Research Paper

The impact of *Helicobacter pylori* infection, eradication therapy and probiotic supplementation on gut microenvironment homeostasis: An open-label, randomized clinical trial

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ABSTRACT

Background: Helicobacter pylori (*H. pylori*) infection is associated with remodeling of gastric microbiota. However, comprehensive analyses of the impact of *H. pylori* infection, eradication therapy and probiotic supplementation on gut microbiota are still lacking. We aimed to provide evidence for clinical decision making.
Methods: Seventy *H. pylori*-positive and 35 *H. pylori*-negative patients (group C) were enrolled. *H. pylori*-positive patients were randomly assigned to group A (14-day bismuth-containing quadruple therapy) and group B (quadruple therapy supplemented with *Clostridium butyricum*). Stool samples of group A and B were collected on day 0, 14 and 56 while stool samples of group C were collected on day 0. Gut microbiota was investigated by 16S rRNA sequencing.
Findings: The Sobs index (richness estimator) was significantly higher in *H. pylori*-positive samples than *H. pylori*-negative samples ($p < .05$). Several metabolic pathways were more abundant in *H. pylori*-positive communities while some disease-associated pathways had higher potential in *H. pylori*-negative community through KEGG pathway analysis. Abundances of most butyrate-producing bacteria significantly decreased, while several detrimental bacteria increased after eradication therapy. Probiotic supplementation was associated with improved gastrointestinal symptoms as well as increased *Bacteroidetes:Firmicutes* ratio.
Interpretation: While *H. pylori* infection may not be necessarily detrimental in all patients, eradication of *H. pylori* was associated with widespread changes in gut microbial ecology and structure. Probiotic supplementation could relieve more gastrointestinal symptoms by inducing alterations in gut microbiota and host immune responses. As such, the decision to eradicate *H. pylori* should be based on comprehensive analysis of individual patients.

1. Introduction

An estimated 4.4 billion individuals were infected with *Helicobacter pylori* (*H. pylori*) worldwide in 2015 [1]. In China, the prevalence of *H. pylori* infection was 66% among rural populations and 47% in urban settings [2]. In infected patients, *H. pylori* is the dominant bacterial species in the gastric microbiota [3]. Infection with *H. pylori* can inhibit gastric acid secretion, induce chronic inflammation of gastric mucosa, and thereby change the gastric microenvironment leading to widespread changes in gastric microbial community [4,5]. In addition, alterations in gut microbiota are associated with a range of gastrointestinal and systemic diseases [6]. Although the stomach has been reported as the exclusive habitat for *H. pylori* [7], it has been detected through 16S rRNA sequencing in stool samples albeit with low relative abundance [8]. Moreover, animal studies have indicated that *H. pylori* results in distinct shifts in gut microbiota in uninflamed distal parts of the gastrointestinal tract but not in the stomach [9]. Similarly in human studies using fluorescence in situ hybridization, fecal samples from *H. pylori*-

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Research in context

Evidence before this study

Infection with *H. pylori* can lead to widespread changes in gastric microbial community. Animal studies have indicated that *H. pylori* results in distinct shifts in gut microbiota in uninflamed distal parts of the gastrointestinal tract but not in the stomach. Similarly, in human studies using fluorescence in situ hybridization, fecal samples from *H. pylori*-infected individuals showed a decrease in abundance of clostridia as well as total anaerobes compared with *H. pylori*-negative individuals.

Approximately 20% of *H. pylori*-infected individuals go on to develop complications. A study in Japan reported an increase in *Bacteroidetes*, *Firmicutes*, and B:F ratio together with a decrease in *Proteobacteria* immediately after eradication treatment.

Preclinical data suggest that a butyrate-producing probiotic, *Clostridium butyricum*, shows promise in treating *H. pylori*. However, data from human studies were mixed. In addition, these studies were conducted with standard triple eradication therapy, which is likely inadequate in most Asian populations.

Added value of this study

Our results suggest that the nitrate-nitrite-NO pathway may play an important regulatory mechanism in pathologic conditions and may be protective against *H. pylori*. Relative abundance of 19 disease-associated or metabolic pathways were significantly different between *H. pylori*-negative and *H. pylori*-positive patients by KEGG pathway analyses.

Abundances of most butyrate-producing bacteria significantly decreased, and several detrimental bacteria increased immediately after therapy. Longitudinal studies are required to determine the consequences of antibiotic-induced disruption of the gut microbiota.

Supplementation with *C. butyricum* could relieve gastrointestinal symptoms by inducing alterations in gut microbiota and host immune responses.

Implications of all the available evidence

The interactions between *H. pylori*, gut microbiota, and host function are complex. The role *H. pylori* plays in human disease and gut microenvironment homeostasis may not be necessarily detrimental.

The decision to eradicate and application of probiotics should be based on comprehensive analyses of individual patients.

infected individuals showed a decrease in abundance of clostridia as well as total anaerobes compared with *H. pylori*-negative individuals [10]. These observations suggest that infection with *H. pylori* leads to widespread changes in host microbial structure and function likely through alterations in the gastric microenvironment. Despite its widespread prevalence, potential for inducing chronic sequelae, and impact on host-microbe interactions, the interactions between *H. pylori*, gut microbiota, and host function are still largely unknown.

According to the Kyoto global consensus report, patients diagnosed with *H. pylori* infection should receive eradication therapy to minimize risk of long-term sequelae, including peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [11]. However, current evidence indicates that eradication with *H. pylori* is associated with major disturbances of the intestinal microbiota. This included a decrease in bacterial diversity as well as reductions

in number of *Bifidobacteria*, *Lactobacilli*, and butyrate producers, such as *Faecalibacterium prausnitzii* [10]. Another study documented that these changes may persist for up to four years after antibiotic treatment is completed [12]. Given that only approximately 20% of *H. pylori*-infected individuals go on to develop complications [13] and the accumulating evidence showing the potential harm of antibiotic administration, there is a pressing need to more fully elucidate the complex *H. pylori*-gut microbiota-host interactions to identify those subjects most at risk for long-term sequelae.

Moreover, supplementation of certain probiotics may have a positive effect on *H. pylori* eradication by immunological and non-immunological mechanisms [14]. A meta-analysis of 14 randomized trials demonstrated that probiotics increased eradication rate (OR 1.84, 95% CI 1.34–2.54) while decreasing adverse events (OR 0.44, 95% CI 0.30–0.66) [15]. Butyrate is a major short chain fatty acid produced by bacterial fermentation of dietary fibers. In addition to being a major energy source for colonocytes, butyrate has been shown to promote mucosal homeostasis likely through beneficial effects on innate and adaptive immune cells as well as epithelial barrier function [16,17]. Butyrate may also have bactericidal effects against *H. pylori*. In vitro studies using butyrate as well as supernatants from butyrate-producing bacteria inhibited growth and were associated with destructive effects on the cell envelope of *H. pylori* [18]. Preclinical data suggest that a butyrate-producing probiotic, *Clostridium butyricum* (*C. butyricum*), shows promise in treating *H. pylori* [19]. However, data from human studies were mixed [20,21]. This may be partially explained by prior studies emphasizing the effect of *C. butyricum* on eradication rate and side effects. In addition, these studies were conducted with standard triple eradication therapy, which is likely inadequate in most Asian populations. Meanwhile, studies of gut microbial alterations were performed using culture-based rather than with more modern metagenomic approaches.

As such, we aimed to comprehensively investigate the impact of *H. pylori* infection, 14-day bismuth-containing quadruple therapy (BQT) and probiotic supplementation on gut microbial homeostasis.

2. Materials and methods

2.1. Patients

This open-label, prospective clinical trial was performed at Sir Run Run Shaw Hospital, Zhejiang Province, China from December 2016 to August 2017. Patients aged between 18 and 70 years were enrolled. Patients were randomly assigned to treatment groups if they were diagnosed as *H. pylori*-positive gastritis in the past month by esophagogastroduodenoscopy and histological examination. Subjects who tested negative for *H. pylori* by esophagogastroduodenoscopy as well as ^{13}C urea breath test were recruited as controls. Exclusion criteria included prior history of treatment for *H. pylori* infection; confirmed or suspected upper gastrointestinal malignant tumor; peptic ulcer or other upper gastrointestinal lesions; the use of antacids or gastric mucosal protective agents in the past two weeks; the use of antibiotics or probiotics in the past month; known allergy to drugs in this study; other known gastrointestinal diseases; history of gastrointestinal surgery; decompensated cardiac, liver, renal, or pulmonary illness; thyroid disease or diabetes mellitus; pregnant and lactating women; and subjects who could not provide informed consent. This study was approved by the Ethics Committee of Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University (20161206-21) and registered at Chinese Clinical Trial Registry (Chictr.org.cn, ChiCTR-IPR-16010286). Written informed consent was obtained from all participants before enrollment.

2.2. Study design

A total of 70 *H. pylori*-positive patients and 35 *H. pylori*-negative patients were enrolled. *H. pylori*-positive patients were randomly assigned to group A and group B. Randomization sequence was generated by a

computer algorithm. Patients in group A received 14-day BQT consisting of pantoprazole 40 mg, amoxicillin 1000 mg, furazolidone 100 mg, colloidal bismuth pectin 0.4 g, all twice a day. This eradication therapy was chosen due to low rates of antibiotic resistance to amoxicillin (0.1%) and furazolidone (0.1%) in southeast China [22]. Patients in group B received BQT supplemented with *C. butyricum* 40 mg three times a day (CBM588, MIYA-BM® tablets, Miyarisan Pharmaceutical, Co., Ltd., Tokyo, Japan) for 14 days. Gastrointestinal symptoms and stool form/frequency were assessed by the Gastrointestinal Symptom Rating Scale (GSRS) [23] and Bristol Stool Scale (BSS) [24] at baseline for all subjects and on day 56 for *H. pylori*-positive patients. *H. pylori*-positive patients also underwent ^{13}C urea breath test on day 56 to assess eradication (Fig. 1).

2.3. Stool sample collection

Fresh stool samples were collected from all subjects at baseline. Additional fresh stool samples were collected prospectively from *H. pylori*-positive patients on day 14 and day 56. All stool samples were immediately frozen and stored at −80 °C.

2.4. DNA isolation, PCR amplification and sequencing

Microbial DNA from stool samples was isolated with TIANamp Stool DNA Kit (TIANGEN BIOTECH, cat. #DP328-02, Beijing, China) according to the manufacturer's instructions. DNA concentration and purification were measured by Nanodrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of 16S rRNA gene were amplified using barcoded primers 338F 5′-ACTCCTACGGG AGGCAGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′ by thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR reactions were conducted using the following program: 3 min of denaturation at 95 °C, 27 cycles of 30s at 95 °C, 30s for annealing at 55 °C, and 45 s for elongation at 72 °C, and a final extension at 72 °C for 10 min. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The amplicons were then extracted from 2% agarose gels, purified by AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified by QuantiFluor™ -ST (Promega, USA).

Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.5. Taxonomy determination

Raw fastq files were demultiplexed and quality-filtered by Trimmomatic and merged by FLASH (Fast Length Adjustment of Short Reads to Improve Genome Assemblies). A sliding window size of 50

base pair (bp) was set and reads with quality score < 20 were truncated from the 3′ end. Next, paired-end reads were merged with a minimum overlap length of 10 bp and a maximum mismatch rate of 20%. No barcode mismatches were allowed, and primers were exactly matched allowing 2 nucleotide mismatching. Based on the barcodes and primers, samples were identified, and the orientations of sequences were adjusted. Sequences were then de-replicated, and singletons were discarded. Operational Taxonomic Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/). Chimeric sequences were identified and removed using UCHIME. The taxonomy of 16S rRNA gene sequence was determined by Ribosomal Database Project(RDP) Classifier algorithm (version 2.2 http://rdp.cme.msu.edu/) with a confidence threshold of 70%, using Silva (Release128 http://www.arb-silva.de) as the taxonomy reference database. Data were analyzed using the software Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1).

2.6. Functional predictions

The 16S rRNA functional prediction is to normalize the OTU abundance table through PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) [25], that is, to remove the influence of 16S marker gene copy number. Then OTUs were categorized into Clusters of Orthologous Groups (COG) [26] and into Kyoto Encyclopedia of Genes and Genome (KEGG) orthology (KO) [27].

According to the COG database, the descriptive information of each COG and its functional information were parsed from the eggNOG database to obtain the functional abundance spectrum. KO, Pathway and Enzyme (EC) information were obtained according to the KEGG database while the abundance of each functional category was calculated according to OTU abundance.

2.7. Statistical analysis

Baseline continuous data were presented as mean ± standard deviation (SD) and were analyzed by one-way ANOVA. Categorical data were described in percentage and compared by χ^2 test or Fisher's exact test. All efficacy analyses were performed on an intention-to-treat (ITT) population where patients who dropped out were considered as treatment failures. Secondary per-protocol (PP) analyses were performed which excluded patients lost to follow-up or prematurely withdrew before completion of the study. Differences of GSRS scores in *H. pylori*-positive groups between day 0 and day 56 were presented as the mean ± standard error (SE) and analyzed by Wilcoxon signed rank test. Both day 0 and day 56 BSS scores were presented as absolute values after subtracting 4 from each value. Wilcoxon signed rank test was subsequently conducted to analyze stool alteration after *H. pylori* eradication treatment. Differences in relative abundance of bacterial taxa between *H. pylori*-positive and *H. pylori*-negative subjects and relative abundance alterations after eradication treatment between group A and group B were compared by Wilcoxon rank sum test.
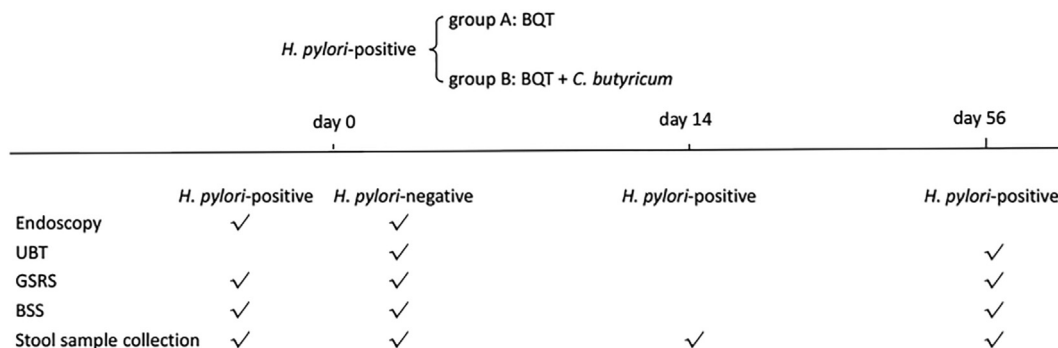

Fig. 1. Study design. BQT, bismuth-containing quadruple therapy; UBT, ^{13}C urea breath test; GSRS, gastrointestinal symptom rating scale; BSS, Bristol stool scale.

Differences among group A, group B and group C were compared by Kruskal-Wallis test.

Mothur (v.1.30.1) was used to calculate indices of alpha diversity (Shannon index and Simpson index), richness (Sobs index and Ace index) and evenness (Shannoneven index and Heip index). Differences in alpha diversity and predicted pathway abundances between *H. pylori*-positive and *H. pylori*-negative subjects were analyzed with Wilcoxon rank sum test. Changes in alpha diversity from day 0 to day 14 and day 56 in group A and group B were compared by Wilcoxon signed rank test. Differences in microbial communities between groups were analyzed using LEfSe (linear discriminant analysis [LDA] coupled with effect size measurements) to avoid high false discovery rates [28]. A *p* value threshold of 0.05 (Wilcoxon rank sum test) and an effect size threshold of 2 were used for all bacterial taxa.

All statistical tests were two-tailed; $p < .05$ was considered statistically significant. Statistical analyses were performed using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Baseline demographics and study follow-up

A total of 118 patients were initially screened for the study and 13 patients were excluded (Fig. 2). Therefore, 105 patients were enrolled, including 70 *H. pylori*-positive patients and 35 *H. pylori*-negative patients. Sixty-three *H. pylori*-positive patients (90%) completed therapy, while 3 patients (8.6%) in group A and 4 patients (11.4%) in group B withdrew from the study. A total of 224 stool samples were collected from 63 *H. pylori*-positive patients and 35 *H. pylori*-negative patients. Baseline characteristics were similar among the three groups (Table 1).

3.2. Differences of gut microbiota between H. pylori-positive and H. pylori-negative populations

We observed a non-statistically significant increase in diversity of *H. pylori*-positive fecal samples compared to *H. pylori*-negative subjects

Table 1
Baseline characteristics of study patients.

Characteristics	Group A (n = 35)	Group B (n = 35)	Group C (n = 35)	P
Age(years)[a]	43.20 ± 12.45	43.89 ± 12.50	40.89 ± 13.80	0.598
BMI[a]	22.66 ± 3.25	22.33 ± 3.04	22.06 ± 2.52	0.690
Gender				0.885
Male[b]	12 (34.3%)	13 (37.1%)	14 (40.0%)	
Female[b]	23 (65.7%)	22 (62.9%)	21 (60.0%)	
Marital status				0.384
Married[b]	28 (80.0%)	29 (82.9%)	26 (74.3%)	
Unmarried[b]	6 (17.1%)	4 (11.4%)	9 (25.7%)	
Divorced/Widowed[b]	1 (2.9%)	2 (5.7%)	0 (0.0%)	
Smoking[b]	4 (11.4%)	6 (17.1%)	6 (17.1%)	0.745
Alcohol[b]	6 (17.1%)	6 (17.1%)	4 (11.4%)	0.745

Group A, *H. pylori*-infected patients treated with bismuth-containing quadruple therapy; Group B, *H. pylori*-infected patients treated with bismuth-containing quadruple therapy and supplemented with *C. butyricum*; Group C, *H. pylori*-negative participants; BMI, body mass index.
[a] Data are presented as mean ± standard deviation (SD).
[b] Data are presented as n (%).

based on number and abundance of OTUs, Shannon index and Simpson index. The observed richness of the community as measured by the Sobs index in *H. pylori*-positive samples was significantly higher relative to *H. pylori*-negative subjects ($p < .05$). However, there were no differences in evenness of the microbial communities by Shannoneven or Heip indices between *H. pylori*-positive and *H. pylori*-negative groups (Supplementary Fig. 1).

Phylum *Nitrospirae* was observed exclusively in *H. pylori*-negative samples ($p = .020$). The *Bacteroidetes:Firmicutes* (B:F) ratios were 0.94 and 0.84 in *H. pylori*-positive and *H. pylori*-negative communities, respectively. Abundances of 22 bacterial genera and 38 bacterial species (Supplementary Table 1) were significantly different between *H. pylori*-positive population samples and that in *H. pylori*-negative population ($p < .05$). There were no significantly different abundances of several putative beneficial taxa, including *Bifidobacterium*, *Lactobacillus*,
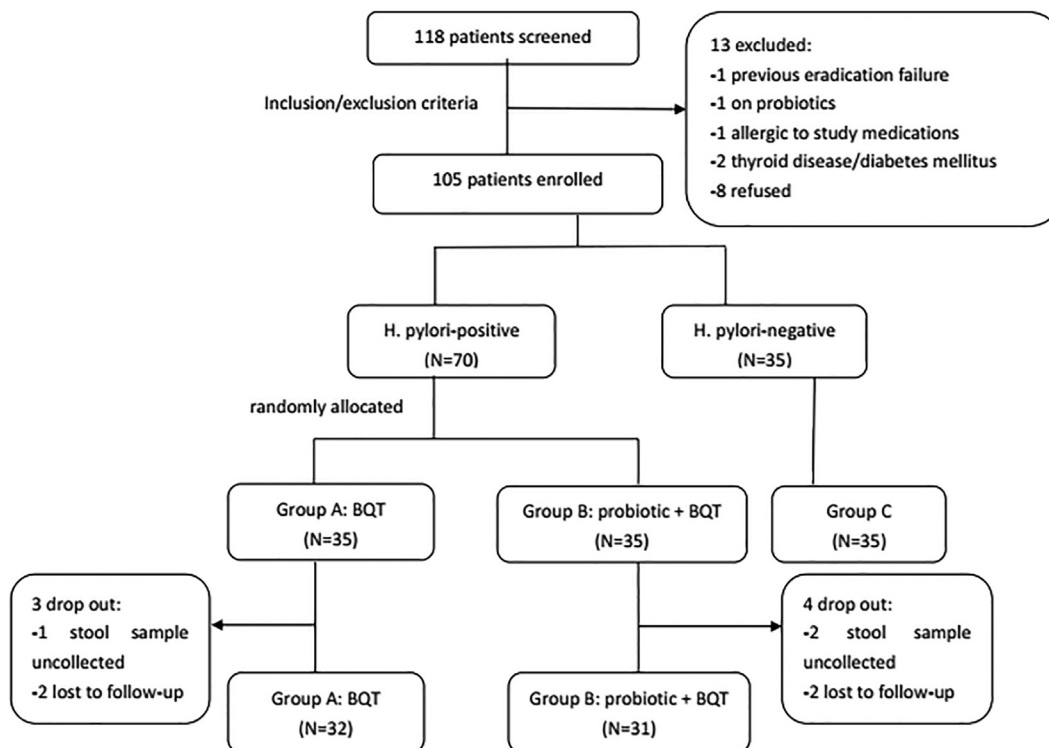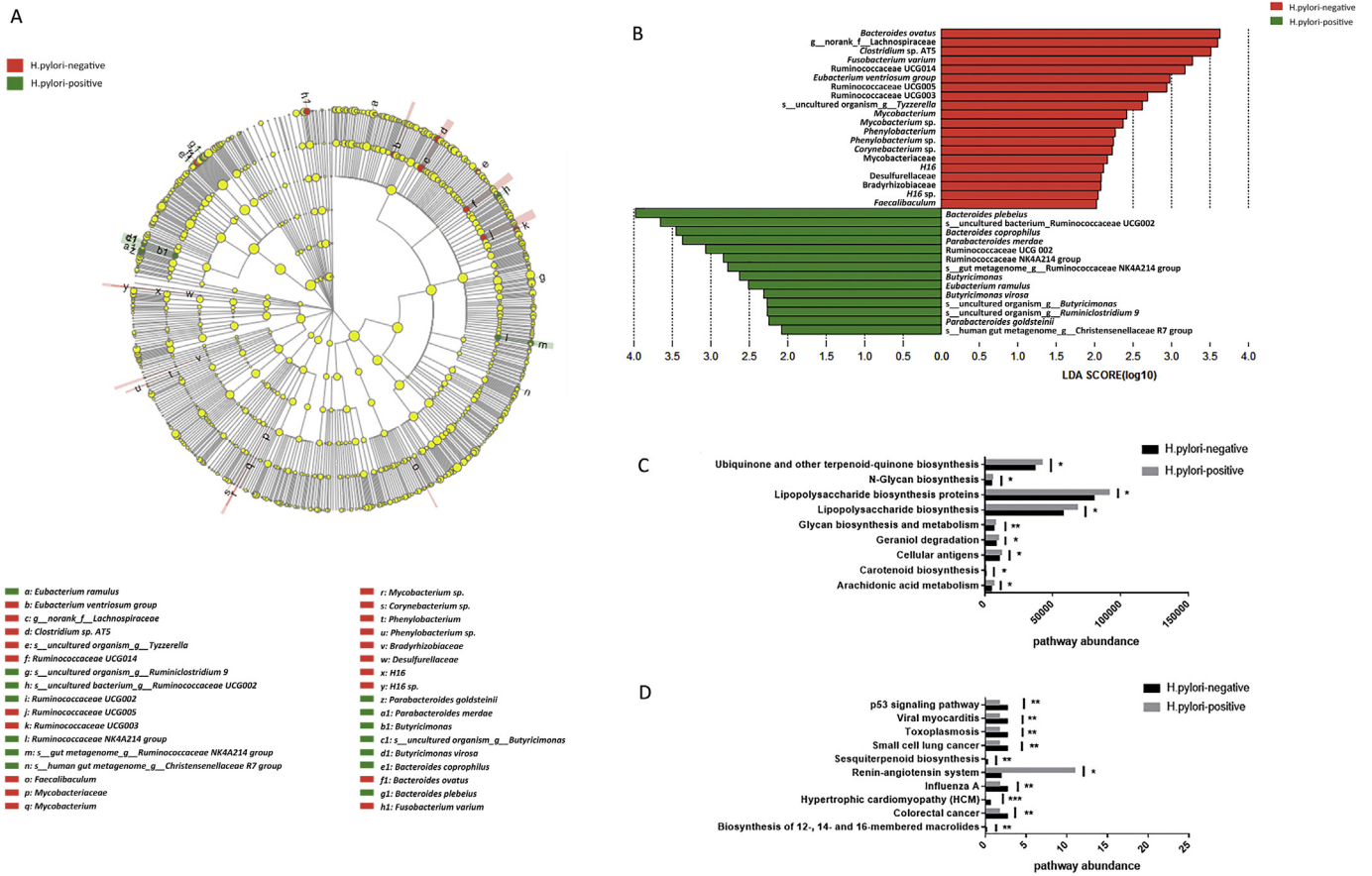

Fig. 2. Patient flowchart. BQT, bismuth-containing quadruple therapy.

Fig. 3. Differences of bacterial taxa and predicted functional pathways between *H. pylori*-positive and *H. pylori*-negative groups. (A) Cladogram representation of gut microbiota taxa differences between *H. pylori*-positive and *H. pylori*-negative groups. (B) Differences of specific bacterial taxa between *H. pylori*-positive group and *H. pylori*-negative group by linear discriminant analysis (LDA) effect size (LEfSe). Red indicates taxa enriched in *H. pylori*-negative group and green indicates taxa enriched in *H. pylori*-positive group. (C, D) Pathways predicted to show significant different abundances between *H. pylori*-positive group and *H. pylori*-negative group according to Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis. *, $p < .05$; **, $p < .01$.

Clostridium butyricum, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, between *H. pylori*-positive and *H. pylori*-negative samples (data not shown). Neither genus *Helicobacter* nor species *Helicobacter pylori* were detected in fecal samples from all patients. There were significant differences in the abundance of different taxa ranging from phyla to species level between these two groups shown by cladogram (Fig. 3A) and LEfSe (Fig. 3B). The relative abundance of 19 pathways were significantly different between *H. pylori*-negative and *H. pylori*-positive patients by KEGG pathway analyses. Several disease-associated pathways were predicted to be more active in *H. pylori*-negative community, such as p53 and colorectal cancer signaling pathways. Many important metabolic pathways were modeled to be more active in patients with *H. pylori*, such as arachidonic acid metabolism, biosynthesis of 12-, 14- and 16-membered macrolides, carotenoid biosynthesis, glycan biosynthesis and metabolism, lipopolysaccharide biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis (Fig. 3C, D).

3.3. Efficacy of H. pylori eradication treatment

The eradication rates of group A and group B were 88.6% and 85.7% by ITT analysis. By PP analysis, the efficacies were 96.9% and 96.8%, respectively. No significant differences in eradication rates between the two groups were observed by ITT or PP analyses (Table 2). After 14-day eradication treatment, there was a significant improvement in overall GI symptoms by GSRS in both group A and group B compared with baseline scores ($p < .05$). Individual symptom scores also improved, including abdominal distention, feeling of incomplete

evacuation, eructation, acid regurgitation and heartburn in group A. Meanwhile, subjects in group B who received probiotics showed improvement in additional symptoms, particularly defecatory function (Supplementary Table 2). This was demonstrated by improvement in BSS scores after treatment indicating an increase in stool frequency and improvement in stool consistency (Supplementary Table 3). There were no significant differences in adverse events or patient compliance between group A and group B (Supplementary Table 4).

3.4. Influence of H. pylori eradication treatment on gut microbiota

There were significant changes in the composition of the fecal microbiota in both group A and group B after 14-day eradication therapy compared with baseline. The alpha diversity indices were strikingly decreased after treatment ($p < .001$). This reduction in alpha diversity, as demonstrated by Sobs index, persisted for up to 6 weeks after completion of treatment ($p < .05$). Although other indices representing richness

Table 2
Efficacy of different eradication regimens.

Analysis	Group A	95% CI	Group B	95% CI	P
ITT	88.6%(31/35)	73.3%–96.8%	85.7%(30/35)	69.7%–95.2%	1.000
PP	96.9%(31/32)	83.8%–99.9%	96.8%(30/31)	83.3%–99.9%	1.000

Group A, *H. pylori*-infected patients treated with bismuth-containing quadruple therapy; Group B, *H. pylori*-infected patients treated with bismuth-containing quadruple therapy and supplemented with *C. butyricum*; ITT, intention to treat; PP, per protocol.

and evenness were not completely recovered on day 56, no statistical significance was observed compared with baseline (Fig. 4A–C).

There were transient changes at the phyla level after 14-day BQT. This included a decrease in relative abundances of *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia* and *Lentisphaerae* as well as an increase in relative abundances of *Proteobacteria* and *Cyanobacteria* on day 14 compared with baseline (Fig. 4D). However, these changes in phyla normalized by day 56 such that no significant difference was detected among these phyla between day 56 and baseline (Fig. 4E). Similarly, there was a decrease in the B:F ratio from 0.98 to 0.34 on day 14 which increased to 0.83 on day 56 (Fig. 4H–I).

There were also marked changes in bacterial community structure at the family, genus and species level. Some of these changes were transient, such as a decrease in the relative abundance of *Lachnospiraceae* and *Ruminococcaceae* on day 14 which recovered by day 56. Others, however, were longer lasting, such as an increase in relative abundance of *Enterobacteriaceae* and *Leuconostocaceae*, together with a decrease of *Rikenellaceae*, *Christensenellaceae*, *Peptococcaceae*, *Clostridiales Family XI*, *Victivallaceae* on day 14 which had not normalized by day 56. There were alterations in the relative abundance of several beneficial anaerobes and some detrimental bacterial taxa on day 14, most of which tended to recover by day 56 (Table 3).

3.5. Impact of H. pylori eradication with probiotic supplementation on gut microbiota

Changes in alpha diversity indices with probiotic supplementation in group B were similar to group A (Fig. 4A–C). The Sobs index had not completely recovered by day 56 ($p < .05$). The B:F ratio decreased from 0.90 to 0.36 on day 14 and increased to 1.23 on day 56 (Fig. 4H–I).

There were significant changes in phyla at day 14 compared with baseline, including reduction in *Firmicutes, Bacteroidetes*, and *Verrucomicrobia* as well as an increased abundance of *Proteobacteria* and *Cyanobacteria*. There were also specific changes in phyla seen only in group B, including a significant decrease in *Fusobacteria* and *Tenericutes* as well as an increase in *Actinobacteria* on day 14 (Fig. 4F). All phylum level changes normalized by day 56 in group B (Fig. 4G).

Overall, subjects in group B demonstrated similar changes in relative abundance of specific taxa compared with group A (Table 3). There was a significant decrease of *Fusobaterium* detected only in group B on day 14 while *Lactococcus raffinolactis, Lactobacillus sakei* and *Acinetobacter baumannii NIPH60* were significantly changed only in group A. Most significant alterations tended to recover by day 56 except some bacteria belonging to *Ruminococcaceae*, *Lachnospiraceae* and *Eubacterium*. The relative abundance of *C. butyricum* after treatment in group B was significantly higher compared to group A on day 14 as well as *H. pylori*-negative subjects ($p = .001$) (Table 4). There was also an increased abundance of *C. butyricum* on day 14 compared to baseline although it did not meet statistical significance.

4. Discussion

We have demonstrated that *H. pylori* infection is associated with widespread alterations in the fecal microbiota compared with *H. pylori*-negative individuals and highlights the intricate and complex interactions between the host and gut microbiota. *H. pylori* has developed mechanisms to co-exist in the harsh gastric microenvironment, whereby it induces mucosal inflammation, immune activation, hypergastrinemia as well as variable effects on gastric acid production. Although the exact mechanisms by which *H. pylori* colonization in the stomach may lead to changes in fecal microbiota are not clearly defined, there is accumulating evidence that interplay between the bacteria and host responses may shape commensal microbiota composition. *H. pylori* has been associated with changes in the gastric microbiota, particularly in patients with advanced premalignant lesions or gastric cancer [29]. Furthermore, this may influence the gut microbiota distally since

changes in luminal pH as well as end products of bacterial fermentation are key regulators in driving the community structure of the gut microbiota [30]. *H. pylori* may further induce other host responses, including inflammatory cytokines or neuroendocrine mediators, which could also conceivably modulate the gut microbiota in non-inflamed locations.

Although *H. pylori* is present in over half of the global populations, sequelae of infection occur in 20% while severe complications, such as gastric cancer, occur in <3% of infected individuals. Indeed, some studies have suggested that humans have developed a symbiotic relationship with *H. pylori*. As *H. pylori* has co-evolved with humans for over 60,000 years [31], there is speculation that this infection may enhance mucosal and systemic immunity to provide a survival benefit to the host. Perry et al. showed *H. pylori* seropositive subjects with latent tuberculosis were more likely to mount an enhanced Th1 response compared with *H. pylori*-negative subjects [32]. Meanwhile, global consensus statements have recommended eradication therapy in all individuals infected with *H. pylori*. However, the consequences of eradicating *H. pylori* and disrupting the gut microbiota on host function are still unknown. There is an inverse relationship between rates of *H. pylori* infection and prevalence of gastroesophageal reflux disease, Barrett's esophagus, and esophageal adenocarcinoma [33]. In addition, there is accumulating evidence linking decreased prevalence of *H. pylori* with increasing rates of asthma and atopic disorders [34]. Taken collectively, *H. pylori* colonization may have beneficial actions on host immunity while eradication may have deleterious consequences in some individuals.

Animal models suggest that the interplay between *H. pylori* infection, host immune response, and commensal microbiota may be associated with development of *H. pylori*-associated diseases. For example, infection with wild-type only, but not mutant strains, of *H. pylori* led to downstream changes in colonic and fecal microbiota in gerbils [9]. Meanwhile, only wild-type strains of *H. pylori* contain the cytotoxin-associated antigen A (*cagA*) virulence factor. Importantly, *H. pylori* strains containing *cagA* genes have been implicated as important prerequisites for long-term sequelae in humans [35]. As such, dynamic and ongoing crosstalk between *H. pylori* and commensal microbiota may influence the host response and susceptibility to complications of *H. pylori* infection.

There were intriguing phyla level changes in fecal microbiota with only *H. pylori*-negative subjects demonstrating presence of *Nitrospirae*. *Nitrospirae* are the most abundant and diverse nitrite-oxidizing bacteria which converts nitrite to nitrate [36]. While risk for *H. pylori*-associated gastric cancer has been associated with increased nitrites and formation of N-nitroso compounds in the stomach [37], there is no data available regarding the relationship between nitrite-oxidizing bacteria in the colon, such as *Nitrospirae*, and *H. pylori*. However, recent evidence suggests that nitrate and nitrite are storage pools for nitric oxide (NO)-like bioactivity which may play important regulatory mechanisms in humans. However, bioactivation of these molecules require presence of commensal bacteria as mammals lack the specific reductase enzymes [38]. Generation of NO is greatly enhanced during periods of hypoxia or acidosis, which allows for vasodilatation and other protective modulatory responses. Indeed, gastric ulcers are common adverse events from taking non-steroidal anti-inflammatory drugs which block generation of NO while administration of dietary nitrate prevented NSAID-induced ulcers in rats [39]. Furthermore, nitrite was shown to have bactericidal effects against *H. pylori* [40]. Thus, the nitrate-nitrite-NO pathway may play an important regulatory mechanism in pathologic conditions and our results suggest may be protective against *H. pylori*.

According to 16S rRNA functional predictions, pathways including lipopolysaccharide biosynthesis were higher in *H. pylori* positive group than in *H. pylori* negative group. As a complex glycolipid of outer membrane of gram-negative bacteria, lipopolysaccharide plays vital roles in outer-membrane integrity and is essential for the viability of bacteria. The capacity to synthesize and export lipopolysaccharide efficiently
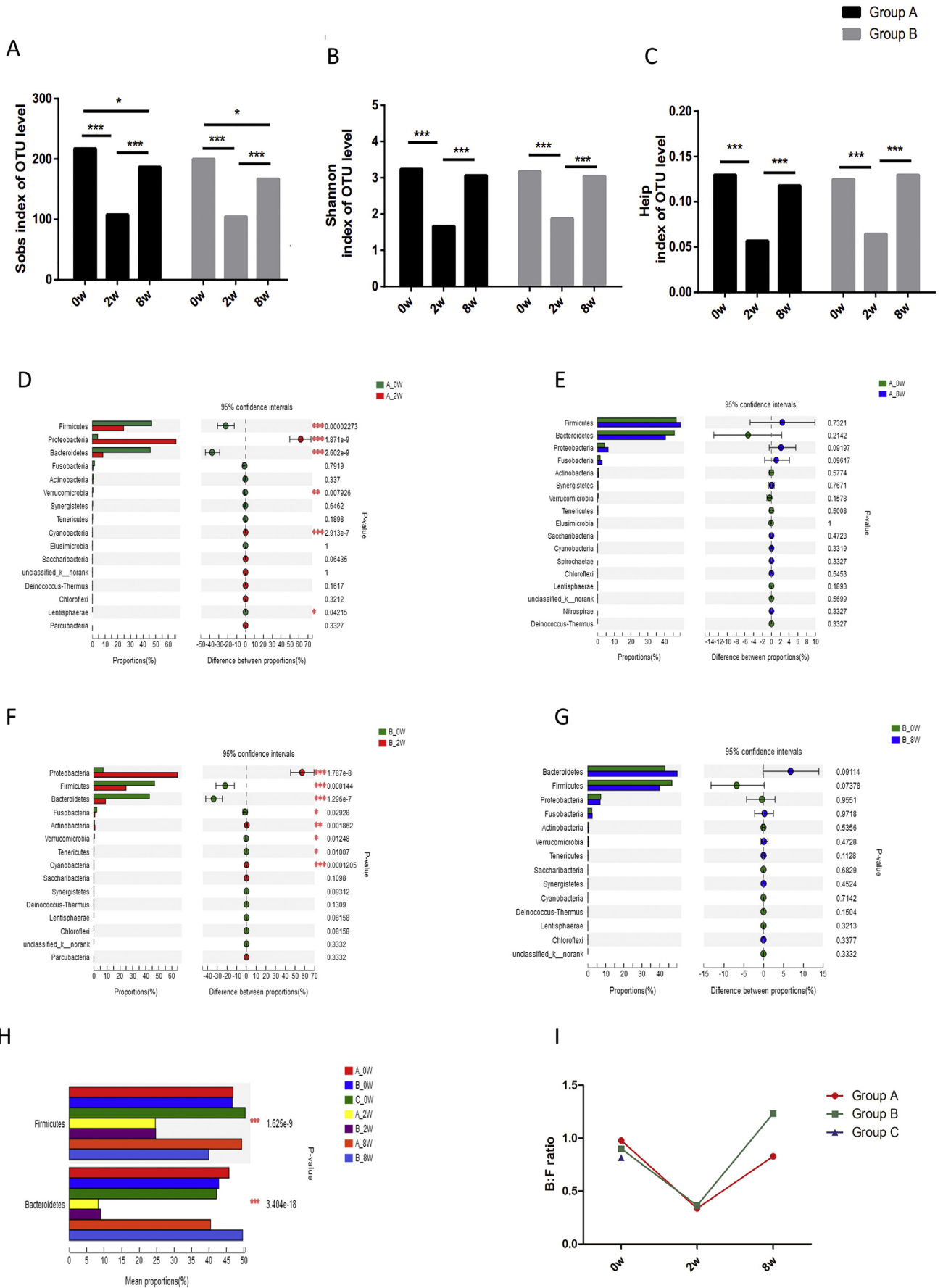
Table 3
Relative abundance alterations on day 14 and day 56 with different eradication regimens.

Bacterial taxa	Group A				Group B			
	Δ1	P	Δ2	P	Δ3	P	Δ4	P
Family								
Lachnospiraceae	−16.06000	***	3.13000	ns	−17.80500	***	−2.53000	ns
Ruminococcaceae	−12.71400	***	−1.08000	ns	−10.62300	***	−1.49000	ns
Neisseriaceae	0.00576	***	0.00034	ns	0.00348	ns	−0.00125	*
Bacteroidales S24–7 group	−0.24065	**	−0.15133	ns	−0.50841	**	−0.29640	ns
Genus								
Alistipes	−2.31950	***	−1.38800	**	−1.49970	***	−0.05800	ns
Coprococcus 1	−0.08053	***	−0.01773	ns	−0.05083	***	−0.00654	ns
Coprococcus 2	−0.29260	**	−0.37186	*	−0.22313	ns	−0.20766	ns
Coprococcus 3	−0.17960	***	−0.11822	*	−0.33450	***	−0.27498	**
Desulfovibrio	−0.31434	***	−0.10010	ns	−0.19677	***	−0.00910	ns
Subdoligranulum	−2.20130	***	−1.11500	**	−2.81630	***	−1.52800	*
Parasutterella	−0.49886	***	−0.10090	ns	−1.16053	***	−0.03500	ns
Klebsiella	16.90610	***	−0.26660	*	25.67100	***	−0.68300	ns
Haemophilus	0.07340	*	−0.12320	ns	−0.11694	*	0.00280	ns
Fusobacterium	−1.06650	ns	1.09700	ns	−1.49460	*	0.23900	ns
Neisseria	0.00409	***	0.00020	ns	0.00052	ns	−0.00083	ns
Species								
Bifidobacterium adolescentis	−0.03811	**	0.07002	ns	−0.04817	**	−0.03470	ns
Faecalibacterium prausnitzii	−0.02807	***	−0.01711	ns	−0.02461	***	0.00366	ns
Akkermansia muciniphila	−0.42931	**	−0.39807	ns	−0.22083	*	0.08520	ns
Intestinimonas butyriciproducens	−0.02137	**	−0.01702	ns	−0.00113	*	0.00679	ns
Roseburia inulinivoran	−0.00566	***	−0.00318	ns	−0.01097	***	−0.00657	ns
Butyricimonas virosa	−0.07121	***	0.00188	ns	−0.08477	***	−0.02939	ns
Dorea formicigenerans ATCC27755	−0.08485	***	−0.02717	*	−0.10362	***	−0.07200	**
Eubacterium rectale	−3.21350	***	0.11800	ns	−2.73848	***	0.32700	ns
Eubacterium eligens	−0.83190	***	0.02850	ns	−0.14570	***	0.86860	ns
Eubacterium coprostanoligenes	−0.41991	***	0.05220	ns	−0.49050	***	−0.09010	*
Eubacterium ruminantium	−0.26003	***	0.05180	ns	−0.16166	**	−0.15800	***
Eubacterium ventriosum	−0.10208	***	−0.01447	ns	−0.09636	***	0.01289	ns
Eubacterium xylanophilum	−0.01384	**	0.02452	ns	−0.06623	**	0.00642	ns
Eubacterium hallii	−0.00939	***	−0.00293	ns	−0.00699	**	−0.00193	**
Eubacterium fissicatena	−0.00294	***	0.00038	ns	−0.00588	**	−0.00197	ns
Clostridium butyricum	0.00278	ns	0.00097	ns	1.23592	ns	0.11712	ns
Lactococcus raffinolactis	0.00176	*	–	–	−0.00039	ns	−0.00028	ns
Lactobacillus sakei	0.00898	*	–	–	0.00026	ns	–	–
Acinetobacter baumannii NIPH60	0.00039	*	0.00027	ns	0.00007	ns	−0.00041	ns

Group A, *H. pylori*-infected patients treated with bismuth-containing quadruple therapy; Group B, *H. pylori*-infected patients treated with bismuth-containing quadruple therapy and supplemented with *C. butyricum*; Δ1, relative abundance difference of Group A between day 0 and day 14; Δ2, relative abundance difference of Group A between day 0 and day 56; Δ3, relative abundance difference of Group B between day 0 and day 14; Δ4, relative abundance difference of Group B between day 0 and day 56; p value was to examine the relative abundance differences between day 0 and day 14 in group A and group B; *, p value < .05; **, p value < .01; ***, p value < .001; ns, no significance; −, not detected in day 0 and day 56.

accounts for rapid growth and cell division [41]. Sobs index, representing richness of community, was significantly higher in *H. pylori*-positive community than *H. pylori*-negative community in our study. This phenomenon may explain the higher lipopolysaccharide biosynthesis pathway abundance in *H. pylori*-positive group. In addition, as a potent endotoxin responsible for triggering mucosal inflammation, lipopolysaccharide of *H. pylori* may interfere with DNA repair system [42]. Interestingly, colorectal cancer pathways were predicted to be higher in *H. pylori*-negative group in our study, which contrasts from several retrospective and prospective studies reporting that *H. pylori* infection was associated with increased risk of colorectal cancer or serrated colonic polyps [43–45]. Further studies are warranted to clarify the relationship between *H. pylori* infection, intestinal inflammation and colorectal carcinogenesis.

We observed striking changes in the gut microbiota after completion of 14-day BQT. This included changes at the phyla level, such as decrease in *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, and *Lentispaerae* as well as an increase in *Proteobacteria* and *Cyanobacteria*, which was in consistent with findings by Ping-I Hsu et al. [46]. Changes in the gut microbial communities, such as decreased B:F ratio and increased abundance of *Proteobacteria*, have been associated with obesity and the metabolic

syndrome [47]. In contrast, Yanagi et al. reported an increase in *Bacteroidetes*, *Firmicutes*, and B:F ratio while *Proteobacteria* decreased immediately after treatment [48]. These discrepant observations may be explained at least partially by different eradication regimens, drug doses and treatment duration lengths between the two studies. Furthermore, many of these changes persisted even up to 3 months after completion of antibiotics. Similar observations were made in a study from Malaysia which showed a decreased B:F ratio in *H. pylori* patients for up to 18 months after treatment [8], indicating long-lasting perturbations in gut microbiota that may have deleterious effects on the host.

There were also important taxonomic changes at the family level after treatment. BQT treatment was associated with decreased relative abundance of *Lachnospiraceae* and *Ruminococcaceae*, which are known to have beneficial effects, such as producing the short chain fatty acid butyrate. Decreases in these bacterial taxa are common after antibiotic exposure, associated with increased risk for *Clostridium difficile* infection, and are restored after fecal microbiota transplantation [49]. Similarly, we also observed reductions in major butyrate-producing bacteria at the genus and species level, including *Eubacterium*, *Coprococcus*, *Faecalibacterium prausnitzii*, *Intestinimonas butyriciproducens*, *Roseburia inulinivoran* and *Butyricimonas virosa*

Fig. 4. Impact of eradication therapy and probiotic supplementation on gut microbiota. (A–I) Alpha diversity indices alterations among day 0, day 14 and day 56. Sobs index (A) represents richness of community; Shannon index (B) represents diversity of community; Heip index (C) represents evenness of community. (D-G) Phyla alterations between day 0 and day 14 in group A (D) and group B (F); phyla alterations between day 0 and day 56 in group A (E) and group B (G). *Bacteroidetes* and *Firmicutes* proportions (H) and *Bacteroidetes:Firmicutes* (B:F) ratios (I) within different groups in different time points. *, p < .05; **, p < .01; ***, p < .001.

Table 4
Relative abundance alterations of *C. butyricum* after eradication treatment.

	Relative abundance (%)				
	Group A	Group B	Group C	P1	P2
Day 0	0.000257	0.005076	0.000106	0.179	0.070
Day 14	0.003032	1.241000		0.025	0.001
Day 56	0.001224	0.122200		0.371	0.168
P3	0.612	0.096			
P4	0.958	0.801			

Group A, *H. pylori*-infected patients treated with bismuth-containing quadruple therapy;
Group B, *H. pylori*-infected patients treated with bismuth-containing quadruple therapy
and supplemented with *C. butyricum*; Group C, *H. pylori*-negative participants.
P1, p values of difference between group A and group B.
P2, p values of difference among group A, group B and group C.
P3, p values of difference between day 0 and day 14.
P4, p values of difference between day 0 and day 56.

immediately after treatment. Other putative beneficial microbes, including *Bifidobacterium adolescentis*, *Akkermansia muciniphila*, *Dorea formicigenerans ATCC27755* and *Subdoligranulum* also decreased after BQT [50,51]. On the other hand, there was an increase in relative abundances of detrimental bacteria, such as *Acinetobacter baumannii NIPH60*, *Klebsiella* and *Haemophilus*. Longitudinal studies are required to determine the consequences of antibiotic-induced disruption of the gut microbiota.

No significant difference in eradication rates were observed with the supplementation of BQT with probiotics (Table 2). This may be related to high eradication rates with both therapeutic regimens (>95% eradication) as well as limited sample size. BQT significantly improved several gastrointestinal symptoms including abdominal distention, feeling of incomplete evacuation, eructation, acid regurgitation and heartburn in *H. pylori*-infected patients. Supplementation with *C. butyricum* also led to improvement of other gastrointestinal symptoms, particularly defecatory function as evidenced by improved BSS scores, such as increased flatus, increased passage of stools and hard stools. Although a statistically significant difference was not observed, supplementation with *C. butyricum* showed a trend towards improving adverse events with antibiotic treatment. Prior systematic reviews and meta-analyses have demonstrated decreased antibiotic-associated adverse events with probiotic supplementation [52,53]. However, most studies reported the effects of *Lactobacillus* strains while our research studied the effect of *C. butyricum*. It is possible that different probiotic strains as well as a larger sample study may explain differences in our results. The relative abundance of *C. butyricum* in group B was strikingly higher when compared to that in group A and *H. pylori*-negative community on day 14. Furthermore, the B:F ratio on day 56 in the probiotic group was significantly higher compared with BQT alone which has previously been shown to correlate with serum IgG and IgM levels [54]. In contrast to group A, relative abundances of *Neisseria* and *Acinetobacter baumannii NIPH60* in group B were not significantly increased while relative abundances of *Haemophilus* and *Fusobacterium* were reduced on day 14. It is possible that probiotic supplementation induced alterations in gut microbiota proportion alteration, leading to changes in host immune response and improved gastrointestinal symptoms. Taken together, eradication of *H. pylori* was associated with significant alterations in the gut microbiota which did not completely recover even 6 weeks after completion of treatment. Administration of a probiotic containing *C. butyricum* improved gastrointestinal symptoms with increased B:F ratio.

Our study had some limitations. First, although this was a prospective study, longer duration of follow-up is necessary to determine consequences of antibiotic-induced alterations in the gut microbiota. Secondly, fecal samples are not representative of the entire gut microbiota as the gut microbiota differs depending on location. In addition, fecal samples provide a measure of luminal microbes as opposed to mucosa-associated microbiota, which are more intimately associated with the host.

In conclusion, *H. pylori* infection may not be necessarily detrimental in all patients. After *H. pylori* eradication treatment gut microenvironment homeostasis may be disturbed with unknown consequences. Our study provided data for effectiveness of probiotic supplementation in improving gastrointestinal symptoms. Collectively, our results argue that the decision to eradicate *H. pylori* should be based on estimating the risk/benefit ratio for each individual.

Conflicts of interests

The authors declare that they have no competing interests.

Author contributions

According to the guidelines of the International Committee of Medical Journal Editors (ICMJE), all authors contributed to the four criteria. Shujie Chen and Jianmin Si conceived and designed the study. Luyi Chen, Wenli Xu, Jiamin He, Wenfang Zheng, Tingting Su, Sanchuan Lai, Yanqin Long, Hua Chu, Yujia Chen, Lan Wang, Kan Wang and Shujie Chen acquired the data. Lan Wang and Bixia Huang analyzed and interpreted the data. Luyi Chen drafted the manuscript. Shujie Chen, Jianmin Si and Allen Lee critically revised the manuscript. All authors read and approved the final manuscript.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.08.028.

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