

RESEARCH ARTICLE

Helicobacter pylori Eradication Causes Perturbation of the Human Gut Microbiome in Young Adults

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Abstract

Background

Accumulating evidence shows that *Helicobacter pylori* protects against some metabolic and immunological diseases in which the development of these diseases coincide with temporal or permanent dysbiosis. The aim of this study was to assess the effect of *H. pylori* eradication on the human gut microbiome.

Methods

As part of the currently on-going ESSAY (Eradication Study in Stable Adults/Youths) study, we collected stool samples from 17 *H. pylori*-positive young adult (18–30 years-old) volunteers. The same cohort was followed up 6, 12 and 18 months-post *H. pylori* eradication. The impact of *H. pylori* on the human gut microbiome pre- and post-eradication was investigated using high throughput 16S rRNA gene (V3-V4 region) sequencing using the Illumina Miseq followed by data analysis using Qiime pipeline.

Results

We compared the composition and diversity of bacterial communities in the fecal microbiome of the *H. pylori*-positive volunteers, before and after *H. pylori* eradication therapy. The 16S rRNA gene was sequenced at an average of 150,000–170,000 reads/sample. The microbial diversity were similar pre- and post-*H. pylori* eradication with no significant differences in richness and evenness of bacterial species. Despite that the general profile of the gut microbiome was similar pre- and post-eradication, some changes in the bacterial

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communities at the phylum and genus levels were notable, particularly the decrease in relative abundance of *Bacteroidetes* and corresponding increase in *Firmicutes* after *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.

Conclusions

Our preliminary stool metagenomics study shows that 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 regimen and be cautious in the clinical management of *H. pylori* infection, particularly in immunocompromised patients.

Introduction

The human body routinely harbors approximately 100 trillion bacteria which outnumber our human cells by 10 to 1 [1]. This bacterial population constitutes the microbiota and the majority of them living in the lower part of gastrointestinal tract. The human gut microbiota is an intricate and dynamic ecosystem that has coevolved with human for millions of years [2]. It has been regarded as a metabolically active “organ” located within the human gastrointestinal tract [3, 4] as it has developed metabolic traits that complement host’s metabolism [5–8]. A healthy gastrointestinal system relies on a balanced commensal biota to regulate processes such as energy metabolism [9–12], elimination of pathogens [13–15] and influences the signaling pathways that range from modulation of the mucosal immune response [16] to development of metabolic diseases [9–11, 17, 18]. 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 [13, 19].

Helicobacter pylori is a Gram-negative, spiral-shaped, microaerophilic bacterium that colonizes the gastric mucosa of humans and non-human primates naturally. More than half of the world’s population is infected with *H. pylori* and its prevalence is as high as 80% in some populations [20]. In a minority of those infected, *H. pylori* can cause peptic ulcers (10%), gastric cancer (1–2%), and rarely mucosa-associated lymphoid tissue (MALT) lymphoma [21]. *H. pylori* is believed to colonize the stomach of humans at least since the initial migration of our ancestors from East Africa approximately 60,000 years ago [22]. It has been postulated that *H. pylori* may be part of the human indigenous microbiome. However, due to socioeconomic development, modern hygienic practices and the advent of the antibiotics, the human gut microbiota is changing and *H. pylori* is gradually disappearing. There is an increasing number of epidemiological and experimental evidence for the protective effect of *H. pylori* infection on the development of obesity [23–25], childhood asthma [26], allergies [27], inflammatory bowel diseases [28], in which the development of these diseases coincide with either temporal or permanent dysbiosis.

Thus, in this study, we hypothesize that the eradication of *H. pylori* may cause perturbation of the GI microbiome, which can indirectly affect the health of human host. We investigated the effect of *H. pylori* eradication on the human gut microbiome using *16S rRNA* gene amplicon metagenomics sequencing up to 18 months post-eradication.

Materials and Methods

Study Population

Our study is part of the currently on-going ESSAY (Eradication Study in Stable Adults/Youths) study in Malaysia, New York and an European Center. In Malaysia, it was conducted at the University of Malaya Medical Centre (UMMC) between June 2012 and May 2014. Healthy young adults between the ages of 18–30 years old were first screened to assess study eligibility. The exclusion criteria for the study were diabetes, hyper or hypothyroidism, prior gastric or bariatric surgery, prior documented treatment of *H. pylori*, antibiotic use within 4 weeks of enrollment, steroid or other immunomodulating drugs use within 4 weeks of enrollment, recent vaccination and Charlson weighed comorbidity index <2. The study protocol was reviewed and approved by Medical Ethics Committee at UMMC (Ref No. 877.1). Written informed consent was obtained from qualified volunteers prior to study participation. *H. pylori*'s status of the qualified candidates was determined as previously described [29].

Sample Collection

As we reported earlier, 57 (9.9%) of the 573 volunteers screened in the ESSAY study were tested positive for *H. pylori* using both non-radioactive ¹³C Urea Breath Test (UBT) and detection of anti-*H. pylori* antibodies and were considered as *H. pylori*-positive. However, only 32 agreed and consented to participate in the study [29]. Of these 32 *H. pylori* positive volunteers, we only managed to collect stool samples from 17 of them. Treatment with a 7-day twice daily regimen and a proton pump inhibitor as current standard of care (amoxicillin 1000 mg, clarithromycin 500 mg, and pantoprazole 40 mg) was given to the volunteers and approximately 6 weeks after completing the treatment protocol, *H. pylori* eradication were ascertained using UBT. The volunteers were subsequently followed up at 6, 12, and 18 months post-*H. pylori* eradication. Stool samples were collected during each visit and frozen immediately at -80°C until DNA extraction.

Nucleic Acid Extraction from Stool Samples

Nucleic acid extraction was done using MoBio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). 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 [30]. Instead of pre-processing the stool specimen with MoBio lysis buffer, approximately 100–200 mg of stool sample was added directly into PowerBead Tubes and vortexed to dispense the sample. The subsequent steps in the manufacturer's protocol were followed accordingly.

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'-NNNNCTACGGGAGGCAGCAG-3') and 1061R (5'-GACTACHVGGGTA TCTAATCC-3') containing the complete Illumina adapter [31, 32].

Briefly, each 50 µL of polymerase chain reaction (PCR) reaction contains 10 ng of fecal 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 following protocol: (1) for the stool samples, an initial denaturation step performed at 98°C for 30 sec followed by 30 cycles of denaturation (98°C, 10 s), annealing (60°C, 10 s) and

extension (72°C, 30 sec), 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) located at the Monash University Malaysia Genomics Facility.

Bioinformatics Analysis

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 [33]. The trimmed paired-end reads were subsequently overlapped using PEAR: Illumina Paired-End reAd mergeR (default setting) [34].

Analysis of quality filtered reads using Qiime. The merged paired-end reads were analyzed using the Quantitative Insights into Microbial Ecology (Qiime) [35] 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 [36] (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 [37], 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 [38] was used to align the OTU representative sequences against the Greengenes core reference alignment [39] with a minimum identity of 75%, and then a phylogenetic tree was built using FastTree [40]. Finally we generated a OTU table (*biom summarize-table*) for downstream diversity analysis by excluding the sequences that had failed to align by PyNAST. We also used the generated OTU table to summarize microbiome communities by taxonomic levels (by default: phylum, class, order, family, genus) based on different time-points (*summarize_taxa_through_plots.py*).

Statistical methods. The raw data of the taxonomy summary results (.txt file) were exported to SPSS software version 20.0 (SPSS Inc., Chicago, IL) 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. Pearson's Correlation Coefficient was also performed to investigate the relationship between the phylum *Bacteroidetes*-to-*Firmicutes* ratio and the Body Mass Index (BMI) of the subjects across different time-points; a two-tailed p-value of < 0.05 was considered significant.

Diversity analysis. We evaluated samples for alpha diversity (microbial diversity within samples) and beta diversity (community diversity between samples) analysis 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. For our study, the rarefaction depth value for the comparison of Baseline and 6 months post-eradication was set as 82,536, the rarefaction depth value for the comparison of Baseline and 12 months post-eradication was set as 84,177, whereas the rarefaction depth value for the comparison of Baseline and 18 months post-eradication was set as 84,333. The alpha diversity was then calculated using both “non-phylogeny-based” (observed species, chao1, Shannon index) and “phylogeny-based” (PD whole tree) matrices for each rarefied OTU table. We compared the alpha diversity between different groups (time-points) of the samples by non-parametric two-sample t-test (*compare_alpha_diversity.py*).

Beta diversity between our samples was calculated using the default beta diversity metrics of weighted and unweighted UniFrac [41] (*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 [42]. 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 and Baseline vs. 12 months eradication).

Results

Demographics of the Study Cohort

Stool samples from 17 *H. pylori*-positive healthy young Malaysian with a mean age of 25 years were collected. The volunteers were followed up after they were given *H. pylori* eradication therapy. Only 17, 10, and 6 stool samples were successfully collected at 6, 12, and 18 months post-eradication, respectively. All the 16S rRNA sequences were deposited in MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST) under the accession numbers as shown in [Table 1](#).

Characterization of Stool Microbiome

A total of 5,834,726 quality-filtered reads were obtained from Baseline vs. 6 months post-eradication group with an average of $171,610 \pm 58,372$ (standard deviation, SD) reads per sample, whilst, a total of 3,077,037 quality-filtered reads were obtained from Baseline vs. 12 months post-eradication group with an average of $153,852 \pm 32,306$ reads per sample. For Baseline vs. 18 months post-eradication group, a total of 2,000,505 quality-filtered reads with an average of $166,709 \pm 69,793$ reads per sample were obtained. 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 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. We used several “phylogeny-based” and “non-phylogeny-based” matrices to calculate alpha diversity, including PD whole tree, chao1,

Table 1. 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
Accession number	Time-point	Sample code
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
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
Accession number	Time-point	Sample code
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

(Continued)

Table 1. (Continued)

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

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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 (Fig 1). 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 (Figs 2 and 3). 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 (S1 & S2 Figs). Non-parametric statistical test analysis of similarity (ANOSIM) also showed that the differences in bacterial 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 summarized in Fig 4 and Fig 5. 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%) (Fig 1A). 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

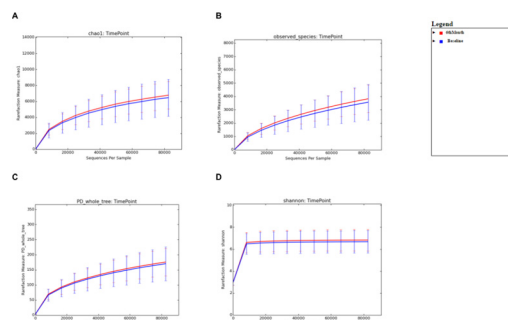


Fig 1. Alpha diversity analysis comparing between baseline and 6-months post-*H. pylori* eradication. Rarefaction curve for **A.** chao1, **B.** observed species, **C.** PD whole tree, and **D.** the Shannon index generated from alpha diversity analysis.

doi:10.1371/journal.pone.0151893.g001

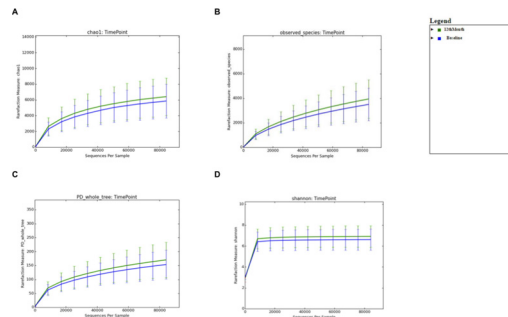


Fig 2. Alpha diversity analysis comparing between baseline and 12-months post-*H. pylori* eradication. Rarefaction curve for **A.** chao1, **B.** observed species, **C.** PD whole tree, and **D.** the Shannon index generated from alpha diversity analysis.

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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 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%) 6 months post-eradication (Fig 1B). 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 (Fig 1C). 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 (Fig 1D) (Table 2). 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

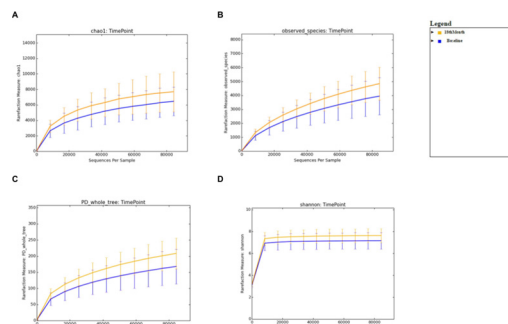


Fig 3. Alpha diversity analysis comparing between baseline and 18-months post-*H. pylori* eradication. Rarefaction curve for **A.** chao1, **B.** observed species, **C.** PD whole tree, and **D.** the Shannon index generated from alpha diversity analysis.

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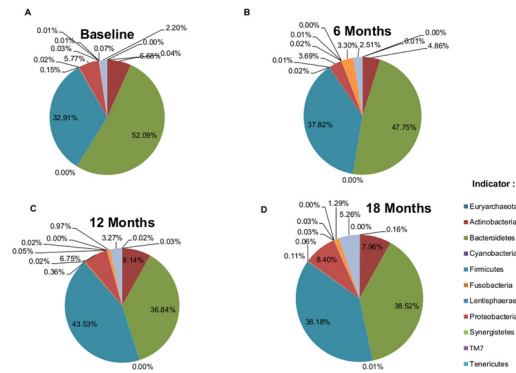


Fig 4. Relative abundance of phyla. Relative abundance of phyla at **A.** Baseline, **B.** 6 months-post eradication, **C.** 12 months-post eradication, and **D.** 18 months-post eradication.

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across time were not statistically significant (Paired-samples t-test, $p \geq 0.05$). The *Bacteroidetes*-to-*Firmicutes* ratio were also not significantly correlated with BMI of the volunteers across different time-points (Person's correlation, $p \geq 0.05$).

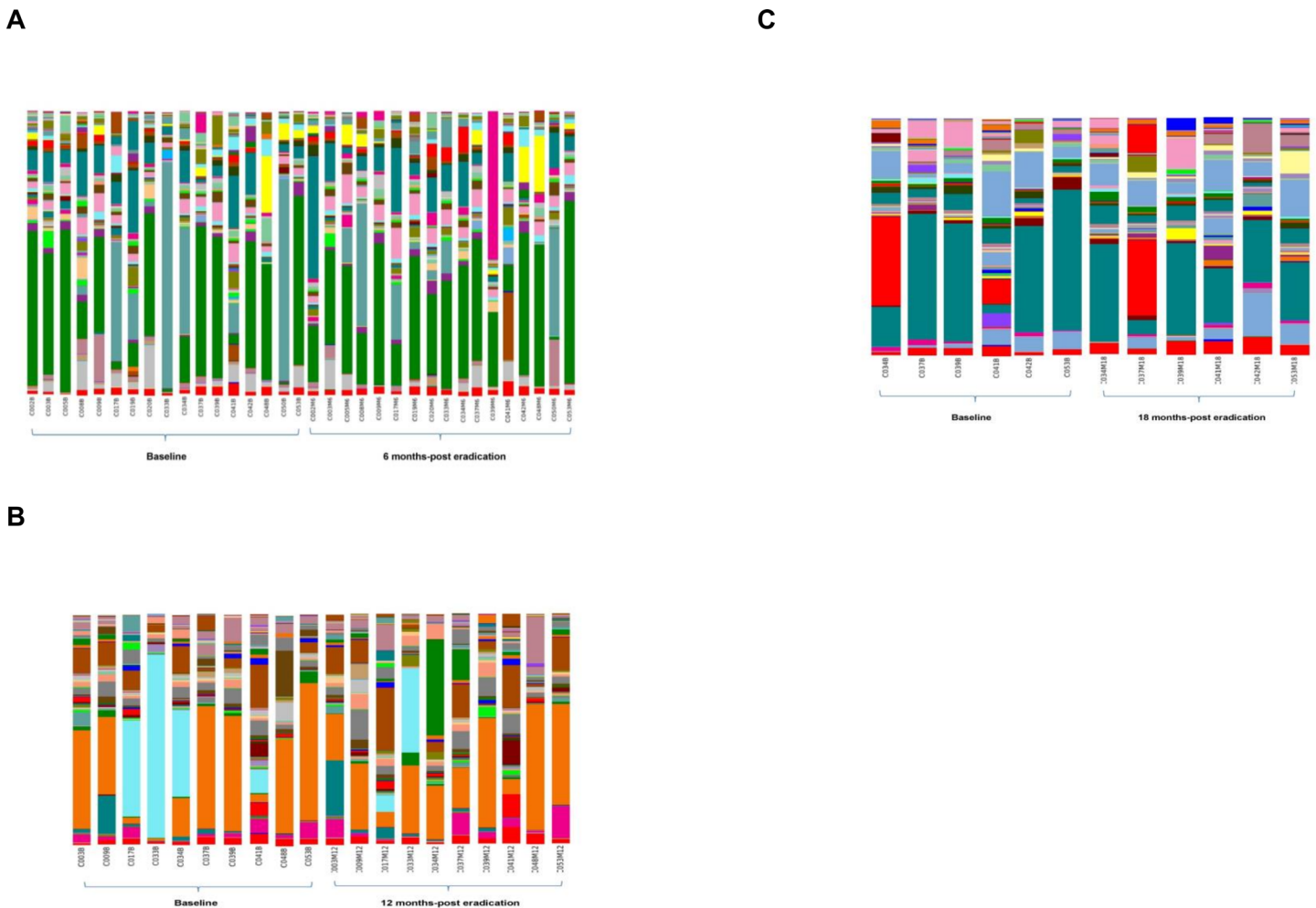


Fig 5. Relative abundance of genera. Comparison of the relative abundance of genera between **A.** Baseline vs. 6 months-post eradication, **B.** Baseline vs. 12 months-post eradication, and **C.** Baseline vs. 18 months-post eradication.

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Table 2. Comparison of Relative Abundance of Phyla of the Gut Microbiome Pre- and Post-*H. pylori* Eradication.

Phylum	Mean relative abundance (%)				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
	(95% CI [*])						
	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
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
Phylum	Mean relative abundance (%)				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
	(95% CI [*])						
	Baseline	6 Months-post eradication	12 Months-post eradication	18 Months-post eradication			
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.

^aPaired-Samples T Test was used in comparing baseline and post-*H. pylori* eradication.

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Table 3. 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	
<i>Anaerofustis</i>	7.70E-4% (1.81E-4%-1.43E-3%)	1.87E-3% (9.17E-4%-2.91E-3%)	0.016
<i>Phascolarctobacterium</i>	0.72% (0.29%-1.26%)	1.76% (0.82%-3.05%)	0.038
<i>Ruminococcus</i> (Family: <i>Lachnospiraceae</i>)	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.

^aPaired-Samples T Test was used in comparing baseline and post-*H. pylori* eradication.

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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 3). 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). *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

Table 4. 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	
<i>Dialister</i>	0.20% (0.06%-0.38%)	0.71% (0.33%-1.13%)	0.033
<i>Agrobacterium</i>	4.34E-4% (1.40E-4%-7.81E-4%)	0%	0.031
<i>Helicobacter</i>	0%	2.17E-4% (4.10E-5%-3.94E-4%)	0.041
(<i>H. pylori</i>)	0%	2.17E-4% (5.80E-5%-4.05E-4%)	0.041

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

^aPaired-Samples T Test was used in comparing baseline and post-*H. pylori* eradication.

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Table 5. Comparison of Relative Abundance of Genera of the Gut Microbiome at Baseline and 18 Months-Post *H. pylori* Eradication.

Genus (species)	Mean relative abundance		Comparison of relative abundance at baseline and 18 months post-eradication (p-value) ^a
	(95% CI*)		
	Baseline	18 Months-Post Eradication	
<i>Helicobacter</i>	0%	5.90E-4% (2.37E-4%-9.27E-4%)	0.033
(<i>H. pylori</i>)	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.

^aPaired-Samples T Test was used in comparing baseline and post-*H. pylori* eradication.

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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 5).

To examine for the presence of enterohepatic *Helicobacter* species (EHS), we used the generated OTU table to further summarize microbiome communities up to species level. The generated 16S rRNA data showed that the only *Helicobacter* species detected was *H. pylori* (Table 4, Table 5 and S1 File)

Discussion

To our knowledge, the effect of *H. pylori* eradication on the gut microbiome has 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. We performed diversity analysis 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, most bacterial species in the human and mouse gut was dominated by phyla *Bacteroidetes* and *Firmicutes* [43]. Less abundant bacteria phyla such as *Actinobacteria*, *Proteobacteria*, *Verrucromicrobia*, and as well as *Euryarchaeota* (mainly methanogenic archae *Methaobrevibacter smithii*) were also present [44, 45]. 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 [46].

Despite that the general profile of the gut microbiome was similar pre- and post-*H. pylori* eradication, our metagenomics study revealed some changes in the bacterial communities at the phylum and genus levels that are notable. Twelve 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 are growing evidences that indicated the inverse relationship between *H. pylori* prevalence and rate of overweight/obesity. Hence, the gradual decrease of the *H. pylori* colonization that has been observed in recent decades could be causally related to the human epidemic obesity [47]. 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* [43]. Study in human 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 normalized to the level observed in lean individuals [48]. *Bacteroidetes* and *Firmicutes* have been associated with the regulation of lipid and bile acid metabolism as well as energy homeostasis in host [49, 50]. Essentially, it has been demonstrated that perturbations of bile acid-mediated signaling pathway influence risk of metabolic complications such as obesity and diabetes [51]. 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 feces 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 [52]. Correspondingly, in our study, the relative abundance of 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* [53, 54]. It was reported that high-level colonization of the human gut by *Verrucomicrobia* following broad-spectrum antibiotic treatment [55]. 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 seems to be restoring to the Baseline level.

Another interesting finding was observed for phylum *Proteobacteria*. The relative abundance of *Proteobacteria* decreased 6 months post-*H. pylori* eradication but then it increased to even higher than the Baseline state at 12 and 18 months post-eradication. This finding may correlate with our observation at the genus level of the gut microbiome where *Helicobacter* was found 12 and 18 months post-eradication but not during Baseline and 6 months post-eradication. It has been reported that besides *H. pylori*, EHS can also colonize the mucosal surfaces of the intestinal tract and/or the liver of humans, mammals and birds [56]. Our study showed that the *Helicobacter* species detected in the stool samples at 12 and 18 months post-eradication was 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 [36]. 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 [57].

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. *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 [58–63]. 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 [63]. All of these studies showed that *H. pylori* may be a facultative intracellular organism [64, 65]. When the *H. pylori*-positive volunteers were given *H. pylori* eradication therapy, most of the *H. pylori* colonized on 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, metabolizing 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 [63]. 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 utilized by *H. pylori* [66]. It is likely that *H. pylori* utilizes the advantage of 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, we performed proteomics analysis 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 (S1 Table) confirming the presence of the bacterium and their viability. In addition, we also managed to call back the volunteers (whom stool samples were detected with *Helicobacter* 16S rRNA) for UBT at 18 months post-*H. pylori* eradication. However, all of them were found to be 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 [67] as they produced low level of urease as compared to spiral forms [68, 69]. These may explain the negative UBT results of these volunteers despite detection of *H. pylori* DNA and proteins in their stool samples. Our finding has also shown that there is a possibility of *H. pylori* can be shed through feces and supported the notion that *H. pylori* may be transmitted through fecal-oral route via contaminated water or food [70].

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 feces. It was found to produce acetate and butyrate as end products of glucose fermentation [71]. 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 [72] 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 [19] through fermentation; the presence of SCFA is believed to be associated with reduced inflammation [73] and has an important effect on colonic health [74, 75]. At 12- & 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 bacteria genus, *Phascolarctobacterium*, also showed significant increment 6 months-post eradication. *Phascolarctobacterium* is also a genus of *Firmicutes* bacteria classified within the class of *Clostridia*. *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 specializing to utilize the succinate generated by other

bacterial species to produce propionate [76], which may also act as a health-promoting microbial metabolite in the human gut [77] post-*H. pylori* eradication to aid in the restoration of the perturbed microbiome. 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 [78–80]. Of the SCFA produced by colonic fermentation of dietary fibre, propionate has the highest affinity for FFAR 2 [81, 82]. 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 [83]. In a study reported recently by our group has also shown that *H. pylori* eradication was associated with long term elevation of active amylin, PYY, and GLP-1 in the serum [29]. 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 [84]. Hence, there is a possibility that eradication of *H. pylori* may cause dysbiosis 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 [85, 86]. Currently, the genus *Ruminococcus* is divided into two phylogenetically separate groups which are categorized under two separate families *Ruminococcaceae* [87] and *Lachnospiraceae* [88] with numerous misclassified *Ruminococcus* species [89]. 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 [90]. Candidate division *TM7* is found in a diverse range of environment habitats [90–93] and human body sites [93–97]. 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 [93, 94]. However, we could not elucidate the effect of *H. pylori* eradication on these organisms in relation to health diseases because they have been uncultivable, with no pure-culture representatives.

In addition to *Helicobacter*, genera *Dialister* and *Agrobacterium* were also found to have changed significantly 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 12 months-post eradication.

Our 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. Nonetheless, high throughput experimental approaches such as whole genome shotgun sequencing and metatranscriptomics with bigger sample size is required to verify the observation of this study and also to reveal the complex gene repertoire of the gut microbiome and consequences of *H. pylori* eradication in modulating human health.

Supporting Information

S1 Fig. 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. (PDF)

S2 Fig. 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.

(PDF)

S1 File. Relative abundance of species of the gut microbiome at Baseline, 6, 12 and 18 Months-Post *H. pylori* eradication.

(XLSX)

S1 Table. List of proteins identified in the four stool samples of 12 months post-*H. pylori* eradication.

(PDF)

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Author Contributions

Conceived and designed the experiments: FF GPP. Performed the experiments: TWCY HMG YPL. Analyzed the data: TWCY. Contributed reagents/materials/analysis tools: JV. Wrote the paper: TWCY HMG FF GPP MFL KLG JV. Carried out the recruitment of volunteers: TWCY AHRL ANA. Carried out the clinical analysis: AHRL KLG. Helped to supervise the laboratory works: MFL JV.

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