Alteration of Gut Microbiota in type 2 Diabetes Complicated with Cholelithiasis Patients

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Research

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Alteration of gut microbiota in type 2 diabetes complicated with cholelithiasis patients

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ABSTRACT

Background： Epidemiological studies showed that diabetes patients are more prone to developing cholelithiasis. Although composition of gut microbiota in type 2 diabetes or cholelithiasis have been studied respectively, the underlying role of gut microbiota in developing from diabetes to cholelithiasis remains unclear. By 16S rRNA gene sequencing, the gut microbial composition of 33 healthy subjects, 53 type 2 diabetes, 31 cholelithiasis and 32 type 2 diabetes complicated with cholelithiasis patients were studied.

Results： Microbial diversity significantly decreased in type 2 diabetes complicated with cholelithiasis patients. In type 2 diabetes patients, phylum *Proteobacteria*, class *Gammaproteobacteria* and order *Lactobacillales* were significantly increased. In cholelithiasis patients, phylum *Bacteroidetes*, class *Bacteroidia*, order *Bacteroidales*, family *Bacteroidaceae* and genus *Bacteroides* were significantly increased. There were also significant increases of phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Lactobacillales*, family *Lactobacillaceae* and genus *Lactobacillus* in type 2 diabetes complicated with cholelithiasis patients accompanied by elevated serum triglyceride and total bile acids.

Conclusions: The results show similar but more intricate gut microbiota dysbiosis in type 2 diabetes complicated with cholelithiasis compared with type 2 diabetes, which might partially explain the mechanism of type 2 diabetes as the risk factor of cholelithiasis from the perspective of gut microbiota.

Keywords: Gut microbiota; Type 2 diabetes; Cholelithiasis; Type 2 diabetes complicated with cholelithiasis; 16S rRNA gene sequencing

INTRODUCTION

Type 2 diabetes and cholelithiasis are both common diseases with a high prevalence. The incidence of type 2 diabetes is about 10.8% in the United States[1]. Cholelithiasis is one of the most common gastrointestinal disease in the Europe and United States, with an incidence between 10-15% among adults[2,3]. In China, the

prevalence of type 2 diabetes is 9.7% and adult cholelithiasis is 8-10%[4]. Obesity, insulin resistance and abnormal metabolism are risk factors of type 2 diabetes, epidemiologic studies suggest that which are also risk factors of cholelithiasis[5]. As a metabolic system disease, type 2 diabetes may cause chronic damage of multiple tissues and organs. Gallstone formation was reported mainly related to the abnormal metabolism of cholesterol and bile acids[6]. Several studies have indicated that diabetes patients are at an increased risk of developing cholelithiasis[7,8]. An animal study has shown that insulin resistance directly promotes the formation of gallstones[9]. However, the underlying mechanism that diabetes patients are more prone to developing cholelithiasis remains unclear.

In recent years，increasing evidences implicated the role of gut microbiota in the development of multiple chronic diseases[10], including non-alcoholic fatty liver disease (NAFLD)[11], obesity[12], colorectal cancer[13], diabetes[14] and gallstone disease[15]. Thus, gut microbiota homeostasis is crucial to human health. Qin et al.'s study[14] found that opportunistic pathogens such as *Escherichia coli*, *Bacteroides caccae* and *Clostridium hathewayi* were more abundant in type 2 diabetes patients, however butyrate-producing bacteria such as *Faecalibacterium prausnitzii* and *Roseburia intestinalis* decreased. Wu et al.'s study[15] showed that the phylum *Proteobacteria* was significantly increased and the genera *Roseburia*, *Lachnospira* and *Faecalibacterium* were significantly decreased in gallstones patients. Maurer KJ[16] found that infection with certain strains of *Helicobacter* promotes gallstone formation in gallstone-susceptible C57L/J mice. The underlying mechanism of gut microbiota in the development of type 2 diabetes and cholelithiasis was not entirely clear. To date, literature has pointed that gut microbiota dysbiosis could lead to the decrease of secondary bile acids, then reduce the activation of bile acid receptors, and finally result in glucose metabolism disorder and type 2 diabetes occurrence[17]. Moreover, the research also indicated that gut microbiota could regulate enterohepatic recycling of bile acids, then disrupt the metabolism of cholesterol, and subsequently contribute to the formation of cholesterol gallstones[6,18,19].

Diabetes patients are at a higher risk of developing cholelithiasis[7,8]. However, the underlying role of gut microbiota in developing from diabetes to cholelithiasis is still unclear. To this end, 16S rRNA gene sequencing was carried out to analyze the microbial composition of gut from healthy subjects, type 2 diabetes patients,

cholelithiasis patients and type 2 diabetes complicated with cholelithiasis patients. Clarifying the gut microbial characteristics of patients with type 2 diabetes complicated with cholelithiasis will help improve our understanding of the potential mechanism that diabetes patients are more prone to developing cholelithiasis from the perspective of gut microbiota. This study provides insights into the role of gut microbiota in the progression from diabetes to cholelithiasis.

RESULTS

Characteristics of clinical samples and data

In this study, feces samples were collected from 33 healthy subjects (D1 group), 53 type 2 diabetes patients (D2 group), 31 cholelithiasis patients (D3 group) and 32 type 2 diabetes complicated with cholelithiasis patients (D4 group) (see Table 1 for detailed patient information). In total, 149 feces samples were obtained. All cholelithiasis patients were classified as cholesterol gallstones according to the definition proposed by Marschall et al.[20], i.e. a cholesterol content of 50-90%. When comparing the typical metabolites levels, we found that type 2 diabetes complicated with cholelithiasis patients (D4 group) have rather similar blood glucose levels with type 2 diabetes patients (D2 group) (Figure 1A), but they have higher triglyceride (Figure 1A) and total bile acids levels (Figure 1B) compared with mere cholelithiasis patients (D3 group).

By 16S rRNA gene sequencing, the microbial composition of gut was analyzed. An average of 83,615 raw reads was obtained for per sample. After quality filtering, a mean of 79,042 high-quality reads were retrieved. The sequencing data is enough to reflect species diversity (Supplementary Figure 1). Sequences were clustered into 3030 operational taxonomic units (OTUs) based on 97% sequence similarity, with 2232 OTUs in healthy subjects (D1 group), 2246 OTUs in type 2 diabetes patients (D2 group), 1971 OTUs in cholelithiasis patients (D3 group) and 2009 OTUs in type 2 diabetes complicated with cholelithiasis patients (D4 group). The OTUs differences among four groups were statistically significant (P<0.05, Kruskal-Wallis test) (Table 1), of which differences between D4 and D1 groups, D4 and D3 groups were statistically significant (D4 vs D1: P=0.008, D4 vs D3: P=0.0051, Mann-Whitney test, data not shown), and OUT richness was least in D4 group. OTUs were annotated at different classification levels using the Silva132 database. At the phylum level, three phyla predominated: *Firmicutes*, *Bacteroidetes* and *Proteobacteria*.

Microbial diversity significantly decreased in type 2 diabetes complicated with cholelithiasis patients

The intra-community alpha diversity, which represents microbial diversity, was analyzed. The observed species, which represent microbial richness, were significantly decreased in type 2 diabetes complicated with cholelithiasis patients (D4 group) compared with cholelithiasis patients (D3 group) (P=0.0031, Wilcoxon rank-sum test) and healthy subjects (D1 group) (P=0.0179, Wilcoxon rank-sum test) (Figure 2A). The shannon index between D4 and D3 groups (P=0.0224, Wilcoxon rank-sum test), D4 and D2 groups (P=0.0159, Wilcoxon rank-sum test), D4 and D1 groups (P=0.0091, Wilcoxon rank-sum test) were also statistically different (Figure 2B). Patients with type 2 diabetes complicated with cholelithiasis (D4 group) presented the lowest shannon index. The results suggested that gut microbiota dysbiosis was more intricate in patients with type 2 diabetes complicated with cholelithiasis. For other alpha diversity indices, such as simpson, chao 1, good's coverage and PD_whole tree, consistent results were obtained (data not shown).

The beta diversity was also analyzed to discover whether gut microbial community differences were significantly existed between groups. We found that unweighted unifrac based-beta diversity between D4 and D3 groups, D4 and D1 groups were significantly different (P<0.01, Wilcoxon rank-sum test), but there was no statistical difference between D4 and D2 groups (P=0.3858, Wilcoxon rank-sum test) (Figure 2C). Moreover, principal co-ordinates analysis (PCoA) using unweighted unifrac distance algorithm showed that the microbial community structureof type 2 diabetes complicated with cholelithiasis patients (D4 group) was similar with type 2 diabetes patients (D2 group)(P=0.051, AMOVA)，whereas was significant different from cholelithiasis patients (D3 group) (P=0.001, AMOVA) (Figure 2D). This finding suggested that type 2 diabetes complicated with cholelithiasis might share common gut microbial characteristics with type 2 diabetes.

Type 2 diabetes, cholelithiasis and type 2 diabetes complicated with cholelithiasis remodeled the gut microbial communities

At the phylum level, the relative abundance of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* was more than 96% in all groups. *Firmicutes* was most common in healthy subjects (D1 group) (approximately 57.75%), but decreased to 48.23%, 44.64% and 44.60% in type 2 diabetes patients (D2 group), cholelithiasis patients (D3 group)

and type 2 diabetes complicated with cholelithiasis patients (D4 group) , respectively (Figure 3A). In contrast, *Bacteroidetes* increased from 32.89% in healthy subjects (D1 group) to 39.24%, 43.87% and 36.44%, *Proteobacteria* increased from 6.91% in healthy subjects (D1 group) to 9.12%, 8.53% and 15.95%, in type 2 diabetes patients (D2 group), cholelithiasis patients (D3 group) and type 2 diabetes complicated with cholelithiasis patients (D4 group) , respectively. *Verrucomicrobia* slightly increased in cholelithiasis patients (D3 group) and type 2 diabetes complicated with cholelithiasis patients (D4 group) (Figure 3A). The gut microbial composition of all samples at the phylum level were shown in Supplementary Figure 2.

Among the top 10 genera, *Bacteroides* predominated, which increased from 26.46% in healthy subjects (D1 group) to 29.46%, 37.00% and 26.55% in type 2 diabetes patients (D2 group), cholelithiasis patients (D3 group) and type 2 diabetes complicated with cholelithiasis patients (D4 group) , respectively. The genera *Faecalibacterium* and *Megamonas* were most common in healthy subjects (D1 group), *Faecalibacterium* decreased from 11.30% in healthy subjects (D1 group) to 5.89%, 7.65% and 5.57%, *Megamonas* decreased from 4.44% in healthy subjects (D1 group) to 0.53%, 0.44% and 0.87%, in type 2 diabetes patients (D2 group), cholelithiasis patients (D3 group) and type 2 diabetes complicated with cholelithiasis patients (D4 group) , respectively (Figure 3B). The relative abundance of genus *unidentified Enterobacteriaceae* increased from 1.47% in healthy subjects (D1 group) to 3.38%, 4.23% and 5.40% in type 2 diabetes patients (D2 group), cholelithiasis patients (D3 group) and type 2 diabetes complicated with cholelithiasis patients (D4 group), respectively (Figure 3B). The gut microbial composition of all samples at the genus level were shown in Supplementary Figure 3.

Similarity and association analysis of gut microbial composition

To further investigate the similarities and associations of gut microbial communities, unweighted pair group method with arithmetic mean (UPGMA, weighted unifrac) clustering analysis at the phylum level was performed. The resulted cluster tree showed that four groups were divided into three different clusters, type 2 diabetes complicated with cholelithiasis patients (D4 group) was in cluster A, type 2 diabetes patients (D2 group) and cholelithiasis patients (D3 group) were in cluster B, and healthy subjects (D1 group) was in cluster C (Figure 3C). The results suggested that the gut microbial composition of mere type 2 diabetes and mere cholelithiasis was

similar at the phylum level, whereas some alterations were generated in type 2 diabetes complicated with cholelithiasis.

Furthermore, Venn diagram analysis showed that 68 OTUs were common to type 2 diabetes (D2 group) and cholelithiasis (D3 group), 94 OTUs were common to type 2 diabetes (D2 group) and type 2 diabetes complicated with cholelithiasis (D4 group), 52 OTUs were common to cholelithiasis (D3 group) and type 2 diabetes complicated with cholelithiasis (D4 group), and 70 OTUs were shared by D2, D3 and D4 groups (Figure 3D). Among the 70 OTUs, the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*, and the genera *Sutterella*, *Bacteroides*, *Dialister* and *Parasutterella*, were significantly different between D2, D3, D4, and D1 groups (data not shown).

Differences in gut microbial components

The linear discriminant analysis (LDA) effect size (LEfSe) analysis according to LDA scores was performed by using non-parametric Kruskal-Wallis rank-sum test to detect species with significant differences between groups. The results showed that as compared with healthy subjects (D1 group), the phylum *Proteobacteria*, class *Gammaproteobacteria* and order *Lactobacillales* were more abundant in type 2 diabetes patients (D2 group), whereas the genus *Faecalibacterium* was lower (Figure 4A and 4B). In cholelithiasis patients (D3 group), the relative abundances of *Bacteroidetes* taxa groups including class *Bacteroidia*, order *Bacteroidales*, family *Bacteroidaceae* and genus *Bacteroides* were significantly increased, and the phylum *Firmicutes* and genus *Faecalibacterium* were significantly decreased (Figure 4C and 4D). In patients with type 2 diabetes complicated with cholelithiasis (D4 group), both *Proteobacteria* taxa groups including class *Gammaproteobacteria*, order *Enterobacteriales*, family *Enterobacteriaceae* and genus *unidentified Enterobacteriaceae*, and class *Bacilli* taxa groups including order *Lactobacillales*, family *Lactobacillaceae* and genus *Lactobacillus*, were significantly enriched. On the contrary, *Firmicutes* taxa groups including class *Clostridia*, order *Clostridiales*, family *Ruminococcaceae*, genera *Faecalibacterium*, *Megamonas* and *Subdoligranulum* were significantly decreased (Figure 4E and 4F).

As compared with type 2 diabetes patients (D2 group), the *Proteobacteria* taxa groups which includes class *Gammaproteobacteria*, order *Enterobacteriales* and family *Enterobacteriaceae* were significantly increased in type 2 diabetes complicated with cholelithiasis patients (D4 group) (Figure 5A and 5B). However, there exists more species with significant differences between cholelithiasis patients (D3 group) and type

2 diabetes complicated with cholelithiasis patients (D4 group) (Figure 5C and 5D). From these results, we found that the tendency of gut microbiota alterations in type 2 diabetes complicated with cholelithiasis patients was more similar with mere type 2 diabetes patients, rather than cholelithiasis patients.

DISCUSSION

Several studies have shown that gut microbiota is partially associated with the pathogenesis of type 2 diabetes[14] and cholelithiasis[15]. Epidemiological studies indicated that diabetes patients have an increased risk of gallstones formation[7,8]. However, no association study has been carried out to clarify the underlying role of gut microbiota in developing from type 2 diabetes to cholelithiasis. This study demonstrated the intricate gut microbiota dysbiosis present with type 2 diabetes complicated with cholelithiasis and discovered an underlying association between type 2 diabetes and type 2 diabetes complicated with cholelithiasis, that is, gut microbiota has the potential to promote type 2 diabetes progressing to type 2 diabetes complicated with cholelithiasis. This is the first study to clarify the microbial composition of gut with type 2 diabetes complicated with cholelithiasis. The results of this study suggested that the gut microbiota alterations of type 2 diabetes complicated with cholelithiasis patients was similar but more intricate compared with mere type 2 diabetes patients, which were supported with methods such as PCoA analysis, UPGMA analysis and LEfSe analysis, as will be discussed in the following.

This study discovered similar but more intricate changes of gut microbial composition in type 2 diabetes complicated with cholelithiasis patients compared with mere type 2 diabetes patients. Unweighted unifrac-based PCoA analysis identified a high degree of microbial community structure similarity between type 2 diabetes complicated with cholelithiasis patients and type 2 diabetes patients (Figure 2D). Meanwhile, 94 OTUs were shared by type 2 diabetes complicated with cholelithiasis patients and type 2 diabetes patients by Venn diagram (Figure 4B). However, weighted unifrac-based UPGMA analysis at the phylum level showed that type 2 diabetes complicated with cholelithiasis patients and type 2 diabetes patients were grouped into

different clusters (Figure 4A), suggesting there exists unique gut microbial components, which might complicate the gut microbiota dysbiosis. Interestingly, serum triglyceride and total bile acids levels were significantly increased compared with mere cholelithiasis patients (Figure 1). The abnormal metabolism condition indirectly reflects the intricate gut microbiota dysbiosis within type 2 diabetes complicated with cholelithiasis patients.

Within the gut of cholelithiasis patients, the *Bacteroidetes* taxa groups which includes various pathogens such as *Bacteroidia*, *Bacteroidales*, *Bacteroidaceae* and *Bacteroides* were significantly increased, the phylum *Firmicutes* and genus *Faecalibacterium* were notably decreased (Figure 5C and D). However, within the gut of type 2 diabetes complicated with cholelithiasis patients, *Proteobacteria*taxa groups including *Gammaproteobacteria*, *Enterobacteriales*, *Enterobacteriaceae* and *unidentified Enterobacteriaceae* were significantly enriched, the phylum *Firmicutes* and genera *Faecalibacterium*, *Megamonas* and *Subdoligranulum* were significantly decreased (Figure 5E and F), which were quite different from mere cholelithiasis patients but partial similar with mere type 2 diabetes patients (Figure 5A and B). Furthermore, we found that there exists an increase of the phylum *Proteobacteria* taxa groups which includes *Gammaproteobacteria*, *Enterobacteriales* and *Enterobacteriaceae* in type 2 diabetes complicated with cholelithiasis patients compared with mere type 2 diabetes patients (Figure 6A and B), which mainly containing opportunistic pathogenic bacteria of human beings. The results suggested that type 2 diabetes complicated with cholelithiasis patients had similar but more intricate gut microbiota dysbiosis compared with mere type 2 diabetes patients. These bacterial species with significant differences might have a potential association with cholelithiasis occurrence in type 2 diabetes patients.

Gut microbiota can regulate bile acids metabolism[21]. The abnormal metabolism of bile acids is the main pathogenesis of cholesterol gallstones[6]. Several studies have indicated that gut microbiota can suppress bile acids synthesis through activating the

nuclear receptor Farnesol X receptor (FXR)[22,23]. The phyla *Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes* can produce hydroxysteroid dehydrogenases (HSDHs), which can catalyze bile acids into oxo- (or keto-) bile acids[24-26]. Some bacteria such as *Lactobacilli*, *Bifidobacteria* and *Bacteroides* can secrete bile salt hydrolase (BSH) and then de-conjugate bile acids, thus regulate the microbial metabolism of bile acids[27-29]. In this study, the relative abundance of *Lactobacillus*, *Bifidobacterium* and *Bacteroides* were increased in the gut of type 2 diabetes patients, suggesting that the changes of bacteria with BSH activity in type 2 diabetes patients might lead to the abnormal metabolism of bile acids and subsequently contribute to the pathogenesis of cholelithiasis, which explained that diabetes patients are at an increased risk of developing cholelithiasis from the perspective of gut microbiota. However, further research is still needed to investigate the effect of these bacteria on gallstone formation.

CONCLUSION

Gut microbial characteristics and abnormal metabolism condition in type 2 diabetes complicated with cholelithiasis patients was first discovered in this study, that is, the gut microbial composition was partial similar with type 2 diabetes patients, whereas present with more intricate gut microbiota dysbiosis. The results of this study suggested that there exists certain bacterial species in type 2 diabetes patients, which are associated with the occurrence of cholelithiasis. Thus, maintaining the gut microbiota homeostasis of type 2 diabetes patients might help reduce the risk of subsequently developing cholelithiasis.

METHODS

Patients and feces collection

From September 2017 to September 2018, 53 patients with mere type 2 diabetes from the department of endocrinology, 31 patients with mere cholelithiasis and 32 patients with type 2 diabetes complicated with cholelithiasis from the department of general surgery, and 33 healthy subjects from the department of physical examination

were enrolled, with average age of 58.5±9.6 and a male ratio of 45.6%. None of patients indicated that they had any other infectious or underlying diseases. None of the healthy subjects indicated that they had suffered type 2 diabetes, cholelithiasis, or any other infectious and metabolic diseases. All participants have not been treated with antibiotics in the last three months. The metabolic indexes were derived from retrospective medical records. Feces samples from all patients and healthy subjects were collected and placed in sterile Eppendorf tube, glycerin was added to final concentration of 20%. All feces samples were stored at −80°C.

DNA preparation, library construction, and sequencing

Genomic DNA was extracted from feces samples using the QIAamp DNA Stool Mini Kit (Qiagen) and then agarose gel electrophoresis was performed to detect the purity and concentration of extracted DNA. As template, the qualified genomic DNA was prepared with concentration of 1 ng/μl to amplify gene sequence in V4 region of bacteria 16S ribosomal RNA. The forward primer (515F) was 5'-GTGCCAGCMGCCGCGGTAA-3'. The reverse primer (806R) was 5'-GGACTACHVGGGTWTCTAAT-3'. Then the PCR products were mixed with equal amounts and purified by GeneJET™ PCR Purification Kit (Thermo). DNA libraries were prepared with Ion Plus Fragment Library Kit (Thermo) and then sequenced on Thermoscher's Ion S5TM XL platform. After shearing the low-quality reads by using Cutadapt (V1.9.1) , the sample data was separated from the obtained reads according to barcode. The barcode and primer sequences were truncated to obtain the raw data (raw reads). Then, the chimeric sequence was detected and removed by comparing raw reads sequence with species annotation database according to UCHIME Algorithm. Finally, the final effective data was obtained (clean reads).

Processing of sequencing data and bioinformatics analysis

All clean reads data were clustered as OTUs according to 97% identity. Species annotation of these OTUs were performed using mothur method (with a threshold of 0.8~1). Classification information at each level were obtained: from kingdom to species.

Finally, the data of each sample were homogenized. QIIME (version 1.9.1) was used to calculate community diversity index and unifrac distance (alpha diversity and beta diversity analysis). The rarefaction curve, rank abundance curve, species accumulation curve, microbial abundance histogram, Venn diagram and UPGMA group clustering tree were drawn using R software (version 2.15.3). LEfSe analysis was performed using LEfSe software with a default setting of LDA Score = 4.

Statistical analyses

SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) and R software (version 2.15.3) were used for statistical analysis. General characteristics are expressed as mean±standard deviation (SD), mean±standard error of mean (SEM) or percentage. Kruskal-Wallis non-parametric test was used for multiple group comparison. Student's t-test and Wilcoxon rank-sum testwere used for two group comparison. $P<0.05$ was considered statistically significant.

Ethics

The study was approved by the Ethics Committee of Peking University Shougang Hospital (SGYYZ201701). All study participants provided informed written consent prior to study enrollment.

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Author contributions

Gu J and Hu SK designed and coordinated the study; Chen JJ, Yan LL and Ma XF performed the experiments; Chen JJ and Yan LL analyzed the data and wrote the manuscript; Yuan P, Zhao F, Han ZH, Liu JS, Wang WB, Zhou DH, Zhao HY, Feng N and Huang DD contributed reagents and materials.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Supplementary Material:

Figure S1 Quality analysis of sequencing data.

Figure S2 Relative abundance of each sample at the phylum level.

Figure S3 Relative abundance of each sample at the genus level.

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Figure Legends

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Figure 3: The composition of gut microbiota in 4 groups. A: Showing the top 10 phyla; B: Showing the top 10 genera. C: Weighted unifrac distance based-UPGMA cluster tree analysis at the phylum level, left: UPGMA clustering tree structure, right: relative abundance distribution of species; D: Unique and shared OTUs of gut microbiota among four groups are represented by Venn diagram. These numbers show the number of OTUs in different groups. There is a total of 3006 OTUs. (D1: Healthy subjects; D2: Type 2 diabetes; D3: Cholelithiasis; D4: type 2 diabetes complicated with cholelithiasis.)

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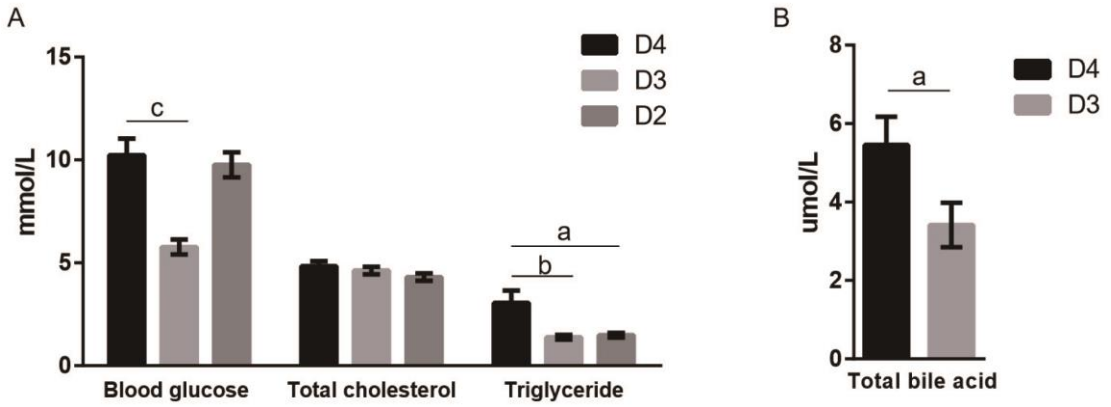
Table 1 Characteristics of clinical samples and data

	D1 group (healthy subjects, n=33)	**D2 group (type 2 diabetes, n=53)**	**D3 group (cholelithiasis, n=31)**	**D4 group (type 2 diabetes complicated with cholelithiasis, n=32)**	**Total (n=149)**
Age (mean ± SD)	51.0±4.5	61.6±9.7	60.1±11.0	59.5±7.8	58.5±9.6
Male (no. ,%)	17, 51.5%	29, 54.7%	8, 25.8%	14, 43.8%	68, 45.6%
Total OTUs	2232	2246	1971	2009	3030
OTUs per samples*	705.0±122.7	661.1±114.5	700.5±94.0	622.9±117.3	670.8±116.2

*P<0.05, using Kruskal-Wallis nonparametric test.



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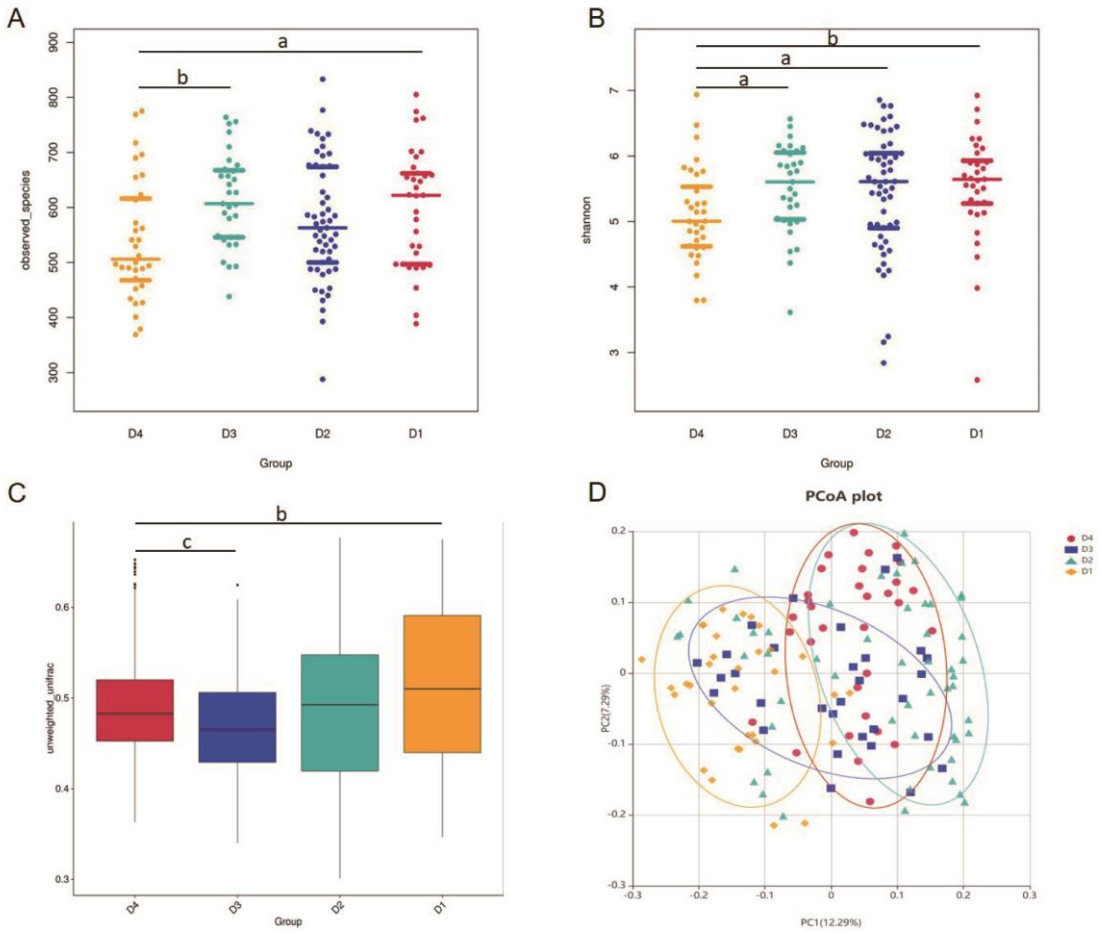

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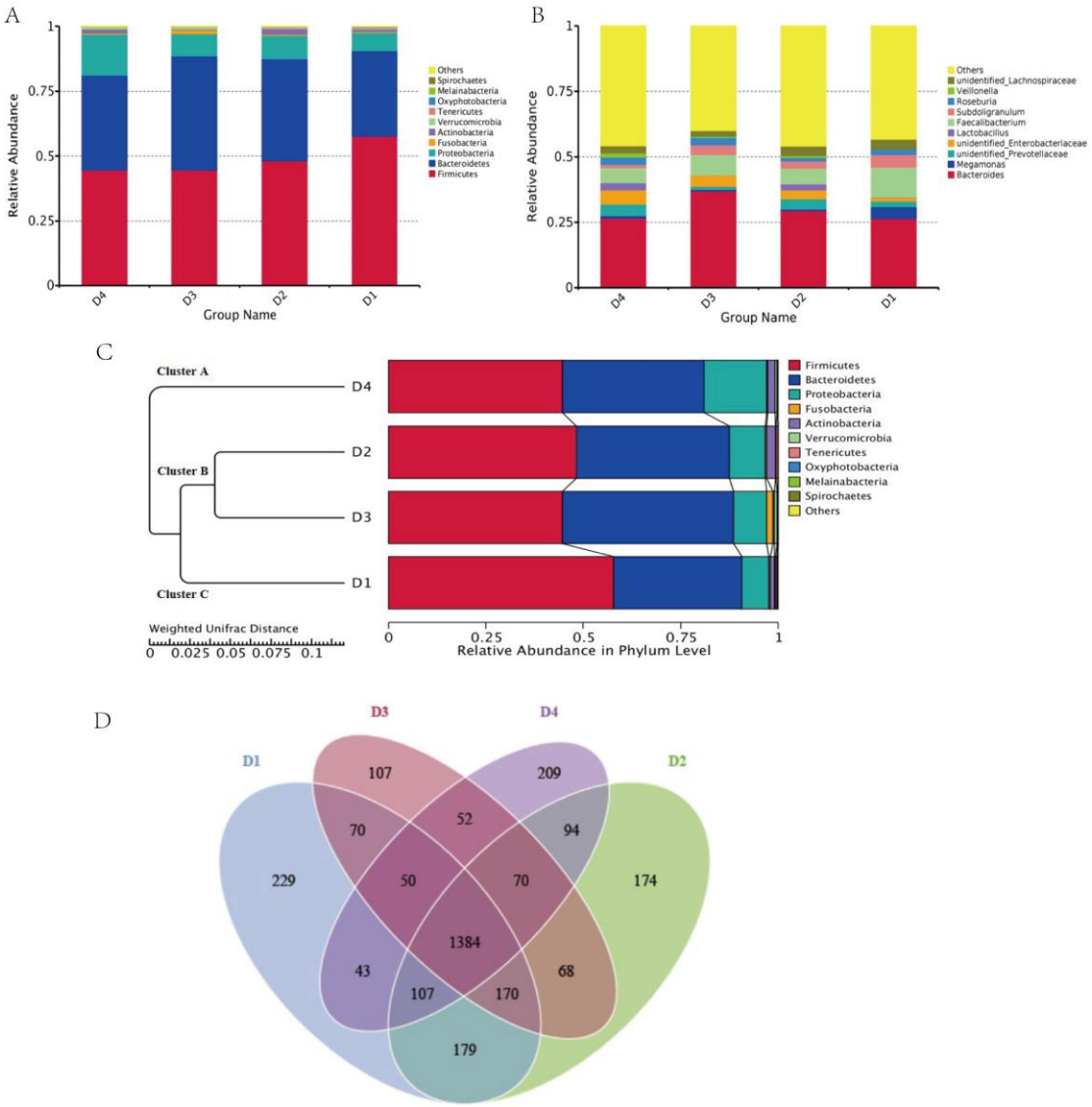

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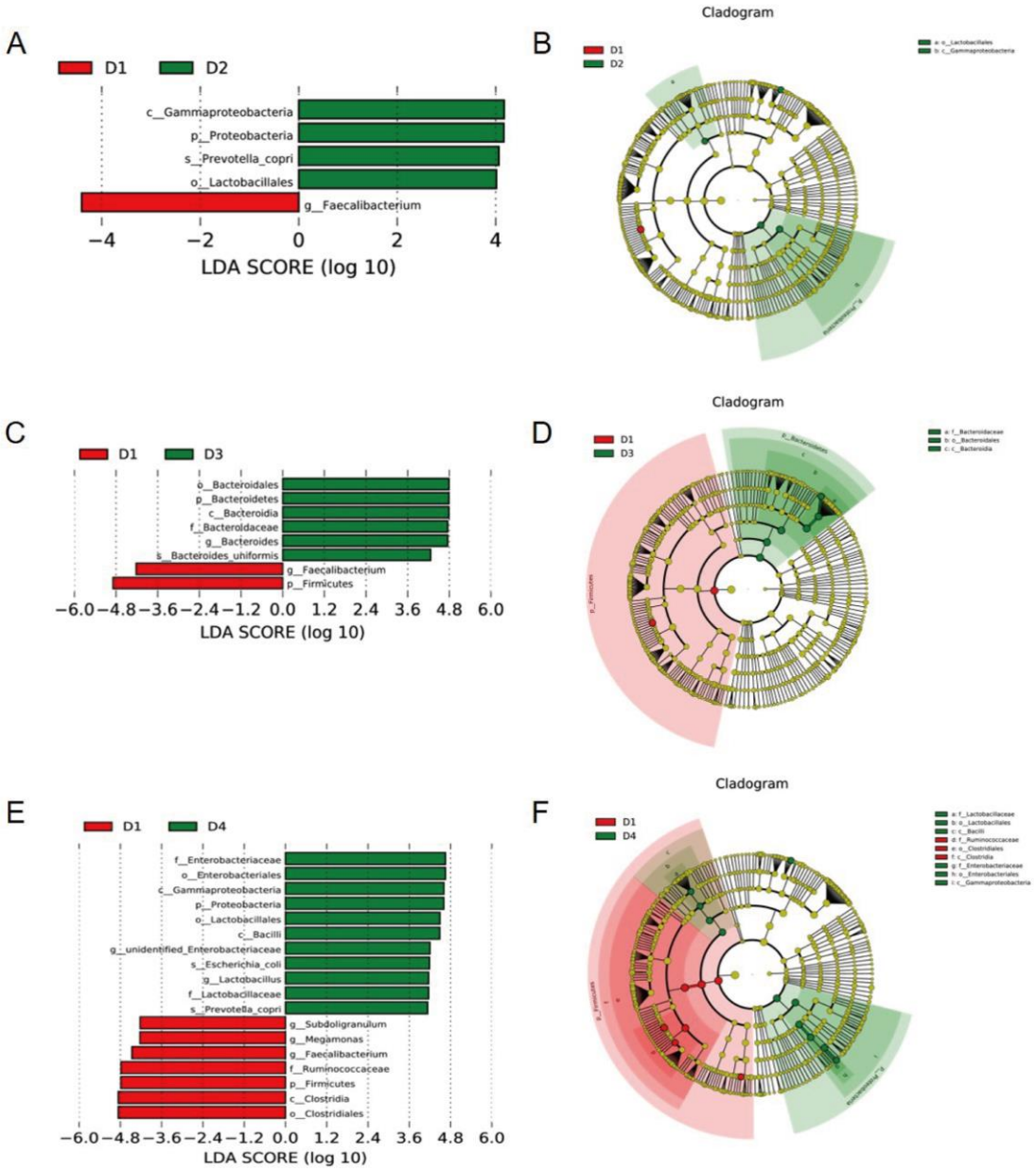

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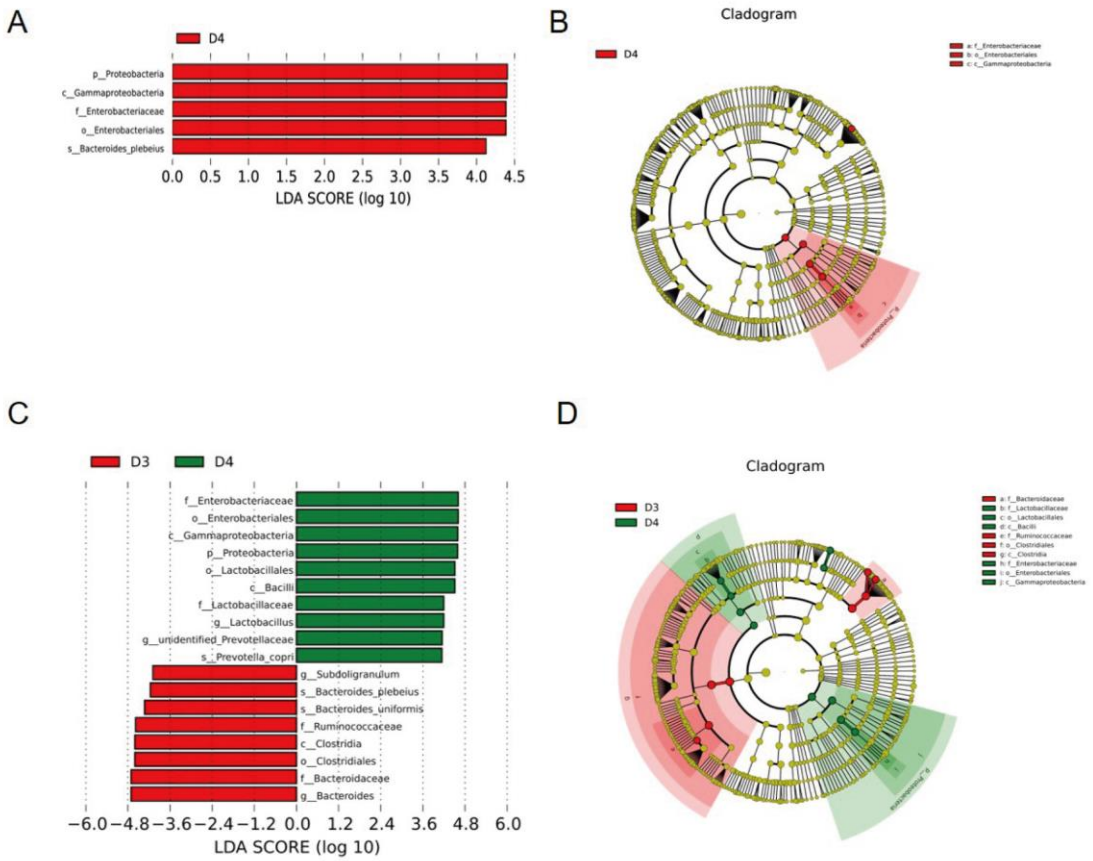

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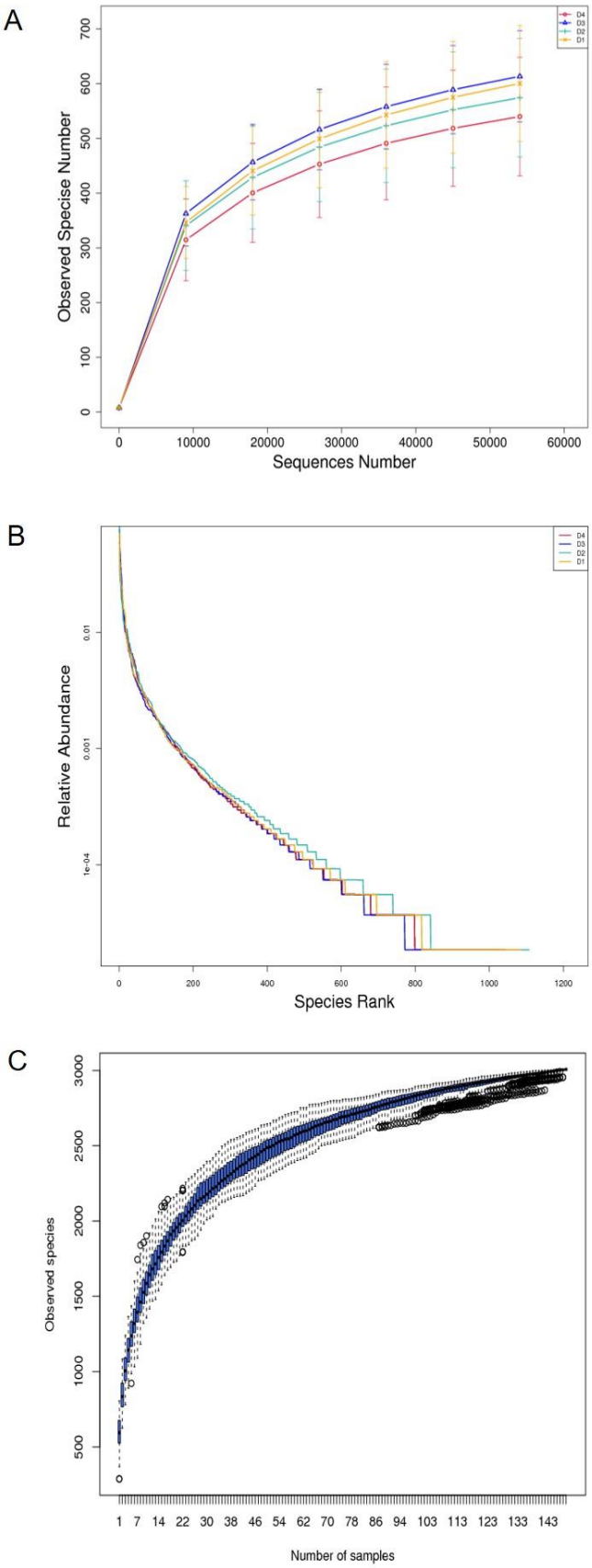
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Sequences Number
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300
400
500
600
700
0
10000
20000
30000
40000
50000
60000
D4
D3
D2
D1
B
Relative Abundance
Species Rank
0.01
0.001
1e-04
0
200
400
600
800
1000
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C
Observed species
Number of samples
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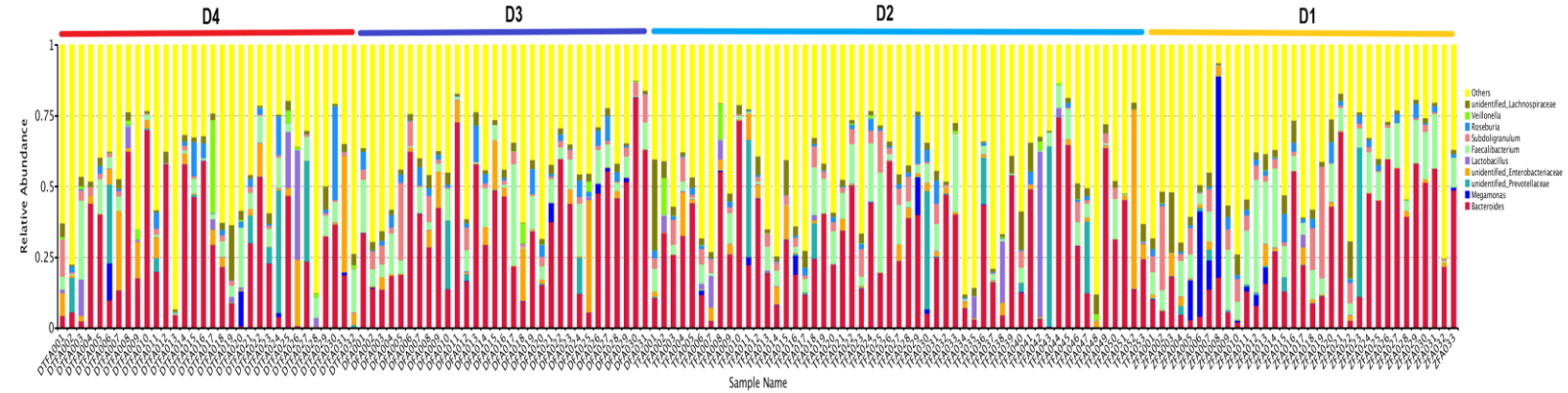
1000
1500
2000
2500
3000
1 7 14 22 30 38 46 54 62 70 78 86 94 103 113 123 133 143

Fig S1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D3
D2
D1
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Sample Name
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Roseburia
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Lactobacillus
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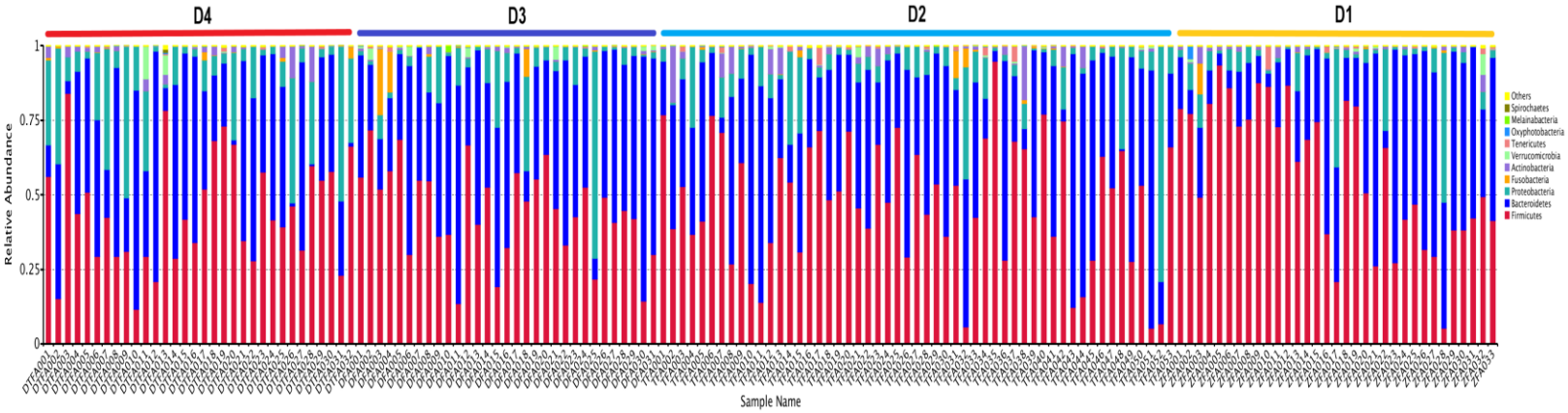
unidentified_Prevotellaceae
Megamonas
Bacteroides

Fig S3


D4
D3
D2
D1
Relative Abundance
Sample Name
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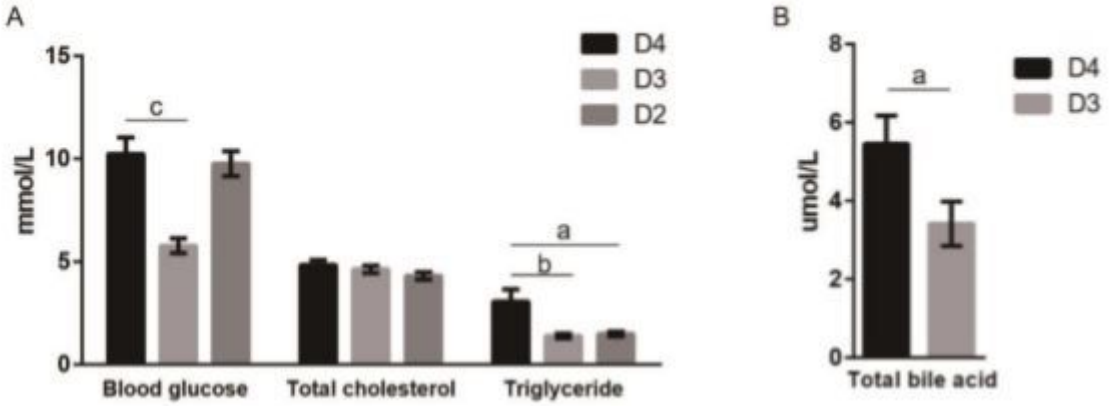
Spirochaetes
Melainabacteria
Oxyphotobacteria
Tenericutes
Verrucomicrobia
Actinobacteria
Fusobacteria
Proteobacteria
Bacteroidetes
Firmicutes

Fig S2

Figures



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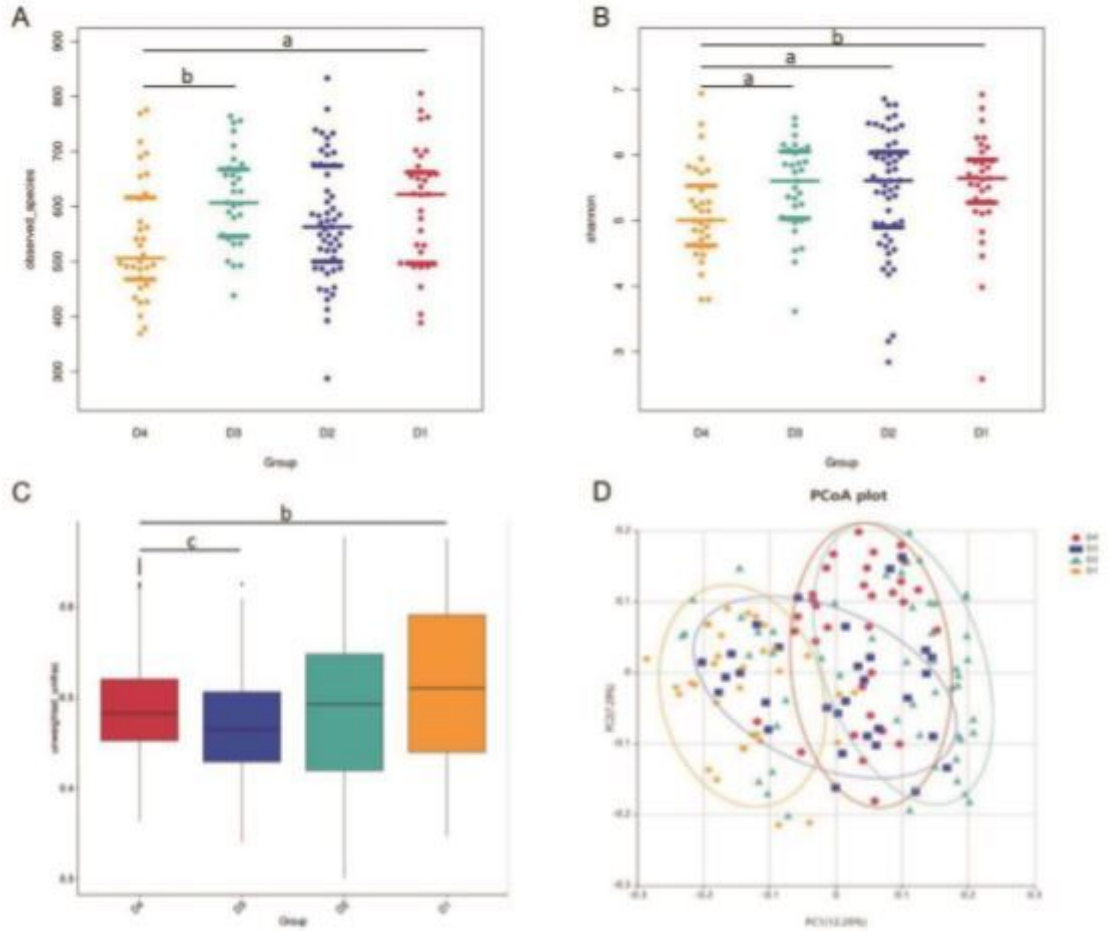

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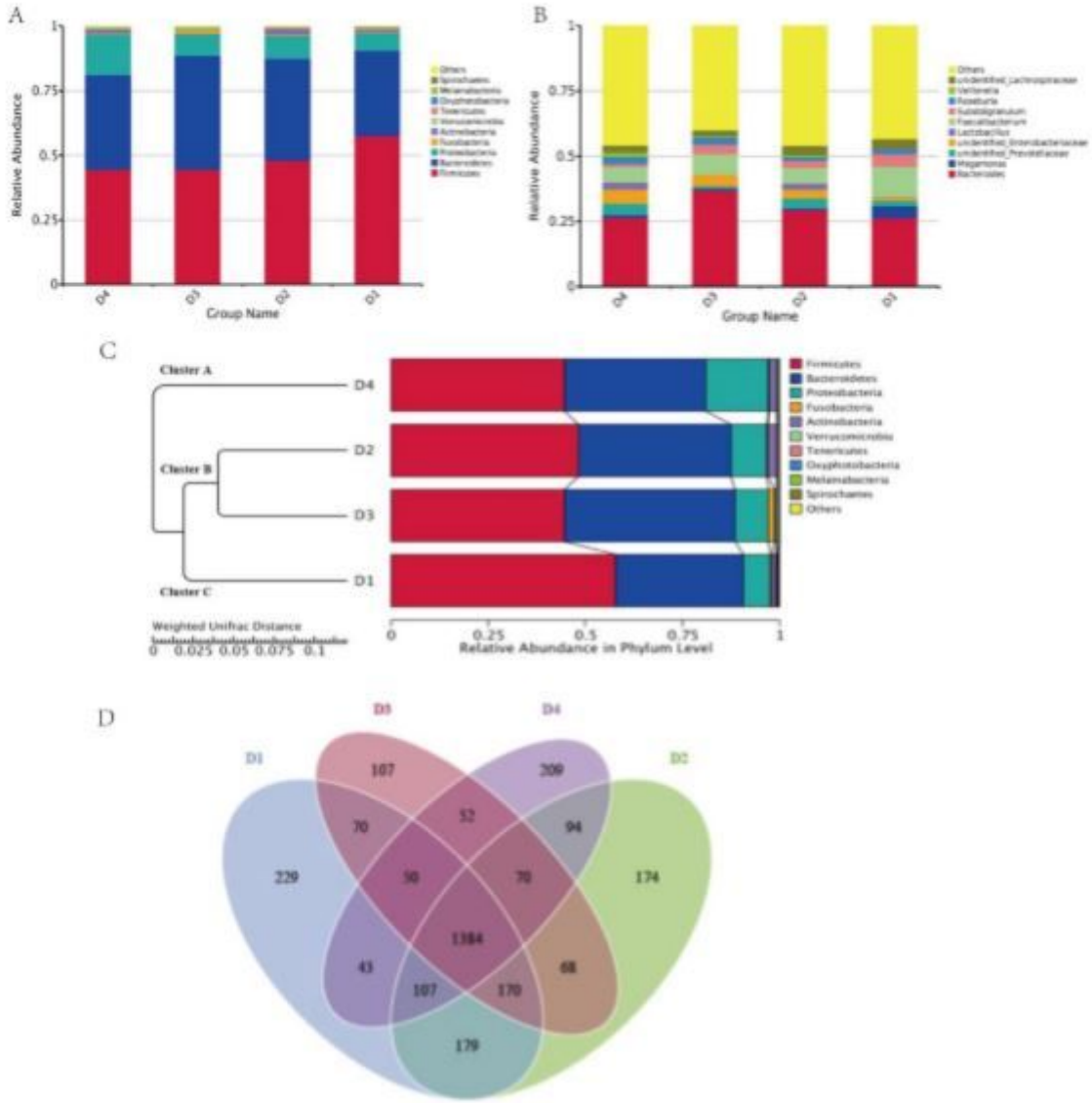

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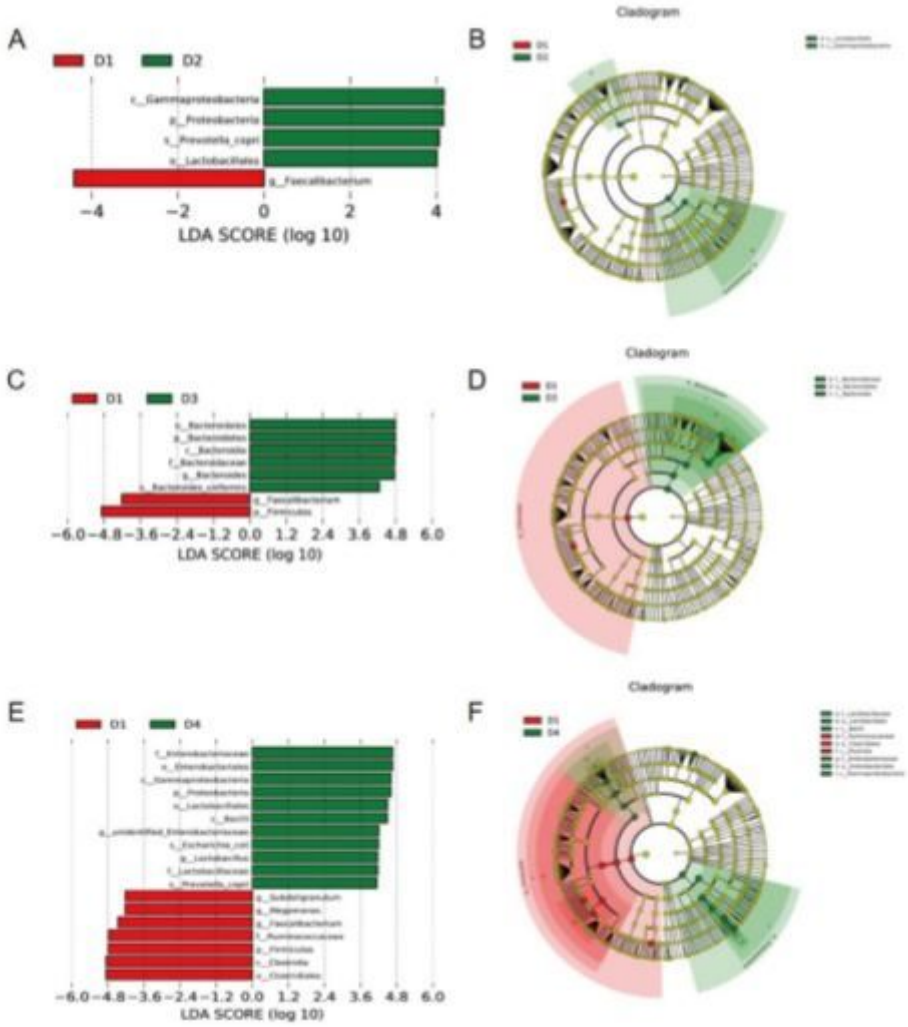

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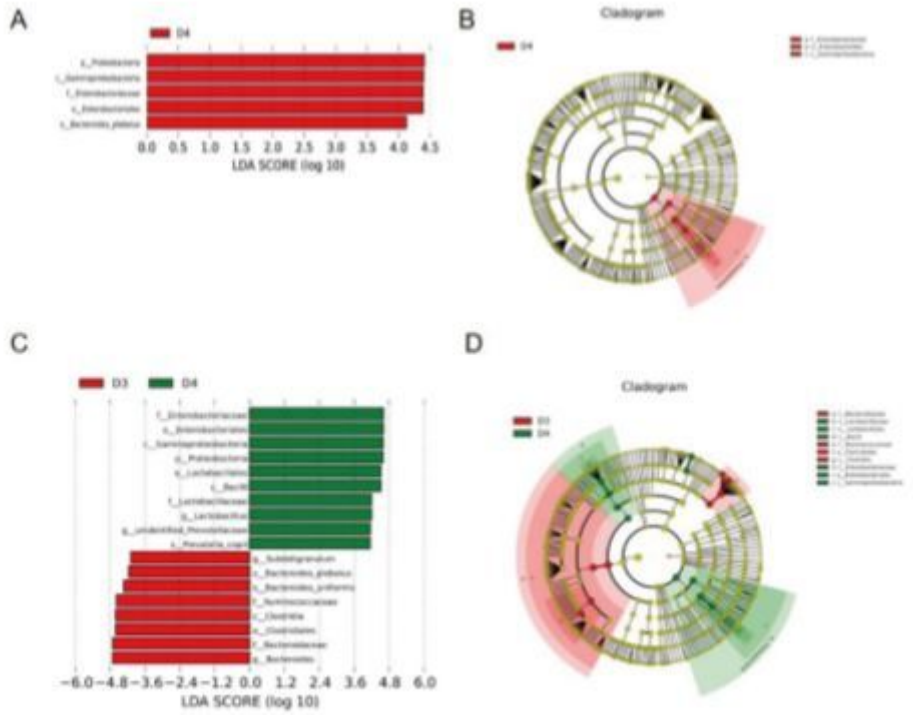

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