UNRAVELLING THE METABOLIC AND IMMUNOLOGICAL IMPACTS OF *HELICOBACTER PYLORI* ERADICATION IN HEALTHY ADULTS/YOUTHS THROUGH OMICS

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ABSTRACT

Helicobacter pylori is an important bacterial aetiological agent of gastroduodenal diseases. *H. pylori* positivity is also a risk factor for gastric adenocarcinoma and MALT lymphoma. Ironically, accumulating evidence demonstrates that *H. pylori* may protect the human host against obesity and atopic disorders. We hypothesised that disappearance *H. pylori* leads to changes in the human gut microbiome resulting in local and systemic changes in metabolism that may contribute to eventual development of undesirable metabolic and immunological disorders. This study was therefore, carried out to investigate the implications of *H. pylori* eradication and the association with metabolic and immunological disorders in a young healthy adult population. From 573 healthy adult volunteers (18-30 years-old) screened, the prevalence of *H. pylori* infection was 9.9%. Eventually, 29 *H. pylori*-positive subjects were enrolled and assessed during baseline followed by 6, 12 and 18 months post-*H. pylori* eradication. Faecal metagenomics and lipidomics were used to evaluate the local effects following *H. pylori* eradication on the gut microbiota and further metabolic, immunological and plasma metabolomic studies were performed to reflect the systemic effects of *H. pylori* eradication. The impact of *H. pylori* on the human gut microbiome pre- and post-eradication was investigated using *16S rRNA* gene (V3-V4 region) sequencing and data analysis using Qiime pipeline. The local and systemic effects of *H. pylori*, post-eradication, were examined through untargeted faecal lipidomics and plasma metabolomics using liquid chromatography mass spectrometry (LC-MS). The effect of *H. pylori* eradication on meal-associated changes on gastrointestinal metabolic hormones, cytokines and Immunolglobulin E (IgE) antibody level were evaluated using a multiplex bead assay and enzyme-linked immunosorbent assay, correspondingly. The microbial diversity was found to be similar pre- and post-*H. pylori* eradication with no

significant differences in bacterial richness and evenness. Despite that, some changes in the bacterial communities at the phylum and genus levels were notable, particularly the decrease in relative abundance of *Bacterioidetes* and the corresponding increase in *Firmicutes* following *H. pylori* eradication. The significant increase of short-chain fatty acids (SCFA)-producing bacteria genera could also be associated with increased risk of metabolic disorders. Faecal lipidomics and plasma metabolomics revealed that eradication of *H. pylori* dramatically changed many global metabolite/lipid features, with the majority of them being down-regulated. The influence of gut microbiota on plasma metabolites profile was also demonstrated. These findings primarily implicate the perturbation of gut microbiota following *H. pylori* eradication in host energy and lipid metabolism which may eventually lead to the development of metabolic disorders. Metabolic studies demonstrated that *H. pylori* eradication was associated with long-term disturbance in active amylin, pancreatic polypeptide and total peptide YY both pre- and post-prandially and glucagon-like peptide-1 post-prandially ($p<0.05$). An inverse association between *H. pylori* infection and allergen specific-IgE antibodies ($p<0.05$) was observed. The predictive metabolic signature of metabolic and immunological disorders following *H. pylori* eradication may give us insights on complex interaction of *H. pylori* with gut microbiota, the importance of biosis of the gut microbiota and their implications in human health. In conclusion, eradication of *H. pylori* demonstrated intricate and complex interactions between *H. pylori* and the gut microbiota in modulating human health and therefore, a point to ponder upon future management of *H. pylori* infection.

ABSTRAK

Helicobacter pylori merupakan etiologi bakteria yang penting untuk penyakit gastrousus. Positif *H. pylori* juga merupakan faktor risiko untuk gastrik adenokarsinoma and limfoma MALT. Ironinya, bukti terdahulu menunjukkan bahawa *H. pylori* dapat melindungi manusia daripada obesiti dan penyakit atopik. Kami menghipotesiskan bahawa kehilangan *H. pylori* membawa perubahan kepada mikrobiom usus manusia dan menyebabkan perubahan setempat dan sistemik metabolisme yang berkemungkinanan menyebabkan gangguan metabolik and imunologi. Oleh itu, kajian ini dijalankan untuk memahami implikasi penyingkiran *H. pylori* dan hubungannya dengan penyakit metabolik dan imunologi di kalangan populasi dewasa awal yang sihat. Daripada 573 sukarelawan dewasa yang sihat (18-30 tahun) yang diperiksa, kadar prevalens jangkitan *H. pylori* adalah 9.9%. Hasilnya, 29 subjek positif *H. pylori* didaftarkan and dinilai semasa penilaian asas dan diikuti dengan penilaian pada 6, 12 dan 18 bulan pasca-penyingkiran *H. pylori*. Metagenomik dan lipidomik tinja digunakan untuk menilai kesan setempat pada mikrobiota usus pasca-penyingkiran *H. pylori*. Kajian metabolik, imunologi dan metabolomik plasma pula djalankan untuk mencerminkan kesan sistemik terhadap penyingkiran *H. pylori*. Impak *H. pylori* kepada mikrobiom usus pra- dan pasca-penyingkiran *H. pylori* dikaji dengan penjujukan gen *rRNA 16S* (rantau V3-V4) dan analisis data dengan perisian Qiime. Kesan setempat dan sistemik pasca-penyingkiran *H. pylori* diperiksa melalui lipidomik tinja dan metabolomik plasma-tidak khusus dengan menggunakan kromatografi cecair spektrometri jisim (LC-MS). Di samping itu, kesan penyingkiran *H. pylori* terhadap hormon gastrousus berkaitan perubahan pemakanan dinilai dengan ujian manik multipleks manakala aras sitokin dan antibodi IgE dinilai dengan ujian ELISA. Kepelbagaian mikrob didapati serupa pra- dan pasca-penyingkiran *H. pylori* iaitu jumlah dan keseragaman spesies bakteria tidak

mempunyai perbezaan yang signifikan. Walaupun demikian, perubahan yang menarik untuk komuniti bakteria pada tahap filum dan genus dapat ditemui. Penurunan bilangan relatif *Bacterioidetes* dan peningkatan yang sama dalam bilangan relatif *Firmicutes* pasca-penyingkiran *H. pylori*. Peningkatan signifikan genus bakteria yang menghasilkan rantai pendek asid lemak (SCFA) juga mungkin dikaitkan dengan peningkatan risiko penyakit metabolik. Kajian lipidomik tinja dan metabolomik plasma mendedahkan bahawa penyingkiran *H. pylori* mengubah ciri-ciri lipid/metabolit global secara dramatik iaitu pengawalaturan untuk majoriti lipid/metabolit diturunkan. Pengaruh mikrobiota usus terhadap profil metabolit di dalam plasma juga ditunjukkan dalam kajian ini. Penemuan dalam kajian ini terutamanya mengimplikasikan bahawa gangguan mikrobiota usus dalam metabolisme tenaga dan lipid perumah pasca-penyingkiran *H. pylori* berkemungkinan menyebabkan penyakit metabolik. Kajian metabolik menunjukkan bahawa penyingkiran *H. pylori* berkait dengan gangguan jangka panjang untuk hormon amilin aktif, polipeptida pankreas dan jumlah peptida YY sebelum dan selepas ujian makanan, serta gangguan terhadap hormon seakan glukagon peptida-1 selepas ujian makanan ($p<0.005$). Perkaitan songsang antara jangkitan *H. pylori* dan antibodi IgE yang spesifik kepada alergen ($p<0.005$) diperhatikan. Penjangkaan tanda-tanda penyakit metabolik dan imunologi selepas penyingkiran *H. pylori* mungkin dapat memberikan kita pemahaman mengenai interaksi kompleks *H. pylori* dengan mikrobiota usus, kepentingan biosis terhadap mikrobiota usus dan juga implikasinya dalam kesihatan manusia. Kesimpulannya, kajian ini menunjukkan hubungan kompleks antara *H. pylori* dan mikrobiota usus dalam pengawalaturan kesihatan manusia. Maka, kita perlu memberi perhatian kepada pengurusan jangkitan *H. pylori* pada masa hadapan.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
°C	degree Celsius
bp	base pair
DOB	delta-over-baseline value
g	gravity
hr	hour
kDa	kilodalton
kV	kilovolt
l	liter
M	molar
mM	millimolar
mm	millimeter
ml	milliliter
mg	milligram
min	minute
nm	nanometer
ng	nanogram
OTUs	Operational Taxonomic Units
pg	picogram
ppm	part per million
psig	pounds per square inch gauge
rpm	rotation per minute
s	second
µg	microgram
µl	microliter

µM	micromolar
µm	micrometer
U	Unit
V	volt
vs.	versus
w/v	weight to volume
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
BAs	bile acids
BCAA	branched-chain fatty acids
BMI	body mass index
BVR	biliverdin reductase
C1P	ceramide-1-phosphate
cagA	cytotoxin-associated gene A
CerS	ceramide synthase
CM	CagA multimerization sequence
CI	confidence interval
CRPIA	conserved repeat responsible for phosphorylation-independent activity
COX2	cyclooxygenase 2
DCs	dendritic cells
DES	dihydroceramide desaturase
DG	diacylglycerols
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
EPIYA	Glu-Pro-Ile-Tyr-Ala motifs
ESI	electrospray ionisation

ESSAY	Eradication in Stable Adults/Youths
FA	fatty acyls
FAD	flavin adenine dinucleotide
FDR	false discovery rate
FT	fourier transform
FXR	farnesoid X receptor
GERD	gastroesophageal reflux disease
GL	glycerolipids
GP	glycerophospholipids
FD	functional dyspepsia
GC	gas chromatography
GI	gastrointestinal tract
GIP	gastric inhibitory polypeptide
GLP-1	glucagon-like peptide-1
H & E	hematoxylin and eosin stained
HGP	Human Genome Project
HO	heme oxygenase
IL	Interleukin
LC	liquid chromatography
LPS	lipopolysaccharides
MALT	mucosa-associated lymphoid tissue
MFI	median Fluorescent Intensity
MG	monoacylglycerols
MG-RAST	MetaGenome Rapid Annotation using Subsystem Technology
MKI	MARK2/PAR1b kinase inhibitor
MPP	Mass Profiler Professional

MS	mass spectrometry/mass spectrometer
MTBE	methyl tert-butyl ether
MSTFA	N-methyltrimethylsilyltrifluoroacetamide
NAD	nicotinamide-adenine dinucleotide
NGS	Next-Generation Sequencing
NHANES	National Health and Nutrition Examination Survey
NSAID	non-steroidal anti-inflammatory drug
NMR	^{1}H nuclear magnetic resonance spectroscopy
NYU	New York University
NAP	neutrophil activating protein
NFκB	nuclear factor κB
PAO	polyamine oxidase
PBQC	pooled biological quality control samples
PC	phosphatidylcholines
PCA	Principle Component Analysis
PCoA	Principal Coordinate Analysis
PCR	polymerase chain reaction
PE	phosphatidylethanolamines
PK	polyketides
PPI	proton-pump inhibitor
PP	pancreatic polypeptide
PR	prenol lipids
PS	phosphatidylserines
PYY	peptide YY
Qiime	Quantitative Insights into Microbial Ecology
QqQ	triple quadrupole

QTOF	quadrupole-time-of-flight
RUT	rapid urease test
S1P	sphingosine 1-phosphate
SCFA	Short-chain fatty acids
SD	standard deviation
SL	saccharolipids
SM	sphingomyelins
SMase	sphingomyelinase
SODA	Validated Severity of Dyspepsia Assessment questionnaire
SP	sphingolipids
SPE	solid phase extraction
SPT	serine palmitoyltransferase
SSA	species-specific antigen
SSAT	spermidine-spermineN1-acetyltransferase
ST	sterol lipids
Th1 or 2	T-helper type 1 or 2
TCA	tricarboxylic acid cycle
TAG/TG	triaglycerides/triacyglycerols
TNF-α	tumor necrosis factor α
TIC	total ion chromatogram
Tregs	regulatory T cells
UBT	urea breath Test
UMMC	University Malaya Medical Centre
vacA	vacuolating cytotoxin A
WMS	whole metagenome shotgun sequencing

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Research Background

Traditionally, it was estimated that he human body contains about 10^{13} cells, routinely harbouring approximately 100 trillion bacteria which outnumber our human cells by 10 to 1 (Savage, 1977). More recent estimates have lowered that ratio to 3:1 or even to approximately the same number (Sender, Fuchs, & Milo, 2016). This bacterial population constitutes the microbiota and majority of them live in the lower part of the gastrointestinal tract. The human gut microbiota is an intricate and dynamic ecosystem that has coevolved with humans for millions of years (Ley et al., 2008). It has been regarded as a metabolically active "organ" located within the human gastrointestinal tract (Hooper, Midtvedt, & Gordon, 2002; O'Hara & Shanahan, 2006) as it has developed metabolic traits that complement the host's metabolism (Cantarel, Lombard, & Henrissat, 2012; Hooper et al., 2001; Li et al., 2008; Rawls, Mahowald, Ley, & Gordon, 2006). A healthy gastrointestinal system relies on a balanced commensal microbiota to regulate processes such as energy metabolism (Cani & Delzenne, 2007, 2009; Harris, Kassis, Major, & Chou, 2012; Tilg & Kaser, 2011), eliminates pathogens (Buffie & Pamer, 2013; Endt et al., 2010; Sekirov, Russell, Antunes, & Finlay, 2010) and influences signalling pathways that range from modulation of the mucosal immune response (Kelly, Conway, & Aminov, 2005) to the development of metabolic diseases (Cani & Delzenne, 2009; DiBaise, Frank, & Mathur, 2012; Harris et al., 2012; Tilg & Kaser, 2011; Tremaroli & Backhed, 2012). Accumulating evidences suggest that dysbiosis, or an abnormal microbiota, has been associated with an increasingly long list of diseases, including inflammatory bowel disease, obesity, and atopic diseases such as eczema and asthma (Sekirov et al., 2010; Sun & Chang, 2014).

Helicobacter pylori is a Gram-negative, spiral-shaped, microaerophilic bacterium that colonises the gastric mucosa of humans. More than half of the world's population is colonised with *H. pylori* in which prevalence rates as high as 80% being reported in some populations (Clyne, Dolan, & Reeves, 2007). All those infected with *H. pylori* develop gastritis, the majority of whom are asymptomatic. Of those infected, approximately 10-20% progress to peptic ulcer disease, 1-3% to gastric cancer and <0.5% to mucosa-associated lymphoid tissue (MALT) (Kusters, van Vliet, & Kuipers, 2006). *H. pylori* is believed to have appeared in the stomach of humans at least since the initial migration of our ancestors from East Africa at least 100,000 years ago (Moodley et al., 2012). It has been postulated that *H. pylori* may be part of the human indigenous microbiota (Blaser, 2006). However, due to socioeconomic development, modern hygienic practices and the advent of antibiotics, the human gut microbiota is further evolving and *H. pylori* is gradually disappearing from the human indigenous microbiota (Blaser, 2006). There is an increasing number of epidemiological and experimental evidence on the protective effect of *H. pylori* against the development of obesity (Francois et al., 2011; Lender et al., 2014; Nwokolo, Freshwater, O'Hare, & Randeva, 2003; Osawa, 2008), childhood asthma (Chen & Blaser, 2008; den Hollander etl al., 2016), allergies (Amberbir et al., 2011), inflammatory bowel diseases (Sonnenberg & Genta, 2012) and other diseases, in which the development of these diseases coincide with transient dysbiosis (Figure 1.1).

Appetite and energy homeostasis in humans are partly regulated by centrally-acting hormones, such as ghrelin and leptin produced in the gastric mucosa, which may be modified by *H. pylori* colonisation (Francois et al., 2011). Plasma ghrelin level increases during fasting and decreases after meal whereas the counterpart hormone, leptin, is produced in higher level following meals to induce satiation (Klok, Jakobsdottir, & Drent, 2007).

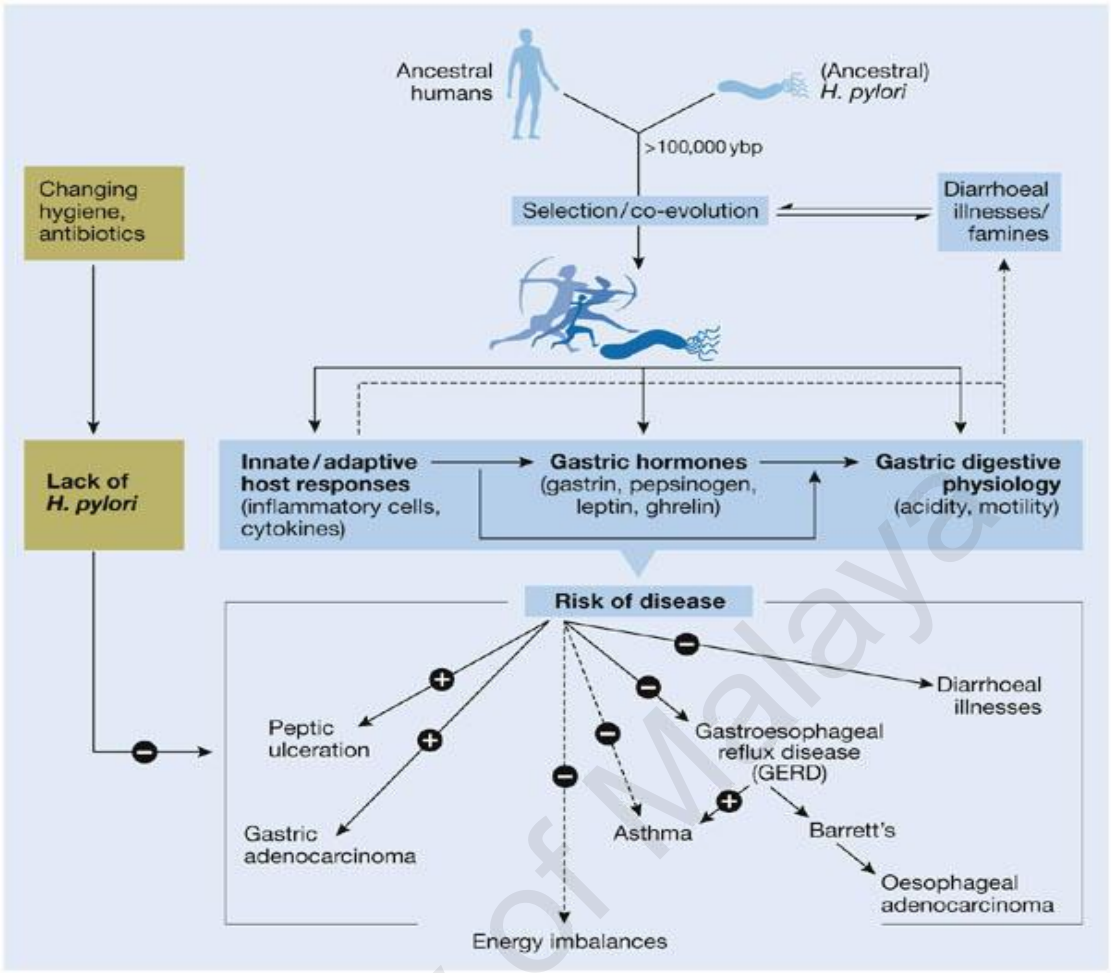


Figure 1.1: Hypothetical relationship of *H. pylori* with human health and disease (Blaser, 2006)

Evidence of the correlation between *H. pylori* colonisation and the regulation of ghrelin and leptin and consequently on the body morphometrics have been shown by several studies but their relationship remains controversial (Chan, Bullen, Lee, Yiannakouris, & Mantzoros, 2004; Cummings et al., 2001; Ikezaki et al., 2002; Tschop, Wawarta, et al., 2001). There are several reports indicating that eradication of *H. pylori* has been associated with an increase in plasma ghrelin level (Francois et al., 2011; Nwokolo et al., 2003). Thus, it has been suggested that the eradication of *H. pylori* might be one of the factors that could fuel epidemic human obesity in developed countries (Francois et al., 2011; Weigt & Malfertheiner, 2009). On the contrary, some studies have also shown that *H. pylori* colonisation has no effect on plasma ghrelin levels (Choe et al., 2007;

Gokcel et al., 2003; Osawa, 2008) or negatively associated with plasma ghrelin level (Kasai et al., 2016; Pacifico et al., 2008).

In another instance, the inverse association between *H. pylori* seropositivity and asthma in children was firstly reported in a cross sectional analysis, using data from 7412 participants from the National Health and Nutrition Examination Survey (NHANES) 1999-2000. In this study, *H. pylori* seropositivity was found to be inversely associated with the onset of asthma before 5 years of age and current asthma in children aged 3–13 years. In addition, colonisation with CagA+ *H. pylori* strains is inversely associated with asthma and is associated with older age of asthma onset in an urban population was reported in a case control study conducted recently in the New York University (NYU)/Bellevue Asthma Registry in New York City. Thus, yet again, the decreasing prevalence of *H. pylori* is related to the presence of asthma; and *H. pylori* is suggested as a marker for protection (Reibman et al., 2008). *H. pylori* seropositivity was also inversely related to wheezing, allergic rhinitis, and dermatitis, eczema, or rash (Chen & Blaser, 2008). Hence, with the substantiation of experimental evidences, there is increasing interest in systems biology research to elucidate the molecular foundations of the gut microbial-mammalian host crosstalk at both the physiological and biochemical levels.

Over the last two decades, systems biology has emerged as a novel way forward to provide insights into the role of mammalian gut microbial metabolic interactions in an individual's susceptibility to health and disease outcomes (Martin, Collino, Rezzi, & Kochhar, 2012). The emergence of systems biology is coinciding with the completion of the Human Genome Project (HGP) (Lander et al., 2001; Venter et al., 2001) and the concomitant emergence of 'omics technologies, namely transcriptomics (Lashkari et al., 1997; Schena, Shalon, Davis, & Brown, 1995), proteomics (Patterson & Aebersold, 2003), metabolomics (Fiehn, 2001; Oliver, Winson, Kell, & Baganz, 1998) and more

recently, lipidomics (Han & Gross, 2003; Wenk, 2005). The metabolome is influenced by genetic variants, epigenetic factors, changes in gene expression, or enzyme activity, as well as by environmental factors (such as diet, physical activity, and pharmaceuticals) and aging (Zierer, Menni, Kastenmüller, & Spector, 2015). Thus, metabolomics is a valuable tool to study environment-related modifications of the genetic susceptibility. Lipidomics, a dominant part of metabolomics, is the detailed analysis and global characterisation, both spatial and temporal, of the structure and function of lipids (the lipidome) within a living system (Harkewicz & Dennis, 2011).

Omics technologies could be utilised to decipher the highly complex metabolic exchange between the diverse biological compartments of *H. pylori* infected human host (including tissues, organs and systemic biofluids) and the gut microbiota and ultimately to help further understand the profound influence exerted by *H. pylori* and gut bacterial flora on the metabolic equilibrium of the host and, as a consequence, on its health status. Therefore, this study was carried out in a young healthy adult population to unravel the implications and underlying mechanisms of *H. pylori* eradication, particularly in the Asian community. Faecal metagenomics and lipidomics enabled us to evaluate the local effects following *H. pylori* eradication on the gut microbiota whereas metabolic study, immunological study and plasma metabolomics reflected the systemic effects of *H. pylori* eradication. Ultimately, our study also provided insights on *H. pylori* infection management and treatment to minimize the undesirable implications on the metabolic and immunological disorders.

1.2 Hypothesis

Disappearance of gastric bacterium, *H. pylori*, leads to changes in the human gut microbiome resulting in local and systemic changes in metabolites that may contribute to eventual development of undesirable metabolic and immunological disorders.

1.3 Objectives

The aim of this study is to elucidate the possible mechanisms underlying the association of *H. pylori* with human metabolic and immunological disorders through -omics profiling, metabolic and immunological study.

The specific objectives are:

1. To determine the prevalence of *H. pylori* infection in healthy young adults in Malaysia.
2. To elucidate possible roles of *H. pylori* in human metabolic and immunological disorders by:
    - Investigating the localised effects of *H. pylori* eradication on the human gut microbiome and faecal lipid profiles.
    - Analysing the systemic effects of *H. pylori* eradication on the plasma metabolite profiles and circulating gut hormones in relation to changes in anthropometric parameters.
    - Correlating the immunological effects of *H. pylori* eradication with serum cytokines and Immunoglobulin E (IgE) antibody level.

1.4 Outline of the research approach

The research approach used to achieve the aim and objectives of this study is summarised in Figure 1.2.

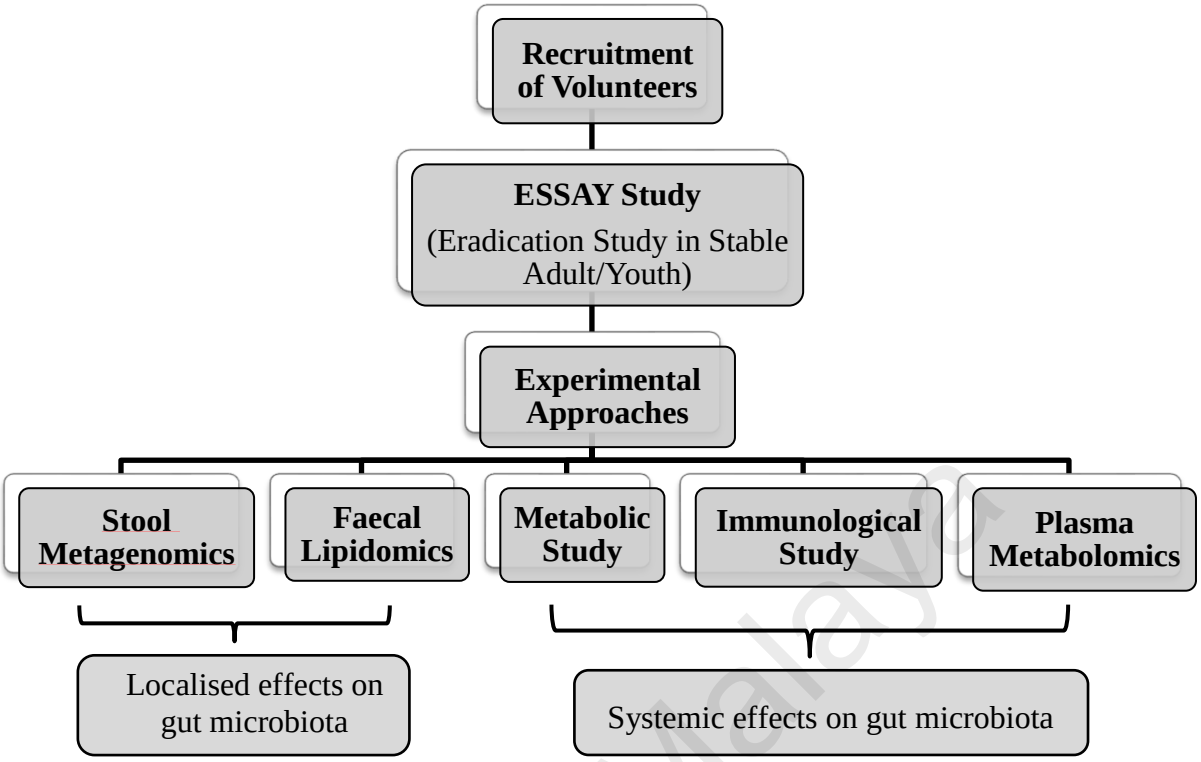


Figure 1.2: Outline of the research approach

CHAPTER 2: LITERATURE REVIEW

2.1 The genus *Helicobacter*

In general, *Helicobacter* is a genus of Gram-negative bacteria within the phylum of epsilon (ε-) *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae* that possesses the characteristic helical/curved shaped with variant forms of short or tapered rods, and they are motile by means of their flagella. The bacteria species in this genus were initially considered to be members of *Campylobacter*, but in 1989, Goodwin and co-workers published substantial experimental evidence to justify for categorising them as an entirely new genus named *Helicobacter* (Goodwin et al., 1989).

The *Helicobacter* genus consists of a group of microorganisms that colonise the mucus layer of epithelial surface of the gastrointestinal tract of humans and a variety of animal species. To date, this genus consists of over 20 recognized species, with many species awaiting formal recognition (Fox, 2002). *Helicobacter* species can be subdivided into two major lineages, the gastric *Helicobacter* species and the enterohepatic (non-gastric) *Helicobacter* species. Both groups demonstrate a high level of organ specificity, such that gastric helicobacters in general are unable to colonise the intestine or liver, and vice versa (Kusters et al., 2006). There are currently 12 validated gastric *Helicobacter* species and 21 validated enterohepatic *Helicobacter* species isolated from human and animals. In addition, there are nine Candidatus and unvalidated species (Mitchell, Rocha, Kaakoush, O'Rourke, & Queiroz, 2014). Some *Helicobacter* species may be commonly (*Helicobacter aurati*) or occasionally (*Helicobacter bilis* and *Helicobacter muridarum*) isolated from both gastric and enterohepatic sites (Solnick, O'Rourke, Vandamme, & Lee, 2006).

Of all the *Helicobacter* species, the most widely known species of the genus is *Helicobacter pylori*, which is the common human pathogen that is strongly associated with gastroduodenal diseases.

2.2 *Helicobacter pylori*

2.2.1 Historical background of the discovery of *Helicobacter pylori*

As early as 1875, a German gastric bacteriologist, G. Bottcher, and his French collaborator, M. Letulle, demonstrated bacterial colonies in the ulcer floor and in its mucosal margins and they suggested a causative bacterial agent in ulcer disease. However, their suggestion was not a popular point of view, despite a similar observation was reported by C. Klebs. In 1889, W. Jaworski first described in detail spiral organisms in the sediment of washings obtained from humans. He suggested that these spiral organisms might play a possible pathogenic role in gastric disease (Kidd & Modlin, 1998). At about the same time, G. Bizzozero noted the presence of similar organism or "spirochetes" in the gastric glands and both in the cytoplasm and vacuoles of parietal cells in the specimens of the gastric mucosa of dogs (Bizzozero, 1893). H. Solomon also reported spirochetes in the gastric mucosa of dogs, cats and rats, although he was unable to identify them in other animals, including man (Solomon, 1896).

In 1909, spiral-shaped bacteria were observed microscopically for the first time in the stomach of a patient with gastric cancer by W. Krienitz (Krienitz, 1909). However, it was generally accepted by the scientific community back then that excessive acid production was the cause of chronic ulcers in the stomach (Kidd & Modlin, 1998). In 1938, Doenges reported spirochettes in about 40% of human stomachs from autopsied individuals (Doenges, 1938). Two years later, Freedburg and Barron reported spirochettes in fresh surgical specimens from human stomachs with ulcer or carcinoma (Freedberg & Baron, 1940). Subsequently, the significance of these

organisms was alternately debated and forgotten until B. Marshall and R. Warren isolated a Gram-negative, microaerophilic bacterium from human gastric biopsies and proposed that the organism may be the cause of chronic gastritis and peptic ulcer disease (Marshall et al., 1984; Marshall & Warren, 1984). Their discovery of the bacterium *H. pylori* and its role in gastritis and peptic ulcer disease had led them to be awarded the highly honoured Nobel Prize in Physiology or Medicine in 2005.

Initially, the bacterium was thought to be a member of the genus *Campylobacter* and named "*Campylobacter pyloridis*" and later, it was revised to "*Campylobacter pylori*". Subsequent *16S rRNA* sequence analysis showed that the distance between *C. pylori* and the true Campylobacters was sufficient to exclude it from the *Campylobacter* genus (Romaniuk et al., 1987), and it was renamed "*Helicobacter pylori*" (Goodwin et al., 1989), the first member of the new Helicobacter genus.

2.2.2 Microbiology of *Helicobacter pylori*

Helicobacter pylori is a microaerophilic, non-spore forming, s- or curved rod-shaped Gram-negative bacterium that colonises primarily the human gastric mucosa. *H. pylori* is typically about 2-4 μm long with a diameter of about 0.5-0.9 μm and can have up to six unipolar sheathed flagella filaments (Anderson & Wadström, 2001; Owen, 1998). The curved morphology of *H. pylori* and the polar motility caused by its flagella at one end caused its screw-like movements, which may enable the organism to penetrate the mucin layer of stomach (Anderson & Wadström, 2001). In contrast to many other pathogens of the gastrointestinal tract, it lacks fimbrial adhesins (Kusters et al., 2006).

H. pylori is a fastidious microorganism and requires complex growth media. Often these media are supplemented with blood or serum. These supplements may act as additional sources of nutrients and possibly also protect against the toxic effects of long-chain fatty acids (Kusters et al., 2006). Commonly used solid media for routine

isolation and primary culture of *H. pylori* from gastric tissue biopsies consist of Columbia or Brucella agar supplemented with either (lysed) horse or sheep blood or, alternatively, newborn or foetal calf serum. While Columbia or Brucella agar is used, many laboratories also use horse blood agar plates (Blood Agar Base No. 2 supplemented with 10% sterile defibrinated horse blood). To prevent the overgrowth of other commensal bacteria in the stomach or oral cavity, a selective medium is usually recommended in addition to the nonselective media for routine culture. The often used selective antibiotic mixture, such as Dent supplement consists of vancomycin, trimethoprim, cefsoludin, and amphotericin B (Dent & McNulty, 1988), whereas the alternatively used Skirrow supplement consists of vancomycin, trimethoprim, polymyxin B, and amphotericin B (Skirrow, 1977). Both selective supplements are commercially available.

Colonies of *H. pylori* from primary culture on supplemented blood agar at 37°C under microaerobic atmosphere (2 to 5% O_2, 5 to 10% CO_2, and 0 to 10% H_2) usually take 3-5 days to appear and are circular (1-2 mm), convex and translucent in appearance. There is slight haemolysis in blood agar around colonies, which are greyish in colour (Anderson & Wadström, 2001; Owen, 1998; Solnick & Vandamme, 2001). Although its natural habitat is the acidic gastric mucosa, *H. pylori* is considered to be a neutralophile (Kusters et al., 2006). In very young cultures, *H. pylori* may appear as almost straight rods on microscopy. After 3 to 5 days of incubation the bacteria look pleomorphic, with irregular curved rods, several being U shaped. In old cultures, *H. pylori* appears as degenerative, possibly viable but non-culturable coccoid forms that Gram stain poorly (Anderson & Wadström, 2001). *H. pylori* is also capable of forming biofilm (Stark et al., 1999). Both conversion to coccoid forms and biofilm formation could be favourable to enhance resistance to host immunity defence mechanism and antibiotics, as well as,

to facilitate the growth and survival of this bacterium (Percival & Suleman, 2014; Sarem & Corti, 2015).

H. pylori has a similar composition of cell envelope to other typical Gram-negative bacteria. Its cell envelope consists of an inner cell membrane, periplasm with peptidoglycan, and an outer membrane (Anderson & Wadström, 2001). The outer membrane is composed by phospholipids and lipopolysaccharides (LPS). The phospholipids moiety of *H. pylori* outer membrane contains cholesterol glucosides, which are very unusual in other bacteria. LPS usually composed of an O side chain and a core oligosaccharide, covalently linked to a lipid moiety termed lipid A (Kusters et al., 2006). To date, the complete structure of *H. pylori* LPS is not available, and the proposed model is a linear arrangement composed of the inner core defined as the hexa-saccharide (Kdo-LD-Hep-LD-Hep-DD-Hep-Gal-Glc), the outer core composed of a conserved trisaccharide (-GlcNAc-Fuc-DD-Hep-) linked to the third heptose of the inner core, the glucan, the heptan and a variable O-antigen, generally consisting of a poly-LacNAc decorated with Lewis antigens (Li et al., 2016). A striking characteristic of *H. pylori* LPS is the significant lower immunological activity compared with enterobacterial LPS as the gold standard (Appelmelk, Negrini, Moran, & Kuipers, 1997). Another striking feature of *H. pylori* LPS is the expression of Lewis and blood group mimicry in the O antigen component of this molecule, in which it is proposed to be associated with gastric adaptation, modulation of immune response and immune evasion (Appelmelk, Negrini, Moran, & Kuipers, 1997). *H. pylori* LPS also displays phase variation through length variation of poly(C) tracts in the genes encoding α-1,3-fucosyltransferases (Appelmelk et al., 1999) and a poly(C) tract and poly(TAA) repeats in the gene encoding the α-1,2-fucosyltransferase (Wang, Rasko, Sherburne, & Taylor, 1999). This LPS phase variation contributes to population heterogeneity and may allow adaptation of *H. pylori* to changing conditions in the gastric mucosa (Moran et al., 2002;

Tannaes, Bukholm, & Bukholm, 2005; Tannaes, Dekker, Bukholm, Bijlsma, & Appelmelk, 2001).

H. pylori possesses five major outer membrane protein families. The largest family includes known and putative adhesins such as 21 Hop and 12 Hor outer membrane proteins (Oleastro & Ménard, 2013). The other four families are porins, iron transporters, flagellum-associated proteins, and proteins of unknown function (Kusters et al., 2006). *H. pylori* produces urease, catalase and superoxide dismutase which are the key enzymes that play important roles for its persistent colonisation in the hostile environment of the stomach. Urease is necessary for the survival of *H. pylori* in the acidic stomach as it provides a pH-neutral microenvironment around the bacteria (Hawtin, Stacey, & Newell, 1990; Perez-Perez, Olivares, Cover, & Blaser, 1992). Catalase protects *H. pylori* against the damaging effects of hydrogen peroxide released from phagocytes (Hazell, Evans, & Graham, 1991). Urease and catalase may also be excreted from *H. pylori* to the surrounding environment and protect this pathogen from the host's humoral immune response (Hawtin et al., 1990). Similarly, superoxide dismutase breaks down superoxide produced in polymorphonuclear leukocytes and macrophages and thereby prevents phagocytosis (Spiegelhalder, Gerstenecker, Kersten, Schiltz, & Kist, 1993). *H. pylori* also produces hydrogenase to obtain energy by oxidising molecular hydrogen (H_2) produced by intestinal microflora (Olson & Maier, 2002).

To date, there are 86 genomes of *H. pylori* that have been completely sequenced. The median of the total length of the complete sequenced of *H. pylori* genomes is 1.63461 Mbp, with a median G+C content of 38.9% (http://www.ncbi.nlm.nih.gov/genome). The genome of different *H. pylori* strains displays a high degree of diversity (Hacker & Kaper, 2013; Jiang, Hiratsuka, & Taylor, 1996). This results in every *H. pylori*-positive subject carrying a distinct strain (Kansau

et al., 1996), although differences within relatives may be small. The genetic heterogeneity is possibly an adaptation of *H. pylori* to the gastric conditions of its host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection (Kuipers et al., 2000; Kusters et al., 2006).

2.2.3 Epidemiology of *Helicobacter pylori* infection

The prevalence of *H. pylori* infection shows large geographical variations, with the largest differences being observed between developed and developing countries. Numerous reports from different parts of the world showing widely differing prevalence rates with the less developed and poorer countries in Asia, Africa and South America carrying a heavier infection burden compared to the more developed countries in Europe and North America (Eusebi, Zagari, & Bazzoli, 2014). In addition, the prevalence of *H. pylori* infection also varies within countries and within subpopulations within the same country. Several studies have shown that there is an increasing prevalence of *H. pylori* infection with age (Graham, Malaty, et al., 1991; Lim et al., 2013). It can be attributed to new acquisition of infection among the adult population or to the presence of different birth cohorts with different rate of acquisition during childhood within the population. The reports suggested that the increase prevalence of *H. pylori* infection with age may be related to different birth cohorts in which each successively younger cohort has had a lower rate of acquisition of *H. pylori* infection than those born earlier (Banatvala et al., 1993; Lim et al., 2013; Parsonnet, Blaser, Perez-Perez, Hargrett-Bean, & Tauxe, 1992).

Furthermore, several studies showed that prevalence of *H. pylori* is inversely related to socio-economic status with the major variable being socio-economic status during childhood, the period of highest risk of acquisition (Cheng et al., 2009; Graham, Malaty, et al., 1991; Malaty, Evans, Evans, & Graham, 1992; Malaty, Kim, Kim, &

Graham, 1996; Malaty, Logan, Graham, & Ramchatesingh, 2001; Malaty et al., 1996; Mitchell et al., 1992; Monno et al., 2008; Sitas et al., 1991). Socio-economic status was defined by occupation, family income level and living conditions (Malaty, 2010).

H. pylori infection has been shown to follow the human route of migration and reflect human ancestry. In Malaysia, ethnic differences in the prevalence of *H. pylori* have been reported. The prevalence of *H. pylori* infection among Indians (29%-62%) is consistently higher than in Chinese (18%-58%) and Malays (7%-29%) in this region (Boey, Goh, Lee, & Parasakthi, 1999; Goh, 1997, 2009; Goh & Parasakthi, 2001; Kaur & Naing, 2003; Sasidharan et al., 2012). This phenomenon leads to the proposal of the "racial cohort" theory (Goh & Parasakthi, 2001). Owing to the relative distinctiveness of all three major racial groups in Malaysia and low level of intermarriages between races, *H. pylori* has remained confined to a particular racial group. The Malays who have a low reservoir of *H. pylori* to begin with, continues to have a low prevalence of infection. The authors suggested that the high prevalence among Chinese and Indians in Malaysia reflected the high *H. pylori* prevalence in Southern China and Southern India from where these races had originally come from. Albeit migration had taken place more than three generations ago, the high *H. pylori* prevalence among the Chinese and Indians remains, with intra-racial/intra-community spread taking place and with low cross-infection occurring between races (Goh & Parasakthi, 2001). This "racial cohort" theory could be supported with several studies reporting that distinctive strains of *H. pylori* have been isolated from different racial groups in Malaysia (Ramelah et al., 2005; Tay et al., 2009). Variation in acquisition of *H. pylori* among different racial groups appears to be essentially related to factors influencing differential exposure to the bacterium, for instance cultural background, social, dietary and environmental factors (Graham, Malaty, et al., 1991; Malaty, Graham, Isaksson, Engstrand, & Pedersen, 1998; Mitchell et al., 1992).

To date, the exact mode of transmission of *H. pylori* is still unclear. Based on the numerous studies reported, it is most likely to be transmitted via person to-person transmission by either the oral-oral or faecal-oral route (Brown, 2000). Transmission occurs mainly within families during early childhood and usually transmitted from either infected father or mother (Malaty, Graham, Klein, Adam, & Evans, 1991; Mitchell, Bohane, Hawkes, & Lee, 1993; Weyermann, Rothenbacher, & Brenner, 2009). However, some studies also reported that *H. pylori* infection is mostly linked to the mother (Escobar & Kawakami, 2004; Kivi, Johansson, Reilly, & Tindberg, 2005; Rothenbacher, Winkler, Gonser, Adler, & Brenner, 2002; Yamaoka, Malaty, Osato, & Graham, 2000) especially when the father has little contact with the children (Malaty et al., 2000). Besides, transmission by birth order has also been reported as well in which *H. pylori* seems to be transmitted most readily among siblings who are close in age, and most frequently from older siblings to younger ones (Goodman & Correa, 2000). All these findings indicate the importance of contact and level of household sanitation in the transmission of *H. pylori* (Nouraie et al., 2009; Weyermann et al., 2009) and it is likely that the routes of transmission are person-to-person and/or exposure to a common source of infection (Malaty, 2010).

The transmission of *H. pylori* through oral-oral route or faecal-oral route is still a subject of debate. The rationale of transmission by the oral-oral route is based on the premise that *H. pylori* is present in the gastric juice and hence it could reach the oral cavity through reflux and vomitus (Malaty, 2010). Indeed, an early study reported that *H. pylori* can be cultivated from vomitus and, occasionally, from saliva and cathartic stools (Parsonnet, Shmuely, & Haggerty, 1999). The organism is potentially transmissible during episodes of gastrointestinal tract illness, particularly with vomiting (Parsonnet et al., 1999; Perry et al., 2006). There is mounting evidence suggesting that *H. pylori* may also be transmitted orally by means of faecal matter through the ingestion

of waste-contaminated water, which is related to poor sanitary practices (Klein, Graham, Gaillour, Opekun, & Smith, 1991; Nurgalieva et al., 2002). Reducing of the rate of *H. pylori* transmission will require improvement in sanitation including waste disposal, clean water and safe food, as well as in household hygiene practices (Nurgalieva et al., 2002). Nonetheless, the lack of established culture methods for the detection of viable *H. pylori* in the environment, particularly the drinking water supplies, has prevented the development of true epidemiological and risk assessments (Malaty, 2010).

2.2.4 *Helicobacter pylori*-associated diseases

Even though gastric colonisation with *H. pylori* induces histologic gastritis in all infected individuals, only a minority develop any apparent clinical signs of this infection. This has been confirmed by the observation that the majority of the *H. pylori* infected individuals do not show any symptoms of the disease and only 10–20% of them have a lifetime risk of developing ulcer disease and a 1-2% risk of developing distal gastric cancer (Ernst & Gold, 2000; Kuipers, 1998, 1999). The main disease caused by *H. pylori* infection is the gastritis (Bartnik, 2008). The risk of development of these disorders and the final clinical implications of *H. pylori* infection depends on various bacterial, host, and environmental factors that mostly relate to the pattern and severity of gastritis (Kusters et al., 2006).

To date, there have been 3 different disease phenotypes associated with the *H. pylori* infection, which are mild gastritis, duodenal ulcer and gastric cancer. The most frequent is the first phenotype, the simple gastritis phenotype, which is not accompanied by significant alterations in gastric acid secretion and does not lead to serious complications. Duodenal ulcers occur in 15% of infected people and are characterised by inflammatory lesions in the antral region of the stomach along with increased gastrin and hydrochloric acid secretion. The most severe type of lesion is the gastric cancer

phenotype with gastric corpus inflammation, mucosal membrane atrophy in this region of the stomach and decreased hydrochloric acid secretion. Such anatomical alterations and functional disorders develop in about 1% of the infected subjects and significantly increase the risk for gastric cancer. The cancer phenotype occurs most frequently in populations of the Asian countries, in which gastric cancer is predominantly common (Bartnik, 2008; Kusters et al., 2006; Naylor et al., 2006). Two malignant gastric cancers including mucosa-associated lymphoid tissue (MALT) lymphoma (LG-MALT) and gastric adenocarcinoma have been reported to be strongly associated with *H. pylori* infection and hence *H. pylori* is classed as group I carcinogen by National Institute of Health (NIH) (Yamada et al., 1994).

Recently, increasing evidence demonstrates possible associations of *H. pylori* infection to diseases in other organ systems, known as the extraintestinal/extragastric disorders. These include coronary heart disease, dermatological disorders such as rosacea and idiopathic urticaria, autoimmune thyroid disease and thrombocytopenic purpura, iron deficiency anaemia, Raynaud's phenomenon, scleroderma, migraine, and Guillain-Barre´ syndrome. The underlying hypothetical mechanisms include chronic low-grade activation of the coagulation cascade, accelerating atherosclerosis, and antigenic mimicry between *H. pylori* and host epitopes leading to autoimmune disorders (Bohr, Annibale, Franceschi, Roccarina, & Gasbarrini, 2007; Franceschi, Zuccala, Roccarina, & Gasbarrini, 2014; Gasbarrini, Carloni, Gasbarrini, & Chisholm, 2004; Moyaert et al., 2008; Nilsson et al., 2005; Pellicano et al., 2009). The postulated role of *H. pylori* in the pathogenesis of extragastric disorders is based on the facts: (1) local inflammation has systemic effects; (2) *H. pylori* is a chronic process that lasts for several decades; and (3) persistent infection induces a chronic inflammatory and immune response that is able to induce lesions both locally and remote to the primary site of infection (Pacifico et al., 2014).

2.2.5 Current clinical management of *Helicobacter pylori* infection

Consensus conference on *H. pylori* infection have been carried out in different regions of the world at 4-5 years' intervals to review and discuss all relevant clinical data to arrive at recommendations for the clinical management of *H. pylori* infection. According to Maastricht IV Consensus Report and Second Asia-Pacific Consensus Guidelines, a test-and-treat strategy should be utilised for uninvestigated dyspepsia in populations where the *H. pylori* prevalence is high (≥20%) (Fock et al., 2009; Malfertheiner et al., 2016). Uninvestigated dyspepsia refers to patients with either new or possibly recurrent dyspeptic symptoms in whom no investigations have previously been undertaken (Jones, 2002). Test and treat is a strategy involving a non-invasive test being carried out in patients with dyspepsia to assess whether *H. pylori* is present and then treatment of the infection if it is found. Therefore, it avoids the cost, inconvenience and discomfort of endoscopy. The test-and-treat strategy is appropriate in situations where the risk of the patient having gastric cancer is low (Malfertheiner et al., 2016). Test and treat must be used cautiously in populations with a low *H. pylori* prevalence (Moayyedi & Axon, 1999) and older population (Niv, Niv, & Koren, 2004) as it becomes less accurate in these settings.

In the Asia-Pacific region, *H. pylori* eradication is also indicated for *H. pylori* positive patients with investigated dyspepsia (non-ulcer dyspepsia) (Fock et al., 2009). Functional dyspepsia (FD) is not the same as uninvestigated dyspepsia. For a diagnosis of FD to be made, a number of investigations will have been performed and will have been found to be normal, notably upper gastrointestinal endoscopy, upper abdominal ultrasonography, and routine haematology and biochemistry screening blood tests (Jones, 2002). However, according to the latest WHO ICD-11β version under development and the Kyoto Global Consensus of *H. pylori* gastritis (Sugano et al., 2015) recommend that the classification of gastritis is based on causative factors, which

includes (a) *H. pylori*-induced, (b) drug-induced, and (c) autoimmune gastritis. *H. pylori* gastritis is a distinct cause of dyspepsia and is therefore an organic disease (Sugano, 2011; Suzuki, Nishizawa, & Hibi, 2011). This is in contradiction to the Rome III consensus that considered *H. pylori*-associated dyspepsia to be 'functional dyspepsia' (Tack et al., 2006). *H. pylori* gastritis has to be excluded before a reliable diagnosis of functional dyspepsia can be made (Malfertheiner et al., 2017).

In addition, *H. pylori* can increase or decrease acid secretion depending on the intragastric distribution of inflammation. In both conditions, treatment of *H. pylori* resolves the gastritis and leads to an, at least partial, correction of the high or low acid state. Though interesting, these changes in acid production after *H. pylori* treatment have no established clinical relevance and they should not be used as an argument in the management of *H. pylori* infection (Malfertheiner et al., 2017).

Epidemiological studies show that the prevalence of *H. pylori* and the severity of gastroesophageal reflux disease (GERD) and incidence of oesophageal adenocarcinoma is inversely associated (Corley et al., 2008; O'Connor, 1999; Rokkas, Pistiolas, Sechopoulos, Robotis, & Margantinis, 2007). Generally, *H. pylori* status has no effect on symptom severity, symptom recurrence and treatment efficacy in GERD. *H. pylori* eradication does not exacerbate pre-existing GERD or affect treatment efficacy (Klinkenberg-Knol et al., 2000; Moayyedi et al., 2001; Qian, Ma, Shang, Qian, & Zhang, 2011; Yaghoobi, Farrokhyar, Yuan, & Hunt, 2010). Routine test and treat for *H. pylori* infection is not recommended for patients with GERD. However, *H. pylori* testing should be considered in patients receiving long-term maintenance treatment with PPI for GERD (Fock et al., 2009; Malfertheiner et al., 2017). Long-term use of PPI causes a deteriorating of the histological grade of gastritis in *H. pylori*-infected patients. There is an accelerated risk of gastric mucosal atrophy that is not seen when PPI are used in uninfected patients or in those in whom eradication therapy has been given prior

to long-term proton-pump inhibitor (PPI) use. There is reason to consider eradicating *H. pylori* infection prior to long-term PPI use, especially in younger patients as gastric mucosal atrophy is known to be a risk factor for the development of gastric adenocarcinoma (Fock et al., 2009).

In non-steroidal anti-inflammatory drug (NSAID)-naive users, *H. pylori* eradication will reduce the risk of peptic ulcer and upper gastrointestinal bleeding. However in patients receiving long-term NSAIDs who have a past history of peptic ulcer disease or complications of peptic ulcer disease, *H. pylori* eradication alone is not sufficient to prevent ulcer recurrence and/or bleeding. Hence, before starting long-term aspirin therapy for patients at high risk for ulcer and ulcer-related complications, test and treat for *H. pylori* infection is recommended (Fock et al., 2009; Malfertheiner et al., 2017). Moreover, some randomised control trials (Chan et al., 2001; Lai et al., 2002) have shown that treating *H. pylori* infection in patients receiving long-term low-dose aspirin therapy and who have a past history of upper gastrointestinal bleeding and perforation will reduce risk of recurrent haemorrhage (Fock et al., 2009).

In patient groups with an increased risk of gastric cancer, the test-and-treat strategy is not recommended and a strategy of 'endoscope and treat' is preferred (Malfertheiner et al., 2017). Screen and treat for *H. pylori* infection in populations with high prevalence of gastric cancer is an effective strategy for prevention of gastric cancer. *H pylori* eradication is the first-line treatment for low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Malfertheiner et al., 2017). Low-grade MALT lymphoma accounts for approximately 50% of cases of gastrointestinal non-Hodgkin's lymphoma. Most are linked to *H. pylori* infection and in the early (lugano I/II) stage low-grade MALT lymphoma can be cured by *H. pylori* eradication in 60-80% of cases (Chen et al., 2005; Stathis et al., 2009; Wotherspoon et al., 1993).

Aforementioned, there is evidence linking *H. pylori* to the aetiology of otherwise unexplained iron-deficiency anaemia (DuBois & Kearney, 2005; Muhsen & Cohen, 2008; Qu et al., 2010), idiopathic thrombocytopenic purpura (Arnold et al., 2009; George, 2009) and vitamin B12 deficiency (Franceschi & Gasbarrini, 2007). It is important to note that despite some studies demonstrated an association between *H. pylori* and iron-deficiency anaemia, a recent meta-analysis of observational studies and randomised controlled trials concluded that iron-deficiency anaemia was not specifically related to *H. pylori* infection (Qu et al., 2010). Nevertheless, *H. pylori* should be sought and eradicated in these disorders (Fock et al., 2009; Malfertheiner et al., 2017). In addition, available evidence shows no unequivocal causative association between *H. pylori* and other extragastric disorders, including cardiovascular and neurological disorders (Charlett et al., 2009; Kountouras et al., 2009; Palm, Urbanek, & Grau, 2009). For asthma and atopy and obesity and related disorders, the evidence available remains contradictory (Chen & Blaser, 2007, 2008; Francois et al., 2011; Fullerton et al., 2009; Ioannou, Weiss, & Kearney, 2005; Nwokolo et al., 2003; Reibman et al., 2008). Hence, further research is necessary to demonstrate these associations before we can establish risk adjusted guidelines for the management of *H. pylori* infection.

2.2.5.1 Diagnosis of *Helicobacter pylori* infection

There are two approaches for the diagnosis of *H. pylori* infection in clinical practice. The first approach involved non-invasive tests, i.e. the urea breath test (UBT) and monoclonal stool antigen test. These tests are accepted as accurate non-invasive tests for initial diagnosis and for the determination of the outcome of *H. pylori* eradication therapy (Fock et al., 2009). Serology is the third method commonly used as a non-invasive method to diagnose *H. pylori* infection. As *H. pylori* infection is a chronic infection, only IgG detection is considered and the favoured method is enzyme-linked

immunosorbent assay (ELISA) (Malfertheiner et al., 2012). The serological tests are not all equivalent. Only validated IgG serology tests should be used owing to variability in the accuracy of different commercial tests (Fock et al., 2009; Malfertheiner et al., 2012). There are a few conditions where a supplementary serological test may aid decision-making, for instance in cases with discordant biopsy-based tests, or in whom biopsies reveal active chronic gastritis in the absence of organisms. Such histology is highly suggestive of infection, and a high titre serological test is helpful in this context. Similarly in bleeding patients, biopsy-based tests for *H. pylori* have a higher false-negative rate, and a high titre serological test is also useful in this context. As PPI and antibiotic use increase the false-negative rate of biopsy, breath and stool antigen tests, a serological test may be helpful when use of these agents is unavoidable (Fock et al., 2009).

The second approach for the diagnosis of *H. pylori* infection in the clinical practice is the endoscopy-based strategy. When an endoscopy is performed, biopsy-based tests such as a rapid urease test (RUT), histology and culture can be carried out. It is imperative to perform culture and standard antimicrobial susceptibility test in a region or population of high clarithromycin resistance before prescription of the first-line treatment if the standard clarithromycin-containing triple therapy is being considered. Furthermore, culture and standard susceptibility antimicrobial susceptibility test should be considered in all regions before second-line treatment if endoscopy is carried out for another reason and usually when a second-line treatment has failed (Malfertheiner et al., 2017). The RUT is a popular, cheap and simple diagnostic test that detects the presence of urease in or on the gastric mucosa. The basis of the test is the ability of *H. pylori* to secrete the urease enzyme, which catalyses the conversion of urea to ammonia and carbon dioxide. The sensitivity and specificity are generally high (Uotani & Graham, 2015). However, the accuracy of the test may be limited by the use of PPI (Saniee,

Shahreza, & Siavoshi, 2016) as well as active gastrointestinal bleeding (Laine, Nathwani, & Naritoku, 2005).

2.2.5.2 Eradication treatment for *Helicobacter pylori* infection

According to Maastricht V/Florence Consensus Report and Second Asia-Pacific Consensus Guidelines, the currently recommended first-line therapy for *H. pylori* infection is PPI, clarithromycin and amoxicillin/metronidazole for 7 days. Fourteen-day triple therapy confers limited advantage over 7-day triple therapy in *H. pylori* eradication rates. There is an increasing rate of resistance to clarithromycin and metronidazole in some regions/populations. This has led to reduced efficacy of PPI-based triple therapy. Bismuth-based quadruple therapy is an effective alternative first-line therapy for *H. pylori* eradication. There are currently insufficient data to recommend sequential therapy as an alternative first line for *H. pylori* therapy in Asia. Salvage therapy for *H. pylori* eradication includes: (i) a standard triple therapy that has not been previously used; (ii) bismuth-based quadruple therapy; (iii) levofloxacin-based triple therapy; and (iv) rifabutin-based triple therapy (Fock et al., 2009; Malfertheiner et al., 2017). It is also worthy to note that CYP2C19 polymorphisms may affect *H. pylori* eradication rates in PPI-based triple therapy. Choice of PPI or increasing the dose is a more practical approach than CYP2C19 genotyping in the clinical setting to overcome CYP2C19 polymorphisms in the context of salvage therapy (Fock et al., 2009).

At the University Malaya Medical Centre (UMMC), the first-line regimen used for *H. pylori* eradication consists of 7-day twice daily regimen and a proton pump inhibitor as per current standard of care (amoxicillin 1g, clarithromycin 500 mg, and pantoprazole 40 mg). Metronidazole is not recommended due to the high metronidazole resistance rate in Malaysia (Ahmad, Zakaria, Abdullah, & Mohamed, 2009; Ahmad, Zakaria, & Mohamed, 2011; Teh et al., 2014). In cases where the first-line eradication

therapy failed, two consecutive rescue regimens are prescribed (Goh, 2012; Goh, Manikam, & Qua, 2012). The first-line rescue regime consists of rabeprazole 20 mg and amoxicillin 1 g, three times daily for two weeks (Goh et al., 2012). Rabeprazole is less susceptible to the influence of genetic polymorphisms for CYP2C19. Thus, it has greater and faster acid suppression compared to other PPIs (Baldwin & Keam, 2009). Amoxicillin is one of the effective antibiotics to *H. pylori* with little side effects and up to date, antibiotic resistance to amoxicillin has not been reported in Malaysia (Goh & Navaratnam, 2011; Teh et al., 2014). In patients who fail to eradicate their *H. pylori* infection, a second-line rescue therapy consisting of rabeprazole 20 mg, levofloxacin 500 mg and amoxicillin 1 g, twice daily for a further 2 weeks is used (Goh et al., 2012).

The optimal treatment of *H. pylori* infection requires careful attention to local antibiotic resistance and eradication patterns. According to the latest Toronto Consensus for the Treatment of *Helicobacter pylori* Infection in adults, the concomitant non-bismuth quadruple therapy (PPI + amoxicillin + metronidazole + clarithromycin [PAMC]) and traditional bismuth quadruple therapy (PPI + bismuth + metronidazole + tetracycline [PBMT]) should play a more prominent role in eradication of *H. pylori* infection, and all treatments should be given for 14 days (Fallone et al., 2016).

2.2.6 Pathogenesis of *Helicobacter pylori* infection

Bacterial virulence factors play a significant role in the outcome and progression of *H. pylori* infection. Two most extensively studied virulence genes of *H. pylori* are vacuolating cytotoxin A (*vacA*) and cytotoxin-associated gene A (*cagA*).

2.2.6.1 Vacuolating Cytotoxin A (VacA)

The cytotoxin gene *vacA* is present in almost all *H. pylori* strains (Hagymasi & Tulassay, 2014; Yamaoka, 2012). The VacA protein plays an important role in the pathogenesis of both peptic ulceration and gastric cancer. The VacA cytotoxin induces the vacuolation, gastric epithelial barrier function disruption, disturbance of late endosomal compartments, and modulation of the inflammatory response. VacA reduces the mitochondrial transmembrane potential, releases cytochrome c from mitochondria, activates caspase 8 and 9, and induces apoptosis (Wroblewski, Peek, & Wilson, 2010; Yamaoka et al., 2006).

Differences in the sequence heterogeneity within the *vacA* gene at the signal (s) region and the middle (m) region contribute to variations in the vacuolating activity of different *H. pylori* strains. The s region of the gene, which encodes the signal peptide, occurs as either an s1 or s2 type, whereas the m region, which contains the p58 cell binding domain, exists as an m1 or m2 type. s1/m1 strains are the most cytotoxic, followed by s1/m2 strains. However, s2/m2 strains have no cytotoxic activity, and s2/m1 strains are rare. The intermediate (i) region also plays role in the vacuolating activity of *H. pylori*. All s1/m1 strains were classified as i1 (vacuolating) type, and all s2/m2 strains were classified as i2 (non-vacuolating) type, while s1/m2 alleles could be i1 or i2, and i1 strains were shown to be more pathogenic. A novel intermediate variant (i3) has been identified. More recently, a fourth disease-related region between the i region and the m region was discovered and named as the deletion (d) region. The d region is divided into d1 without a deletion and d2 with a 69 to 81bp deletion (Wen & Moss, 2009; Yamaoka, 2012).

The variants in s and m regions seem to be a good indicator of clinical outcomes. The s1 or m1 strains can induce greater vacuolation, and are associated with peptic ulcer disease and gastric cancer in Western countries (Wen & Moss, 2009; Wroblewski et al.,

2010). In East Asia, most of the *H. pylori* strains possess the *vacA* s1 genotype; therefore, the type of s region is independent of clinical outcomes (Yamaoka, Kodama, et al., 1999; Yamaoka et al., 2002). In contrast, the m1 genotype is common in areas of Northeast Asia, such as Japan and South Korea, whereas the m2 genotype is predominant in areas of Southeast Asia, such as Taiwan and Vietnam (Yamaoka, 2010; Yamaoka et al., 2002). Because the incidence of gastric cancer is higher in the northern regions than in the southern regions of East Asia, the *vacA* m region may play a role in the regional differences in the disease pattern in East Asia (Yamaoka, 2012). Similarly, *vacA* m1 genotype was reported to be more prevalent in Hanoi than in Ho Chi Minh City in Vietnam, and the incidence of gastric cancer was also reported to be higher in Hanoi than in Ho Chi Minh City (Uchida et al., 2009). These findings support the possibility that the *vacA* m region is related to clinical outcomes in East Asia (Yamaoka, 2012).

vacA i1 strains were associated with gastric cancer in Iranian patients but not in the East Asian or Southeast Asian populations (Rhead et al., 2007). i1 genotype appeared to be a better predictor of carcinoma-associated *H. pylori* strains than the s or m genotype (Ogiwara, Graham, & Yamaoka, 2008). In Western countries, d1 strains without the deletion of the d region are predictors of histological inflammation, atrophy, and an increased risk of peptic ulceration and gastric cancer, compared with the presence of the *vacA* s-, m-, and i-region strains (Ogiwara et al., 2009). Nonetheless, the roles of i and d regions should be further investigated (Yamaoka, 2012). The genotypes of the s and the m regions seem to currently serve as good markers of clinical outcomes.

2.2.6.2 Cytotoxin-associated gene A (CagA)

CagA is the most extensively studied *H. pylori* virulence factor. There are two types of clinical *H. pylori* isolate: CagA-producing (*cagA* positive) strains and CagA-non-

producing (*cagA* negative) strains (Kusters et al., 2006; Yamaoka, 2010). Several animal studies have shown that CagA is a bacteria-derived carcinogen which is associated with the development of gastric cancer (Franco et al., 2005; Franco et al., 2008; Ohnishi et al., 2008). In Western countries, it has been reported that individuals infected with *cagA*-positive strains of *H. pylori* are at a higher risk of peptic ulcer or gastric cancer than those infected with *cagA*-negative strains (van Doorn et al., 1998). In East Asia, however, most strains of *H. pylori* have the *cagA* gene irrespective of the disease. Thus, the pathogenic difference in East Asia is difficult to explain solely in terms of the presence or absence of the *cagA* gene alone (Yamaoka, Kodama, et al., 1999). It is worthy to note that almost all *cagA*-positive strains are classified as the *vacA* s1 strain (either m1 or m2), whereas almost all *cagA*-negative strains are classified as the *vacA* s2/m2 strain (Atherton et al., 1995).

The *cagA* is a polymorphic gene (Yamaoka, 2010) and it is located at one end of the cag pathogenicity island (cag PAI), which is an approximately 40kbp region that is believed to have been integrated into the *H. pylori* genome by horizontal transfer from an unknown source (Censini et al., 1996). There are different numbers of repeat sequences located in the 3' region of the *cagA* gene of different *H. pylori* strains (Yamaoka, El-Zimaity, et al., 1999; Yamaoka et al., 2000; Yamaoka, Kodama, Kashima, Graham, & Sepulveda, 1998). The repeat regions were initially classified into two types: the first repeat and the second repeat, and the sequence of the second repeat region was found to have considerable difference between East Asian strains and Western strains of *H. pylori* (Yamaoka et al., 1998; Yamaoka, El-Zimaity, et al., 1999; Yamaoka et al., 2000; Yamaoka et al., 2002). Each repeat region of the CagA protein contains Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, including a tyrosine phosphorylation site. Lately, it has been more common to name the first repeat regions as EPIYA-A and EPIYA-B segments and the second-repeat region in Western and East Asian strains as EPIYA-C

and EPIYA-D segments, respectively (Hatakeyama, 2004). As such, each CagA is assigned a sequence type consisting of the names of the EPIYA segments in its sequence, that is, ABC, ABCC or ABCCC for Western-type CagA and ABD for East-Asian-type CagA. CagA binds to and activates or inactivates multiple signalling proteins in both a phosphorylation-dependent and phosphorylation-independent manner (Yamaoka, 2010).

There have been many reports that the site of tyrosine phosphorylation in the EPIYA motif of CagA plays a direct role in the pathogenicity of *H. pylori*. At least eighteen of the cag PAI-encoded proteins serve as building blocks of a type IV secretion apparatus, which forms a syringe like structure capable of penetrating the gastric epithelial cells and facilitating the translocation of CagA, peptidoglycan, and possibly other bacterial factors into host cells (Asahi et al., 2000; Christie & Vogel, 2000; Covacci et al., 1993; Fischer et al., 2001; Odenbreit, Gebert, Puls, Fischer, & Haas, 2001; Odenbreit et al., 2000; Segal, Cha, Lo, Falkow, & Tompkins, 1999). Once delivered inside the cell, the CagA protein is phosphorylated at tyrosine residues in EPIYA motifs (Asahi et al., 2000; Higashi et al., 2002; Odenbreit et al., 2000; Segal et al., 1999; Stein, Rappuoli, & Covacci, 2000) by host Abl and Src family kinases which results in the impairment of a variety of intracellular signalling systems. Studies from Mueller and colleagues reported that the two kinases c-Src and c-Abl phosphorylate CagA sequentially, and in a tightly regulated and coordinated manner. c-Src is the first kinase to become activated upon cytoplasmic CagA delivery by the cag PAI–encoded type IV secretion system. It phosphorylates CagA exclusively on EPIYA motifs flanked by C/D segment sequences; this first phosphorylation event primes the subsequent c-Abl–mediated phosphorylation of EPIYA-A, -B, -C, or -D motifs later in infection. One molecule of CagA is never phosphorylated on more than two EPIYA motifs simultaneously, and phosphorylation of a single motif is not sufficient to induce

downstream effects. Interestingly, the preferred combination of phosphorylatable motifs is A+C (and B+D in East Asian strains), which, strikingly, may be located on separate CagA molecules (Mueller et al., 2012; Müller, 2012).

Currently, there are 20 known cellular binding partners of CagA, 10 of which form phosphorylation-dependent interactions with CagA (Backert, Tegtmeyer, & Selbach, 2010; Selbach et al., 2009). The number of EPIYA motifs present is related to the level of CagA phosphorylation that occurs in epithelial cells infected by either East Asian (Hirata et al., 2004) or Western strains (Argent et al., 2004). The best studied of the host signalling factor that interacts with phosphorylated CagA is the Src homology-2 domain-containing phosphatase 2 (SHP-2) (Higashi et al., 2002). SHP2 is able to bind EPIYAB, EPIYA-C and EPIYA-D segments. Importantly, however, CagA with an EPIYA-D segment has a higher binding affinity for SHP2 than CagA with an EPIYA-C segment (Hatakeyama, 2004). The sequence flanking the tyrosine phosphorylation site of the EPIYA-D segment (EPIYATIDF), but not the EPIYA-C segment (EPIYATIDD), matches perfectly the consensus high-affinity binding sequence for the SH2 domains of SHP-2 (Yamaoka, 2010). SHP-2 is known to have oncogenic activity and results in morphological changes to the cells (Hatakeyama, 2004).

In addition, numerous studies have suggested that the number of EPIYA segments in the second repeat region is associated with gastric cancer (Yamaoka et al., 1998; Yamaoka, El-Zimaity, et al., 1999). In Western countries, the incidence of gastric cancer is markedly higher in patients infected with strains containing multiple EPIYA-C segments than in patients infected with strains containing a single EPIYA-C segment (that is, ABCCC versus ABC) (Argent et al., 2004; Azuma et al., 2002; Yamaoka et al., 1998; Yamaoka, El-Zimaity, et al., 1999). However, as almost all East Asian strains contain a single EPIYA-D segment (Xia, Yamaoka, Zhu, Matha, & Gao, 2009), it is

therefore difficult to differentiate between simple gastritis and gastric cancer merely by considering the number of repeated sequences in East Asia.

CagA also impairs the host intracellular signalling systems via the phosphorylation-independent manner. Currently, there are at least 10 known phosphorylation-independent CagA host interaction partners (Backert et al., 2010). CagA forms dimers in cells in a phosphorylation-independent manner, and the CagA multimerization (CM) sequence (Ren, Higashi, Lu, Azuma, & Hatakeyama, 2006) (also named MARK2/PAR1b kinase inhibitor [MKI] (Neišić et al., 2010) or the conserved repeat responsible for phosphorylation-independent activity [CRPIA] (Suzuki et al., 2009)) was identified as the site responsible for dimerization, for inhibition of MARK2/PAR1b kinase and for the interaction of CagA with activated c-Met. It is subsequently led to the loss of cell polarity (Ren et al., 2006; Saadat et al., 2007), the loss of the elongation phenotype of host cells (Umeda et al., 2009), the upregulation of β-catenin and nuclear factor κB (NFκB) transcriptional activities, which promoted proliferation and inflammation, respectively (Suzuki et al., 2009).

2.3 Human gut microbiota

The human gastrointestinal tract is not homogenous and it contains a myriad collection of microorganisms. The number of bacterial cells present in the mammalian gut shows a continuum that goes from 10^1 to 10^3 bacteria per gram of contents in the stomach and duodenum, progressing to 10^4 to 10^7 bacteria per gram in the jejunum and ileum and culminating in 10^{11} to 10^{12} cells per gram in the colon (O'Hara & Shanahan, 2006). Moreover, the microbial composition varies between these sites (Frank et al., 2007; Swidsinski, Loening-Baucke, Lochs, & Hale, 2005). The trillions of commensal microorganisms that constitute the gut microbiota are primarily composed of five bacterial phyla, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and

Fusobacteria (Eckburg et al., 2005). *Bacteroidetes* and *Firmicutes* predominate and represent ~90% of the total gut microbiota (Rajilić-Stojanović, Smidt, & De Vos, 2007).

It has been suggested that the initial colonisation during birth is one of the major factors in shaping the composition of the human gut microbiota. Evidence has shown that there are distinct differences in gut microbiota between vaginally delivered infants and infants delivered through caesarean (Huurre et al., 2008; Mandar & Mikelsaar, 1996). After the initial establishment of the gut microbiota and during the first year of life, the microbial composition of the human intestine is relatively simple and varies widely between different individuals and also with time. However, after one year of age, the gut microbiota of children starts to resemble that of a young adult and stabilises (Mackie, Sghir, & Gaskins, 1999; Mandar & Mikelsaar, 1996). Parental inoculation or kinship seems to be also another factor in shaping adult gut microbiota community. Ley and co-workers have shown that, in mice, the microbiota of offspring is closely related to that of their mothers (Ley et al., 2005). A study in human has also revealed that the microbiota of adult monozygotic and dizygotic twins were equally similar to that of their siblings, suggesting that the colonisation by the microbiota from a shared mother was more decisive in determining their adult microbiota than their genetic makeup (Zoetendal, Akkermans, Akkermans-van Vliet, de Visser & de Vos, 2001).

The gut microbiota has been regarded as a metabolically active "organ" located within the human gastrointestinal tract (Hooper et al., 2002; O'Hara & Shanahan, 2006) as it has developed metabolic traits that complement host's metabolism (Cantarel et al., 2012; Hooper et al., 2001; Li et al., 2008; Rawls et al., 2006). A healthy gastrointestinal system relies on a balanced commensal biota to regulate processes such as energy metabolism (Cani & Delzenne, 2007, 2009; Harris et al., 2012; Tilg & Kaser, 2011), elimination of pathogens (Buffie & Pamer, 2013; Endt et al., 2010; Sekirov et al., 2010). In addition, it also influences the signalling pathways that range from modulation of the

mucosal immune response (Kelly et al., 2005) to development of metabolic diseases (Cani & Delzenne, 2009; DiBaise et al., 2012; Harris et al., 2012; Tilg & Kaser, 2011; Tremaroli & Backhed, 2012). Accumulating evidence suggests that dysbiosis, or an abnormal microbiota, has been associated with an increasingly long list of diseases, including inflammatory bowel disease, obesity, and atopic diseases such as eczema and asthma (Sekirov et al., 2010; Sun & Chang, 2014). Interestingly, the developments of these diseases coincide temporally with the disappearing of *H. pylori* infection.

2.4 Protective effects of *Helicobacter pylori* infection

H. pylori is considered the most successful human pathogen and it is even categorised as carcinogen. With this simple view, eradication seems an apparent choice. In actual fact, however, the relationship between *H. pylori* and disease is more nuanced. There is a growing number of evidence suggesting that the absence of *H. pylori* might also be associated with an increased risk of atopic diseases such as asthma and allergy as well as metabolic disorders such as obesity. An absence of *H. pylori* indicates that an individual was never colonised or that the organism was present in earlier life and subsequently eradicated. Interestingly, it is worthy to note that the development of these diseases coincide with transient dysbiosis.

2.4.1 Obesity

Obesity has become epidemic in the United States and other developed countries (Nguyen & El-Serag, 2010; Ogden, Carroll, Kit, & Flegal, 2014; Ogden, Yanovski, Carroll, & Flegal, 2007). The prevalence of obesity has increased so rapidly over the past three decades that a very strong environmental cause must be present. Although obesity could also be attributed by other contributing factors such as diet and lifestyle, they are inadequate to explain this epidemic. Differences in the GI microbiome of obese and lean individuals may affect their metabolic potential, which may confer greater

capacity to harvest energy, contributing to obesity (DiBaise et al., 2008). Therefore, it is possible that the changes in the GI microbiome, especially involving the major gastric bacterium *H. pylori,* are associated with changes in the regulation of metabolic hormones and consequently, fuelling epidemic human obesity. Indeed, several epidemiological and experimental studies support a protective effect of *H. pylori* against the development of obesity (Francois et al., 2011; Lender et al., 2014; Nwokolo et al., 2003; Osawa, 2008). Chronic persistent damage of the gastric mucosa by *H. pylori* may affect the regulation of ghrelin and leptin, as well as other hormones that may influence human metabolism, leading to changes in food intake and body weight.

Leptin and ghrelin are two centrally-acting hormones that have a major influence on energy homeostasis in mammals (Klok et al., 2007). Leptin, produced mainly by adipose tissue, is a long-term mediator of energy balance. It may play a role in reducing appetite, and increasing energy utilisation. In contrast, ghrelin is a fast-acting hormone mainly secreted by the stomach that may play a role in feeding behaviour and decreased energy expenditure (Francois et al., 2011; Klok et al., 2007). Ghrelin serum levels are high before meals and decrease post-prandially (Ariyasu et al., 2001; Cummings et al., 2001). Although it has been hypothesised that leptin and ghrelin have opposing effects on human energy homeostasis (Inui et al., 2004; Klok et al., 2007), several studies have produced conflicting results (Chan et al., 2004; Cummings et al., 2001; Ikezaki et al., 2002; Tschop, Weyer, et al., 2001).

Evidence of the correlation between *H. pylori* colonisation and the regulation of ghrelin and leptin and consequently on the body morphometric have been shown by several studies. An early cohort study conducted in the United Kingdom showed that six weeks following cure of *H. pylori,* plasma ghrelin increased profoundly by 75% in healthy asymptomatic subjects and the authors proposed that increased ghrelin following *H. pylori* eradication may play a role in obesity (Nwokolo et al., 2003).

Subsequent studies also showed that gastric ghrelin expression (Tatsuguchi et al., 2004), gastric ghrelin mRNA levels (Osawa et al., 2006) and plasma acyl-ghrelin levels (Kawashima et al., 2009) increased significantly post-*H. pylori* eradication and these could lead to the increased appetite and weight gain seen following *H. pylori* eradication. In addition, *H. pylori* infection was also found to be significantly and independently associated with metabolic syndrome in a Japanese population (Gunji et al., 2008). In another Japanese population, cure of *H. pylori* infection significantly reduced gastric leptin expression, with a concomitant increase in BMI, although serum leptin levels did not change significantly. (Azuma et al., 2001). In prepubertal children in Italy, long-term eradication of *H. pylori* infection was associated with a significant increase in BMI, lean and fat mass along with a significant decrease in circulating ghrelin levels and an increase in leptin levels (Pacifico et al., 2008). In a recent study involving veterans referred for upper GI endoscopy at New York University Langone Medical Center, Francois and co-workers reported that post-prandial acylated ghrelin and median integrated leptin levels were significantly increased after a median of seven months following eradication. Furthermore, BMI was also significantly increased over 18 months in the initially *H. pylori*-positive individuals, but was not significantly changed in those who were *H. pylori*-negative or indeterminant at baseline (Francois et al., 2011). All of these findings have provided evidence that *H. pylori* colonisation may be involved in ghrelin and leptin regulation, with consequent effects on body morphometry. However, further investigations are required to examine these associations as some research groups have reported otherwise (Ioannou et al., 2005; Jang et al., 2008).

2.4.2 Atopic disorders

Over the last decades, there has been a drastic increased prevalence of asthma and other atopic disorders, especially in children (Beasley, Crane, Lai, & Pearce, 2000; Eder, Ege,

& von Mutius, 2006; Pacifico et al., 2014; Wong, Rayner-Hartley, & Byrne, 2014). Although the underlying reason is still unknown, clinical, epidemiological and experimental evidence indicate that infectious diseases can influence the development of allergic disorders (Strachan, 1989). The "hygiene hypothesis" states that exposure to certain infectious agents may protect against the development of allergic diseases (Strachan, 2000). This notion has been recently revised by Blaser and Falkow (Blaser & Falkow, 2009), who suggest that the important factor in modern allergic and metabolic diseases might not be our decreased exposure to the microorganisms as has been postulated by the "hygiene hypothesis", but instead could reflect the specific loss of ancestral microorganisms due to modern health practices (including exposure to antibiotics) and lifestyle changes. According to the "disappearing microbiota" hypothesis, alterations in human macroecology have progressively affected the composition of our indigenous microbiota, which in turn has affected human physiology and, ultimately, disease risk. Thus the loss of our ancestral indigenous organisms is not entirely beneficial and has consequences that might include post-modern conditions such as asthma (and obesity). *H. pylori* appears like a potential candidate as it chronically infects more than 50% of the world's population, it is usually acquired during childhood, and it causes lifelong chronic inflammation if it is left untreated. Subsequently, several studies have investigated the association of *H. pylori* infection and allergic disease, and increasing data are indicative of a negative association of *H. pylori* with asthma and allergy.

In a case-control study, it was shown that atopy was inversely related to markers of infections transmitted through the orofaecal route or borne by contaminated hands or foods (*Toxoplasma gondii*, *H. pylori*, hepatitis A virus) but not to those mainly transmitted through other routes (measles, mumps, rubella, chickenpox, cytomegalovirus, herpes simplex virus type 1) (Matricardi et al., 2000). A further

investigation was carried out to determine the prevalence of allergen-specific IgE antibodies to the four most common allergens in Finland in two cross-sectional adult populations, using sera samples collected in 1973 and 1994 for which *H. pylori* serostatus was known. Between 1973 and 1994, a rise in the allergen specific-IgE antibodies had occurred, with the increase mainly observed in the *H. pylori* seronegative subjects. Hence, they hypothesised that *H. pylori* could be one of the microbes counteracting atopy (Kosunen et al., 2002).

A cross-sectional study was conducted to study the prevalence of three atopic disorders in 3244 subjects participating in a community-based, prospective, randomised, controlled trial of *H. pylori* eradication. *H. pylori* infection was reported to be associated with a substantially reduced risk of three common atopic disorders (asthma, eczema and allergic rhinitis). There was a 30% reduction in the prevalence of all three atopic disorders in people who had active *H. pylori* infection. These provide further indirect evidence of the importance of childhood infections in influencing the development of a normal immune response (McCune et al., 2003).

The first major study to investigate the associations of *H. pylori* status with history of asthma and allergy and with skin sensitization using data from 7663 adults in the Third National Health and Nutrition Examination Survey (NHANES III) was conducted by Chen and Blaser. An inverse association between *H. pylori cagA*-positive strains and asthma was reported, with a stronger association being observed in younger age group (Chen & Blaser, 2007). Moreover, the colonisation with CagA+ *H. pylori* strains is inversely associated with asthma and is associated with an older age of asthma onset in an urban population was reported in a case control study conducted in the New York University (NYU)/Bellevue Asthma Registry in New York City. Thus, yet again, the decreasing prevalence of *H. pylori* is related to the presence of asthma and *H. pylori* is suggested as a marker for protection (Reibman et al., 2008). In a second study

conducted by Chen and Blaser, the inverse association between *H. pylori* seropositivity and asthma in children was again reported from a cross sectional analyses, using data from 7412 participants in the NHANES 1999-2000. In this study, *H. pylori* seropositivity was found to be inversely associated with onset of asthma before 5 years of age and current asthma in children aged 3–13 years. *H. pylori* seropositivity also was inversely related to recent wheezing, allergic rhinitis, and dermatitis, eczema, or rash (Chen & Blaser, 2008).

On the contrary, other studies have reported neutral and even positive associations between infection and asthma (Annagur, Kendirli, Yilmaz, Altintas, & Inal, 2007; Bodner, Anderson, Reid, & Godden, 2000; Fullerton et al., 2009; Holster et al., 2012; Jun, Lei, Shimizu, Dobashi, & Mori, 2005). Recent meta-analyses have been performed in order to clarify these controversial findings. Wang and co-workers reviewed 19 studies and found a weak inverse association between asthma and *H. pylori* infection (Wang, Yu, & Sun, 2013). Similarly, Zhou and co-workers found a significantly lower rate of *H. pylori* infection in asthmatics in a review of 14 studies (Zhou, Wu, & Zhang, 2013). At present, this inverse relationship is fairly well supported in the literature, especially in childhood asthma.

The most supported pathogenic mechanism of this protective effect is the ability of *H. pylori* to stimulate the T-helper type 1 (Th1) immune response. Allergic diseases are driven by T cells that produce T-helper type 2 (Th2) cytokines and are inhibited by Th1 responses (Amedei, Codolo, Del Prete, de Bernard, & D'Elios, 2010). One of the suggested underlying molecular mechanisms of this possible protective effect of *H. pylori* is that the neutrophil activating protein of *H. pylori* (HP-NAP) not only plays a key role in driving Th1 inflammation but is also able to inhibit Th2-mediated bonchial inflammation of allergic bronchial asthma (D'Elios et al., 2009). A number of investigations have been reported in support of this hypothesis (Amedei et al., 2006;

Codolo et al., 2008; Oertli & Muller, 2012). Furthermore, systemic HP-NAP also significantly resulted in the reduction of total serum IgE responses (Codolo et al., 2008; D'Elios et al., 2009). Based on these properties, NAP was identified as a vaccine candidate as a preventive strategy against allergic diseases (Amedei et al., 2006) and NAP might be important *H. pylori* molecule which confers protective effect in allergic diseases (Codolo et al., 2008; D'Elios et al., 2009).

Another hypothesis for the inverse association between *H. pylori* and asthma is that high levels of regulatory T cells (Tregs) associated with *H. pylori* infection may contribute to the prevention of allergic diseases, while impaired expansion of natural and/or adaptive Tregs might lead to the development of allergy and asthma (Umetsu & DeKruyff, 2006). Several studies have indicated that Tregs play an important role in controlling exaggerated Th2-biased immune responses (Arnold, Hitzler, & Muller, 2012; Umetsu & DeKruyff, 2006), and that *H. pylori*-positive people have higher levels of gastric Tregs than those without the organism (Lundgren, Trollmo, Edebo, Svennerholm, & Lundin, 2005; Robinson et al., 2008).

In a study utilising mouse models of allergic airway disease induced by ovalbumin or house dust mite allergen to experimentally examine a possible inverse correlation between *H. pylori* and asthma, *H. pylori* infection was found to have efficiently protected mice from airway hyper-responsiveness, tissue inflammation, and goblet cell metaplasia, which are hallmarks of asthma, and prevented allergen-induced pulmonary and bronchoalveolar infiltration with eosinophils, Th2 cells, and Th17 cells. Protection against asthma was most robust in mice infected neonatally and was abrogated by antibiotic eradication of *H. pylori*. Asthma protection was further associated with impaired maturation of lung-infiltrating dendritic cells (DCs) and the accumulation of highly suppressive Tregs in the lungs. Systemic Treg depletion abolished asthma protection; conversely, the adoptive transfer of purified Treg

populations was sufficient to transfer protection from infected donor mice to uninfected recipients (Arnold et al., 2011). Accumulation of Tregs and semi-mature DCs in the lungs of *H. pylori* infected mice may suggest that *H. pylori* infection actively prevents DCs maturation and efficiently re-programs DCs toward a tolerance-promoting phenotype (Arnold et al., 2012; Oertli & Muller, 2012). *H. pylori*-experienced DCs fail to induce T-cell effector functions, but efficiently induce FoxP3 expression in naive T-cells in vitro and in vivo (Oertli & Muller, 2012).

2.5 Systems Biology

Comprehending the factors that influence human health and diseases has always been one of the major driving forces of biological research. With the remarkable progresses in quantitative techniques, large-scale measurement methods and with the intimate integration between experimental and computational approaches, biology has recently acquired new technological and conceptual tools to investigate, model and understand living organisms at the system level (Lemberger, 2007). The term "systems biology" was coined and emerged to describe the frontier of cross-disciplinary research in biology (Ideker, Galitski, & Hood, 2001; Kitano, 2002a, 2002b; Stelling, 2004; Westerhoff & Palsson, 2004). The advent of this term in the biological research about a decade ago (Ideker et al., 2001; Kitano, 2002a, 2002b), coinciding with the completion of the Human Genome Project (HGP) (Lander et al., 2001; Venter et al., 2001) and the concomitant emergence of 'omics technologies, namely transcriptomics (Lashkari et al., 1997; Schena et al., 1995), proteomics (Patterson & Aebersold, 2003), metabolomics (Fiehn, 2001; Oliver et al., 1998) and most recently, lipidomics (Han & Gross, 2003; Wenk, 2005). It is therefore linked to the post-genomic era and the development of global molecular profiling methods collectively known as 'omics technologies (Dunn et al., 2011).

In this study, 'omics technologies could be utilised to decipher the highly complex metabolic exchange between the diverse biological compartments of the *H. pylori* infected human host (including tissues, organs and systemic biofluids) and the gut microbiota and ultimately to help further understanding of the profound influence exerted by *H. pylori* and gut bacterial flora on the metabolic equilibrium of the host and, as a consequence, on its health status.

2.5.1 Metabolomics

One core area of systems biology research is metabolomics which is the study of low molecular weight organic and inorganic (typically <1500 Da) metabolites (Dunn et al., 2011). Unlike genomics, metabolomics (just like the transcriptomics and proteomics) is dynamic and it changes the living systems in response to genetic differences, environmental influences and disease or drug perturbations. Metabolites are the products and by-products of many intricate biosynthetic and catabolic pathways and hence, monitoring the resulting metabolic variations provides insight into intra- and extra-cellular regulatory processes involved in our metabolic homeostasis (Martin et al., 2012).

Generally, metabolomics analysis require sample preparation methods that are fast, reproducible, cover a wide range of analytes and also compatible with the instrumental technique. Metabolomics studies still largely utilise the general extraction protocols, often in modified versions, which were introduced by Folch et al. (Folch, Lees, & Stanley, 1957) and Bligh-Dyer (Bligh & Dyer, 1959). The Folch method uses approximately a 20-fold excess of a mixture of chloroform/methanol (2:1, v/v) for the extraction, whereas the Bligh–Dyer method is also based on a mixture of chloroform/methanol (1:2, v/v), but uses a subsequent addition of 1 volume of chloroform and 1 volume of water.

Metabolomics employs primarily two analytical techniques based on high resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) with different ion sources and mass analysers. MS is usually coupled with either gas chromatography (GC) or liquid chromatography (LC) (Heather, Wang, West, & Griffin, 2013; Roux, Lison, Junot, & Heilier, 2011). Both NMR and MS provide complimentary but sometimes redundant information, and they have their respective pros and cons. NMR was one of the first techniques used in metabolomics analysis (Lenz, Bright, Wilson, Morgan, & Nash, 2003; Lindon & Nicholson, 2008; Nicholson, Connelly, Lindon, & Holmes, 2002; Nicholson, Higham, Timbrell, & Sadler, 1989). It is a non-destructive, fast and robust technique which produces informative structural information. Despite the robustness of NMR, this technique is much less sensitive than MS with a low limit of detection and signal-to-noise ratio, and therefore, larger amounts of samples are required. NMR is often used without coupleing to any separation method and does not require method development as in the case with chromatography. However, as each metabolite participates to the NMR spectra, the deconvolution of signals is often a tedious process (Roux et al., 2011).

GC-MS has a big advantage over NMR spectroscopy in which it has the ability to discriminate individual fatty acids (Heather et al., 2013). In addition, a wide range of polar metabolites can be detected following N-methyltrimethylsilyltrifluoroacetamide (MSTFA) derivatisation including amino acids, glycolytic intermediates, sugars, TCA cycle intermediates and nucleotides (Gullberg, Jonsson, Nordström, Sjöström, & Moritz, 2004). The most common form of ionisation used in GC-MS is electron ionisation and this produces characteristic fragmentation patterns for many metabolites, allowing their identification in databases such as NIST and the Golm GC-MS database (Kopka et al., 2005). Although GC-MS is a robust and versatile technique, it can only analyse volatile

or derivatised compounds. Furthermore, the derivatisation step can introduce variability into the analysis and hence it is better to avoid this where possible (Heather et al., 2013).

LC-MS has emerged as a popular and powerful tool in which the development of LC-MS significantly impacted biological research including metabolomics. By far the most commonly utilised technique in LC-MS based metabolomics is electrospray ionisation (ESI) which relies on forming droplets of solvent containing the analyte. For LC-MS, samples are introduced in solution and then loaded onto an LC column containing the solid phase, and chromatography is performed between this solid phase and the liquid phase of the solvents running through the column. During this process, metabolites are retained on the stationary solid phase of the column while the bonding between the metabolite and the stationary phase is stronger than that between the metabolite and the solvent. However, if the composition of the solvent is varied there may come a point when the bonding is stronger between the metabolite and the solvent and at this stage the metabolite is eluted from the column. It is subsequently desolvated by ion source and lastly detected by the mass analyser (Heather et al., 2013).

High-resolution mass spectrometers such as time-of-flight (TOF), quadrupole-time-of-flight (QTOF) and Fourier transform (FT) mass spectrometers are becoming increasingly popular in the field of metabolite profiling because they provide accurate mass measurements which are useful for the differentiating between isobaric ions, and even isomers if their fragmentation patterns are different (Madalinski et al., 2008), leading to the detection of a higher number of signals than that obtained with low-resolution analysers such triple quadrupole (QQQ) or ion trap. Furthermore, accurate mass measurements also enable the determination of elemental compositions of metabolites for further identification (Madalinski et al., 2008).

2.5.2 Lipidomics

Lipids are defined as hydrophobic or amphipathic small molecules that might originate entirely or in part by carbanion based condensations of ketoacyl thioesters and/or by carbocation based condensations of isoprene units by the LIPID MAPS consortium (Fahy et al., 2005). Lipids have been divided into eight categories: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK) (Fahy, Cotter, Sud, & Subramaniam, 2011). Lipids have many vital biological and cellular functions such as structural components of cell membranes, energy storage sources and participating in cell growth, multiplication, and death (Hyötyläinen & Orešič, 2014; Li, Yang, Bai, & Liu, 2014). Some lipids are intermediate in cell signalling transduction processes (Dobrosotskaya, Seegmiller, Brown, Goldstein, & Rawson, 2002; Pawson & Nash, 2003) and can be utilised as biomarkers of some diseases (Alarcon et al., 2006; Hodge et al., 2007; Schaefer et al., 2006). Therefore, it is not surprising that altered lipid metabolism plays pivotal roles in the pathogenesis of most of the common diseases including *H. pylori* infection.

Lipidomics was first introduced as a discipline of metabolomics in 2003 (Han & Gross, 2003) and was defined as "the full characterisation of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation" (Spener, Lagarde, Géloên, & Record, 2003). Lipidomics involves the mapping of the entire spectrum of cellular pathways and lipid-lipid interaction (Gross & Han, 2011). Hence, it complements genomics, proteomics and metabolomics to provide a more comprehensive understanding of system biology, which allowed us to understand at a fundamental level the interaction of various molecules and how they implicated in health and disease (Griffiths & Wang, 2009).

As in metabolomics, most lipidomic studies still depend on the general extraction protocols, introduced by Folch and co-workers (Folch et al., 1957) and Bligh-Dyer (Bligh & Dyer, 1959). Recently, a novel lipids extraction procedure employing methyl tert-butyl ether (MTBE) was introduced. The advantage of MTBE extraction over conventional biphasic chloroform-containing solvent systems came from the low density of the lipid-containing organic phase that forms the upper layer during phase separation which significantly simplified its collection and minimized dripping losses. Furthermore, compared with chloroform, MTBE is non-toxic and non-carcinogenic, which reduces the environmental burden as well as the health risks for exposed personnel (Matyash, Liebisch, Kurzchalia, Shevchenko, & Schwudke, 2008).

Advances in lipid analysis have made MS a principal of choice for lipid analysis (Cajka & Fiehn, 2014; Hyötyläinen & Orešič, 2014; Zhao, Wu, Liu, Zhang, & Lin, 2014). MS can be used either by using direct infusion, *i.e.*, by the so-called shotgun MS, or, aforementioned, in combination with chromatographic separation-typically LC and sometimes also with GC (Hyötyläinen & Orešič, 2014; Li et al., 2014). The LC-MS based method is a more popular choice and it has several advantages over direct infusion techniques, such as more reliable identification of individual lipid species, even at trace levels, separation of isomers and isobars, or reduced ion-suppression effects. For global non-targeted lipidomic analysis, high resolution accurate MS systems capable of tandem mass measurements are typically applied, particularly Orbitrap MS and quadrupole time-of-flight (QTOF) MS (Cajka & Fiehn, 2014; Hyötyläinen & Orešič, 2014). On the other hand, triple quadrupole (QqQ) MS instruments are a good option for sensitive quantitative targeted analysis using multiple reaction monitoring and also can be used for species-specific lipid detection by precursor ion and neutral loss scanning (Balgoma, Checa, Sar, Snowden, & Wheelock, 2013; Han, Yang, & Gross, 2012; Xiao, Zhou, & Ressom, 2012).

2.5.3 Metagenomics

Classical studies of the gut microbiota are dependent on cultivation techniques. However, traditional culture methods can only cultivate 10%-30% of the total gut microbiota (Sokol & Seksik, 2010; Suau et al., 1999; Tannock, 2001). This low rate of successful cultivation may be due to the unknown growth requirements of the bacteria, the selectivity of the media used, the stress imposed by the cultivation procedures, the necessity of strict anoxic conditions. Culture-independent methods are, thus, required to circumvent these limitations.

The advent of high-throughput technological platforms such as the Next-Generation Sequencing (NGS) overcomes the shortcomings of classical approaches and improves the analytical performance for viable but uncultivable microorganisms (Hamady & Knight, 2009; Zoetendal, Rajilic-Stojanovic, & de Vos, 2008). NGS enables affordable and high-throughput DNA sequencing and it thus, leads to the remarkable growth in the field of metagenomics, particularly, the initiation of The European project, MetaHIT (Qin et al., 2010), and the American Human Microbiome Project (Arumugam et al., 2011; The Human Microbiome Project Consortium, 2012) which have contributed to the availability of the reference gene catalogue.

The main metagenomics approaches are 16S ribosomal RNA gene amplicon sequencing and whole metagenome shotgun (WMS) sequencing (Mande, Mohammed, & Ghosh, 2012; Preidis & Hotez, 2015). *16S rRNA* gene amplicon sequencing generally involves extraction of nucleic acid from samples, amplification of bacterial DNA using universal 16S polymerase chain reaction (PCR) primers, cloning, sequencing using Sanger, 454, or Illumina platforms, and alignment to libraries of known *16S* sequences (Preidis & Hotez, 2015), such as the Greengenes database or Ribosomal Database Project (Cole et al., 2014). *16S rRNA* metagenome data analysis usually utilising bioinformatics pipelines such as MG-RAST (Meyer et al., 2008) or QIIME (Caporaso,

Kuczynski, et al., 2010). This approach is attractive because of their affordability and tendency to be highly automated. However, it can detect only the bacterial members of the microbiome, often fail to achieve species-level taxonomic identification, and are subject to both PCR amplification bias (which might omit entire bacterial clades) and to copy-number variation of the *16S rRNA* gene. Alternatively, WMS sequencing involves all nucleotides in a sample, identifying bacteria, along with any archaea, viruses, and fungi that maybe present, to a species or even strain level while also providing functional databased on genome content. The drawbacks of WMS are higher cost (in terms of both nucleotide number and resources needed to analyse the larger data set) and contamination with host nucleic acid (Preidis & Hotez, 2015).

Metagenomics could help to decipher the impact of disappearance of *H. pylori* to the commensal community and human host particularly in health and disease. Consequences of changes in the gastric microbiota are yet not well understood as well as the influence of gastric *H. pylori* infection on changes in the microbiota of the distal GI tract. Thus, the availability of metagenomics provides the opportunity for new medical research fields by redefining 'diseased' and 'healthy', giving more credit to the 'microbiota' in shaping the immune system and therefore being beneficial for new medical approaches. The possibility to analyse microbiota throughout the GI tract independent of cultural techniques will significantly impact our knowledge on bacterial key players in the stomach environment and will bring insights on their interactions within a complex ecological system.

CHAPTER 3: MATERIALS AND METHODS

3.1 Recruitment of volunteers

This study is part of the on-going ESSAY (Eradication Study in Stable Adults/Youths) study in Kuala Lumpur and New York (New York University Langone Medical Center). In Kuala Lumpur, it was conducted at the University of Malaya Medical Centre (UMMC), Malaysia. Malaysian young adult volunteers (age range from 18 to 30 years) were consecutively recruited between June 2012 and November 2013, and were screened to assess eligibility for the study based on a priori exclusion criteria, including diabetes, hyper or hypothyroidism, prior gastric or bariatric surgery, prior documented *H. pylori* treatment, antibiotic, steroid or other immunomodulating drug use within 4 weeks of enrolment, recent vaccination, and Charlson weighed comorbidity index <2. Demographic information collected via a baseline questionnaire (Appendix A), included ethnic designation (self-reported as Malay, Chinese, Indian or Other). Height and weight were measured and body mass index (BMI) was calculated. The study protocol was reviewed and approved by the UMMC Medical Ethics Committee (Ref No. 877.1). Written informed consent was obtained from qualified candidates prior to study participation.

3.2 Screening of volunteers

Fifteen ml of blood was collected from the qualified candidates. The blood sample was centrifuged at 4000 *xg* for 20 mins and stored as serum at -80°C until examined. Urea Breath Test (UBT) and *H. pylori* serology were performed to determine *H. pylori* status of the volunteers.

3.2.1 ^{13}C-Urea Breath Test

H. pylori testing was performed using the non-radioactive ^{13}C-UBT (Isotope-Selective Infrared Spectroscopy method), shown to have a sensitivity of 91% and a specificity of 93% in our local population (Rushdan, Ong, Anwar, & Ramelah, 2005). The technique involves the consumption of 75 mg of a ^{13}C-urea solution after collection of a baseline breath sample. Breath samples then were collected 10, 20 and 30 min post ^{13}C-urea administration, and the concentrations of isotope-labelled carbon dioxide analysed using an IRIS infrared isotope analyser (Wagner Analysen Technik, Bremen, Germany). A delta-over-baseline value (DOB) >4% indicates *H. pylori* positivity.

3.2.2 *Helicobacter pylori* serology

Anti-*Helicobacter pylori* IgA & IgG antibodies in serum were determined quantitatively using the Pyloriset® EIA-A III (sensitivity 96.1%; specificity 91.5%) & EIA-G III (sensitivity 100%; specificity 94.3%) (Orion Diagnostica, Espoo, Finland) according to manufacturer's protocol. Briefly, sera were diluted 1 to 201 with serum diluting buffer. Then, 100 µl of calibrator sera and diluted serum samples were dispensed into appropriate well coated with inactive *H. pylori* antigen. The microtiter plate was incubated at 18-25°C for 30 mins using a shaking speed of 700-1000 rpm. Each well was then aspirated and washed three times with washing buffer. Subsequently, enzyme conjugate (peroxidase conjugated anti-human IgA/IgG (rabbit)) was added and the incubation and washing steps were repeated. Next, 100 µl of TMB-substrate (1.25 mmol/l 3.3'5'5'-Tetramethylbenzidine) was dispensed into each well and the plate was incubated for 10 mins. Lastly, 100 µl of stopping solution (0.5 M H_2SO_4) was added to stop the enzymatic reaction. The absorbance of each well was read at 450nm using Varioskan™ Flash Multimode Reader (ThermoFisher Scientific, Waltham, MA, USA). A standard curve was constructed by plotting the mean absorbance obtained for each

calibrator against its concentration in U/ml. Using the mean absorbance value for each sample, the U/ml of the sample was obtained from the standard curve. Result interpretation was as follows: Positive ≥20 U/ml; Negative <20 U/ml. Volunteers were considered as *H. pylori*-positive, if UBT and serology tests were both positive.

3.3 Enrollment of *Helicobacter pylori*-positive volunteers into ESSAY Study

Only volunteers who tested positive in UBT and *H. pylori* serology were considered as *H. pylori*-positive. *H. pylori*-positive volunteers were consented to participate in the ESSAY Study and undergo gastroscopy. Biopsy samples were obtained to further confirm their *H. pylori* infection status. The workflow of enrolment *H. pylori*-positive volunteers into ESSAY Study was summarised in Figure 3.1.

3.3.1 Endoscopy

Each volunteer fasted for 12 hours overnight prior to endoscopy. After intravenous administration of meperidine and midazolam, complete endoscopic evaluation of the upper gastrointestinal tract was performed in standard fashion to the second portion of the duodenum, as described (Francois et al., 2008). Gastric inflammation was graded using the Sydney-Houston system by the attending pathologist (Tytgat, 1991). Using standard forceps, two biopsies were obtained from the gastric antrum for histopathological analysis, two for rapid urease testing (RUT), and two for *H. pylori* culture. Positivity by RUT, histological examination, and culture were considered as confirmatory for *H. pylori* positivity.

3.3.2 Histological analysis

Biopsy specimens from the antrum were fixed in 10% formalin, embedded in paraffin, sectioned, and hematoxylin and eosin (H & E) stained (Ho, Windsor, Snowball, &

Marshall, 2001). A single experienced GI pathologist, blinded to the data, graded the extent of gastritis based on the presence or absence of *H. pylori*, chronic inflammation (mononuclear cells), polymorphonuclear neutrophil activity, glandular atrophy, and intestinal metaplasia based on a mild, moderate or marked scale, according to the updated Sydney classification (Dixon, Genta, Yardley, & Correa, 1996). The presence of polymorphonuclear neutrophil activity indicates active gastritis while presence of both polymorphonuclear neutrophil activity and mononuclear cells indicates chronic active gastritis (Dixon et al., 1996). *H. pylori* was detected as spiral or curved organisms near the mucous layer.

3.3.3 *Helicobacter pylori* culture

Gastric biopsy samples obtained from the antrum of the stomach were homogenized and plated onto non-selective and selective chocolate agar supplemented with 7% lysed horse blood (Oxoid, Hampshire, UK). Selective chocolate agar contained vancomycin (10 μg/ml), amphotericin B (5 μg/ml), trimethoprim (5 μg/ml) and nalidixic acid (20 μg/ml) (Corry, Curtis & Baird, 2003). The inoculated agar plates were incubated for 3-10 days in a humidified 10% CO_2 incubator at 37^{o}C. Bacterial growth was identified as *H. pylori* by typical morphology and positive reactions to urease activity through rapid urease test. Two colonies were isolated and saved for each gastric biopsy samples. All strains were harvested by suspension in preservative medium made of brain heart infusion broth (Lab M Limited, UK), yeast extract (Oxoid, Hampshire, UK), 20% glycerol (Friendemann Schmidt, Australia), and supplemented with 10% fetal calf serum (Biowest, Kansas City, MO, USA) and stored in -80°C until used. The *SSA* gene encoding a 26-kDa species-specific antigen was chosen as target house-keeping gene to further confirm the presence of *H. pylori* by Polymerase Chain Reaction (PCR).

The *cagA* and *vacA* status as virulence factors were also determined in all strains by PCR method.

3.3.4 Molecular confirmation of *Helicobacter pylori* isolates

3.3.4.1 Extraction of DNA from *Helicobacter pylori* isolates

DNA was extracted from *H. pylori* isolates using the RTP Bacteria DNA Mini Kit (STRATEC Molecular GmbH, Germany) according to manufacturer's protocol. Briefly, 400 µl Resuspension Buffer R was added to the pellet by pipetting up and down. The re-suspended sample was transferred into the Extraction Tube L and vortexed shortly. The sample was then incubated in a thermomixer at 65°C for 10 min. The Extraction Tube L was placed into the thermomixer and incubated at 95°C for 5-10 min. Next, 400 µl Binding Buffer B6 was added to the sample and vortexed briefly. The sample was loaded onto the RTA Spin Filter Set and incubated for 1 min and subsequently, centrifuged at 13,400 *xg* for 1 min. The filtrate was discarded and the RTA Spin Filter was placed back into the RTA Receiver Tube. 500 µl Wash Buffer I was added and centrifuged at 9,300 *xg* for 1 min. The filtrate and the RTA Receiver Tube were discarded. The RTA Spin Filter was placed into a new RTA Receiver Tube. 600 µl Wash Buffer II was added and centrifuged at 9,300 *xg* for 1 min. The filtrate was discarded and the RTA Spin Filter was placed back into the the RTA Receiver Tube and centrifuged for 3 min at maximum speed to remove the ethanol completely. Lastly, the RTA Spin Filter was placed into a new 1.5 ml Receiver Tube and 50 µl of Elution Buffer D was added. It was incubated for 1 min at room temperature and finally centrifuged for 1 min at 5,900 *xg* to elute the DNA. The DNA concentration was measured using Micro UV-Vis/Fluorescence Spectrophotometer e-Spect ES-2 (Malcom Co., Japan).

3.3.4.2 Detection of 26-kDa species-specific antigen (SSA) gene

PCR amplification of 26-kDa species-specific antigen (SSA) gene was performed in a 25 µl standard reaction with 1X DreamTaq Buffer (Thermo Scientific, Waltham, MA, USA), 2.5U DreamTaq DNA Polymerase (Thermo Scientific, Waltham, MA, USA), 200 µM dNTP Mix (Thermo Scientific, Waltham, MA, USA), 0.2 µM of each forward and reverse primer and 20 ng of template DNA, using a Bio-Rad T100™ Thermal Cycler (Hercules, CA, USA) according to the protocol of Hammar and colleagues (Hammar, Tyszkiewicz, Wadström, & O'Toole, 1992). The primers used for SSA gene, the expected PCR fragment length and PCR conditions were listed in Table 3.1. The PCR products together with a GeneRuler 100 bp DNA Ladder (Thermo Scientific, Waltham, MA, USA) and a no template control were separated by electrophoresis 1.5% (w/v) agarose gel containing 0.5X SYBR® Safe DNA Gel Stain (Invitrogen, Waltham, MA, USA) at 110 V for 45 min and examined under UV illumination using Bio-Rad Molecular Imager® ChemiDoc™ XRS+ with Image Lab™ Software, version 5.1 Beta (Hercules, CA, USA).

3.3.4.3 Genotyping of virulence factors: *cagA* gene and *vacA* alleles

Typing of *cagA* gene and *vacA* for signal sequence (s) region alleles, middle (m) region alleles and intermediate (i) region alleles were carried out using the primers and PCR conditions as described by Atherton and colleagues for s1a, s1b, s1c and s2 (Atherton et al., 1995), and Schmidt and colleagues for *cagA* gene, m1 or m2 and i1 and i2. These eight pairs of primers, the expected PCR fragment lengths and PCR conditions were listed in Table 3.1. PCR amplification of the genes, gel electrophoresis and gel visualization were performed according to the protocols mentioned above.

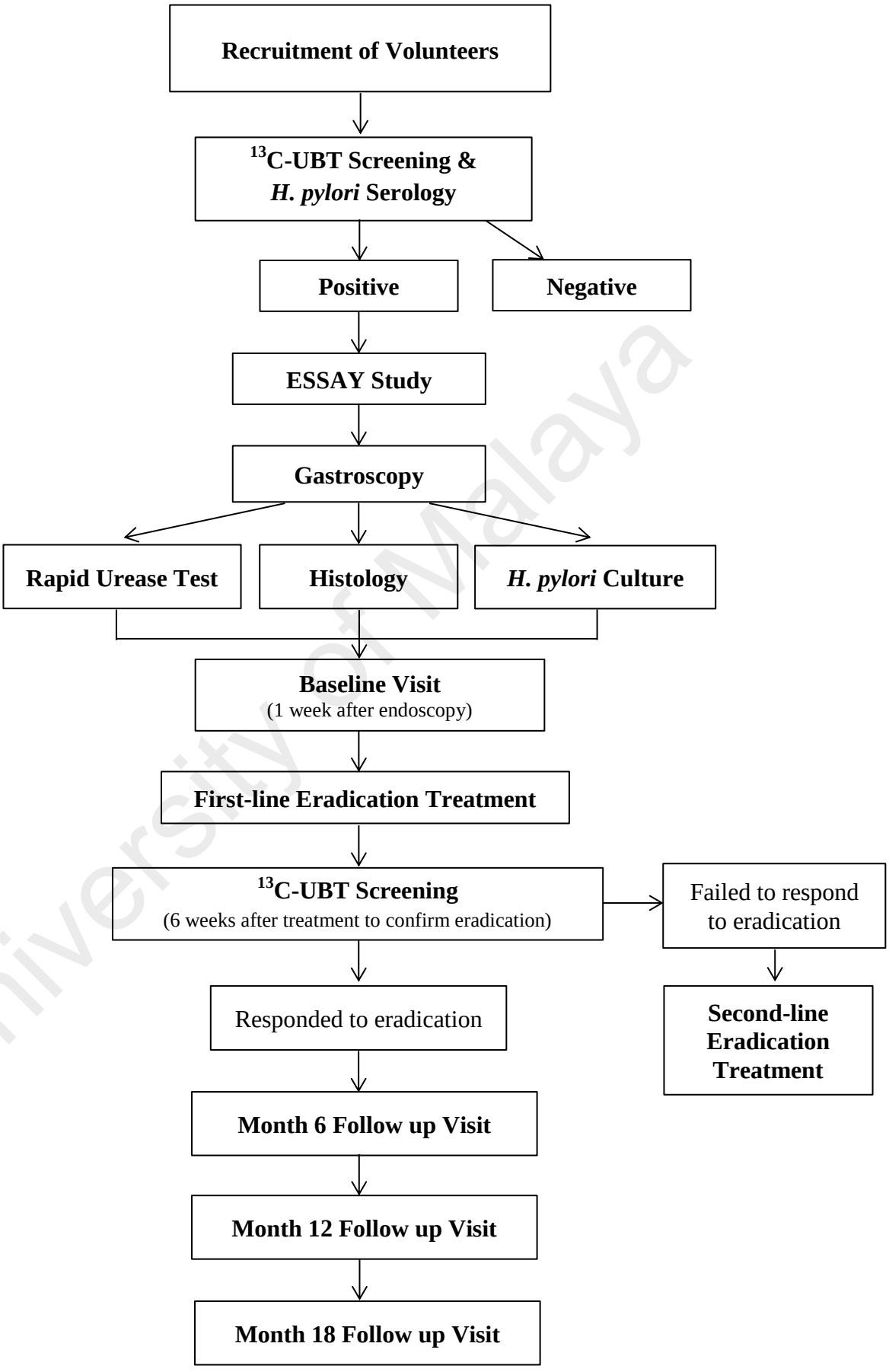


Figure 3.1: Summary of ESSAY Study

Table 3.1: PCR primer pairs and PCR conditions used for genotyping of *H. pylori* isolates (Atherton et al., 1995; Hammar et al., 1992; Schmidt et al., 2010)

Primer	Sequence	PCR Conditions	PCR Product Size (bp)
SSA-F **SSA**-R	5' TGG CGT GTC TAT TGA CAG CGA GC 3' 5' CCT GCT GGG CAT ACT TCA CCA G 3'	98°C, 10 min; 37 x (92°C, 30 s; 68°C, 1 min); 6 x (92°C, 30 s; 68°C, 1 min; 72°C, 2 min)	303
CagA-F ***CagA***-R	5' GAT AAC AGG CAA GCT TTT GAG 3' 5' CTG CAA AAG ATT GTT TGG CAG 3'	95°C, 5 min; 35 x (95°C, 30 s; 50°C, 30 s; 72°C, 1 min); 72°C, 7 min	349
VacA			
s1a-F s1a-R	5' GTC AGC ATC ACA CCG CAA C 3' 5' CTG CTT GAA TGC GCC AAA C 3'	95°C, 5 min; 35 x (95°C, 1 min; 52°C, 1 min; 72°C, 1 min); 72°C, 7 min	190
s1b-F s1b-R	5' AGC GCC ATA CCG CAA GAG 3' 5' CTG CTT GAA TGC GCC AAA C 3'	95°C, 5 min; 35 x (95°C, 1 min; 52°C, 1 min; 72°C, 1 min); 72°C, 7 min	187
s1c-F s1c-R	5' CTC TCG CTT TAG TGG GGY T 3' 5' CTG CTT GAA TGC GCC AAA C 3'	95°C, 5 min; 35 x (95°C, 1 min; 52°C, 1 min; 72°C, 1 min); 72°C, 7 min	213

Table 3.1, continued

Primer	Sequence	PCR Conditions	PCR Product Size (bp)
m1/m2-F	5’ CAA TCT GTC CAA TCA AGC GAG 3’	95°C, 5 min; 35 x (95°C, 20 s; 52°C, 20 s; 72°C, 40 s); 72°C, 7 min	570/645
m1/m2-F	5’ GCG TCT AAA TAA TTC CAA GG 3’		
i1-F	5’ GTT GGG ATT GGG GGA ATG CCG 3’	95°C, 5 min; 35 x (95°C, 20 s; 55°C, 20 s; 72°C, 40 s); 72°C, 7 min	426
i1-R	5’ TTA ATT TAA CGC TGT TTG AAG 3’		
i2-F	5’ GTT GGG ATT GGG GGA ATG CCG 3’	95°C, 5 min; 35 x (95°C, 20 s; 55°C, 20 s; 72°C, 40 s); 72°C, 7 min	432
i2-R	5’ GAT CAA CGC TCT GAT TTG A 3’		

3.4 Baseline visit

H. pylori-positive volunteers were instructed to arrive at the Endoscopic Unit of UMMC at 8am after 12 hours of fasting. Anthropometric measurements (height, weight, waist & hip circumference, and triceps-skinfold) were obtained. Validated Severity of Dyspepsia Assessment questionnaire (SODA) and SF-36 Health Survey were administrated to volunteers to assess baseline symptoms and quality of life profile (Appendix B & C).

Five ml of fasting blood was collected in EDTA-coated tube. The protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma-Aldrich, St. Louis, MO, USA), was added to the blood collection tubes at a concentration of 1 mg/ml. Tubes were kept on ice until centrifuged, and plasma was frozen at -80°C for correlation study, metabolic study and metabolomics analysis. Stool samples were also collected and frozen immediately at -80°C for metagenomic and lipidomic analysis.

3.4.1 Test meal

Following the fasting blood draw, volunteers consumed a standard 16-oz liquid meal totalling 700 calories (2 cans of Ensure Plus®) (Abbott Laboratories, Abbott Park, IL, USA). The contents of the meal provide 100 g carbohydrate, 26 g protein, and 22 g fat (as reported by the manufacturer). Five ml of blood was subsequently collected at one hour post-prandially.

3.4.2 *Helicobacter pylori* eradication therapy

Treatment with a 7-day twice daily regimen and a proton pump inhibitor as per current standard of care (amoxicillin 1000 mg, clarithromycin 500 mg, and pantoprazole 40 mg) were offered to volunteers who tested positive for *H. pylori*. *H. pylori* eradication was ascertained using the non-radioactive ^{13}C Urea Breath Test, ≥ 6 weeks after completion

of the treatment protocol. Volunteers who failed the first-line eradication regime were given second-line eradication therapy with a 2-week twice daily regime (amoxicillin 1000 mg, levofloxacin 500 mg and rabeprazole 20 mg).

3.5 Follow-up assessment

Twenty-nine volunteers in whom *H. pylori* was successfully eradicated were recruited into the ESSAY Study. These volunteers returned for follow-up assessment at 6, 12, and 18 months, paralleling that described for the baseline visit. The number of volunteers returned for follow-up assessment was 29, 18, and 12 respectively (Figure 3.1). All the plasma samples were successfully collected during each visit. However, only 23, 14, and 11 faecal samples were able to be collected at 6, 12, and 18 months post-eradication follow-up visit respectively.

3.6 Stool metagenomic analysis

3.6.1 Nucleic acid extraction from stool samples

Nucleic acid extraction was done using MoBio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). The DNA extraction protocol was adopted from Section 7.9 Specimen Processing For Extraction of Bacterial Genomic DNA taken from Manual of Procedures for Human Microbiome Project: Core Microbiome Sampling Protocol A (HMP Protocol #07-001), Version 12.0 (http://www.hmpdacc.org/tools_protocols/tools_protocols.php) with slight modification (Peterson et al., 2009).

Approximately 100-200 mg of stool sample was added directly into PowerBead Tubes provided and vortexed for 30-40 s to dispense the sample. Sample was then heated at 65°C and then at 95°C for 10 mins. Subsequently, 60 µl of Solution C1 was added and vortexed at maximum speed for 10 mins. The tube was then centrifuged at

10,000 *xg* for 30 s at room temperature. Following the centrifugation, supernatant was transferred to a clean Collection Tube provided. Next, 250 µl of Solution C2 was then added vortexed for 5 s and incubated at 4°C for 5 mins. The tube was again centrifuged at room temperature for 1 min at 10,000 *xg*. By avoiding the pellet, supernatant was transferred to another clean Collection Tube. Then, 200 µl of Solution C3 was added and incubated at 4°C for 5 mins. The tube was then centrifuged at room temperature for 2 mins at 10,000 *xg*. By avoiding the pellet, supernatant was again transferred to another clean Collection Tube and 1200 µl of Solution C4 was added to the supernatant and vortexed for 5 s. Supernatant was then loaded onto a Spin Filter and centrifuged at 10,000 *xg* for 1 min and this process was repeated for 3 times. Subsequently, 500 µl of Solution C5 was added to wash the DNA. It was then centrifuged twice at room temperature for 30 s and 1 min, respectively, at 10,000 *xg*. Finally DNA elution was done using 100 µl of Solution C6 and stored at -20°C until analysis.

3.6.2 *16S rRNA* gene amplification and sequencing

The V3-V4 region of the bacterial *16S rRNA* gene sequences were amplified using the primer pair 338F* (5′-NNNNCCTACGGGAGGCAGCAG-3′) and 1061R (5′-GACTACHVGGGTATCTAATCC-3′) containing the complete Illumina adapter (Bartram, Lynch, Stearns, Moreno-Hagelsieb, & Neufeld, 2011; Klindworth et al., 2013).

Briefly, each 50 µl of PCR reaction contains 10 ng of faecal genomic DNA as template, 25 µl NEBNext High-Fidelity 2x PCR Master Mix (New England Biolabs, Ipswich, MA) and 1 µl of 10 µM of each primer. PCR reactions were carried out using the respective protocols: (1) for the stool samples, an initial denaturation step performed at 98°C for 30 s followed by 30 cycles of denaturation (98°C, 10 s), annealing (60°C, 10 s) and extension (72°C, 30 s), and a final elongation of 1 min at 72°C. PCR products

~600 bases in size were gel-excised and purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The libraries were quantified using KAPA library quantification kit (KAPA Biosystems, Capetown, South Africa), normalized, pooled and sequenced (2 x 250 bp paired-end read setting) on the MiSeq (Illumina, San Diego, CA, USA) located at the Monash University Malaysia Genomics Facility.

3.6.3 Bioinformatics analysis

3.6.3.1 Sequence pre-processing and quality filtering

Demultiplexing and generation of raw fastq files for each individual library was performed on-board by the MiSeq Reporter Software. The forward and reverse 16S primer sequence located at the 5' end of the forward and reverse reads, respectively, were trimmed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The trimmed paired-end reads were subsequently overlapped using PEAR: Illumina Paired-End reAd mergeR (default setting) (Zhang, Kobert, Flouri, & Stamatakis, 2014).

3.6.3.2 Analysis of quality filtered reads using Qiime

The merged paired-end reads were analysed using the Quantitative Insights into Microbial Ecology (Qiime) (Caporaso, Kuczynski, et al., 2010) pipeline. To perform detection and clustering of 16S rRNAs, an open-reference Operational Taxonomic Units (OTUs) picking approach was used. pick_open_reference_otus.py is the primary interface for open-reference OTU picking in QIIME, and includes taxonomy assignment, sequence alignment, and tree-building steps. In this open-reference OTU picking process, reads were firstly clustered against a Greengenes 13_8 reference sequence collection (McDonald et al., 2012) (available at http://qiime.org/home_static/dataFiles.html) through closed-reference OTUs picking. Subsequently, 0.1% of the reads which failed to hit the reference sequence collection

were randomly subsampled and clustered de novo using UCLUST (Edgar, 2010), with an OTU cluster defined at a sequence similarity of 97%. Each cluster centroid was then chosen as a "new reference sequence" for another round of closed-reference OTU picking. OTU assignments for read that failed to hit the reference database were picked by an additional round of de novo clustering. The PyNAST alignment algorithm (Caporaso, Bittinger, et al., 2010) was used to align the OTU representative sequences against the Greengenes core reference alignment (DeSantis et al., 2006) with a minimum identity of 75%, and then a phylogenetic tree was built using FastTree (Price, Dehal, & Arkin, 2010). Finally an OTU table (*biom summarize-table*) was generated for downstream diversity analysis by excluding the sequences that had failed to align by PyNAST. The generated OTU table was also used to summarise microbiome communities by taxonomic levels (phylum, class, family, etc.) based on different time-points (summarize_taxa_through_plots.py).

3.6.3.3 Statistical methods

The raw data of the taxonomy summary results (.txt file) were exported to SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA) for statistical analysis. The mean abundance in percentage (%) and the 95% confidence interval (95% CI) for the phyla of stool microbiome at different time-points were calculated. Parametric paired-samples t-test was performed to compare the genera of the stool microbiome between Baseline vs. 6 months, Baseline vs. 12 months and Baseline vs. 18 months post-*H. pylori* eradication; a two-tailed p-value of < 0.05 was considered significant.

3.6.3.4 Diversity analysis and Statistical analysis

Alpha diversity (microbial diversity within samples) and beta diversity (community diversity between samples) analysis were evaluated using Qiime. Alpha diversity

analysis (*alpha_rarefaction.py*) involves rarefaction analysis by subsampling OTU table on the basis of a minimum rarefaction depth value that is chosen depending on the minimum number of sequences/sample obtained. In this study, the rarefaction depth value for the comparison of Baseline and 6 months post-eradication was set as 82536, whereas the rarefaction depth value for the comparison of Baseline and 12 months post-eradication was set as 84177. The alpha diversity was then calculated using both "non-phylogeny-based" (observed species, chao1, Shannon index) and "phlogeny-based" (PD whole tree) matrices for each rarefied OTU table. The alpha diversity between different groups (time-points) of the samples were compared by non-parametric two-sample t-test (*compare_alpha_diversity.py*).

Beta diversity between the samples was calculated using the default beta diversity metrics of weighted and unweighted UniFrac (Lozupone & Knight, 2005) (*beta_diversity_through_plots.py*) on even subsampled OTU table. The resulting UniFrac distance matrices were used to perform Principal Coordinate Analysis (PCoA) to determine the similarity between groups of samples/time-points. The PCoA plots in three dimensions were visualized using the Emperor tool (Vazquez-Baeza, Pirrung, Gonzalez, & Knight, 2013). Non-parametric statistical analysis ANOSIM was performed via QIIME (*compare_categories.py --method anosim*) to test the statistical significance between different time-points (Baseline vs. 6 months post-eradication, Baseline vs. 12 months post-eradication and Baseline vs. 18 months eradication).

3.7 Faecal lipidomics

3.7.1 Faecal sample preparation and lipid extraction

All the organic solvents used for faecal lipids extraction were of HPLC-grade. Methanol, acetonitrile and isopropanol were purchased from Friendemann Schmidt (Australia), methyl *tert*-butyl ether/hexafluoroisopropanol (MTBE) was purchased from Sigma-

Aldrich (St. Louis, MO, USA), LCMS-grade water from a Milli-Q water purification system (EMD Millipore, Billerica, MA, USA). Firstly, faecal slurry for each faecal sample was prepared by combining 150 mg solid faecal material per three ml of water. The faecal slurry was then diluted with water in 1:3 ratio. The diluted faecal slurry were then pooled into different groups according to gender, body mass index (BMI), and race (Appendix D) to ascertain whether these variables are the confounding factors of this study. 50 µl of each diluted faecal slurry sample was placed into 2 ml centrifuge tube. Five hundred microliters of methanol was added to each tube and vortexed for 30 s to ensure all components were well mixed. The mixed samples were then vortexed intermittently for 15 min and centrifuged at 12000 *xg* for 10 min to pellet insoluble faecal material. The supernatant was then transferred to 15 ml polypropylene centrifuge tubes. MTBE, 2 ml per sample, was added to each tube, vortexed to mix, and incubated at room temperature for 10 min to precipitate proteins. The lipid-containing supernatants were transferred to new 15 ml tubes, and 1.5 ml of water was added to induce phase separation. Samples were vortexed 30 s to mix, then centrifuged at 5000 *xg* for 5 min. After phase separation, the lipid-containing MTBE phase forms the top layer, whilst the methanol and water forms the bottom layer. The top layer lipid extracts were aspirated and transferred to new tubes (Gregory et al., 2013). The lipid extracts were dried in a CentriVap Concentrator Systems (LABCONCO, Kansas City, MO, USA) at 4°C and then resuspended to a 500 µl of acetonitrile:isopropanol:water (65:30:5), vortexed for 30 s and then centrifuged again at 12,000 *xg* for 5 min before injecting into liquid chromatography system.

3.7.2 High-Performance Liquid Chromatography Mass Spectrometry (LC-MS)

LC-MS analyses on the faecal lipids were performed on a 1260 Infinity High Performance Liquid Chromatography system coupled with a 6540 UHD Accurate-Mass

Q-TOF mass spectrometer from Agilent Technologies (Santa Clara, CA, USA) with a Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI) source.

Analysis was performed utilising both positive ionisation and negative ionisation mode of the Dual AJS ESI source and using All Ions MS/MS technique. All Ions MS/MS alternates between high and low energy scans during acquisition: high energy scans create fragment ions, low energy scans preserve the precursor ions. Optimization of several LC parameters such as injection volume, flowrate, and LC gradient were conducted. To ensure the reproducibility and robustness of the data acquired, a pooled biological quality control samples (PBQC) (constituted of pooled of all experimental faecal samples) and a lipid standards mixture (Avanti Polar Lipids, Alabaster, AL, USA) (Appendix E) were periodically injected throughout the duration of analysis. The sample run order was also assigned randomly to avoid any systematic bias.

The aqueous mobile phase (mobile phase A) was 60% acetonitrile and 40% water with 10 mM ammonium acetate (ThermoFisher Scientific, Waltham, MA, USA) and the organic mobile phase (mobile phase B) was 10% acetonitrile and 90% isopropanol with 10 mM ammonium acetate. A 10 µl of extracted faecal lipids was loaded onto Zorbax Eclipse Plus C18, 2.1 x 100 mm, 1.8 µm reverse phase column (Agilent Technologies, Santa Clara, CA, USA) with 70% mobile phase B at 0.20 ml/min. Lipids were eluted from the column with a gradient of 70–100% mobile phase B over 8 min at 0.20 ml/min followed by a 7 min rinse of 100% mobile phase B. The column was immediately re-equilibrated under the initial conditions (isocratic hold at 70% mobile phase B) for 10 min.

Consistent mass accuracy (<2 ppm) was maintained through a constant infusion (2 µl/min) of reference calibrants, methyl stearate and HP-122l (with reference mass of 299.294457 and 1221.990637 *m/z*, respectively), via a reference nebulizer. Data were collected in both positive and negative ESI mode acquiring in centroid mode from 100-

1700 *m/z* with an acquisition rate of 1 spectrum per second in 2 GHz extended dynamic range. The Dual AJS ESI capillary voltage was set at 3.5 kV and nozzle voltage was set at 1 kV; The gas temperature, drying gas flow, nebulizer pressure, sheath gas temperature and sheath gas flow were set at 300°C, 8 l/min, 35 psig, 350°C and 11 l/min, respectively. The fragmentor and skimmer voltage were set at 175 V and 65 V, correspondingly. For All Ions MS/MS, collision energy was ramped from 0 V to 40 V during each 4 s data collection cycle. All the acquired mass spectral data was collected in a *.d* format.

3.7.3 Data processing and Statistical analysis

The first step in data analysis for faecal lipidomic profiling workflows is to extract molecular features from the raw mass spectral data (*.d* files) where features are defined by retention time and accurate mass. A feature condenses the abundances from all the specified adducts and isotopes of a compound into a single compound. Molecular features were extracted (Find by Molecular Feature) with Agilent MassHunter Workstation Qualitative Analysis software, version B.06.00 (Agilent Technologies, Santa Clara, CA, USA). The extracted data was exported as compound exchange file (*.cef* files) and imported into Mass Profiler Professional (MPP) software, version 12.1 (Agilent Technologies, Santa Clara, CA, USA) for the first round of data filtering, alignment and normalisation. The generated list of compounds was then exported as a recursive list. Subsequently, a targeted feature finding (Find by Formula-Options) was performed with MassHunter Workstation Qualitative Analysis software by using the recursive list as source of formula to confirm the identified compounds; a mass tolerance of 5 ppm was set. The recursed data was then exported as compound exchange file (*.cef* files). The recursed *.cef* files were then imported into MPP to perform data processing, compounds identification and annotation, and differential and statistical

analysis between baseline and post-*H. pylori* eradication groups. MPP integrated with ID Browser was used for identification and annotation of compounds using LC/MS Personal Compound Databases (METLIN database).

A one-way ANOVA and Tukey's honest significant difference (HSD) post-hoc analysis were performed to compare the lipids between Baseline vs. 6 months, Baseline vs. 12 months post-*H. pylori* eradication and Baseline vs. 18 months post-*H. pylori* eradication; a two-tailed p-value of < 0.01 and false discovery rate (FDR) of $< 1\%$ were considered significant. A multivariate statistical analysis, i.e. Principle Component Analysis (PCA), was performed to observe clustering, trends and outliers in our data sets and to examine whether the Baseline and post-*H. pylori* eradication groups could be differentiated using lipid profiles. The significant differentially expressed lipids were then mapped into pathways using Pathway Analysis integrated in MPP. Identifying small molecules/lipids that showed significant changes between Baseline and post-eradication groups is the most laborious and time-consuming aspect of non-targeted metabolomics/lipidomics. Each identified entities were manually checked through to filter out wrongly annotated or ambiguous lipids as well as non-annotated entities and sorted them into different categories. The significant differentially expressed lipids were then mapped to pathways using Pathway Analysis integrated in MPP.

3.8 Metabolic study

3.8.1 Metabolic hormone testing

MILLIPLEX® MAP Kit-Human Metabolic Hormone Magnetic Bead Panel (EMD Millipore, Billerica, MA, USA) was used to simultaneously quantify gut hormones that are important regulators of food intake, energy expenditure, and body weight via the gut-brain axis in plasma. The hormones include acyl-ghrelin (active), leptin, active amylin, insulin, glucagon-like peptide-1 (GLP-1), total gastric inhibitory polypeptide

(GIP), total peptide YY (PYY), and pancreatic polypeptide (PP). Since it has been reported that serum ghrelin levels change one hour post-prandially (Cummings et al., 2001), only plasma samples collected before the test meal (pre-prandial) and 1 hr after the test meal (post-prandial) were tested. This immunoassay was performed according to manufacturer's protocol. Briefly, 25 µl of assay buffer was added to background wells and sample wells. Then, 25 µl of standards, quality controls and plasma samples were added into their respected wells. Next, 25 µl of matrix solution was added into background, standards and quality controls. Lastly, 25 µl of mixed beads was added to each well before the plate was incubated with agitation on a plate shaker for overnight in dark at 4°C. On the next day, the well contents were removed and washed 3 times with 200 µl of wash buffer using hand-held magnet. Then, 50 µl of detection antibodies was added into each well and the plate was incubated in dark with agitation for 30 min at room temperature (20-25°C). Subsequently, 50 µl of streptavidin-phycoerythrin was added to each well. The plate was again incubated in dark with agitation for 30 min at room temperature (20-25°C). Following incubation, the washing step was repeated for 3 times using hand-held magnet. Finally, 100 µl of sheath fluid was added to all wells and the beads were resuspended on a plate shaker for 5 min. Measurement was performed on Luminex 200™ system with xPONENT™ software, version 3.1 (Luminex, Austin, TX, USA). The median Fluorescent Intensity (MFI) data was saved and analysed using a weighted 5-parameter logistic method for calculating the analyte concentrations in samples.

3.8.2 Statistical analysis

Depending on their distribution, data were expressed as mean ± Standard Deviation (SD), or median and 95% confidence interval (CI). Continuous variables were compared using the *t*-test, or one-way ANOVA, and pairwise analyses (e.g. pre-prandial

vs. post-prandial, baseline vs. 6 months post-eradication, baseline vs. 12 months post-eradication) were performed using non-parametric tests (Wilcoxon's signed rank test, Mann-Whitney U test), as appropriate. Statistical analysis was performed using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA); a two-tailed p-value of <0.05 was considered significant (Francois et al., 2011). Baseline vs. 18 months post-eradication results were not included as the number of volunteers that returned for follow-up assessment at 18 months was too small to be statistically significant.

3.9 Immunological Study

3.9.1 Determination of Immunoglobulin E (IgE) concentration in plasma

Anti-human IgE antibody in serum was determined quantitatively using the Abnova IgE (Human) ELISA Kit (Taipei, Taiwan) according to manufacturer's protocol. Briefly, 20 µl of standards, controls and samples were dispensed into appropriate well coated with monoclonal anti-IgE antibody. The plate was thoroughly mixed for 30 s and incubated at room temperature (18-25°C) for 30 mins. Each well was then aspirated and washed five times with deionized water. Subsequently, 150 µl enzyme conjugate (horseradish peroxidase conjugated anti-human IgE (goat)) was added and gently mixed for 10 s. The incubation and washing steps were then repeated. Next, 100 µl of TMB-substrate (3.3'5'5'-Tetramethylbenzidine) was dispensed into each well. The microtiter plate was gently mixed for 10 s and incubated in dark for 20 mins at room temperature. Lastly, 100 µl of stop solution (1 N HCl) was added to stop the enzymatic reaction. The absorbance of each well was read at 450nm. A standard curve was constructed by plotting the mean absorbance obtained for each calibrator against its concentration in U/ml. Using the mean absorbance value for each sample, the U/ml of the sample was obtained from the standard curve. The results of the IgE ELISA were interpreted as follows: Positive >100 U/ml; Negative <100 U/ml (sensitivity/specificity not available).

3.9.1.1 Statistical analysis

Data were expressed as median ± 95% confidence interval (CI). Pairwise analyses (baseline vs. 6 months post-eradication, baseline vs. 12 months post-eradication and baseline vs. 18 months post-eradication) were performed using non-parametric Wilcoxon's signed rank test. Statistical analysis was performed using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA); a two-tailed p-value of <0.05 was considered significant.

3.9.2 Quantitative determination of cytokine concentration

SunRed Human Tumor Necrosis Factor α (TNF-α) ELISA Kit and Human Interleukin-4 (IL-4) ELISA Kit (SunRed Biological Technology Co., Shanghai, China) were used to determine the concentration of TNF-α and IL-4 quantitatively. Briefly, 50 µl of standards were added into standard wells. Then, 40 µl of diluted plasma and 10 µl of TNF-α/IL-4 labelled with biotin were added into sample wells. Subsequently, 50 µl of streptavidin-HRP conjugate was added into both standard and sample wells and incubated with gentle shaking at 37C for 1 hr. Each well was then aspirated and washed three times with 1X wash solution. Next, 50 µl of Chromogen Solution A and 50 µl of Chromogen Solution B were then added into blank, standard and sample wells. The plate was gently mixed and incubated for 10 min at 37°C in dark. Lastly, 50 µl of Stop Solution was added to stop the enzymatic reaction. The absorbance of each well was read at 450nm. A standard curve was constructed by plotting the mean absorbance obtained for each calibrator against its concentration in U/ml. Using the mean absorbance value for each sample, the U/ml of the sample was obtained from the standard curve.

3.9.2.1 Statistical analysis

Data were expressed as median ± 95% confidence interval (CI). Pairwise analyses (baseline vs. 6 months post-eradication, baseline vs. 12 months post-eradication and baseline vs. 18 months post-eradication) were performed using non-parametric Wilcoxon's signed rank test. Statistical analysis was performed using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA); a two-tailed p-value of <0.05 was considered significant.

3.10 Plasma metabolomics

3.10.1 Plasma sample preparation and metabolite extraction

All the organic solvents used for plasma metabolites extraction were also of HPLC-grade. Methanol and acetonitrile were purchased from Friendemann Schmidt (Australia), LCMS-grade water from a Milli-Q water purification system (EMD Millipore, Billerica, MA, USA) and formic acid from Sigma-Aldrich (St.Louis, MO, USA). Prior to the experimental sample extraction, we optimized the ratio of methanol to sample (v/v) for plasma metabolite extraction as well as the resuspension volume of 95:5 water:acetonitrile. Similar to faecal lipids extraction, the experimental plasma samples were pooled into different groups according to gender, body mass index (BMI), and race (Appendix D) to ascertain whether these variables are the confounding factors of this study. A methanol precipitation of proteins was conducted by adding 500 µl aliquots of ice cold methanol to 100 µl aliquots of plasma samples. The samples were immediately vortexed for 30 sec and allowed to rest on ice for 20 min. After centrifugation at 12,000 *xg* for 10 min, the metabolite containing supernatant was removed from the precipitated protein pellet and transferred to fresh tubes. The supernatant samples were dried in a CentriVap Concentrator Systems (LABCONCO, Kansas City, MO, USA) at 4°C and then resuspended to a 100 µl of water: acetonitrile

(95:5), vortexed for 30 s and then centrifuged again at 12,000 *xg* for 5 min before injecting into liquid chromatography system (Denery, Nunes, Hixon, Dickerson, & Janda, 2010).

3.10.2 High-Performance Liquid Chromatography Mass Spectrometry (LC-MS)

The platform and methodology for LC-MS analyses on the plasma metabolomics were the same as faecal lipids mentioned above except that instead of using All Ions MS/MS technique, we were using only MS. Analysis was performed utilising both positive ionisation and negative ionisation mode of the Dual AJS ESI source. Optimization of LC parameters was conducted, and a quality control sample (constituted of pooled of all experimental plasma samples) and an external commercial Waters MetID Small Molecule Standard Mix (constituted of 5-hydroxy omeprazole and omeprazole sulfone) (Waters Corporation, Milford, MA, USA) were periodically injected throughout the duration of analysis. The sample run order was also assigned randomly to avoid any systematic bias.

For positive ionisation, the aqueous mobile phase (mobile phase A) was water with 0.1% formic acid and the organic mobile phase (mobile phase B) was acetonitrile with 0.1% formic acid. For negative ionisation, the aqueous mobile phase (mobile phase A) was water with 1 mM Ammonium fluoride (Sigma-Aldrich, St. Louis, MO, USA) and the organic mobile phase (mobile phase B) was acetonitrile only. The instrument control and data acquisition were conducted using the MassHunter Workstation Data Acquisition software, version B.05.01 (Agilent Technologies, Santa Clara, CA, USA). A five µl of extracted plasma metabolites was loaded onto Zorbax Eclipse Plus C18, 2.1 x 100 mm, 1.8 µm reverse phase column (Agilent Technologies, Santa Clara, CA, USA) with 5% mobile phase B at 0.45 ml/min. Metabolites were eluted from the column with a gradient of 5–100% mobile phase B over 22 min at 0.45 ml/min followed by a 5 min

rinse of 100% mobile phase B. The column was immediately re-equilibrated under the initial conditions (isocratic hold at 5% mobile phase B) for 5 min.

Data were collected in both positive and negative ESI mode acquiring in profile mode from 70-1700 *m/z* with an acquisition rate of 2 spectra per second in 2 GHz extended dynamic range. The Dual AJS ESI capillary voltage was set at 3 kV; The gas temperature, drying gas flow, nebulizer pressure, sheath gas temperature and sheath gas flow were set at 300°C, 10 l/min, 45 psig, 250°C and 8 l/min, respectively. For the MS Q-TOF, the fragmentor and skimmer voltage were set at 140 V and 60 V, correspondingly. All the acquired mass spectral data was collected in a *.d* format.

3.10.3 Data processing and Statistical analysis

The first step in data analysis for plasma metabolomics profiling workflows is to extract molecular features from the raw mass spectral data (*.d* files) where features are defined by retention time and accurate mass. A feature condenses the abundances from all the specified adducts and isotopes of a compound into a single compound. Molecular features were extracted (Find by Molecular Feature) with Agilent MassHunter Workstation Qualitative Analysis software, version B.06.00 (Agilent Technologies, Santa Clara, CA, USA). The extracted data was exported as compound exchange file (*.cef* files) and imported into Mass Profiler Professional (MPP) software, version 12.1 (Agilent Technologies, Santa Clara, CA, USA) for the first round of data filtering, alignment and normalisation. The generated list of compounds was then exported as a recursive list. Subsequently, a targeted feature finding (Find by Formula-Options) was performed with MassHunter Workstation Qualitative Analysis software by using the recursive list as source of formula to confirm the identified compounds; a mass tolerance of 5 ppm was set. The recursed data was then exported as compound exchange file (*.cef* files). The recursed *.cef* files were then imported into MPP to perform data

processing, compounds identification and annotation, and differential and statistical analysis between baseline and post-*H. pylori* eradication groups. MPP integrated with ID Browser was used for identification and annotation of compounds using LC/MS Personal Compound Databases (METLIN database).

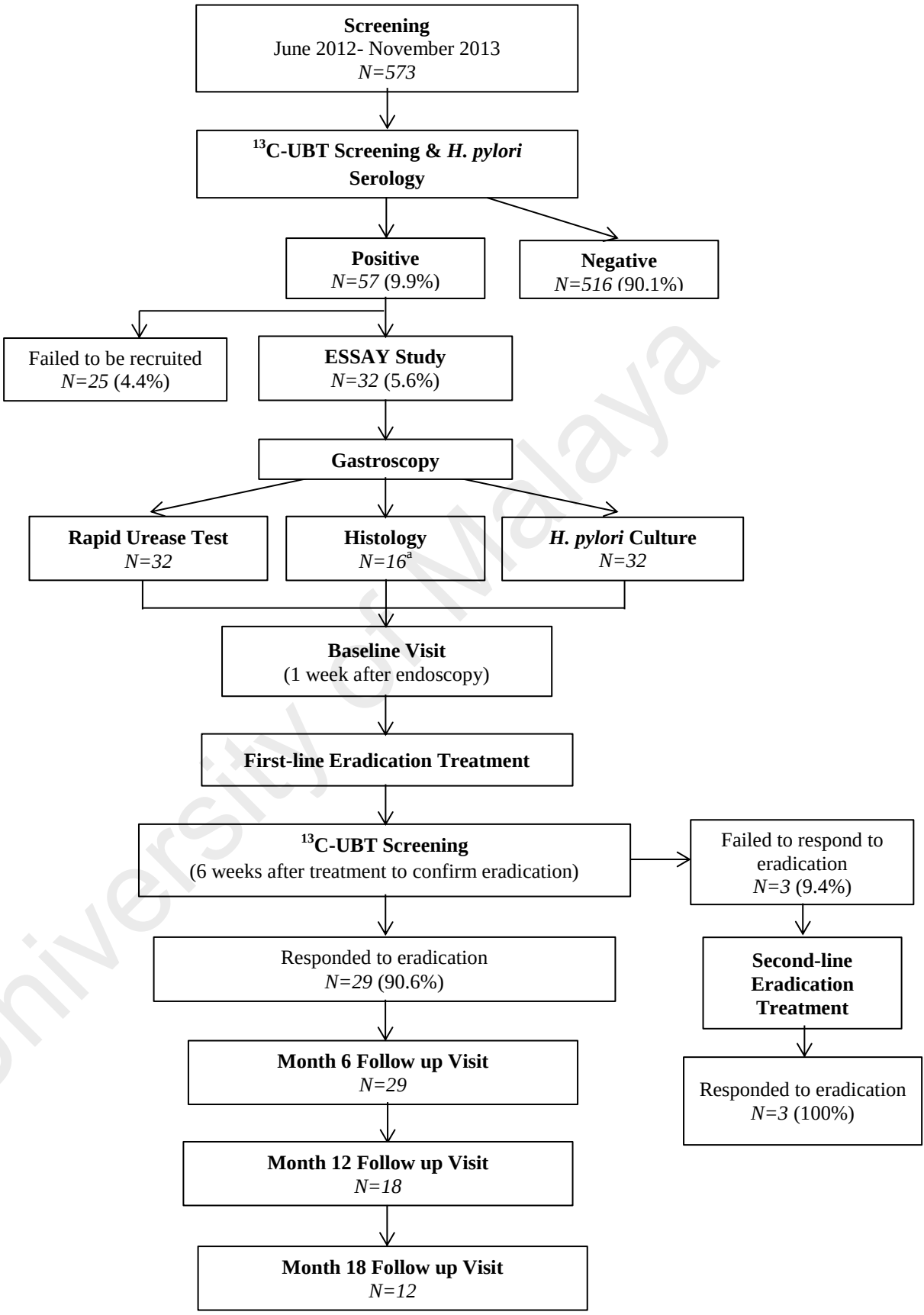
A one-way ANOVA and Tukey's HSD post-hoc analysis were performed to compare the metabolites between Baseline vs. 6 months, Baseline vs. 12 months post-*H. pylori* eradication and Baseline vs. 18 months post-*H. pylori* eradication; a two-tailed p-value of < 0.01 and FDR of $< 1\%$ were considered significant. A multivariate PCA was performed to observe clustering, trends and outliers in our data sets and to examine whether the Baseline and post-*H. pylori* eradication groups could be differentiated using metabolite profiles. The significant differentially expressed metabolites were then mapped to pathways using Pathway Analysis integrated in MPP.

CHAPTER 4: RESULTS

4.1 Subject demographics and Clinical analysis

Initially, 573 healthy adult volunteers participated in this study. Of the 573 volunteers recruited, 67 (11.7%) tested positive using UBT. The volunteers were also screened for *H. pylori* serology in which a large fraction was found to be IgA, IgG, or both IgA/IgG positive, respectively. Of the 573 volunteers screened in this study, 57 (9.9%) tested positive using both UBT and detection of anti-*H. pylori* antibodies and were considered as *H. pylori*-positive (Table 4.1). However, only 32 (5.6%) consented to participate in the ESSAY Study (Figure 4.1). They were consented to undergo gastroscopy and biopsy samples were obtained. Their *H. pylori* infection status was further confirmed using RUT, histological examination, and culture. Out of 32 of them, only 16 biopsy samples were able to be submitted for histological analysis. Biopsy samples were not collected for the remaining 16 volunteers who did not consent for gastroscopy. The histology results showed the presence of both neutrophils and mononuclear cells in all the samples which indicated that these *H. pylori* positive volunteers all had chronic active gastritis (Table 4.2). Of 32 of them, 31 were RUT positive whilst 27 were culture positive. First-line eradication treatment was given to the 32 *H. pylori* positive volunteers but was successful for only 29 (90.6%) (see Chapter 3, pg 57 for explanation). Twenty-nine of these volunteers were recruited into the ESSAY Study. For the remaining three (9.4%) volunteers who failed to respond, second-line eradication treatment was prescribed. They were excluded from this study, since their number was small and they differed from the primary eradication group. Summary of the recruitment was shown in Figure 4.1.

The majority of the 29 volunteers eventually recruited into the ESSAY Study were medical students of the University of Malaya. The mean age of the volunteers was



[a]Biopsy sample was submitted for histology for 16 cases only.

Figure 4.1: Summary of ESSAY Study

Table 4.1: Summary of *H. pylori* status screening

Classification	^{13}C-UBT N (%)		*H. pylori* Serology N (%)					Overall *H. pylori* Status	
	Positive	**Negative**	**Positive (IgA only)**	**Positive (IgG only)**	**Positive (IgA+IgG)**	**Seropositive**	**Seronegative**	**Positive** [a]	**Negative**
Total	**67 (11.7)**	**504 (88.0)**	**120**	**39**	**60**	**219 (38.2)**	**354 (61.8)**	**57 (9.9)**	**516 (90.1)**
Ethnicity									
Malay	12 (17.9)	225 (44.6)	46	8	10	64 (29.2)	173 (48.9)	9 (15.8)	228 (44.2)
Chinese	15 (22.4)	160 (31.8)	25	19	23	67 (30.6)	109 (30.8)	12 (21.0)	164 (31.8)
Indian	37 (55.2)	112 (22.2)	48	12	24	84 (38.4)	66 (18.6)	33 (57.9)	117 (22.7)
Other	3 (4.5)	7 (1.4)	1	0	3	4 (1.8)	6 (1.7)	3 (5.3)	7 (1.3)
Gender									
Male	28 (41.8)	217 (43.1)	64	12	20	96 (43.8)	149 (42.1)	22 (38.6)	223 (43.2)
Female	39 (58.2)	287 (56.9)	56	27	40	123 (56.2)	205 (57.9)	35 (61.4)	293 (56.8)
Indeterminate	**2 (0.3)**		-	-	-	-	-	-	-

[a]UBT with IgA and/or IgG positive were considered as *H. pylori* positive.

Table 4.2: Summary of histopathology findings

Histology[a]	Scale, N (%)			
	Absent	**Mild**	**Moderate**	**Marked**
Neutrophils	1 (6)	5 (31)	7 (44)	3 (19)
Mononuclear cells	-	-	4 (25)	12 (75)
Atrophy	16 (100)	-	-	-
Intestinal metaplasia	15 (93.8)	1 (6.2)	-	-

[a]Only sixteen RUT positive antral biopsy samples were evaluated for histology analysis.

24.9 years and 23 (79%) were female. The volunteers consisted of 5 (17%) Malay, 8 (28%) Chinese, 13 (45%) Indian while the remaining 3 (10%) came from other ethnicities. The mean BMI at baseline was 22.45 kg/m^2 which was consistent with the normal weight category (World Health Organization) ("Obesity: preventing and managing the global epidemic. Report of a WHO consultation", 2000). The 29 volunteers were followed up to 6 months post-*H. pylori* eradication. However, 11 and 17 volunteers dropped out of the study at 12 and 18 months post- *H. pylori* eradication respectively. Thus, we could only manage to obtain follow-up to one year with 18 of them and only follow-up to 18 months with 12 of them (Figure 4.1).

4.1.1 Medical history and Risk factors associated with *Helicobacter pylori* infection

According to the medical history survey in the baseline questionnaire, the majority of the volunteers in this study did not present symptoms such as indigestion, heartburn, regurgitation and nausea at all over the last 2 months. In addition, the majority of the volunteers also expressed that these symptoms did not interfere with their normal activities (eating, sleeping, work, leisure) over the last 2 months (data not shown).

A binomial logistic regression was performed to ascertain the effects of gender, race, method of birth, birth order, family household income, smoking habit and asthma on the likelihood that volunteers were infected with *H. pylori*. However, no significant relationship was found between them except for race. Race was a factor associated with an increased likelihood of volunteers infected with *H. pylori* (p=0.000, odd ratio=2.85; 95% CI=1.87-2.89). As shown in Table 4.1, in this study, the prevalence of *H. pylori* infection of Indians (57.9%) is higher than in Chinese (21%), Malays (15.8%) and other ethnic groups (5.3%). Although a significant relationship was not found between gender

and *H. pylori* infection status of the volunteers, the prevalence of *H. pylori* infection in female (61.4%) was higher than that of male (38.6%) in this study (Table 4.1).

4.1.2 *Helicobacter pylori* isolates genotypes

Of the 29 volunteers that eventually recruited into the ESSAY Study, only 24 of them were *H. pylori* culture positive. However, only 19 *H. pylori* strains were successfully isolated from the biopsy samples. The status of *SSA* gene, *cagA* gene and *vacA* alleles of *H. pylori* strains isolated from the ESSAY volunteers are shown in Table 4.3. All the *H. pylori* strains isolated possessed the *SSA* gene as predicted. All the *H. pylori* strains were found to be *cagA*- and *vacA*-positive. Genotyping of *vacA* alleles also revealed that all of these *H. pylori* isolates expressed s1a allele, none of them expressed s1b allele and 47.4% of them expressed the hybrid of s1a/s1c alleles in their signal region. For middle region of *VacA* gene, the frequency of m1 and m2 alleles was 73.7% and 26.3% respectively whereas for intermediate region of *vacA* gene, the frequency of i1 and i2 alleles was 84.7% and 5.3% correspondingly. To sum up, the *H. pylori* strains isolated from the biopsy samples could be categorised in five different groups that expressed the *vacA* s1a/m1/i1, s1a/m2/i1, s1a/m2/i2, s1a/s1c/m1/i1, and s1a/s1c/m2/i1 alleles, of which their frequency was 42.1%, 5.3%, 5.3%, 31.6%, 15.8% respectively (Table 4.3).

Table 4.3: Summary of genotyping of *H. pylori* isolates

Gene	No. of subjects N (%)
26 kDa *SSA*	19 (100.0)
cagA	19 (100.0)
vacA	19 (100.0)
s1a/m1/i1	8 (42.1)
s1a/m1/i2	-
s1a/m2/i1	1 (5.3)
s1a/m2/i2	1 (5.3)
s1a/s1c/m1/i1	6 (31.6)
s1a/s1c/m1/i2	-
s1a/s1c/m2/i1	3 (15.8)
s1a/s1c/m2/i2	-

4.1.3 Severity of Dyspepsia Assessment (SODA) questionnaire and SF-36 Health Survey pre- and post-*Helicobacter pylori* eradication

The Severity of Dyspepsia Assessment (SODA) questionnaire was given to the volunteers at baseline and at the follow-up visits to assess dyspeptic symptoms following *H. pylori* eradication. The SODA questionnaire demonstrated that the intensity of abdominal discomfort (or stomach-ache) 7 days prior to the survey, on average, had dropped significantly (Mann Whitney U-Test, $p < 0.05$) when post-eradication values were compared with baseline values. Following *H. pylori* eradication, the satisfaction level of volunteers towards the current level of abdominal discomfort (or stomach-ache) due to dyspepsia had increased significantly (Mann Whitney U-Test, $p < 0.05$). Other signs and symptoms associated with dyspepsia (bad breath, burping/belching, heartburn, bloating, passing gas, sour taste, and nausea) did not significantly present problems to the volunteers before or after *H. pylori* eradication (data not shown).

Assessing the quality of life profile and volunteers' health condition, using the SF-36 Health Survey, showed that, the volunteers expressed that their health condition had improved significantly at 6 and 18 months post-eradication of *H. pylori,* compared to the previous year (Mann Whitney U-Test, $p < 0.05$). The survey also indicated that both the physical and emotional health of volunteers four weeks prior to the survey, (pre- versus post-*H. pylori* eradication groups), had not affected their performance at work or other regular daily activities and did not interfere with their normal social activities (data not shown).

4.2 Stool metagenomic analysis

4.2.1 Demographics of the study cohort

The DNA of the collected stool samples was extracted. The V3-V4 region of the *16S rRNA* of the extracted samples were amplified and sequenced. All the *16S rRNA* sequences were deposited in MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST) under the accession numbers as shown in Table 4.4.

Table 4.4: Accession number of the *16S rRNA* sequences deposited in MG-RAST

Accession number	Time-point	Sample code
4562320.3	Baseline	C002
4562322.3	Baseline	C003
4562324.3	Baseline	C005
4562326.3	Baseline	C008
4562328.3	Baseline	C009
4562330.3	Baseline	C017
4562332.3	Baseline	C019
4562334.3	Baseline	C020
4562340.3	Baseline	C033
4562342.3	Baseline	C034
4562344.3	Baseline	C037
4562346.3	Baseline	C039
4562348.3	Baseline	C041
4562350.3	Baseline	C042
4562352.3	Baseline	C048
4562354.3	Baseline	C050
4562356.3	Baseline	C053
4562321.3	6 months post-eradication	C002
4562323.3	6 months post-eradication	C003
4562325.3	6 months post-eradication	C005
4562327.3	6 months post-eradication	C008
4562329.3	6 months post-eradication	C009
4562331.3	6 months post-eradication	C017
4562333.3	6 months post-eradication	C019
4562335.3	6 months post-eradication	C020
4562341.3	6 months post-eradication	C033
4562343.3	6 months post-eradication	C034
4562345.3	6 months post-eradication	C037
4562347.3	6 months post-eradication	C039
4562349.3	6 months post-eradication	C041
4562351.3	6 months post-eradication	C042
4562353.3	6 months post-eradication	C048
4562355.3	6 months post-eradication	C050
4562357.3	6 months post-eradication	C053

Table 4.4, continued

Accession number	Time-point	Sample code
4626552.3	12 months post-eradication	C003
4626553.3	12 months post-eradication	C009
4626556.3	12 months post-eradication	C017
4626557.3	12 months post-eradication	C033
4626558.3	12 months post-eradication	C034
4626560.3	12 months post-eradication	C037
4626562.3	12 months post-eradication	C039
4626564.3	12 months post-eradication	C041
4626567.3	12 months post-eradication	C048
4626568.3	12 months post-eradication	C053
4626559.3	18 months post-eradication	C034
4626561.3	18 months post-eradication	C037
4626563.3	18 months post-eradication	C039
4626565.3	18 months post-eradication	C041
4626566.3	18 months post-eradication	C042
4626569.3	18 months post-eradication	C053

4.2.2 Characteristics of stool microbiome

Stool microbiome of baseline samples prior of eradication of *H. pylori* were compared with stool microbiome of 6, 12, and 18 months post-*H. pylori* eradication samples. A total of 5,834,726 quality-filtered reads were obtained from Baseline vs. 6 months post-eradication group with an average of 171,610 ± 58,372 (standard deviation, SD) reads per sample, whilst, a total of 3,077,037 and 2,000,505 quality-filtered reads with an average of 153,852 ± 32,306 and 166,709 ± 69,793 reads per sample were obtained from Baseline vs. 12 months and 18 months post-eradication group, respectively. These reads were clustered into 45,875 unique OTUs at 97% sequence similarity in Baseline vs. 6 months post-eradication group with an average of 1349 OTUs per sample. In Baseline vs. 12 months post-eradication group, these reads were clustered into 31,351 unique OTUs at 97% sequence similarity with an average of 1568 OTUs per sample. In Baseline vs. 18 months post-eradication group, they were clustered into 28,157 unique OTUs at 97% sequence similarity with an average of 2346 OTUs per sample.

For the comparison of microbial biodiversity within Baseline and 6 months post-eradication stool samples, alpha diversity analysis was performed after rarefaction to

82,536 sequences/sample (minimum sampling depth). For the comparison of microbial diversity within Baseline and 12 months post-eradication stool samples, alpha diversity analysis was performed after rarefaction to 84,177 sequences/sample whereas for the comparison of microbial diversity within Baseline and 18 months post-eradication stool samples, alpha diversity analysis was performed after rarefaction to 84,333 sequences/sample. Several "phylogeny-based" and "non-phylogeny-based" matrices were used to calculate alpha diversity, including PD whole tree, chao1, observed species for microbial richness and the Shannon index for microbial evenness. When Baseline samples was compared with 6 months post-eradication samples, the rarefaction curves generated for all four matrices showed that the stool microbiome in 6 months-post eradication samples demonstrated greater diversity than Baseline samples (Figure 4.2). However, non-parametric two-sample t-test performed on the four matrices showed that there was no significant difference of the microbial diversity within baseline and 6 months post-eradication stool samples ($p>0.05$). Similarly, when Baseline samples was compared with 12 or 18 months post-eradication samples, the rarefaction curves generated for all four matrices showed that the stool microbiome in 12 and 18 months-post eradication samples demonstrated greater diversity than Baseline samples (Figure 4.3 and 4.4). However, non-parametric two-sample t-test performed on the four matrices showed that the microbial biodiversity within baseline and 12 months post-eradication as well as within baseline and 18 months post-eradication stool samples also did not differ significantly ($p>0.05$).

The PCoA plots generated from both weighted and unweighted UniFrac distance metrics in beta diversity analysis for Baseline vs. 6 months post-eradication, Baseline vs. 12 months post-eradication and Baseline vs. 18 months post-eradication did not show distinct clustering between the time-points (Figure 4.5 & 4.6). Non-parametric statistical test analysis of similarity (ANOSIM) also showed that the differences in bacterial

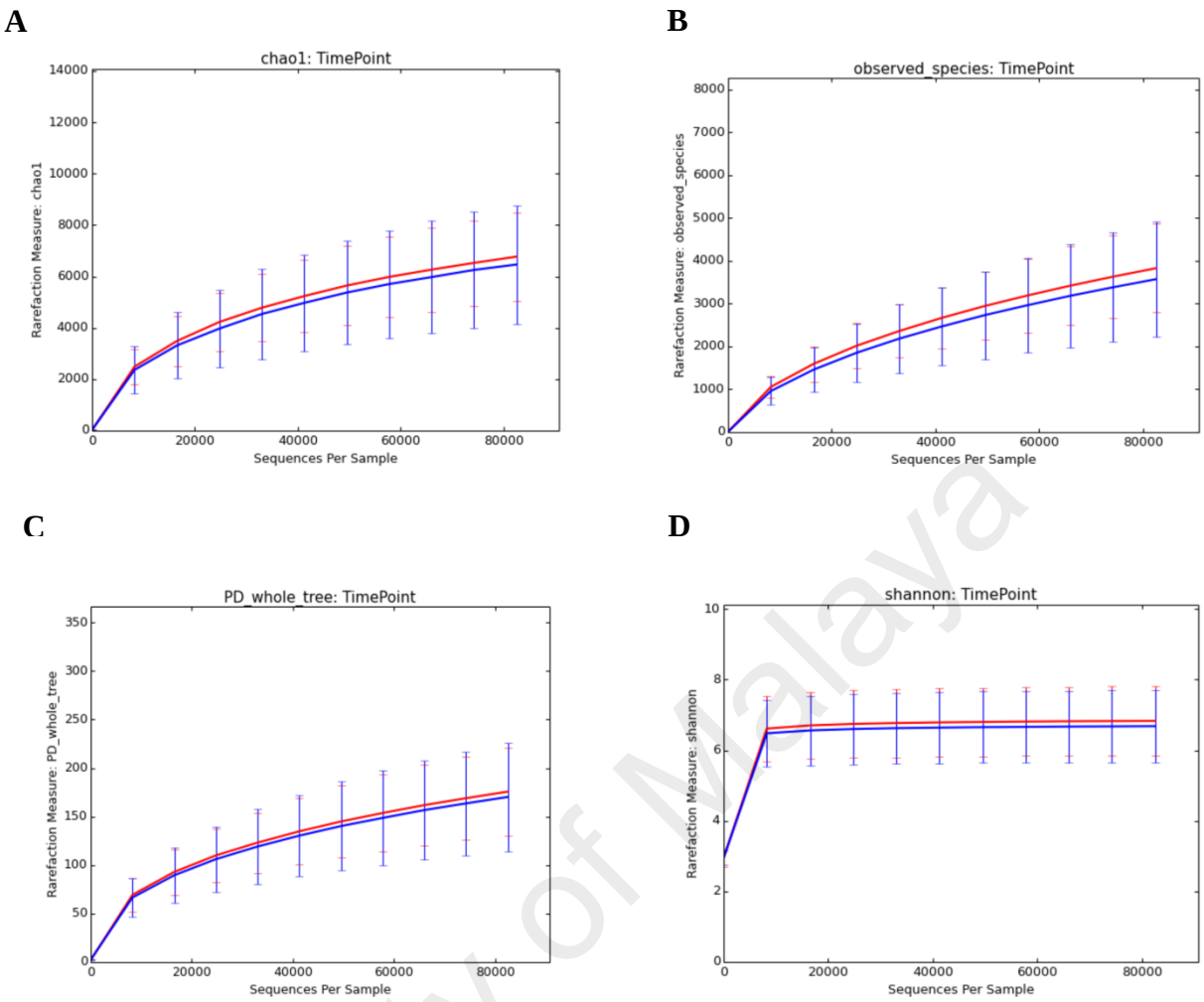


Figure 4.2: Alpha diversity analysis comparing between baseline and 6 months post-*H. pylori* eradication. Rarefaction curve for **A.** chao1, **B.** observed species, **C.** PD whole tree, and **D.** the Shannon index generated from alpha diversity analysis

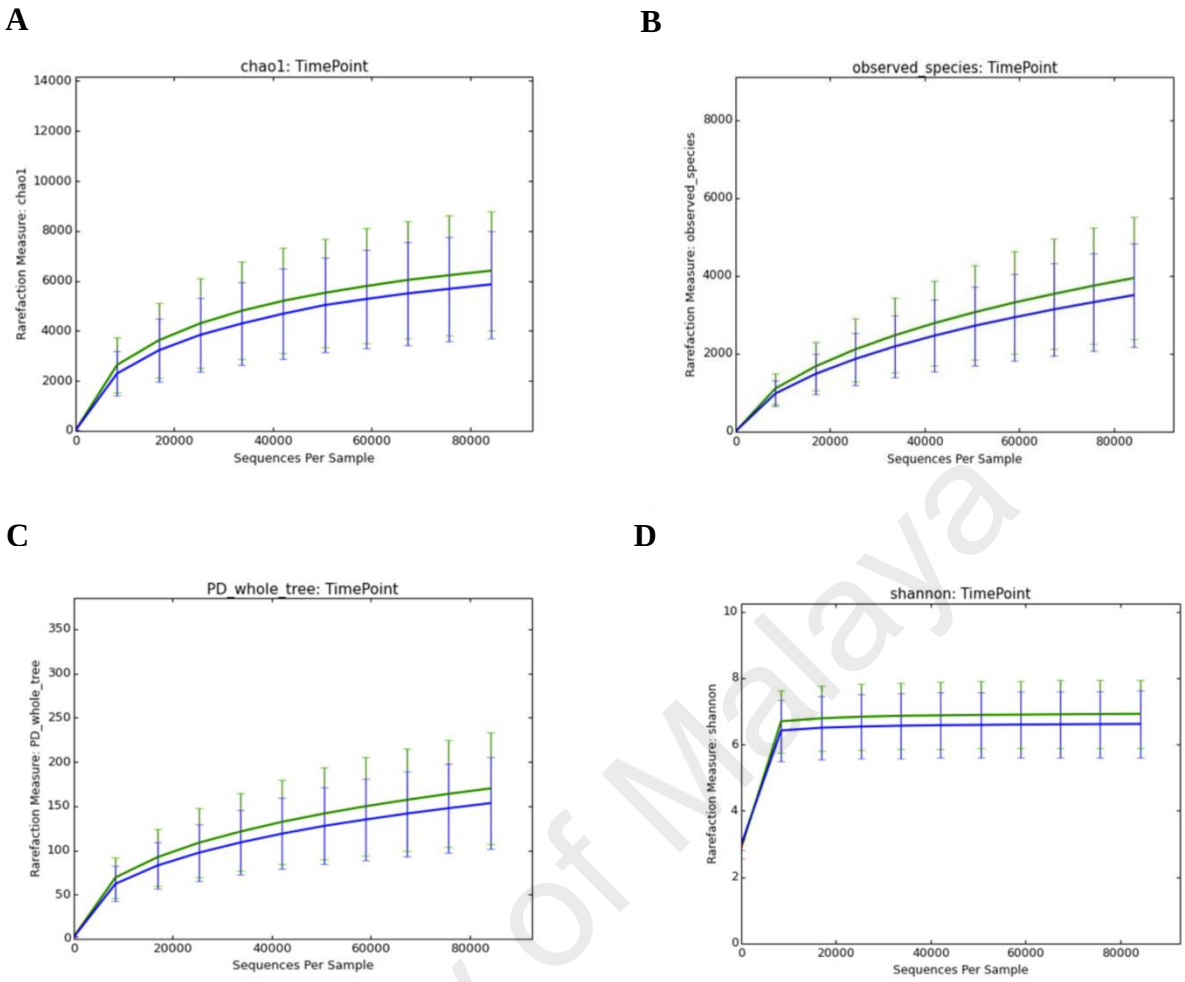


Figure 4.3: Alpha diversity analysis comparing between baseline and 12 months post-*H. pylori* eradication. Rarefaction curve for **A.** chao1, **B.** observed species, **C.** PD whole tree, and **D.** the Shannon index generated from alpha diversity analysis

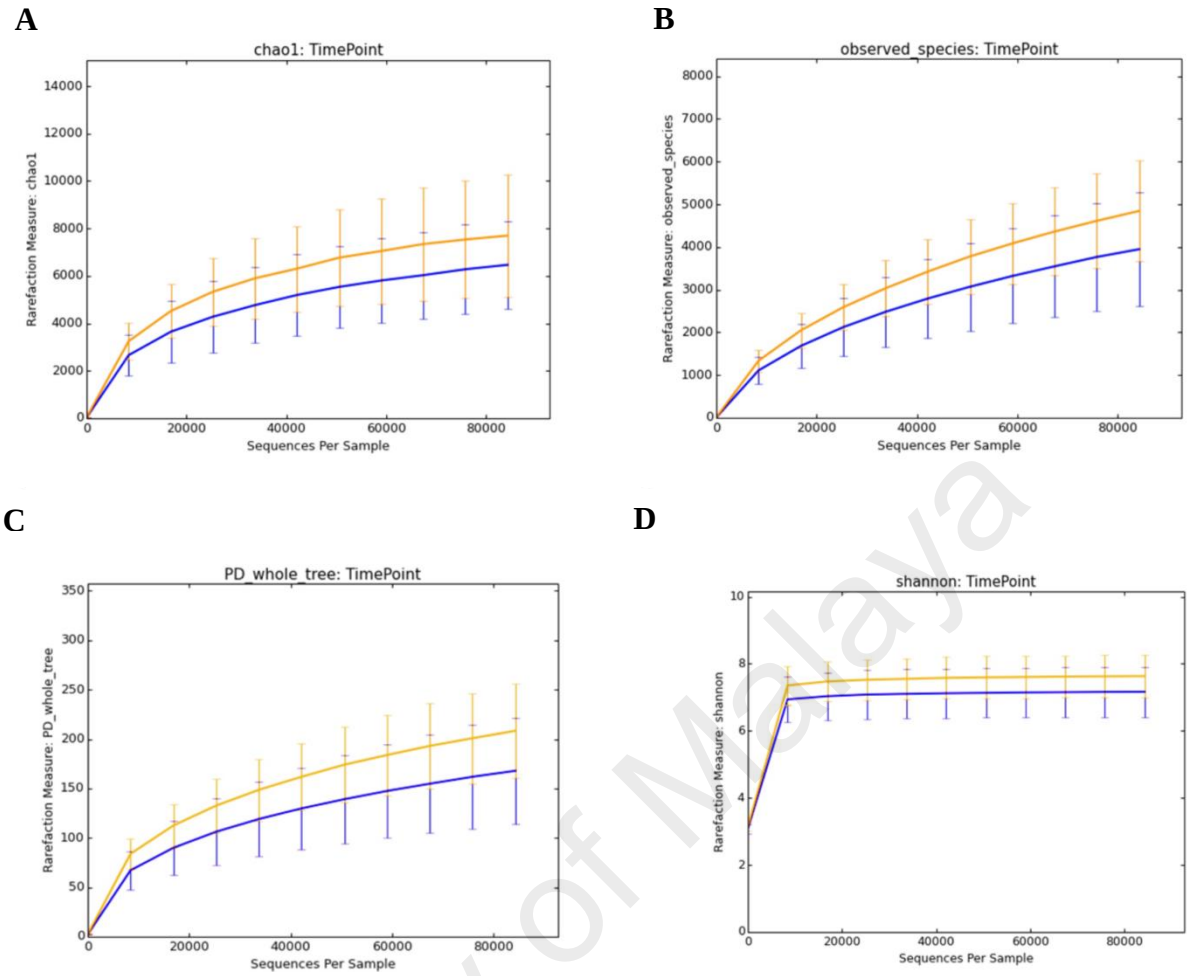
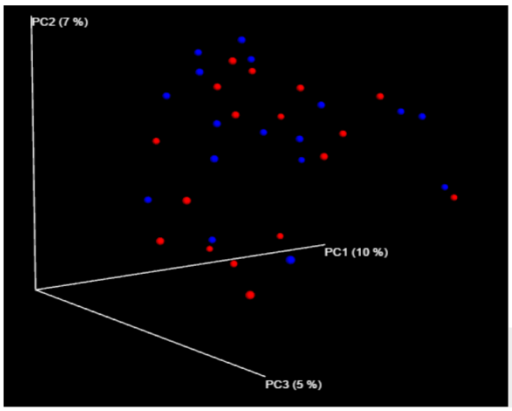


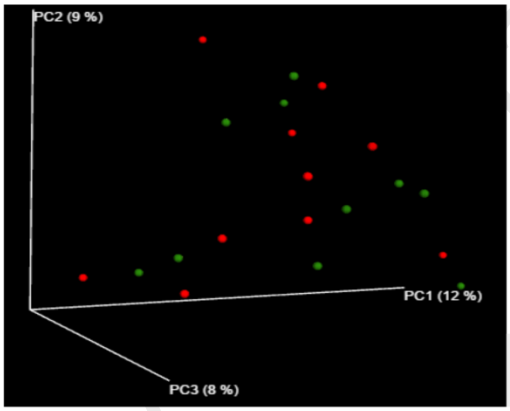
Figure 4.4: Alpha diversity analysis comparing between baseline and 18 months post-*H. pylori* eradication. Rarefaction curve for **A.** chao1, **B.** observed species, **C.** PD whole tree, and **D.** the Shannon index generated from alpha diversity analysis

A



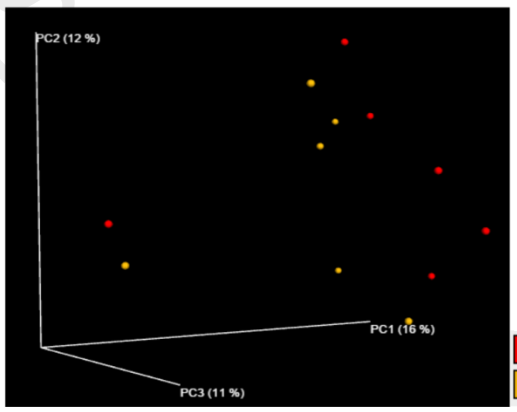
Indicator:
Baseline
6 months post-eradication

B



Indicator:
Baseline
12 months post-eradication

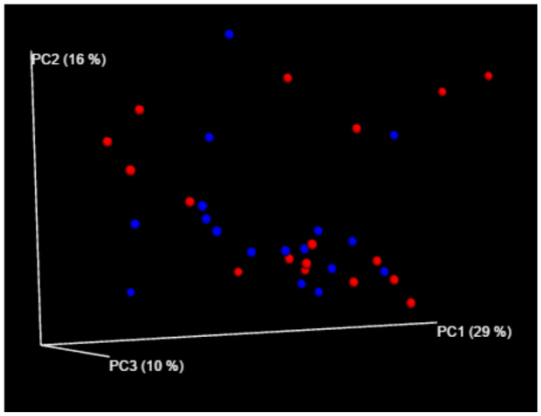
C



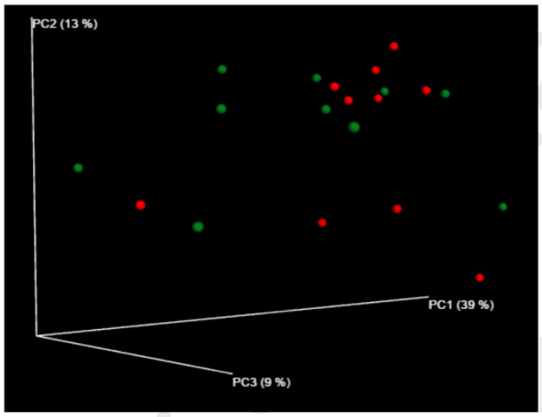
Indicator:
Baseline
18 months post-eradication

Figure 4.5: Unweighted Principal Coordinate Analysis (PCoA) plots. Unweighted PCoA plots generated in beta diversity analysis for **A.** Baseline vs. 6 months post-eradication, **B.** Baseline vs. 12 months post-eradication, and **C.** Baseline vs. 18 months post-eradication

A



B



C

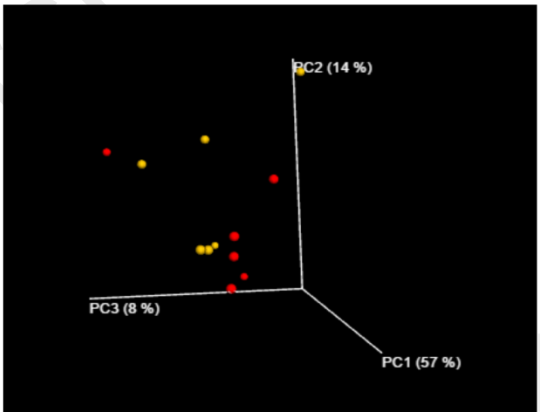


Figure 4.6: Weighted Principal Coordinate Analysis (PCoA) plots. Weighted PCoA plots generated in beta diversity analysis for **A.** Baseline vs. 6 months post-eradication, **B.** Baseline vs. 12 months post-eradication, and **C.** Baseline vs. 18 months post-eradication

communities between the time-points were not significant ($p>0.05$).

The taxonomy summary of the phyla and genera of stool microbiome in healthy young Malaysian adults pre- and post-eradication of *H. pylori* were summarised in Figure 4.7 and Figure 4.8. Before the eradication *H. pylori*, the most abundant phyla were *Bacteroidetes* (52.09%; 95% CI, 44.85%-60.07%), *Firmicutes* (32.91%; 95% CI, 26.67%-39.06%), *Actinobacteria* (6.68%; 95% CI, 4.03%-9.68%), and *Proteobacteria* (5.77%; 95% CI, 3.94%-8.03%) (Figure 4.7A). At 6 months post-*H. pylori* eradication, the relative abundance of *Bacteroidetes* decreased to 47.82% (95% CI, 42.24%-52.94%) and *Firmicutes* increased to 37.82% (95% CI, 32.19%-43.71%), as compared to Baseline. In addition, the relative abundance of both phyla *Actinobacteria* (4.86%; 95% CI, 2.65%-7.27%), and *Proteobacteria* (3.69%; 95% CI, 2.58%-4.92%) also reduced at 6 months post-eradication. Interestingly, we also observed that the relative abundance of *Verrucomicrobia* increased markedly, from 0.07% (95% CI, 0.01%-0.17%) at Baseline to 3.30% (95% CI, 0.08%-9.53%) at 6 months post-eradication (Figure 4.7B). At 12 months post-*H. pylori* eradication, *Firmicutes* (43.53%; 95% CI, 31.66%-54.29%) replaced *Bacteroidetes* (36.84%; 95% CI, 26.45%-49.26%), as the most abundant phylum in the stool microbiome. Similar patterns were observed in *Actinobacteria* (8.14%; 95% CI, 3.34%-14.48%), *Proteobacteria* (6.75%; 95% CI, 2.93%-12.39%), and *Fusobacteria* (0.36%; 95% CI, 0%-1.07%) in which their relative abundances higher than Baseline. The relative abundance of *Verrucromicrobia* (0.97%; 95% CI, 0.2%-2.1%), on the other hand, seems to be restoring to the Baseline level though its abundance was still higher than that of Baseline (Figure 4.7C). At 18 months-post *H. pylori* eradication, both *Bacteroidetes* and *Firmicutes* had the highest relative abundance which was approximately 38% for both phyla. Enrichment of the relative abundance of *Proteobacteria* (8.40%; 95% CI, 3.77%-13.03%) and *Actinobacteria* (7.96%; 95% CI, 3.11%-14.57%) were observed where their relative abundance

increased to higher than Baseline, 6 months, and also 12 months post-eradication. The relative abundance of *Verrucomicrobia* (1.29%; 95% CI, 0%-3%) at 18 months-post eradication was still higher than Baseline whereas the relative abundance of *Fusobacteria* (0.11%; 95% CI, 0%-0.32%) had been restored to Baseline level (Figure 4.7D) (Table 4.5). The *Bacteroidetes*-to-*Firmicutes* ratio at Baseline, 6 months, 12 months and 18 months were 8:5, 13:10, 5:6, and 1:1, respectively. Despite the observed trend in *Bacteroidetes*-to-*Firmicutes* ratio, these changes across time were not statistically significant (Paired-samples t-test, $p \geq 0.05$). The *Bacteroidetes*-to-*Firmicutes* ratio was also not significantly correlated with BMI of the volunteers across different time-points (Person's correlation, $p \geq 0.05$).

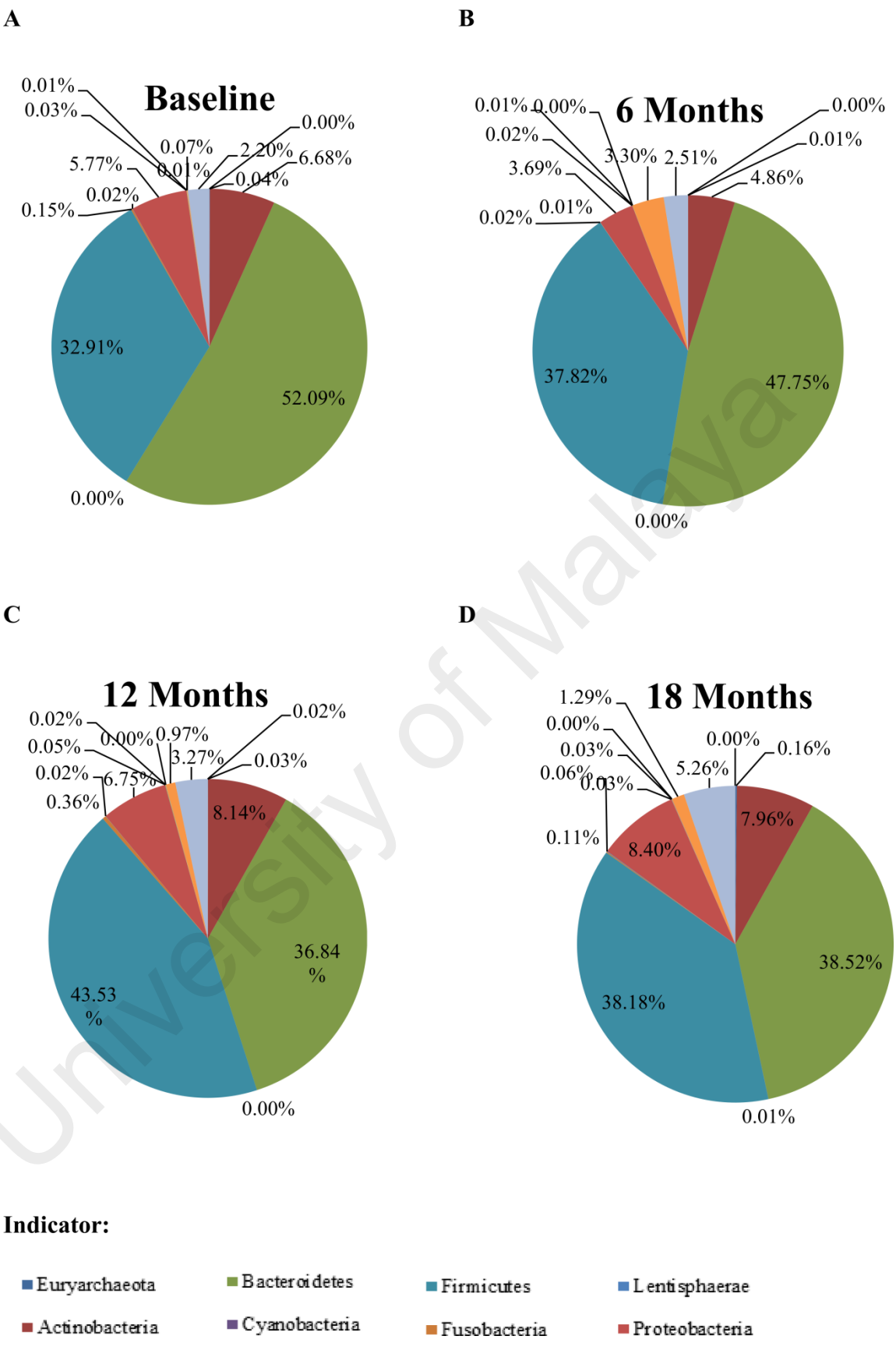
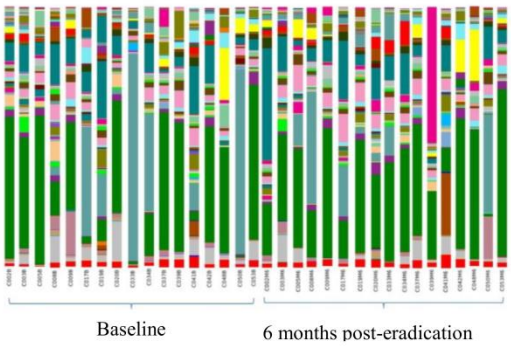
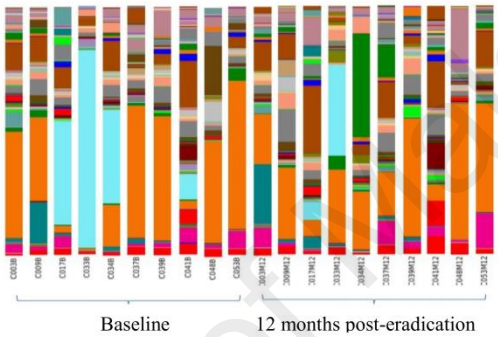


Figure 4.7: Relative abundance of phyla. Relative abundance of phyla at **A.** Baseline, **B.** 6 months post-eradication, **C.** 12 months post-eradication and **C.** 18 months post-eradication

A



B



C

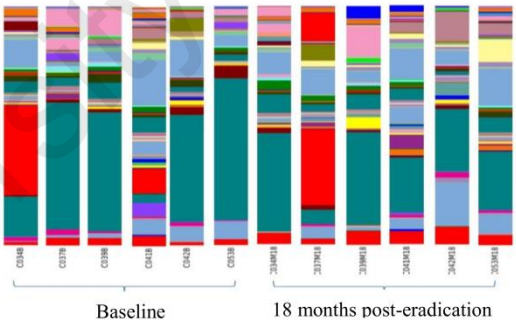


Figure 4.8: Relative abundance of genera. Comparison of the relative abundance of genera between **A.** Baseline vs. 6 months post-eradication, **B.** Baseline vs. 12 months post-eradication and **C.** Baseline vs. 18 months post-eradication. Indicators see Appendix F.

Table 4.5: Comparison of relative abundance of phyla of the gut microbiome pre- and post-*H. pylori* eradication

Phylum	Mean relative abundance (%) (95% CI*)				Comparison of the relative abundance at baseline and 6 months post-eradication (p-value)[a]	Comparison of the relative abundance at baseline and 12 months post-eradication (p-value)[a]	Comparison of the relative abundance at baseline and 18 months post-eradication (p-value)[a]
	Baseline	6 months post-eradication	12 months post-eradication	18 months post-eradication			
Firmicutes	32.91 (26.67-39.06)	37.82 (32.19-43.71)	43.53 (31.66-54.29)	38.17 (31.53-44.66)	0.31	0.12	0.42
Bacteroidetes	52.09 (44.85-60.07)	47.75 (42.24-52.94)	36.84 (26.45-49.26)	38.52 (33.22-43.77)	0.41	**0.02**	0.13
Actinobacteria	6.68 (4.03-9.68)	4.86 (2.65-7.27)	8.14 (3.34-14.48)	7.96 (3.11-14.57)	0.41	0.54	0.36
Proteobacteria	5.77 (3.94-8.03)	3.69 (2.58-4.92)	6.75 (2.93-12.39)	8.40 (3.77-13.03)	0.17	0.85	0.69
Fusobacteria	0.15 (0-0.41)	0.02 (0-0.05)	0.36 (0-1.07)	0.11 (0-0.32)	0.23	0.77	0.35
Verrucomicrobia	0.07 (0.01-0.17)	3.30 (0.08-9.53)	0.97 (0.20-2.11)	1.29 (0-3)	0.31	0.12	0.21
Euryarcheota	0.04 (0-0.11)	0.01 (0-0.02)	0.03 (0-0.07)	0.16 (0-0.48)	0.21	0.73	0.37
Synergistetes	0.03 (0-0.07)	0.02 (0-0.06)	0.05 (0-0.10)	0.03 (0-0.08)	0.60	0.87	0.29

Table 4.5, continued

Phylum	Mean relative abundance (%) (95% CI*)				Comparison of the relative abundance at baseline and 6 months post-eradication (p-value)[a]	Comparison of the relative abundance at baseline and 12 months post-eradication (p-value)[a]	Comparison of the relative abundance at baseline and 18 months post-eradication (p-value)[a]
	Baseline	6 months post-eradication	12 months post-eradication	18 months post-eradication			
Lentisphaerae	0.02 (0-0.04)	0.01 (0-0.02)	0.02 (0-0.05)	0.06 (0-0.16)	0.30	0.41	0.36
TM7	0.01 (0-0.01)	0.01 (0-0.01)	0.02 (0-0.04)	0.03 (0.01-0.05)	0.05	0.3	**0.08**
Tenericutes	0.01 (0-0.02)	0.00	0.00	0.00	0.26	0.26	0.36
Cyanobacteria	0.00	0.00	0.00	0.01 (0-0.02)	0.39	0.24	0.10
Other (bacteria)	0.00	0.00	0.02 (0-0.04)	0.00	0.82	0.36	0.18
Other (not assigned)	2.2 (1.70-2.70)	2.51 (2.05-3.06)	3.27 (2.23-4.57)	5.26 (4.02-6.42)	0.23	0.17	0.05

p-values< 0.05 were indicated in bold.
*Bootstrapped 95% confidence interval (CI) was based on 1000 replicates.
[a]Paired-Samples T Test was used in comparing baseline and post-*H. pylori* eradication.

When the genera of stool microbiome between Baseline and 6 months post-*H. pylori* eradication group were compared, the relative abundance of *Anaerofustis*, *Phascolarctobacterium*, and *Ruminococcus* (Family: *Lachnospiraceae*) in the stool microbiome were found to have increased significantly whereas the relative abundance of an unnamed genus under Candidate Division TM7 phylum (Order & Family: unnamed) decreased significantly (Paired-samples t-test, $p<0.05$) (Table 4.6). The relative abundance of three genera in the stool microbiome changed significantly ($p<0.05$) when baseline was compared with 12 months post-eradication groups. The relative abundance of *Dialister* ($p=0.033$) and *Helicobacter* ($p=0.041$) increased while the relative abundance of *Agrobacterium* ($p=0.031$) decreased 12 months post-*H. pylori* eradication (Table 4.7). *Agrobacterium* was detected in Baseline and 6 months post-eradication samples but disappeared in 12 and 18 months post-eradication samples. When the genera of stool microbiome between Baseline and 18 months post-*H. pylori* eradication group were compared, genus *Helicobacter* ($p=0.033$) and another unnamed genus under Candidate Division TM7 phylum (Family: *Rs-045*) was found to have increased significantly (Table 4.8).

To examine for the presence of enterohepatic *Helicobacter* species (EHS), we used the generated OTU table to further summarise microbiome communities up to species level. The generated *16S rRNA* data showed that the only *Helicobacter* species detected was *H. pylori* (Table 4.7, Table 4.8 and Appendix G).

Table 4.6: Comparison of relative abundance of genera of the gut microbiome at Baseline and 6 months post-*H. pylori* eradication

Genus	Mean relative abundance (95% CI*)		Comparison of relative abundance at baseline and 6 months post-eradication (p-value)[a]
	Baseline	6 months post-eradication	
Anaerofustis	7.70E-4% (1.81E-4%-1.43E-3%)	1.87E-3% (9.17E-4%- 2.91E-3%)	0.016
Phascolarctobacterium	0.72% (0.29%-1.26%)	1.76% (0.82%-3.05%)	0.038
Ruminococcus (Family: *Lachnospiraceae*)	0.60% (0.37%-0.89%)	1.25% (0.76%-1.88%)	0.030
Unknown (Phylum: *TM7*; Class: *TM7-3*)	0.01% (4.84E-3%-0.01%)	4.22E-3% (2.54E-3%-5.82E-3%)	0.027

*Bootstrapped 95% confidence interval (CI) was based on 1000 replicates.

[a]Paired-Samples T Test was used in comparing baseline and post-*H. pylori* eradication

Table 4.7: Comparison of relative abundance of genera of the gut microbiome at Baseline and 12 months post-*H. pylori* eradication

Genus (species)	Mean relative abundance (95% CI*)		Comparison of relative abundance at baseline and 12 months post-eradication (p-value)[a]
	Baseline	12 months post-eradication	
Dialister	0.20% (0.06%-0.38%)	0.71% (0.33%-1.13%)	0.033
Agrobacterium	4.34E-4% (1.40E-4%-7.81E-4%)	0%	0.031
Helicobacter	0%	2.17E-4% (4.10E-5%-3.94E-4%)	0.041
(*H. pylori*)	0%	2.17E-4% (5.80E-5%-4.05E-4%)	0.041

*Bootstrapped 95% confidence interval (CI) was based on 1000 replicates.
[a]Paired-Samples T Test was used in comparing baseline and post-*H. pylori* eradication.

Table 4.8: Comparison of relative abundance of genera of the gut microbiome at Baseline and 18 months post-*H. pylori* eradication

Genus (species)	Mean relative abundance (95% CI*)		Comparison of relative abundance at baseline and 18 months post-eradication (p-value)[a]
	Baseline	18 months post-eradication	
Helicobacter	0%	5.90E-4% (2.37E-4%-9.27E-4%)	0.033
(*H. pylori*)	0%	5.86E-4% (2.05E-4%-9.04E-4%)	0.033
Unknown (Phylum: *TM7*; Class: *TM7-3*; Order: *I025*; Family: *Rs-045*)	2.02E-4% (0%-4.3E-4%)	1.28E-3 (6.13E-4%-1.86E-3%)	0.036

*Bootstrapped 95% confidence interval (CI) was based on 1000 replicates.
[a]Paired-Samples T Test was used in comparing baseline and post-*H. pylori* eradication.

4.3 Faecal lipidomic analysis

The numbers of faecal samples collected from Baseline, 6 months, 12 months and 18 months post-eradication group were 29, 23, 14 and 11 samples, respectively. In order to minimize bias as well as to identify confounding factors that may affect our findings, the collected faecal samples were pooled into different groups according to gender, body mass index (BMI), and race for LC-MS analysis (Appendix D).

The LC-ESI-TOF total ion chromatograms (TIC) of faecal lipidomics for Baseline and post-eradication groups, acquired under ESI positive and negative ionisation, correspondingly, demonstrated several peaks with different intensities, (marked by asterisks) in the TIC, that were readily observed between the Baseline and post-*H. pylori* eradication groups (Figure 4.9 and 4.10). Data analysis using one-way ANOVA and Tukey's honest significant difference (HSD) post-hoc analysis of the faecal lipidome revealed a large number of significantly changed molecular features between Baseline and post-*H. pylori* eradication groups and a total of approximately 1300-1600 molecular features were extracted from a typical positive and negative LC-ESI-TOF chromatogram between Baseline and post-*H. pylori* eradication groups (Table 4.9). Of the total molecular features, 513 and 434 of these features were significantly changed between Baseline and post-eradication groups, acquired under positive and negative ionisation respectively.

A multivariate statistical principal component analysis (PCA) was subsequently employed to examine whether samples belonging to Baseline and post-eradication groups could be differentiated based on their lipids profiles. The three-dimensional PCA scores plots showed clear separation of samples of Baseline and post-*H. pylori* eradication groups, under both positive and negative ionisation modes (Figure 4.11, 4.12, and 4.13). However, the three-dimensional PCA scores plots did not show distinct

separation between the different pooled faecal groups which indicated that gender, BMI and race were not the confounding factors in this study.

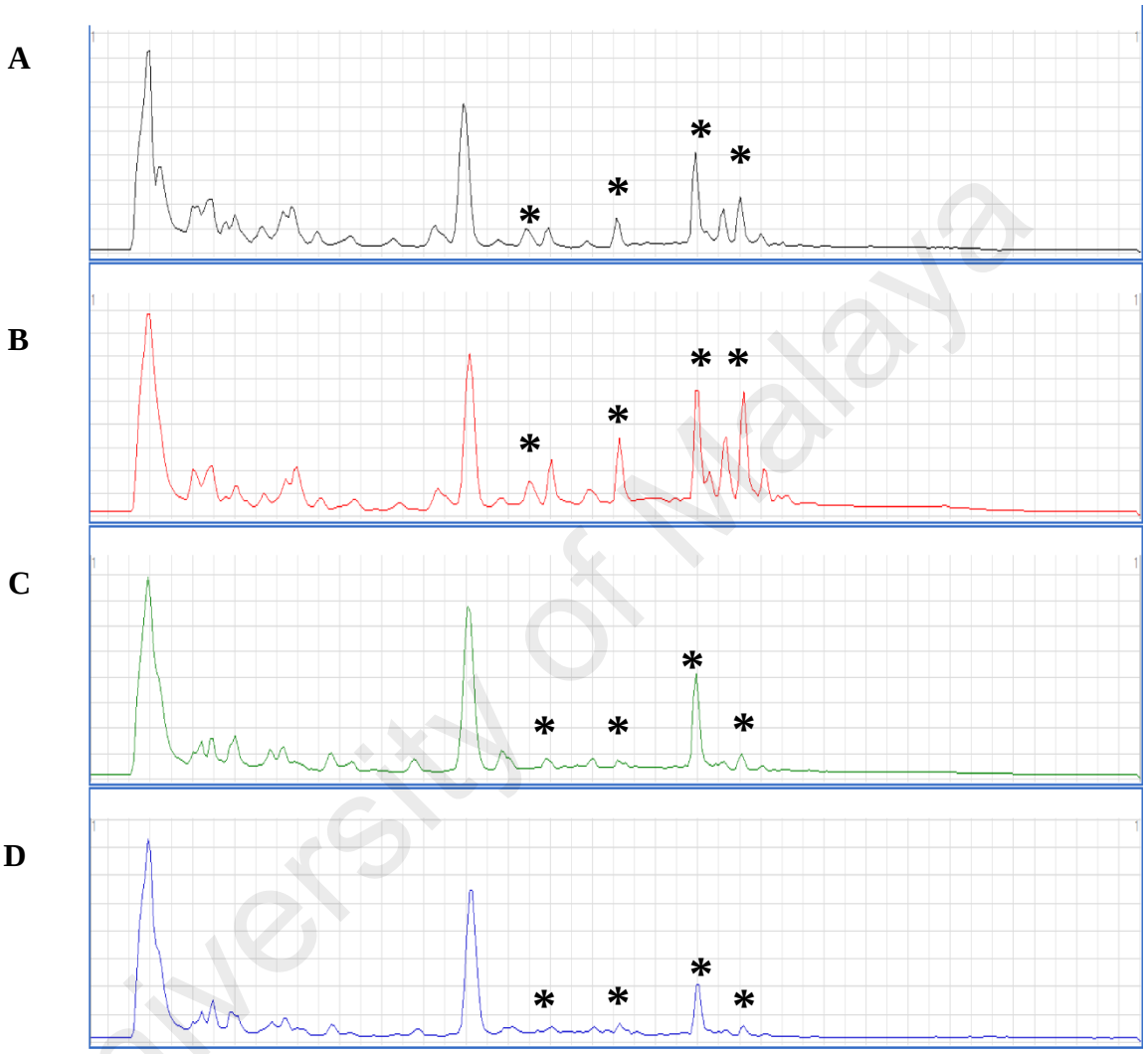


Figure 4.9: Typical LC-ESI-TOF total ion chromatogram of faecal lipid extract from **A.** Baseline, **B.** 6 months, **C.** 12 months, and **D.** 18 months post-eradication group, acquired under ESI positive ionisation

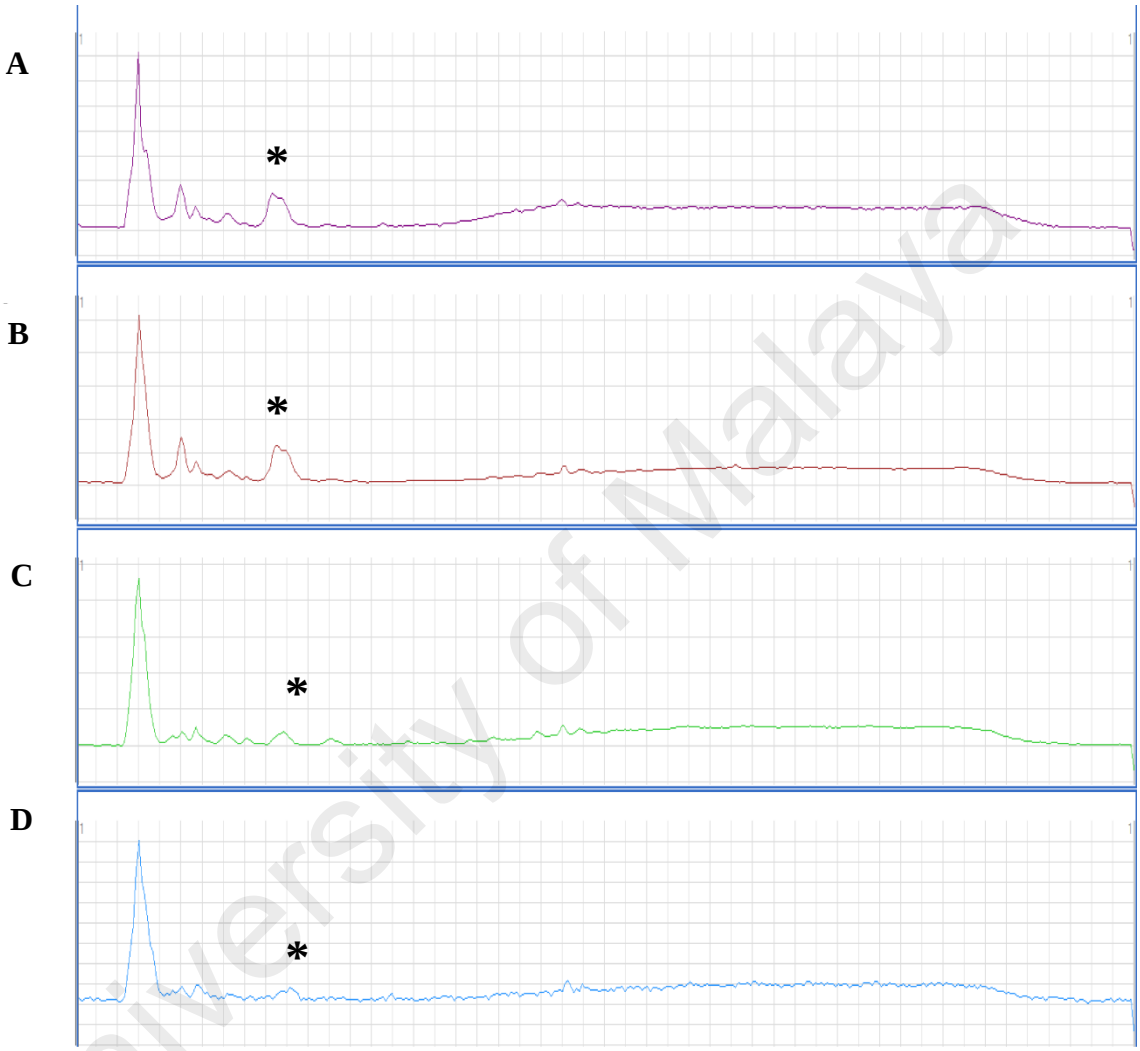
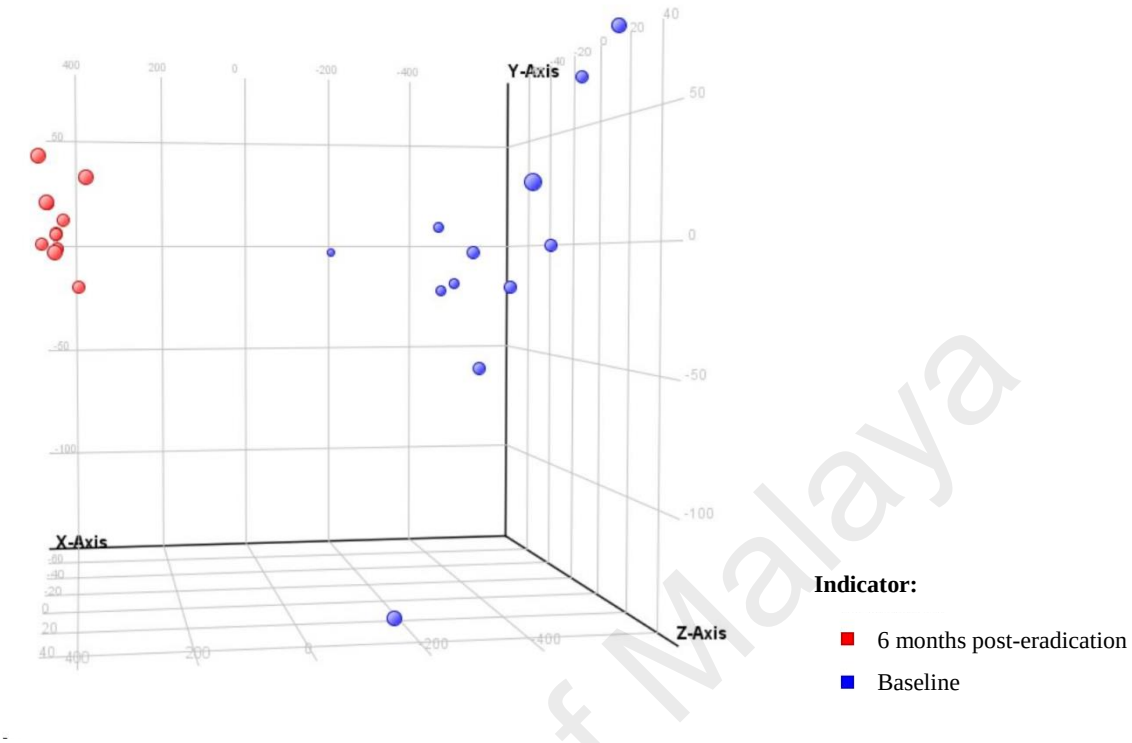


Figure 4.10: Typical LC-ESI-TOF total ion chromatogram of faecal lipid extract from **A.** Baseline, **B**. 6 months, **C.** 12 months, and **D.** 18 months post-eradication group, acquired under ESI negative ionisation

A



B

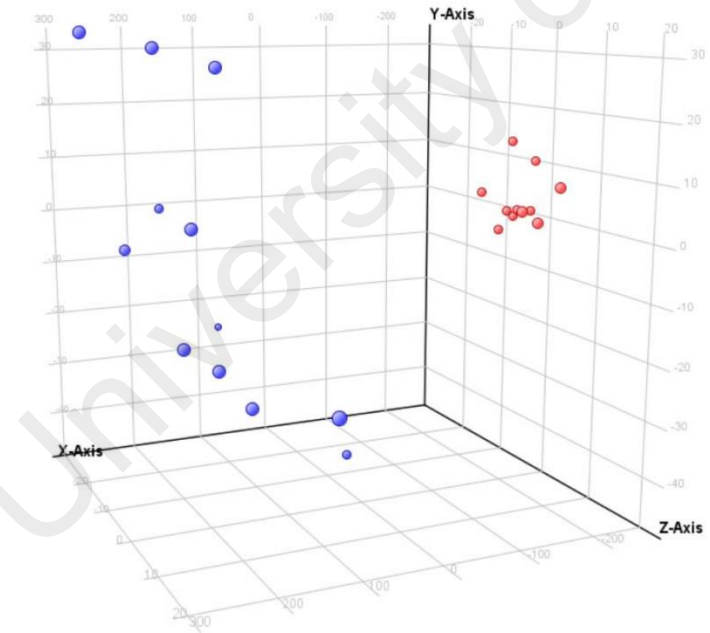
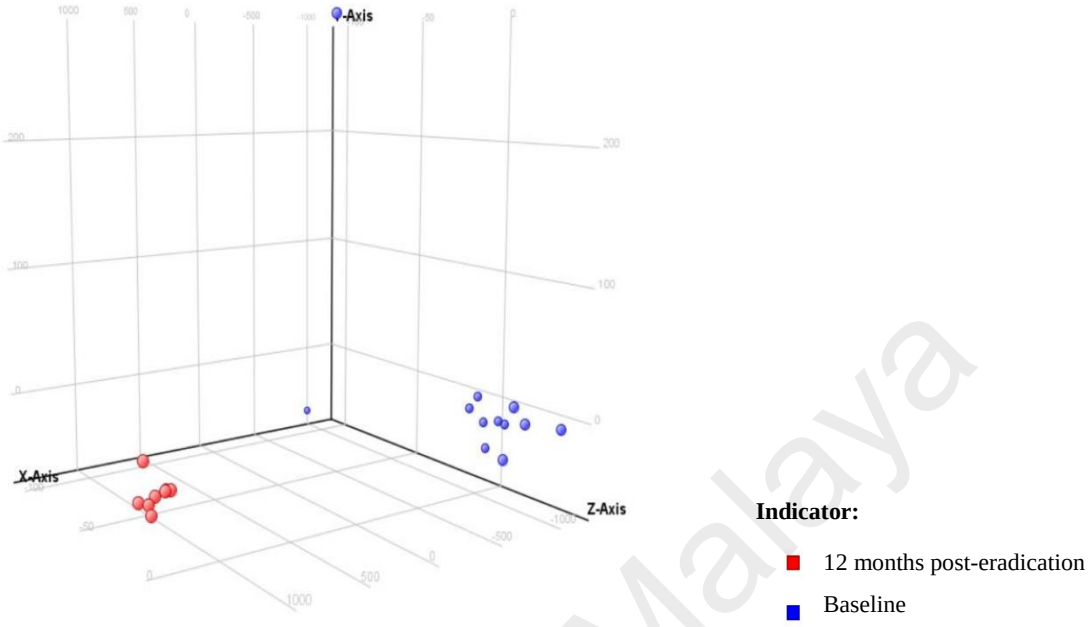


Figure 4.11: Three-dimensional PCA scores plot of faecal lipidomics for Baseline vs. 6 months post-*H. pylori* eradication, acquired under both **A.** positive and **B.** negative ionisation modes

A



B

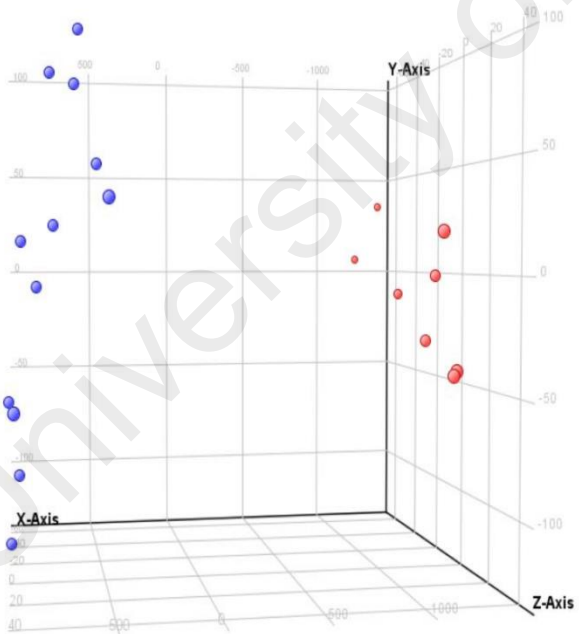
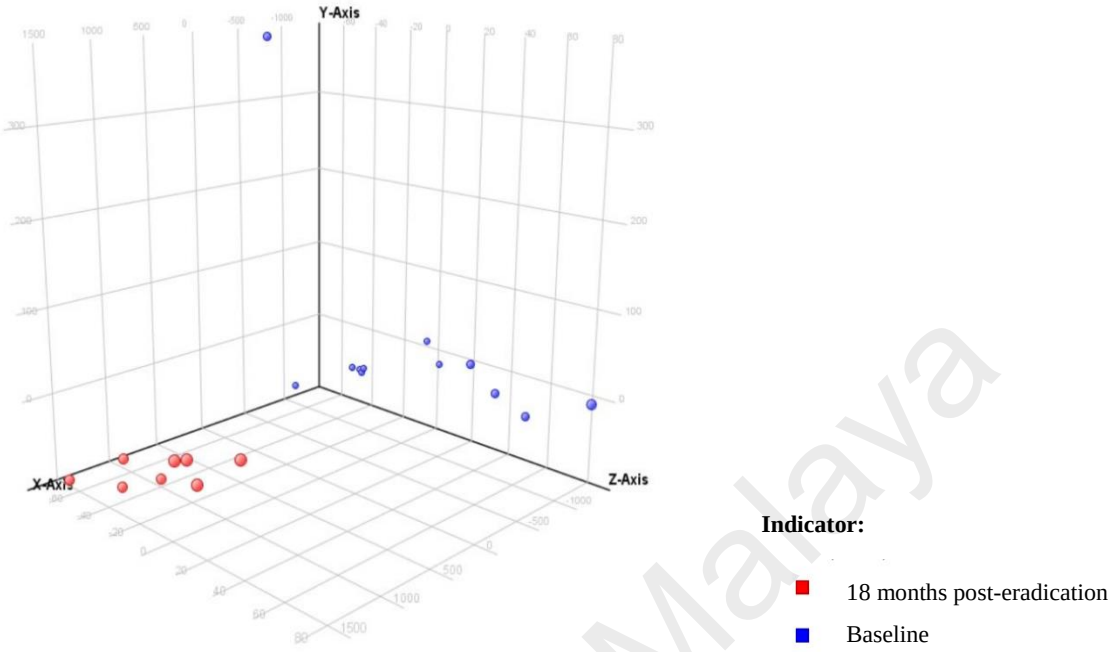


Figure 4.12: Three-dimensional PCA scores plot of faecal lipidomics for Baseline vs. 12 months post-*H. pylori* eradication, acquired under both **A**. positive and **B.** negative ionisation modes

A



B

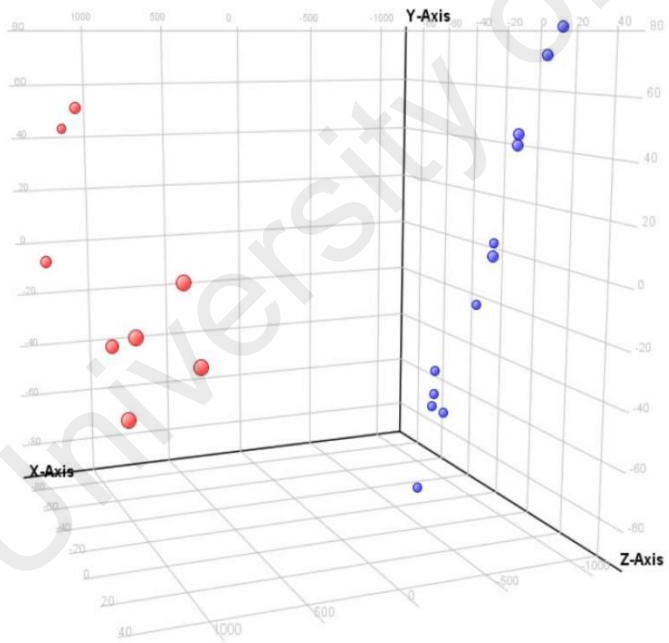


Figure 4.13: Three-dimensional PCA scores plot of faecal lipidomics for Baseline vs. 18 months post-*H. pylori* eradication, acquired under both **A**. positive and **B.** negative ionisation modes

Table 4.9: Global characterisation of the faecal lipidomics data

Group	LC-ESI ionisation mode	Total features	Total significantly altered features[§]	Total decreased features*	Total increased features*
Baseline	+	1686	-	-	-
	-	1447	-	-	-
6 months-post eradication	+	1628	513	180	333
	-	1431	434	66	368
12 months-post eradication	+	1473	513	335	178
	-	1421	434	289	145
18 months-post eradication	+	1462	513	347	166
	-	1316	434	322	112

[§]Significantly altered features denote those with more than 2 fold changes, $p<0.001$, FDR<1%.
*Decreased and increased features are relative to the Baseline group.

4.3.1 Potential faecal lipids

In order to identify the potential faecal lipids associated with *H. pylori* eradication, the significantly altered molecular features were identified and annotated as entities using Mass Profiler Professional (MPP) integrated with ID Browser and LC/MS Personal Compound Databases (METLIN database). In brief, 161, 271, and 269 entities were significantly changed when we compared Baseline vs. 6 months, Baseline vs. 12 months, and Baseline vs. 18 months post-*H. pylori* eradication, respectively (Appendix H). It is important to note that although the same metabolite/lipid could be identified by ID Browser as several different entities, but they are actually the components of same compound. They are differentiated due to factors such as different retention time. In addition, certain compounds could be detected in both ionisation modes. After excluding these redundancies, it was found that when Baseline was compared with 6 months-post eradication, 34 and 13 lipids were up-regulated and down-regulated respectively. Conversely, more lipids were down-regulated when Baseline was compared to the 12 and 18 months-post eradication groups. When Baseline was compared with 12 months-post eradication, 29 and 43 lipids were up-regulated and down-regulated respectively. When Baseline was compared with 18 months-post eradication, 25 and 45 lipids were up-regulated and down-regulated correspondingly

(Table 4.10). These significant lipids could be categorised into lipid classes such as fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids and saccharolipids, of which five were mapped to different biochemical pathways which include retrograde endocannabinoid signaling and sphingolipid metabolism (Table 4.11).

Table 4.10: Summary of the differentially expressed faecal lipids

Comparison group	Total significantly expressed metabolites[§]	Total down-regulated metabolites	Total up-regulated metabolites
Baseline vs. 6 months	47	13	34
Baseline vs. 12 months	72	43	29
Baseline vs. 18 months	70	45	25

[§]Significantly expressed metabolites denote those with more than 2 fold changes, $p<0.001$, FDR<1%.

Table 4.11: Selected significantly expressed lipids between Baseline and post-*H. pylori* eradication groups

KEGG/ HMDB/ LMID	Lipid	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C19913/ LMFA08020002	Anandamide 0-phosphate	Fatty acyls	Retrograde endocannabinoid signalling	445.2819	14.79	+11.72	+3.40	-.3.31
C00836/ HMDB00269/ LMSP01020001	Sphinganine	Sphingolipids	Sphingolipid metabolism	302.3567	1.56	+1.77	+1.30	+0.82
C12144/ HMDB04610/ LMSP01030001	Phytosphingosine	Sphingolipids	Sphingolipid metabolism	318.2937	1.45	-19.48	-18.77	-4.25
HMDB13244/ LMSL01040002	Lipid A -disaccharide-1-P	Saccharolipids	-	661.4385	10.03	+0.38	+3.98	+16.15
LMPR04000022	32,35-anhydrobacteriohopaneterol	Prenol lipids	-	527.4471	11.54	+0.25	+0.21	-13.32

*Expression level indicates log fold change(FC). Log_2 FC≥1 (FC≥2) is considered as significant; + indicates up-regulated in post-eradication group; - indicates down-regulated in post-eradication group.

4.4 Metabolic study

4.4.1 Meal-associated changes of metabolic hormones and effects of *Helicobacter pylori* eradication

The levels of the eight studied gut metabolic hormones varied markedly among the subjects in both the pre-prandial and post-prandial sera (Table 4.12). The studied hormones responded to the test meal, as predicted (Holst, 2007; Holzer, Reichmann, & Farzi, 2012; Lutz, 2006; Mazzaferri, Starich, & St Jeor, 1984; Wynne, Stanley, & Bloom, 2004). Insulin, active amylin, total GIP, GLP-1 and PP levels rose physiologically and significantly ($p<0.05$) after the test meal, at all three time points (baseline, 6 months and 12 months post-*H. pylori* eradication). At baseline, post-prandial total PYY levels did not rise significantly, but did at 6 months and 12 months after *H. pylori* eradication. At baseline and 12 months post-*H. pylori* eradication, but not at 6 months post-eradication, levels of acyl-ghrelin decreased significantly following the test meal. Leptin decreased significantly post-prandially at baseline and 6 months post-*H. pylori* eradication but not at 12 months post-eradication.

The levels of the eight gut metabolic hormones did not differ significantly pre-prandially between baseline and 6 months post-eradication. However, when baseline was compared with the 12 months post-eradication values, pre-prandial active amylin, total PYY and PP levels were significantly elevated ($p<0.05$). Median pre-prandial active amylin was increased by 5-fold from 7.3 pg/ml increased to 37.8 pg/ml (Baseline 95% CI: 0-31.1 pg/ml vs. 12 Months 95% CI: 0-84.1 pg/ml). Median pre-prandial total PYY was increased by 3-fold from 30.7 pg/ml increased to 96.3 pg/ml (Baseline 95% CI: 0-44.6 pg/ml vs. 12 Months 95% CI: 0-150.2 pg/ml). Median pre-prandial PP was increased by 2-fold from 16.1 pg/ml elevated to 33 pg/ml (Baseline 95% CI: 0-39.5 pg/ml vs. 12 Months 95% CI: 5.6-62.9 pg/ml).

The post-prandial hormonal changes between baseline and 6 months post-eradication also did not show significant difference except for total PYY (Baseline, median= 65.9 pg/ml; 95% CI: 0-83.1 pg/ml vs. 6 Months, median= 86.5 pg/ml; 95% CI: 77.2-95.7 pg/ml), which was approximately 1.3-fold higher 6 months post-eradication than baseline ($p<0.05$). However, by 12 months post-eradication, post-prandial levels of four of the gut metabolic hormones (GLP-1, total PYY, active amylin, PP) were significantly higher than at baseline ($p<0.05$). Median post-prandial total PYY was increased by 2.6-fold (Baseline, median= 65.9 pg/ml; 95% CI: 0-83.1 pg/ml vs. 12 Months, median= 174.4 pg/ml; 95% CI: 140.9-219.5 pg/ml). Median post-prandial active amylin was increased by 2.2-fold (Baseline, median= 44.3 pg/ml; 95% CI: 34.2-52 pg/ml vs. 12 Months, median= 98.6 pg/ml; 95% CI: 54.8-136.4 pg/ml). Median post-prandial PP was increased by 1.8-fold (Baseline, median= 75.5 pg/ml; 95% CI: 52.4-100.2 pg/ml vs. 12 Months, median= 136.7 pg/ml; 95% CI: 77.9-229.2 pg/ml). Median post-prandial GLP-1 was increased by 1.4-fold (Baseline, median= 44.4 pg/ml; 95% CI: 25.6-55.5 pg/ml vs. 12 Months, median= 63.5 pg/ml; 95% CI: 47.2-84.2 pg/ml) (Table 4.12).

4.4.2 Effects of *Helicobacter pylori* eradication on body mass index and anthropometry

Twelve months following eradication of *H. pylori*, the BMI of volunteers did not significantly change. Correspondingly, waist, hip, and triceps circumference values also did not change significantly. Triceps circumference was significantly increased at 6 months post-*H. pylori* eradication (Table 4.13).

Table 4.12: Eight metabolic hormone levels in studied subjects according to *H. pylori* eradication status

	Median (95% CI*) hormone concentration (pg/ml)									Comparison of hormone concentration at baseline and 6 months post-eradication (p-value)[a]		Comparison of hormone concentration at baseline and 12 months post-eradication (p-value)[a]	
	Baseline (N=29)			6 months post-eradication (N=29)			12 months post-eradication (N=18)						
Hormone	**Pre-prandial**	**Post-prandial**	**p-value[b]**	**Pre-prandial**	**Post-prandial**	**p-value[b]**	**Pre-prandial**	**Post-prandial**	**p-value[b]**	**Pre-prandial**	**Post-prandial**	**Pre-prandial**	**Post-prandial**
Active Amylin	7.3 (0-31.1)	44.3 (34.2-52)	**0.013**	18.5 (0-24.9)	46.1 (31.1-53.6)	**0.006**	37.8 (0-84.1)	98.6 (54.8-136.4)	**0.008**	0.24	0.48	**0.036**	**0.017**
Acyl-ghrelin	12.7 (6.5-19.4)	10.2 (7.1-16.4)	**0.022**	11.8 (7.1-17.1)	12 (6.5-16.5)	0.45	15.4 (8.8-27.1)	7.6 (6.5-19.6)	**0.022**	0.26	0.29	0.17	0.72
Total GIP	25.4 (15.4-52.5)	281.3 (190.8-302.5)	**0.005**	35.1 (20.7-48.5)	222.2 (200.8-317.8)	**0.002**	41.1 (25.1-70.7)	276 (237-430.4)	**0.005**	0.88	0.64	0.09	**0.022**
GLP-1	17.4 (13.6-32.1)	44.4 (25.6-55.5)	**0.013**	17.4 (0-29.1)	49.6 (37-59.6)	**0.002**	22.5 (0-47.5)	63.5 (47.2-84.2)	**0.005**	0.21	**0.041**	0.68	**0.005**
Insulin	293.3 (209.5-392.2)	2235.4 (1842.5-3198.5)	**0.003**	292.1 (251.4-445.3)	1937.9 (1518.5-2436.6)	**0.002**	504 (310-559.4)	3455.3 (2252.8-4449.2)	**0.005**	0.88	0.16	0.14	0.09

Table 4.12, continued

	Median (95% CI*) hormone concentration (pg/ml)									Comparison of hormone concentration at baseline and 6 months post-eradication (p-value)[a]		Comparison of hormone concentration at baseline and 12 months post-eradication (p-value)[a]	
	Baseline (N=29)			6 months post-eradication (N=29)			12 months post-eradication (N=18)						
Hormone	**Pre-prandial**	**Post-prandial**	**p-value[b]**	**Pre-prandial**	**Post-prandial**	**p-value[b]**	**Pre-prandial**	**Post-prandial**	**p-value[b]**	**Pre-prandial**	**Post-prandial**	**Pre-prandial**	**Post-prandial**
Leptin	11091.8 (7318.1-13249.7)	9510.6 (6427.7-10939.4)	**0.016**	10917.7 (5477.9-13279.9)	9805 (5124.9-13090.1)	**0.012**	6059.6 (3000.1-16842.4)	7947 (5216.5-14507.5)	0.17	0.42	0.16	0.86	0.44
PP	16.1 (0-39.5)	75.5 (52.4-100.2)	**0.004**	16.4 (0-34)	88.3 (55.8-157.8)	**0.002**	33 (5.6-62.9)	136.7 (77.9-229.2)	**0.005**	0.65	0.29	**0.028**	**0.009**
Total PYY	30.7 (0-44.6)	65.9 (0-83.1)	0.05	35.6 (0-53.4)	86.5 (77.2-95.7)	**0.002**	96.3 (0-150.2)	174.4 (140.9-219.5)	**0.007**	1	**0.006**	**0.021**	**0.005**

p-values< 0.05 were indicated in bold.
*Bootstrapped 95% confidence interval (CI) was based on 1000 replicates.
[a]Wilcoxon's signed rank test was used in comparing baseline and post-*H. pylori* eradication.
[b]Wilcoxon's signed rank test was used in comparing pre-prandial and post-prandial values.

Table 4.13: Comparison of BMI, and waist, hip, and triceps circumference values according to *H. pylori* eradication status

Characteristic	Mean BMI (kg/m^2)/Waist Circumference (cm)/ Hip Circumference (cm)/Triceps Skinfold (cm)			Comparison between baseline and 6 months post-eradication (p-value)[a]	Comparison between baseline and 12 months post-eradication (p-value)[a]
	Baseline (N=29)	6 months post-eradication (N=29)	12 months post-eradication (N=18)		
BMI	22.45	22.75	23.55	0.093	0.061
Waist Circumference	76.67	74.56	75.35	0.209	0.130
Hip Circumference	94.83	94.81	97.96	0.992	0.831
Triceps Skinfold	1.57	1.89	1.98	**0.024**	0.059

p-values< 0.05 were indicated in bold.

[a]Paired t-test was used to compare BMI, and waist, hip, and triceps circumference values at baseline to post-eradication.

4.5 Immunological study

4.5.1 Effects of *Helicobacter pylori* eradication on Immunoglobulin E (IgE) concentration in plasma

The median concentration of IgE for Baseline, 6, 12 and 18 months post-*H. pylori* eradication groups were 67.1, 103.2, 231.8 and 208.5 U/ml respectively (Table 4.14). According to manufacturer's protocol, IgE concentration more than 100 U/ml is considered as IgE positive. In this study, it was found that the median plasma level of IgE was significantly higher in post-*H. pylori* eradication groups than Baseline group with p-values <0.05. This result indicated that the volunteers were IgE positive following the eradication of *H. pylori* and an inverse association between *H. pylori* infection and IgE concentration was observed.

4.5.2 Effects of *Helicobacter pylori* eradication on IL-4 and TNF-α

The median value of IL-4 concentrations were rather constant pre- and post-*H. pylori* eradication. There was no significant change of IL-4 concentrations in the plasma at baseline and post-*H. pylori* eradication groups and the IL-4 level remained fairly constant across all time points. On the other hand, the median TNF-α concentrations were only significantly reduced when Baseline was compared with 18 months post-*H. pylori* eradication group (Table 4.15).

Table 4.14: Comparison of IgE concentration according to *H. pylori* eradication status

Median (95% CI*) IgE concentration (U/ml)				Comparison between baseline and 6 months post-eradication (p-value)[a]	Comparison between baseline and 12 months post-eradication (p-value)[a]	Comparison between baseline and 18 months post-eradication (p-value)[a]
Baseline (N=29)	**6 months post-eradication (N=29)**	**12 months post-eradication (N=18)**	**18 months post-eradication (N=12)**			
67.1 (72.7-226.7)	103.2 (99.7-202.3)	231.8 (145.4-540.8)	208.5 (123.1-400.4)	**0.003**	**0.000**	**0.002**

p-values< 0.05 were indicated in bold.
*Bootstrapped 95% confidence interval (CI) was based on 1000 replicates.
[a]Wilcoxon's signed rank test was used in comparing baseline and post-*H. pylori* eradication.
[b]Wilcoxon's signed rank test was used in comparing pre-prandial and post-prandial values.

Table 4.15: Cytokine levels in studied subjects according to *H. pylori* eradication status

Cytokine	Median (95% CI*) cytokine concentration (pg/ml)				Comparison between baseline and 6 months post-eradication (p-value)[a]	Comparison between baseline and 12 months post-eradication (p-value)[a]	Comparison between baseline and 18 months post-eradication (p-value)[a]
	Baseline (N=29)	**6 months post-eradication (N=29)**	**12 months post-eradication (N=18)**	**18 months post-eradication (N=12)**			
IL-4	818.6 (664.6-2936.3)	787.8 (729.4-3185.5)	992.0 (785.4-2893.8)	802.9 (734.1-2057.5)	1.000	0.646	0.214
TNF-α	348.3 (151.1-1185.9)	206.2 (165.5-1111.4)	181.3 (142.2-477.3)	149.2 (133.3-596.0)	0.859	0.093	**0.028**

p-values< 0.05 were indicated in bold.
*Bootstrapped 95% confidence interval (CI) was based on 1000 replicates.
[a]Wilcoxon's signed rank test was used in comparing baseline and post-*H. pylori* eradication.
[b]Wilcoxon's signed rank test was used in comparing pre-prandial and post-prandial values.

114

4.6 Plasma metabolomic analysis

4.6.1 Pre- and post-prandial plasma metabolomics data

Plasma metabolomics profiling was performed on samples collected at Baseline, 6, 12, and 18 months-post *H. pylori* eradication. The number of plasma samples collected at Baseline, 6, 12, and 18 months-post *H. pylori* eradication were 29, 29, 18, and 12 samples respectively. Parallel to faecal lipidomic study, the collected plasma samples were pooled into different groups according to gender, body mass index (BMI), and race for LC-MS analysis (Appendix D).

Several dominant differences of peak intensities could be noted from the LC-ESI-TOF TIC of plasma metabolites of Baseline and post-eradication groups, acquired under ESI positive and negative ionisation (Figure 4.14 and 4.15). One-way ANOVA and Tukey's HSD post-hoc analysis showed that approximately a total of 7000-20,000 molecular features could be extracted from a typical positive LC-ESI-TOF chromatogram whereas only approximately 2000-5000 features could be extracted from a typical negative LC-ESI-TOF chromatogram (Table 4.16 and Table 4.17). Of this enormous amount of molecular features detected, only 5932 and 1867 of these features were markedly changed between Baseline and post-eradication groups, both pre- and post-prandially, acquired under positive and negative ionisation respectively.

A multivariate statistical principal component analysis (PCA) was subsequently employed to examine whether samples belonging to Baseline and post-eradication groups could be differentiated based on their metabolite profiles. Three-dimensional PCA scores plots for Baseline vs. 6 months, Baseline vs. 12 months, and Baseline vs. 18 months post-*H. pylori* eradication, both pre- and post-prandially, showed clear separation of samples between the Baseline and post-*H. pylori* eradication groups, under both positive and negative ionisation modes, as illustrated in Figure 4.16, 4.17, 4.18 and Figure 4.19, 4.20, 4.21. Parallel to faecal lipidomic analysis, the three-dimensional PCA

scores plots did not show distinct separation between the different pooled plasma groups which indicated that gender, BMI and race were not the confounding factors.

Table 4.16: Global characterisation of the pre-prandial plasma metabolomics data

Group	LC-ESI ionisation mode	Total features	Total significantly altered features[§]	Total decreased features*	Total increased features*
Baseline	+	18179	-	-	-
	-	4894	-	-	-
6 months-post eradication	+	19323	5932	4165	1767
	-	4824	1867	679	1188
12 months-post eradication	+	13828	5932	3743	2189
	-	3963	1867	821	1046
18 months-post eradication	+	6789	5932	5340	592
	-	2257	1867	1670	197

[§]Significantly altered features denote those with more than 2 fold changes, $p<0.001$, FDR<1%.
*Decreased and increased features are relative to the Baseline group.

Table 4.17: Global characterisation of the post-prandial plasma metabolomics data

Group	LC-ESI ionisation mode	Total features	Total significantly altered features[§]	Total decreased features*	Total increased features*
Baseline	+	19055	-	-	-
	-	3963	-	-	-
6 months-post eradication	+	16009	5932	3776	2156
	-	3778	1867	1119	748
12 months-post eradication	+	14021	5932	4306	1626
	-	3998	1867	855	1012
18 months-post eradication	+	10709	5932	4552	1380
	-	3284	1867	1018	849

[§]Significantly altered features denote those with more than 2 fold changes, $p<0.001$, FDR<1%.
*Decreased and increased features are relative to the Baseline group.

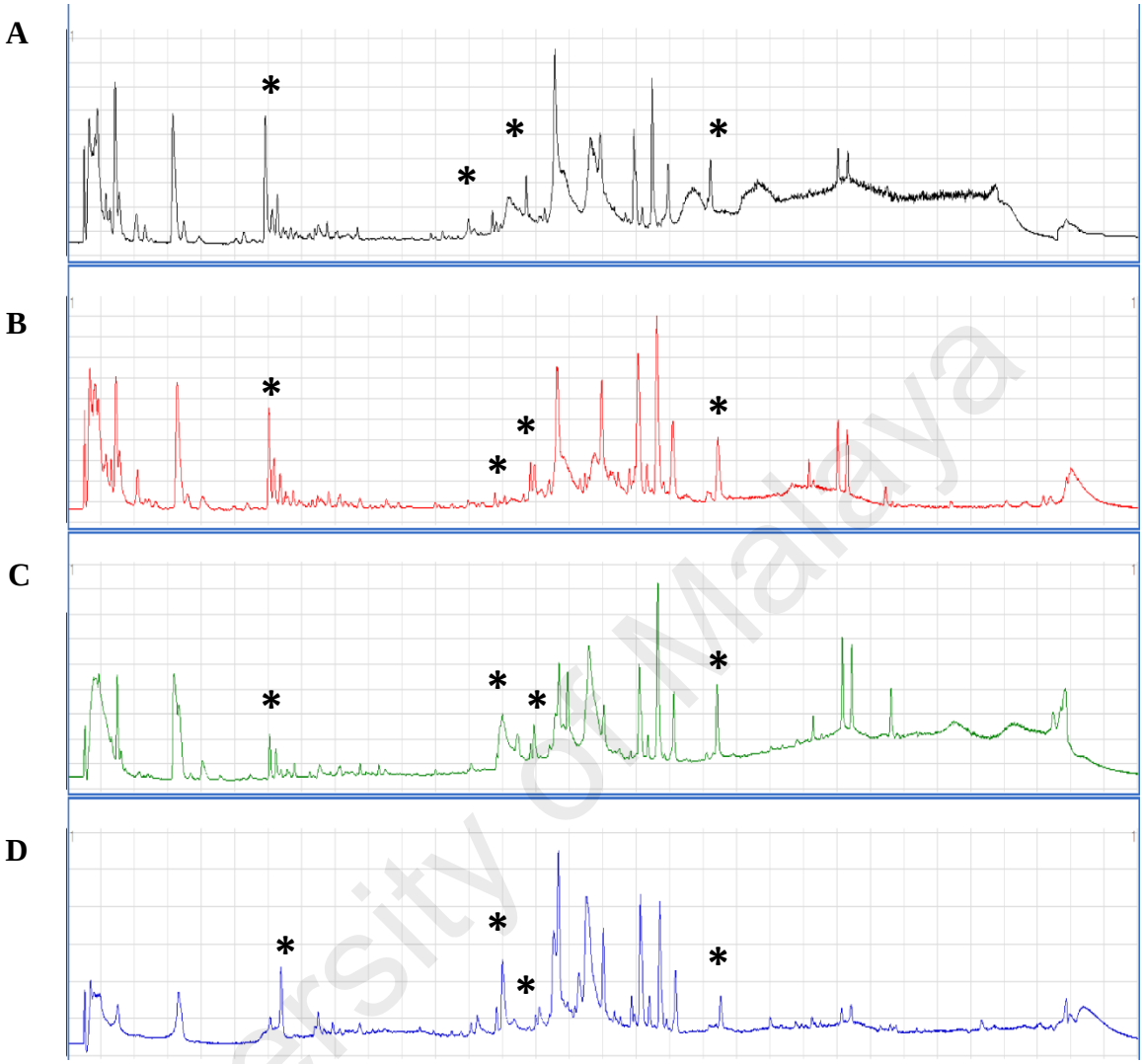


Figure 4.14: Typical LC-ESI-TOF total ion chromatogram of plasma metabolite extract from **A.** Baseline, **B.** 6 months, **C.** 12 months, and **D.** 18 months post-eradication group, acquired under ESI positive ionisation

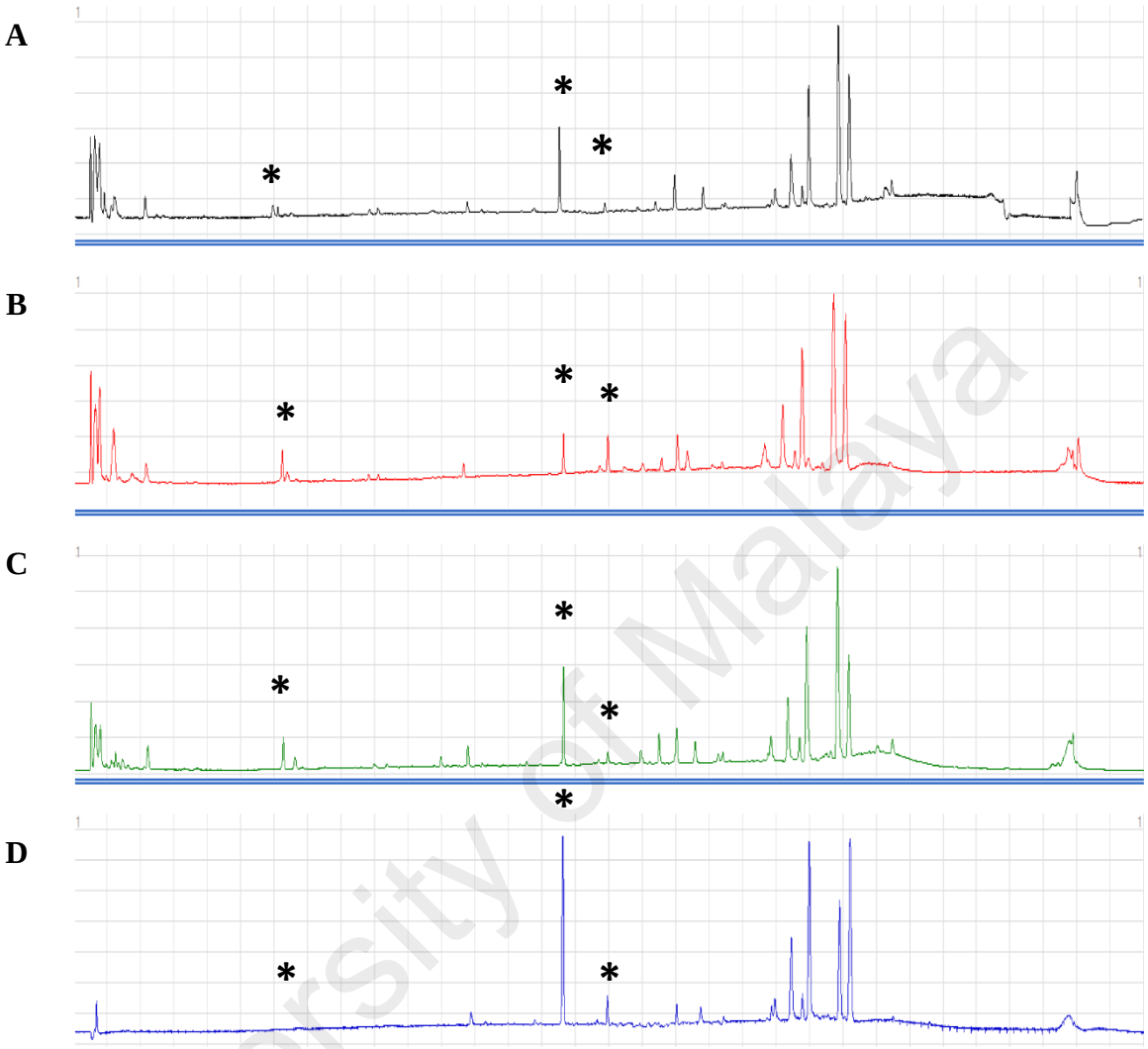
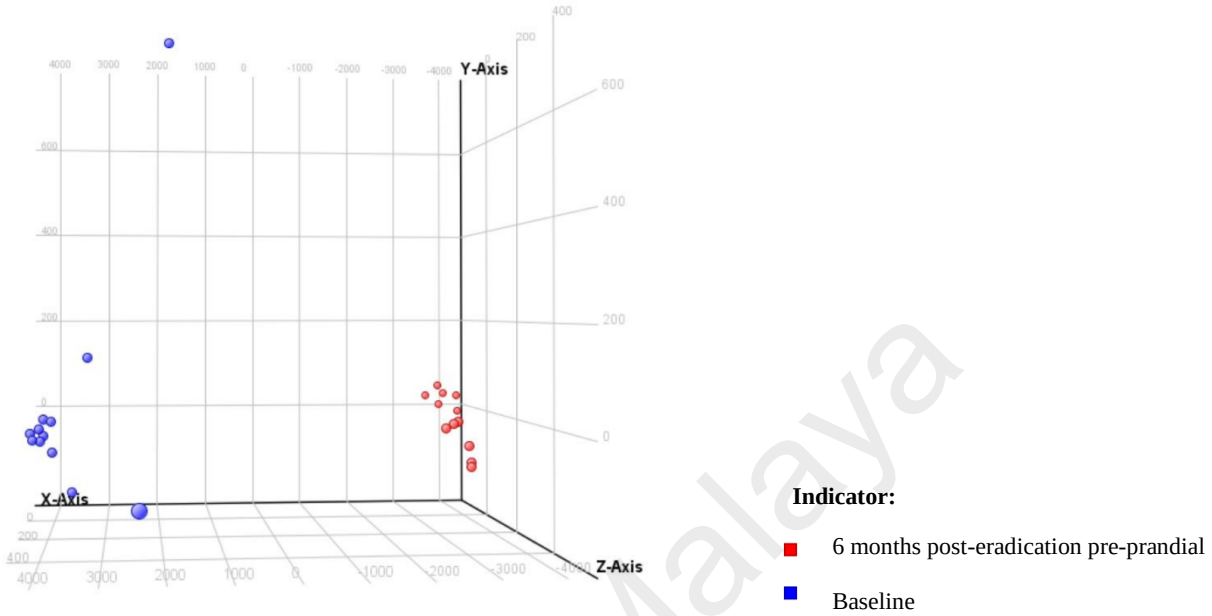


Figure 4.15: Typical LC-ESI-TOF total ion chromatogram of plasma metabolite extract from **A.** Baseline, **B.** 6 months, **C.** 12 months, and **D.** 18 months post-eradication group, acquired under ESI negative ionisation

A



B

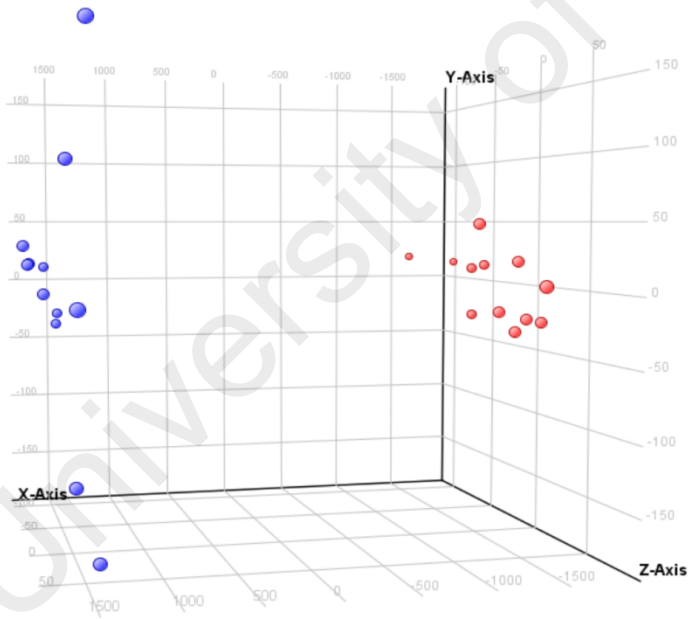
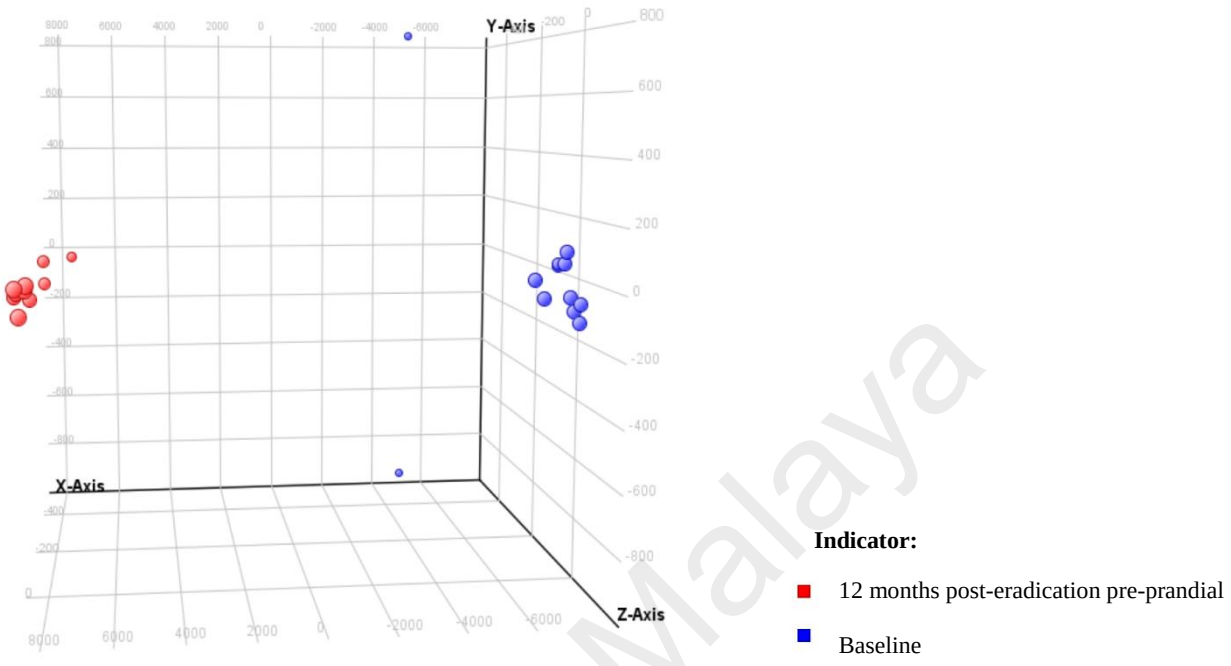


Figure 4.16: Three-dimensional PCA scores plot of plasma metabolomics for Baseline vs. 6 months post-*H. pylori* eradication pre-prandial, acquired under both **A.** positive and **B.** negative ionisation modes

A



B

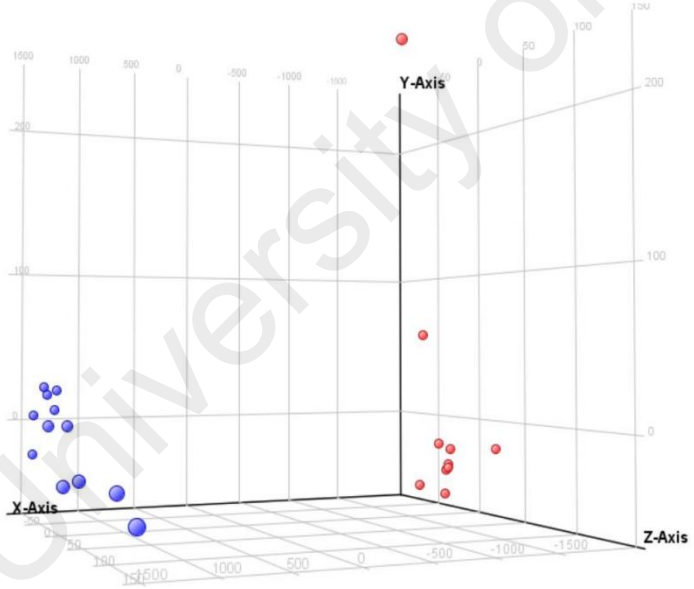
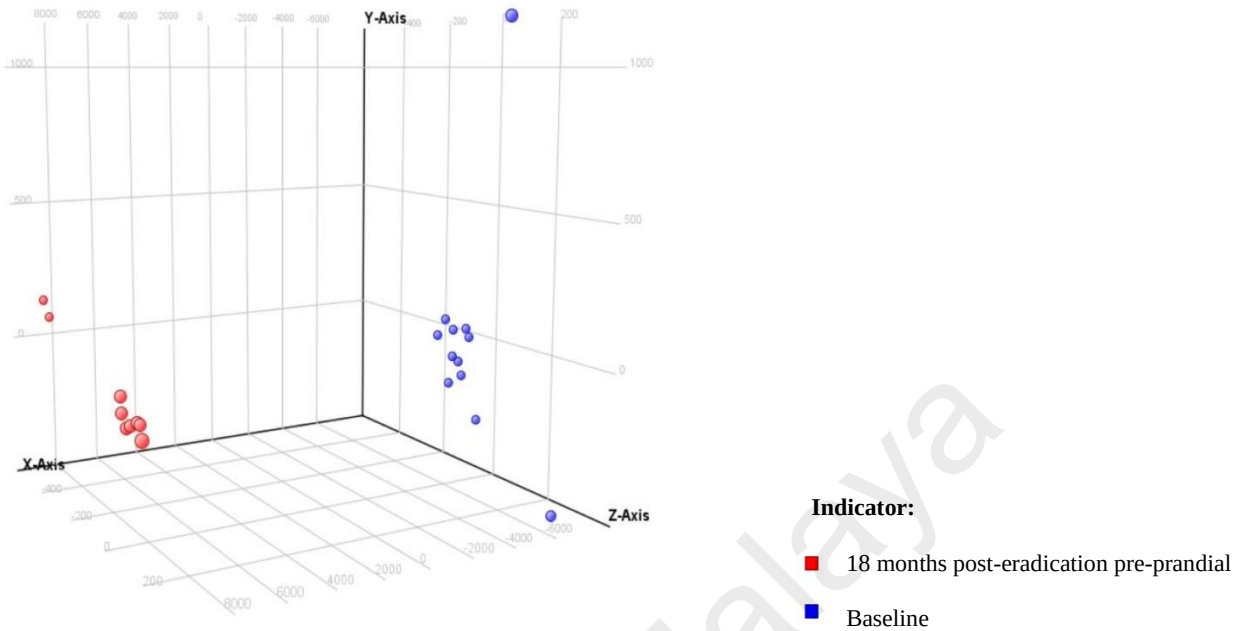


Figure 4.17: Three-dimensional PCA scores plot of plasma metabolomics for Baseline vs. 12 months post-*H. pylori* eradication pre-prandial, acquired under both **A.** positive and **B.** negative ionisation modes

A



B

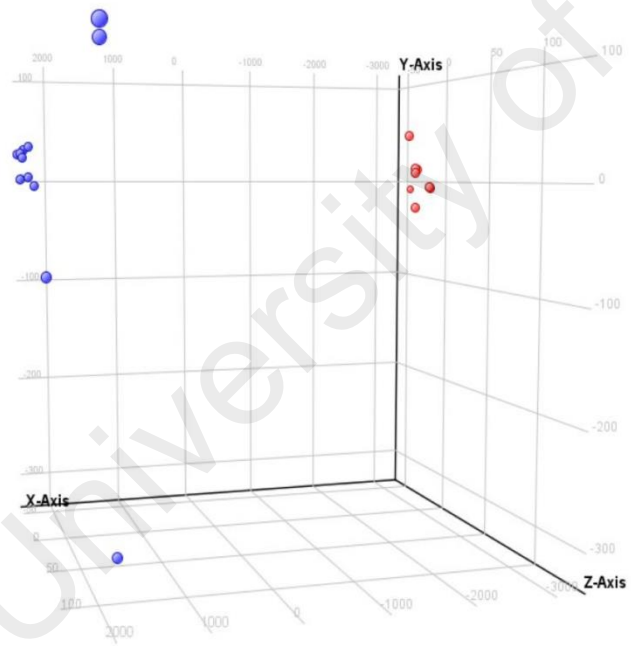
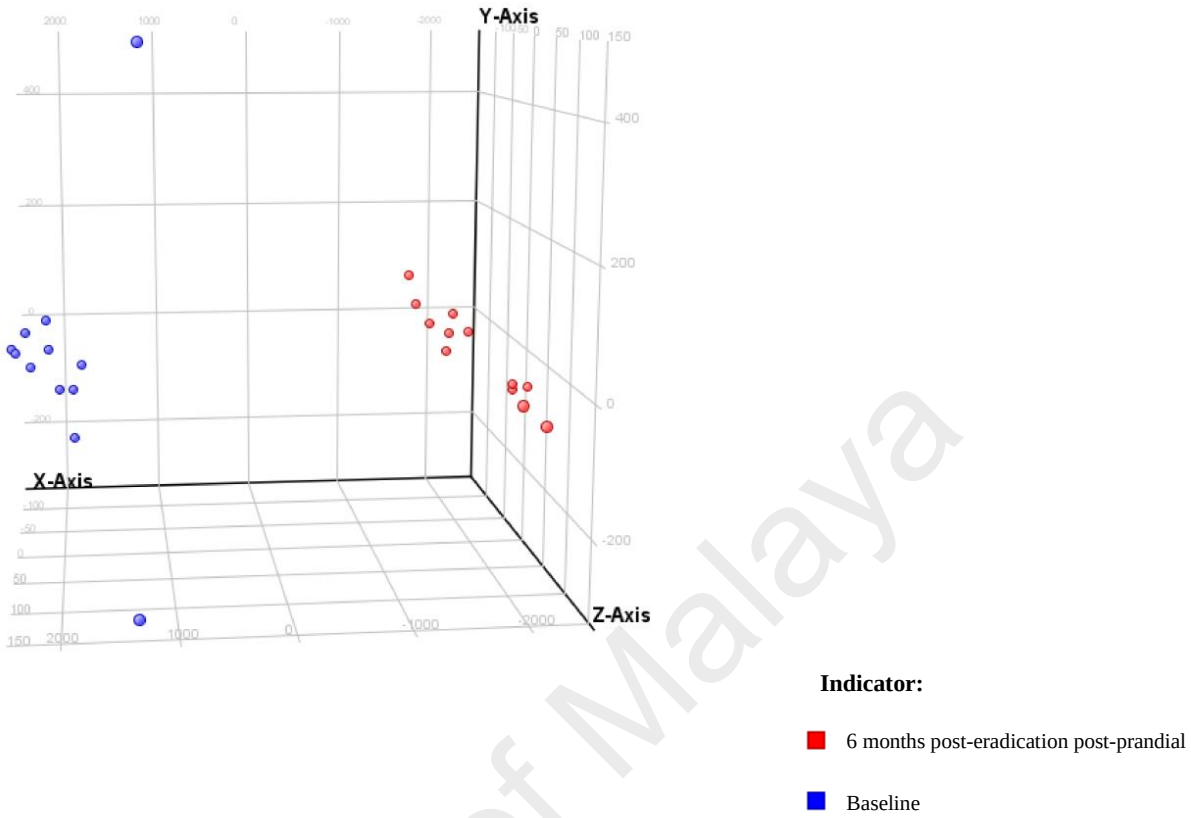


Figure 4.18: Three-dimensional PCA scores plot of plasma metabolomics for Baseline vs. 18 months post-*H. pylori* eradication pre-prandial, acquired under both **A.** positive and **B.** negative ionisation modes

A



B

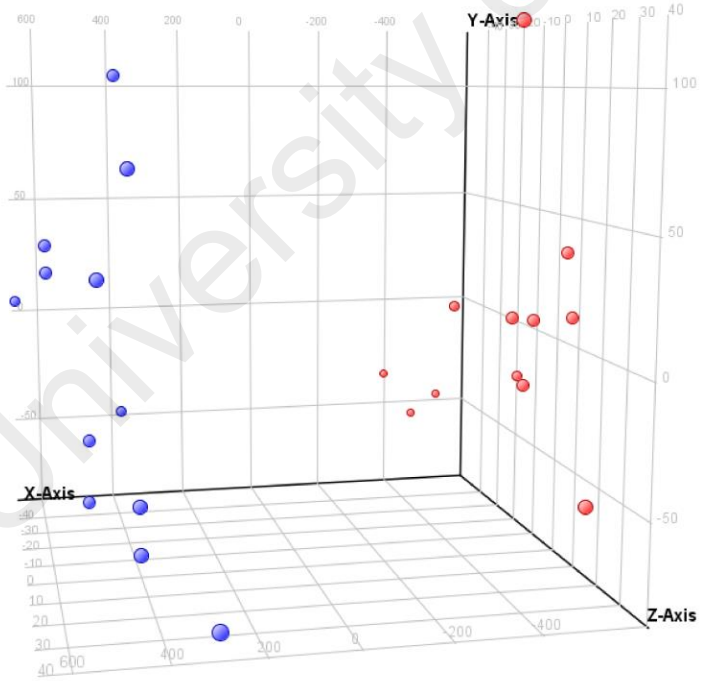
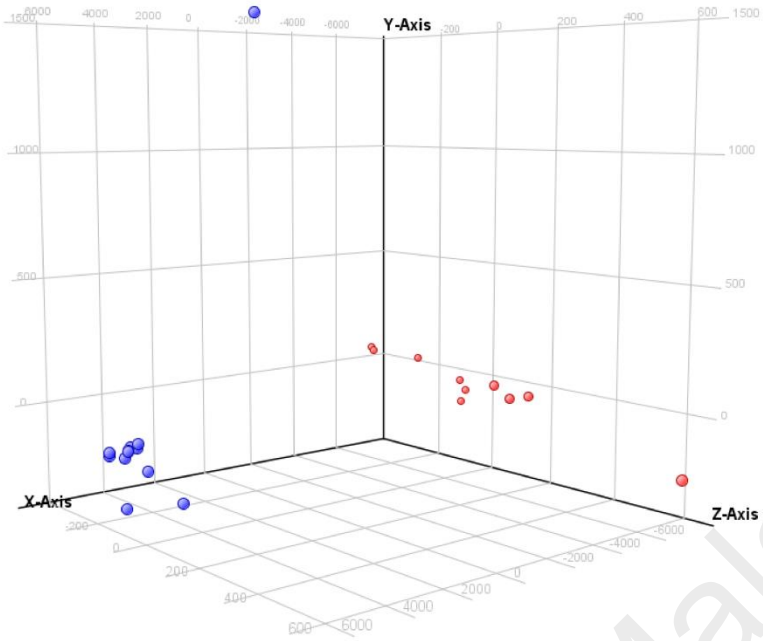


Figure 4.19: Three-dimensional PCA scores plot of plasma metabolomics for Baseline vs. 6 months post-*H. pylori* eradication post-prandial, acquired under both **A.** positive and **B.** negative ionisation modes

A



Indicator:

12 months post-eradication post-prandial

Baseline

B

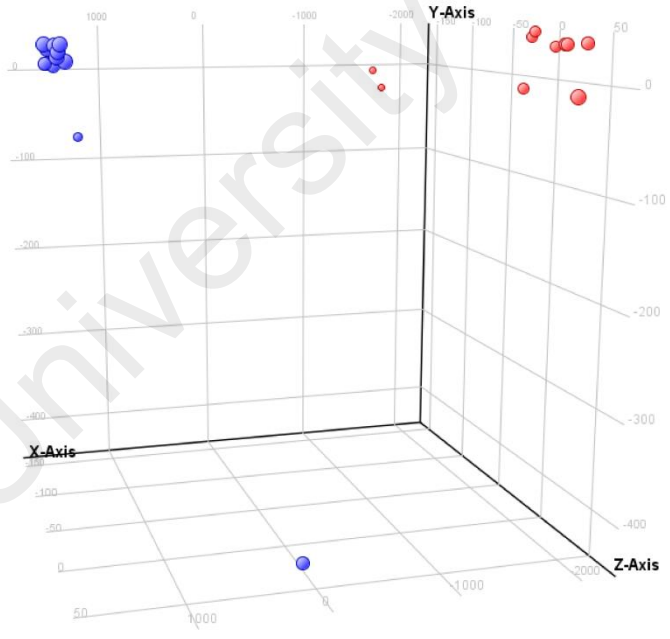
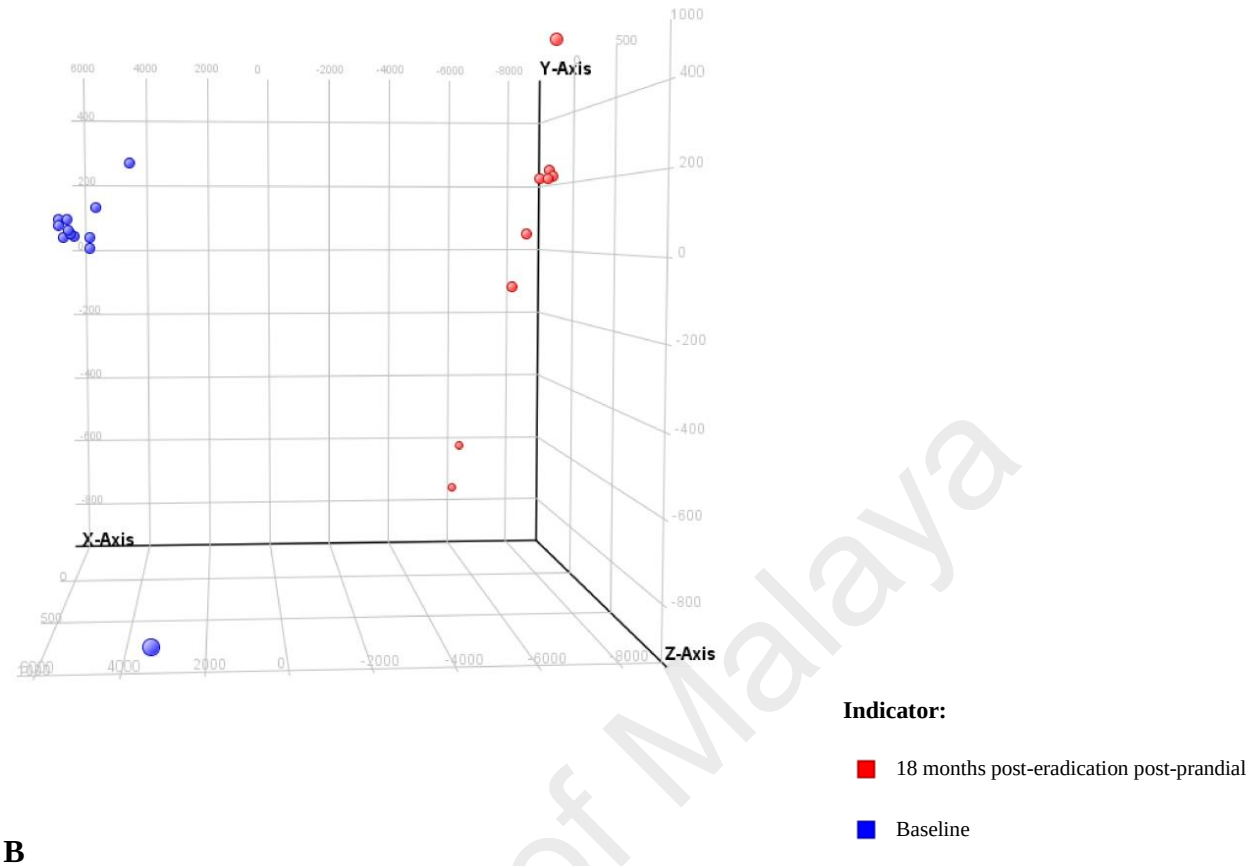


Figure 4.20: Three-dimensional PCA scores plot of plasma metabolomics for Baseline vs. 12 months post-*H. pylori* eradication post-prandial, acquired under both **A.** positive and **B.** negative ionisation modes

A



B

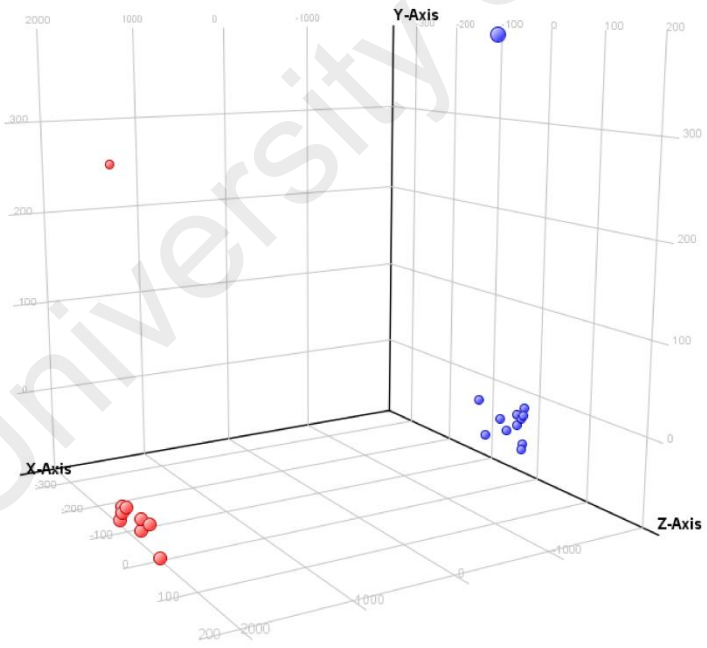


Figure 4.21: Three-dimensional PCA scores plot of plasma metabolomics for Baseline vs. 18 months post-*H. pylori* eradication post-prandial, acquired under both **A.** positive and **B.** negative ionisation modes

4.6.2 Potential small molecules associated with *Helicobacter pylori* eradication pre- and post-prandially

The same identification and annotation approaches were used for both lipid and metabolite datasets. Exogenous metabolites and xenobiotics were also filtered out as the current study was only interested to investigate the effect of *H. pylori* eradication pre- and post-prandially on the human endogenous plasma metabolomics. To sum up, a total of 1080, 1125 and 1262 entities were significantly changed for comparison of pre-prandial Baseline vs. 6 months, Baseline vs. 12 months and Baseline vs. 18 months post-*H. pylori* eradication, respectively (Appendix I). As for post-prandial, a total of 974, 1179 and 1150 entities were significantly changed for comparison Baseline vs. 6 months, Baseline vs. 12 months and Baseline vs. 18 months post-*H. pylori* eradication, respectively (Appendix J).

For pre-prandial plasma metabolomics, it was found that when Baseline was compared with 6 months-post eradication, there were 222 and 348 metabolites were up-regulated and down-regulated respectively. When Baseline was compared with 12 months-post eradication, the up- and down-regulated metabolites were 239 and 359 correspondingly. Conversely, only 45 metabolites were up-regulated but there were 551 metabolites down-regulated when Baseline was compared with 18 months-post eradication group (Table 4.18). Similarly, for post-prandial plasma metabolomics, it was found that when Baseline was compared with 6 months-post eradication, there were 228 and 324 metabolites were up-regulated and down-regulated respectively. When Baseline was compared with 12 months-post eradication, the up- and down-regulated metabolites were 224 and 397 correspondingly. Conversely, only 147 metabolites were up-regulated but there were 456 metabolites down-regulated when Baseline was compared with 18 months-post eradication group (Table 4.19). Among these significantly expressed metabolites, 71 of them could be categorised into 22 major

classes of metabolites and mapped into different biochemical pathways (Table 4.20 and Table 4.21). From the Pathway Analysis, porphyrin and chlorophyll metabolism we found to be the major perturbed pathway followed by tryptophan metabolism and bile acid biosynthesis.

For pre-prandial plasma metabolomic analysis, when Baseline was compared with post-*H. pylori* eradication groups, a number of fatty acids and sphingolipids involved in biosynthesis of unsaturated fatty acids and sphingolipid metabolism respectively were down-regulated post-*H. pylori* eradication. Two metabolites belong to the class of organic compounds of indoles and derivatives, melatonin and 5-Hydroxytryptophan, which involved in the melatonin biosynthesis were also found to be down-regulated in post-*H. pylori* eradication groups. Propinol adenylate that involved in propanoate metabolism, adenosylcobalamin that involved in riboflavin metabolism as well as UDP L-rhamnose and 2,4-Bis(acetamido)-2,4,6-trideoxy-beta- L-altropyranose that involved in amino sugar and nucleotide sugar metabolism were also down-regulated in post-*H. pylori* eradication groups. Conversely, pseudouridine, oxytocin, angiotensin I and prequalene diphosphate, involved in pyrimidine metabolism, cAMP signaling pathway, renin-angiotensin system and cholesterol biosynthesis respectively, were found to be up-regulated following *H. pylori* eradication. 6-phosphogluconic acid that involved in the pentose phosphate pathway was found to be up-regulated at 6 months post-eradication but it remained unchanged at 12 and 18 months post-eradication. On the other hand, taurocholic acid and taurochenodeoxycholic acid that involved in primary bile acid biosynthesis were up-regulated at 6 and 12 months-post eradication but they were down-regulated at 18 months-post eradication. Interestingly, there were five metabolites that belong to the class of tetrapyrroles and derivatives which involved in bilirubin degradation down-regulated at 12 and 18 months-post *H. pylori* eradication. Besides, four porphyrins which are also belong to the class of

tetrapyrroles and derivatives that involved in heme biosynthesis was also found to be down-regulated at 18 months post-eradication. Metabolites that involved in tricarboxylic acid cycle (TCA), citric acid and flavin adenine dinucleotide (FAD), were found to be up-regulated at 6 months post-eradication but they were down-regulated at 12 and 18 months post-eradication. N1,N12-Diacetylspermine and 3-Acetamidopropanal are intermediates that involved in polyamine metabolism. Both of these metabolites were up-regulated at 6 and 12 months post-*H. pylori* eradication. At 18 months post-eradication, N1,N12-Diacetylspermine remained unchanged but 3-Acetamidopropanal was found to be down-regulated (Table 4.20).

Similarly, for post-prandial plasma metabolomic analysis, when Baseline was compared with post-*H. pylori* eradication groups, several fatty acids and sphingolipids involved in biosynthesis of unsaturated fatty acids and sphingolipid metabolism respectively were down-regulated post-*H. pylori* eradication. Melatonin and 5-Hydroxytryptophan were also found to be down-regulated in post-*H. pylori* eradication groups, parallel to pre-prandial plasma metabolome results. Besides, propinol adenylate (propanoate metabolism), adenosylcobalamin (riboflavin metabolism), UDP-L-rhamnose and 2,4-Bis(acetamido)-2,4,6-trideoxy-beta-L-altropyranose (amino sugar and nucleotide sugar metabolism), N-Succinyl-2-amino-6-ketopimelate (lysine biosynthesis), 3-Acetamidopropanal (spermine and spermidine degradation), p-Hydroxybenzyldesulphoglucosinolate (glucosinolate biosynthesis/2-Oxocarbocylic acid metabolism), isovaleryl-CoA (valine, leucine and isoleucine degradation) and 4a-Hydroxytetrahydrobiopterin (tryptophan, phenylalanine and tyrosine metabolism) were also down-regulated in post-eradication groups. On the contrary, pseudouridine, which was found to be up-regulated following *H. pylori* eradication in pre-prandial plasma metabolomic analysis, was found to be down-regulated in post-eradication groups in post-prandial plasma metabolome instead. Correspond to pre-prandial plasma

metabolome results, oxytocin, angiotensin I and presqualene diphosphate involved in cAMP signalling pathway, renin-angiotensin system and cholesterol biosynthesis respectively, were found to be up-regulated following *H. pylori* eradication in post-prandial plasma metabolome. Intermediate of pentose phosphate pathway, 6-phosphogluconic acid, was found to be down-regulated at 6 months post-eradication, followed by a transient up-regulation at 12 months post-eradication and it was down-regulated again at 18 months post-eradication. Taurocholic acid and taurochenodeoxycholic acid that involved in primary bile acid biosynthesis were down-regulated at 12 and 18 months-post eradication. Five metabolites which involved in bilirubin degradation were found to be down-regulated significantly only at 18 months-post H. pylori eradication. For the two metabolites that involved in TCA cycle, citric acid was found to be up-regulated at 12 months post-eradication and then down-regulated at 18 months post-eradication whereas FAD was down-regulated at 12 and 18 months post-eradication. As for metabolites involved in polyamine metabolism, N1,N12-Diacetylspermine was found to be up-regulated in post-eradication groups and on the contrary, 3-Acetamidopropanal was found to be down-regulated in post-eradication groups (Table 4.21).

Table 4.18: Summary of the differentially expressed pre-prandial plasma metabolites associated with *H. pylori* eradication

Comparison group	Total significantly expressed metabolites[§]	Total down-regulated metabolites	Total up-regulated metabolites
Baseline vs. 6 months	570	348	222
Baseline vs. 12 months	598	359	239
Baseline vs. 18 months	596	551	45

[§]Significantly expressed metabolites denote those with more than 2 fold changes, p<0.001, FDR<1%.

Table 4.19: Summary of the differentially expressed post-prandial plasma metabolites associated with *H. pylori* eradication

Comparison group	Total significantly expressed metabolites[§]	Total down-regulated metabolites	Total up-regulated metabolites
Baseline vs. 6 months	552	324	228
Baseline vs. 12 months	621	397	224
Baseline vs. 18 months	603	456	147

[§]Significantly expressed metabolites denote those with more than 2 fold changes, p<0.001, FDR<1%.

Table 4.20: Selected significantly expressed pre-prandial metabolites between Baseline and post-*H. pylori* eradication groups

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C00345/ HMDB01316	6-Phosphogluconic acid	Carbohydrates and carbohydrate conjugates	Pentose Phosphate Pathway	139.0196	3.12	+13.24	-	-
C01598/ HMDB01389	Melatonin	Indoles and derivatives	Tryptophan metabolism Melatonin biosynthesis	233.1282	5.00	-10.26	-12.06	-12.06
C01017/ HMDB00472	5-Hydroxytryptophan	Indoles and derivatives	Tryptophan metabolism Melatonin biosynthesis	221.0916	1.70	-1.14	-6.60	-9.78
C00463/ HMDB00738	Indole	Indoles and derivatives	Tryptophan metabolism	116.0507	2.13	+2.19	+1.07	-7.52
C05635/ HMDB00763	5-Hydroxyindoleacetic acid	Indoles and derivatives	Tryptophan metabolism	190.0509	1.21	+1.10	-4.38	-13.99
C00328/ HMDB00684	Kynurenine	Benzene and substituted derivatives	Tryptophan metabolism	209.0918	1.43	-5.78	+2.60	-15.71
C05647/ HMDB12948	Formyl-5-hydroxykynurenamine	Benzene and substituted derivatives	Tryptophan metabolism	207.0770	1.22	+2.72	+0.912	-15.74
C01829/ HMDB00248	Thyroxine	Benzene and substituted derivatives	Tyrosine metabolism Thyroid hormone synthesis	777.6942	12.64	+2.39	-0.43	-9.25

Table 4.20, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C15522/ HMDB02281	4a-Hydroxytetrahydrobiopterin	Pteridines and derivatives	Tryptophan metabolism Phenylalanine and tyrosine metabolism	302.0840	5.93	-14.40	-7.80	-18.57
C09332/ HMDB06825	Tetrahydrofolyl-[Glu](2)	Pteridines and derivatives	Folate biosynthesis	597.2033	3.21	+16.98	+11.44	-8.54
C05843/ HMDB04194	N-Methyl-4-pyridone-3-carboxamide	Pyridines and derivatives	Nicotinate and nicotinamide metabolism	153.0653	0.96	+2.96	-8.30	-8.30
C00791/ HMDB00562	Creatinine	Azolines	Arginine and proline metabolism	112.0517	0.67	+2.26	-0.57	-19.87
C00262/ HMDB00157	Hypoxanthine	Imidazopyrimidines	Purine metabolism	137.0452	0.80	-2.69	+10.19	-4.32
C00147/ HMDB00034	Adenine	Imidazopyrimidines	Purine metabolism	134.0471	0.93	+3.20	-14.88	-14.88
C04376/ HMDB01308	5'-Phosphoribosyl-N-formylglycinamide (FGAR)	Glycinamide ribonucleotides	Purine metabolism	332.0851	12.29	-13.98	-9.94	-18.75
C01261/ HMDB01340	P1,P4-Bis(5'-guanosyl) tetraphosphate (GppppG)	(5'->5')-dinucleotides	Purine metabolism	869.0477	14.65	-13.65	-6.68	-11.45
C00366/ HMDB00289	Uric acid	Alkaloids and derivatives	Purine metabolism	167.0212	0.59	+3.56	+1.92	-17.50
C02067/ HMDB00767	Pseudouridine	Nucleoside and nucleotide analogues	Pyrimidine metabolism	243.0624	0.76	+3.00	+1.64	+1.64
C05983/ HMDB06806	Propinol adenylate	Purine nucleotides	Propanoate metabolism	426.0801	0.62	-1.85	-12.52	-17.24

Table 4.20, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C02199/ HMDB12305	UDP-L-rhamnose	Pyrimidine nucleotides	Amino sugar and nucleotide sugar metabolism	573.0505	0.69	-14.98	-14.98	-14.98
C19972	2,4-Bis(acetamido)-2,4,6-trideoxy-beta-L-altropyranose	-	Amino sugar and nucleotide sugar metabolism	247.1287	2.33	-6.10	-2.55	-15.88
C00158/ HMDB00094	Citric acid	Carboxylic acids and derivatives	Tricarboxylic acid cycle (TCA)	215.0154	0.71	+4.41	-3.21	-8.38
C02237/ HMDB00805	R-(+)-Pyrrolidone-5-carboxylic acid	Carboxylic acids and derivatives	D-glutamine and D-glutamate metabolism	130.0496	6.13	+3.74	-14.97	-14.97
C11684	MET-Enkephalin	Carboxylic acids and derivatives	Neuroactive ligand-receptor interaction	591.2605	16.84	-6.63	+4.61	-4.97
C04462/ HMDB12266	N-Succinyl-2-amino-6-ketopimelate	Carboxylic acids and derivatives	Lysine biosynthesis	285.1320	7.80	+2.53	-5.42	-12.29
C04148/ HMDB06344	Alpha-N-Phenylacetyl-L-glutamine	Carboxylic acids and derivatives	Phenylalanine metabolism	265.1183	6.12	+1.65	+11.41	-12.21
C11588	cis-3-(Carboxy-ethyl)-3,5-cyclo-hexadiene-1,2-diol	Carboxylic acids and derivatives	Phenylalanine metabolism	183.0670	1.11	+22.34	+4.13	-1.87
C00746/ HMDB02865	Oxytocin	Carboxylic acids and derivatives	cAMP signalling pathway	521.2544	7.79	+8.94	+16.89	+1.99
C03413/ HMDB02172	N1,N12-Diacetylspermine	Carboxylic acids and derivatives	Polyamine metabolism	309.2256	9.80	+4.63	+16.58	-
C00016/ HMDB01248	Flavin adenine dinucleotide (FAD)	Flavin nucleotides	-	786.1643	6.36	+2.04	-9.58	-9.58

Table 4.20, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
HMDB11612	6-Hydroxy Flavin adenine dinucleotide	Flavin nucleotides	-	819.1830	9.30	+1.34	-1.75	-7.44
C00194/ HMDB02086	Adenosylcobalamin	-	Porphyrin and chlorophyll metabolism Riboflavin metabolism	693.3397	18.96	-18.21	-18.21	-18.21
C03262/ HMDB01261	Coproporphyrinogen III	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism Heme biosynthesis	329.1504	1.19	+2.57	+2.23	-11.87
C02191/ HMDB00241	Protoporphyrin IX	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism Heme biosynthesis	580.2920	17.64	+1.31	+5.32	-6.3
HMDB00668	Hematoporphyrin	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism Heme biosynthesis	599.2856	12.36	+3.58	-5.65	-9.73
HMDB00683	Harderoporphyrin	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism Heme biosynthesis	626.2958	19.40	-6.89	+5.69	-3.25
C00500/ HMDB02309	Biliverdin IX	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism	583.2551	14.19	+1.94	-2.85	-10.57
C05793/ HMDB04159	L-Urobilin	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism: Bilirubin degradation	595.3480	14.93	+10.82	-3.16	-4.68
C05794/ HMDB04160	I-Urobilin	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism: Bilirubin degradation	591.3163	15.08	-0.85	-12.83	-14.40
C05790/ HMDB01898	Mesobilirubinogen	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism: Bilirubin degradation	615.3149	15.05	+3.64	-9.60	-9.60
C05789/ HMDB04157	L-Urobilinogen	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism: Bilirubin degradation	597.3633	12.72	-6.60	-4.69	-8.13

Table 4.20, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C18170/ HMDB12880	3-Acetamidopropanal	Carbonyl compounds	Spermine and spermidine degradation Polyamine metabolism	114.0562	0.51	+3.57	+3.27	-13.06
C00873/ HMDB61196	Angiotensin I	Polypeptides	Renin-angiotensin system	1318.6613	22.50	+17.68	+14.72	+11.76
C17935	Cysteinyldopa	-	Tyrosine metabolism	317.0817	0.67	+6.54	+8.32	-8.28
C17240	p-Hydroxybenzyldesulphoglucosinolate	-	Glucosinolate biosynthesis 2-Oxocarboxylic acid metabolism	346.0939	1.20	-2.41	+1.35	-14.42
C06428/ HMDB01999/ LMFA01030761	Eicosapentaenoic acid (EPA (d5))	Fatty acyls	Biosynthesis of unsaturated fatty acids	330.2456	15.55	-7.59	-8.13	-15.94
C16531	Docosenoyl-CoA	Fatty acyls	Biosynthesis of unsaturated fatty acids	1088.4354	14.69	-3.94	-11.05	-10.94
C02939/ HMDB01113	Isovaleryl-CoA	Fatty acyls	Valine, leucine and isoleucine degradation	874.1622	0.63	-10.55	+5.22	-14.14
C14827/ LMFA02000012	9(S)-HpODE	Fatty acyls	Linoleic acid metabolism	311.2229	16.66	+4.12	+10.26	-8.32
C04717/ LMFA02000034	13(S)-HpODE	Fatty acyls	Linoleic acid metabolism	335.2206	13.73	+1.52	-3.24	+2.96
C16318/ LMFA02020016	methyl (+)-7-isojasmonate	Fatty acyls	alpha-Linoleic acid metabolism	223.1337	15.58	+1.89	+5.78	-8.27
C05984/ HMDB00008	DL-a-Hydroxybutyric acid	Fatty acyls	Propanoate metabolism	103.0403	0.61	+0.57	-0.26	-19.02

Table 4.20, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C01120/ HMDB01383/ LMSP01050002	Sphinganine 1-phosphate	Sphingolipids	Sphingolipid metabolism	382.2716	16.16	-10.38	-17.50	-17.50
C06124/ LMSP01050001	Sphingosine-1-phosphate	Sphingolipids	Sphingolipid metabolism	378.2405	15.51	-14.12	-14.13	-2.72
C12144/ HMDB04610/ LMSP01030001	Phytosphingosine	Sphingolipids	Sphingolipid metabolism	318.3004	13.94	-5.91	-12.93	-17.48
C03033/ HMDB10320	Cortolone-3-glucuronide	Sterol lipids	Estrogen metabolism	543.2802	10.63	+3.49	+2.33	-10.54
C05464/ HMDB00631	Glycodeoxycholate	Sterol lipids	Secondary bile acid biosynthesis	450.3207	14.86	+4.81	-4.45	-2.52
C05122/ HMDB00036/ LMST05040001	Taurocholic acid	Sterol lipids	Primary and Secondary bile acid biosynthesis	533.3256	12.03	+7.73	+8.27	-1.35
C05465/ HMDB00951/ LMST05040005	Taurochenodeoxycholic acid	Sterol lipids	Primary and Secondary bile acid biosynthesis	500.3040	13.60	+2.64	+2.92	-3.82
C03033/ HMDB02579/ LMST05010048	Glycochenodeoxycholic acid 3-glucuronide	Sterol lipids	Pentose and glucoronate interconversions Starch and sucrose metabolism Bile secretion	626.3539	12.97	-5.68	-17.01	-17.01

Table 4.20, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C03033/ HMDB10351	11-beta-hydroxyandrosterone-3-glucuronide	Sterol lipids	Pentose and glucoronate interconversions Starch and sucrose metabolism Bile secretion	481.2431	9.22	+4.18	+3.50	-11.59
C03033/ HMDB10357	Tetrahydroaldosterone-3-glucuronide	Sterol lipids	Pentose and glucoronate interconversions Starch and sucrose metabolism Bile secretion	558.2907	13.37	+6.04	-7.40	-11.88
C03642/ HMDB02580/ LMST05020003	Taurolithocholic acid 3-sulfate	Sterol lipids	Bile secretion	562.2492	11.66	+0.53	-14.56	-14.56
C05300/ HMDB00335/ LMST02010041	16alpha-Hydroxyestrone	Sterol lipids	Steroid hormone biosynthesis	287.1655	12.57	-6.01	-6.01	-6.01
C11136/ LMST05010014	Etiocholan-3α-ol-17-one 3-glucuronide	Sterol lipids	Steroid hormone biosynthesis	465.2477	11.09	+2.20	-3.61	-14.8
C18043/ HMDB00653/ LMST05020016	Cholesterol sulfate	Sterol lipids	Steroid hormone biosynthesis	465.3037	21.47	-8.71	+1.65	-4.43
C18044/ HMDB00774/ LMST05020014	Pregnenolone sulfate	Sterol lipids	Steroid hormone biosynthesis	395.1891	13.20	-1.32	+12.43	-1.32
C05473/ LMST02030187	11b,21-Dihydroxy-3,20-oxo-5b-pregnan-18-al	Sterol lipids	Steroid hormone biosynthesis	363.2171	11.08	-5.78	-9.27	-8.89

Table 4.20, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C05471/ HMDB03259/ LMST02030204	Dihydrocortisol	Sterol lipids	Steroid hormone biosynthesis	365.2318	10.68	+0.83	+2.46	-6.44
C05683/	2-Aminoethylphosphocholate	Sterol lipids	Phosphonate and phosphinate metabolism	516.3065	17.26	+4.70	+16.68	-
C03428/ HMDB01278/ LMPR0106010003	Presqualene diphosphate	Prenol lipids	Cholesterol biosynthesis	604.3531	8.34	+7.97	+8.54	+3.66

*Expression level indicates log fold change (FC). Log_2 FC≥1 (FC≥2) is considered as significant; + indicates up-regulated in post-eradication group; - indicates down-regulated in post-eradication group.

Table 4.21: Selected significantly expressed post-prandial metabolites between Baseline and post-*H. pylori* eradication groups.

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C00345/ HMDB01316	6-Phosphogluconic acid	Carbohydrates and carbohydrate conjugates	Pentose Phosphate Pathway	139.0196	3.12	-4.56	+4.13	-6.11
C01598/ HMDB01389	Melatonin	Indoles and derivatives	Tryptophan metabolism Melatonin biosynthesis	233.1282	5.00	-11.19	-17.76	-17.76
C01017/ HMDB00472	5-Hydroxytryptophan	Indoles and derivatives	Tryptophan metabolism Melatonin biosynthesis	221.0916	1.70	-3.94	-16.21	-17.73
C00463/ HMDB00738	Indole	Indoles and derivatives	Tryptophan metabolism	116.0507	2.13	-2.44	+2.14	-4.50
C05635/ HMDB00763	5-Hydroxyindoleacetic acid	Indoles and derivatives	Tryptophan metabolism	190.0509	1.21	-4.66	-6.98	-13.59
C00328/ HMDB00684	Kynurenine	Benzene and substituted derivatives	Tryptophan metabolism	209.0918	1.43	+4.78	+2.92	-6.35
C05647/ HMDB12948	Formyl-5-hydroxykynurenamine	Benzene and substituted derivatives	Tryptophan metabolism	207.0770	1.22	-2.85	+1.44	-15.53
C01829/ HMDB00248	Thyroxine	Benzene and substituted derivatives	Tyrosine metabolism Thyroid hormone synthesis	777.6942	12.64	-1.19	+0.08	-3.59

Table 4.21, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C05843/ HMDB04194	N-Methyl-4-pyridone-3-carboxamide	Pyridines and derivatives	Nicotinate and nicotinamide metabolism	153.0653	0.96	+13.00	-3.37	-3.37
C00791/ HMDB00562	Creatinine	Azolines	Arginine and proline metabolism	112.0517	0.67	-1.18	+0.56	-1.06
C00262/ HMDB00157	Hypoxanthine	Imidazopyrimidines	Purine metabolism	137.0452	0.80	-7.68	+1.18	-9.12
C00147/ HMDB00034	Adenine	Imidazopyrimidines	Purine metabolism	134.0471	0.93	+2.54	-13.07	-13.07
C04376/ HMDB01308	5'-Phosphoribosyl-N-formylglycinamide (FGAR)	Glycinamide ribonucleotides	Purine metabolism	332.0851	12.29	+1.15	-6.08	-2.43
C01261/ HMDB01340	P1,P4-Bis(5'-guanosyl) tetraphosphate (GppppG)	(5'->5')-dinucleotides	Purine metabolism	869.0477	14.65	-3.07	-7.00	-13.71
C00366/ HMDB00289	Uric acid	Alkaloids and derivatives	Purine metabolism	167.0212	0.59	-5.13	+7.26	+1.02
C02067/ HMDB00767	Pseudouridine	Nucleoside and nucleotide analogues	Pyrimidine metabolism	243.0624	0.76	-0.98	-2.65	-8.40
C05983/ HMDB06806	Propinol adenylate	Purine nucleotides	Propanoate metabolism	426.0801	0.62	-8.59	-6.49	-15.87
C02199/ HMDB12305	UDP-L-rhamnose	Pyrimidine nucleotides	Amino sugar and nucleotide sugar metabolism	573.0505	0.69	-1.71	-16.62	-16.62
C03413/ HMDB02172	N1,N12-Diacetylspermine	Carboxylic acids and derivatives	Polyamine metabolism	309.2256	9.80	+1.33	+11.64	+1.67

Table 4.21, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C19972	2,4-Bis(acetamido)-2,4,6-trideoxy-beta-L-altropyranose	-	Amino sugar and nucleotide sugar metabolism	247.1287	2.33	-0.75	-7.67	-11.13
C00158/ HMDB00094	Citric acid	Carboxylic acids and derivatives	Tricarboxylic acid cycle (TCA)	215.0154	0.71	-0.48	+7.26	-6.68
C02237/ HMDB00805	R-(+)-Pyrrolidone-5-carboxylic acid	Carboxylic acids and derivatives	D-glutamine and D-glutamate metabolism	130.0496	6.13	+2.86	-15.86	-13.91
C11684	MET-Enkephalin	Carboxylic acids and derivatives	Neuroactive ligand-receptor interaction	591.2605	16.84	-6.56	+1.57	+0.14
C04462/ HMDB12266	N-Succinyl-2-amino-6-ketopimelate	Carboxylic acids and derivatives	Lysine biosynthesis	307.1127	7.83	-7.41	-8.24	-6.71
C04148/ HMDB06344	Alpha-N-Phenylacetyl-L-glutamine	Carboxylic acids and derivatives	Phenylalanine metabolism	287.0998	6.13	+2.09	+8.32	-17.07
C11588	cis-3-(Carboxy-ethyl)-3,5-cyclo-hexadiene-1,2-diol	Carboxylic acids and derivatives	Phenylalanine metabolism	183.0670	1.11	+11.95	-9.03	-9.03
C00746/ HMDB02865	Oxytocin	Carboxylic acids and derivatives	cAMP signalling pathway	521.2544	7.79	+4.55	+11.41	+15.22
C00016/ HMDB01248	Flavin adenine dinucleotide (FAD)	Flavin nucleotides	-	786.1643	6.36	+4.63	-8.63	-8.63
HMDB11612	6-Hydroxy Flavin adenine dinucleotide	Flavin nucleotides	-	819.1830	9.31	-12.20	-7.14	-15.73
C00194/ HMDB02086	Adenosylcobalamin	-	Porphyrin and chlorophyll metabolism Riboflavin metabolism	693.3397	18.96	-6.39	-15.21	-15.21

Table 4.21, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C03262/ HMDB01261	Coproporphyrinogen III	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism Heme biosynthesis	329.1504	1.19	+3.21	+13.21	-
C02191/ HMDB00241	Protoporphyrin IX	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism Heme biosynthesis	580.292	17.64	+1.20	+1.58	+7.32
HMDB00668	Hematoporphyrin	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism Heme biosynthesis	599.2860	12.36	+1.60	-9.64	-10.67
HMDB00683	Harderoporphyrin	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism Heme biosynthesis	626.2958	19.40	+1.88	-1.88	+1.81
C00500/ HMDB02309	Biliverdin IX	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism	1165.5023	14.20	-2.08	+1.93	-13.58
C05793/ HMDB04159	L-Urobilin	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism: Bilirubin degradation	595.3480	14.93	-0.65	+5.98	-8.98
C05794/ HMDB04160	I-Urobilin	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism: Bilirubin degradation	591.3163	15.08	-0.49	+0.90	-12.49
C05790/ HMDB01898	Mesobilirubinogen	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism: Bilirubin degradation	615.3149	15.05	-0.30	-12.10	-15.29
C05789/ HMDB04157	L-Urobilinogen	Tetrapyrroles and derivatives	Porphyrin and chlorophyll metabolism: Bilirubin degradation	597.3633	12.72	-3.32	-8.40	-10.24
C18170/ HMDB12880	3-Acetamidopropanal	Carbonyl compounds	Spermine and spermidine degradation Polyamine metabolism	114.0562	0.51	-11.11	-2.28	-10.60
C00873/ HMDB61196	Angiotensin I	Polypeptides	Renin-angiotensin system	1318.6613	22.50	+5.99	-	+17.82

Table 4.21, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C17935	Cysteinyldopa	-	Tyrosine metabolism	317.0817	0.67	+2.33	-6.95	-8.5
C17240	p-Hydroxybenzyldesulphoglucosinolate	-	Glucosinolate biosynthesis 2-Oxocarboxylic acid metabolism	346.0939	1.20	-6.67	-3.34	-17.21
C06428/ HMDB01999/ LMFA01030761	Eicosapentaenoic acid (EPA (d5))	Fatty acyls	Biosynthesis of unsaturated fatty acids	330.2455	15.55	+1.14	-0.12	-0.5
C16531	Docosenoyl-CoA	Fatty acyls	Biosynthesis of unsaturated fatty acids	1088.4354	14.69	+2.13	-9.32	-12.77
C02939/ HMDB01113	Isovaleryl-CoA	Fatty acyls	Valine, leucine and isoleucine degradation	830.1976	0.64	-3.51	-15.50	-17.35
C14827/ LMFA02000012	9(S)-HpODE	Fatty acyls	Linoleic acid metabolism	311.2229	16.66	+3.07	+9.62	-1.74
C04717/ LMFA02000034	13(S)-HpODE	Fatty acyls	Linoleic acid metabolism	335.2194	15.12	+5.95	-7.98	-6.22
C16318/ LMFA02020016	methyl (+)-7-isojasmonate	Fatty acyls	alpha-Linoleic acid metabolism	223.1340	16.02	+2.21	+3.72	-1.21
C05984/ HMDB00008	DL-a-Hydroxybutyric acid	Fatty acyls	Propanoate metabolism	103.0403	0.61	-12.53	+6.18	+2.92

Table 4.21, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C01120/ HMDB01383/ LMSP01050002	Sphinganine 1-phosphate	Sphingolipids	Sphingolipid metabolism	382.2716	16.16	-1.02	-17.13	-15.46
C06124/ LMSP01050001	Sphingosine-1-phosphate	Sphingolipids	Sphingolipid metabolism	378.2407	15.51	-3.46	-14.55	-14.55
C12144/ HMDB04610/ LMSP01030001	Phytosphingosine	Sphingolipids	Sphingolipid metabolism	318.3002	13.94	+4.77	+4.83	-8.28
C03033/ HMDB10320	Cortolone-3-glucuronide	Sterol lipids	Estrogen metabolism	560.3068	10.88	-1.55	-1.71	-10.95
C05464/ HMDB00631	Glycodeoxycholate	Sterol lipids	Secondary bile acid biosynthesis	450.3207	14.86	-5.55	-5.08	-15.56
C05122/ HMDB00036/ LMST05040001	Taurocholic acid	Sterol lipids	Primary and Secondary bile acid biosynthesis	533.3256	12.03	+2.17	-9.24	-9.24
C05465/ HMDB00951/ LMST05040005	Taurochenodeoxycholic acid	Sterol lipids	Primary and Secondary bile acid biosynthesis	500.3040	13.60	-4.37	-12.65	-12.65
C03033/ HMDB02579/ LMST05010048	Glycochenodeoxycholic acid 3-glucuronide	Sterol lipids	Pentose and glucoronate interconversions Starch and sucrose metabolism Bile secretion	626.3537	13.06	-2.07	-18.33	-14.24

Table 4.21, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C03033/ HMDB10351	11-beta-hydroxyandrosterone-3-glucuronide	Sterol lipids	Pentose and glucoronate interconversions Starch and sucrose metabolism Bile secretion	481.2431	9.22	-10.00	+2.61	-8.12
C03033/ HMDB10357	Tetrahydroaldosterone-3-glucuronide	Sterol lipids	Pentose and glucoronate interconversions Starch and sucrose metabolism Bile secretion	558.2907	13.37	-0.65	-8.65	-10.06
C03642/ HMDB02580/ LMST05020003	Taurolithocholic acid 3-sulfate	Sterol lipids	Bile secretion	562.2492	11.66	-2.13	-14.41	+0.71
C05473/ LMST02030187	11b,21-Dihydroxy-3,20-oxo-5b-pregnan-18-al	Sterol lipids	Steroid hormone biosynthesis	363.2159	11.08	+0.50	-4.14	-1.51
C05471/ HMDB03259/ LMST02030204	Dihydrocortisol	Sterol lipids	Steroid hormone biosynthesis	365.2318	10.68	-2.41	+0.78	-11.51
C05300/ HMDB00335/ LMST02010041	16alpha-Hydroxyestrone	Sterol lipids	Steroid hormone biosynthesis	287.1655	12.57	-14.26	-19.57	-19.57
C11136/ LMST05010014	Etiocholan-3α-ol-17-one 3-glucuronide	Sterol lipids	Steroid hormone biosynthesis	465.2477	11.09	-2.28	-4.74	-1.17
C18043/ HMDB00653/ LMST05020016	Cholesterol sulfate	Sterol lipids	Steroid hormone biosynthesis	465.3041	21.48	+2.78	+12.40	-4.69

Table 4.21, continued

KEGG/ HMDB/ LMID	Metabolite	Class	Pathway	m/z	RT (min)	Comparison of expression level at 6 months post-eradication to Baseline*	Comparison of expression level at 12 months post-eradication to Baseline*	Comparison of expression level at 18 months post-eradication to Baseline*
C18044/ HMDB00774/ LMST05020014	Pregnenolone sulfate	Sterol lipids	Steroid hormone biosynthesis	395.1891	13.01	+2.13	+0.04	+5.08
C05683/	2-Aminoethylphosphocholate	Sterol lipids	Phosphonate and phosphinate metabolism	516.3065	17.26	-	+13.28	-
C03428/ HMDB01278/ LMPR0106010003	Presqualene diphosphate	Prenol lipids	Cholesterol biosynthesis	587.3264	8.31	+6.13	+12.21	+13.67

*Expression level indicates log fold change (FC). Log_2 FC≥1 (FC≥2) is considered as significant; + indicates up-regulated in post-eradication group; - indicates down-regulated in post-eradication group.

CHAPTER 5: DISCUSSION

5.1 Subject demographic

This study focused on a young and healthy Malaysian population with low risk of diseases, based on the premise that they have normal and balanced microbiota and energy homeostasis, making them an excellent target population for investigating the local as well as systemic effects of *H. pylori* eradication on the human body. This notion was further substantiated by the medical history survey in the baseline questionnaire which indicated that the subject population of this study were generally asymptomatic.

In this study, the prevalence of *H. pylori* infection of health young Malaysian adults was 9.9%. The prevalence reported earlier in Malaysia under different demographic and clinical settings range from 10%-30% (Boey et al., 1999; Chieng, Pan, & Loong, 2015; Kaur & Naing, 2003; Sasidharan et al., 2012). Earlier studies have also shown that the prevalence of *H. pylori* could be associated with several factors such as gender, race, birth order and social economy status (Chapter 2). Nevertheless, in this study, only race was shown to be a risk factor associated with *H. pylori* infection. Ethnic differences in the prevalence of *H. pylori* in this region have been reported. In Malaysia, the prevalence of *H. pylori* infection of Indians (25%-62%) is consistently higher than in Chinese (17%-58%) and Malays (6%-29%) in both symptomatic and asymptomatic subjects (Boey et al., 1999; Chieng et al., 2015; Goh, 1997, 2009; Goh & Parasakthi, 2001; Kaur & Naing, 2003; Sasidharan et al., 2012). Hence, the racial distribution of volunteers recruited into the ESSAY study reflects the *H. pylori* prevalence among the various ethnic groups in Malaysia. Previous studies have shown that *H. pylori* infection is predominantly seen in males (de Martel & Parsonnet, 2006; Sasidharan et al., 2012) but it was indicated otherwise in the current study.

5.1.1 *Helicobacter pylori* isolates genotypes

All the *H. pylori* strains isolated expressed the 26kDa species-specific antigen (*SSA*) gene which encodes a 26-kDa cell surface protein of *H. pylori.* This was another confirmatory result indicated that the bacteria strains isolated from the ESSAY volunteers were indeed *H. pylori*. Subsequent genotyping of *H. pylori* virulence factors has shown that all the *H. pylori* strains isolated from the volunteers were *cagA*- and *vacA*-positive. It is known that most East-Asia strains of *H. pylori* have *cagA* gene (Yamaoka, Kodama, et al., 1999) while *vacA* is present in almost all *H. pylori* strains (Hagymasi & Tulassay, 2014; Yamaoka, 2012). Additionally, all the *H. pylori* strains in this study were found to be expressed *vacA* s1a allele with either m1 or m2 allele. This observation was in accordance to a prior study which reported that almost all *cagA*-positive strains are classified as the *vacA* s1 strain (either m1 or m2) (Atherton et al., 1995). Moreover, *vacA* s hybrid (s1a/s1c) genotype was also detected in the isolated *H. pylori* strains which were supported by previous genotyping studies in other Asia countries (Choe et al., 2002; Linpisarn et al., 2007). In addition, a previous study conducted in Malaysian and Singaporean subjects also demonstrated that the isolated *H. pylori* strains were *vacA* subtypes s1/m1/i1 and s1/m2/i1 (Schmidt et al., 2010).

5.1.2 Dyspepsia symptoms and quality of life profile pre- and post-eradication of *Helicobacter pylori*

According to the SF-36 Healthy Survey, the *H. pylori*-positive volunteers who participated in this study and completed the test meal and follow-up visits were healthy and fit physically and emotionally. *H. pylori* eradication did not affect their physical or emotional health, and their personal perception of their health status. However, in this unblinded study, the SODA questionnaire has revealed that *H. pylori* eradication was associated with decreased dyspeptic symptoms. The decrease in symptoms should be

interpreted with caution because of the importance of placebo effects in clinical trials of dyspepsia (Mearin, Balboa, Zarate, Cucala, & Malagelada, 1999; Musial, Klosterhalfen, & Enck, 2007).

5.2 Stool metagenomic analysis

5.2.1 Effects of *Helicobacter pylori* eradication on gut microbiome

To our knowledge, the effects of *H. pylori* eradication on the gut microbiome have yet to be investigated in *H. pylori*-positive healthy young Malaysian adult. *H. pylori*-positive volunteers were given eradication therapy and the same cohort of volunteers was subsequently followed up for 6, 12, and 18 months post-eradication. Diversity analysis was performed to study the effect of *H. pylori* eradication on the gut microbial communities. The lack of significance within each time-point group as well as between the different time-point groups demonstrated that the microbial diversity of the gut microbiome of the volunteers was equally rich and proportional. In addition, following the eradication of *H. pylori*, the bacterial communities were not affected. The bacterial communities were similar pre- and post-*H. pylori* eradication. Therefore, these results suggested that eradication of *H. pylori* may not remarkably interrupt the composition and structure of the gut microbiome.

Irrespective of the *H. pylori* eradication status, the general profile of the gut microbiome of the volunteers in our ESSAY study was in accordance with previous findings. As reported elsewhere, the bacterial species in the human and mouse gut was dominated by phyla *Bacteroidetes* and *Firmicutes* (Ley et al., 2005). Less abundant bacteria phyla such as *Actinobacteria*, *Proteobacteria*, *Verrucromicrobia*, and as well as *Euryarcheaota* (mainly methanogenic archae *Methaobrevibacter smithii*) were also present (Eckburg et al., 2005; Qin et al., 2010). It has been reported that the general profile of the bacteria community of an individual at different body habitats seems to be reasonably stable over time (Costello et al., 2009).

Although the general profile of the gut microbiome was similar pre- and post-*H. pylori* eradication, the metagenomic analysis in this study revealed some changes in the bacterial communities at the phylum and genus levels that are notable. At 12 months post-*H. pylori* eradication, the relative abundance of *Bacteroidetes* in the stool microbiome of the volunteers decreased 15% with relative increase in *Firmicutes*, as compared to stool microbiome at Baseline. There is increasing evidence supporting the view that indicated the inverse relationship between *H. pylori* prevalence and rate of overweight/obesity. Hence, the gradual decrease of the *H. pylori* colonisation that has been observed in recent decades could be causally related to the human epidemic obesity (Lender et al., 2014). Studies in human and mice have shown that obesity is associated with changes in the composition of the gut microbiome. An early study reported that genetically obese ob/ob mice had a 50% reduction in the abundance of *Bacteroidetes* and proportional increase in *Firmicutes* (Ley et al., 2005). Human studies have also demonstrated enrichment in *Firmicutes* and a corresponding reduction in *Bacteroidetes* levels in the microbiota of obese individuals; after weight loss, the *Bacteroidetes*-to-*Firmicutes* ratio normalised to the level observed in lean individuals (Ley, Turnbaugh, Klein, & Gordon, 2006). *Bacteroidetes* and *Firmicutes* have been associated with the regulation of lipid and bile acid metabolism as well as energy homeostasis in host (Turnbaugh et al., 2006; Van Eldere, Celis, De Pauw, Lesaffre, & Eyssen, 1996). Essentially, it has been demonstrated that perturbations of bile acid-mediated signalling pathway influence risk of metabolic complications such as obesity and diabetes (Martin et al., 2007). Eighteen months post-eradication, however, the relative abundance of *Bacteroidetes* and *Firmicutes* seems to be restoring to the Baseline levels with the enrichment of *Proteobacteria*.

In a recent study that investigated the short- and long-term effects of clarithromycin and metronidazole treatment, a dramatic decline in *Actinobacteria* in

both throat and faeces was reported immediately after *H. pylori* eradication therapy. Although the diversity of the microbiome subsequently recovered to resemble the pre-treatment states, the microbiota remained perturbed in some cases for up to four years post-treatment (Jakobsson et al., 2010). Correspondingly, in this study, the relative abundance of the phylum *Actinobacteria* decreased 6 months-post eradication, and at 12 and 18 months post-eradication, it had increased to resemble to or higher than the Baseline level. This result indicated that broad-spectrum antibiotics used in *H. pylori* eradication treatment are also capable of inhibiting a range of Gram-positive and Gram-negative bacteria as well as other bacteria besides eradicating *H. pylori* (Elliott & Stone, 1990; Peters & Clissold, 1992). It was reported that high-level colonisation of the human gut by *Verrucomicrobia* occurred following broad-spectrum antibiotic treatment (Dubourg et al., 2013). Thus, the increase of phylum *Verrucomicrobia* 6 months post-eradication could be also attributed to the broad-spectrum antibiotic treatment used in *H. pylori* eradication therapy. However, at 12 and 18 months post-eradication, it appeared to be returning to Baseline levels.

Another interesting finding was observed for the phylum *Proteobacteria*. The relative abundance of *Proteobacteria* decreased at 6 months post-*H. pylori* eradication. However, at 12 and 18 months post-eradication, it was found to be increased as compared with Baseline levels. This finding may correlate with our observation at the genus level of the gut microbiome where *Helicobacter* was found at 12 and 18 months post-eradication but not at Baseline and 6 months post-eradication. It has been reported that besides *H. pylori,* EHS can also colonise the mucosal surfaces of the intestinal tract and/or the liver of humans, mammals and birds (Schauer, 2001). The finding in this study showed that the *Helicobacter* species detected in the four stool samples at 12 and 18 months post-eradication were not EHS but *H. pylori*. The sole identification of *H. pylori* is not due to the lack of taxonomic representation for *Helicobacter* species in

Greengenes database (McDonald et al., 2012). A study published recently showed that *16S rRNA* gene can be used to differentiate between gastric *Helicobacter* and EHS although it is not sufficient to distinguish between different EHS (Ménard, Buissonnière, Prouzet-Mauléon, Sifré, & Mégraud, 2016).

Before the *H. pylori*-positive volunteers were given the eradication regimen, *H. pylori* was still attached to the gastric mucosa of the stomach and therefore, it may be the reason why it was not detected in their stool samples. Although *H. pylori* is generally viewed as a non-invasive pathogen, some *in vivo* and *in vitro* studies have demonstrated otherwise. For example, *H. pylori* was found to reside in the vacuole in the cytoplasm, replicate on the cell membrane to form a microcolony, multiply in macrophages and bone marrow-derived dendritic cells, replicate in epithelial cells, and repopulate the extracellular space after the extracellular bacterial population has been killed by gentamicin for up to 3 days (Amieva, Salama, Tompkins, & Falkow, 2002; Chu, Wang, Wu, & Lei, 2010; Kwok, Backert, Schwarz, Berger, & Meyer, 2002; Tan, Tompkins, & Amieva, 2009; Wang, Gorvel, Chu, Wu, & Lei, 2010; Wang, Wu, & Lei, 2009). Chu et al. also reported that some coccoid forms of *H. pylori* were present on the plasma membrane of epithelial cells 18 hours post-*H. pylori* infection (Chu et al., 2010). All of these studies showed that *H. pylori* may be a facultative intracellular organism (Dubois & Boren, 2007; Petersen & Krogfelt, 2003). When the *H. pylori*-positive volunteers were given *H. pylori* eradication therapy, most of the *H. pylori* colonising the gastric mucosa may be killed, but some of them may have invaded the gastric epithelial cells and/or antigen-presenting cells and turned into non-culturable but viable, metabolising coccoid forms under the stress of antibiotics. The dormant coccoid form is resistant to antibiotic and can spread to infect other cells in the absence of an effective concentration of antibiotic (Chu et al., 2010). In addition, Tan et al. recently published a report suggesting that even at low to moderate multiplicity of infection (MOI 10), *H.*

pylori may impede the proliferation of macrophages by disrupting the cell cycle-associated genes and such disruption may be an immunoevasive strategy utilised by *H. pylori* (Tan et al., 2015). It is likely that *H. pylori* utilises the advantage of this ecological niche to replicate intracellularly and survive the antibacterial therapy. This may explain the transient disappearance of *H. pylori* immediately following *H. pylori* eradication therapy but reappeared at 12-18 months later.

To further strengthen our findings, proteomic analysis was performed on these stool samples using LC-MS approach. Consistent with the detection of *H. pylori 16S rRNA* in these stool samples, *H. pylori* proteins were also detected (Appendix K) confirming the presence of the bacterium and their viability. In addition, the volunteers (whose stool samples were positive for *Helicobacter 16S rRNA*) were called back for UBT at 18 months post-*H. pylori* eradication. However, all of four of them were negative for *H. pylori* by UBT (data not shown). Negative UBT results at 18 months post-*H. pylori* eradication ruled out the possibility of recrudescence or reinfection of *H. pylori* in the volunteers. A previous report showed that coccoid forms of *H. pylori* may give false negative result for UBT (Weingart et al., 2004) as they produced low level of urease as compared to spiral forms (Hua & Ho, 1996; Nilius, Strohle, Bode, & Malfertheiner, 1993). These may explain the negative UBT results of these volunteers despite detection of *H. pylori* DNA and proteins in their stool samples. This finding has also shown that there is a possibility of *H. pylori* can be shed through faeces and supported the notion that *H. pylori* may be transmitted through faecal-oral route via contaminated water or food (Malaty, 2010).

The genus *Anaerofustis* was proposed and classified as *A. stercorihominis* sp. nov. under phylum *Firmicutes* and class *Clostridia* to accommodate a phylogenetically distinct Gram-positive, strictly anaerobic, catalase-negative, rod-shaped organism isolated from human faeces. It was found to produce acetate and butyrate as end

products of glucose fermentation (Finegold et al., 2004). The significant increment of the relative abundance of *Anaerofustis* 6 months-post eradication could be attributed to the anti-inflammatory and antimicrobial properties possessed by butyrate-producing bacteria (Hamer et al., 2008) that may play a role in restoring the delicate balance between human host and the perturbed gut microbiome. Butyrate producing bacteria produce SCFA such as acetate, butyrate, and propionate (Sun & Chang, 2014) through fermentation; the presence of SCFA is believed to be associated with reduced inflammation (Karlsson, Tremaroli, Nielsen, & Backhed, 2013) and has an important effect on colonic health (Cummings & Macfarlane, 1997a; Szylit & Andrieux, 1993). At 12 and 18 months-post eradication, the relative abundance of *Anaerofustis* was returned to Baseline level after human gut microbiome was restored.

Interestingly, the relative abundance of another SCFA-producing bacterial genus, *Phascolarctobacterium,* also significantly increased 6 months post-eradication. *Phascolarctobacterium* is a genus within the phylum *Firmicutes* and classified within the class of *Negativicutes* (https://www.ncbi.nlm.nih.gov/genome/13721). *P. succinatutens* sp. nov. isolated by Watanabe and co-workers recently from human feces. *P. succinatutens* sp. nov. is distributed broadly in the gut as subdominant members that may adapt to the intestinal environment by specialising to utilise the succinate generated by other bacterial species to produce propionate (Watanabe, Nagai, & Morotomi, 2012), which may also act as a health-promoting microbial metabolite in the human gut (Hosseini, Grootaert, Verstraete, & Van de Wiele, 2011) post-*H. pylori* eradication to aid in the restoration of the perturbed microbiome. In a recent study, the abundance of propionate/acetate producing species such as *Phascolarctobacterium* was reported to be increased in rats fed with high fat diet in which their increase in abundance was strongly correlated with adiposity and deterioration of metabolic factors (Lecomte et al., 2015).

On the side note, SCFA was also reported to stimulate the release of hormone PYY and GLP-1 from rodent enteroendocrine L cells via activation of the G-protein-coupled free fatty acid receptor (FFAR) 2 (Anini et al., 1999; Cherbut et al., 1998; Tolhurst et al., 2012). Of the SCFA produced by colonic fermentation of dietary fibre, propionate has the highest affinity for FFAR 2 (Brown et al., 2003; Le Poul et al., 2003). Recently, the first-in-human study also demonstrated that direct delivery of propionate to the colon acutely increases the release of PYY and GLP-1 (Chambers et al., 2014). A metabolic study of the same study population has also shown that *H. pylori* eradication was associated with long term elevation of active amylin, PYY, and GLP-1 in the serum (Yap et al., 2015) (see Section 5.4). By this mechanism of SCFA-linked G-protein-coupled receptor activation, the gut microbiota may contribute markedly to increased nutrient uptake and deposition, contributing to the development of metabolic disorders (Erejuwa, Sulaiman, & Ab Wahab, 2014). Hence, there is a possibility that eradication of *H. pylori* may cause dybiosis which in turn influence the human energy metabolism and lead to the development of obesity.

The genus *Ruminococcus* belongs to phylum *Firmicutes* and corresponds to 5–15% of the total bacterial population in the colon (Chassard et al., 2008; Ramirez-Farias et al., 2009). Currently, the genus *Ruminococcus* is divided into two phylogenetically separate groups which are categorised under two separate families *Ruminococcaceae* (Rainey, 2009b) and *Lachnospiraceae* (Rainey, 2009a) with numerous misclassified *Ruminococcus* species (Liu, Finegold, Song, & Lawson, 2008). Thus, although the relative abundance of this genus was found to have increased significantly 6 months-post eradication, we could not decipher the effect of *H. pylori* eradication on this bacteria genus. Similarly, genus TM7 is a recently described candidate division of the domain Bacteria, which is currently known only from environmental *16S* ribosomal DNA sequence data (Hugenholtz, Tyson, Webb, Wagner,

& Blackall, 2001). Candidate division TM7 is found in a diverse range of environment habitats (Brinig, Lepp, Ouverney, Armitage, & Relman, 2003; Hugenholtz et al., 2001; Kumar et al., 2003; Ouverney, Armitage, & Relman, 2003) and human body sites (Brinig et al., 2003; Dewhirst et al., 2010; Dinis et al., 2011; Kuehbacher et al., 2008; Pei et al., 2004). These microorganisms have been suggested to play an important role in the early stages of inflammatory mucosal processes, probably by modifying growth conditions for competing bacterial populations (Brinig et al., 2003; Kuehbacher et al., 2008). However, the effect of *H. pylori* eradication on these organisms in relation to health and diseases could not be elucidated because TM7 have been uncultivable, with no pure-culture representatives.

In addition to *Helicobacter*, the genera *Dialister* and *Agrobacterium* were also found to have changed significantly at 12 months post-*H. pylori* eradication. Although the clinical significance of *Dialister* spp. and *Agrobacterium* associated with any disease or infection following *H. pylori* eradication remains unknown for the time being, it is noteworthy that the significant changes of the relative abundance of these genera at 12 months-post eradication.

This preliminary stool metagenomics study has shown that the eradication of *H. pylori* caused perturbation of the gut microbiome and may indirectly affect the health of human. Clinicians should be aware of the effect of broad spectrum antibiotics used in *H. pylori* eradication regime and be more cautious in the clinical management of *H. pylori* infection, particularly patients from the immunocompromised group.

5.3 Faecal lipidomic analysis

5.3.1 Biological significance of the faecal lipidomic analysis

Lipidomic analysis of faecal material allows us to assess the human GI functions broadly and non-invasively and hence, it should reflect the GI health and functions

(Gregory et al., 2013). Lipids are central to intestinal biology, more stable than many metabolites, and more conserved across microbiota (Gregory et al., 2013). Moreover, lipidomics can complement microbiome studies, integrating information from microbial genomes, the host, and environmental factors (Gregory et al., 2013). A series of lipids, which could elucidate the local effects of *H. pylori* eradication on the gut microbiota and also emphasize the vital role of gut microbiota in the modulation of energy and lipid metabolism, were identified.

The endocannabinoid system is an important endogenous signalling system that consists of the cannabinoid receptors, their endogenous ligands (the endocannabinoids), and the enzymes catalysing endocannabinoid formation and degradation (Di Marzo, Bifulco, & De Petrocellis, 2004). The endocannabinoid system is well-known to be involved in a broad range of physiological functions and pathophysiological conditions including energy metabolism and metabolic disorders such as obesity (Di Marzo, 2008; Engeli, 2008; Engeli et al., 2005; Pacher, BÁTkai, & Kunos, 2006; Pataky, Bobbioni-Harsch, Carpentier, & Golay, 2013). Obesity is characterised by altered gut microbiota, low grade inflammation and dysregulation of endocannabinoid system, in majority of the cases overactive of the system (Geurts et al., 2011; Muccioli et al., 2010). Anandamide (also known as *N*-arachidonoylethanolamine or AEA) is an endocannabinoids that synthesised from N-arachidonoyl phosphatidylethanolamine (NAPE) by multiple pathways or it can be synthesised from free arachidonic acid and ethanolamine by the action of a fatty acid amide hydrolase (FAAH) acting in reverse (Sugiura, 2008; Wang & Ueda, 2009). Accumulating evidence has shown that anandamide is synthesised mainly by the former pathway rather than the latter in various mammalian tissues and cells (Sugiura, 2008). Anandamide 0-phosphate is one of the intermediates in the biosynthesis of anandamine (KEGG: map04723). Anandamide 0-phosphate was found to be up-regulated at 6 and 12 months following *H.*

pylori eradication but it was then down-regulated at 18 months post-eradication. The marked changes in expression of this fatty acid could probably be linked with the perturbed of gut microbiota post-*H. pylori* eradication which in turn causes dysregulation of the endocannabinoid system and subsequently affect the regulation of energy metabolism and therefore, lead to the development of obesity.

Sphingolipids are important signal molecules that mediate many biological functions such as cell proliferation, apoptosis, and inflammation (Futerman & Hannun, 2004; Hannun & Obeid, 2008; Morad & Cabot, 2013). Both phytosphingosine and sphinganine are involved in sphingolipid metabolism (KEGG: map00600). Ceramide is a major molecule in sphingolipid metabolism and a precursor of complex sphingolipids (Hannun & Obeid, 2008; Sugiura et al., 2002). Phytosphingosine detected in faecal samples was down-regulated post-*H. pylori* eradication and we believe that it could probably be related to the reduced inflammation of the stomach lining after eradication of *H. pylori*. On the contrary, sphinganine (also known as dihydrosphingosine) detected in the faecal samples was found to be up-regulated significantly at 6 and 12 months post-eradication. The biological significance of this conflicting observation is unknown. Significant changes of ceramides were also observed but there were no consistent pattern of changes among different ceramides (Appendix H).

One of the most interesting findings of this study is Lipid A-disaccharide-1-P was significantly elevated at 12 and 18 months post-*H. pylori* eradication. Lipid A, one of the three structural components of the lipopolysaccharide (LPS) molecule, is responsible for the pathophysiological effects associated with Gram-negative bacteria infections (Lodowska, Wolny, Weglarz, & Dzierzewicz, 2007). Gram-negative bacteria such as *Bacteroidetes* and other *Proteobacteria* (including *H. pylori*) are dominant microorganisms in the gut (Ley et al., 2005; Yap et al., 2016). The up-regulation of Lipid A at 12 and 18 months post-*H. pylori* eradication could probably be associated

with the perturbation of microbiota in the gut following *H. pylori* eradication therapy. In previous faecal metagenomics study (see Section 5.2), a transient disappearance of *H. pylori* was found immediately following *H. pylori* eradication therapy but reappeared at 12–18months (Yap et al., 2016). The overlapping of time points may be worthy of note as it has been discussed previously, there is a possibility that *H. pylori* utilises the advantage of ecological niche to replicate intracellularly and survive the anti-bacterial therapy (Yap et al., 2016). Nevertheless, we could not prove that the Lipid A originated from *H. pylori*.

A hopanoid named 32,35-anhydrobacteriohopaneterol produced by *Proteobacteria* (Talbot, Rohmer, & Farrimond, 2007) was found to be down-regulated at 18 months post-eradication. Hopanoids are bacterial pentacyclic triterpenoids that are structurally and biosynthetically similar to eukaryotic steroids (Doughty et al., 2011; Ourisson, Rohmer, & Poralla, 1987; Welander et al., 2012), but their cellular roles are poorly understood (Doughty, Dieterle, Sessions, Fischer, & Newman, 2014). In many bacteria, hopanoids may play roles in the adjustment of cell membrane permeability in adaptation to extreme environmental conditions. They are formed in the aerial hyphae (spore bearing structures) of the prokaryotic soil bacteria *Streptomyces*, where they are thought to minimize water loss across the membrane to the air (Poralla, Muth, & Hartner, 2000). In the actinomycete *Frankia*, the hopanoids in diazovesicle membranes likely restrict the entry of oxygen by making the lipid bilayer more tight and compact (Berry et al., 1993). The *Proteobacteria* in the gut including *H. pylori* may be expressing hopanoids such as 32,35-anhydrobacteriohopaneterol to adapt the hostile environment in the gut. The perturbation of gut microbiota following *H. pylori* eradication therapy may be the reason of the reduced expression of this hopanoid.

5.4 Metabolic study

5.4.1 Meal-associated changes of metabolic hormones and effects of *Helicobacter pylori* eradication

Normal energy homeostasis in humans is controlled by signalling pathways that involve hormones (leptin and acyl-ghrelin) with prominent central nervous system activity and hormones (insulin, active amylin, incretins (total GIP, GLP-1), PP, and total PYY) that have major activity in the GI tract and in other peripheral tissues. Due to its intimate relationship with the gastric epithelium, *H. pylori*, the dominant member of the gastric microbiota, has the potential to affect the regulation of normal energy homeostasis of humans (Choe et al., 2007; Francois et al., 2011; Jang et al., 2008; Liew, Lee, Lee, & Chen, 2006; Nwokolo et al., 2003; Osawa, 2008; Osawa et al., 2006).

Ghrelin is a circulating peptide that serves as one of the key regulators of energy homeostasis (Kirchner, Heppner, & Tschop, 2012; Klok et al., 2007). Through multiple synthesis steps, ghrelin is acylated to form acyl-ghrelin, believed to be the most active form of ghrelin that induces positive energy balance (Asakawa et al., 2005). The majority of human acyl-ghrelin is produced by X/A-like cells in the gastric oxyntic mucosa (Date et al., 2000). Ghrelin has substantial roles in energy homeostasis including meal initiation, adipogenesis, and body weight gain, mediated through the hypothalamus (Francois et al., 2011; Kirchner et al., 2012; Klok et al., 2007). The secretion of ghrelin by the gastric mucosa depends largely on nutritional state and the levels of leptin (Klok et al., 2007). In contrast, leptin is produced primarily by adipose tissue and has important hypothalamus-dependent roles in regulating food intake and energy expenditure (Francois et al., 2011; Klok et al., 2007).

The effects of leptin and ghrelin on energy homeostasis may be opposing; leptin induces weight loss by suppression of food consumption, while ghrelin acts as an appetite-stimulatory signal (Francois et al., 2011; Klok et al., 2007). In this study, *H.*

pylori eradication had a transient effect on acyl-ghrelin levels, with the loss of meal-induced suppression, but by 12 months, normal physiology was restored. In contrast, the regulation of leptin appeared to be disturbed at 12 months post-*H. pylori* eradication. Under normal circumstances, the levels of leptin decreased post-prandially. However, leptin levels were stimulated in response to the standard meal at 12 months post-*H. pylori* eradication. These data provided evidence that eradication of *H. pylori* may have long-term effects on both acyl-ghrelin and leptin levels. Furthermore, this could also suggest that inter-meal ghrelin levels may display a diurnal rhythm, that is in phase with that of leptin in healthy humans (Cummings et al., 2001). A recent study also reported that leptin administration to healthy volunteers did not regulate ghrelin levels over several days (Chan et al., 2004). The results obtained in this study are consistent with prior findings, providing evidence that the circulating acyl-ghrelin level is not regulated by leptin, but instead both hormones may have independent roles in regulating energy homeostasis.

Whether the eradication of *H. pylori* is associated with increased or decreased levels of ghrelin remains a subject of controversy (Francois et al., 2011; Nwokolo et al., 2003; Pacifico et al., 2008; Ulasoglu et al., 2013). In this study, *H. pylori* eradication had no significant effect on the levels of acyl-ghrelin, consistent with an earlier report that both pre-prandial and post-prandial plasma ghrelin levels remain unchanged after *H. pylori* eradication (Nwokolo et al., 2003). As with ghrelin, conflicting findings have been reported on the effect of *H. pylori* eradication on meal-associated leptin level (Azuma et al., 2001; Francois et al., 2011; Pacifico et al., 2008; Shimzu, Satoh, & Yamashiro, 2002). Similarly, both pre-prandial and post-prandial plasma leptin levels in this study remained unchanged post-*H. pylori* eradication.

Other studies found concomitant changes in ghrelin and leptin levels with change in BMI and a positive correlation between ghrelin, leptin and BMI in patients

after *H. pylori* eradication (Francois et al., 2011; Shimzu et al., 2002). However, since our study showed that the BMI of volunteers did not significantly change following *H. pylori* eradication, correlation analysis could not be performed.

The contradicting results between metabolic and anthropometric measures across different studies may reflect methodological differences including variation in demographic characteristics (health status, gastrointestinal disease conditions, socio-economic status, age, gender, ethnicity), geographic settings (Western vs. Eastern populations), diet, lifestyle, and length of post-*H. pylori* eradication therapy follow-up. The extent and location of *H. pylori*-induced inflammation at baseline may be associated with differences in ghrelin physiology that develop with *H. pylori* eradication (Francois et al., 2011; Kawashima et al., 2009; Liew et al., 2006). In one study, subjects with only antral gastritis showed the largest increases in ghrelin levels obtained pre- and post-prandial, and across the meal ghrelin levels (Francois et al., 2011). Without biopsies from the gastric corpus, determine inflammation status could not be determined, and thus could not confirm or refute these findings.

In this study, as expected, gastrointestinal hormones (insulin, active amylin, incretins (total GIP, GLP-1), PP and total PYY) increased significantly after the consumption of the standard meal. Following a meal, the blood glucose level rises and insulin is released to increase glucose uptake (Thorens, 2011). Amylin, co-localized with insulin, is simultaneously secreted to assist with glycemic regulation by slowing gastric emptying and promoting satiety, thus minimizing post-prandial spikes in the blood glucose level (Aronoff, Berkowitz, Shreiner, & Want, 2004; Koda et al., 1992). GIP and GLP-1, synthesised primarily by K cells at the mucosa of the duodenum and jejunum and ileal L-cells respectively, are incretins (Holst, 2007; Yamada et al., 2006). Incretins promote insulin secretion (Yamada et al., 2006), reduce the rate of nutrient absorption into the bloodstream and slow gastric emptying (Aronoff et al., 2004). PP

and PYY increase post-prandially and aid in metabolic regulation by regulating pancreatic secretion (Batterham et al., 2003; Holzer et al., 2012). These gut hormones also play important roles in energy homeostasis through their effects on neuroendocrine signalling within the brain (Aronoff et al., 2004; Holzer et al., 2012). Through this intricate cross-regulated system, the gastrointestinal tract, adipose tissue, and brain can communicate efficiently in regulating energy intake.

H. pylori eradication was associated with long-term disturbances in gut hormones not secreted by the stomach, three (active amylin, PP and total PYY) both pre- and post-prandially and one hormone (GLP-1) only post-prandially. The mechanisms for these changes are unknown, but could be associated with perturbation of gastric hormones, including gastrin (Kaneko, Konagaya, & Kusugami, 2002), or host immunity (Cadamuro, Rossi, Maniezzo, & Silva, 2014), and/or the gut microbiota compositions (Sekirov et al., 2010; Sun & Chang, 2014) following *H. pylori*-eradication, that in turn might modulate the human gut hormones and metabolism. The faecal *16S* metagenomic analysis of the same cohort of volunteers has shown that the relative abundance of short-chain fatty acid (SCFA)-producing bacteria was elevated post-*H. pylori* eradication (see Section 5.2). Interestingly, SCFA also have been reported to stimulate the release of hormone PYY and GLP-1 in both rodent and human via activation of the G-protein-coupled receptor (Anini et al., 1999; Chambers et al., 2014; Cherbut et al., 1998; Hosseini et al., 2011; Tolhurst et al., 2012). Therefore, the antibiotic eradication therapy used for *H. pylori* eradication may also cause perturbation of the gut microbiota, which is in turn may interfere with the regulation of human energy homeostasis.

In conclusion, this study indicates that eradication of *H. pylori* affects the regulation of human metabolic hormones involved in appetite-control and energy homeostasis, particularly the long term elevation of hormone PP and PYY could be

associated with the perturbation of gut microbiome and eventually lead to the development of metabolic disorders. Longer monitoring of volunteers from the ESSAY cohort is necessary to investigate the impact of *H. pylori*-eradication-associated hormonal changes on weight maintenance and other physiologic measures.

5.5 Immunological study

In the current study, an inverse association between *H. pylori* infection and allergen specific-IgE antibodies was observed. This observation was supported by numerous studies that reported the similar findings (Arram, Shahin, & Sherif, 2012; Kosunen et al., 2002; Lee et al., 2015). Furthermore, as mentioned in Chapter2, the inverse relationship of *H. pylori* infection and atopic disorders such as asthma and allergy is fairly well supported in the literature by a lot of epidemiology and experimental studies (Chen & Blaser, 2007, 2008; Kosunen et al., 2002; Matricardi et al., 2000; McCune et al., 2003; Reibman et al., 2008). Hence, the finding in this study was another instance that shows the *H. pylori* infection may confer protective effect against atopic disorders.

H. pylori generally persists in host for lifetime and could possibly evade and modify of host inflammatory, innate, and adaptive immune responses. The ability of *H. pylori* to stimulate the T-helper type 1 (Th1) immune response and inhibit T-helper type 2 (Th2) responses could be the possible pathogenic mechanism of the protective effect against atopic disorders. Neutrophil activating protein of *H. pylori* (HP-NAP) plays a key role in driving this polarised Th1/Th2 immune response. As mentioned in Chapter 2, systemic HP-NAP also significantly resulted in the reduction of total serum IgE responses (Codolo et al., 2008; D'Elios et al., 2009). In this study, total plasma IgE antibodies in the volunteers were significantly lower before the eradication of *H. pylori*. Thus, this result may further support the molecular mechanism of the possible protective effect of *H. pylori* using HP-NAP.

Allergic diseases and asthma are driven by T cells that produce Th2 cytokines (Amedei et al., 2010; Babu, Davies, & Holgate, 2004). Cytokines help to orchestrate the airway inflammation in asthma by promoting the development, differentiation, recruitment, priming, activation, and survival of inflammatory cells in which different cytokines may have overlapping cell regulatory action and function through complex cytokine networks. Asthma is perceived as a Th2 disease with a cytokine profile that is characterised by IL-4, IL-5, and IL-13 (Babu et al., 2004). IL-4 induces differentiation of naive helper T cells (Th0 cells) to Th2 cells and overproduction of IL-4 is associated with allergies (Bradding et al., 1994; Hershey, Friedrich, Esswein, Thomas, & Chatila, 1997; Saggini et al., 2011). Evidence suggests that other cytokines that are considered to be associated with a Th1-type profile such as TNF-α also are related to the inflammatory response in asthma (Babu et al., 2004; Berry, Brightling, Pavord, & Wardlaw, 2007). Studies have shown that increased TNF-α in the airways of asthmatic patients (Bradding et al., 1994; Ying et al., 1991). Furthermore, the administration TNF-α to normal subjects as well as patients asthma led to the development of airway hyperresponsiveness (Thomas & Heywood, 2002; Thomas, Yates, & Barnes, 1995).

Since *H. pylori* infection may confer protection against allergic diseases and asthma and consequently, the production of IL-4 and TNF- α should be lower before *H. pylori* eradication. However, in this study, the level of IL-4 was rather constant pre- and post-*H. pylori* eradication. Conversely, TNF- α was found to be decreasing instead of increasing post-*H. pylori* eradication.

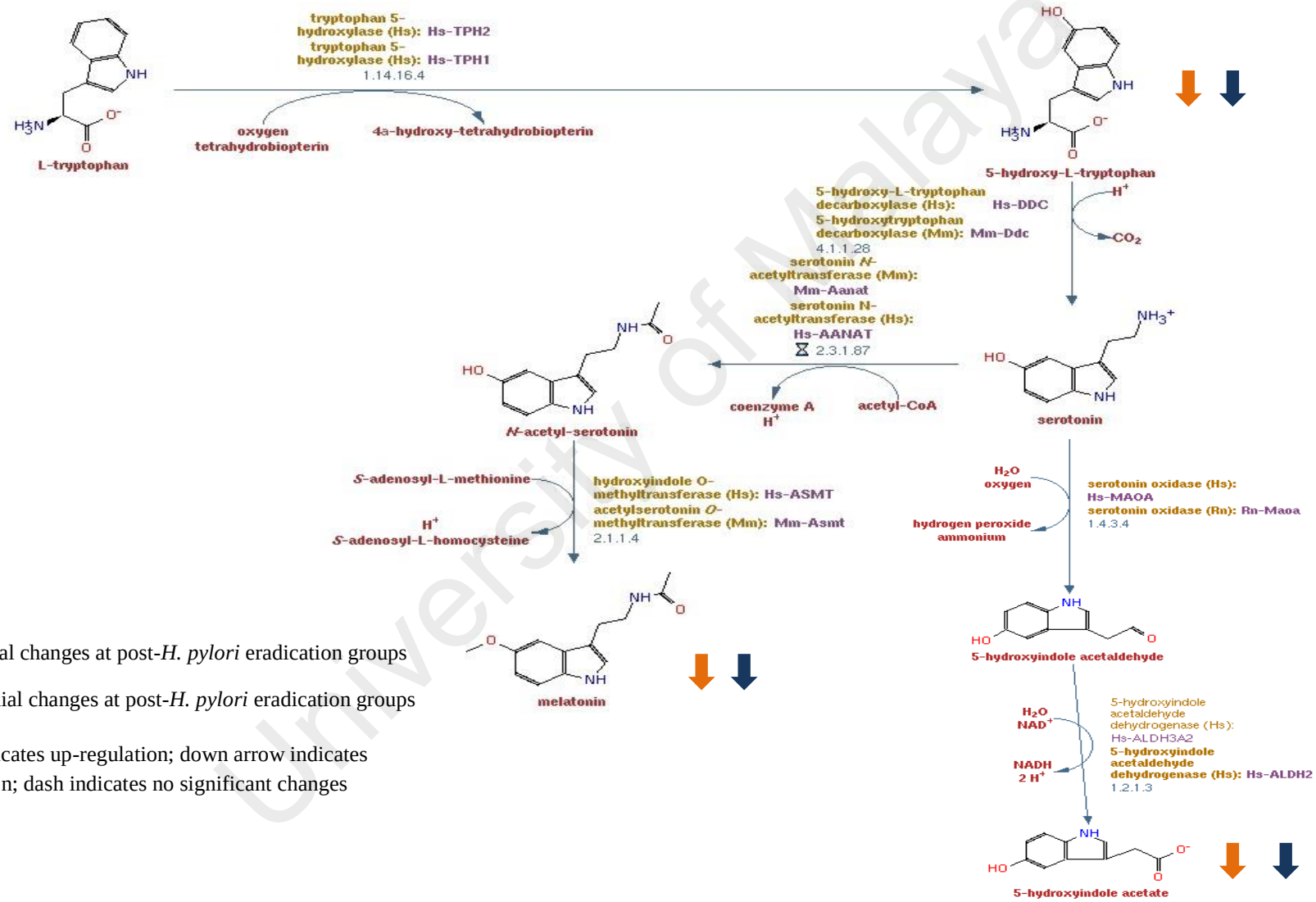
5.6 Plasma metabolomic analysis

5.6.1 Biological significance of the plasma metabolomic analysis

In this study, plasma metabolites instead of gastric tissues metabolites were studied because blood sample collection is minimally invasive compared with the collection of

tissue biopsy samples. In addition, blood is an integrative biofluid that incorporate the functions and phenotypes of many different parts of body in one sample and hence provide an overview of 'metabolic footprint' of many areas of metabolism in the human body (Dunn et al., 2011). The identified potential small molecules covered a representative part of the main metabolic pathways, thus allowing the determination of main intermediates of heme biosynthesis and bilirubin degradation, amino acid metabolisms, lipid metabolism, and energy metabolism (tricarboxylic acid cycle). A large number of metabolites in the plasma were significantly changed following *H. pylori* eradication, with majority being down-regulated in the post-*H. pylori* eradication groups, both pre- and post-prandially. A sophisticated interaction of how the changes in the gut microbiota (local) after the eradication of *H. pylori* influence the systemic changes of a human body could be observed.

Pathway analysis revealed that melatonin and 5-Hydroxytryptophan involved in melatonin biosynthesis were down-regulated following *H. pylori* eradication, both pre- and post-prandially. Melatonin is the primary regulatory hormone of circadian rhythm and plays an important role in initiating and maintaining sleep. Recent experimental evidences suggested that melatonin may influence food intake, energy expenditure, the accumulation of energy in adipose tissue, insulin secretion and glycemic control (Amaral, Castrucci, et al., 2014; Peschke et al., 2006; Picinato et al., 2008; Prokopenko et al., 2009). It has been proposed that the reduction in melatonin production may induce insulin resistance, glucose intolerance, sleep disturbance, and metabolic circadian disorganization characterising a state of chrono-disruption leading to metabolic disorders such as obesity (Amaral, Turati, et al., 2014; Cipolla-Neto, Amaral, Afeche, Tan, & Reiter, 2014; Pulimeno et al., 2013; Sharma, Singh, Ahmad, Mishra, & Tiwari, 2015). Taken together, the down-regulation of 5-Hydroxytryptophan and melatonin in plasma post-*H. pylori* eradication, both pre- and post-prandially, may be



Indicators:

Pre-prandial changes at post-*H. pylori* eradication groups

Post-prandial changes at post-*H. pylori* eradication groups

*Up arrow indicates up-regulation; down arrow indicates down-regulation; dash indicates no significant changes

Figure 5.1: Changes in melatonin biosynthesis pathway post-*H. pylori* eradication (adopted from (Caspi et al., 2014))

associated with the protective effect of *H. pylori* against metabolic disorders.

6-phosphogluconic acid is an intermediate in the pentose phosphate pathway. It is formed from 6-phosphogluconolactone and catalysed by phosphogluconate dehydrogenase to produce ribulose 5-phosphate. The pentose phosphate pathway is also resulted in the generation of NADPH which is necessary for fatty acid synthesis. Previous hepatic gene expression study reported that pentose phosphate pathway was up-regulated in obese patients with type 2 diabetes (Takamura et al., 2008). Another in vivo study also demonstrated that G6PD expression and activity, NADPH levels, and 6-phosphogluconic acid generation were significantly increased in the liver of adult male Zucker fa/fa rats, a prototype model of hyperglycemia and type 2 diabetes (Gupte et al., 2009). The elevation of 6-phosphogluconic acid at 6 months post-eradication in the pre-prandial plasma metabolome could be a metabolic signature of metabolic disorders. However, it seems like the expression of this metabolite was restored at 12 and 18 months post-eradication. On the other hand, the inconsistent changes of 6-phosphogluconic at 6, 12 and 18 months post-*H. pylori* eradication in the post-prandial plasma metabolome was unknown.

N-Methyl-4-pyridone-3-carboxamide is one of the end products of nicotinamide-adenine dinucleotide (NAD) degradation. This metabolite was initially up-regulated at 6 months post-eradication but it was down-regulated at 12 and 18 months post-eradication, both pre- and post-prandially. NAD is one of the cofactors essential for redox reactions. NAD and NADP play vital roles in metabolic conversions as signal transducers and in cellular defence systems. Both coenzymes participate as electron carriers in energy transduction and biosynthetic processes (Pollak, Dölle, & Ziegler, 2007). This observation in this study indicated that eradication of *H. pylori* may affect the biosynthesis of NAD in nicotinate and nicotinamide metabolism and hence, it

corroborated our previous finding that eradication of *H. pylori* may affect the regulation of energy metabolism in the human body (Yap et al., 2015).

This plasma metabolomic analysis has also shown that an intermediate and a cofactor of tricarboxylic acid cycle (TCA), citric acid and flavin adenine dinucleotide (FAD), were changed post-*H. pylori* eradication, both pre- and post-prandially. It is known that the TCA cycle, in conjunction with oxidative phosphorylation, provides the vast majority of energy used by aerobic cells—in human beings, greater than 95% (Berg , 2002). In addition, accumulating evidence has highlighted the important roles of gut microbiota in energy harvest and metabolism in hosts (Backhed et al., 2004; Musso, Gambino, & Cassader, 2010; Velagapudi et al., 2010; Venema, 2010), which are thought to be associated with a number of diseases such as obesity and diabetes. The dysregulation of these metabolites could probably reflect the long term effect of the eradication of *H. pylori* on regulation of energy metabolism in which the eradication of this bacterium causes the perturbations of the gut microbiota and therefore, it affects regulation of energy metabolism and subsequently leads to development of metabolic disorders such as obesity. This result could be an example to support the role of gut bacteria on blood metabolite profile.

Uric acid is the final product of purine metabolism in humans. The final two reactions in its production catalysing the conversion of hypoxanthine to xanthine and the latter to uric acid are catalysed by the enzyme, xanthine oxidoreductase (Glantzounis, Tsimoyiannis, Kappas, & Galaris, 2005). There is increasing experimental and clinical evidence showing that uric acid has an important role in *in vivo* as an antioxidant.(Becker, 1993; Glantzounis et al., 2005). Infection by *H. pylori* increases the production of reactive oxygen and nitrogen species in the human stomach (Ding et al., 2007; Handa, Naito, & Yoshikawa, 2011) and thus, following *H. pylori* eradication at 6 months, the oxidative stress in the stomach has relieved and this may

led to the down-regulation of hypoxanthine and uric acid, both pre- and post-prandially. However, the reason behind the transient increases of these metabolites at 12 months post-eradication, both pre- and post-prandially was unknown.

Spermine is acetylated to N1-acetylspermine by spermidine-spermineN1-acetyltransferase (SSAT) using acetyl-CoA as substrate. N1-acetylspermine can be oxidised by polyamine oxidase (PAO) to produce spermidine and 3-Acetamidopropanal. In addition, N1-acetylspermine can be acetylated for the second time, also by SSAT, to form N1,N12-Diacetylspermine (Morgan, 1998; Pegg, 2008). Polyamines (putrescine, spermine and spermidine) are organic polycations that are essential for various cellular functions affecting cell growth, cancer and ageing of which its metabolism is tightly regulated (Pegg & Casero, 2011). Both *in vivo* and *in vitro* studies have shown that polyamine pathway plays a major role in energy homeostasis (Jell et al., 2007; Kraus et al., 2014). Studies suggested that the increase of SSAT acetylation of spermine and spermidine increase the metabolic flux and thus it may lead to metabolic consequences (Jell et al., 2007; Kramer et al., 2008). The up-regulation of N1,N12-Diacetylspermine, both pre- and post-prandially, and the up-regulation of 3-Acetamidopropanal, pre-prandially, in post-eradication groups may indicate the increase of SSAT which could be a predictive signature of metabolic disorders such as obesity. Nonetheless, the down-regulation of 3-Acetamidopropanol, post-prandially, in post-eradication groups could not be elucidated.

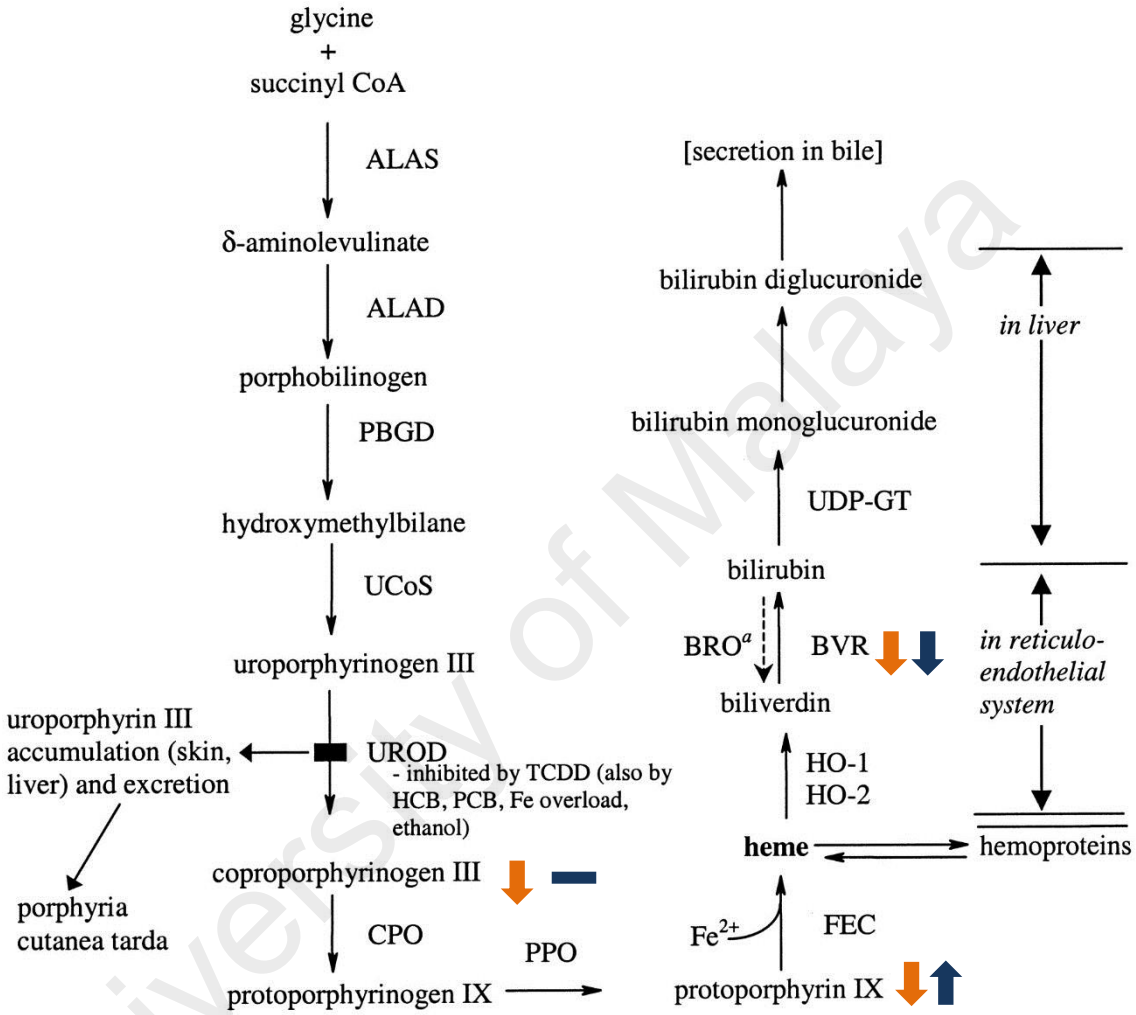
Studies also showed that the increase in blood concentrations of selected essential amino acids and their derivatives, in particular, branched-chain amino acids (BCAA), aromatic amino acids (Adams, 2011), and the decreases in the metabolism of essential fatty acids (linoleic and α-linoleic acids) and polyunsaturated fatty acids (such as eicosapentaenoic acid (EPA)) (Das, 2006), are believed to play an important role in the pathophysiology of several diseases including obesity. Correspondingly, in this

study, a cofactor involved in the metabolism of BCAA, isovaleryl-CoA, as well as numerous small molecules involved in aromatic amino acid metabolisms (tryptophan, tyrosine and phenylalanine metabolisms) were found to be largely perturbed following *H. pylori* eradication and these small molecules were all down-regulated at 18 months-post eradication, both pre- and post-prandially. The decrease in the metabolisms of BCAA and aromatic amino acids following *H. pylori* eradication could indirectly lead to the increase of the concentrations of these amino acids in the blood. The intermediates involved in the essential fatty acid metabolisms (linoleic and α-linoleic acids metabolism), both pre- and post-prandially, and the polyunsaturated fatty acid EPA, pre-prandially, were found to be down-regulated at 18 months post-*H. pylori* eradication as well. Therefore, these dysregulated fatty acid and amino acid metabolisms may be associated with the future onset of metabolic disorders.

Another important change in pre-prandial plasma metabolome following *H. pylori* eradication was the down-regulation of tetrapyrroles and derivatives that are involved in heme biosynthesis and bilirubin degradation. Coproporphyrinogen III, protoporphyrin IX, hematoporphyrin and harderoporphyrin are naturally occurring porphyrins, intermediates that play an important role in heme biosynthesis. Heme is critical also for the biological functions of several enzymes, such as cytochromes (Ajioka, Phillips, & Kushner, 2006). The down-regulation of porphyrins is another observation that may support our previous findings that eradication of *H. pylori* may affect the regulation of energy metabolism and hence, possibly lead to the development of metabolic disorders such as obesity (Yap et al., 2015) (see Section 5.4). However, such consistent pattern of expression of these metabolites was not observed in the post-prandial plasma metabolome probably due to the consumption of high protein test meal that changed the energy metabolism in the body.

The down-regulation of Biliverdin IX, which acts as the precursor of biosynthesis of bilirubin, as well as uribilinoids (Mesobilirubinogen and L-Urobilinogen) and urobilins (I-Urobilin and L-Urobilin), which are by-products of bilirubin degradation, both pre- and post-prandially, at 18 months post-eradication could be associated with the down-regulation of cellular antioxidant activity. Bilirubin is a lipophilic linear tetrapyrrole, abundant in blood plasma, which occurs uniquely in mammals. It is the final product of heme catabolism, as heme oxygenase (HO) cleaves the heme ring to form the water-soluble biliverdin, which is reduced by biliverdin reductase (BVR) to bilirubin (Baranano, Rao, Ferris, & Snyder, 2002). Studies have shown that bilirubin is a major physiologic antioxidant cytoprotectant (Baranano et al., 2002; Kapitulnik, 2004; Sedlak et al., 2009; Stocker, Yamamoto, McDonagh, Glazer, & Ames, 1987) which can protect cells from a 10,000-fold excess of H_2O_2 (Baranano et al., 2002; Sedlak et al., 2009). The down-regulation of biliverdin IX in plasma post-*H. pylori* eradication may indicate low concentration of bilirubin in tissues. Cellular depletion of bilirubin markedly increases tissue levels of reactive oxygen species and causes apoptotic cell death (Baranano et al., 2002; Sedlak et al., 2009). In addition, it has been reported that oxidative stress may play a crucial role in the pathogenesis of asthma. For example, Ohrui et al. reported a case of significant relief of asthma symptoms during jaundice (Ohrui, Yasuda, Yamaya, Matsui, & Sasaki, 2003). On the other hand, increasing evidence suggests that bilirubin also possesses immunomodulatory properties (Haga, Tempero, Kay, & Zetterman, 1996; Kirkby & Adin, 2006; Liu et al., 2008; Nejedlá, 1970; Větvička, Miler, Šíma, Táborský, & Fornůsbk, 1985) and may protect mammals against autoimmune diseases (Liu et al., 2008). Therefore, the down-regulation of Biliverdin IX and by-products of bilirubin following *H. pylori* eradication could be related to the negative association of *H. pylori* with asthma and allergy (Amedei et al., 2006; Amedei et al., 2010; Blaser, Chen, &

Reibman, 2008) and autoimmune diseases (Ram et al., 2013; Sawalha, Schmid, Binder, Bacino, & Harley, 2004) where *H. pylori* infection may have protective effect against some of the immunological disorders.



Indicator:

Pre-prandial changes at 18 months post-*H. pylori* eradication when compared to Baseline

Post-prandial changes at 18 months post-*H. pylori* eradication when compared to Baseline

*Up arrow indicates up-regulation; down arrow indicates down-regulation; dash indicates no significant changes

Figure 5.2: Changes in heme biosynthesis pathway and bilirubin degradation pathway at 18 months post-*H. pylori* eradication (adopted from (Niittynen et al., 2003))

Up-regulation of prequalene diphosphate, an intermediate involved in the cholesterol biosynthesis, was probably linked with the increment of bile acid level in the plasma post-*H. pylori* eradication. The synthesis of bile acids is the major pathway of cholesterol catabolism in human. Bile acids (BAs) are now discussed as metabolic integrators of whole-body energy homeostasis (Du, Virtue, Wang, & Yang, 2013). BAs can influence glucose and lipid metabolism through the activation of farnesoid X receptor (FXR), a member of the superfamily of ligand-activated nuclear receptor transcription factors, lowers plasma triglyceride (TAG) synthesis by a mechanism that may involve the repression of hepatic sterol regulatory element-binding protein (SREBP)-1c expression and/or the modulation of glucose-induced lipogenic genes (Lefebvre, Cariou, Lien, Kuipers, & Staels, 2009). Thus, BAs can be a potential biomarker of the development metabolic syndrome. Besides, it is also worthy to note that the marked changes of expression of BAs and TAG in plasma are another examples that indicate gut microbiota plays a role in blood metabolite profile in which studies have shown that intestinal bacteria play an important role in the metabolism of BAs (Cummings & Macfarlane, 1997b) and modulation of lipid metabolism (Velagapudi et al., 2010). In this study, two BAs, taurocholic acid and taurochenodeoxycholic acid were identified in pre-prandial plasma metabolome in which they were up-regulated 6 and 12 months post-*H. pylori* eradication but down-regulated at 18 months-post eradication. Consistently, majority of the TAG in our study were found to be down-regulated at 6 and 12 months post-*H. pylori* eradication (Appendix File I). However, at 18 months post-eradication, the bile acids in plasma were down-regulated instead, and strangely, majority of the plasma TAG were still down-regulated as compared to Baseline. Conversely, in post-prandial plasma metabolome, taurocholic acid and taurochenodeoxycholic acid were down-regulated at 12 and 18 months post-eradication and majority of the TAG were also down-regulated. Hence, the dysregulation of BAs

and TAG in the plasma could also be the indication of possibility of future onset of metabolic disorders.

The main bioactive sphingolipids includes ceramide, sphingosine, sphingosine 1-phosphate (S1P), and ceramide-1-phosphate (C1P) act as signalling molecules regulating a number of physiological events such as cell proliferation, apoptosis, and inflammation (Futerman & Hannun, 2004; Hannun & Obeid, 2008; Morad & Cabot, 2013). Ceramide is a major molecule in sphingolipid metabolism and a precursor of complex sphingolipids. S1P has strong proinflammatory properties. It activates neutrophils and macrophages and further induces mast cells degranulation. S1P also stimulates cyclooxygenase 2 (COX2), thus leading to production of inflammatory mediators (Takeuchi et al., 2006). It is known that colonisation of the stomach by *H. pylori* can result in chronic gastritis, an inflammation of the stomach lining, and in minority cases, it causes the development of peptic ulcers at the site of infection (Kusters et al., 2006). Eradicating *H. pylori* infection usually can permanently cure of most gastric and duodenal ulcers. In this study, following the eradication of *H. pylori*, the down-regulation of S1P, sphinganine 1-phosphate and phytosphingosine could probably be related to the reduced inflammation of the stomach lining after the cure of the ulcers in the stomach. These observations were probably complimentary with the down-regulation of phytosphingosine in post-eradication groups in faecal lipidomic analysis. It is important to note that we also observed significant changes of ceramides and other more complex sphingolipids such as glucosylceramides and lactosylceramide in the plasma but they did not display a consistent pattern of expression (Appendix I and J). Studies have shown that lipid metabolism in blood could be impacted by the changes of gut microbiota (Velagapudi et al., 2010). Thus, changes of expression of sphingolipids in this study may reflect another aspect of the influence of gut microflora on biochemical reactions in blood.

On filtering out exogenous plasma metabolites that were not produced by the human body, two hopanoids produced by *Proteobacteria* in the plasma, bacteriohopane-,32,33,34-triol-35-cyclitolguanine and 2-methyl-32,35-anhydrobacteriohopanetetrol, were detected, in both pre- and post-prandial plasma metabolome. These hopanoids may be correlated with the *Proteobacteria* hopanoid (32,35-anhydrobacteriohopaneterol) found in faecal lipidome. Similar to the hopanoid found in faecal lipidome, these two plasma hopanoids were also down-regulated post-*H. pylori* eradication (Appendix L). This observation was yet again another instance that probably indicates that the changes in the gut microbiota (local) impact the systemic changes of human body following eradication of *H. pylori*.

5.7 Significance of this study on the clinical management of patients

In this study, disappearance of *H. pylori* was found to lead to changes in the human gut microbiome resulting in local and systemic changes in metabolites that may contribute to eventual development of undesirable metabolic and immunological disorders. Although further research should be carried out to establish the mechanisms underlying the association of *H. pylori* with asthma and atopic as well as obesity and related illness, the findings in this study supported the recommendation by the Maastricht IV/Florence Consensus Report and the Second Asia-Pacific Consensus Guidelines that *H. pylori* eradication to prevent gastric cancer should only be undertaken in patients presenting with symptoms of dyspepsia or peptic ulcer disease, as well as those at high risk of developing gastric cancer (Malfertheiner et al., 2012).

5.8 Limitations of the study

One of the major limitations of this study was the high dropout rate (approximately 40-60%) of volunteers between 12 and 18 months post-eradication follow-up due to various reasons unrelated to the study. Volunteers who failed the first-line eradication therapy, as well as *H. pylori*-negative volunteers in the control group were also planned to be recruited into this study. However, some failed to recognise the benefit of joining the study and thus, both groups were not sufficiently populated to carry out a well-powered study. Moreover, the *H. pylori*-negative individuals are healthy individuals without gastrointestinal symptoms. Therefore it was not feasible and ethical to perform invasive gastroscopy, prescribe eradication treatment and follow them up to 18 months post-eradication. It is known that metabolic and immunological disorders are multifactorial diseases. Diet may be one of the factors that may influence the outcomes of the study. However, diet and calorie intake information was not included as part of the survey, thus another limitation of this present study. Some may suggest that comparison of gut microbiome of the *H. pylori*-positive volunteers post-eradication and gut microbiome of the healthy subjects deposited in the NIH human microbiome project database can be performed to ascertain whether the perturbation of gut microbiome that was observed in this study was due to *H. pylori* eradication. However, the study populations of the human gut microbiome in the database were heterogeneous (different geographical region, age group, ethnicity and medical history) which could be the confounding factors for comparison study.

CHAPTER 6: CONCLUSION

6.1 Conclusion

Faecal metagenomics and lipidomics enabled us to evaluate the local effects following *H. pylori* eradication on the gut microbiota whereas metabolic study, immunological study and plasma metabolomics reflected the systemic effects of *H. pylori* eradication. The local and systemic effects of *H. pylori* eradication and the proposed mechanisms underlying the association of *H. pylori* eradication with human metabolic and immunological disorders have been addressed in this study (Figure 6.1).

The stool metagenomic analysis in this study has shown that eradication of *H. pylori* caused perturbation of the gut microbiome and may indirectly affect the health of human. Perturbation of the gut microbiome, particularly the decrease in relative abundance of *Bacterioidetes* and the corresponding increase in *Firmicutes* after *H. pylori* eradication could be linked to obesity. The significant increase of SCFA-producing bacteria genera in the stool metagenomics study could also be associated with increased risk of metabolic disorders. SCFA have been reported to stimulate the release of hormone PYY and GLP-1 via activation of the G-protein-coupled receptor (Anini et al., 1999; Chambers et al., 2014; Cherbut et al., 1998; Hosseini et al., 2011; Tolhurst et al., 2012). Correspondingly, the metabolic study also indicates that *H. pylori* eradication was associated with long-term disturbance in active amylin, PP and total PYY both pre- and post-prandially and GLP-1 post-prandially. Perturbation of the gut microbiota may in turn interfere with the regulation of human energy homeostasis. An inverse association between *H. pylori* infection and allergen specific-IgE antibodies was found in immunological study, and hence, this study has demonstrated that *H. pylori* infection may confer protective effect against atopic disorders. Conversely, the levels of

cytokines IL-4 and TNF-α, which are associated with allergic diseases, were found to be otherwise.

Furthermore, non-targeted faecal lipidomics and plasma metabolomics revealed that eradication of *H. pylori* infection dramatically changed many global metabolite/lipid features, with the majority of metabolites being down-regulated. The influence of gut microbiota on the systemic changes of human body was demonstrated. The findings in this study primarily implicate the perturbation of gut microbiota following *H. pylori* eradication may affect the energy and lipid metabolism in human which eventually may lead to the development of metabolic disorders.

In conclusion, the predictive signatures of metabolic and immunological disorders following *H. pylori* eradication gave us insights into the intricate and complex interaction of *H. pylori* and gut microbiota in modulating human health. However, further research is needed to verify the findings in this study and investigate the protective effect of *H. pylori* against metabolic and immunological disorders Nevertheless, findings in this study supported the recommendation by the Maastricht IV/Florence Consensus Report and the Second Asia-Pacific Consensus Guidelines that *H. pylori* eradication to prevent gastric cancer should only be undertaken in patients presenting with symptoms of dyspepsia or peptic ulcer disease, as well as those at high risk of developing gastric cancer (Malfertheiner et al., 2012).

6.2 Suggestions for future research

By reason of resources and time constraints, the findings in this study could not be further verified. High throughput experimental approaches such as performing metagenomics analysis using whole genome shotgun sequencing, metatranscriptomic and targeted metabolomic/lipidomic studies with bigger sample size is required in the future research to verify the observations in this study and also to reveal the complex

gene repertoire of the gut microbiota and consequences of *H. pylori* eradication in modulating human health.

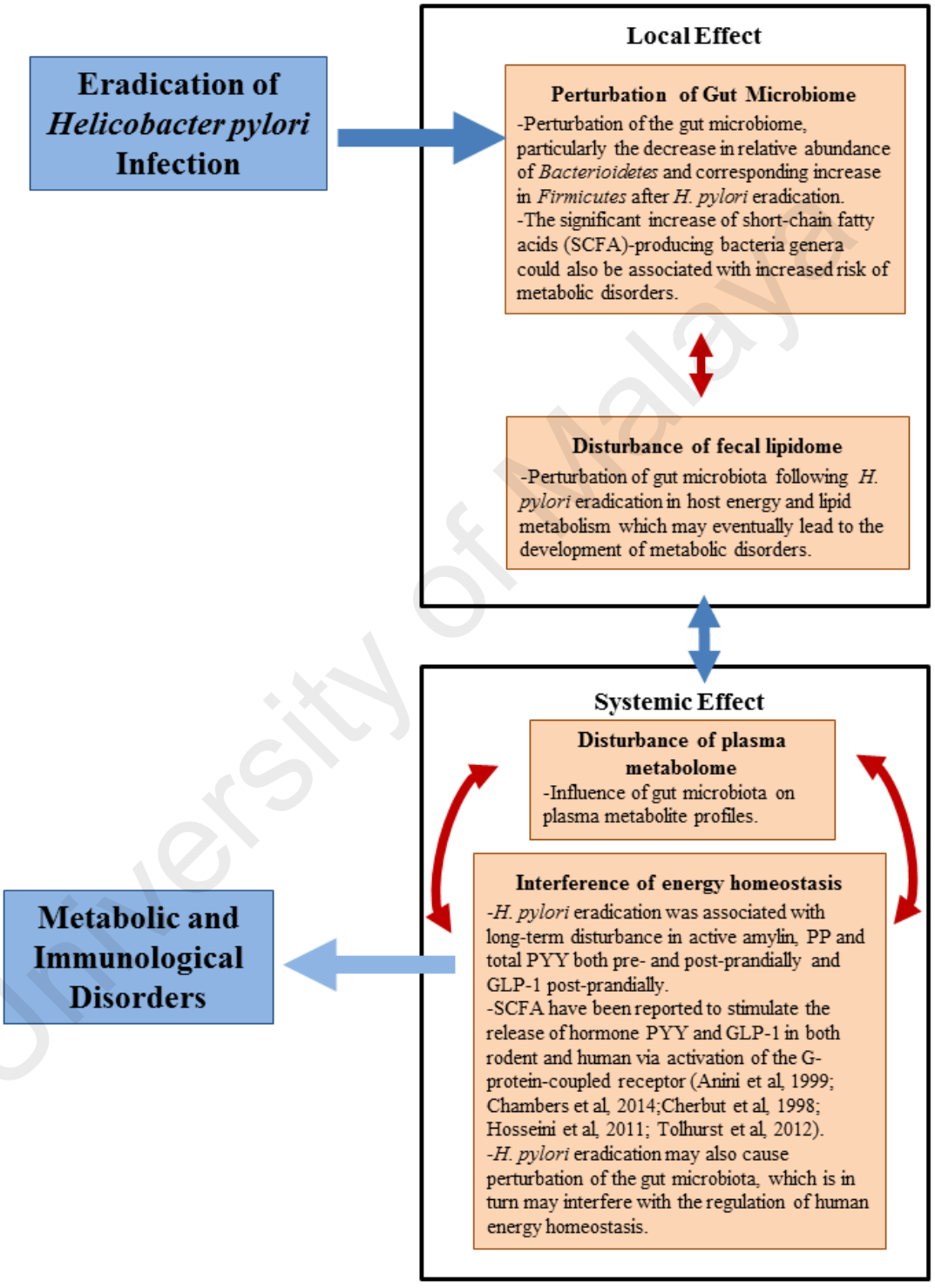


Figure 6.1: The local and systemic effects of *H. pylori* eradication

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PUBLICATONS

1. Yap, T. W.-C., Chan, W.-K., Leow, A. H.-R., Azmi, A. N., Loke, M.-F., Vadivelu, J., & Goh, K.-L. (2015). Prevalence of serum celiac antibodies in a multiracial Asian population-a first study in the young Asian adult population of Malaysia. *PLoS One, 10*(3), e0121908. doi:10.1371/journal.pone.0121908

2. Yap, T. W.-C., Leow, A. H.-R., Azmi, A. N., Francois, F., Perez-Perez, G. I., Blaser, M. J., . . . Vadivelu, J. (2015). Changes in metabolic hormones in Malaysian young adults following *Helicobacter pylori* eradication. *PLoS One, 10*(8), e0135771. doi:10.1371/journal.pone.0135771

3. Yap, T. W.-C., Gan, H.-M., Lee, Y.-P., Leow, A. H.-R., Azmi, A. N., Francois, F., . . . Vadivelu, J. (2016). *Helicobacter pylori* eradication causes perturbation of the human gut microbiome in young adults. *PLoS One, 11*(3), e0151893. doi:10.1371/journal.pone.0151893

4. Yap, T. W.-C., Leow, A. H.-R., Azmi, A. N., Callahan, D. L., Francois, F., Perez-Perez, G. I., Loke, M.-F., Goh, K.-L. & Vadivelu, J. Global fecal and plasma metabolite dynamics associated with eradication of *Helicobacter pylori*. *Microbiome* (submitted).

5. Yap, T. W.-C., Leow, A. H.-R., Azmi, A. N., Callahan D.L., Perez-Perez, G. I., Loke, M.-F., Goh, K.-L. & Vadivelu, J. (2017). Global fecal and plasma metabolic dynamics related to *Helicobacter pylori* eradication. *Frontiers in Microbiology*, 8: 536. doi: 10.3389/fmicb.2017.00536

CONFERENCE PAPER

Leow, A. H.-R., Yap, T. W.-C., Azmi, A. N., Loke, M. F., Vadivelu, J. , Goh, K. L. (2013). Differential rates of disappearance of *Helicobacter pylori* in young multiethnic population in Malaysia. *J Gastroenterol and Hepatol* 28(3):23-693.

INTERNATIONAL WORKSHOP/SHORT COURSE

1. 7th HKU-Pasteur Immunology Course, Hong Kong University, 20 November-12 December 2014.

2. SLING Training Course "i c lipid"- Immersion Course into Mass Spectrometry based Lipidomics, National University of Singapore, 6-10 May 2013.

AWARDS

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