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(i) Title: Short-term and long-term impacts of Helicobacter pylori eradication with reverse hybrid therapy on the gut microbiota

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ABSTRACT

Background and Aims: Anti-*H. pylori* therapy may lead to the growth of pathogenic or antibiotic-resistant bacteria in the gut. The study aimed to investigate the short-term and long-term impacts of *H. pylori* eradication with reverse hybrid therapy on the components and macrolide resistance of the gut microbiota.

Methods: *H. pylori*-related gastritis patients were administered a 14-day reverse hybrid therapy. Fecal samples were collected before treatment and at the end of week 2, week 8, and week 48. The V3-V4 region of the bacterial *16S rRNA* gene in fecal specimens was amplified by polymerase chain reaction and sequenced on Illumina Miseq platform. Additionally, amplification of *erm*(B) gene (encoding erythromycin resistance methylase) was performed.

Results: Reverse hybrid therapy resulted in decreased relative abundances of Firmicutes (from 62.0% to 30.7%; $P < 0.001$) and Actinobacteria (from 3.4% to 0.6%; $P = 0.032$) at the end of therapy. In contrast, the relative abundance of Proteobacteria increased from 10.2% to 49.1% ($P =$

0.002). These microbiota alterations did not persist but returned to the initial levels at week 8 and week 48. The amount of *erm*(B) gene in fecal specimens was comparable to the pretreatment level at week 2 but increased at week 8 ($P = 0.025$) and then returned to the pretreatment level by week 48.

Conclusions: *H. pylori* eradication with reverse hybrid therapy can lead to short-term gut dysbiosis. The amount of *erm*(B) gene in the stool increased transiently after treatment and returned to the pretreatment level 1-year post-treatment.

KEYWORDS: *Helicobacter pylori*, treatment, microbiome, dysbiosis, *erm*(B)

INTRODUCTION

Human gut microbiota influence essential biological functions of the hosts including energy metabolism, immune modulation, and host defense against pathobionts.[1,2] Numerous studies have reported that abnormal alterations of the gut microbiota (a.k.a., dysbiosis) may promote the development illness including colorectal cancer, inflammatory bowel disease, obesity, type 2 diabetes mellitus, asthma, rheumatoid disorders, and neurodegenerative diseases.[2-4]

Helicobacter pylori (*H. pylori*) infection is the major cause of chronic gastritis, gastric ulcer, duodenal ulcer, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (MALToma).[5,6] Eradication of *H. pylori* not only can prevent the recurrence of peptic ulcers but also may decrease the incidence of gastric adenocarcinoma.[7,8] However, most *H. pylori* eradication regimens contain antibiotics and proton pump inhibitor (PPI),[9-11] which may deplete gut resident commensal microbes resulting in dysbiosis. Antibiotic treatment is known to alter the composition of the normal human gut microbiota. Adverse events such as pseudomembranous colitis associated with *Clostridium difficile* (*C. difficile*) infection have been reported after anti-*H. pylori* therapy.[12,13] In addition, gastric acid suppression by PPI is a known risk factor for *C. difficile*-associated diarrhea in hospitalized patients.[14,15] Another concern with the administration of antibiotics is the selection of antibiotic-resistant strains. It is known that gut microbial macrolide resistance is mediated by erythromycin-resistance methylases encoded by *erm* genes.[16] The *erm* genes have been found in different genera of bacteria and *erm*(B) has the largest host range.[16] Highly macrolide-resistant enterococci have been identified after anti-*H. pylori* therapy with clarithromycin-containing regimen and an increase in *erm*(B) levels in enterococci has been reported following the treatment.[17] Therefore, a comprehensive investigation of the effects of *H. pylori* eradication treatment on the growth of pathogenic or antibiotic-resistant bacteria is quite important before

recommending global anti-*H. pylori* therapy for cancer prevention in asymptomatic subjects.

Previous studies[18] showed that standard triple therapy containing amoxicillin and clarithromycin led to a reduction of the relative abundance of Firmicutes phylum. In contrast, bismuth quadruple therapy containing metronidazole and tetracycline resulted in a dramatic decrease in the relative abundance of Bacteroidetes.[19] Hybrid therapy is a recommend anti-*H. pylori* treatment in areas of either high or low clarithromycin resistance in the Taiwan *H. pylori* Consensus Report.[20] and also in the American College of Gastroenterology (ACG) guideline and the Bangkok *H. pylori* Consensus Report.[21,22] Our group has recently shown that a modified hybrid therapy, called reverse hybrid therapy consisting of a PPI and amoxicillin for 14 days and clarithromycin and metronidazole in the initial 7 days, is a more simplified hybrid therapy regimen.[23] It achieves a higher eradication rate than standard triple therapy with similar tolerability and at a lower cost.[24] Additionally, reverse hybrid therapy has comparable efficacy as bismuth quadruple therapy in the treatment of *H. pylori* infection and was also found to have fewer side effects.[25]

Because the impact of reverse hybrid therapy on the gut microbiota is unknown, the aims of our study are (1) to clarify the short-term and long-term impacts of reverse hybrid therapy on the components of the gut microbes, and (2) to examine the short-term and long-term impacts of reverse hybrid therapy on the amount of *erm*(B) gene in the fecal microbiota.

METHODS

Study population

H. pylori-infected adult patients (age ≥ 20 years) with gastritis documented by esophagogastroduodenoscopy were recruited. *H. pylori* infection was confirmed by at least two positive test results (e.g., rapid urease test, histology, and culture). Subjects with any of the

following criteria were excluded from this study: (a) previous eradication therapy, (b) allergy to any antibiotic of our study, (c) previous gastrectomy, (d) the coexistence of severe concomitant illness (e.g., decompensated cirrhosis, uremia, congestive heart failure, chronic obstructive pulmonary disease, and cancer), (f) pregnancy or lactating women, (g) the use of antibiotics within the previous 8 weeks, and (h) taking PPI or histamine-2 receptor antagonist within previous 8 weeks. This trial was approved by the Institutional Review Board of the Kaohsiung Veterans General Hospital (VGHKS15-CT2-10).

Sample collection procedures

The eligible subjects received a 14-day reverse hybrid therapy consisting of pantoprazole 40 mg plus amoxicillin 1 g twice daily for 14 days and clarithromycin 500 mg plus metronidazole 500 mg twice daily for the initial 7 days. Patients were asked to return in 2 weeks to check drug adherence and adverse events. They underwent a urea breath test to assess post-treatment *H. pylori* status at week 8.[25] Fecal samples for gut microbiota analysis were collected the morning of day 1 before anti-*H. pylori* therapy and at the end of week 2, week 8, and week 48. Patients collected fecal samples at home and stored them at 4 °C. The samples were sent to our laboratory within 6 hours after collection and were immediately stored at -70 °C until DNA extraction.

16S rRNA gene amplification and sequencing by MiSeq

Bacterial DNA in fecal samples was extracted with Bacterial DNA Extraction Kits (Topgen Biotechnology Co. LTD, Kaohsiung, Taiwan). The *16S rRNA* gene was amplified in a 50 ul reaction containing bacterial DNA (5ng/ul), hotStart Taq (Qiagen, Hilden, Germany) and polymerase chain reaction (PCR) primers (Rd1-16S-V3-V4-Forward /Rd2-16S-V3-V4-Reverse) as previously

described.[19] The PCR conditions used were 95℃ for 3 min, 25 cycles of 95℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 40 sec followed by 72℃ for 5 min. The PCR products with a length of ~550 base pairs (bp) were further purified using AMPure XP beads. Then PCR products were subjected to 2nd PCR amplification in a 50 ul reaction containing 1st PCR products (5 ul), hotStart Taq and PCR primers (Nextera XT index primer 1-N7XX and primer2-S5XX) (Illumina, San Diego, CA). Finally, the 2nd PCR products were purified using AMPure XP beads and the DNA concentration and quality were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). Equal DNA amounts of samples with different specific barcode sequences were pooled and sequencing was performed using MiSeq V3 reagent kit (600 cycles) (Illumina, San Diego, CA, USA).

Bioinformatics Analysis

De-multiplexing and generation of raw fastq files for each library were performed with the MiSeq Reporter Software.[27] Trimmomatic was applied to trim the forward and reverse 16S primer sequence located at the 5' end of the forward and reverse reads.[28] PEAR was used to merge the trimmed paired-end reads.[29] The Quantitative Insights into Microbial Ecology (Qiime) was applied to analyze the merged paired-end reads.[30] An open-reference Operational Taxonomic Units (OTUs) picking approach was used to perform detection and clustering of 16S rRNAs.[31] OTU assignments for reads that failed to hit the reference database were picked by an additional round of de novo clustering.[32] The OTU representative sequences against the Greengenes core reference alignment was then aligned by the PyNAST alignment algorithm with a minimum identity of 75%.[31]

Statistical methods.

The raw data of the taxonomy summary results were exported to R version 3.4.1 (R

Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0) for statistical analysis.[19] Nonparametric Wilcoxon signed-ranks test was applied to compare the relative abundances of phyla and genera of the fecal microbiota at different time points. Additionally, the Benjamini-Hochberg procedure for multiple testing was used to correct *P* values. A *P* value less than 0.05 was considered statistically significant.

Diversity analysis.

The alpha diversity was calculated using PD whole tree. A nonparametric Wilcoxon signed-ranks test was used to compare the alpha diversity between fecal microbiota at different time-points.[19] Beta diversity between fecal samples was assessed using the default beta diversity metrics of weighted UniFrac.[32] The resulting UniFrac distance matrices were applied to perform Principal Coordinate Analysis (PCoA) to determine the similarity between groups of samples/time-points. Non-parametric statistical analysis ANOSIM was conducted via Qime to test the statistical significance between different time-points.

Estimation of fecal erm(B) gene

The amount of *erm*(B) gene in feces was analyzed according to previous studies.[33] The *16S rRNA* gene was used as a reference gene. The *erm*(B) gene was amplified using ermBf/ermBr and a TaqMan probe (ABI). The *16S rRNA* gene was amplified using 16Sf/16Sr and a TaqMan probe. The fluorescent reporter dye at the 59 end of the probe is 6-FAM; the quencher at the 39 end was a black-hole quencher-1 (BHQ-1). All primers were synthesized using Invitrogen (Carlsbad, California) and the probes were synthesized using Thermo Electron GmbH (Ulm, Germany). The cycling program was performed on an ABI Prism 7900HT (ABI). The PCR mixture without

template DNA was included in each run as a negative control. The results were analyzed using the software SDS 2.1 (ABI). In the present study, we normalized the *erm*(B) gene copies to the number of *16S rRNA* copies and preparation of curves and calculations were carried out as previously described.[34]

RESULTS

From August 2015 to February 2017, 12 adult patients were recruited and received 14-day reverse hybrid therapy. All the patients completed a fecal sample collection on enrollment and at each follow-up time point. The mean age of these 12 patients was 53.5 ± 14.5 years (mean ± standard deviation). Supplementary Table 1 shows the demographic data of each patient. A total of 3,856,172 quality-filtered reads were obtained from all the fecal samples with an average of 80,337 ± 40,669 reads per sample. All the *16S rRNA* sequences were deposited in the National Center for Biotechnology Information Short Read Archive (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP171595) (**Supplementary Table 2)**.

Diversity analysis.

Alpha diversity analysis with PD whole tree for microbial richness was performed after rarefaction to 2,310 sequences/sample (minimum sampling depth). The rarefaction curves showed that the fecal microbiota at week 2 had less richness than that at baseline (P = 0.018; **Figure 1**). However, the microbial richness at week 8 and 1-year post-eradication did not significantly differ from that at baseline.

Figure 2 and **Supplementary Figure 1** show the PCoA plots generated from weighted UniFrac distance metrics in beta diversity analysis for stool samples in baseline *vs.* end-of-therapy

(week 2), baseline *vs.* 6-week post-eradication (week 8), and baseline *vs.* 1-year post-eradication (week 48). Distinct clustering was noted between the gut microbiota at baseline and at week 2 (**Figure 2A**). However, the differences in bacterial compositions were not significant between baseline and week 8 (**Figure 2B**) and between baseline and 1-year post-eradication (**Figure 2C**).

Sequential changes in relative abundance of bacteria at phylum taxonomy level

Figure 3 shows the relative abundance of phyla of the gut microbiota at baseline, week 2, week 8, and week 48. Before the eradication of *H. pylori*, the most abundant phyla were Firmicutes (62.0%; 95% confidence interval [CI], 52.5% - 71.4%), Proteobacteria (14.1%; 95% CI, 3.3% - 24.9%), Bacteroidetes (10.2%; 95% CI, 3.0% - 17.4%), and Actinobacteria (3.4%; 95% CI, 0.5% - 6.2%) (**Table 1**). At the end of reverse hybrid therapy, the relative abundances of Firmicutes and Actinobacteria decreased to 30.7% (95% CI, 19.2% - 42.2%; P = < 0.001) and 0.6% (95% CI, 0.2% - 1.0%; P = 0.024), respectively. In contrast, the relative abundance of Proteobacteria increased to 49.0% (95% CI, 29.2% - 68.8%; P = 0.011). At week 8, the relative abundances of Firmicutes, Actinobacteria, and Proteobacteria returned baseline levels. The relative abundances of all phyla at 1-year follow up were not significantly different from baseline.

Sequential changes in relative abundance of bacteria at genus taxonomy level

Next we compared the microbiota impact of reverse hybrid therapy at the genus level. At week 2, a significant decrease of relative abundances in Firmicutes phylum was observed in *Clostridium*, *Coprococcus*, *Lachnospira*, *Roseburia*, and *Ruminococcus* (**Table 2**). In Actinobacteria, the relative abundance of *Collinsella* was significantly decreased, while the relative abundances of many genera of Proteobacteria including *Klebsiella*, *Proteus*, *Serratia*, and *Trabulsiella* were

increased. At week 8, the relative abundances of gut microbiota at the genus level were comparable to baseline. Nonetheless, the relative abundance of *Staphylococcus* genus in the Firmicutes phylum was lower than that at baseline (**Supplementary Table 2**). At 1 year following eradication therapy, the relative abundances of most genera were similar to those at baseline. However, the relative abundances of *Brochothrix*, *Lysinibacillus*, *Solibacillus* genera in the Firmicutes phylum and the relative abundances of *Enhydrobacter*, *Psychrobacter*, and *Pseudomonas* genera in the Proteobacteria were lower than those at baseline (**Table 3**).

Sequential changes in fecal erm(B) gene

The total amount of *erm*(B) gene in the feces at the end of week 2 was comparable to that at baseline (P = 0.850). However, the amount was increased at week 8 (P = 0.025) and returned to pretreatment level at week 48 (P = 0.120; **Figure 4**).

DISCUSSION

In the current study, we conducted the first cohort study to assess the effect of reverse hybrid therapy on the gut microbiota. The data clearly demonstrated that microbial richness was decreased after reverse hybrid therapy. The Bacteroidetes and Actinobacteria phyla rapidly declined following treatment whereas the Proteobacteria phylum increased. Additionally, the abundance level of *erm*(B) gene in the feces was significantly increased 6 weeks after eradication therapy and returned to the initial level 1-year post-treatment. These findings indicate that reverse hybrid therapy can lead to a short-term dysbiosis and a transient increase in the amount of clarithromycin-resistant genes in the feces.

In the current study, reverse therapy containing pantoprazole, amoxicillin, clarithromycin, and

metronidazole was used to eradicate *H. pylori*. Dramatic changes of the gut microbiota at the phylum level were notable. The relative abundances of Firmicutes and Actinobacteria were markedly reduced. In contrast, the relative abundance of Proteobacteria increased rapidly. The change of phylum profile of the gut microbiota by reverse hybrid therapy was consistent with a previous study investigating the impacts of standard triple therapy on the composition of gut microbiota.[18] The study showed that standard triple therapy with lansoprazole, clarithromycin, and amoxicillin led to a reduction in the relative abundance of Firmicutes and an increase of the relative abundance of Proteobacteria.[18] The effect of bismuth quadruple therapy on the gut microbiota was different from that of clarithromycin-based eradication therapies. Our previous study demonstrated that bismuth quadruple therapy consisting of a PPI, bismuth, tetracycline, and metronidazole led to a decrease in the relative abundances of Bacteroidetes, Actinobacteria, and Verrucomicrobia and an increase of the relative abundance of Proteobacteria and Cyanobacteria.[19] Overall, both clarithromycin-based anti-*H. pylori* therapy and bismuth quadruple therapy can lead to an increase of the relative abundance of Proteobacteria in the gut. The impacts of the two commonly used anti-*H. pylori* therapies on the other phyla are noted to be different.

In this study, a dramatic decrease of the relative abundance of Firmicutes phylum from 62.0% to 30.7% was noted after reverse hybrid therapy. Both amoxicillin and clarithromycin may contribute to the reduction of Firmicutes following eradication therapy. A metagenomic study has demonstrated that both amoxicillin and azithromycin can decrease the abundance of Firmicutes.[35] Therefore, the decrease of Firmicutes following reverse hybrid therapy was most likely due to the effects of amoxicillin and clarithromycin. Long-term erythromycin therapy has also been shown to decrease the relative abundances of members of the Actinomyces genus in the oropharyngeal microbiota.[35] Thus, clarithromycin in reverse hybrid regimen may contribute to the decrease of the

relative abundance of Actinobacteria. In this study, a dramatic increase in the relative abundance of Proteobacteria, a major phylum of gram-negative bacteria, was observed after reverse hybrid therapy. These include a wide variety of pathogens, such as *Escherichia*, *Proteus*, *Salmonella*, *Klebsiella*, and *Morganella*. Since amoxicillin, clarithromycin, and metronidazole all have limited activity against Proteobacteria, it is likely that Proteobacteria may rapidly increase due to inhibition of other commensal bacteria by reverse hybrid therapy.

Currently, the impact of eradication therapy-induced alterations of the gut microbiota remains unclear. Murata *et al*. revealed that showed anti-*H. pylori* therapy improved symptoms of chronic constipation.[36] On the other hand, Imase *et al.* showed that eradication therapy induced antibiotic-associated diarrhea due to dysbiosis with the growth of *C. difficile*.[37] Another recent study demonstrated that dysbiosis characterized by an increased relative abundance of Proteobacteria during bismuth quadruple therapy may contribute to the development of adverse effects such as nausea, vomiting, and fatigue.[19] Additionally, several studies revealed that probiotic supplementation could reduce the antibiotic-induced dysbiosis and decrease the frequency of adverse effects of *H. pylori* eradication therapy.[18,38] In addition, there is emerging experimental and epidemiological evidence suggesting that *H. pylori* may be beneficial to its carriers by preventing the development of inflammatory bowel disease.[39,40] Future research is warranted to clarify the benefits and risk of eradication therapy in the development of intestinal disorders and the relationships between alterations of the gut microbiota following eradication therapy and the risk of developing intestinal diseases.

In this study, the richness of the gut microbiota declined at the end of eradication therapy and returned to the level before treatment at week 8. Additionally, there were no significant differences in the richness between microbiota at baseline and week 48. The relative abundances of all phyla

at week 8 returned to the levels at baseline. These data suggest that reverse hybrid therapy did not permanently alter the richness and major composition of the gut microbiota. Nonetheless, it is still worthy to note that the relative abundances of some genera in the Firmicutes including *Brochothrix*, *Lysinibacillus*, *Solibacillus* and some genera in the Proteobacteria including *Enhydrobacter*, *Psychrobacter*, and *Pseudomonas* at week 48 were lower than those at baseline. Because the relative abundances of all aforementioned genera in the Firmicutes and Proteobacteria with decreased relative abundances 1-year post-treatment did not significantly change at the end of eradication therapy (week 2), whether the changes of relative abundances in this small subset of gut microbiota was due to aging, change in diet habit, or eradication therapy needs further investigation.

Anti-*H. pylori* therapy can result in antibiotic resistance development among *H. pylori* strains[41] and also in normal intestinal microbiota.[42] The increase of resistant strains can be due to point mutation, clonal expansion of resistant strains, or resistance acquisition by new populations via horizontal gene transfer following antibiotic treatment.[43] In the current study, the amount of *erm*(B) gene in feces at the end of eradication therapy was comparable to that before treatment. However, its amount increased at 6-week post-treatment. The delayed impact of reverse hybrid therapy on the amount of *erm*(B) gene in gut microbiota was most likely due to the inhibition of bacterial growth during eradication therapy. However, it is important to note that the amount of *erm*(B) gene in feces returned to the initial level 1-year post-treatment. Our data suggest that reverse hybrid therapy can lead to an increase of the total amount of *erm*(B) gene in gut microbiota but its impact on the amount of *erm*(B) gene is transient.

This study has some limitations. First, the study did not include a placebo arm for comparison, thus, whether the long-term changes in the gut microbiota was only due to eradication therapy

remain to be clarified. Second, resistance to macrolides in gut microbiota can be mediated by methylation of 23S rRNA *via* erm(B) methylase, drug efflux *via* mef(A), and point mutations in 23S rRNA genes or ribosomal proteins.[43,44] The current study only investigated the impacts of eradication therapy on the amount of *erm*(B) gene in the gut microbiota. Nonetheless, the current study is the first study to investigate the short-term and long-term effects of reverse hybrid therapy on the composition and clarithromycin resistance of gut microbiota.

In conclusion, *H. pylori* eradication with reverse hybrid therapy can lead to transient gut dysbiosis with an increased relative abundance of Proteobacteria and decreased relative abundances of Firmicutes and Actinobacteria. The abundance of *erm*(B) gene in the gut microbiota temporarily increases following treatment but returns to the initial level 1-year post-treatment.

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Table 1. Comparison of the relative abundance of phyla of the gut microbiota between baseline (week 0) and end of eradication therapy (week 2), baseline and 6-weeks post-eradication (week 8), and baseline and 1-year post-eradication (week 48)

Phylum	*Mean relative abundance (%)*				*P value*		
	Week 0	Week 2	Week 8	Week 48	Week 0 *vs* Week 2	Week 0 *vs* Week 8	Week 0 *vs* Week 48
Bacteroidetes	10.23 (3.02-17.44)	13.24 (1.65-24.83)	17.24 (6.97-27.52)	10.86 (1.58-20.13)	0.977	0.948	1
Firmicutes	61.99 (52.54-71.44)	30.71 (19.21-42.20)	53.80 (42.88-64.73)	60.13 (45.44-74.82)	< 0.001*	0.948	1
Proteobacteria	14.14 (3.33-24.94)	49.04 (29.24-68.84)	13.19 (3.68-22.70)	14.67 (5.61-23.72)	0.011*	1	1
Actinobacteria	3.36 (0.48-6.23)	0.59 (0.15-1.03)	5.60 (0.17-11.03)	2.45 (0.47-4.42)	0.024*	1	1
Cyanobacteria	7.21E-03 (0-0.01)	0.01 (4.27E-03-0.01)	0.02 (-0.01-0.05)	0.02 (-7.2E-03-0.06)	0.270	1	0.997
Fusobacteria	0.17 (-0.18-0.53)	0.02 (-6.10E-03-0.05)	0.24 (-0.29-0.78)	1.95 (-2.4-6.31)	0.731	1	0.997
Verrucomicrobia	0.33 (-0.42-1.09)	7.76E-04 (-5.23E-04-2.08E -03)	2.02 (-2.24-6.30)	0.58 (-0.73-1.91)	0.177	1	1

Euryarchaeota	3.08E-04 (-1.82E-04-7.99E-04)	0	2.02E-03 (-2.4E-03-6.49E-03)	3.19E-04 (-4.24E-04-1.06E-03)	0.270	1	1
Synergistetes	5.18-E04 (-3.17E-04-1.35E-03)	0	1.12E-03 (-7.39E-04-2.98E-03)	0.02 (-0.03-0.07)	0.177	1	1
TM7	0.01 (1.09E-04-0.02)	4.84-E03 (-4.39E-03-0.01)	4.75E-03 (1.43E-03-8.08E-03)	5.18E-03 (-3.64E-04-0.01)	0.101	1	0.997
Tenericutes	0.06 (-0.08-0.21)	0	0.06 (-0.08-0.20)	0.01 (-0.01-0.03)	0.467	1	1
Others	5.08E-03 (-1.65E-03-0.01)	0.01 (-2.61E--0.02)	0.01 (-6.72E-03-0.04)	0.01 (-6.71E-03-0.03)	0.760	1	1

* denotes $P < 0.05$

Table 2. Sequential changes of the proportions of the genera with significant differences in the relative abundances of the bacteria at the end of reverse hybrid therapy (week 2) compared with those at baseline.

Genus	*Proportion of microbiota*				*P value*		
	Week 0	Week 2	Week 8	Week 48	Week 0 *vs* Week 2	Week 0 *vs* Week 8	Week 0 *vs* Week 48
Other (Family: Carnobacteriaceae)	8.35E-06	5.13E-05	1.09E-05	3.51E-06	0.044*	0.790	0.422
Other (Family: other)	0.008	0.001	0.011	0.006	0.004*	0.537	0.058
Other (Family: Clostridiaceae)	0.001	1.97E-04	0.001	0.001	0.042*	0.554	0.588
Clostridium (Family: Clostridiaceae)	0.012	7.66E-04	0.002	0.005	0.020*	0.061	0.269
Other (Family: Lachnospiraceae)	0.014	0.002	0.009	0.010	0.003*	0.204	0.302
Collinsella (Family: Coriobacteriaceae)	0.002	3.31E-06	0.006	0.002	0.043*	0.389	0.7119
Coprococcus (Family: Lachnospiraceae)	0.018	7.24E-04	0.009	0.006	0.017*	0.172	0.077
Lachnospira (Family: Lachnospiraceae)	0.006	2.52E-05	0.007	0.009	0.046*	0.883	0.578
Roseburia (Family: Lachnospiraceae)	0.002	1.95E-04	0.002	0.005	0.019*	0.588	0.113
Ruminococcus (Family: Ruminococcaceae)	0.016	6.85E-04	0.008	0.027	0.028*	0.227	0.357
Other (Family: other)	5.87E-05	6.66E-06	3.09E-05	3.92E-05	0.025*	0.194	0.444
Other (Family: Other)	1.35E-05	3.75E-04	2.05E-04	5.53E-05	0.010*	0.262	0.214
Other (Family: Aeromonadaceae)	8.76E-07	3.57E-05	2.61E-06	1.12E-05	0.049*	0.439	0.274
Other (Family: Enterobacteriaceae)	0.072	0.322	0.071	0.064	0.006*	0.983	0.810
Klebsiella (Family: Enterobacteriaceae)	0.002	0.017	0.002	0.002	0.022*	0.822	0.976
Proteus (Family: Enterobacteriaceae)	3.02E-04	0.009	0.001	2.33E-04	0.041*	0.355	0.756
Serratia (Family: Enterobacteriaceae)	5.13E-05	5.84E-04	4.06E-05	2.27E-06	0.039*	0.562	0.193

Trabulsiella (Family: Enterobacteriaceae)	1.65E-05	6.00E-04	2.71E-05	8.96E-05	0.041*	0.166	0.435
Other (Family: Other)	1.03 E-06	1.19E-05	2.49 E-06	0	0.016*	0.338	0.338
Other (Family: Pseudomonadaceae)	6.73E-04	0.001	8.21E-04	6.44E-05	0.004*	0.730	0.015*

Table 3. Sequential changes of the proportions of the genera with significant differences in the relative abundances of the bacteria 1 year post-eradication (week 48) compared with those at baseline.

	Proportion of microbiota				*P value*		
Genus	Week 0	Week 2	Week 8	Week 48	Week 0 *vs* Week 2	Week 0 *vs* Week 8	Week 0 *vs* Week 48
Other (Family: Micrococcaceae)	1.65 E-04	2.56 E-04	9.48E-05	5.97E-07	0.468	0.368	0.020*
Other (Family: other)	2.65E-05	5.23 E-05	2.52E-05	0	0.442	0.945	0.010*
Arthrobacter (Family: Micrococcaceae)	5.42E-04	0.001	4.30E-04	2.89E-06	0.237	0.717	0.004*
Flavobacterium (Family: Flavobacteriaceae)	4.66E-05	6.02E-05	3.19E-05	1.25E-06	0.552	0.573	0.007*
Myroides (Family: Flavobacteriaceae)	1.11E-04	1.91E-04	1.17E-04	0	0.289	0.938	0.016*
Other (Family: other)	5.43E-05	4.30E-05	3.59E-05	1.81E-06	0.590	0.424	0.018*
Geobacillus (Family: Bacillaceae)	2.50E-06	1.79E-05	8.38E-06	4.11E-05	0.218	0.151	0.024*
Brochothrix (Family: Listeriaceae)	4.30E-04	7.76E-04	3.16E-04	4.92E-05	0.277	0.616	0.009*
Paenibacillus (Family: Paenibacillaceae)	0	3.70E-06	0	3.65E-6	0.166	—	0.044*
Lysinibacillus (Family: Planococcaceae)	1.57E-04	2.01E-04	9.26E-05	2.17E-05	0.607	0.424	0.016*
Solibacillus (Family: Planococcaceae)	2.68E-05	8.28E-05	2.50E-05	0	0.232	0.920	0.026*
Other (Family: Moraxellaceae)	0.001	0.001	0.001	3.23E-06	0.157	0.940	0.003*
Enhydrobacter (Family: Moraxellaceae)	9.06E-05	1.44E-04	7.50E-05	1.51E-05	0.316	0.782	0.017*
Psychrobacter (Family: Moraxellaceae)	2.08E-04	1.97E-04	1.89E-04	0	0.884	0.881	0.007*
Other (Family: Pseudomonadaceae)	1.31E-04	1.60E-04	6.98E-05	9.77E-06	0.564	0.366	0.039*
Pseudomonas (Family: Pseudomonadaceae)	3.27E-03	0.003	0.002	3.13E-04	0.606	0.637	0.013*

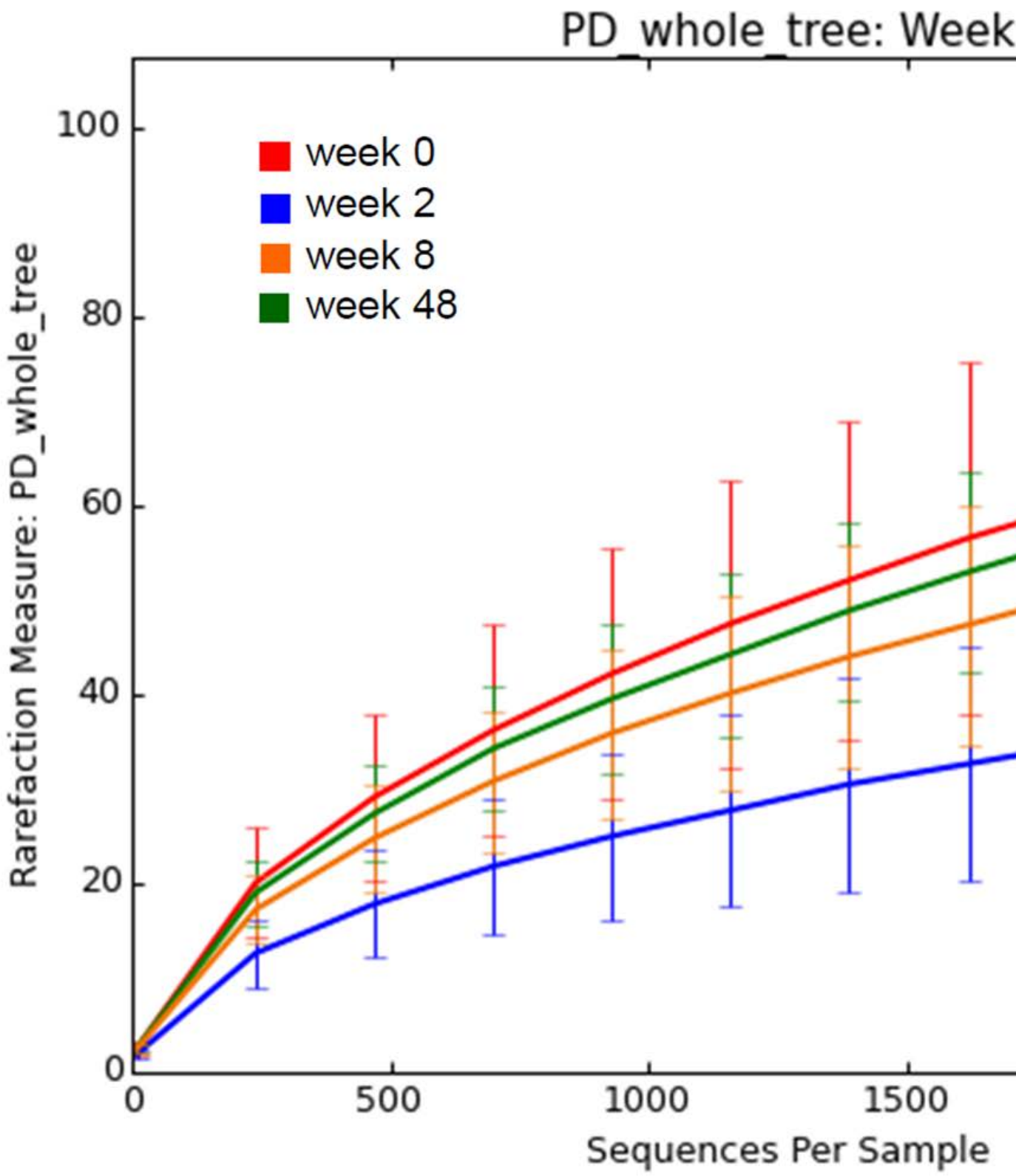
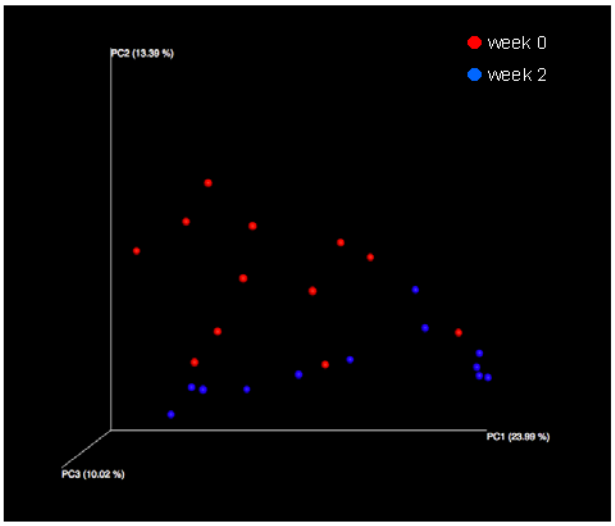


Figure 1. Alpha diversity analysis for the richness of the gut microbiota at baseline, week 2, week 8 and week 48.

A



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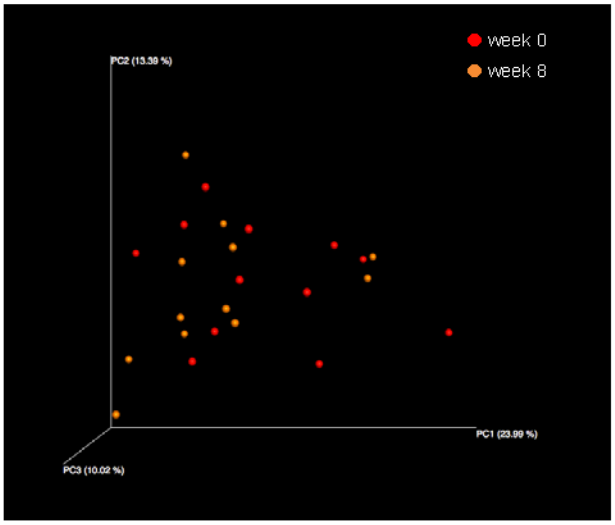

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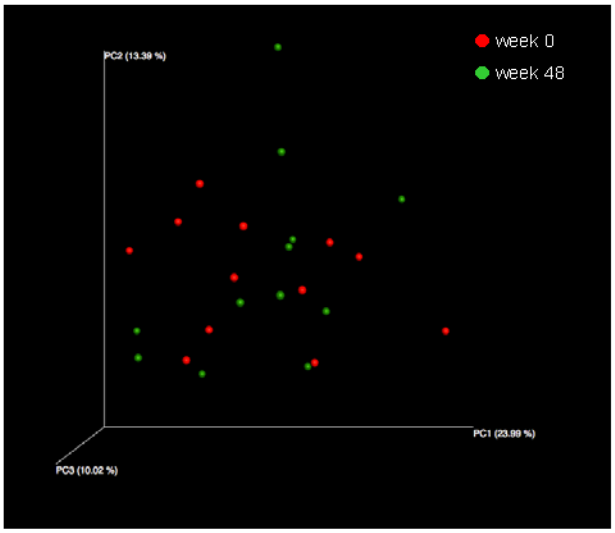


Figure 2. The PCoA plots generated from weighted UniFrac distance metrics in beta diversity analysis for (A) baseline (week 0) *vs.* end of eradication therapy (week 2), (B) baseline *vs.* 6 weeks post-eradication (week 8) and (C) baseline *vs.* 1-year post-eradication (week 48). Distinct clustering was noted between the gut microbiota at baseline and week 2 ($P = 0.011$; Figure A). However, the differences between the distance metrics at baseline and week 8 (Figure B) and between those at baseline and 1-year post-eradication (Figure C) were not significant.

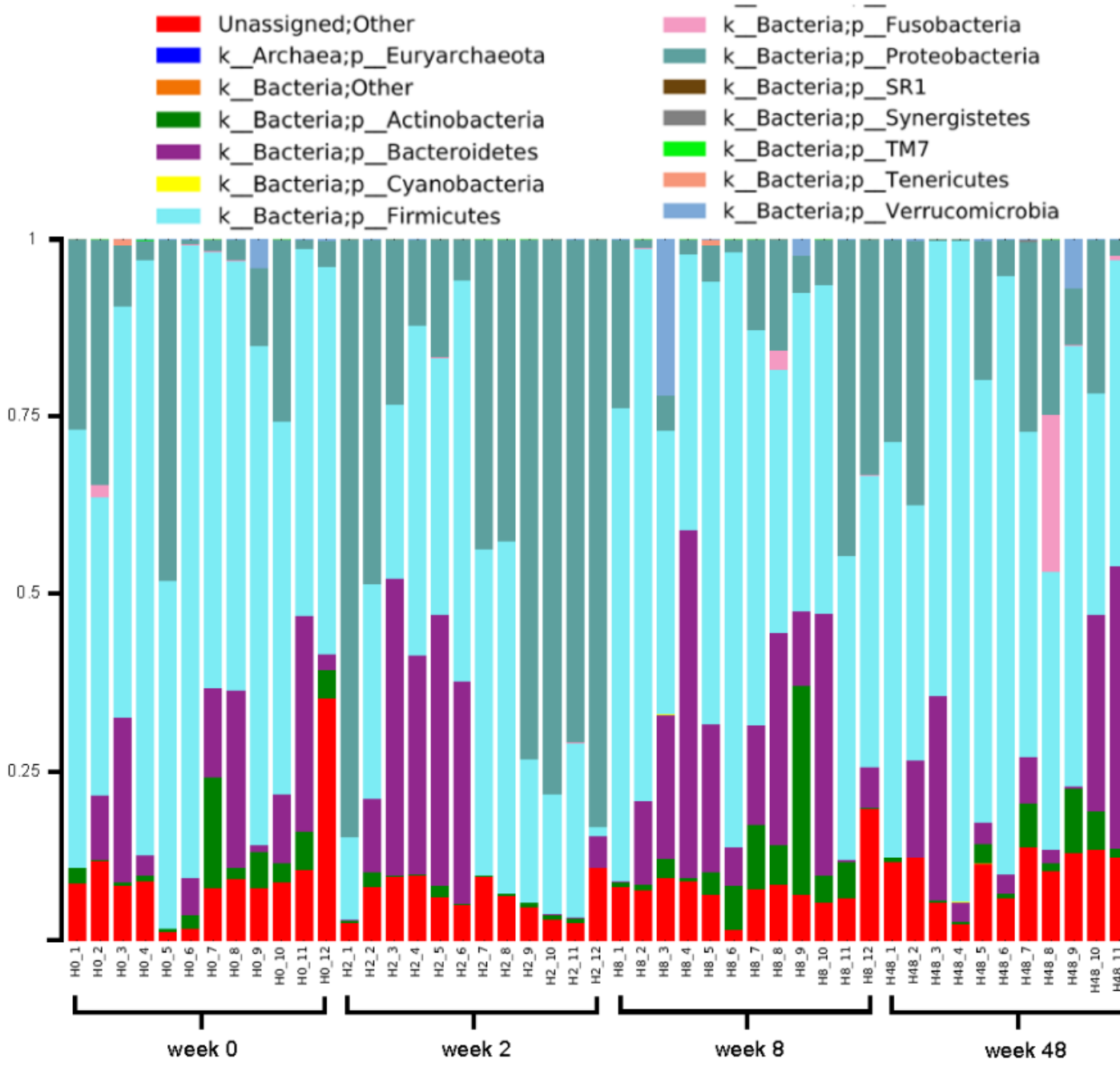


Figure 3. Relative abundance of phyla of gut microbiota at the baseline, the end of reverse hybrid therapy (week 2), 6 weeks post-eradication (week 8), and 1-year post-eradication (week 48). At the end of reverse hybrid therapy, the relative abundances of Firmicutes, and Actinobacteria decreased. In contrast, the relative abundance of Proteobacteria increased.

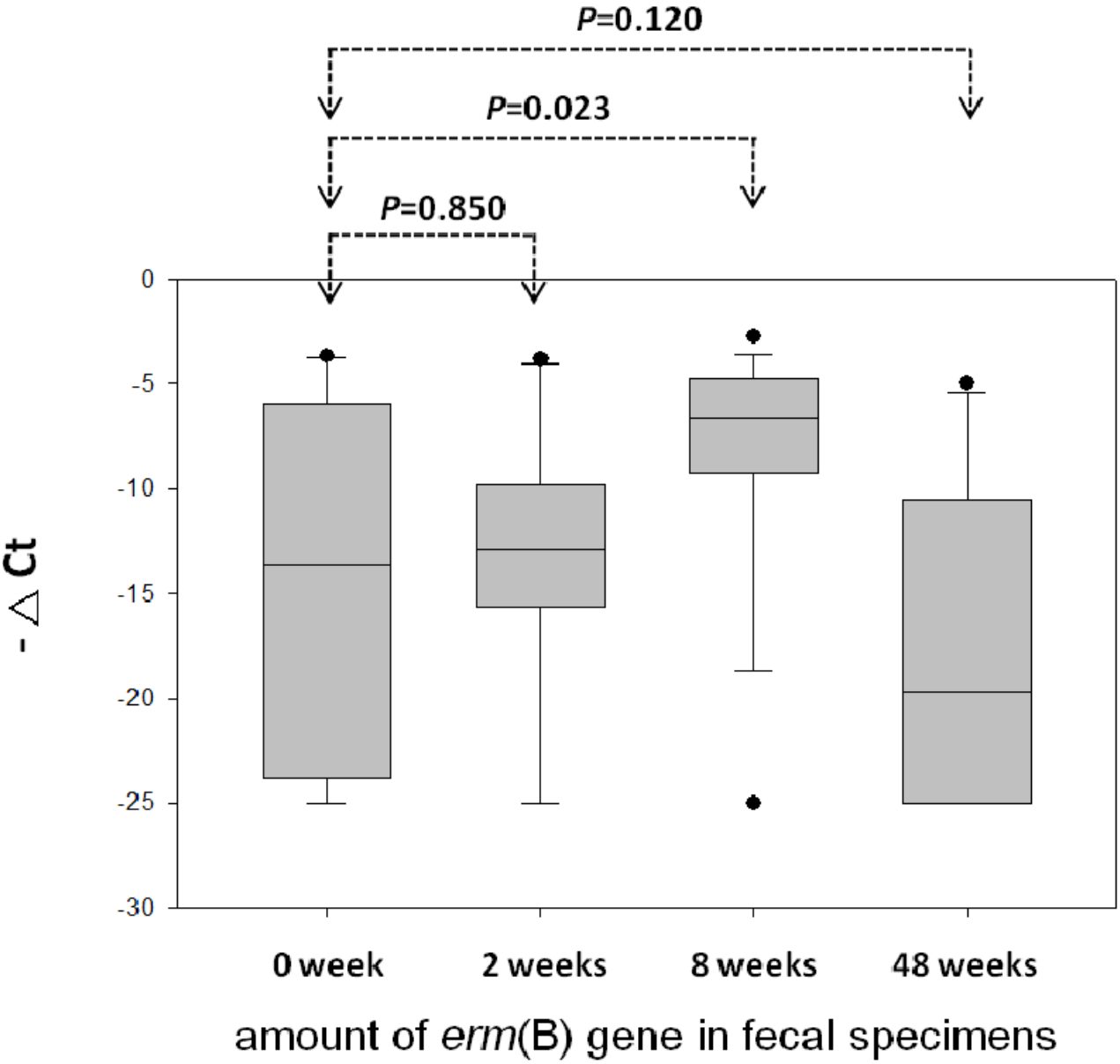
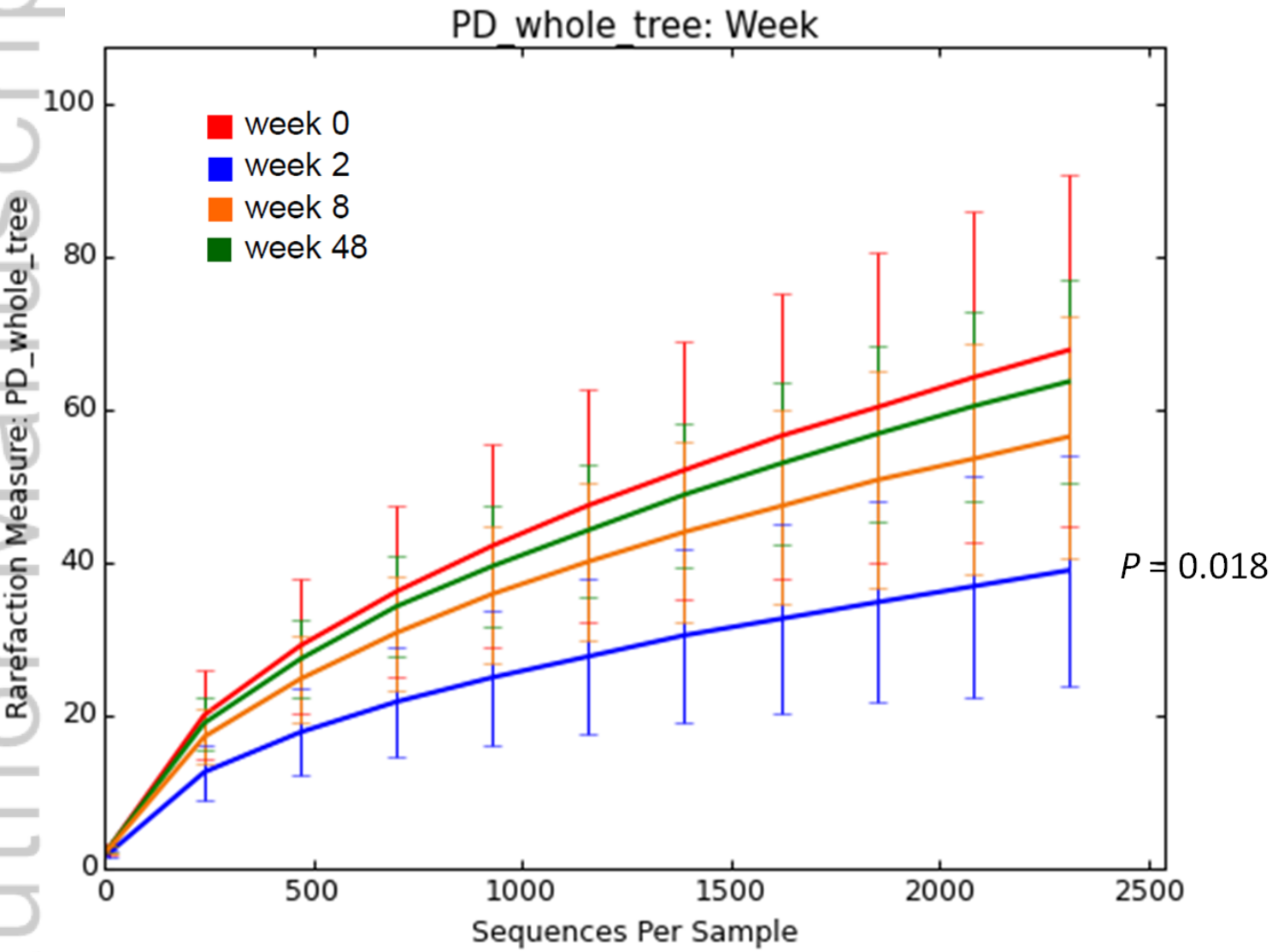


Figure 4. The sequential change of the amount of *erm*(B) gene in fecal specimens after *H pylori* eradication with reverse hybrid therapy. The abundance amount of *erm*(B) gene at the end of week 2 was comparable to that at week 0. Its level increased at week 8 (P = 0.023) and restored to the level before treatment.



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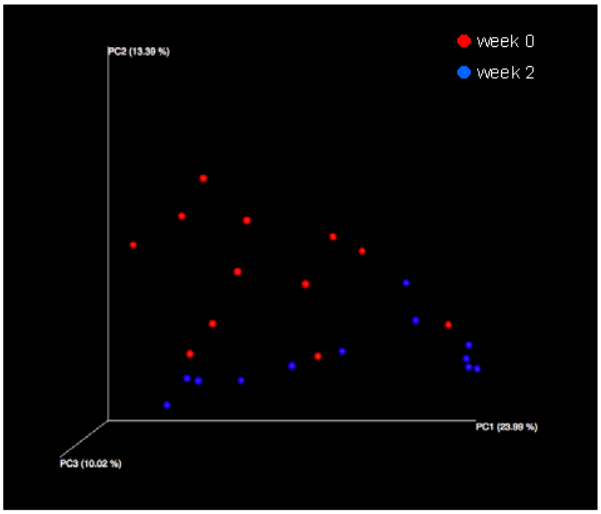
A



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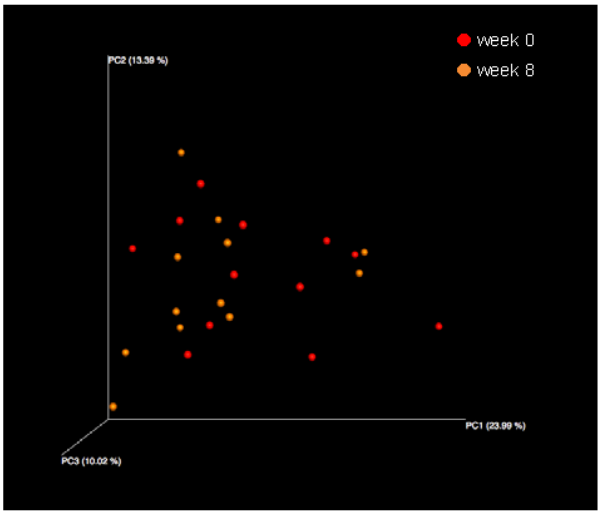

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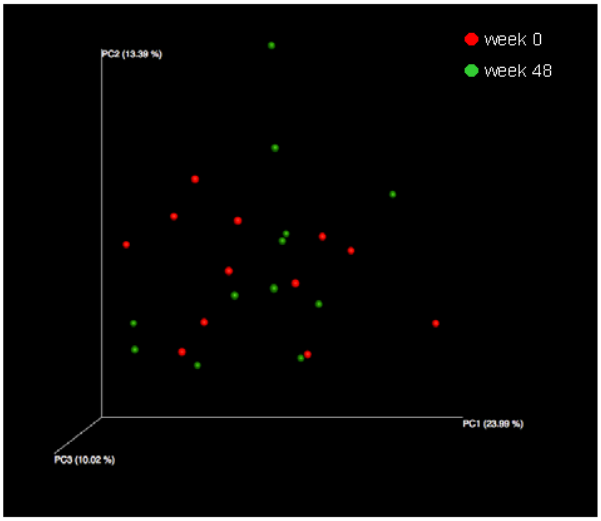

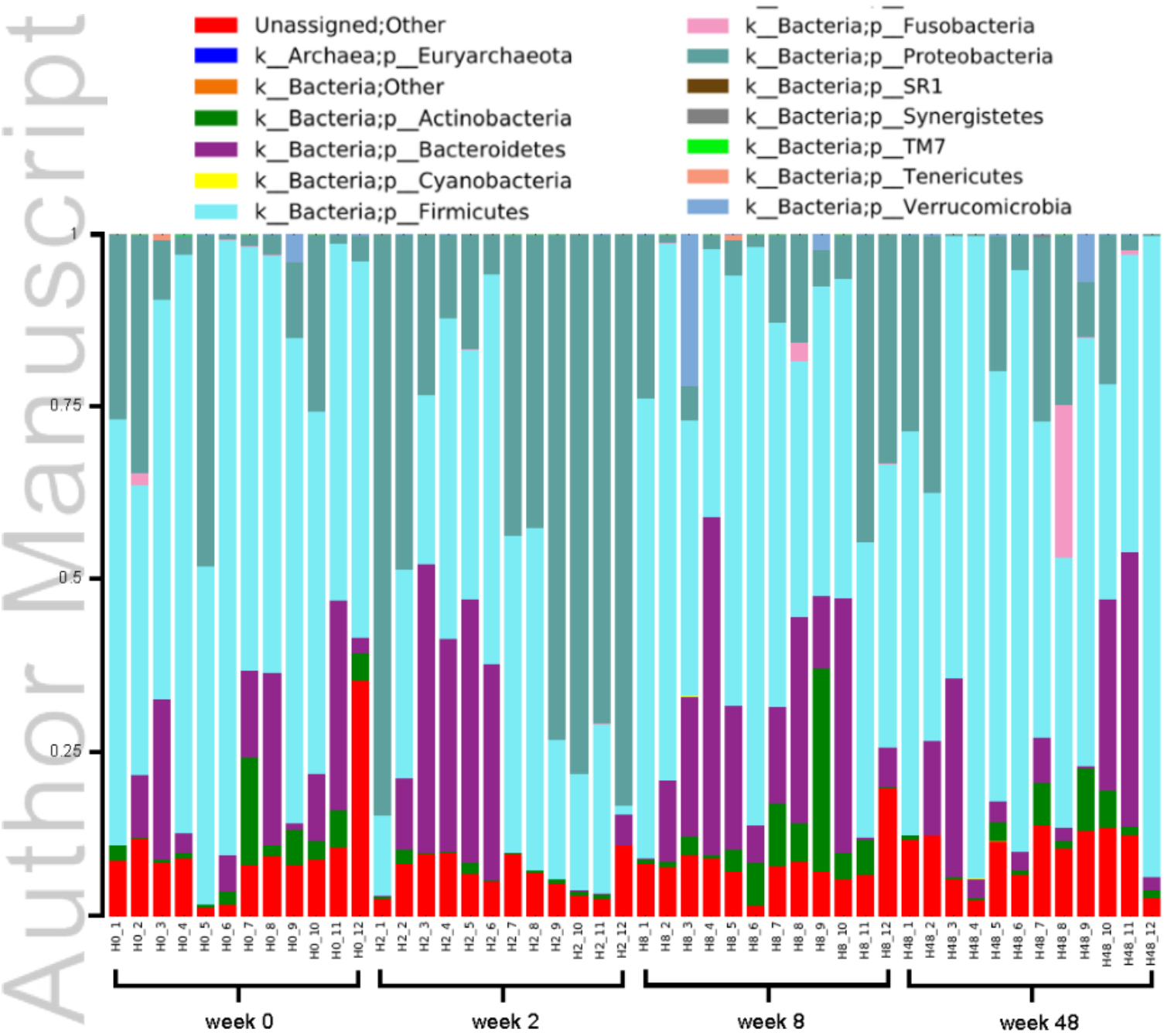

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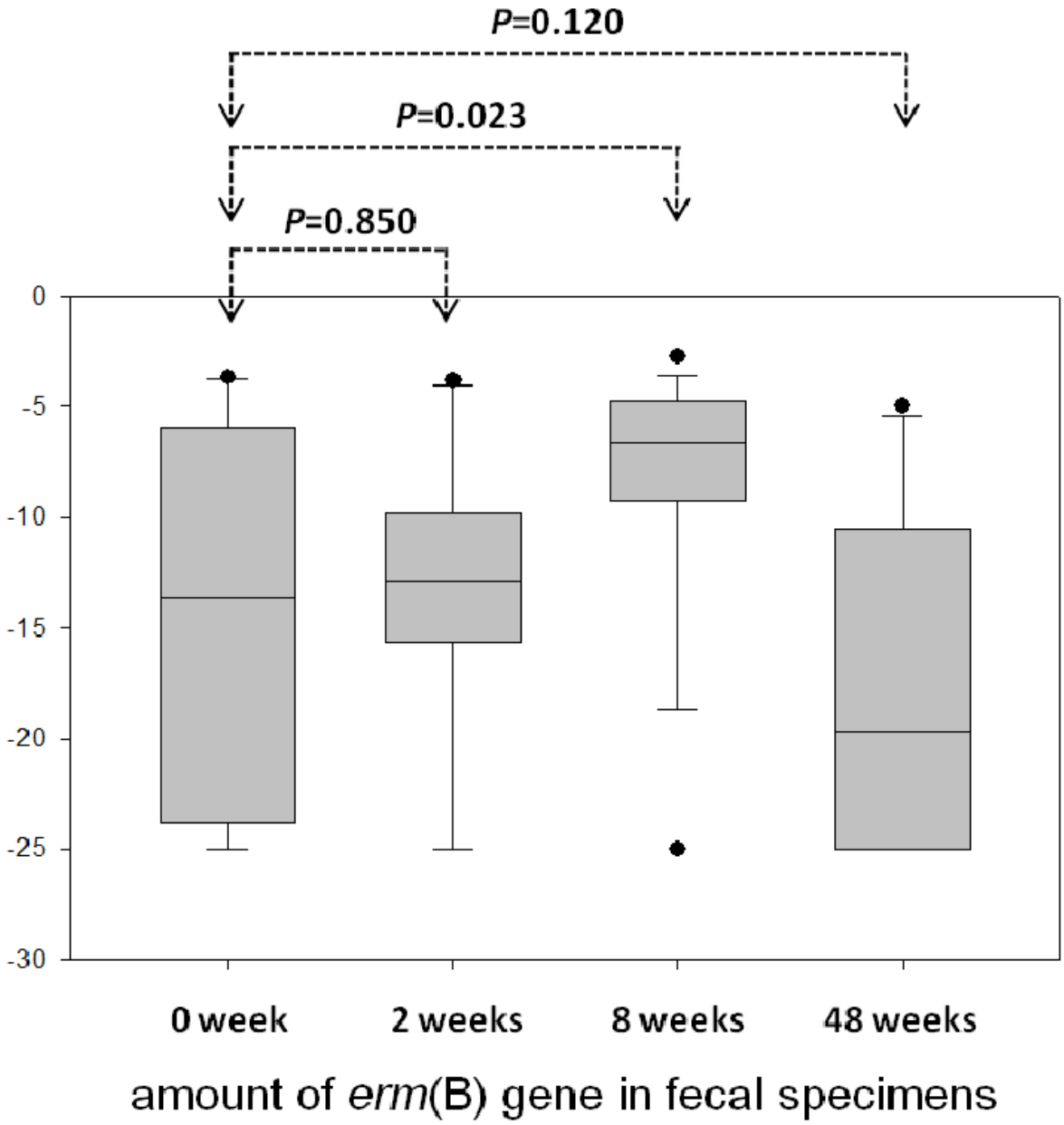

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