 2010). This bacterium can easily be ingested in small numbers and then become part of the transitory human intestinal flora. *B. cereus* infection is characterized by tissue destructive exoenzyme production which include hemolysins, phospholipases, emesis inducing toxin and pore forming enterotoxins (Bottone, 2010). Although *B. cereus* infection has increased, it is still considered to be a contaminant when isolated from clinical specimens as it is widely available in soil, dust, water and the hospital environment; on the equipment used.

1.6.3 *B. cereus* infection and cancer

To our knowledge, there have been no studies that explicitly correlate *B. cereus* to cancer formation. This does not mean *B. cereus* may not play a role in perhaps the activation or exacerbation of any cancer. Additionally, bacterial nitric oxide synthase (NOS) has been found to present in many Gram-positive bacterial species such as *B. cereus* (Tsuji *et al*., 1996). The NO production is believed to select mutant p53 cells and contribute to tumor angiogenesis (Weiming *et al*., 2002). Much like other gram-positive bacteria, *B. cereus* has NO synthase activity (Adak *et al*., 2002).

1.7 Nitric oxide synthase activity

Nitric oxide is an important chemical within the human body, it acts as a signaling molecule, and also plays a role in blood pressure regulation in humans. A range of bacteria are capable of producing NO from different pathways, genomic sequencing has identified prokaryotic genes with high sequence similarity to the oxygenase domains (NOSox) of mammalian NOS. These NOSox-like proteins have been found in Gram-positive bacteria (Sudhamsu and Crane, 2009).

In bacteria however, NOS activity has been associated with pathogenicity. NOS are heme-based monooxygenases that oxidize arginine to NO (Sudhamsu and Crane, 2009). NOS activity has been found to be present in insects, parasites, fungi and bacteria among other organisms. Their amino acid sequences have also been found to be similar to mammalian NOSs (Adak *et al*., 2002). NOS in mammals have been known to catalyze the oxidation of L-arginine to citrulline and NO. Additionally, NO is also responsible for the dispersion of bacterial biofilms (Caranto, 2019). NOS activity is important in bacteria as NO generated by NOS have been shown to increase bacterial resistance against antibiotics (Gusarov *et al*., 2009), thus allowing the bacteria to survive.

1.8 Rationale for study

Gastric cancer is one of the leading causes of morbidity and mortality in Southern Africa. The bacterium *H. pylori* has been associated with the increase in gastric cancer cases. Similarly, it is well known that carcinogens, such as N-nitrosamines, cause cancer. However, it is not known how these nitrosamines are formed in the human GIT. This study set to investigate the role that bacterial organisms such as *H. pylori* and *B. cereus* play in the formation of nitrosamines and subsequently; to understand how gastric cancer can be activated or exacerbated by these factors.

1.9 Aim

The aim of the study was to determine if nitrites and nitrates are converted to N-nitrosamines by *H. pylori* and *B. cereus* alone, and when the two bacteria are in combination.

1.10 Objectives

- To culture *H. pylori* and *B. cereus* bacteria, separately and in combination, on agar and in liquid cultures.
- To assay *H. pylori, B. cereus* and a combination of both for nitrosation (to mimic what could possibly happen in the gut).
- To extract, detect and identify the possible nitrosamines formed using GC-MS.

Chapter 2: Materials and Methods

2.1 Ethics

An ethics waiver was obtained to use the *H. pylori* and *B. cereus* clinical isolates. The waiver was obtained through the Human Research Ethics Committee of the University of the Witwatersrand (Ref: W-CBP-200221-02).

2.2 Materials

Tables B1-B3(Appendix B) show the materials used in this experiment. These include consumables, software and equipment.

2.3 Bacteria Culture

H. pylori cultures grown included a) reference strains (J99 and B8) from the DSMZ German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany);
and b) *H. pylori* strains (6202C, 623C, and 538C). The latter had previously been isolated under an unrelated project from clinical patients at the Chris Hani Baragwanath Academic Hospital (Ethical clearance number: Rec-270710-028-RA level 01 from University of Fort Hare Eastern Cape) and under Clearance M160228 (Chris Hani Baragwanath Academic Hospital). The bacterial samples were cultured on Columbia Blood Agar (CBA) plates, and these CBA plates were prepared using 39g/l of CBA powder as per instructions by the manufacturer. CBA was the agar of choice as *H. pylori* grows well on CBA compared to other agar media (Andersen and Wadstrom, 2001). The CBA was supplemented with 5% Fetal Bovine Serum (FBS), as well as culture media supplement (Vitolex, Becton Dickinson) and antibiotics (Skirrows supplement-vancomycin, trimethoprim and polymyxin B) which selects for *B. cereus* and pathogenic *Campylobacter* microorganisms including *H. pylori*.

After streaking the plates with the bacteria, plates were incubated under micro-aerobic conditions (85% N_2, 10% CO_2, 5% O_2) for 24 hours at 37°C; using an anaerobic jar and a gas pack (MicroAero-Aeropack NGC). After incubation, isolated colonies from each of the strains were

inoculated onto 37g/l Brain Heart Infusion (BHI) powder, supplemented with the antibiotic cocktail (Skirrows supplement – vancomycin, trimethoprim and polymyxin B) to eliminate competing non-*Helicobacter species (spp)*. The BHI was also enriched with vitamin mix and 7% FBS. These supplements were used to ensure the effective growth of *H. pylori* and *B. cereus*.

The liquid cultures were then incubated under micro aerobic conditions again for 24 hours, with rotation of 100rpm.

To prepare the samples that contained the combination of bacteria; the *B. cereus* liquid cultures were centrifuged at 4500 rpm for 15 minutes. Thereafter, the supernatants of these liquid cultures (2ml) were added into the *H. pylori* (reference and clinical strains) liquid cultures. These cultures were allowed to incubate for an additional 24 hours before undergoing the nitrosation assay.

2.4 Biochemical tests

Following growth of the bacteria, biochemical tests were run including the urease test, catalase test and the Gram test for bacterial identification.

The urease test was used for as it is widely used for *H. pylori* identification. *H. pylori* has an active urease enzyme which catalyzes the conversion of urea to ammonia and CO_2. The urea broth was made according to the instructions from Brink (2010). The broth was then distributed into different test tubes (10 ml) and thereafter inoculated with the respective bacteria colonies. The test tubes were then incubated at 37°C and were monitored every 5 minutes for a color change. Ammonia produced raises the pH of the medium and the phenol red pH indicator changes from yellow (negative) to red (positive).

The second test used was the catalase test. Catalase is an enzyme produced by microorganisms that is used to neutralize toxic forms of oxygen metabolites such as H_2O_2. Catalase breaks down H_2O_2 into H_2O and O_2. Adding the bacteria sample in a hydrogen peroxide solution creates O_2 bubbles. The different bacterial colonies were smeared onto different slides, thereafter, a drop of hydrogen peroxide solution (3%) was added onto each smear. The slides were then observed for bubble formation.

The Gram stain test was used to determine whether the bacteria cultured was Gram-positive or gam-negative. The Gram stain is used to identify the peptidoglycan type that is present in the bacteria. A thick peptidoglycan is present in Gram-positive bacteria and allows bacteria to take up dye. In contrast, Gram-negative bacteria have a thinner wall and are unable to retain color.

For this test, a colony of bacteria was smeared onto a slide and spread to the size of a small coin. Thereafter, the smears were fixed onto the slides by gently waving them over a light flame. This was done to ensure that the bacteria were not lost during rinsing. After fixation, crystal violet stain was added onto each slide and allowed to sit for a minute. The stain was then poured off and rinsed with water to wash off the excess dye. This was followed by the addition of iodine, which was again allowed to sit for a minute and then poured off the slide. Ethanol was then used to assist in the decolorization of the samples. Lastly, safarin was added to the slides, this acted as a counterstain. The slides were then viewed under a microscope, Gram-positive bacteria were expected to look purple, while Gram-negative bacteria were expected to have a pink color.

Table 2.1: Interpretation of biochemical tests

Biochemical Test	**Positive results (+)**	**Negative results (-)**
Urease test	Red	Yellow
Catalase test	Bubble formation	No bubbles formed
Gram stain	Purple/violet color	Pink color

2.5 Matrix-Assisted laser desorption/ionization-Time of flight Mass Spectrometry (MALDI-TOF MS) identification of *H. pylori*/ *B. cereus*

MALDI-TOF MS allows for rapid identification of microorganisms based on the mass spectral pattern of bacterial proteins. Microorganisms including bacteria, fungi and yeast can be identified by comparison of the mass spectra of their proteins with a reference database (Zhou *et al*., 2017). MALDI-TOF MS was therefore used to confirm the identity of bacterial growth and was undertaken at the National Institute for Communicable Diseases (NICD; Sandringham Campus, Johannesburg).

To prepare for the MALDI-TOF MS analysis, pure colonies of both the *H. pylori* and *B. cereus* strains were isolated using Loop last® inoculation loops and were then washed with 70% ethanol. Forty microliters of 70% formic acid and pure acetone were added in a 1:1 (v/v) ratio to the bacterial pellet. The mixture was then vortexed for 30 seconds and thereafter centrifuged on a micro-centrifuge for 2 minutes at 1300 rpm. The supernatant was transferred into new Eppendorf tubes and spotted and smeared in duplicate on a 96-well stainless steel MALDI target plate (Bruker, MSP 96 target ground steel BC #8280799) and allowed to dry at room temperature to dry. To reconstitute the matrix (HCCA), a α-cyano-4- hydroxycinnamic acid (CHCA) (5 mg/ml) in a 50:48:2 acetonitrile (ACN): water: trifluoroacetic acid (TFA) matrix solution (1 µl) was overlaid on the spots and colony smears and left at room temperature to dry. Once dry, the target plate was placed in the MALDI-TOF MS for identification.

2.6 Nitrosation Assay

After identifying of the bacteria, a resting cell assay was used to assay the bacteria for nitrosation (Calmels *et al*., 1988).

Briefly; individual bacterial strains were cultured as described in Section 2.3 for 24 hours in a suspension (50 ml) in Erlenmeyer flasks. Two ml of the culture supernatants were added to a reaction mixture to generate nitrosamine containing 0.1M Tris-HCL buffer (pH ~7.2), 0.15g of

$NaNO_2$, 0.15g of $NaNO_3$ and 0.05g of diphenylamine in a total of 8ml. These reaction mixtures were then incubated at 37°C with shaking (100 rpm) for 1 hour in sealed Falcon tubes. To stop the reaction, 1% (w/v) NaOH (1ml) was added to the mixture.

Negative controls included the complete assay mixture and

a) saline solution (NaCl 0.9% w/v) in place of the bacterial suspension;
b) without $NaNO_2$;
c) without $NaNO_3$;
d) without diphenylamine;
e) only $NaNO_2$;
f) only $NaNO_3$;
g) only diphenylamine.

This is summarized in Table 2.2., and the experimental assay scheme is depicted in Figure 2.1. These controls were introduced to ensure that the nitrosamines formed were formed from the bacteria and not from chemical reactions caused by the interaction of the reaction mixture.

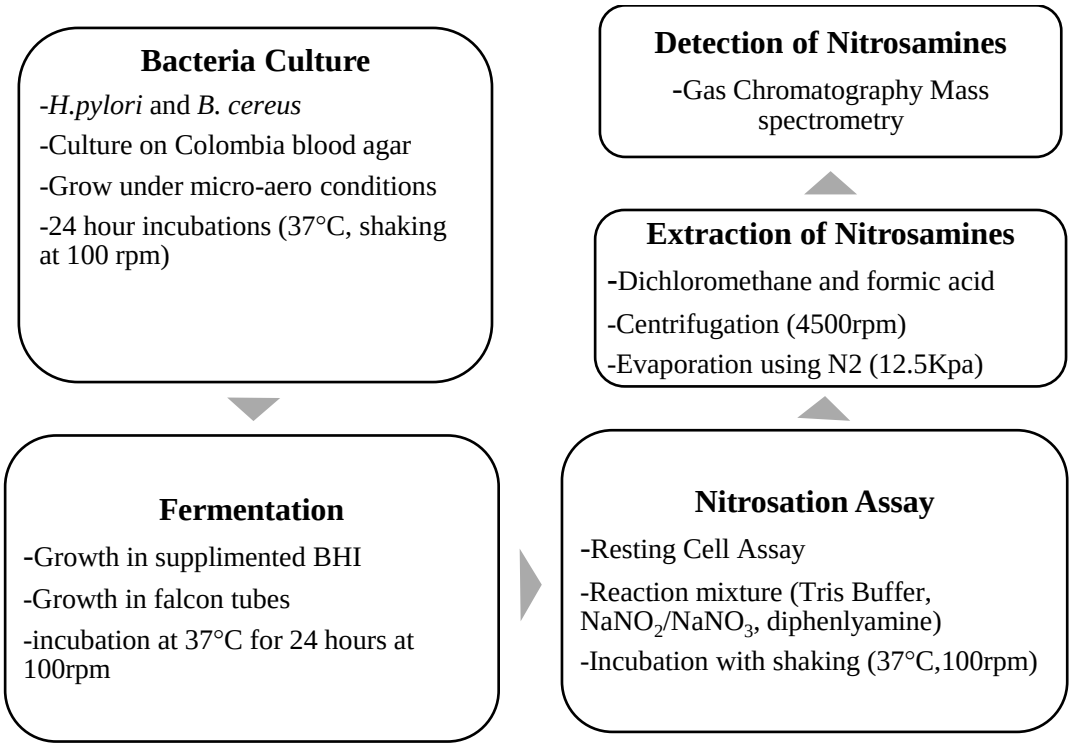


Figure 2.1 Schematic diagram of the experimental assay to determine the production of nitrosamines by *B. cereus* and *H. pylori*.

Table 2.2. A summary of the conditions used

Condition	No bacteria	*H. pylori* (6202C)	*H. pylori (623C)*	*H. pylori (538C)*	*H. pylori (B8)*	*H. pylori (J99)*	B. *cereus*	*H. pylori* + *B. cereus*
$NaNO_2$	X	X	X	X	X	X	X	X
$NaNO_3$	X	X	X	X	X	X	X	X
Diphenylamine	X	X	X	X	X	X	X	X
$NaNO_2$+Diphenylamine	X	X	X	X	X	X	X	X
$NaNO_3$+Diphenylamine	X	X	X	X	X	X	X	X
$NaNO_2$+$NaNO_3$+Diphenylamine	X	X	X	X	X	X	X	X

2.7 Extraction of nitrosamines

To extract nitrosamines from cultures, 2.5ml of the mixtures from section 2.6 (above) were added to 7.5ml of acetonitrile in Falcon tubes. Thereafter, 750µl of formic acid (1% of the volume of acetonitrile) was added to each Falcon tube and vortexed until a homogeneous mixture formed. These mixed solutions were transferred to 3 clean 2ml microtubes and were then centrifuged at 4500 rpm for 10 minutes. The supernatants were transferred to 3 clean 2ml micro tubes and frozen at -80˚C for 15 minutes, thereafter, the extracts were defrosted and centrifuged again at 4500 rpm for 10 minutes. The supernatants were transferred to 3 clean 2ml micro tubes. The acetonitrile in the samples was evaporated to a volume of 0.25ml by applying a gentle flow of N_2 (~12.5Kpa) at room temperature in a fume hood. The remaining volumes were adjusted to 1 ml using acetone and stored at -80℃ until they are ready to be analyzed (Calmels *et al*., 1988).

2.8 Nitrosamine standard calibration

A nitrosamine standard (EPA 8270/Appendix IX Nitrosamines Mix, Sigma) was diluted in acetone to a concentration of 100ppb. One-hundred micro liters of the diluted nitrosamine standard was added to each of the final samples from section 2.7 (above) to ensure that our GC-MS detection was reliable and accurate. Serial dilutions of the standard were also prepared, these dilutions included concentrations of 100ppb, 250ppb, 500ppb, 600ppb, 700ppb, 800ppb, 900ppb, 1000ppb, 1250ppb, 1500ppb and 2000ppb. These dilutions were used to create a standard calibration curve (Figure 3.3). The calibration curves were used to determine the concentrations of each analyte in the samples.

2.9 Detection of nitrosamines

Gas Chromatography Mass Spectrometry (GC-MS) was used to detect nitrosamines using the parameters described in Table 2.3, adapted from Chen *et al.* (2012). The P*egasus 4D GCxGC-TOFMS* instrument from the Department of Chemistry (University of the Witwatersrand was used for analysis. This method was appropriate for separating small and volatile molecules in complex mixtures by gas chromatography with targeting and identifying these molecules by mass spectrometry.

Volatile molecules in the sample were volatized and separated by a capillary column packed with a stationary (solid) phase with the gas chromatograph (Agilent 7890B). The volatile analytes were carried by helium carrier gas and eluted from the column at different retention times. Once eluted, these were ionized by the mass spectrometer using electron ionization. Ionized molecules were then accelerated through the instrument's mass analyzer. Ion fragments were separated according to their different mass-to-charge (*m/z)* ratios. A mass range of 45 to 300Da was used with the signal-noise ratio being set at 5 (Chen *et al*., 2012).

The peak areas of the analytes in the chromatogram would be related to the standard curves to calculate concentrations of the nitrosamines from each bacteria strain.

Table 2.3. The experimental conditions used on the GC-MS instrument, autosampler as well as the system, adapted from ThermoScientific (Chen *et al*., 2012).

GC-MS instrument:	
iC injector module	Split/splitless
Injector temperature	250°C
Injection mode	Splitless
Surge mode	300Kpa
Splitless time	1.0min
Analytical column	TG-WAX MS (30m×0.25mm×0.5µm)
Carrier gas	He (99.999% purity)
Flow rate	1.0 mL/min, constant flow
Oven program	45°C for 3min 25°C/min to 130°C 12°C/min to 230°C, 1min hold
Transfer line temperature	250°C
Total analyses time	15min
Total cycle time	17min
Autosampler:	
Injection volume	1µl
Solvent	Acetone
Standard runs	2 replicate of injections each
GC-MS system:	
Ionisation mode	EI
Source temperature	220°C

Mass resolution setting	Normal
Scan mode	MRM mode
Nitrosamine standard:	
Dilutions of standard mix	100ppb, 250ppb, 500ppb, 600ppb, 700ppb, 800ppb, 900ppb, 1000ppb, 1250ppb, 1500ppb and 2000ppb
Nitrosamine standard	EPA 8270/Appendix IX Nitrosamines Mix, Sigma added to each calibration level at 100ppb

2.10 Data analysis

The mass fragmentation of each component peak was determined as a function of their *m/z* ratios. These were compared, and matched to mass spectra of different nitrosamine compounds in database libraries to identify and name the nitrosamine. Nitrosamines produced in the absence and presence of the bacteria, nitrogen oxides (nitrite, nitrate and the combination, in the presence of diphenylamine) were listed. Nitrosamines not in the Sigma standard were also listed

Average peak areas of each nitrosamine in the commercial standard were used to determine the concentrations of the nitrosamine. Average nitrosamine concentrations produced by individual bacteria with a) nitrate OR nitrite (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the complete reference nitrosamine assay); and d) as c) with *B. cereus* AND the *H. pylori* strain (BC+HP) were reported as mean±std in graphs. See experimental section for details. Statistical differences between the nitrosamine concentrations produced by the complete reference nitrosamine assay and other reaction conditions and the *B. cereus* with the *H. pylori* strain were determined using two-sided difference of means test (Statistica Version 13.5).

Chapter 3: Results

3.1 Biochemical tests and MALDI-TOF MS identification of bacteria

Biochemical tests were used to verify the characteristics of the bacteria that was cultured. These tests included the catalase test, urease test as well as the Gram staining. Table 3.1 below describes results (positive or negative) obtained from these tests. It was found that *H. pylori* was positive for the urease, catalase and oxidase tests. *B. cereus* was negative for the urease and positive for the catalase test. This confirmed the culture Gram-negative (*H. pylori*) and Gram-positive (*B. cereus*) bacteria.

Table 3.1: Results of the biochemical tests on each bacteria type.

Bacteria	**Urease test**	**Catalase test**	**Gram stain**
B. cereus	-	+	+
H. pylori (6202C)	+	+	-
H. pylori (623C)	+	+	-
H. pylori (538C)	+	+	-
H. pylori (B8)	+	+	-
H. pylori (J99)	+	+	-

+ represents that the results were positive and – represents that the results were negative

In addition to the biochemical tests, the cultured bacteria strains were taken to the NICD for identification where *H. pylori spp*. were identified by MS.

3.2 Calibration curves for nitrosamines

A hundred micro liters (100ppb) of a nitrosamine standard (EPA 8270/Appendix IX Nitrosamines Mix) obtained from Sigma was added to each sample. The nitrosamine standards contained nine nitrosamine analytes as listed in Table 3.2. Figure 3.1 shows the chromatogram of the nitrosamine standard diluted in acetone. The chromatogram was obtained using a nitrosamine concentration of 2000ppm.

The nitrosamine standard was run as a blank to ensure that all analytes could be detected by the GC-MS machine. Indeed, all nine nitrosamines were detected by the GC-MS instrument. This is visible in Figure 3.1, where all nine analytes are seen as peaks. Figure 3.2 shows the chromatograph of one of the samples. All nine standard analytes are seen, along with additional peaks and compounds added during sample preparation.

Table 3.2: Nine analytes and the names identified by the GC-MS library recovered from standards. The analytes are shown in the order in which they eluted as seen in Figure 3.1.

Nitrosamine name	**Retention times (minutes)**	**Nitrosamine name from GC-MS library**
N-Nitrosodimethylamine	5:20	N-Nitrosodimethylamine
N-nitrosomethylethylamine	5:40	Ethanamine, N-methyl-N-nitroso
N-Nitrosodiethylamine	5:50	Ethanamine, N-ethyl-N-nitroso
N-nitrosodi-n-propylamine	6:50	1-Propanamine, N-nitroso-N-propyl
1-Nitrosopyrrolidine	8:00	Pyrrolidine,1-nitroso
Nitrosomorpholine	8:14	Morpholine,4-Nitroso
1-Nitrosopiperidine	8:25	Piperidine,1-nitroso
N-Nitrosobutylamine	8:42	1-Butanamine, N-butyl-N-nitroso
N-Nitrosophenylamine	12:40	N-Nitrosodiphenylamine

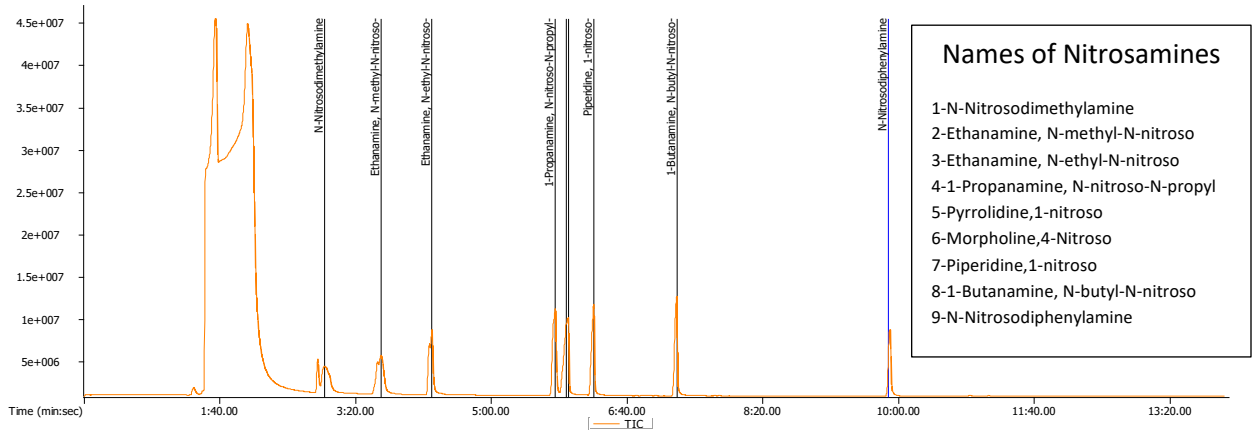


Figure 3.1: Chromatograph of the nitrosamine standard (2000ppm). The nitrosamine analytes are shown at each major peak and named in the legend. The first peak represents acetone; the solvent used to dilute the standard and the samples. y axis unit; peak intensity, x axis units; time (100 second intervals).

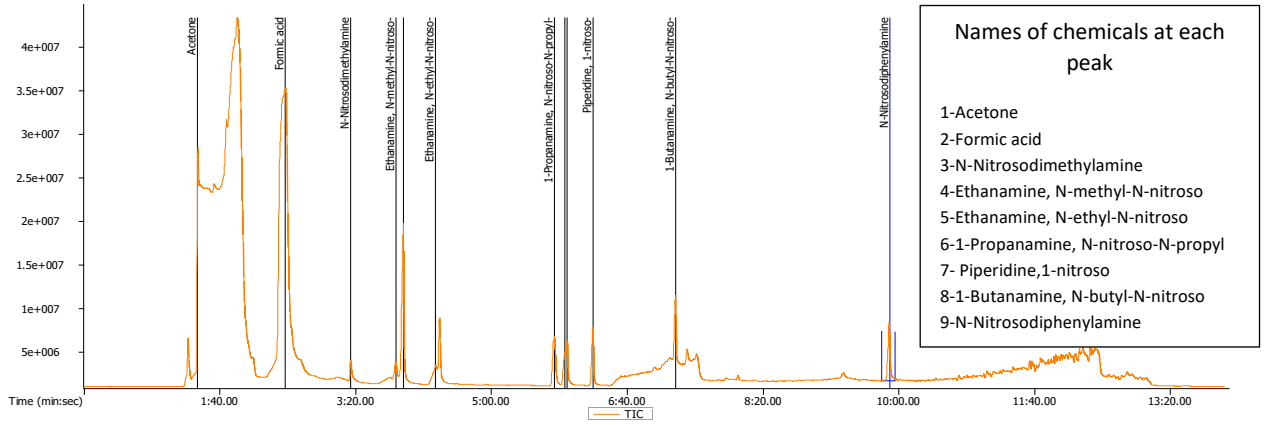


Figure 3.2: Chromatograph of a typical sample spiked with 1ppm nitrosamine standard. More peaks are seen due to the availability of more compounds used during sample preparation. The chemicals at each peak are named in the legend. y axis unit; peak intensity, x axis units; time (100 second intervals)

The nitrosamine standard was used to determine concentration calibrations and to create calibration curves. Some concentrations outside the calibration line were excluded from the

calibration process. Figure 3.3 shows an example of the calibration curve of the analyte N-nitrosodimethylamine. Each of the 9 analytes within the standard had a calibration curve generated (See Appendices).

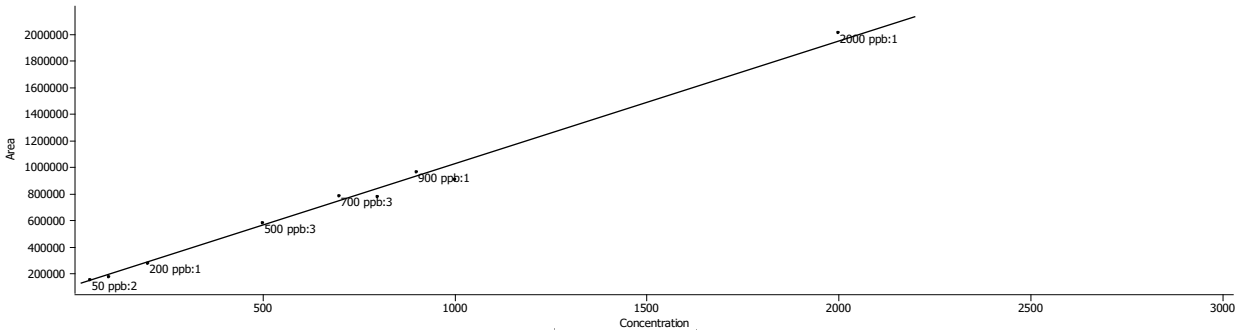


Figure 3.3: Calibration curve of the analyte N-nitrosodimethylamine. Curve shows the different concentrations used to obtain the curve. y=918.06x+97267.5, r=0.99599.

After calibration with the nitrosamine standard, the individual concentrations of the analytes were determined from eighteen control samples containing no bacteria.

3.3 Identification of Nitrosamines using GC-MS

The samples were spiked with the nitrosamine standard at 100ppb (Figure 3.2) and the analytes of the standard were identified in these samples using the conditions described. However, new nitrosamines were also formed. A number of nitrosamines were detected in most of the samples, including controls that contained no bacteria. These nitrosamines were assumed to result from non-biochemical reaction between the nitrite/nitrate and secondary amine precursors. Table 3.3 shows a list of the nitrosamines detected in the control samples that contained no bacteria. These were subsequently assumed to have not been formed from any bacteria.

Table 3.3: A list of common reoccurring nitrosamines found in control samples without bacteria and nitrosamine standard (see Table 2.2).

Name of nitrosamine
(+-)-N-Benzyl-alpha-methyl-N-nitrosobenzylamine
1-Butanamine, N-ethyl-N-nitroso-
1H-Azepine, hexahydro-1-nitroso-
1-Propanamine, N,2-dimethyl-N-nitroso-
1-Propanamine, N-ethyl-N-nitroso-
1-Propanamine, N-methyl-N-nitroso-
2-Propanamine, N-(1-methylethyl)-N-nitroso-
2-Propanamine, N-ethyl-N-nitroso-
2-Propanamine, N-methyl-N-nitroso-
3-Piperidinol, 1-nitroso-
4-Piperidinol, 1-nitroso-
Glycine, N-methyl-N-nitroso-
Methanamine, N-methyl-N-nitro-
Methane, nitroso-
N-Isopentyl-N-nitroso-pentylamine
N-Nitroso-2-methyl-oxazolidine
1,4-Dinitrosopiperazine
Piperazine, 1-nitroso-
Pyrimidine-2,4(1H,3H)-dione, 5-amino-6-nitroso-
Urea, N-methyl-N-nitroso-

Table 3.4 shows the nitrosamines formed by each bacterium after the exclusion of the commonly reoccurring nitrosamines in the controls. These nitrosamines were assumed to be formed by the bacteria present in the sample. The combination of *H. pylori* and *B. cereus* yielded the most nitrosamine production, eluding to the fact that the two bacteria may have a synergistic relationship with respect to nitrosamine production. These reactions may be of importance in cancer formation.

Table 3.4: Nitrosamines identified from *B. cereus* and *H. pylori* samples

Nitrosamines	*B. cereus*	*H. pylori* 620C	*H. pylori* 623C	*H. pylori* 538C	*H. pylori* B8	*H. pylori* J99	*H. pylori* +*B. cereus*	*No standard*
11-Acetyl-N-nitrosohemanthidine							■	
1H-Azepine, hexahydro-1-nitroso-				■				
Benzaldehyde, 2-nitroso-							■	
Benzene, nitroso-							■	
Benzenemethanamine, N-nitroso-N-phenyl-							■	■
2-Butanol, 2-nitroso-, acetate (ester)				■				
Carbamic acid, methylnitroso-, ethyl ester	■			■			■	■
Carbamic acid, nitrosopropyl-, ethyl ester								■
1H-1,4-Diazepine, hexahydro-1,4-dinitroso-	■						■	
3,5-Dimethyl-4-nitrosomorpholine						■		
1,4-Dinitrosopiperazine					■			■
Dipentylamine, N-nitroso-			■					
Ethanol, 2,2'-(nitrosoimino)bis-					■		■	
Ethylnitrosocarbamic acid ethyl ester	■	■	■	■	■		■	■
Hexahydro-1,3,5-trinitroso-1,3,5-triazine			■			■	■	
N-Isopentyl-N-nitroso-pentylamine							■	
Methanamine, N-methoxy-N-nitroso-				■			■	
3-Methyl-1-nitrosopiperidine			■			■		
3-Piperidinol, 1-nitroso-oxazolidine							■	
3-Thiophenamine, tetrahydro-N-methyl-N-nitroso-, 1,1-dioxide								■
4-Nitrosothiomorpholine	■							
4-Nitrosothiomorpholineoline, 4-nitroso-							■	
4-Piperidinol, 1-nitroso-ine, 1-nitroso-					■			
N,N'-Bis(2-methyl-2-nitrosopentan-4-one)			■					
N-methyl-N-nitroso-1-pentanamine	■						■	■
N-Methyl-N-nitroso-1-phenylmethanamine								
1-Nitrosoazetidine							■	
N-Nitroso-2-(1-phenyl-2-propyl)aminopropionitrile							■	
N-Nitroso-2-methyl-oxazolidine			■					
N-Nitroso-2-methyl-Piperidine, 2,6-dimethyl-1-nitroso-							■	
N-Nitroso-2-methylthiazolidine							■	
Piperaz-3-Piperidinol, 1-nitroso-					■			
Piperazine, 1-nCarbamic acid, ethylnitroso-, ethyl esteritroso-				■				
Piperidine, 2,6-dimethyl-1-nitroso-							■	
Piperidine, 2-methyl-1-nitroso-								■
Piperidine, 4-methyl-1-nitroso-	■						■	■
Pyrimidine-2,4(1H,3H)-dione, 5-amino-6-nitroso-							■	
2-Pyrrolidinone					■			
Urea, N-ethyl-N-nitroso-							■	
Urea, trimethylnitroso-	■				■			

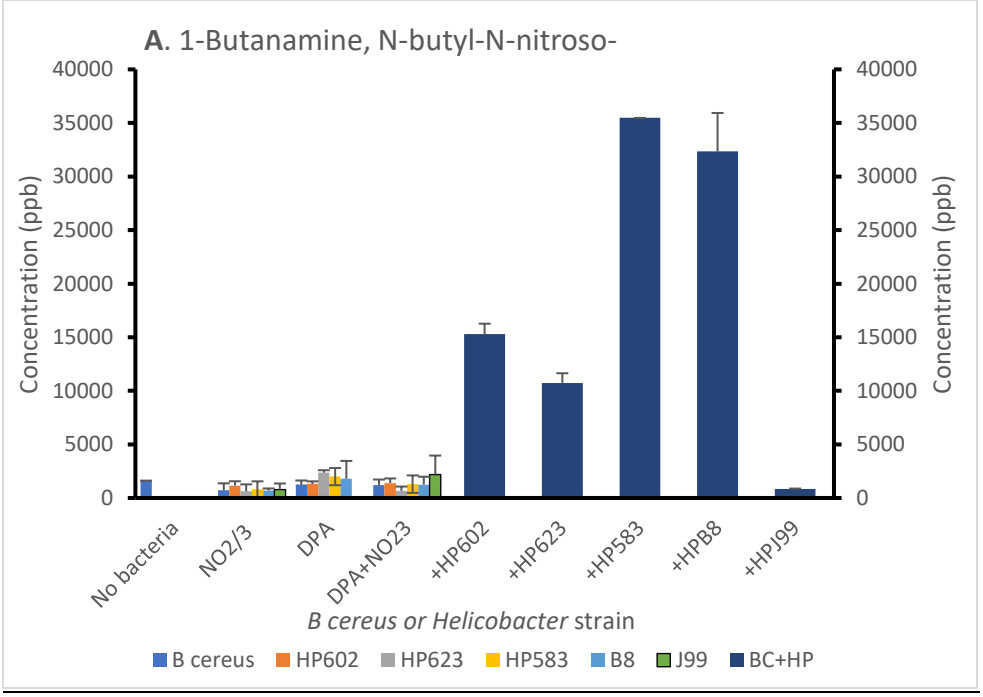


Figure 3.4A: Production N-butyl-N-nitroso- 1-butanamine by individual bacteria with (left half of the graph) a) nitrate OR nitrite (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5A (below).

Table 3.7A. Statistical analysis of differences (p-values) in Fig 3.4A (above) between N-butyl-N-nitroso-1-butanamine concentrations formed in the nitrosamine assay (DPA+NO2/3) by *B. cereus* or *H. pylori* strains alone (DPA+NO2/3), compared to reactants a) nitrate OR nitrite (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

DPA+NO23 assay vs:	***B. cereus***	**HP602**	**HP623**	**HP583**	**B8**	**J99**
DPA+NO23	1	1	1	1	1	1
No bacteria	nd	nd	nd	nd	nd	nd
NO2/3	0.38	0.55	0.94	0.06	0.39	0.19
DPA	0.88	0.95	**0.0135**	0.12	0.64	nd
DPA+NO2/3	0.92	0.71	nd	nd	nd	0.26
BC+HP602	**<0.00001**	**<0.00001**	**0.0001**	**0.0002**	**<0.0001**	**0.0026**
BC+HP623	**0.0002**	**<0.0001**	**0.0004**	**0.0005**	**0.0002**	**0.0133**
BC+HP583	nd	nd	nd	nd	nd	nd
BC+B8	**<0.00001**	**<0.0001**	**<0.0001**	**0.0005**	**<0.0001**	**0.0002**
BC+J99	0.62	0.07	0.57	0.51	0.51	0.31

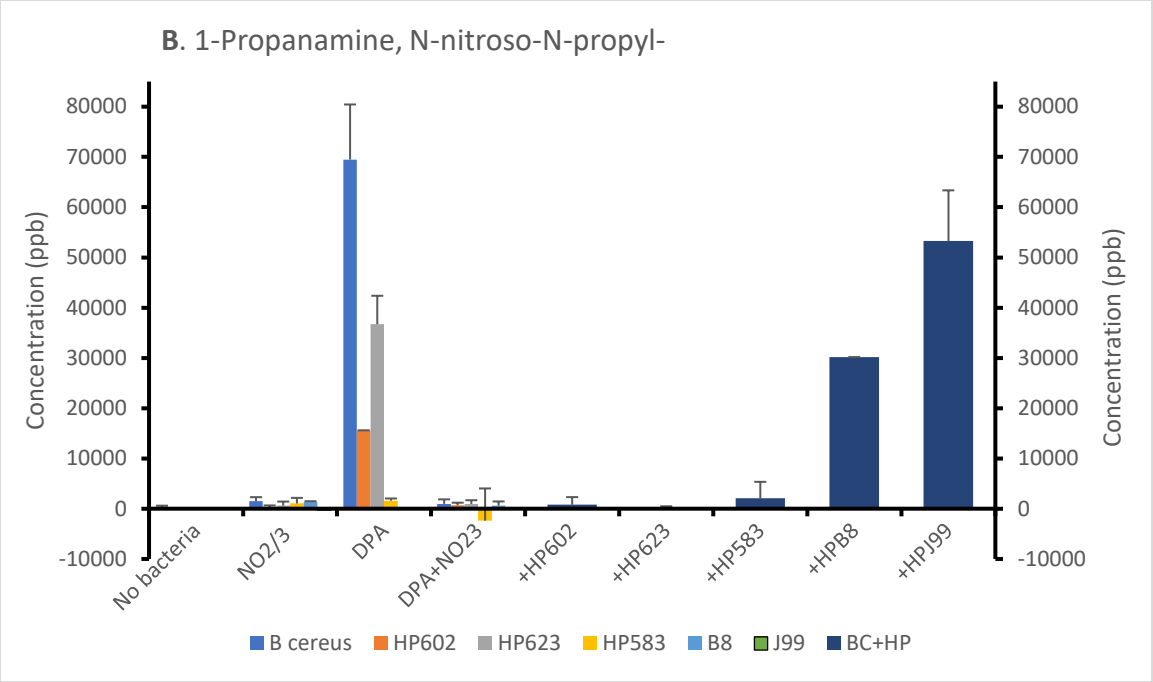


Figure 3.4B: Production N-nitroso-N-propyl-1-propanamine, by individual bacteria with (left half of the graph) a) nitrate OR nitrate (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5B (below).

Table 3.5B. Statistical analysis of differences (p-values) in Fig 3.4B (above) between N-nitroso-N-propyl-1-propanamine concentrations formed in the nitrosamine assay (DPA+NO23) by *B. cereus* or *H. pylori* strains alone (DPA+NO23), compared to reactants a) nitrite OR nitrate (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

DPA+NO23 assay vs:	***B cereus***	**HP602**	**HP623**	**HP583**	**B8**	**J99**
DPA+NO2+3	1	1	1	1	1	1
No bacteria	**0.0172**	**0.0076**	0.25	0.33	0.47	0.20
NO23	0.67	**0.007**	0.62	0.11	0.48	**0.0046**
DPA	**0.0006**	nd	**0.0013**	0.24	0.34	nd
DPA+NO2/3	**0.0165**	**0.0473**	nd	0.22	0.72	0.53
BC+HP602	0.28	0.44	0.88	0.29	0.97	0.85
BC+HP623	**0.0142**	**0.0028**	0.26	0.33	0.48	0.15
BC+HP583	0.83	0.65	0.59	0.23	0.46	0.47
BC+B8	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
BC+J99	**0.0003**	**0.0003**	**0.0023**	**0.0012**	**0.0003**	**0.0022**

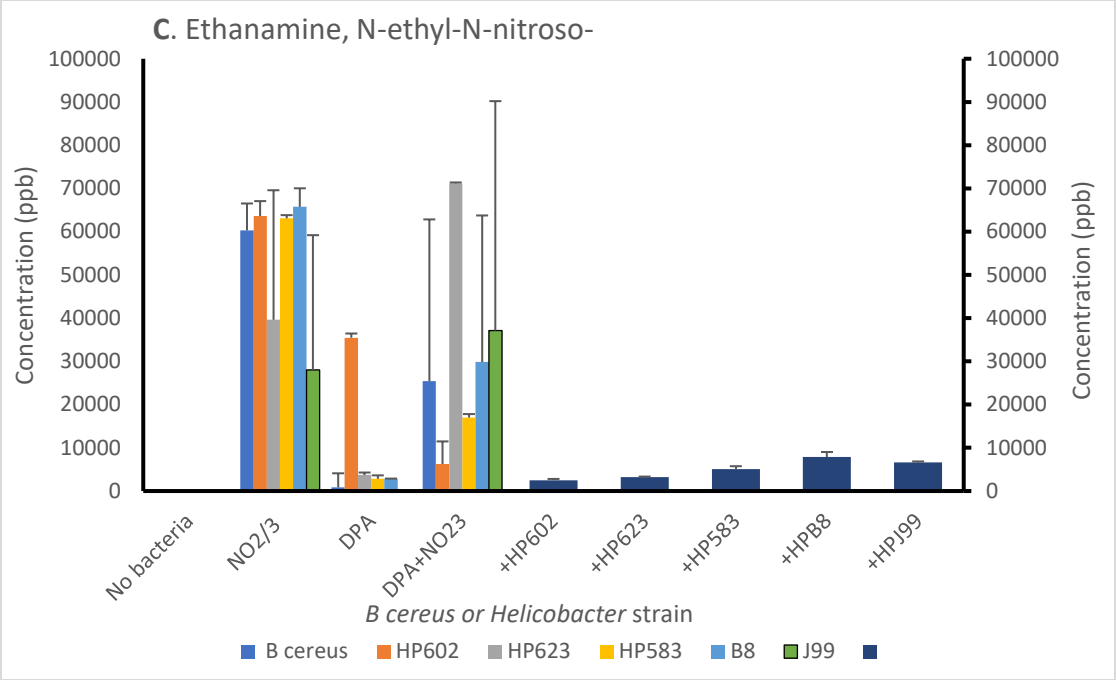


Figure 3.4C: Production N-ethyl-N-nitroso-ethanamine by individual bacteria with (left half of the graph) a) nitrate OR nitrate (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5C (below).

Table 3.5C. Statistical analysis of differences (p-values) in Fig 3.4C (above) between N-ethyl-N-nitroso-ethanamine concentrations formed in the nitrosamine assay (DPA+NO23) by *B. cereus* or *H. pylori* strains alone (DPA+NO23), compared to reactants a) nitrite OR nitrate (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

DPA+NO23 assay vs:	***B cereus***	**HP602**	**HP623**	**HP583**	**B8**	**J99**
DPA+NO2+3	1	1	1	1	1	1
No bacteria	0.34	0.23	0.31	0.14	0.32	0.28
NO23	**0.21**	**<0.0001**	0.87	**<0.0001**	0.27	0.97
DPA	0.35	0.0033	0.35	0.23	0.35	nd
DPA+NO2/3	0.47	0.91	nd	0.19	0.33	0.63
BC+HP602	0.37	0.43	0.33	0.23	0.35	0.32
BC+HP623	0.38	0.51	0.35	0.25	0.35	0.33
BC+HP583	0.41	0.77	0.37	0.35	0.38	0.37
BC+B8	0.45	0.79	0.41	0.55	0.41	0.43
BC+J99	0.43	0.98	0.39	0.45	0.4	0.40

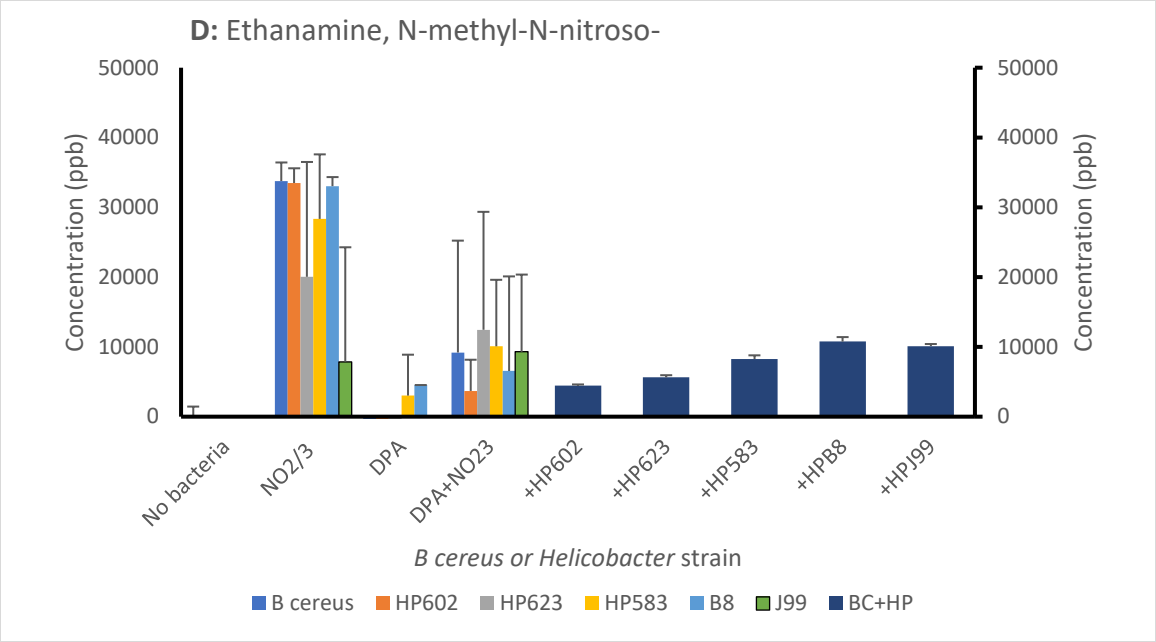


Figure 3.4D: Production N-methyl-N-nitroso-ethanamine by individual bacteria with (left half of the graph) a) nitrate OR nitrate (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5D (below).

Table 3.5D. Statistical analysis of differences (p-values) in Fig 3.4D (above) between N-methyl-N-nitroso-ethanamine concentrations formed in the nitrosamine assay (DPA+NO23) by *B. cereus* or *H. pylori* strains alone (DPA+NO23), compared to reactants a) nitrite OR nitrate (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

	B cereus	**HP602**	**HP623**	**HP583**	**B8**	**J99**
DPA+NO2+3	1	1	1	1	1	1
No bacteria	0.08	**0.0182**	0.30	0.26	0.59	0.21
NO23	0.35	**<0.0001**	0.71	**0.0113**	**0.0451**	0.54
DPA	0.19	0.16	0.27	0.9	0.71	nd
DPA+NO2/3	0.06	0.89	nd	0.26	nd	0.18
BC+HP602	0.32	0.71	0.44	0.59	0.79	0.36
BC+HP623	0.35	0.45	0.48	0.33	0.85	0.41
BC+HP583	0.44	0.14	0.60	0.12	0.99	0.55
BC+B8	0.55	**0.0484**	0.72	0.06	0.89	0.71
BC+J99	0.52	0.06	0.72	0.07	0.92	0.66

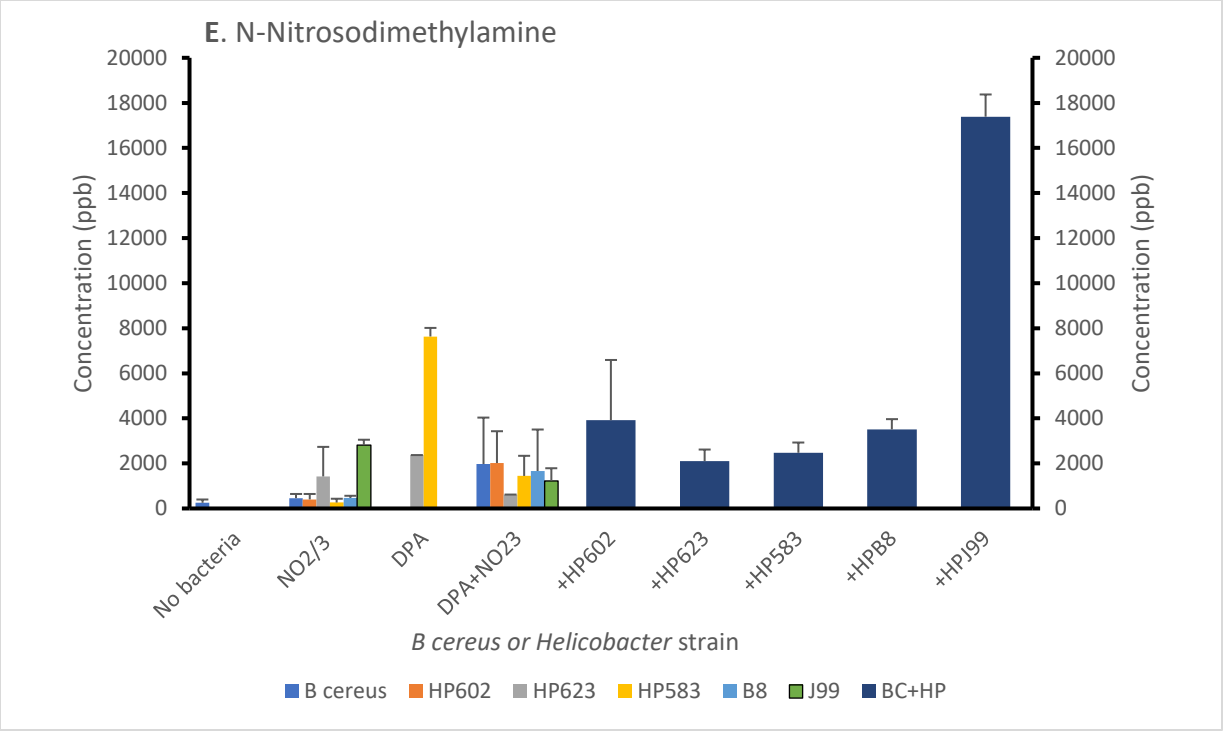


Figure 3.4E: Production N-nitrosodimethylamine by individual bacteria with (left half of the graph) a) nitrate OR nitrate (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5E (below).

Table 3.5E. Statistical analysis of differences (p-values) in Fig 3.4E (above) between N-nitrosodimethylamine concentrations formed in the nitrosamine assay (DPA+NO23) by *B. cereus* or *H. pylori* strains alone (DPA+NO23), compared to reactants a) nitrite OR nitrate (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

	B cereus	**HP602**	**HP623**	**HP583**	**HPB8**	**HPJ99**
No bacteria	0.19	**0.0152**	**0.0224**	**0.0451**	0.37	0.06
NO2/3	0.15	**0.0010**	0.11	**0.0023**	0.21	**0.0103**
DPA	nd	nd	nd	**0.0077**	0.79	nd
DPA+NO23	nd	0.8	nd	0.21	nd	0.68
+HP602	0.25	0.38	nd	0.45	0.34	0.08
+HP623	0.10	0.49	nd	0.95	0.52	0.08
+HP583	0.06	0.19	nd	0.59	0.37	**0.0298**
+HPB8	**0.0224**	**0.0404**	nd	0.12	0.16	**0.0042**
+HPJ99	**0.0020**	**0.0021**	nd	**0.0028**	**0.0053**	**<0.0001**

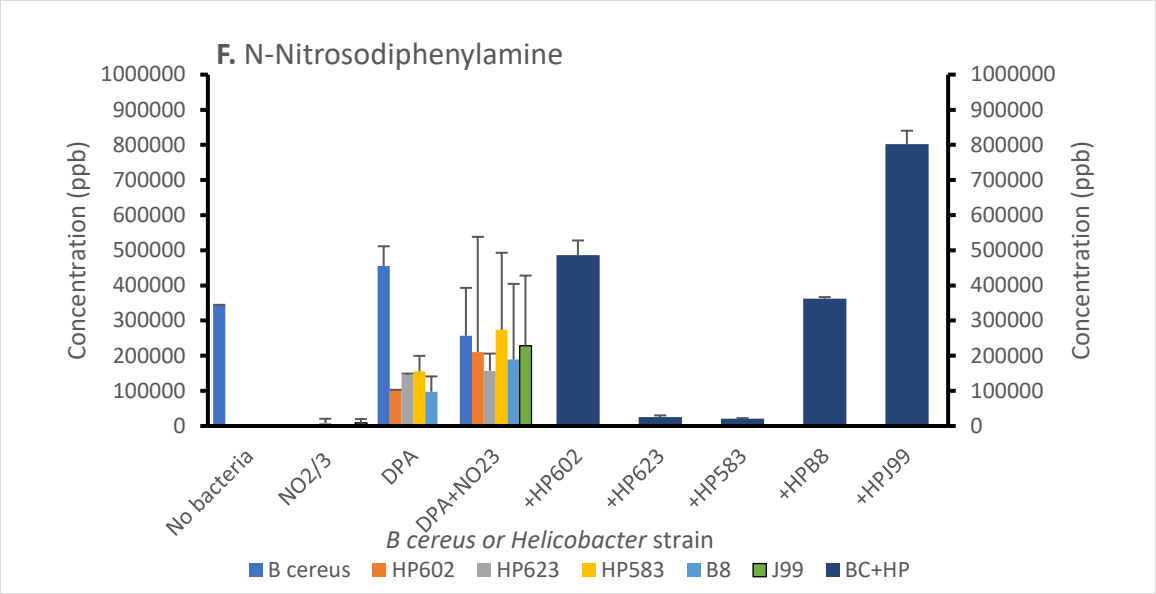


Figure 3.4F: Production of N-Nitrosodiphenylamine by individual bacteria with (left half of the graph) a) nitrate OR nitrate (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5F (below).

Table 3.5F. Statistical analysis of differences (p-values) in Fig 3.4F (above) between N-Nitrosodiphenylamine concentrations formed in the nitrosamine assay (DPA+NO23) by *B. cereus* or *H. pylori* strains alone (DPA+NO23), compared to reactants a) nitrite OR nitrate (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

	B cereus	**HP602**	**HP623**	**HP583**	**HPB8**	**HPJ99**
No bacteria	0.13	0.98	**0.0011**	0.06	**0.0255**	0.18
NO2/3	**0.039**	0.34	**<0.0001**	**0.0279**	0.20	0.16
DPA	**0.0053**	0.49	0.26	0.71	0.92	nd
DPA+NO23	0.25	0.47	nd	0.07	0.31	0.25
BC+HP602	**0.0271**	0.65	**0.0004**	**0.0217**	**0.0058**	0.053
BC+HP623	0.18	0.38	**0.0026**	0.31	0.31	0.4
BC+HP583	0.17	0.37	**0.0023**	0.29	0.29	0.39
BC+HPB8	0.10	0.93	**0.0011**	0.057	**0.0201**	0.15
BC+HPJ99	**0.0024**	0.21	**<0.0001**	**0.0037**	**0.0006**	**0.0064**

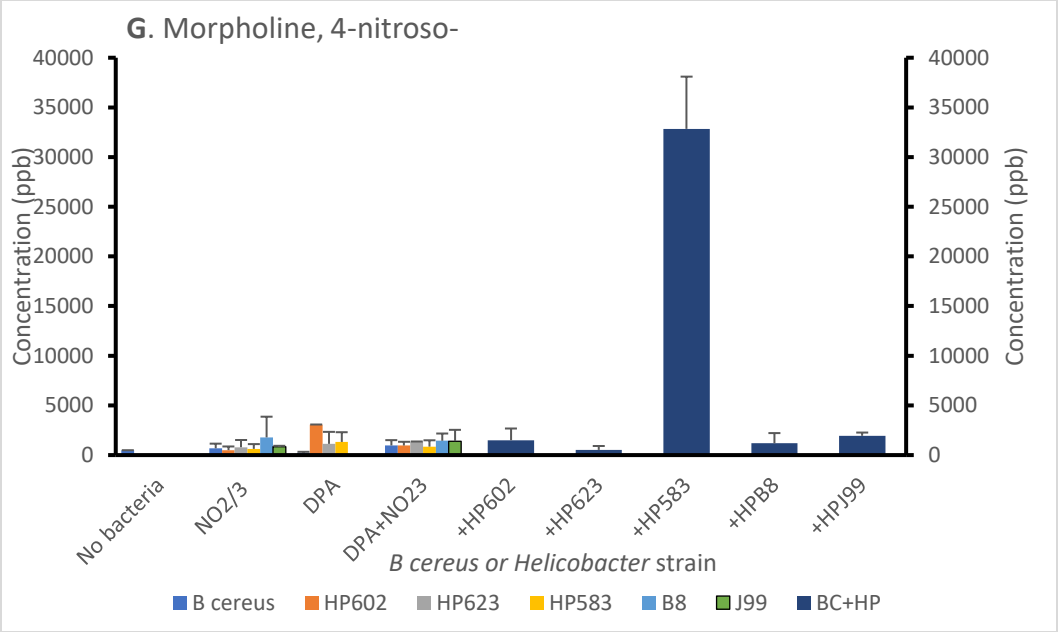


Figure 3.4G: Production of 4-nitroso-morpholine by individual bacteria with (left half of the graph) a) nitrate OR nitrate (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5G (below).

Table 3.5G. Statistical analysis of differences (p-values) in Fig 3.4G (above) between 4-nitroso-morpholine concentrations formed in the nitrosamine assay (DPA+NO23) by *B. cereus* or *H. pylori* strains alone (DPA+NO23), compared to reactants a) nitrite OR nitrate (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

DPA+NO23 assay vs:	***B cereus***	**HP602**	**HP623***	**HP583**	**HPB8***	**HPJ99**
DPA+NO2+3	1	1	1	1	1	1
No bacteria	nd	nd	nd	nd	nd	nd
NO23	0.28	0.07	nd	0.35	nd	0.53
DPA	**0.0458**	0.99	nd	0.99	nd	nd
DPA+NO2/3	0.62	0.44	nd	0.21	nd	0.5
BC+HP602	0.72	0.72	nd	0.90	nd	0.92
BC+HP623	0.21	0.15	nd	0.40	nd	0.35
BC+HP583	**0.0135**	**0.0135**	nd	**0.0141**	nd	**<0.0001**
BC+B8	0.97	0.97	nd	0.89	nd	0.82
BC+J99	0.12	0.07	nd	0.51	nd	0.72

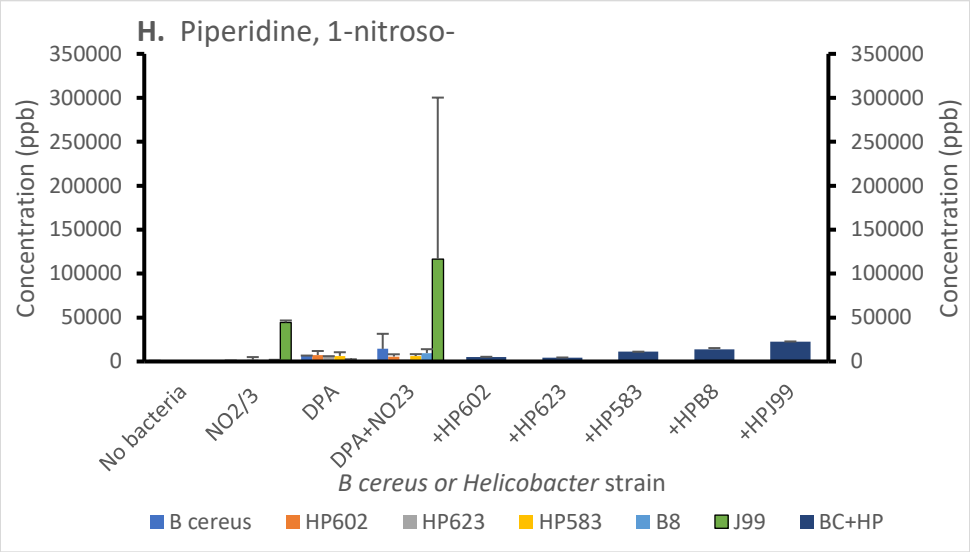


Figure 3.4H. Production of 1-nitroso-piperidine by individual bacteria with (left half of the graph) a) nitrate OR nitrate (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5H (below).

Table 3.5H. Statistical analysis of differences (p-values) in Fig 3.4H (above) between 1-nitroso-piperidine concentrations formed in the nitrosamine assay (DPA+NO23) by *B. cereus* or *H. pylori* strains alone (DPA+NO23), compared to reactants a) nitrite OR nitrate (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

	B cereus	**HP602**	**HP623**	**HP583**	**HPB8**	**HPJ99**
No bacteria	0.78	0.12	0.65	0.06	0.10	0.49
NO2/3	0.48	0.10	0.47	0.06	0.12	0.64
DPA	nd	0.68	**0.0151**	0.97	0.15	0.93
DPA+NO23	0.19	nd	nd	0.85	nd	0.96
BC+HP602	**0.0129**	0.75	**0.0062**	0.42	0.28	0.51
BC+HP623	**0.0109**	0.59	**0.0028**	0.31	0.24	0.50
BC+HP583	**0.0013**	0.09	**0.0004**	0.09	0.68	0.52
BC+HPB8	**0.0056**	0.05	**0.0049**	0.05	0.31	0.53
BC+HPJ99	**0.0003**	**0.01**	**0.0001**	**0.0100**	0.05	0.56

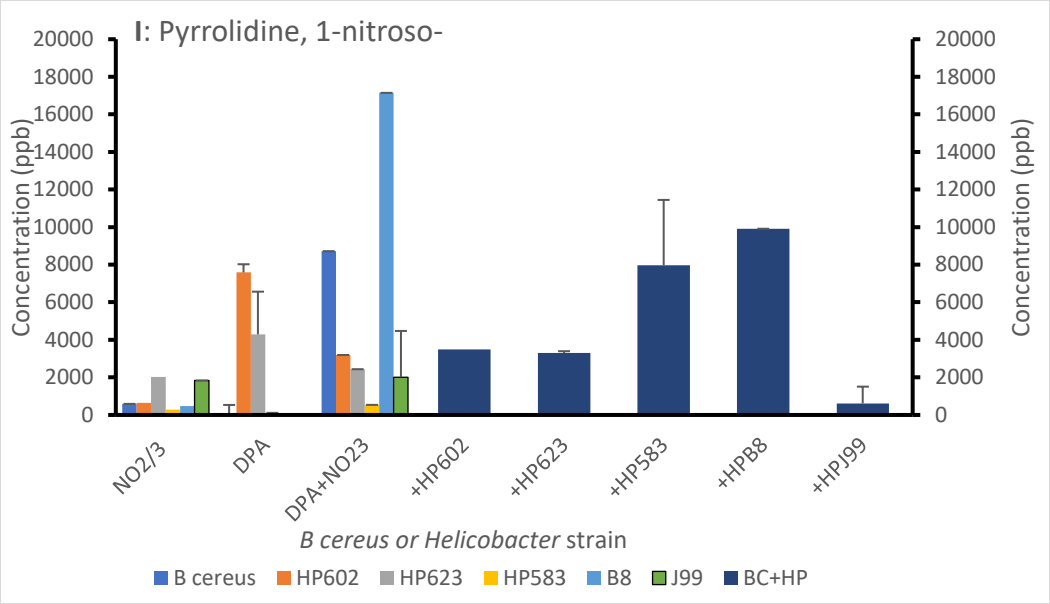


Figure 3.4I: Production of Pyrrolidine, 1-nitroso- by individual bacteria with (left half of the graph) a) nitrate OR nitrate (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23; i.e. the reference nitrosamine assay); and (right half of the graph: broad bars) d) as c) with *B. cereus* AND the *H. pylori* (BC+HP). See experimental section for details. Data as mean±std (error bars) with statistical differences shown in Table 3.5I (below).

Table 3.5I. Statistical analysis of differences (p-values) in Fig 3.4I (above) between Pyrrolidine, 1-nitroso- concentrations formed in the nitrosamines assay (DPA+NO23) by *B. cereus* or *H. pylori* strains alone (DPA+NO23), compared to reactants a) nitrite OR nitrate (NO2/3); b) diphenylamine (DPA) alone; c) DPA with nitrate OR nitrite (DPA+NO2/3); and d) the equivalent nitrosamine assays with both bacteria.

	B cereus	**HP602**	**HP623**	**HP583**	**HPB8**	**HPJ99**
No bacteria	nd	nd	nd	nd	nd	nd
NO2/3	1	0.85	0.85	0.22	0.14	nd
DPA	nd	0.93	0.82	nd	0.65	nd
DPA+NO23	0.2	nd	nd	nd	0.66	nd
BC+HP602	**0.01**	0.93	0.69	**0.00**	0.42	**0.01**
BC+HP623	0.39	0.97	0.82	0.09	0.41	0.25
BC+HP583	nd	nd	nd	nd	nd	nd
BC+HPB8	**0.01**	0.16	0.87	**0.00**	0.37	**0.0010**
BC+HPJ99	nd	nd	nd	nd	nd	nd

Table 3.6. Summary of results in Tables 3.7 (A-I) showing changes in nitrosamine concentrations according to the assay conditions. Differences in nitrosamines depended on the presence of nitrates and nitrites, the presence of DPA as well as the stain type.

		1-Butanamine, N-butyl-N-nitroso-	**1-Propanamine, N-nitroso-N-propyl-**	**Ethanamine, N-ethyl-N-nitroso-**	**Ethanamine, N-methyl-N-nitroso-**	**N-Nitrosodi-methylamine**	**N-Nitrosodi-phenylamine**	**Morpholine, 4-nitroso-**	**Piperidine, 1-nitroso-**	**Pyrrolidine, 1-nitroso-**
NO2/3	***B. cereus***	-	-	-	-	-	↓	-	-	-
	HP602	-	↓	↑↑↑	↑↑↑	↑↑	-	-	-	-
	HP623	-	-		-	-	↓↓↓	nd	-	-
	HP583	-	-	↑↑↑	↑	↑↑	↓	-	-	↓↓
	HPB8	-	-		↑	-	-	nd	-	↓↓
	HPJ99	-	↓↓		-	↑	-	-	-	nd
DPA	***B. cereus***	-	↑↑	-	-	nd	↑	↑	nd	-
	HP602	-	nd	↑↑	-	nd	-	-	-	-
	HP623	↑	↑↑	-	nd	nd	-	nd	↑	-
	HP583	-	-	-	-	↑	-	-	-	nd
	HPB8	-	-	-	nd	-	-	nd	-	nd
	HPJ99	-	nd	-	nd	nd	nd	-	-	nd
DPA+ NO23	***B. cereus***	-	↓	-	-	-	-	-	-	-
	HP602	-	↓	-	-	-	-	-	nd	-
	HP623	-	nd	nd	nd	-	nd	nd	nd	-
	HP583	-	-	-	-	-	-	-	-	↓
	HPB8	-	-	-	nd	-	-	nd	nd	-
	HPJ99	-	-	-	-	-	-	-	-	nd
B. cereus	**+HP602**	↑↑↑	-	-	-	-	-	-	-	↑
	+HP623	↑↑↑	-	-	-	-	-	nd	↑↑	-
	+HP583	↑↑↑	-	-	-	-	↑↑	↑	-	↑
	+HPB8	↑↑↑	↑↑↑	-	↑	-	↑	nd	-	-
	+HPJ99	-	↑↑	-	-	↑↑↑	↑↑	-	-	nd

Production nitrosamines by individual bacteria in the presence of a) nitrate OR nitrite (NO2/3); b) diphenylamine (DPA); c) DPA with nitrite AND nitrate (DPA+NO23 (i.e. the reference nitrosamine assay) and d) *B. cereus* AND the *H. pylori* (BC+HP) has been shown in Figures 3.4 (A-I) and Tables 3.5 (A-I). Nitrosamine production varied according to the experimental conditions and between strains. The presence of nitrate and nitrite tended to increase the concentrations of several nitrosamines, and where decreases were noted, the concentrations were already low. N-methyl-, N-ethyl- N-nitroso-ethanamine and N-nitrosodimethylamine increased in the presence of nitrate and nitrate and the combination, without differences between the nitrogen species.

N-butyl-N-nitroso-1-butanamine was significantly increased by incubating *H. pylori* in *B. cereus* supernatants over that of the assays of *B. cereus* alone, and the *H. pylori* strains 602, 623, 583 and type strain B8 but not strain J99.

Increases in N-nitroso-N-propyl-1-propanamine, N-nitrosodimethylamine, N-nitrosodiphenyl-amine were increased more in type stains. Finally, there did not appear to be differences between *cag*+ strains and the *cag*- 583 strain.

Chapter 4: Discussion

Overview

This study investigated whether carcinogenic compounds; nitrosamines, can be formed by *H. pylori*, *B. cereus* and by the combination of the two bacteria. We found that nitrosamine compounds were formed from the *H. pylori* and *B. cereus* individually, and certain nitrosamines were produced at higher concentrations when *H. pylori* strains were cultured in supernatant from *B. cereus*.

The *B. cereus* samples contained 7 unique nitrosamines. On the other hand, the clinical strains and reference strains of *H. pylori* both produced an average of 5 nitrosamines. More interestingly, the combination of *H. pylori* and *B. cereus* produced 22 unique nitrosamines.

Overall, the findings suggest that nitrosamines are abundant and may be produced by combinations of gastrointestinal microorganisms which may work synergistically to produce these potential carcinogens.

4.1 Experimental approach

Nitrosamines form in the presence of nitrate, nitrate and nitric oxide, with an amine. *B. cereus* has a nitrite reductase and nitric oxide synthase enzyme (Tsuji *et al*., 1996) but, in contrast to related *Campylobacter jejuni* and *Helicobacter hepaticus*, *H. pylori* appears to have no homologue of nitrate reductase (Sparacino-Watkins *et al.*, 2014). The combination of these bacteria was hypothesized to provide conditions for nitrosamine formation. Furthermore, the observation that *B cereus* was often co-cultured when attempting to isolate *H. pylori* suggested there might be a synergistic or symbiotic relationship between these bacteria.

The ready growth of *B. cereus* under the same conditions as *H. pylori* was not surprising as similar culture conditions have been used with polymyxin and trimethoprim (Tallent *et al*., 2001; Chon *et al*., 2012) as selective antibiotics to isolate *B. cereus*. Although, *B. cereus* strains may be susceptible to vancomycin, concentrations used (30ug/L; Amor *et al*., 2019) appear higher than the Skirrows antibiotic mix used in the current study.

A standard approach was used to assay for nitrosamine production (Calmels *et al*., 1988) with a described GC-MS/MS measurement (Chen *et al*., 2012). Although a commercially available nitrosamine mixture standard was used, the approach was untargeted, with many nitrosamines identified in the databases which were not in the standard mixture. Indeed, formation of nitrosamines was also observed in control samples that contained no bacteria. Without standards, these nitrosamines were identified but not quantified.

4.2 Nitrosamine formation by other bacteria

It has been well established that nitrosamines are carcinogenic compounds that are formed from the reaction between nitrites or nitrates with secondary amines. However, to our knowledge, other origins of the compounds have not been thoroughly investigated.

Nitrates, the precursors to nitrosamine formation, are part of many people's daily food intake as they are normal dietary components found in vegetables (Neurath *et al*., 1976). Nitrates are also used to preserve meat products, and can be found in water (Hawksworth and Hill, 1971; Ayanaba and Alexander, 1972). Additionally, secondary amines have been suggested to be produced by intestinal bacteria and they have been found in human urine and feces (Hawksworth and Hill, 1971).

The majority of bacteria that produced nitrosamines contained a nitrate reductase activity (Hawksworth and Hill, 1971). Fong and Chan (1973) found an increase in the nitrosamine dimethyl nitrosamine produced in the presence of *Staphylococcus aureus* (*S. aureus*) that was isolated from processed Chinese fish. This is when compared to fish samples that were not inoculated with the bacterium. Similar results were obtained by Driessens (1973), who found appreciable amounts of nitrosamines (diethyl nitrosamine and dimethyl nitrosamine) in marine fish. The presence of these nitrosamines was attributed to the bacteria reactions of *S. aureus* and the nitrate that is found in the salt used to preserve these fish. Much like *B. cereus*, *S. aureus* is a Gram-positive bacterium that causes food poisoning (Burke and Lascelles, 1975). *S. aureus* is able to grow in different temperatures ranging from 7°C to 48.5°C, allowing it to grow on

different foods. More importantly, *S. aureus* has nitrate reducing activity, allowing for the production nitric oxide precursors (Burke and Lascelles, 1975).

Hawksworth and Hill (1971) found that *Escherichia coli (E. coli)* strains of human intestinal origin were also able to form nitrosamines when incubated with secondary amines; diphenylamine, dimethyl amine, diethylamine, piperidine, pyrrolidine and N-methyl aniline. (Neurath *et al*., 1976). Additionally, they found that strains of *Enterococci, Clostridia, Bacteroides* and *Bifidobacterial* also formed the nitrosamine diphenylnitrosamine when a nitrite was replaced by a nitrate. However, none of the above-mentioned bacteria had nitrate reductase activity (Hawksworth and Hill, 1971), and this is also characteristic of *H. pylori*. Even though it has been established that bacteria containing the nitrate reductase activity was responsible for nitrosamine production, bacteria with no reductase activity may have a different mechanism of conversion. Interestingly, all the bacterial strains that produced nitrosamines from nitrate and secondary amines could also form nitrosamines if the nitrate was replaced by nitrite (Hawksworth and Hill, 1971).

Nitrosamine synthesis occurs in the gut (Fong and Chan, 1973) and has largely been attributed to the gut microbiome. Calmels and colleagues (1988) sought to determine the mechanism by which the nitrosamine, nitrosomorpholine, was formed by the bacteria: *E. coli*, *Proteus morganii* (*P. morganii*), *Pseudornonas aeruginosa* (*P. aeruginosa*) and *Paracoccus denitrificans* (*P. denitrificans*) isolated from human intestines. These bacteria were exposed to nitrate and morpholine to act as precursors and were able to have nitrosating activity. However, no induction of nitrosating activity or formation was induced in the *E. coli* strains that did not have the nitrite reductase activity. Nitrosating activity was seen in in *P. aeruginosa* in the presence of both nitrates and nitrites. Additionally, it was found that a low nitrate concentration inhibited in vitro nitrosation. Moreover, the presence of nitrate during culture was required for the induction of nitrosating activity in bacteria. Both *E. coli* and *P. morganii* showed high levels of nitrosation. In contrast, the bacteria *P. aeruginosa* and *P. denitrificans* had different induction of nitrosating activity. No significant nitrosating activity was observed under anaerobic conditions and *P. aeruginosa* exhibited the highest activity in the presence of nitrite rather than of nitrate (Calmels, *et al*., 1988).

Studies have shown that there are higher levels of nitrosamines detected in the gut of rats with a normal microbial flora compared to those who were germ free. This highlights the importance of the gut microbiota is nitrosamine formation (Kobayashi, 2018). Only a few bacteria that are involved in nitrosamine formation have been identified. With that being said, it is clear that the bacteria that induce nitrosamine production are bacteria that are able to use nitrite or nitrate for respiratory denitrification by reducing nitrate to nitrite (Kobayashi, 2018). Nitrate and nitrite serve as electron acceptors to generate energy for anerobic bacteria, and this allows them to thrive in a bacterial community.

It is suggested that bacteria can aid in nitrosamine formation in one of three ways:

1) by reducing nitrate to form nitrite-and this is believed to be aided by oral bacteria, resulting in the swallowing of the formed nitrite compounds (Lundberg and Weitzberg, 2012);

2) by synthesis in the stomach, where nitrosating agents are generated from nitrite and thus forming nitrosamines from diet derived amines; and

3) by infection with pathogens, causing inflammation involving the induction of inducible NOS and then causing the inducible NOS derived NO.

It is believed that nitrosation of nitroso compounds usually occurs in the stomach caused by an acid catalyzed reaction or in achlorhydric individuals who have a profuse bacterial flora in the stomach (Hawksworth and Hill, 1974; Forsythe *et al*., 1988). We have seen that it is possible for bacteria to have nitrosating activity and to produce nitrosamines. It is therefore plausible that *H. pylori* and *B. cereus* may also be able to form nitrosamines in a similar manner in the human gut.

4.2.1 Nitrosamines formed by *B. cereus*

Several nitrosamines which were not in the standard were detected using the GC-MS/MS method as per Chen *et al*., 2012.

N-Methyl-N-nitroso-1-pentanamine formed from the reaction with amine compounds (Newsom *et al*., 2019) has been shown to induce esophageal carcinomas in rats (Iizuka *et al*., 1980; Kondoh *et al*., 2007). Iizuka and colleagues administered a) 15ppm of N-methyl-N-nitroso-1-

pentanamine to rats by adding it to drinking water for 90 days or b) at 30ppm for 60 days, or c) injected through the tail rats, for four weeks. Results showed that the rats developed esophageal papillomata. After 8 weeks of exposure, esophageal carcinomas started to develop (Kondoh *et al*., 2007). This highlights the importance of this nitrosamines in esophageal cancer formation.

Ethylnitrosocarbamic acid ethyl ester, commonly known as N-ethyl-N-nitrosourethane (Schoental ,1965) was found to induce tumors of the colon by repeated rectal application on rats. Administration of the nitrosamine in 50% ethanol resulted in liver tumors. Additionally, when administered intraperitoneally, 3 out of 6 rats developed adenomas and adenocarcinomas of the ileum (Schoental, 1965). These adenocarcinomas occurred in 2 male rats and 1 female rat. These tumors developed after a year of exposure. Similarly, a previous study found that administration of 2.5ppm and 10ppm of ethylnitrosocarbamic acid ethyl ester resulted in tumor formation in 100% of the rat population used, with an inverse relationship between nitrosamine dosage and survival (Maekawa *et al*., 1989). The tumors observed from administration of ethylnitrosocarbamic acid ethyl ester were located in the uterus, mammary glands, endocrine organs, as well as the small intestine, the forestomach and the spleen (Schoental and Bensted, 1967; Maekawa *et al*., 1989). Interestingly, the incidences of these tumors were low in the rat groups that received the highest dose of nitrosamine (10ppm). Upper digestive tract tumors, specifically in the oral cavity and the forestomach, were also observed (Maekawa *et al*., 1989; Onodera and Maekawa, 1992) after administration of this nitrosamine. These tumors were induced after administration of concentrations of 10ppm, 20ppm and 40ppm.

4-Nitrosothiomorpholine identified in the *B. cereus* sample, has been reported to cause brain hemorrhage and interalveolar hemorrhage in the lungs, as well as a tumor in the esophagus of rats after oral administration (Garcia *et al*., 1970).

The other nitrosamines detected in *B. cereus* cultures were hexahydro-1,4-dinitroso-1H-1,4-diazepine which can be formed from diphenylpiperidine (Jeyaraman and Senthilkumar, 1995). 4-methyl-1-nitrosopiperidine, methylnitroso-carbamic acid ethyl ester and trimethylnitroso- urea were also detected. To our knowledge, no research that has been done on these latter nitrosamine compounds.

4.2.2 Nitrosamines formed from clinical strains (620C, 623C and 538C) of *H. pylori*

Ethylnitrosocarbamic acid ethyl ester was the only nitrosamine, not found in the commercial standard, formed from the *H. pylori* (620C) clinical strain. This nitrosamine was also present in the *H. pylori* (623C) clinical strain and the *B. cereus* sample, as discussed above.

The *H. pylori* (623C) clinical strain also produced N-nitroso-dipentylamine, which is commonly known as dipentlynitrosamine. This nitrosamine has been implicated in the production of liver tumors. A study by Davis *et al* (1981) incorporated dipentlynitrosamine in rat food and found that rats that ingested the nitrosamine at a concentration of 2000ppm resulted in weight loss as well as a bile duct carcinoma. In addition to this, a hepatoma was observed in male rats that received a concentration of 1500ppm. Moreover, the incidence of cell proliferation in the bile ducts was higher in males than in it was in females when they were exposed to a concentration of 750ppm. When exposed to a concentration of 2000ppm, over half of the rats of both sexes showed bile duct carcinomas. This study concluded that feeding rats the nitrosamine dipentylnitrosamine at 2000ppm constantly for 8 weeks results in tumor formation. When administered once off, the nitrosamine exposure did not result in the formation of any cancer, whereas exposure to the nitrosamine for 4 weeks resulted in moderate bile duct cell proliferation in all the rats (Davis *et al*., 1981). However, dipentylnitrosamine administration did not result in deaths and there could mean this nitrosamine is not lethal.

Hexahydro-1,3,5-trinitroso-1,3,5-triazine has been found to contaminate areas of explosive manufacturing (Smith *et al*., 2006). When rats were orally exposed to Hexahydro-1,3,5-trinitroso-1,3,5-triazine in drinking water at different concentrations varying from 0.001, 0.010 and 0.1ppm, results showed that the nitrosamine was absorbed by the GIT of the mice. The level of absorption within the GIT and the liver was dependent on the dose administered (Smith *et al*., 2006). However, although exposure to the nitrosamine did not affect the number of offspring produced, offspring mortality was affected as many of the offspring died at birth or within 4 days (Smith *et al*., 2006). The researchers suggest that the toxicity if the nitrosamine could have been passed down through intrauterine and milk exposure. An increase in toxicity in the offspring was

also attributed to the underdevelopment of the kidney glomerular filtration. Additionally, it was found that there was a dose dependent weight loss seen in the mice, but this was only seen during the early stage of life (birth to weaning).

Other nitrosamines identified in *H. pylori* (623C) samples included N, N'-bis-(2-methyl-2-nitrosopentan-4-one), 3-methyl-1-nitrosopiperidine and N-nitroso-2-methyl-oxazolidine. No further information on these nitrosamines could be found.

Six nitrosamines were identified in *H. pylori* (538C) cultures: ethylnitrosocarbamic acid ethyl ester and the ethyl ester of methylnitroso-carbamic acid which were also detected in the previous sample and have been discussed above. Additionally, the nitrosamines: 2-nitroso-2-butanyl acetate, N-methoxy-N-nitrosomethanamine and ethylnitroso-1-N-carbamic acid piperazine were also formed but no further information on these compounds was found.

Hexahydro-1-nitroso-1H-azepine, was also formed by the *H. pylori* (538C) strain. Althoff *et al*. (1973) investigated the effects of this nitrosamine in hamsters and shows high dose subcutaneous administration of the nitrosamine (32mg/kg and 64mg/kg) resulted in bleeding and necrosis of the kidneys in 20 hamsters of each sex. The groups of hamsters that received low doses showed no differences from the control group whereas survival was short in the high exposure group (Althoff *et al*., 1973). Other tumors of different organs were found in 67% of the animals used. These tumors were found in the nasal cavity, the larynx, the lungs and majority in the trachea. Most of these tumors were seen in the male hamsters. Interestingly, a squamous cell papilloma of the forestomach in a male hamster and a papillary adenoma of the thyroid in a male hamster were found after 74 weeks and 94 weeks respectively. Moreover, the tumors resulted from constant exposure to the nitrosamine (Althoff *et al*., 1973).

4.2.3 Nitrosamines formed from reference strains of *H. pylori* (B8 and J99)

In the reference strain of *H. pylori* (B8), six nitrosamines were produced. These included Ethylnitrosocarbamic acid ethyl ester, which we encountered in a previous sample. Other nitrosamines formed were; 4-Piperidiol,1-nitroso-ine,1-nitroso-, and Piperaz3-Piperidinol,1-nitroso-; nitrosamines that nitrosamines that appear to have no literature studies linked to them.

The other nitrosamine formed was Ethanol,2,2'(nitrosoimino)bis-, commonly known as N-nitrosodiethanolamine or NDELA. This nitrosamine has been found to be one of the most widespread nitrosamine compounds in the environment. It is formed through the reaction between sodium nitrite and diethanolamine (Jarvholm *et al*., 1986) and is commonly found in cosmetic products as well as in tobacco products. Ethanol,2,2'(nitrosoimino)bis- is a weak carcinogen, only high doses result in carcinogenicity. A study by Dittberner and colleagues (1988) found that exposing the nitrosamine Ethanol,2,2'(nitrosoimino)bis- to human blood microcultures resulted in chromosome aberrations as seen by sister chromatid exchanges. In this regard, the number of sister chromatid exchanges increased with an increase in nitrosamine concentration (Dittberner *et al*., 1988). The increase in sister chromatid exchanges was seen at a concentration of 25umol. This study highlighted that this nitrosamine was a mutagen in human lymphocytes.

Men exposed to NDELA in industrial environments and working with cutting fluids which were mainly composed of sodium carbonate and sodium nitrite as well as alkonolamines ended up developing tumors. Exposure to NDELA did not cause any differences in mortality rates and cancer formation in people exposed to the nitrosamine were not different from the general population who were not exposed, (Jarvholm *et al.,* 1986). There was, however, no way of determining the concentration of NDELA that the men were exposed to

Preussmann *et al*., (1981) administered NDELA to rats in doses between 70-75mg on the shaved backs of the rats using a small brush. Additionally, the rats were fed 2000ppm sodium nitrite in drinking water for 6 days, thereafter, the rats were exposed to the diethanolamine at day 6 on the skin. NDELA was excreted at high percentages following both parenteral and oral

administration, suggesting vivo formation of NDELA after percutaneous application of the amine and oral application of the nitrite in the drinking water. A subsequent study by Preussmann *et al.*, (1982) found that administering the NDELA in drinking water to male Sprague-Dawley rats resulted in tumors in the liver and the nasal cavity. This experiment involved feeding the different groups of rats' different doses of the NDELA (1.5, 6, 25, 100 and 400mg/kg) of the rats' body weight. This caused loss of body weight in the groups with a high dosage (≥6, 25, 100 and 400mg/kg) as well as development of tumors in the liver which included hepatocellular adenocarcinomas and adenomas (Preussmann *et al*., 1982). These tumors occurred in the low dose groups whereas no liver tumors were formed in the non-exposed control group. The data suggests NDELA is a potent carcinogen.

1,4-Dinitrosopiperazine identified from the cultures has been identified to cause malignancy in rats after 6 months (Pai *et al*., 1981). The tumors formed included tumors of the forestomach and it was also found that male rats developed more stomach cancers than their female counterparts (Pai *et al.*, 1981). Lastly, the nitrosamine from this sample was Urea, trimethylnitroso which also has no cancer related literature.

The second reference strain of *H. pylori* (J99) only produced 3 unique nitrosamines, two which were previously mentioned (Hexahydro-1,3,5-trinitroso-1,3,5-triazine and 3-Methyl-1-nitrosopiperidine). The third nitrosamine formed was 3,5-Dimethyl-4-nitrosomorpholine which appears to have no literature describing its origin or toxicity.

4.2.4 Nitrosamines formed from a combination of *H. pylori* and *B. cereus*

In the sample that contained the combination of *B. cereus* and *H. pylori*, 22 nitrosamines were formed. These included the nitrosamines that were formed in previous samples namely; 1H-1,4-Diazepine, hexahydro-1,4-dinitroso-, N-methyl-N-nitroso-1-pentanamine, Ethylnitrosocarbamic acid ethyl ester, Ethanol, 2,2'-(nitrosoimino)bis-,Hexahydro-1,3,5-trinitroso-1,3,5-triazine, Methanamine, N-methoxy-N-nitroso-,Piperidine, 4-methyl-1-nitroso-,4-Nitrosothiomorpholine , and Urea, N-ethyl-N-nitroso-. All these nitrosamines were formed and previously identified in

previous samples that only contained *B. cereus* or *H. pylori*. The reoccurrence of the nitrosamines highlights the fact that the bacteria may indeed be independently be mediating the formation of these nitrosamine compounds.

In addition to these, more nitrosamines were formed, including; 11-Acetyl-N-nitrosohemanthidine, 3-Piperidinol, 1-nitroso-oxazolidine, Benzaldehyde, 2-nitroso-,Benzene, nitroso-,Benzenemethanamine, N-nitroso-N-phenyl-,N-Isopentyl-N-nitroso-pentylamine, N-Nitroso-2-(1-phenyl-2-propyl)aminopropionitrile, N-Nitroso-2-methyl-Piperidine,2,6-dimethyl-1-nitroso-,N-Nitroso-2-methylthiazolidine, Piperidine, 2,6-dimethyl-1-nitroso-,Propane, 1-ethylamino-2,2-dinitro-, Pyrimidine-2,4(1H,3H)-dione, 5-amino-6-nitroso- which have all not been scientifically studied.

1-Nitrosoazetidine was also detected from the combination of *H. pylori* and *B. cereus*. Lijinsky and Taylor (1977) determined carcinogenicity of this nitrosamine by feeding rats different concentrations of 1-nitrosoazetidine (0.17mmol, 0.67mmol and 2mmol). They found that at the lower concentration (0.17mmol), 2 out of 30 of the rats developed liver tumors; at the 0.67mmol concentration, 6 out of 21 rats developed liver tumors. Additionally, at the highest concentration; all of the rats died with hepatocellular carcinomas by the end of 62 weeks, whereas the rats exposed to 0.17mmol and 0.67mmol of 1-nitrosoazetidine lived for longer. Even then, not all animals had died by the 120^{th} week. All the liver tumors seen in this experiment were hepatocellular carcinomas (Lijinsky and Taylor, 1977). From this study, the researchers concluded that the nitrosamine is a potent liver carcinogen (Lijinsky *et al*., 1967; Lijinsky and Taylor, 1977). It is however unclear what effects bacteria may have on this carcinogen.

N-Methyl-N-nitroso-1-phenylmethanamine was detected in the sample that contained both *H. pylori* and *B. cereus*. This carcinogen has been associated with esophageal cancer (Schweinsberg and Burkle, 1985). Feeding rats this nitrosamine combined with $NaNO_2$ has been seen to result in cancer formation such as micropapillomas of the esophagus. However, only 1 animal developed a tumor when treated with N-Methyl-N-nitroso-1-phenylmethanamine alone. This study concluded that the administration of the nitrosamine with $NaNO_2$ exacerbates esophageal cancer tumor formation. The tumors were larger in volume and number in the higher dosage groups (Schweinsberg and Burkle, 1985).

Other nitrosamines identified included : N-methyl-N-nitroso-1-pentanamine; 3-thiophenamine, tetrahydro-N-methyl-N-nitroso-, 1,1-dioxide; benzenemethanamine, N-nitroso-N-phenyl-; carbamic acid, ethylnitroso-, ethyl ester; carbamic acid, methylnitroso-, ethyl ester; carbamic acid, nitrosopropyl-, ethyl ester; nitrosodiphenylamine; 1,4-dinitrosopiperazine; piperidine, 2-methyl-1-nitroso-; piperidine, 4-methyl-1-nitroso- which were previously discussed.

Hirono *et al*. (1982) found that the administration of tetrahydro-N-methyl-N-nitroso-3-thiophenamine to rats resulted in tumors of the tongue, pharyngeal tumors, oesophageal tumors. These tumors were increased in number when the administration was accompanied by the rats being fed braken, a type of plant (Hirono *et al*., 1982). However, tumors were also seen when the braken diet was administered alone, in the absence of the accompanying nitrosamine.

Other nitrosamines produced included; tetrahydro-N-methyl-N-nitroso-3-thiophenamine-1,1-dioxide, 2-methyl-1-nitroso-piperidine, which to our knowledge, has no literature associated with cancer formation.

4.3 Nitrosamine formation by *H. pylori* and *B. cereus*

Although our study showed the production of nitrosamines by *H. pylori* and *B. cereus*, a study by Vermeer *et al*., (2002) tested a number of *H. pylori* strains and concluded that the bacterium does not mediate the formation of carcinogenic nitrosamines. Our findings are in agreement with this study with respect to the lack of formation of nitrosomorpholine by several strains of *H. pylori* even in the presence of nitrate, nitrite and DPA and combinations of these. However, *H. pylori* strain 583, and no other strains tested, when cultured in the supernatant of *B. cereus* produced significant concentrations of nitrosomorpholine (Figure 3.4G). This strain was the only strain we tested which was cag-.

H. pylori infection together with nitrosomorpholine was seen to result in intestinal type and diffuse type adenocarcinomas (Vermeer *et al*., 2002) and therefore *H. pylori* may indirectly affect the production of nitrosamines. It was suggested that nitrosamine formation from *H. pylori* could be a result of the bacteria eliciting an inflammatory response which could result in the activation of leukocytes and macrophages, which we are well known and inflammatory cells are

capable of nitrosamine formation via production of nitric oxide (Grisham *et al*., 1992). Additionally, *H. pylori* causes chronic gastritis (Taylor and Blaser, 1991) which leads to atrophy and hypochlorhydria (Goldstone *et al*., 1996) and results in the increase in stomach pH. Nitrosating bacteria are thus able to proliferate in the acidic environment and produce nitrosamines (Suzuki and Mitsuoka, 1984). Unfortunately, the pathway or enzyme responsible for nitrosation in *H. pylori* are still not known.

Although little is known about the production of nitrosamines by *B. cereus,* this bacterium has the nitrate reductase (Hackenthal 1966), and nitric oxide synthase enzymes (Adak *et al*., 2002) and complement amines produced in the presence of *H. pylori*, to generate nitrosamines.

4.4 Cancer formation by *H. pylori* and *B. cereus*

It has been established that chronic *H. pylori* infection plays a role in the production of gastric cancer, however, the mechanism is not clearly known. It has been suggested that *H. pylori* infection leads to reduced acid secretion which may in turn make way to the growth of several other gastric bacteria (Ferreira *et al*., 2018). Ferreira *et al*., (2018) sought to evaluate the composition of gastric microbiota in chronic gastric carcinoma patients. They found that the microbiota was reduced in diversity, mainly due to the presence of *H. pylori.*

Research has been done in Mongolian gerbils where the rodents were inoculated with *H. pylori* and either a nitrosamine; N-methyl-N-nitrosourea, or with the nitrosamine alone. The animals that were inoculated with both developed adenocarcinomas (Sugiyama *et al*., 1998). It is clear that *H. pylori* plays a pivotal role in cancer formation as seen in previous studies, this is however not known for *B. cereus*.

4.5 Nitric oxide

Nitrogen is an important component of biology, specifically amino acids, which are responsible for protein buildup. NO is synthesized from the catalysis of NOS which also produces L-citrulline. Inducible NOS is calcium-independent and activated by inflammatory cytokines such as interleukin-1 and interferon as well as bacterial toxins (Lundberg and Weitzberg, 2012). The half-life of NO in biological systems is extremely short lasting only a couple of milliseconds. The oxidation of NO produces more stable nitrogen oxides nitrite and nitrate. It has been found that bacteria generate NO in the denitrification part of the nitrogen cycle. This reaction is catalyzed by nitrite reductases and the NO formed is reduced by NO reductases. This is done to prevent the buildup of potentially toxic levels of NO for the bacteria. Bacteria that reside on mucosal surfaces in the GIT may participate in NO formation by using an alternative reductive pathway. This pathway involves serial reductions of endogenous and dietary-derived nitrate and nitrite to form NO.

4.6 Nitroso compounds

Nitrosamines have been seen to be carcinogenic in animal models (Althoff *et al*., 1973; Iizuka *et al*., 1980; Kondoh *et al*., 2007). It has been well established that nitrite compounds react with secondary amines to form nitrosamine compounds

The importance of nitroso compounds is because they are easily formed, and are both soluble in water soluble and organic solvents, suggesting they could easily diffuse through epithelial barriers. Additionally, humans are regularly exposed to the precursors of nitrosamines as nitrites are regularly used as preservatives. Nitrosamine formation can also be formed through the preparation, specifically, the heating of food products before consumption. It is also vital to note that nitrosamine formation does not necessarily occur over night as we are not exposed to the precursors in large amounts or concentrations. It has been suggested that specific tumors are formed and locations of tumors are dependent on the chemical structure of the nitrosamine compound as well as the route of administration and the host used (Schmahl and Habs, 1980).

Formation of nitrosamine compounds in animal models may mostly occur in the GIT, however, it has also been found that nitrosamine formation can be observed in the urinary bladder of rats (Hawksworth and Hill, 1974). Nitrosamines are required to be metabolically activated in order to exert their carcinogenic effects (Arranz *et al*., 2007), with the key activation pathway being cytochrome p450.

Nitrosamine compounds are believed to show carcinogenic properties through oxidation via the cytochrome P450 enzymes and through alkylation of general macromolecules (Smith *et al*., 2006). Additionally, nitrosamine compounds are known to require metabolic activation before they can begin their carcinogenic effects (Harris, 1987). It has been proposed that the metabolism of nitrosamines, specifically nitrosodimethylamine, gives rise to two products that can react with nucleophilic sites in cellular molecules. The products are methyldiazonium which is created by alkalation and the second being formaldehyde that is formed from unstable methylol derivatives with amine groups. Formaldehyde may then be oxidized in the cell to form formate and then carbon dioxide (Harris, 1987). More recently it has been realized that nitrosamine compounds are DNA alkylating agents that can transfer alkyl groups to DNA and leading to DNA damage. These DNA changes include chromatin breaks, supercoiling or gene promoter specific structure alterations, as well as histone modifications (Chen *et al*., 2016). The study by Chen and colleagues (2016) found that exposing gastric cells to the nitrosamine N-methyl-nitroso resulted in the increased phosphorylation of the DNA histones and resulted in DNA damage.

It has been found that nitrosamines can be metabolized by cultured human epithelial tissues and cells (Harris, 1987). However, there are differences seen with regards to alkylation of DNA in individuals and the between different organs within an individual. Furthermore, the present study has suggested combinations of bacteria catalyzed the formation of nitrosamines. The same argument could suggest other combinations will work against the formation of such nitrosamines or inactive them. This could account for why not everyone goes on to develop gastric cancer.

Vitamin-C plays a role against the formation of nitrosamines (Mirvish, 1986; Arranz *et al*., 2007). Vitamin-C is a co-factor for some enzymes and is an antioxidant (Arranz *et al*., 2007). It works by scavenging reactive oxygen and nitrogen species and protecting cells against free radical mediated damage (Arranz *et al*.,2007). Apart from directly exerting antioxidant effects, vitamin-C can promote the removal of oxidized DNA through the upregulation of repair

enzymes (Cooke *et al*., 1998). When the effects of vitamin-C against the nitrosamines N-nitrosodimethylamine, N-nitrosopyrrolidine, N-nitrosopiperidine and N-nitrosodibutylamine were investigated, it was found that vitamin-C protected the human liver cells against oxidative damage induced by the nitrosamines (Arranz *et al*.,2007).

4.7 Limitations of the study

Limitations of the study include the fact that not all bacteria could be identified by MALDI-TOF MS. As such, not all nitrosamine formation can be attributed solely to the presence of *H. pylori* and *B. cereus*. In the same light, bacterial contamination could not be completely ruled out in the samples that contained no bacteria.

4.8 Conclusion

The aim of our study was to attempt to gain an understanding of the formation of nitrosamine compounds by gut bacteria, specifically *H. pylori* and *B. cereus*. This was to understand the possible role of these bacteria in formation of cancer by determining their role in the production of endogenous carcinogenic nitrosamine compounds known to be mutagenic or carcinogenic.

As hypothesized, particular nitrosamines were formed by *H. pylori* strains and *B. cereus*, or the combination of these. This production was dependent on the presence of nitrates, nitrites and the amine; diphenylamine. The combination of these bacteria resulted in the increased concentrations of particular nitrosamines suggesting these two bacteria may indeed have a relationship which, under particular conditions, results in the mutagenic or indeed carcinogenic nitrosamine compounds. The study used an untargeted approach and identified a number of nitrosamines produced under the reaction conditions used.

Further work is still needed to determine a) the routes by which these nitrosamines are formed; b) if other bacteria may be responsible for nitrosamine production or breakdown of the nitrosamines; c) the induction of cancers using bacterial culture supernatants *in vivo* in animal

models. Further work also needs to be done on the chemistry and role of nitrosamines as carcinogens, where much of the information available was generated in the 1980's and 1990's. This study may serve as the starting point for future work to elucidate the role of the microbiome in nitrosamine formation and metabolism.

Chapter 5

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Appendices

Appendix A



UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

21/02/2020

Ref: W-CBP-200221-02

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Ms M Phakati
Student No. (if appropriate): 794404
Staff No. (if appropriate):

Supervisor: Professor G Candy

School: Clinical Medicine
Surgery
Medical School
University

Project title: *The production of nitrosamine compounds by gut bacteria*

Reason: Laboratory study.
No human participants will be involved in the study.

Dr CB Penny
Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
Tel Nos. +27 (0)11-717-1234/2656/2700/1252
Office E-mail: HREC-MedicalResearchOffice@wits.ac.za
Website http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Appendix B

Table B1: Microbiological inoculation and culture supplies

Product	**Manufacturer**	**Catalogue number**
Disposable inoculating loops	ThermoFisher™	QL10
Petri-dishes	ThermoFisher™	172931
Microaero Gas packs	ThermoFisher™	R681005
Anaerobic jars	Oxoid™	AG0025A
Fetal bovine serum	Thermo Scientific™	16140089
Growth media/Agar	Thermo Scientific™	CM0331R
50ml Falcon tubes	Thermo Scientific™	339652
15ml Falcon tubes	Thermo Scientific™	339650
2ml Microcentrifuge tubes	Thermo Scientific™	69720

Table B2: Biochemical tests consumables

Product	**Manufacturer**	**Catalogue number**
Gram stain Kit	Sigma-Aldrich	77730
Catalase test solution	Sigma-Aldrich	88597
Urease	Sigma-Aldrich	U4883

Table B3: MALDI-TOF MS consumables and equipment

Name	**Manufacturer**	**Catalogue number**
Reusable polished steel MALDI target plate	Bruker Daltonics	8280799
Bruker Matrix HCCA, portioned	Bruker Daltonics	8255344

Appendix C

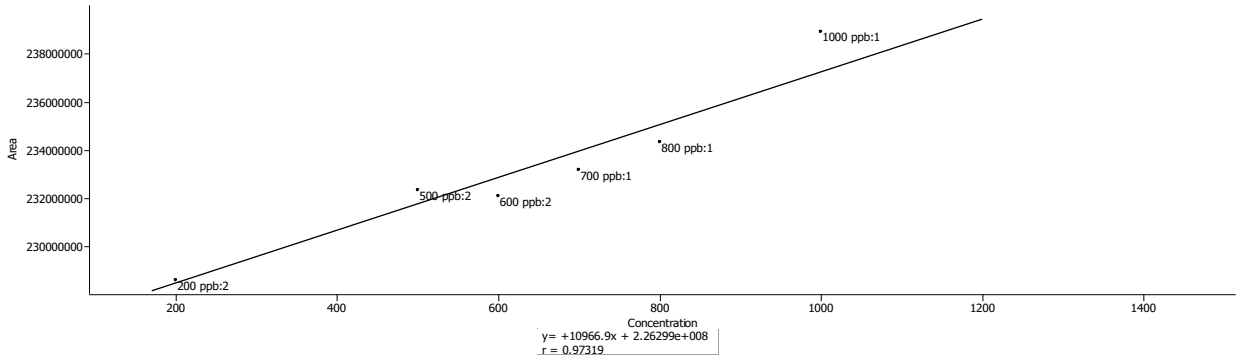


Figure C1: Calibration curve of the nitrosamine standard analyte; Ethanamine, N-methyl-N-nitroso-. Curve shows the different concentrations used to obtain the curve. y=10966.9x+2.26299*e*+008, r=0.97319.

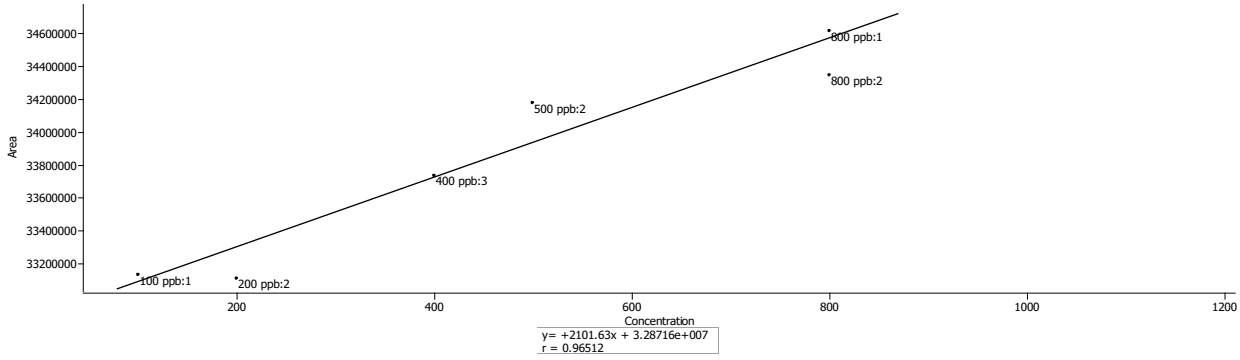


Figure C2: Calibration curve of the nitrosamine standard; Ethanamine, N-ethyl-N-nitroso-. Curve shows the different concentrations used to obtain the curve. y=2101.63x+3.2871*e*+007, r=0.96512.

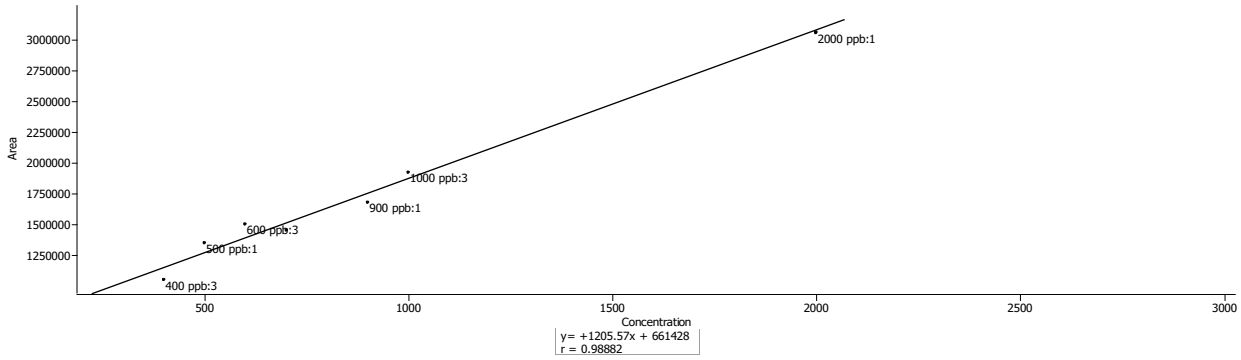


Figure C3: Calibration curve of the nitrosamine standard analyte ;1-Propanamine-N-nitroso-N-propyl. Curve shows the different concentrations used to obtain the curve. y=1205.57x+661428, r=0.98882.

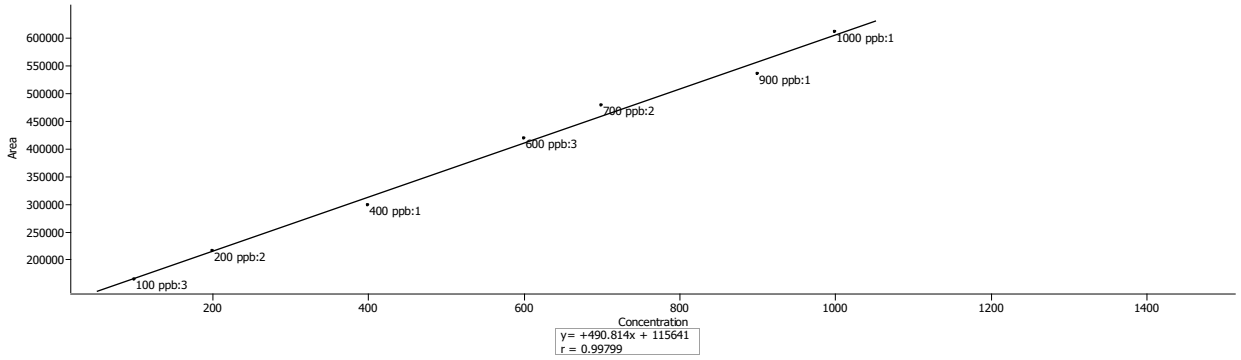


Figure C4: Calibration curve of the nitrosamine standard analyte;1-Butanamine, N-butyl-N-nitroso. Curve shows the different concentrations used to obtain the curve. y=490.814x+115641, r=0.99799.

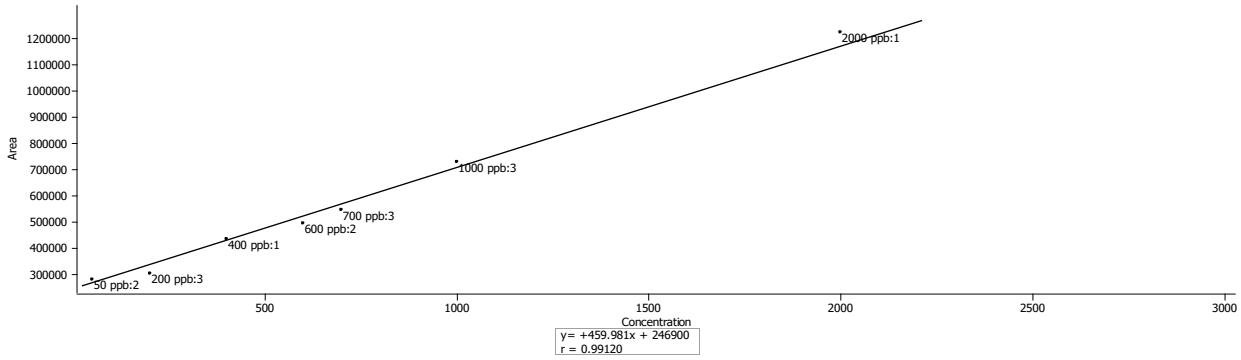


Figure C5: Calibration curve of the nitrosamine standard analyte; Piperidine,1-nitroso-. Curve shows the different concentrations used to obtain the curve. y=459.981x+246900, r=0.99120.

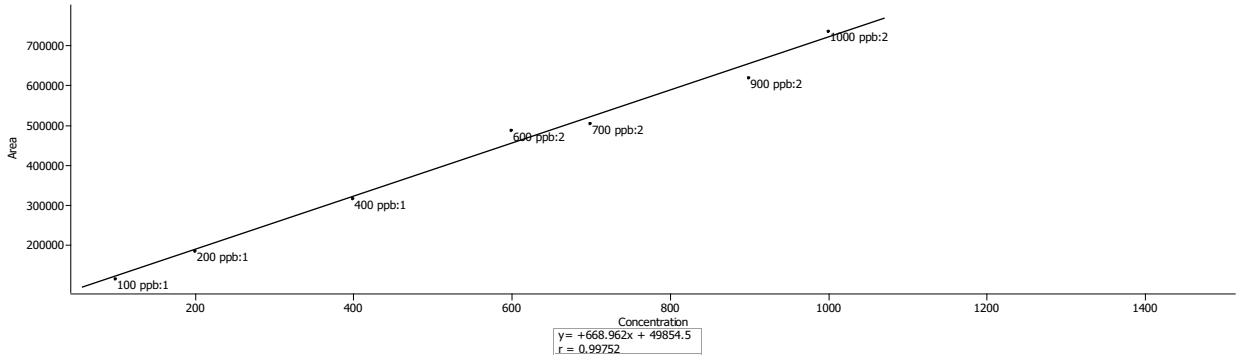


Figure C6: Calibration curve of the nitrosamine standard analyte; Pyrrolidine,1-nitroso-. Curve shows the different concentrations used to obtain the curve. y=668.962x+49854.5, r=0.99752.

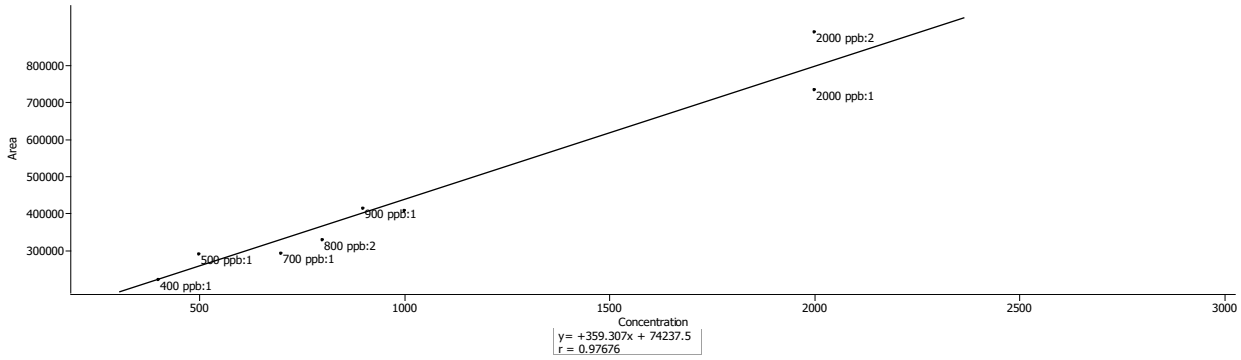


Figure C7: Calibration curve of the nitrosamine standard analyte Morpholine,4-nitroso-. Curve shows the different concentrations used to obtain the curve. y=359.307x+74237.5, r=0.97676.

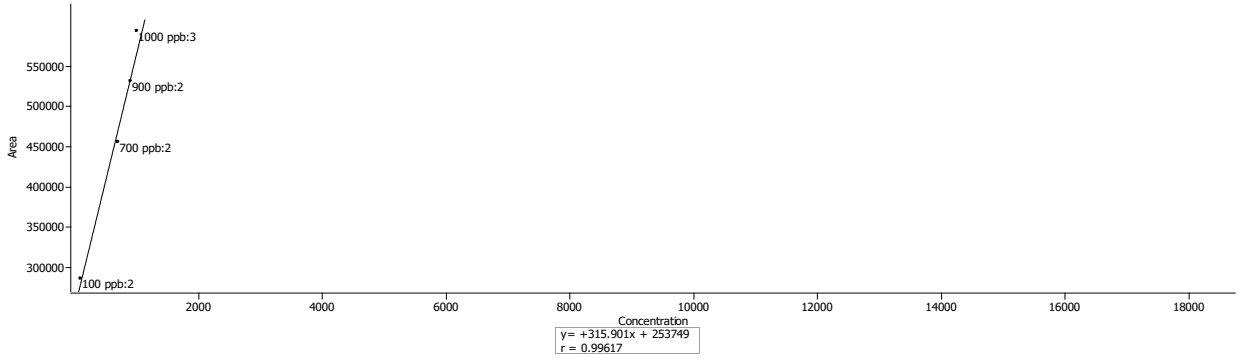


Figure C8: Calibration curve of the nitrosamine standard analyte N-Nitrosodiphenylamine. Curve shows the different concentrations used to obtain the curve. y=315.901x+253749, r=0.99617.

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