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ORIGINAL RESEARCH

Characteristics of Gut Microbiota in Cerulein-Induced Chronic Pancreatitis

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Background: Although clinical trials and animal models have evaluated the alterations of the microbiome in chronic pancreatitis (CP), the gut microbiota composition and diversity in cerulein-induced CP is unknown. This study aimed to evaluate the changes of gut microbiota in a CP mice model, and to determine whether these gut microbiota changes were consistent with those in patients with CP.

Methods: A total of ten male C57BL/6j mice were randomly divided into two groups. The experimental group were injected intraperitoneally with cerulein, while the normal control group received comparable injections of saline, the entire molding process lasted 6 weeks. Histology analysis was used to assess pancreatic morphological changes and fibrosis, meanwhile the gut microbiota composition and diversity were analyzed by high throughput sequencing. Spearman correlation analysis was used to determine whether body weight and weight changes were associated with changes in gut microbial abundance.

Results: The bacterial richness and diversity of CP mice decreased, and the gut microbiota changed, including lower levels of *Firmicutes*, decreased *Firmicutes/Bacteroidetes* ratio and increased abundance of *Bacteroidetes, Actinobacteria* and *Verrucomicrobia*. We found statistically significant differences in body weight and weight changes between the two groups. However, there was no significant correlation between alterations of gut microbiota and in body weight and weight changes.

Conclusion: Our results showed that the gut microbiota in cerulein-induced CP was changed.

Keywords: chronic pancreatitis, cerulein, gut microbiota, body weight, weight changes

Introduction

Gut microbiota plays an important role in human physiology by influencing metabolism, regulating the mucosal immune system, producing vitamins, promoting digestion and regulating intestinal architecture.[1,2] Gut dysbiosis is associated with the pathogenesis of various gastrointestinal diseases such as inflammatory bowel disease and irritable bowel syndrome,[3] as well as other diseases such as the metabolic syndrome, obesity, diabetes[4–6] and pancreatic diseases including chronic pancreatitis (CP).[7] CP is a chronic inflammatory disease of the pancreas, which is characterized by irreversible morphological changes that usually result in pain and/or permanent loss of function. This progressive, irreversible disease causes the destruction of healthy pancreatic tissue and the formation of fibrous scar tissue. This is followed by a gradual loss of exocrine and endocrine function, as well as clinical manifestations such as increased fat, abdominal pain and diabetes.[8] Evidence for gut microbial dysbiosis is suggested by the frequent observation of small intestinal bacterial

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overgrowth in CP patients.[9] In addition, higher levels of *Enterobacteriaceae* are found in CP, suggesting a relative abundance of *Enterobacteriaceae* could promote a systemic inflammatory response thus contributing to the development of CP.[10] Gut microbiota alterations in the microbiome may have pathogenic effects, while normalization of the microbiome may have therapeutic effects. Nevertheless, functional roles of identified bacterial species and relevance to pathogenesis in CP remain unclear.

Much of the evidence that gut microbial dysbiosis is thought to contribute to the pathogenesis of CP comes from animal models and these models will continue to be valuable for testing hypotheses. Developing appropriate animal models is an essential step in gut microbiota of CP research. A variety of experimental models of CP[11–13] have been established. A good model of CP recapitulating human disease is repeated intraperitoneal injection of cerulein, which is a cholecystokinin analog.[14] This decapeptide stimulates smooth muscles and increases digestive secretions, resulting in parenchymal fibrosis and CP criteria to be prominently observed. The C57BL/6 mouse is the most commonly used inbred mouse in biomedical research. Although clinical trials and animal models have evaluated the alterations of the microbiome in CP,[15,16] the gut microbiota composition and diversity in cerulein-induced CP of C57BL/6j mouse is unknown. This study aimed to evaluate the changes of gut microbiota in the feces of cerulein-induced CP mice model, and to determine whether these gut microbiota changes were consistent with those in patients with CP.

Materials and Methods

Animals

A total of ten male C57BL/6j mice, aged 6–8 weeks, weighing 20–22 g, were purchased from Animal Laboratory of Nanjing Medical University (Nanjing, China). At the time of purchase, the manufacturer provided a production license and a cage quality certificate, and the animal quality standards met the GB 14922.1–2001 microbiology and parasitological quality standards. Once in our experimental center, animals were kept strictly in accordance with the rules and regulations of Southeast University Experimental Animal Center. The SPF status of the facility was continuously monitored with sentinel animals by the Veterinary Service of Southeast University Laboratory Animal Center. Each group of animals was housed in a single ventilated cage. After 1 week of acclimation, the mice were randomly divided into an experimental group (CP) and a normal control group (CON) (n = 5 mice in each group) and maintained at a temperature of 18–26 °C for a 12-hour light/12-hour dark cycle, with free access to water and food. The water was made by a water purifier, and the food was provided by Jiangsu Synergy Bioengineering Co., Ltd (order number XC201910000003). Its composition: moisture 98 g/kg, crude ash 54.8 g/kg, crude protein 192.1 g/kg, crude fat 46.5 g/kg, crude fiber 30.3 g/kg, calcium 17 g/kg, total phosphorus 8.0 g/kg. All studies were performed according to the protocols approved by the Animal Experimental Ethical Inspection Form of Southeast University (No. 20190225003). We also followed the guidelines of the Administrative Measures for Experimental Animals of Jiangsu Province for the welfare of the laboratory animals.

Experimental Model of CP

For the CP group, mice were injected intraperitoneally with cerulein (Bubendorf, Switzerland) (50 μg/kg) once every hour for a total of 6 hours, three times a week for six consecutive weeks, as described previously.[17] At the same time, normal control mice received comparable injections of saline. The weight of all mice was recorded before each intervention. After the modeling was completed, using clean cotton swabs 1–2 pieces of each mouse's uncontaminated feces were added into the corresponding sample preservation liquid, stirred well and sent to Hangzhou Guhe Information Technology Co., Ltd (Zhejiang Province, China) for fecal flora detection. Then, the mice were sacrificed and their pancreas and colon were collected and divided into two sections, one section was fixed in 10% neutral buffered formalin (Sigma-Aldrich) solution and the rest was immediately frozen in liquid nitrogen.

Evaluation of Pancreas Morphology and Fibrosis

Pancreatic tails were fixed in 10% neutral buffered formalin solution embedded in paraffin, and hematoxylin and eosin (HE) and Masson's trichrome staining were performed according to standard histological examination methods. Then, the morphological changes and fibrosis were observed under a light microscope (Nikon, Japan).

DNA Extraction and 16S rDNA Amplicon Pyrosequencing

A PowerMax (fecal/soil) DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) was used to extract total

bacterial genomic DNA samples from all samples according to the instructions and the DNA stored at −20 °C until further analysis. PCR amplification of the bacterial 16S rRNA genes V4 region was performed using the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′).

Bioinformatics and Statistical Analysis

The sequencing reaction was performed by Hangzhou Guhe Information and Technology Co., Ltd (Zhejiang, China). Sequence data analyses were mainly performed using QIIME and R packages (v3.2.0). Sequencing reads were clustered to operational taxonomic units (OTUs) with a 97% similarity threshold. According to the OTU clustering results, a Venn diagram was generated using the R package "VenDiagram" to visualize the shared and unique OTU between the two groups, based on the presence of OTUs in each group regardless of their relative abundance.[18] OTU-level ranked abundance curves were generated to compare the richness and evenness of OTUs between the two groups. OTU-level alpha diversity indices, such as observe_species, Shannon diversity index, Simpson index and Chao1 richness estimator, were calculated using the OTU table in QIIME. We analyzed beta diversity using UniFrac distance metrics to study the structural variation of microbial communities among samples[19] and visualized via principal coordinate analysis (PCoA), principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS).[20] Taxa abundances at the phylum, class, order, family, genus and species levels were statistically compared between the two groups by Kruskal.test from the R stats package. Linear discriminant analysis effect size (LEfSe) was determined with default parameters to detect differentially abundant taxa between the two groups.[21]

Data were presented as mean ± standard deviation (SD) and the two groups' differences were analyzed using the Wilcoxon–Mann–Whitney test. Adjusting *p*-values controlled the false discovery rate (FDR) using the Benjamini–Hochberg method. The possible correlation between changes in gut microbial abundance and both weight and weight changes were assessed by the Spearman test (*r*). The adjusted *p*-value was no more than 0.05, which was statistically significant.

Results

Pancreatic Histopathological Assessment

HE staining of pancreatic tissue in the CP group showed severe loss of acinar cells, infiltration of inflammatory cells' enlarged interstitial spaces, atrophy of the glands, degeneration and parenchyma fibrosis (Figure 1A,B). Masson's trichrome staining (Figure 1C,D) showed extensive trichrome-positive staining in the CP group.

Richness and Diversity of the Gut Microbiota

At the end of the experiment, we found that the feces of CP mice were yellow, almost the color of the feed, and felt softer than the feces of normal mice (Figure 2A). After Illumina MiSeq sequencing analysis, a Venn diagram showed shared and unique OTUs between the two groups (Figure 2B). OTU-level ranked abundance curves showed the gut microbiota of the CP group had a significantly lower richness and evenness than the control group (Figure 2C).

The diversity of the fecal microbiota was determined by the alpha diversity analysis. Although there was no statistically significant difference in the Chao1 index between the two groups, the observed species and the Shannon and Simpson indexes in the CP group were lower than those in the control group (Figure 3A,B). Beta diversity between the two groups was also evaluated using unweighted UniFrac distances. A scatter plot based on PCoA scores showed a clear separation of the community composition between the two groups. PC1 and PC2 explained 42.94% and 15.47% of total variance, respectively (Figure 3C). PCA and NMDS showed similar results (Figure 3D,E).

Alterations of the Gut Microbiota

To determine the structural changes in the gut microbiota of the CP group and the control group, and the analysis of differences between the groups, we compared the relative abundance of the dominant strains from the phylum to genus level in the two groups. However, we found there were no differences between the CP group and the control group in class and order levels.

Bacteroidetes, Firmicutes, Proteobacteria and *Actinobacteria* were dominant at the phylum level. *Bacteroidetes* accounted for approximately 50% and *Firmicutes* accounted for approximately 46% of the total bacteria in the control group, and *Bacteroidetes* accounted for approximately 82% while *Firmicutes* accounted for about 7% in the CP group (Figure 4A). *Bacteroidetes, Actinobacteria* and *Verrucomicrobia* were more abundant in the CP group compared with the control group. While the

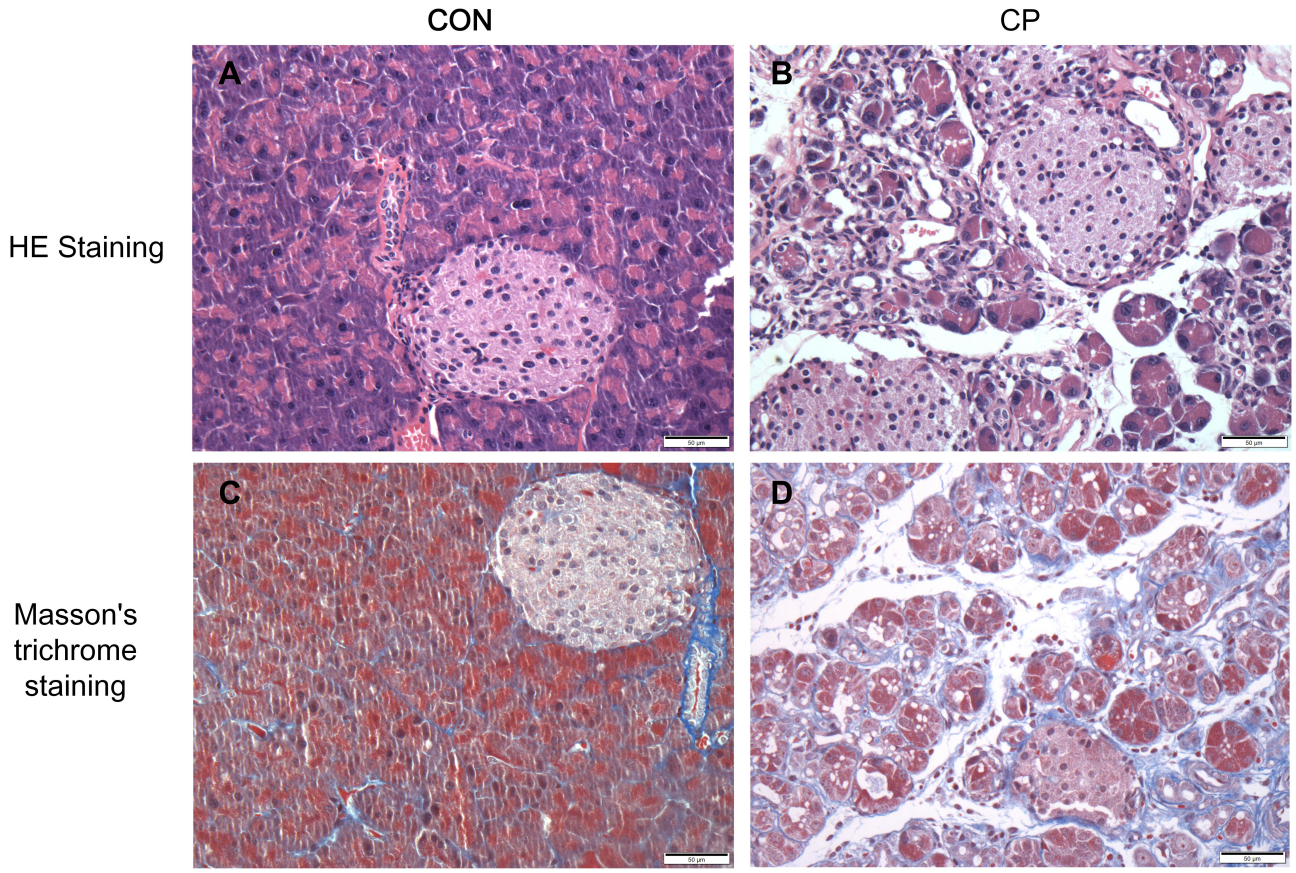

Figure 1 Hematoxylin and eosin staining (HE staining) (**A**,**B**), and Masson's trichrome staining (**C**,**D**) in the two groups of mice (original magnification × 400).
Abbreviations: CP, chronic pancreatitis group; CON, control group.

levels of *Firmicutes* and the *Firmicutes*/*Bacteroidetes* ratio were lower in the CP group than in the control group (Table 1).

At the family level, the microbiota was mainly composed of *S24-7, Bacteroidaceae, Ruminococcaceae* and *Lachnospiraceae* (Figure 4B). *Bifidobacteriaceae, Bacteroidaceae, Porphyromonadaceae* and *Verrucomicrobiaceae* were found to be more abundant in the CP group than the control group (Table 1).

At the genus level, *Bacteroides, Prevotella* and *Odoribacter* were the dominant bacteria (Figure 4C). The abundance of *Bifidobacterium, Bacteroides, Parabacteroides* and *Akkermansia* was higher in the CP group. However, the abundance of *Odoribacter* significantly reduced in the CP group (Figure 4D and Table 1). We also used LEfSe to detect differences in the microbiota profile between the two groups. The CP group was dominated by *Actinobacteria, Verrucomicrobia, Bifidobacteriaceae, Bacteroidaceae, Porphyromonadaceae* and *Verrucomicrobiaceae*. The control group was dominated by *Firmicutes, Clostridia, Clostridiales* and *Rikenellaceae*. (Figure 4E,F).

Association of Gut Microbiota Composition with Weight and Weight Changes

Before each intraperitoneal injection of the cerulein or saline, we measured the body weight of the two groups of mice, and obtained a total of 18 sets of data. We found that there was a statistical difference in body weight between the two groups at the twelfth injection. That is, at the last injection in the fourth week of the experiment, the weight of mice in the experimental group was lower than that in the control group, and the weight continued to be lower than the control group until the end of the experiment (Supplemental Figure 1).

We also found statistically significant differences in weight change between the two groups at the end of the study. To determine whether body weight and weight changes are associated with changes in gut microbial abundance, we additionally performed Spearman correlation analysis. However, there

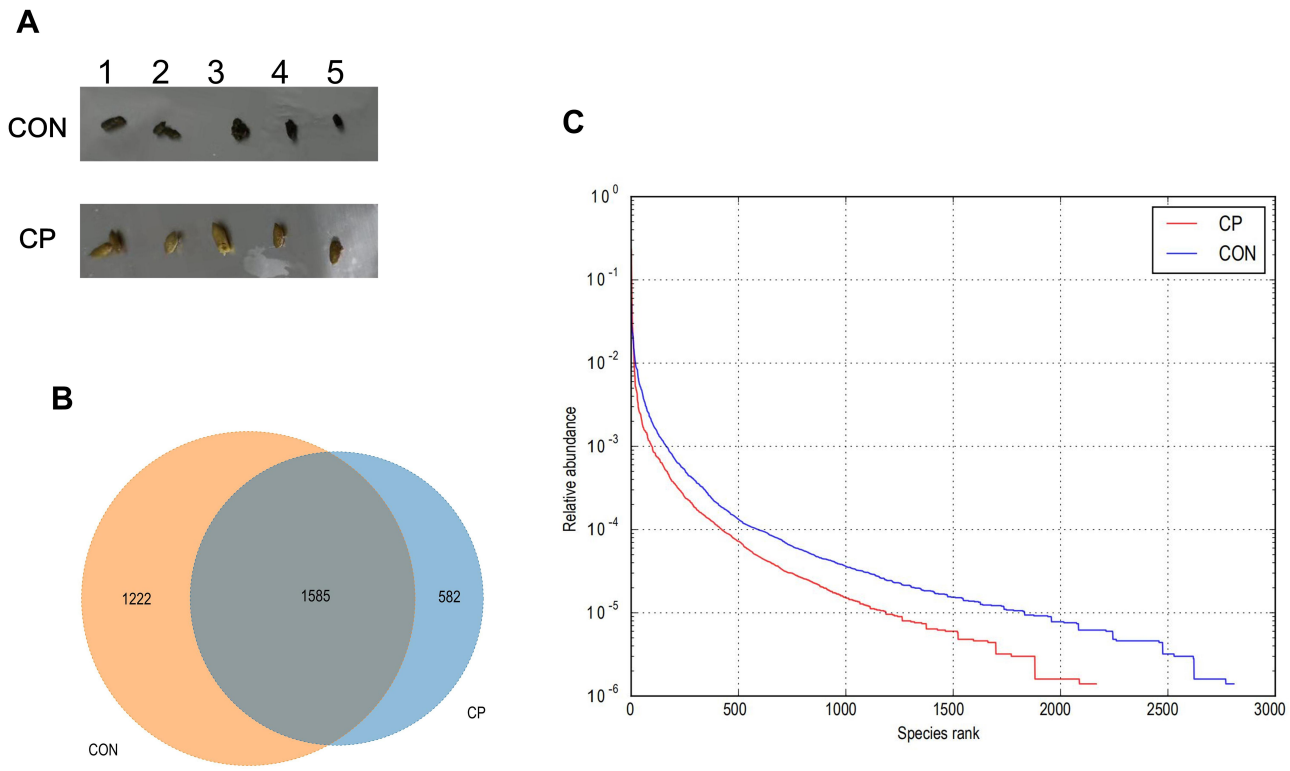

Figure 2 The appearance of the two groups of mice feces after modeling was completed (**A**), Venn diagram (**B**) and OTU-level ranked abundance curves (**C**).
Abbreviations: CP, chronic pancreatitis group; CON, control group.

was no significant correlation between alterations of gut microbiota and in body weight and weight changes (Supplemental Tables 1–2).

Discussion

The cerulein-based model used in our study was an excellent histomorphological model of CP caused by recurrent acute pancreatitis. Despite the lack of a direct etiology correlation, this model is useful because the histological characteristics of different CP etiology are basically indistinguishable. However, the gut microbiota of this model has not been studied, and whether it is consistent with the distribution of gut microbiota in CP patients is unclear. To our knowledge, this was the first study to assess the gut microbiota in cerulein-induced CP.

Decreased gut microbiota diversity has been reported in CP animal models and CP patients. In this study, we also observed that the gut microbial diversity of cerulein-induced CP was significantly lower than that of healthy controls. This result suggested that gut microbiota diversity may be an important factor in regulating host metabolism and therefore can be used as a simple indicator of health status.

Bacteroidetes and *Firmicutes* are the two main intestinal phyla and they have been linked to obesity in both human and animal research.[22,23] Turnbaugh et al[22] observed that the microbiota of obese individuals displayed a higher level of the phylum *Firmicutes* and lower levels of *Bacteroidetes*. A possible explanation for this finding was that *Firmicutes* was more effective as an energy source than *Bacteroidetes*, which promoted more efficient absorption of calories and subsequent weight gain. Interestingly, obese people lost weight due to calorie restrictions and physical exercise[24] as well as reduced fat or carbohydrate diets[23] and laparoscopic sleeve gastrectomy[25], which led to a distinct increase in *Bacteroidetes* and a decline in the abundance of *Firmicutes* and a reduction of the *Firmicutes/Bacteroidetes* ratio. Our results showed lower levels of *Firmicutes* and higher levels of *Bacteroides*, and the *Firmicutes/Bacteroidetes* ratio was significantly decreased in the gut microbiotas of CP mice. Although there was a statistically significant difference in body weight and weight changes between the two groups at the end of the study, there was no significant correlation between the *Firmicutes*/*Bacteroidetes* ratio and body weight and body

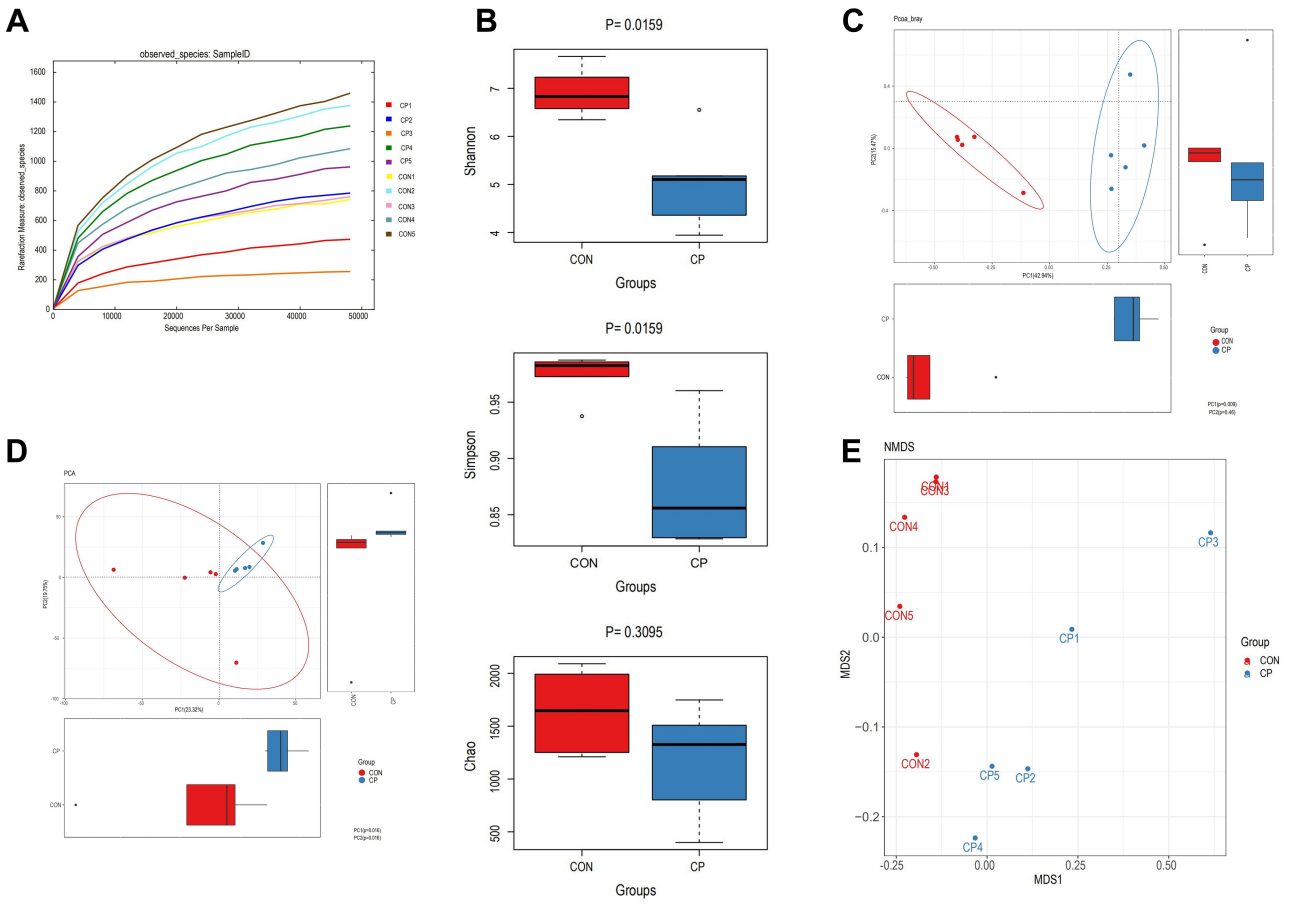

Figure 3 OTU-level alpha diversity indices, such as observed species (**A**), Shannon diversity index, Simpson index and Chao1 richness of each group (**B**), and beta diversity analysis visualized via principal coordinate analysis (PCoA) (**C**), principal component analysis (PCA) (**D**) and nonmetric multidimensional scaling (NMDS) (**E**).
Abbreviations: CP, chronic pancreatitis group; CON, control group.

weight changes in the CP and control groups. Therefore, a driver of changes in the composition of fecal microbiota may be the adaptability of bacteria to caloric restriction, as *Bacteroides* was less effective than *Firmicutes* as an energy source, thus inhibiting more efficient absorption of calories and subsequent slow weight gain.

As common probiotics, *Bifidobacterium* and *Lactobacillus* have received widespread attention. Surprisingly, our study found that *Bifidobacterium* was significantly higher in the CP group than in the control group. However, *Lactobacillus* was no different between the two groups. Our results seemed to be in conflict with previous data, which showed lower levels of *Bifidobacterium* or *Lactobacillus* in CP patients.[7] *Bifidobacterium* increased significantly in prebiotic treatment of obese or overweight children.[5] Although our study found that CP mice gained significantly less weight than healthy controls, both weight and weight changes have no relationship for this change in gut microbial abundance. *Bifidobacterium* was also found increased in active inflammatory bowel disease patients, and it may play a role in promoting inflammation.[26] However, the role of increased *Bifidobacterium* in CP is unclear.

Another interesting taxon identified in our study was that *Akkermansia* abundance increased and *Odoribacter* abundance decreased. *Akkermansia*, which belongs to the *Verrucomicrobia* phyla, has been proposed as a potential biomarker for the state of healthy gut status. It has been determined that the intestinal *Akkermansia* abundance decreased in obesity or other signs of metabolic disorder.[27] The antiobesity effects of pterostilbene[28] induced an increase in *Verrucomicrobia, Akkermansia* and *Odoribacter* and a decrease in the levels of *Firmicutes*, and it also confirmed that there was a strong inverse correlation between *Akkermansia muciniphila* and body weight. However, no significant correlation between higher abundance of *Akkermansia* with weight and weight changes was observed in our study. The reason for the higher abundance of Akkermansia in the CP

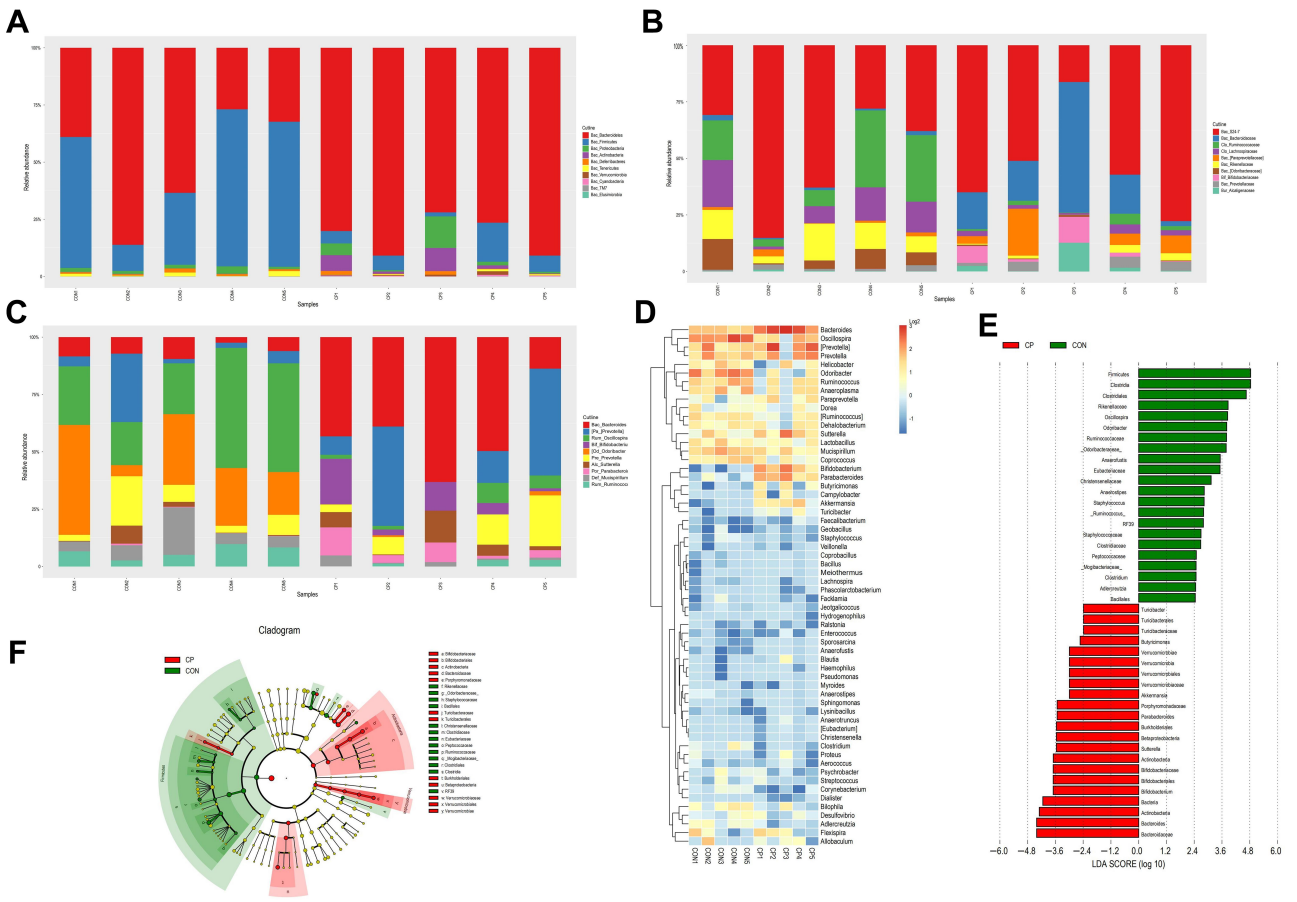

Figure 4 Relative abundances of the gut microbiota in the CP and CON groups at the phylum level (**A**), family level (**B**) and genus level (**C**), heatmap cluster at genus levels (**D**), cladogram of the LDA scores showing the abundant genera in CP (red) and CON (green) (**E**) and cladogram generated by LEfSe analysis showing the enriched taxa in CP (red) and CON (green) (**F**).

Abbreviations: CP, chronic pancreatitis group; CON, control group; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size.

group may be due to the low utilization of intestinal nutrients in favor of the growth of *Akkermansia*. Genus *Odoribacter*, which belongs to the family *Porphyromonadaceae*, was found to decrease in our CP group. The genera of this taxa are often associated with opportunistic infections, particularly intra-abdominal and systemic infections.[29] *Odoribacter splanchnicus* also showed a negative correlation with adiposity;[28] nevertheless, the relevance of this change in our studies was unclear.

Although numerous CP animal models have been introduced, the gut microbiota of those model is scarcely studied. So far, only gut microbiota of CP induced by ethanol and cerulein[11] and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) have been reported.[12,13] Han et al[11] found that the bacterial richness and diversity of CP mice induced by ethanol and cerulein were reduced, and the gut microbiota changed, including the relative abundance of *Lachnospiraceae_NK4A136, Ruminiclostridium* and *Roseburia* were reduced, while *Bacteroides* and *Alloprevotella* were higher. Several possible reasons can explain the difference between our main results and theirs. Although all experiments were conducted in the same animal experiment center and used the same animal, namely male C57BL/6 mice, our CP model was different from this study. We used cerulein for modeling, they used cerulein in combination with alcohol for modeling. In addition, the location of our materials was different, colon feces were collected in their research, 1–2 pieces of uncontaminated anus feces from each mouse were collected in our study. Surprisingly, it was found[12] that the DDC-induced CP model had significantly increased gut microbiota diversity and richness, DDC inducement decreased the proportion of *Lactobacillus, Bacteroides, Roseburia* and *Prevotella*, but increased the proportion of *Alistipes, Incertae_Sedis, Helicobacter, Parabacteroides* and *Rikenella* in the CP group compared with that in the control group. Li et al[13] also observed that the diversity and evenness of the DDC-induced CP group was higher. They found the phylum

Table 1 Relative Abundance of Gut Bacterial Taxa in Two Groups

Bacterial Group (%)	CON	CP	$p_{Bonferroni}$value
Phylum			
Firmicutes	46.4 ± 24.2	7.5 ± 5.7	0.037
Bacteroidetes	49.5 ± 24.7	81.9 ± 8.6	0.037
Firmicutes/Bacteroidetes	109 ± 97.8	9.2 ± 7.6	0.037
Actinobacteria	0.1 ± 0	4.0 ± 4.3	0.037
Verrucomicrobia	0 ± 0	0.6 ± 0.6	0.037
Family			
Bifidobacteriaceae	0 ± 0	3.8 ± 4.2	0.047
Bacteroidaceae	0.8 ± 0.4	19.5 ± 18.1	0.047
Porphyromonadaceae	0 ± 0	2.6 ± 2.8	0.047
Verrucomicrobiaceae	0 ± 0	0.6 ± 0.6	0.047
Genus			
Bifidobacterium	0 ± 0	3.8 ± 4.2	0.042
Bacteroides	0.8 ± 0.4	19.5 ± 18.1	0.042
Parabacteroides	0 ± 0	2.6 ± 2.8	0.042
Odoribacter	3.6 ± 2.7	0.1 ± 0.2	0.042
Akkermansia	0 ± 0	0.6 ± 0.6	0.042

Notes: Values are means ± standard deviations. Adjusting *p*-values to control the false discovery rate (FDR) using the Benjamini–Hochberg method ($p_{Bonferroni}$value).
Abbreviations: CP, chronic pancreatitis; CON, control.

Verrucomicrobia was unique in the CP group and DDC induced the proliferation of *Verrucomicrobia*. The CP group contained less *Firmicutines* but more *Bacteroidetes* when compared with the control group, and the decreased ratio of *Firmicutes*/*Bacteroidetes* suggested that the progression of CP resulted in the disproportions of *Firmicutes* and *Bacteroidetes* in the gut. In our study, we observed that the abundance of *Bacteroides, Actinobacteria and Verrucomicrobia* in the CP model group increased, but the abundance of *Firmicutes* decreased.

In summary, although changes in the gut microbiota of the three CP animal models have been reported, the results are inconsistent. This may be due to the different species of mice. Two of the studies[12,13] used ICR mice. This study and the other study[11] used C57BL/6 mice. The above-mentioned modeling methods were different in these four studies, and in two of the studies[12,13] the gut microbiota comparison between multiple groups was performed. Different statistical data analysis methods may also affect the results.

Numerous studies have focused on microbial associations with CP in humans,[15] and the results of these studies have been reviewed.[7] This review reported that three studies showed lower levels of *Bifidobacterium* or *Lactobacillus* and higher levels of *Enterobacteriaceae* in CP. One of the studies[15], which compared patients with endocrine and exocrine insufficiency, reported that patients with both CP and type-3c diabetes (T3cDM) had higher levels of *Bacteroidetes* and lower levels of *Faecalibacterium* compared to those without T3cDM, and pancreatic exocrine insufficiency had lower amounts of *Bifidobacterium* compared to those without pancreatic exocrine insufficiency. While *Bifidobacteria* levels were higher in CP and T3cDM without exocrine insufficiency. Recently, Zhou et al[30] found in patients with CP, not including CP patients with diabetes, their gut microbiota dysbiosis with decreased diversity and richness. Compared with the control group, the intestinal microbiome of the CP group had a lower abundance of *Firmicutes* and *Actinobacteria*, and a higher abundance of *Proteobacteria*. Our research was partially similar to this research. However, the gut microbiota of the three CP animal models mentioned above was not completely consistent with CP patients. Most likely, these differences can be due to different environmental influences, such as diet, physical activity, as well as socioeconomic impacts.[31] Therefore, none of these three models of CP can fully simulate patients with CP. This should be taken into account in future studies of the mechanisms involved in gut microbiotas in CP animal models and finding suitable animal models is worthy of further research.

There are some limitations to our research. First, the observation of gut microbiota and CP was independent. Therefore, the interaction between them remains unclear. We did not understand causality. In addition, we evaluated only a small number animals, it is necessary to analyze the gut microbiota of more samples, and the metabolism of a single bacteria and the correlation between the gut microbiota and CP deserve further study. Second, we did not analyze inflammatory biomarkers and internal and external secretions of the pancreas, which may explain changes in the intestinal flora. Finally, we only observed the changes in the gut microbiota of the two groups after the experiment was completed, and did not detect the dynamic changes, such as weight detection. This may have guiding significance for us to discover that the gut microbiota begins to change.

In summary, our results showed the bacterial richness and diversity of CP mice induced by cerulein were reduced, and the gut microbiota changed, including lower levels of *Firmicutes*, decreased *Firmicutes/Bacteroidetes* ratio and increased abundance of *Bacteroidetes, Actinobacteria* and *Verrucomicrobia*. Although we found statistically significant differences in body weight and weight change between the two groups at the end of the study, there was no significant correlation between

alterations of gut microbiota and in body weight and weight changes. In short, more research is needed to confirm our results.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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