

Altered Gut Microbiota Associated with Hemorrhage in Chronic Radiation Proctitis

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Research

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Abstract

Background: Pelvic cancer radiotherapy may cause chronic radiation proctitis (CRP) that adversely affects patient's quality of life, especially in patients with prolonged hematochezia. However, previous studies of radiation enteropathy mainly focused on acute irradiation hazards, and the detail pathogenesis process and mechanism of prolonged hematochezia associated with radiation-induced toxicity remain unclear.

Methods: The 16S DNA of gut microbiota of 32 CRP patients with or without hematochezia were sequenced. The diversities and densities of gut microbiota were analyzed.

Findings: Differential patterns of dysbiosis were observed. The abundance of *Peptostreptococcaceae*, *Eubacterium* and *Allisonella* were significantly higher in CRP patients with hematochezia, while the compositions of the *Lachnospiraceae*, *Megasphaera*, *Megamonas* and *Ruminococcaceae* were lower in the microbiota of non-hematochezia patients. Functional prediction suggested significant difference in the expression of mineral absorption and the Arachidonic Acid metabolism proteins between hematochezia and non-hematochezia patients, possibly interdependent on radiation-induced inflammation.

Interpretation: This study provides new insight to the function of gut microbiota in hemorrhage symptom of CRP patients. Further study is required to select probiotics and prebiotics to improve intestinal homeostasis and relieve prolonged hematochezia in CRP patients.

Research In Context

Evidence before this study

The colorectal cell is highly sensitive to radiation and the injury would last for months to years after radiotherapy. Gut microbial dysbiosis were previously reported associated to acute pathophysiological change in gastrointestinal disorders and short-term radiation-associated conditions. Some altered compositions of microbiota in radiation enteritis are similar with that in inflammatory bowel diseases. Attempt of applying probiotics has been demonstrated efficient in protecting the murine small intestinal epithelium from acute radiation injury.

Added value of this study

Chronic radiation proctitis commonly manifests with hemafesia which leads to anemia, weakness and impaired quality of life. Our pioneer study explored the rectal microbiota in Chronic Radiation Proctitis in perspective of hemorrhage. We identified enrichment of *Peptostreptococcaceae* and diminishment of *Lachnospiraceae* were associated with rectal hemorrhage. Functional modules including Arachidonic Acid metabolism, Glutathione peroxidase and Glutaredoxin were altered in CRP microbiome, which may change the inflammatory status via regulating biosynthesis of prostaglandins in host cells.

Implications of all the available evidence

Accumulating evidence sheds light on the mechanism of rectal hemorrhage after pelvic radiotherapy, and strategies deployed in patients with severe symptoms to modulate homeostasis of microbiota which may suppress progress of CRP.

Background

Pelvic radiotherapy is one of the most important treatments of Gynecologic tumors. Chronic Radiation Proctitis (CRP) is a well-known complication of pelvic radiotherapy that occurs in 5 % to 20 % of patients 6 months to several years later(1). Unlike acute radiation enteropathy (RE) which presents with acute diarrhea and requires immediate termination of radiotherapy(2), CRP commonly manifests hematochezia which adversely affects patient's quality of life in long term. Current treatments of CRP, such as high doses of 5-aminosalicylic acid enemas, endoscopic thermal formalin therapy and antibiotics, are palliative(3–5). Prolonged, recurrent rectal inflammatory and bleeding lead to weakness, anemia, and a series of mental effects including anxiety and depression, especially in female patients(6,7).

Gut microbial dysbiosis has known to be crucial for a series of gastrointestinal disorders including inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS) and RE. A multi-omics study of the gut microbial ecosystem described both host and microbial activities with characteristic taxonomic, functional and biochemical shifts in IBD(8). However, IBD may not be an exact blueprint for studying radiation-induced colorectal injury. Previous research applied DNA fingerprinting and cloning-sequencing techniques and revealed that acute postradiotherapy diarrhea might be linked to patient's initial microbial composition(9). Higher counts of *Clostridium IV*, *Roseburia*, and *Phascolarctobacterium* are significantly associated with RE(10). *Lactobacillus rhamnosus* GG (LGG) protection of intestinal epithelium from radiation injury was illustrated by rodent model. The LGG-mediated radioprotection is reported to dependent on Myeloid Differentiation factor MyD88, Toll-like receptor TLR-2 and Cyclooxygenase COX-2(11). However, little is known about the interaction between gut microbiota and prolonged proctorrhagia after irradiation.

In the present study, we set out to characterize the gut microbiota profiles of CRP patients and demonstrated dysbiosis in CRP patients with hematochezia.

Materials And Methods

Ethics statement and patient information

This study was approved by the Ethics Board at Guangzhou Hospital of TCM. Samples were collected from 30 female outpatient under proctoscope. Informed consent was obtained from all subjects. The patients received pelvic radiotherapy one to two years prior to the onset of chronic radiation proctitis. No antibiotic has been adopted by any patient for at least 3 months. 13 patients complained hematochezia for at least 3 times per day and bleeding was observed under proctoscope. These patients were classified

as H group. 16 patients complained diarrhea with no or little blood in stool were classified as NH group (**Table 1**). Samples from 3 healthy volunteers were collected in parallel. Vegetarian, patients complicated with irritable bowel disease or genetic hemorrhagic disease, and patients who have received antibiotics or steroid treatment in the past 3 months were excluded from this study.

DNA extraction

Total DNA was purified from 0.25g sample by QIAamp PowerFecal DNA Kit (QIAGEN, DE). The DNA concentration and purity were determined by Multiskan™ GO (Thermo Fisher Scientific, US), and the integrity of the DNA was determined by agarose gel electrophoresis.

16S rRNA gene sequencing

PCR was performed on the V4 region (515F-806R) of the 16S rRNA gene of the sample bacteria by priming different indexes at both ends. The reaction system of PCR was performed using 10 µl of KAPA HiFi HotStart ReadyMix (KAPA Biosystems, USA), 2 µl of DNA (30 ng/µl), and 1µl of forward and reverse primer (10 µM). The forward primer of 16S was 5'-gtgccagcmgcccgcggtaa-3' and the reverse primer was 5'-ggactacnvgggtwtctaat-3'. The following thermal cycling program was used: an initial denaturation step at 95°C for 3 mins followed by 30 cycles of 95°C for 20s, 60°C for 30s (annealing / synthesis step), 72°C for 30s (extension) and finally 72°C for 10min extension(12,16,28).

Table 1. Clinical characteristics of the patients

Characteristics	NH (n = 16)	H (n = 13)	p value
Age(y)	58.5 (18.25)	57 (22)	0.26
Female	16 (100%)	13 (100%)	1
Type of malignancy			1
Cervix cancer	14 (87.5%)	12 (92.3%)	
Uterus cancer	2 (12.5%)	1 (7.7%)	
Heamafecia frequency (times per month)	2 (1.875)	30 (39)	0.020621
Erosive mucosa and rectal bleeding	0 (0)	13 (100%)	0
DRE, Fresh blood on glove	1 (6.3%)	13 (100%)	<0.0001

Notes: Results presented as frequency (%) or median (interquartile range). DRE: Digital Rectal Examination.

The PCR products were purified with the AxyPrep™ PCR Cleanup Kit (Axygen, USA), and the concentration was determined with Qubit 3.0 (Thermo Fisher Scientific, USA). Equal amounts of each sample were mixed together to form a sequencing library. QSEP100 (Bioptic, CHN) and ABI7300 quantitative PCR (Thermo Fisher Scientific, US) were used to detect and quantify the insertion fragment and concentration of the library, respectively. The tested libraries were Paired-End 150 bp (PE150) sequenced using Illumina MiniSeq platform.

Bioinformatic analysis

Sequences generated from Illumina sequencing were analyzed with MOTHUR (version 1.39.5) for data cleaning and chimera removal, identification of operational taxonomic units (OTU), taxonomic assignment and community comparison by adapting its standard operational procedure(29). Sequences were realigned with the SILVA-compatible alignment database (<http://www.mothur.org/w/images/9/98/Silva.bacteria.zip>). The 3% dissimilarity cut-off value was used for assigning an OTU. Shannon's diversity, Simpson's diversity, ACE, and Chao I richness indices were generated with the MOTHUR program. Output matrixes were further analyzed by principal coordinates analysis (PcoA) based on UniFrac distance(30,31), and linear discriminant analysis (LDA) effect size (LEfSe) to identify differences in relative abundance at different taxonomic levels(32). Gene function and metabolic pathway prediction was conducted by PICRUST(31).

Statistics and data visualization

The analysis of variance (ANOVA) test was used to assess the differences in the similarity index and the number of bands among the three groups: controls, patients with, and patients without hematochezia. The unpaired Students' t-test was used when we analyzed between two groups using Graphpad prism 8. Krona was used for visualization of microbiome community.

Data sharing

Metagenomic data is available in <http://dx.doi.org/10.17632/c72ygv58wt.1>

Results

Patients and sample collection

32 female participants were assigned into 2 groups: group NH, meaning chronic radiative proctitis (CRP) patients with no or mild hematochezia (hematochezia frequency < twice per week and/or no fresh blood observed under proctoscope, n = 16); and group H, meaning CRP patients with hematochezia (hematochezia frequency > twice per week and/or fresh blood was found under proctoscope, n = 13) (**Table 1**). Three patients were excluded as presented with rectal ulcers (diameter > 1 mm) and / or symptoms of bacterial infection. Age of the 30 patients ranged from 28y to 71y and no significant difference was found between the two groups. Patients have received total dose of 48 to 54 Gy irradiation as for radiotherapy of uterus or cervical carcinoma but have not received antibiotic treatment for at least

3 months. Frequency of uterus or cervix cancer were similar between two groups. 3 samples from healthy adults were collected and analyzed in parallel.

α - Diversity of gut microbiota in CRP patients.

To investigate the ecological complexity of the gut microbiota of CRP patients, a diversity analysis within each group was conducted using Community richness calculators Chao1 estimator and ACE estimator as well as Community diversity calculators the Simpson index and the Shannon index in MOTHUR. As shown in **Figure 1**, the Community richness in CRP patients with hematochezia (Mean of Chao1 = 1065; Mean of ACE = 1738) as compare with CRP patients without hematochezia (Mean of Chao1 = 943.0; Mean of ACE = 1488) were not statistically different ($p > 0.1$). Neither Community richness in healthy volunteer was distinguished (**Supplementary Figure 1**). This result suggests that the overall complexity of gut microbiota may not be a sensitive indicator for CRP.

Altered β -diversity of gut microbiota in CRP patients

The 16S rRNA gene amplicon data contained 1005 OTUs, 133 families and 342 genera. Venn diagram was generated to illustrate the assigned OTUs in each group. About 70% of the OTUs were shared in CRP patients while 27% and 29% of the taxonomic units were unique in H group and NH group, respectively (**Figure 2a**). Ordination analysis including Principal Component Analysis (PCA), Principal Co-ordinates Analysis (PCoA) and Non-Metric Multi-Dimensional Scaling (NMDS) were conducted to measure the difference in bacterial community composition in two-dimension. The first principle component (PC1) of PCA and PCoA largely distinguished changes among healthy controls and CRP patients with hematochezia (**Figure 2b, Supplementary Figure S2**). Inter-individual variation accounted for part of the variance, which was consistent with previous studies(8,12). In addition, the heatmaps demonstrate differential composition of microbiota in three levels (**Supplementary Figure S3**). The β -diversity of gut microbiota in CRP patients suggests that a proportion of bacteria population is related to hematochezia.

Different bacterial taxa between hematochezia and non-hematochezia group

To further study the effect of microbiota composition on CPR symptom, the OTU matrixes were clustered into three cohorts. Hematochezia and non-hematochezia groups were isolated and visualized by Krona (**Supplementary Figure S4**). Taxonomy of the filtered bacteria with more than 2% of total abundance is shown in **Figure 3**. Changed compositions of more than two folds were observed in *Fusobacteriales* and *Selenomonadales* Orders; *Peptostreptococcaceae*, *Fusobacteriaceae*, *Verrucomicrobiaceae*, *Prevotellaceae* and *Veilonelaceae* Families; as well as *Peptostreptococcus*, *Akkermansia*, *Prevotella* and *Megamonas* Genera.

With the linear discriminant analysis (LDA) on effective size (LefSe), we generated a global view of gut microbiota and revealed 25 bacteria taxa with differential relative abundance in both NH and H groups (LDA score > 2 , **Figure 4**). The LDA result was consistent with the Krona plots. The *Peptostreptococcaceae*, *Eubacterium* and *Allisonella* were enriched from the hematochezia group, while

the *Ruminococcaceae*, *Megasphaera* and *Megamonas* were distinguished from non-hematochezia group. Dominant population of *Bacteroidaceae* and *Lachnospiraceae* in each group depend on species. OTU 35, 54, 162, 134, 212 of *Lachnospiraceae* were significant in non-hematochezia group. OTU 797 and 993 of *Bacteroidaceae* were found in hematochezia group.

Peptostreptococcus is the most abundant genus within the *Peptostreptococcaceae* Family (account for > 70% of the Family in the H group; 84% in the NH group, **Supplementary Figure S4**), which met the highest LDA score in hematochezia group. We compared the relative abundance of *Peptostreptococcus* OTU45 from each group and found the data distribution was similar with that of *Peptostreptococcaceae* (**Figure 5**), which indicated that the abundance of *Peptostreptococcus* contributed to dysbiosis in hematochezia patients. In contrary, the relative abundance of *Lachnospiraceae* OTU212 was significantly higher in non-hematochezia group, occupying 0.1% of the rectal microbiome. In addition, *Bacteroidaceae* OTU993, *Lachnospiraceae* OTU1449, *Fusobacteriaceae* OTU1586 and *Clostridiales* OTU1275 were uniquely found from hematochezia group. *Bacteroidaceae* OTU217 and *Megamonas* OTU899 were identified from non-hematochezia group (**Figure 5, Supplementary figure S5**).

These data imply that the bacterial taxa in hematochezia group is interfered and may alter the microenvironment in colorectum, leading to progression of chronic radiation proctitis.

Microbial functional dysbiosis in Hematochezia group

As the composition of microbiota community altered, functional and metabolic changes could be predicted based on previous molecular studies(13,14). Four Clusters of Orthologous Groups (COG) with $p < 0.01$ and four Kyoto Encyclopedia of Genes and Genome (KEGG) orthology with $p < 0.05$ were identified (**Figure 6**). The Protease E (COG3340, **Supplementary Table S1**) were significantly increased in hematochezia group, while the β -galactosidase β subunit (COG2731), the Mannitol-1-phosphate/altronate dehydrogenases (COG246) and the Thymidine phosphorylase (COG213) were down-regulated (**Figure 6a**). Notably, mineral absorption and the metabolism of Arachidonic Acid (ARA) were significantly higher in Hematochezia group according to the annotation of the KEGG orthology (**Figure 6b**). It is known that ARA is not only an important composition of bacterial cell membrane but also interacts with mammalian cell to improve biosynthesis of inflammatory mediators prostaglandin (PGE2 and PGI2). Also, phenylpropanoid biosynthesis and cyanoaminoacid metabolism showed significant difference. The functional dysbiosis in CRP patients with hematochezia suggested an exacerbated inflammation state caused by rectal dysbiosis.

Functional analysis of rectal microbiota was also compared with healthy volunteers (**Supplementary figure S6**). The Fe-S protein (COG1600) Glutaredoxins (COG4545) and Glutathione peroxidase (COG386) (15) were predicted to be significantly increased in both of the CRP groups. Mineral absorption and ARA metabolism in healthy controls were further reduced, compared with samples from CRP patients.

Discussion

Rectal bleeding is the most common symptom of Chronic Radiation Proctitis (CRP) that lays heavy medical burden to the patients. In this pilot study, we took gut microbiota samples from CRP patients under proctoscope and revealed an altered microbial ecosystem in CRP patients with hematochezia.

CRP patients are mostly outpatients in Surgery clinic seeking for remission of rectal irritation and hematochezia. It is believed that the sign and symptom of CRP are largely contributed by interstitial fibrosis and occlusive endovascular inflammation(3,4). Proctoscope examination provides clearer vision of rectal mucus in a relatively convenient and cost-effective way(1). It is suggested that regular proctoscope could be beneficial to patients with radiation proctitis(6). Here we included patients with an array of criteria including patients' observation of blood in stool, digital rectal examination (DRE) and doctors' observation under proctoscope. A systematic history collection with multiple categories is helpful for not only research purpose but also outpatient management.

Dysbiosis has been demonstrated radiation-induced enteric inflammatory, although most of the researches focused on acute enteritis. In previous study on irradiation-inferred microbiome, *Clostridium IV*, *Roseburia*, and *Phascolarctobacterium*(10) were enriched. To our knowledge, this is the first study digging into the radiation-induced dysbiosis with chronic hemorrhage. Relative abundances of these bacterium were similar between H and NH groups, implying other bacterium involve in prolong bleeding. A limitation is the lack of pre-radiotherapy microbiome data, which could provide the baseline individual variation for better causative analysis and facilitate identification of biomarker for prognosis of pelvic cancer radiotherapy.

In this population of CRP patients, hematochezia is associated with an increased proportion of *Peptostreptococcaceae* and *Peptostreptococcus* OTU45 is of most abundance in this family. High density of *Peptostreptococcaceae* was displayed in the intestine of humans with non-alcoholic fatty liver disease, possibly deal to inflammation and impaired mucosal immune function(16). Dis-regulated inflammation could be resulted from stress response. Gao et. al utilized chronic stress mouse model to investigate the role of gut microbiota in stress-induced inflammatory bowel disease (IBD). They found that *Peptostreptococcaceae* were one of the inflammation-promoting OTUs and could be eliminated by antibiotic(17), which had been considered as radiation response modifier. Clathrin adapter AP-1B is the epithelium-specific basolateral targeting factor that polarize the epithelial cells, maintaining homeostasis of the gastrointestinal immune system. AP-1B was demonstrated to be the key factor of *Peptostreptococcaceae* abundance which was significantly increased in *Aplm2^{-/-}* colitis mice, but the detail mechanism remains unclear(18). These evidences suggest that *Peptostreptococcaceae* is highly associated with gastrointestinal inflammation, which may subsequently influence epithelial cell repair capacity or proliferation rate.

Diminished composition of *Lachnospiraceae* is also known to be related to gastrointestinal inflammation. In patients with IBD and acute colitis, *Lachnospiraceae*, *Ruminococcus spp.*, *Faecalibacterium spp.*, and *Roseburia spp.* were consistently depleted(19), suggesting they are crucial gastrointestinal probiotics. Here we observed *Lachnospiraceae* OTU212 in hematochezia group was

significantly reduced, compared to that in non-hematochezia group. Our LDA result also showed that genus *Lachnospiraceae_FCS20* was higher in healthy controls, compared with CRP patients. Interestingly, *Lachnospiraceae* bacterium may contribute to the development of diabetes in obese mice(20) and calorie restriction resulted in lower *Lachnospiraceae* proportion(21). CRP with prolong hematochezia and radiation enteritis patients have relatively thin physique. However, the causation of specific *Lachnospiraceae* insufficient and the decrease of body weight / mass index, or diet intake remains to be elucidated.

Microbiota produce vitamins, energy sources and amino acids by degradation and processing of diet-derived substrates. The differential mineral absorption, ARA metabolism and Protease E between H and NH groups were predicted by PICRUSt. One possible explanation is that the micro-environment with continuous bleeding is favor for high metabolic activity bacterium. The Grxs, FeS and GPx upregulated in CRP patients are key regulators of cell redox and electron transport that involve in various processes of biosynthesis and detoxification(22). Glutaredoxins (Grxs) ligated Fe-S cluster participates in oxidative signaling(23) and haem synthesis(24), which may be compensatory to the prolonged anemia. The Grxs system also acts as an efficient electron donor to plasma glutathione peroxidase (GPx)(15). GPx is known as one of the major enzymes that prevent oxidative stress by catalyzing glutathione (GSH) into glutathione disulfide (GSSG). The increase of Grxs, FeS and GPx may result from high oxidative micro-environment after irradiation. In addition, GPx together with Arachidonic Acid (ARA) enriched in hematochezia group is capable of regulating prostaglandin PGE2 and PGI2 which promote gut inflammation(25). A prospective study of ARA and / or GPx effect on inflammation and hemorrhage in post-irradiation model is expected in the future.

The dysbiosis could be related to the change of gene expression in glandular and/or epithelial cells induced by irradiation and inflammation(26). Thus, combination of larger scale metagenomic data, transcriptome profiles and proteomic profile of inflammatory factors study is expected to provide comprehensive view of post-irradiation gut-microbiome interaction(27). In summary, this is a pilot study revealing the altered gut microbiota in CRP with hematochezia, providing a fundamental evidence for developing and evaluating treatments for chronic radiation proctitis.

Conclusions

In this study, we show for the first time that the gut microbiome is associated with hematochezia in CRP. In accordance with published data, we find the differentiated microbial ecosystem in hematochezia patients where Akkermansia is dominant in rectal bacterial community. The shifted gut microbiota of CRP patients with hematochezia toward that of non-hematochezia patients is consistent with the hypothesis that gut microbiota may be responsible for CRP symptoms. Metabolites related to Akkermansia such as FeS, Grxs and GPx could further induce expression of inflammatory factor that interact with irradiated rectal epithelial cells and exacerbate inflammation. Introduction of Enterobacteriales to restore microbial balance could be a possible solution to improve hematochezia and

anemia. The findings in the current study require validation in a larger cohort and the development of probiotic therapy warrant further investigation.

Declarations

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Declaration of interests

All authors declare no competing interests.

Contributors

JJL and LZL conceived the study. LZL contributed to the literature search, preparation of figures and panels, and writing of the manuscript. CYC, BCC and XL contributed to sample collection clinical data analysis. CD contributed to programming data analysis. All authors have seen and agreed on the final submitted version of the manuscript.

Abbreviations

ARA: Arachidonic Acid

CRP: Chronic Radiation Proctitis

Grxs: Glutaredoxins

GPx: Glutathione Peroxidase

GSH: glutathione

GSSG: glutathione disulfide

LefSe: linear discriminant analysis effect size

NMDS: Nonmetric multidimensional scaling

OTUs: operational taxonomic units

PCoA: principal coordinates analysis

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Table

Table 1. Patients information.

	Number	Age(y)	Gender	Site of cancer	Erosive mucosa and rectal bleeding	DRE: fresh blood on glove
NH	1	62	F	Cervix	-	-
	2	48	F	Uterus	-	-
	3	49	F	Cervix	-	-
	4	32	F	Cervix	-	-
	5	67	F	Cervix	-	-
	6	65	F	Cervix	-	-
	7	56	F	Cervix	-	-
	8	50	F	Cervix	-	-
	9	48	F	Cervix	-	-
	10	60	F	Cervix	-	-
	11	68	F	Cervix	-	-
	12	41	F	Uterus	-	+
	13	57	F	Cervix	-	-
	14	68	F	Cervix	-	-
	15	67	F	Cervix	-	-
	16	68	F	Cervix	-	-
H	1	59	F	Cervix	+	+
	2	56	F	Cervix	+	+
	3	71	F	Cervix	+	+
	4	61	F	Cervix	+	+
	5	56	F	Cervix	+	+
	6	32	F	Cervix	+	+
	7	37	F	Cervix	+	+
	8	28	F	Cervix	+	+
	9	58	F	Cervix	+	+
	10	58	F	Cervix	+	+
	11	57	F	Cervix	+	+
	12	61	F	Cervix	+	+
	13	34	F	Cervix	+	+

Notes: NH: Non-hematochezia group; H: hematochezia group; F: Female; DRE: Digital Rectal Examination; -: not observed; +: observed.

Figures

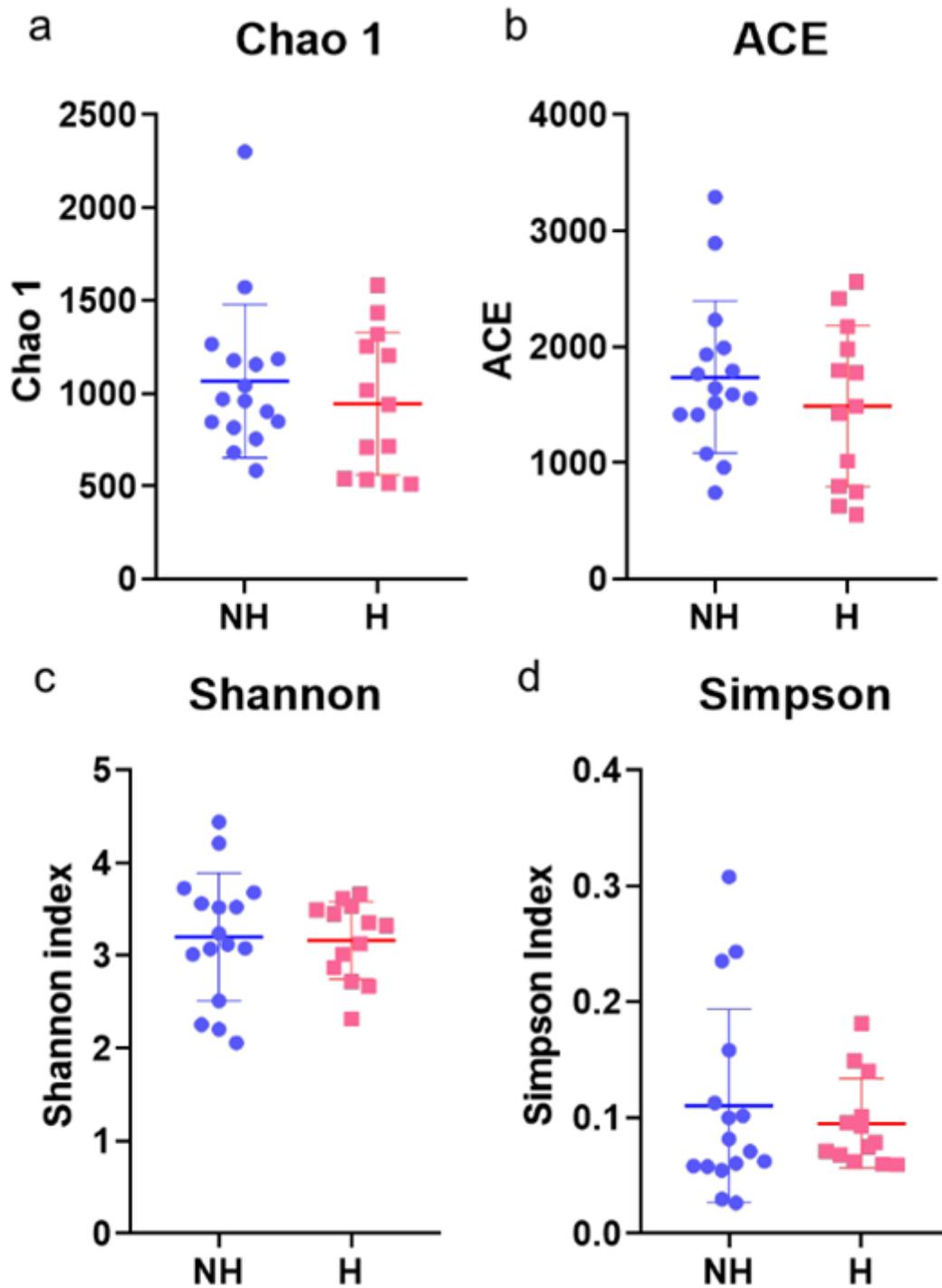


Figure 1

Comparative α -diversity analysis by (a) Chao1, (b) ACE estimator, (c) Shannon and (d) Simpson Index indicating microbiome differences between Non-Hematochezia (NH) and Hematochezia (H) subjects. Error bars indicate SEM.

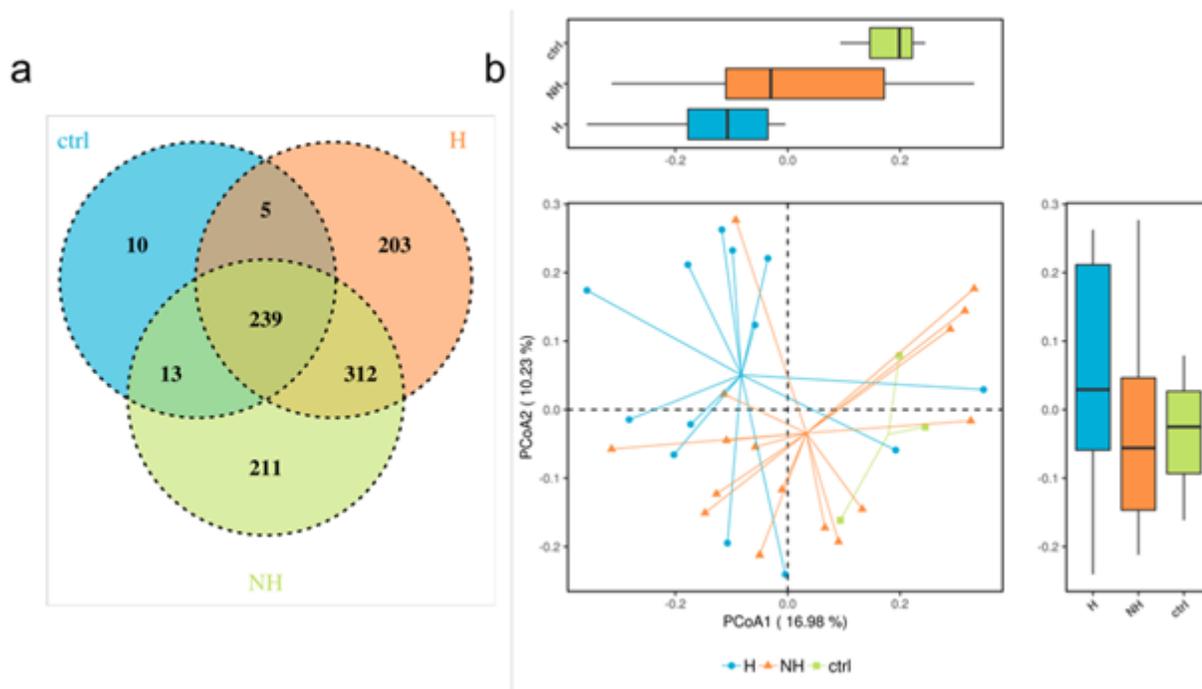


Figure 2

Differential composition of gut microbiome in CRP patients. (a) Venn diagram representing the operational taxonomic units (OTUs) in samples obtained in healthy volunteers (ctrl), CRP patients with hematochezia (H) and without hematochezia (NH). (b) PCoA based on UniFrac matrix showing the differential microbiota composition. Boxes represent the 25th –75th percentile of the distribution; the median is shown as a thick line in the box.

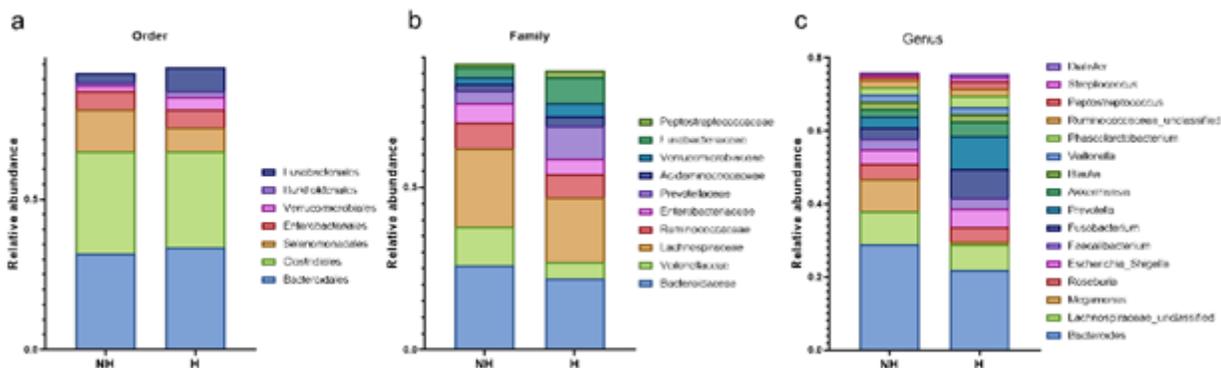


Figure 3

The relative abundance of the bacterial taxa with more than 2% of the abundance. (a) The seven most abundance bacteria in Order level. (b) The ten most abundance bacteria in Family level. (c) The 16 most abundance bacteria in Genus level.

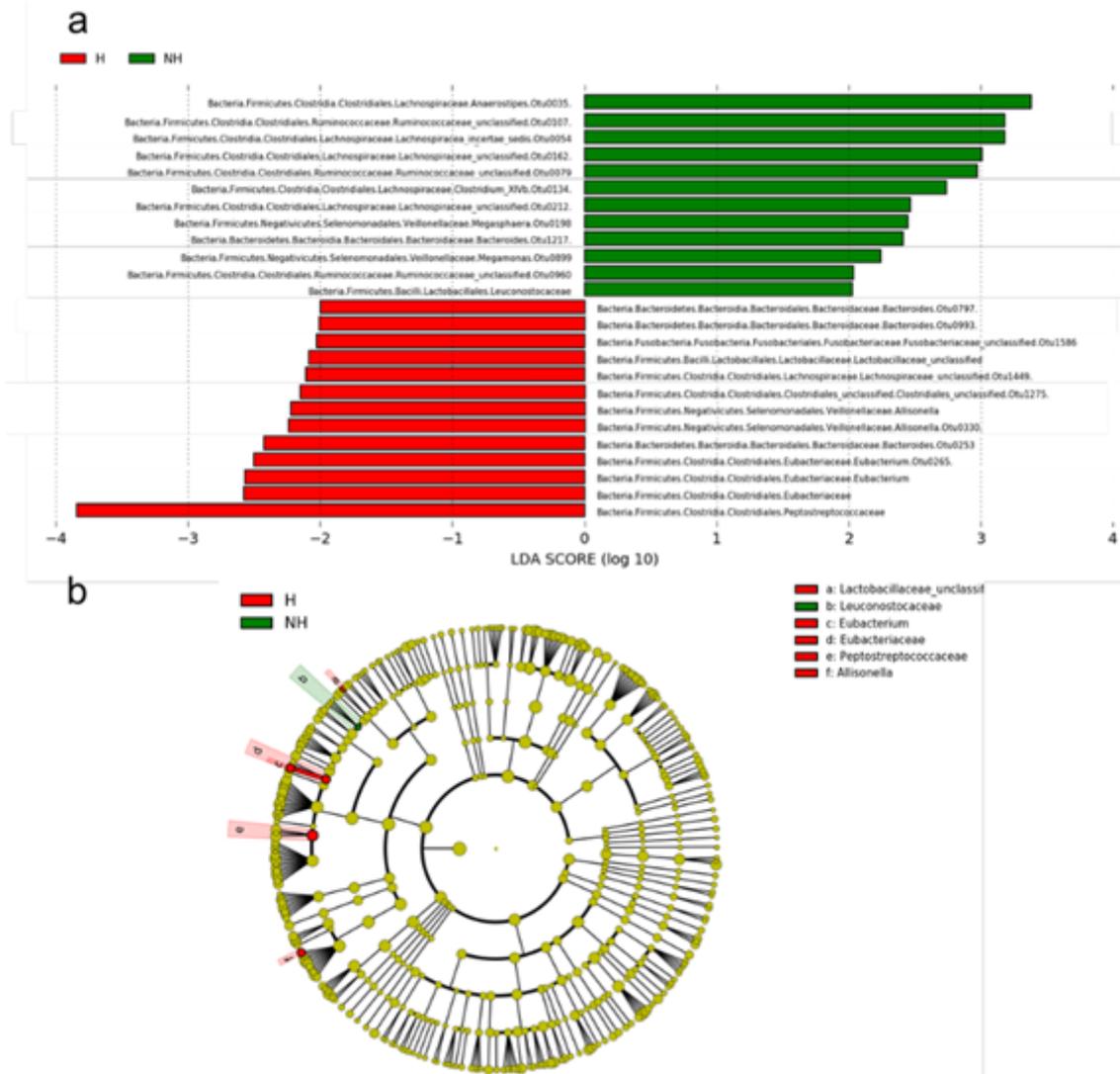


Figure 4

LDA score (a) and evolutionary diagram (b), with circles representing taxonomic levels from phylum to genus / species. Each small circle at a different classification level represents a classification at that level, and the diameter of the small circle is proportional to the relative abundance. The species with no significant difference were yellow, while the differential species were colored as indicated.

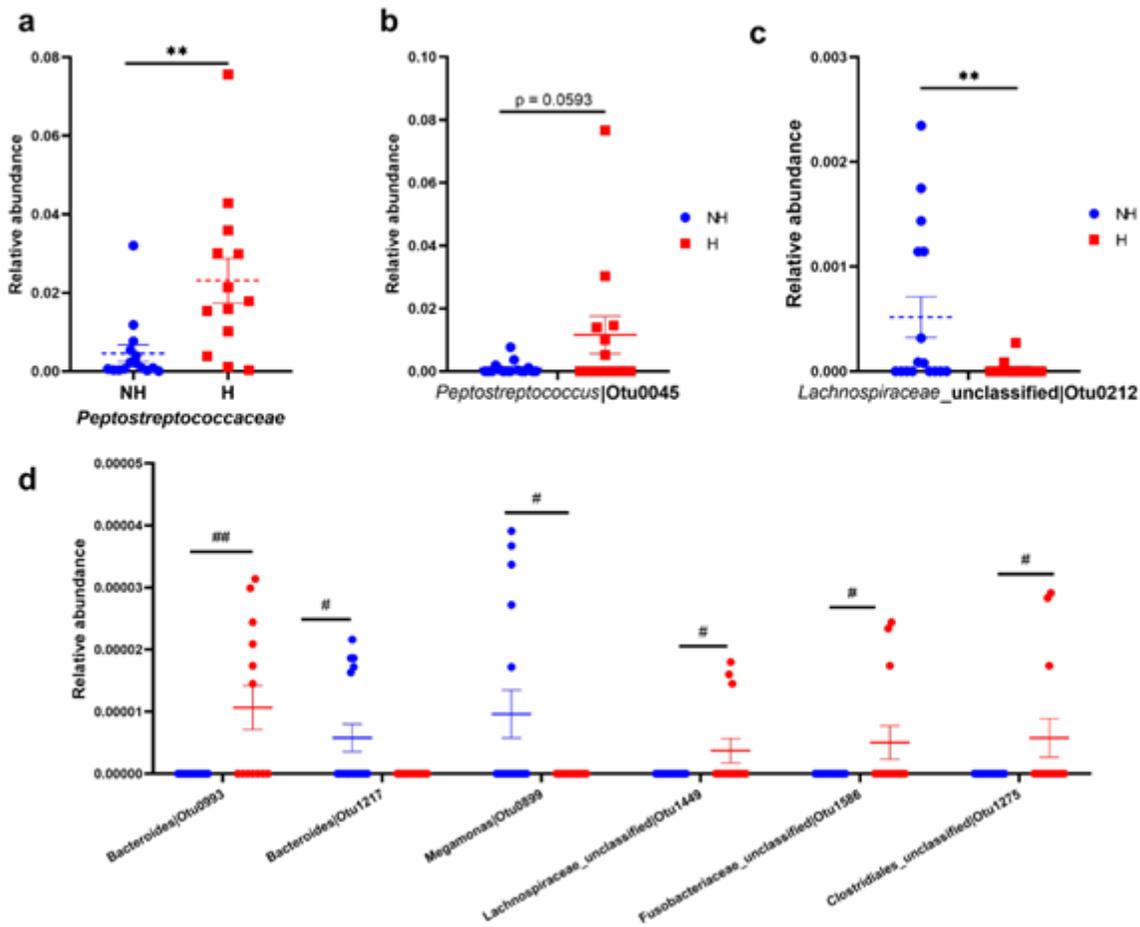


Figure 5

. Significantly differential relative abundance of bacteria in Hemafecia patients. Error bars indicate SEM.
 *: $p < 0.05$, **: $p < 0.01$, #: $qFDR < 0.1$, ##: $qFDR < 0.05$.

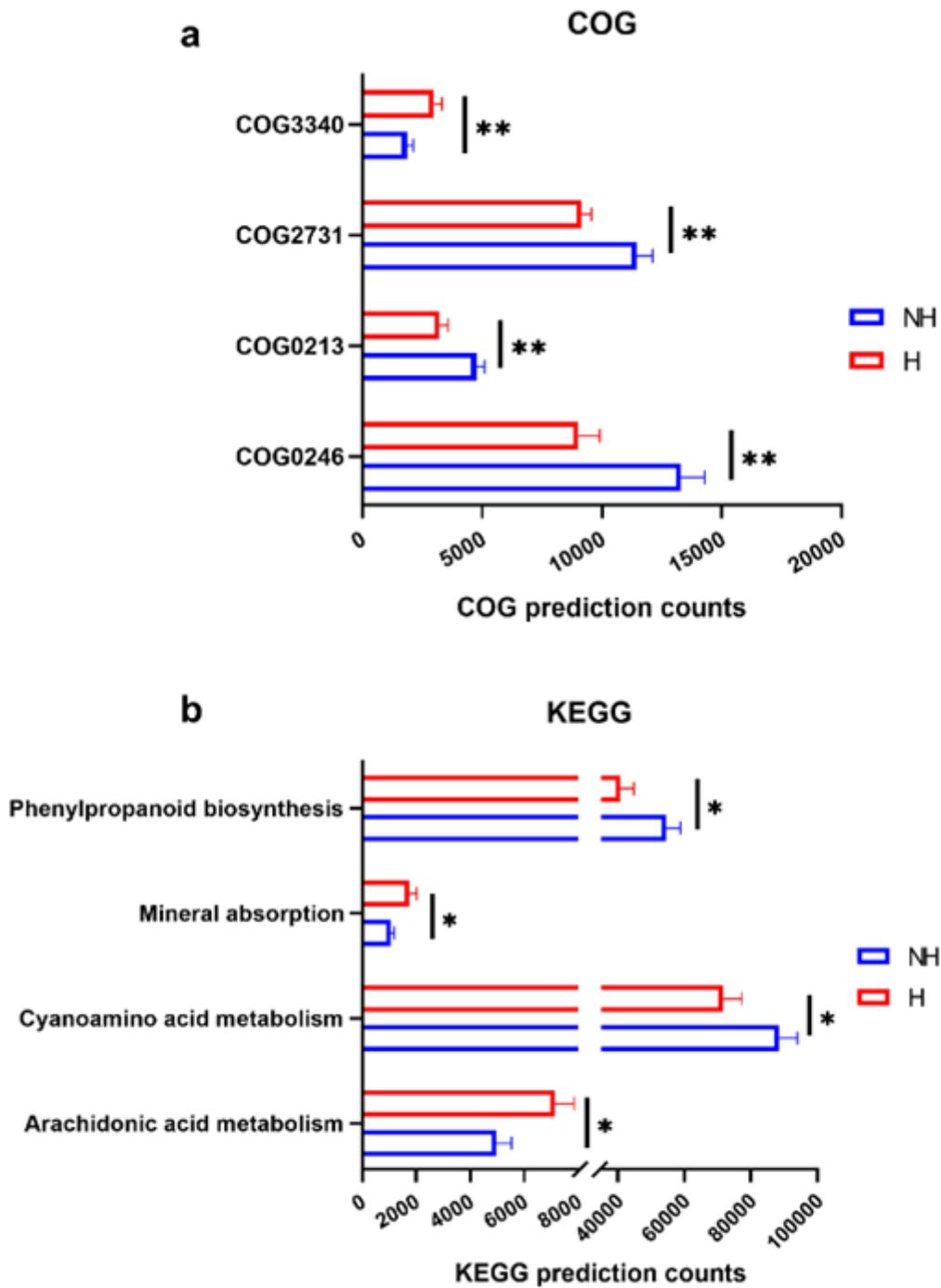


Figure 6

The distribution of functional categories. The distribution of (a) COG and (b) KEGG function with significant difference between two groups. The column length represents the corresponding samples in this metabolic pathway, and error bar represents the standard error of abundance. *: $p < 0.05$, **: $p < 0.01$.