Analysis of Gut Microbiota Characteristics Across Different Age Groups in Chronic Fatigue Syndrome: A Postprint

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Abstract

Background Chronic Fatigue Syndrome (CFS) is a chronic debilitating disease of unknown etiology, associated with intestinal microecological dysbiosis and bacterial metabolic disorders. Significant differences exist in gut microbiota composition and intestinal permeability between CFS patients and healthy populations; however, reports on gut microbiota characteristics in CFS populations of different ages are scarce.

Objective To investigate the characteristics of gut microbiota in CFS patients across different age groups and to provide a scientific basis for risk prevention and control of CFS in different age groups.

Methods CFS patients from the outpatient clinic of Sichuan Provincial Hospital of Integrated Traditional Chinese and Western Medicine between February and October 2021 were selected as study subjects. Diagnosis was conducted by three attending physicians. A total of 60 cases were included and divided into three groups: youth group (18–34 years) with 20 cases, middle-aged group (35–55 years) with 20 cases, and elderly group (56–80 years) with 20 cases. Basic information of the cases was collected, fecal specimens were obtained, and 16S rRNA high-throughput sequencing was used to detect gut microbiota, followed by bioinformatics and statistical analysis.

Results Species composition and relative abundance differed among CFS patients of different age groups. The Shannon index and Pielou_e index of gut microbiota in the elderly group were higher than those in the middle-aged group ($P<0.05$). The clustered microbiota in the middle-aged group showed significant separation from the other two groups ($P<0.01$). The core genera in the youth group were Coprococcus, Megamonas, Dialister, and Acinetobacter, while the core genus in the elderly group was Ruminococcus. The relative abundances of

Pseudomonadales, Moraxellaceae, and Acinetobacter in the youth group were higher than those in the middle-aged and elderly groups (P<0.05). The relative abundances of Erysipelatoclostridium and Sellimonas in the middle-aged group were higher than those in the youth and elderly groups (P<0.05). The relative abundances of Eggerthellaceae and Bilophila in the elderly group were higher than those in the youth and middle-aged groups (P<0.05).

Conclusion The diversity and composition of gut microbiota differed among CFS patients of different age groups. Acinetobacter and Erysipelatoclostridium were preliminarily identified as key bacterial genera that could distinguish gut microbiota among elderly, middle-aged, and youth CFS patients.

Full Text

Characterization of Intestinal Flora in Different Age Groups with Chronic Fatigue Syndrome

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Abstract

Background: Chronic fatigue syndrome (CFS) is a chronic debilitating disease of unknown etiology associated with intestinal microecological dysregulation and disturbances in bacterial metabolism. While significant differences exist in intestinal flora composition and intestinal permeability between CFS patients and healthy populations, the characteristic gut microbiota features of CFS across different age groups remain poorly documented.

Objective: To investigate the characteristics of intestinal flora in CFS patients across different age groups and provide a scientific basis for age-specific risk prevention and control strategies.

Methods: CFS patients from outpatient clinics of Sichuan Integrative Medicine Hospital between February 2021 and October 2021 were recruited as study subjects. Three attending physicians were responsible for diagnosis. A total of 60 cases were included and divided into three groups: 20 cases in the young group (18–34 years), 20 cases in the middle-aged group (35–55 years), and 20 cases in the old-aged group (56–80 years). Basic demographic information was collected, fecal specimens were obtained, and intestinal flora was analyzed using 16S rRNA high-throughput sequencing followed by bioinformatics and statistical analyses.

Results: Differences in species composition and relative abundance were observed across age groups in CFS patients. The old-aged group exhibited higher Shannon and Pielou's indices of intestinal flora compared to the middle-aged group ($P<0.05$). The aggregated flora in the middle-aged group showed significant separation from the other two groups ($P<0.01$). The core genera in the young group were *Coprococcus*, *Megamonas*, *Dialister*, and *Acinetobacter*, while *Ruminococcus* was the core genus in the old-aged group. The relative abundance of Pseudomonadales, Moraxellaceae, and *Acinetobacter* was higher in the young group compared to the middle-aged and old-aged groups ($P<0.05$). The relative abundance of *Erysipelatoclostridium* and *Sellimonas* was higher in the middle-aged group compared to the young and old-aged groups ($P<0.05$). The relative abundance of Eggerthellaceae and *Bilophila* was higher in the old-aged group compared to the young and middle-aged groups ($P<0.05$).

Conclusion: Significant differences exist in the diversity and composition of intestinal flora among CFS patients of different age groups. *Acinetobacter* and *Erysipelatoclostridium* may serve as key discriminatory taxa distinguishing intestinal flora profiles in old-aged, middle-aged, and young CFS patients.

Key words: Fatigue syndrome, chronic; Age groups; Intestinal flora; *Acinetobacter*; *Erysipelatoclostridium*; Southwest China; 16S rRNA

Introduction

The gut microbiota plays a crucial role in maintaining human health [1]. Research has demonstrated that intestinal microecological imbalance is closely associated with various diseases, including cancer [2], digestive disorders [3], immune dysfunction [4], and psychiatric conditions [5]. Chronic fatigue syndrome (CFS) is a chronic debilitating illness of unknown etiology, with common symptoms including sleep deprivation, cognitive impairment, orthostatic intolerance, and pain. These symptoms persist or recur over extended periods, leading to significantly reduced functional capacity [6]. Patients with gastrointestinal infections or inflammatory bowel disease show markedly increased risk of developing CFS [7-8]. Significant differences in gut microbiota composition and intestinal permeability exist between healthy individuals and CFS patients, and CFS is associated with intestinal microecological dysbiosis and distinct bacterial metabolic disturbances [9]. With advancing age, particularly accompanied by dietary changes, the gut microbiota undergoes corresponding alterations, and gut microbiota diversity increases across different age groups [10]. In diseases such as autism [11], major depressive disorder [12], and non-alcoholic fatty liver disease [13], studies have confirmed that gut microbiota composition may differ according to age even when the same disease is present. CFS affects a broad population and can occur in children, adolescents, middle-aged, and elderly individuals [14-16], with different disease phenotypes emerging across age groups [17-18]. However, whether gut microbiota characteristics differ among

CFS patients of various ages remains rarely reported. This study explores the gut microbiota features of CFS patients across different age groups to provide a scientific basis for age-specific risk prevention and control.

Methods

1.1 Study Subjects

CFS cases from Sichuan Integrative Medicine Hospital between February and October 2021 were screened by three attending physicians. Based on patient age, cases were divided into three groups: young (18–34 years), middle-aged (35–55 years), and old-aged (56–80 years) [19-20]. Among patients meeting CFS diagnostic criteria, 20 cases were randomly selected from each age group, totaling 60 CFS patients for retrospective analysis.

Inclusion criteria: (1) Met CFS diagnostic criteria [21]; (2) Aged 18–80 years, regardless of gender; (3) No severe cognitive impairment, able to correctly understand questionnaire content; (4) Voluntary participation with signed informed consent.

Exclusion criteria: (1) Primary diseases that could cause chronic fatigue; (2) Diagnosed mood disorders or various mental illnesses; (3) Severe chronic debilitating diseases with liver or kidney damage; (4) Positive pregnancy test or lactating women, or those with pregnancy plans; (5) Use of medications causing fatigue symptoms within the past month (including amoxicillin capsules, roxithromycin tablets, etc.); (6) Use of anti-fatigue medications within the past month; (7) Participation in any other clinical trials within the past month; (8) Inability or unwillingness to cooperate with regular follow-up or intervention plans; (9) Subjects deemed unsuitable by researchers. This study was approved by the Ethics Committee of Sichuan Integrative Medicine Hospital (Approval No.: 2019KY-011).

1.2 Methods

1.2.1 Fatigue Self-Assessment Scale (FSAS) The FSAS was used to evaluate fatigue type, severity, and characteristics in CFS patients, including four factors (general fatigue, physical fatigue, mental fatigue, and fatigue consequences) and two characteristic dimensions (fatigue response to sleep/rest and situational fatigue) [22]. Standard scores for each fatigue factor ranged from 0 to 100, with higher scores indicating more severe physical and mental fatigue, more serious fatigue consequences, and more pronounced characteristics of unrelieved fatigue by sleep/rest and situational fatigue.

1.2.2 Fecal Sample Collection and DNA Extraction Physicians instructed subjects to collect fresh fecal samples (approximately 5 g) using specialized sterile fecal collection tubes. Collected samples were stored at -80°C. Microbial total DNA was extracted from fecal samples using the QIAGEN

PowerFecal® RNA Isolation kit. DNA integrity was assessed using agarose gel electrophoresis.

1.2.3 Library Construction and High-Throughput Sequencing The V4 hypervariable region of the 16S rRNA gene was amplified from fecal samples [23] using primers F515: 5'-ACTCCTACGGGAGGCAGCA-3' and R806: 5'-GGACTACHVGGGTWTCTAAT-3', with MiSeq sequencing adapters added to the 5' ends. Polymerase chain reaction (PCR) amplification was performed. PCR products were purified using a PCR purification kit. Libraries were constructed using Illumina's TruSeq DNA PCR-Free Library Preparation Kit. Library quality was assessed using the Qubit® 2.0 Fluorometer and Agilent Bioanalyzer 2100 system. Finally, sequencing was completed on the NovaSeq 6000 platform. Raw reads from Illumina NovaSeq sequencing were assembled, and data underwent chimera filtering and other noise reduction processes. Sequences with abundance <5 were removed for further quality control to obtain valid data for subsequent analysis.

1.2.4 Bioinformatics Analysis The Qiime 2 platform [24] was used to analyze valid data from Illumina NovaSeq sequencing. First, DADA2 software [25] was employed to filter out low-quality (<Q20), chimeric, and erroneous reads to obtain amplicon sequence variants (ASVs). Taxonomic classification of ASVs was determined using BLAST against representative sequences from the SILVA reference database (version 132). Prior to α- or β-diversity analysis, sequence counts in the ASVs table were normalized to the minimum read count across samples to eliminate bias from different sequencing depths. α-diversity was analyzed using Pielou's evenness index, Observed_{ASVs} index, and Shannon index. β-diversity was analyzed using principal coordinates analysis (PCoA) based on Bray-Curtis distance matrices. Species composition and differential analysis were performed at the phylum level. Linear discriminant analysis effect size (LEfSe) analysis was conducted to identify statistically significant biomarker taxa between groups, with P<0.05 (Kruskal-Wallis test) and lg[LDA]$ $2.0 considered significantly different microorganisms.

1.3 Statistical Analysis

Statistical graphs were generated using GraphPad 8.0 software and R software (version 4.2) packages including "boxplot", "barplot", and "ggplot2" [26]. SPSS 26.0 software was used for statistical analysis. Normality and homogeneity of variance tests were performed on measurement data. Normally distributed data were expressed as mean±standard deviation ($\bar{x}\pm s$) and compared among multiple groups using one-way ANOVA, with pairwise comparisons using LSD-t test. Non-normally distributed data were expressed as median (P25, P75) and compared between two groups using Mann-Whitney U test and among multiple groups using Kruskal-Wallis H test. Count data were expressed as relative frequencies and compared using ^{2} test. P<0.05 was considered statistically significant.

Results

2.1 General Information

Among the 60 CFS patients, 16 were male and 44 were female, with a mean age of (44.5$±$19.0) years. No statistically significant differences were observed among the three groups in gender distribution, general fatigue, physical fatigue, mental fatigue, fatigue consequences, fatigue response to sleep/rest, or situational fatigue scores (P>0.05). However, age differed significantly among the three groups (P<0.05), as shown in Table 1 .

2.2 Gut Microbiota Analysis

2.2.1 Species Diversity Analysis α-diversity refers to species diversity within an ecosystem or specific environment. Significant differences in Pielou's evenness index were observed among the three groups (P<0.05). No statistically significant differences were found in Shannon index or Observed_{ASVs} index among the three groups (P>0.05). The old-aged group showed higher Pielou's evenness index and Shannon index compared to the middle-aged group (P<0.05), as shown in Table 2 .

β-diversity represents comprehensive diversity (i.e., species differences) between different environmental communities. Bray-Curtis distance-based principal coordinates analysis (PCoA) revealed separation among aggregated flora in the three groups (R^2=0.071, P=0.001). PCoA1 and PCoA2 accounted for 12.63% and 9.61% of the total variation, respectively. The distinct clustering patterns indicated substantial differences in species composition among the three groups, with the middle-aged group showing significant separation from the other two groups (P<0.01), as shown in Figure 1 [Figure 1: see original paper].

2.2.2 Distribution of ASVs Analysis of gut microbiota ASVs revealed 1,226 ASVs in the young group, 1,820 in the middle-aged group, and 1,052 in the old-aged group, with 384 ASVs shared among all three groups. The young and middle-aged groups shared 485 ASVs, the young and old-aged groups shared 470 ASVs, and the middle-aged and old-aged groups shared 476 ASVs (Figure 2A). Core flora generally refers to microbial taxa present in the majority ($ $50%) of individual samples. Screening for core flora in each group identified four core genera in the young group: *Coprococcus*, *Megamonas*, *Dialister*, and *Acinetobacter*, while the old-aged group had one core genus: *Ruminococcus* (Figure 2B).

2.2.3 Community Composition Analysis In CFS patients, the top 10 phyla by relative abundance were Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Verrucomicrobia, Euryarchaeota, Chloroflexi, Acidobacteria, Tenericutes, and Gemmatimonadetes (Figure 3A). The dominant phyla across all age groups were Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes. Chloroflexi and Gemmatimonadetes were absent in the old-aged

group but present in both young and middle-aged groups. The relative abundance of Verrucomicrobia was higher in the young group compared to the middle-aged and old-aged groups. The old-aged group showed higher relative abundance of Firmicutes and Bacteroidetes compared to the middle-aged group, higher relative abundance of Bacteroidetes compared to the young group, and significantly lower relative abundance of Acidobacteria compared to the middle-aged group ($P<0.05$) (Figure 3B). No statistically significant differences were observed in Firmicutes/Bacteroidetes (F/B) ratio among the three groups ($P>0.05$) (Figure 3C).

2.2.4 Differential Flora Analysis LEfSe analysis was performed to identify differential gut microbiota across age groups in CFS patients. The LDA plot (Figure 4A), P values and LDA scores (Figure 4B), and evolutionary tree (Figure 4C) are presented. Taxa meeting $P<0.05$ and lg[LDA]$ $2 were considered biomarker species. The young group showed significantly higher relative abundance of Pseudomonadales, Moraxellaceae, and *Acinetobacter* compared to the middle-aged and old-aged groups ($P<0.05$). The middle-aged group exhibited significantly higher relative abundance of *Erysipelatoclostridium* and *Sellimonas* compared to the young and old-aged groups ($P<0.05$). The old-aged group demonstrated significantly higher relative abundance of Eggerthellaceae and *Bilophila* compared to the young and middle-aged groups ($P<0.05$).

Further random forest model analysis identified key differential flora among the three groups (Figure 5 [Figure 5: see original paper]), with larger values indicating greater species importance. The most important taxa included *Acinetobacter*, *Erysipelatoclostridium*, *Weissella*, *Holdemanella*, *Alcaligenes*, unidentified genus in Burkholderiaceae, *Ruminococcaceae*, *Megamonas*, *Akkermansia*, *Coprococcus*, *Sellimonas*, unidentified genus in Enterobacteriaceae, *Adlercreutzia*, *Megasphaera*, unidentified genus in Anaerolineaceae, *Rothia*, *Slackia*, *Pediococcus*, *Barnesiella*, *Prevotella*, unidentified genus in Aminicenantales, *Haemophilus*, unidentified genus in Nitrososphaeraceae, *Collinsella*, *Faecalitalea*, unidentified genus in Acidobacteriales, *Eubacterium*, *Sulfurimonas*, and *Mogibacterium*. *Acinetobacter* and *Erysipelatoclostridium* could serve as key taxa distinguishing gut microbiota profiles among old-aged, middle-aged, and young CFS patients.

Discussion

3.1 Differences in Gut Microbiota Across Age Groups in CFS

This study investigated the composition and diversity of gut microbiota in CFS patients across different age groups using 16S rRNA high-throughput sequencing. The results indicate that *Acinetobacter* and *Erysipelatoclostridium* may serve as key taxa distinguishing gut microbiota profiles in young, middle-aged, and old-aged CFS patients. Additionally, the young group had four core genera: *Coprococcus*, *Megamonas*, *Dialister*, and *Acinetobacter*, while *Ruminococcus* was the core genus in the old-aged group. *Megamonas* belongs to Firmicutes and

shows significantly increased abundance in obese populations [30] and is also abundant in 19.2% of early-stage colorectal cancer patients [31]. These findings suggest that different core genera exist across age groups and may play roles in the pathogenesis and progression of CFS in different age populations. Furthermore, the old-aged group showed higher relative abundance of Firmicutes and Bacteroidetes compared to the other groups, with a decreased F/B ratio relative to the young group. A decreased F/B ratio is considered indicative of intestinal dysbiosis and is commonly observed in inflammatory bowel disease [32], suggesting that elderly CFS patients may be more prone to intestinal ecological imbalance compared to younger patients.

LEfSe analysis revealed differential flora across age groups. The young group showed significantly higher relative abundance of Pseudomonadales, Moraxellaceae, *Acinetobacter*, and unidentified genus in Burkholderiaceae compared to the middle-aged and old-aged groups. These biomarker taxa in the young group all belong to Proteobacteria, with *Acinetobacter* being a unique core genus in this age group. *Acinetobacter* is currently the most important opportunistic pathogen in nosocomial infections, with pathogenic species such as the *Acinetobacter calcoaceticus-baumannii* complex capable of surviving for extended periods in hospital environments and transmitting through patient contact and shared medical equipment, causing infections and even death in some patients [33]. The old-aged group showed significantly higher relative abundance of Eggerthellaceae and *Bilophila* compared to the other groups. *Bilophila* is associated with colorectal cancer [34], while Eggerthellaceae belongs to Actinobacteria and shows higher abundance in severe COVID-19 compared to mild disease [35]. *Acinetobacter*, as a unique core genus in the young group, may also be a dominant genus causing CFS in young populations, while elderly CFS patients warrant attention to Eggerthellaceae and *Bilophila*.

3.2 Focus on Middle-Aged CFS Population

Previous studies have indicated that middle-aged populations have higher risk of CFS [36]. Analysis of fatigue indices revealed that the middle-aged group had significantly lower scores for fatigue consequences and fatigue response to sleep/rest compared to the young group ($P<0.05$). α- and β-diversity analyses showed that the middle-aged group had significantly lower gut microbial richness and evenness compared to the old-aged group ($P<0.05$), with significant separation of aggregated flora from the other two groups ($P<0.05$). LEfSe analysis demonstrated that the middle-aged group had significantly higher relative abundance of *Erysipelatoclostridium* and *Sellimonas* compared to the young and old-aged groups. Studies have shown that *Sellimonas* abundance is higher in ulcerative colitis patients with comorbid depression/anxiety [37], while *Erysipelatoclostridium* is associated with colonic disease and represents a potential pathogen [38]. Moreover, *Erysipelatoclostridium* can serve as a key taxon distinguishing gut microbiota profiles among young, middle-aged, and old-aged CFS patients.

3.3 Limitations and Future Directions

All outpatients in this study were from Southwest China, and we excluded factors affecting gut microbiota such as medication use and diseases. However, patients' dietary habits were not strictly analyzed. Additionally, substantial inter-individual variation exists in gut microbiota, and microbiota composition changes with age within the same individual. This study could not avoid inter-individual differences in gut microbiota caused by dietary structure and other factors. Future studies should implement more stringent inclusion and exclusion criteria, particularly regarding factors influencing gut microbiota including dietary habits. Furthermore, this study focused primarily on gut microbiota in CFS patients. To more clearly establish causality between microbiota changes and CFS, samples could be collected from the same patients before and after treatment to compare microbiota differences, as microbiota remains relatively stable within the same individual over time, allowing for clearer understanding of the association between gut microbiota and CFS. Additionally, animal models and fecal microbiota transplantation techniques could be employed to more directly validate the impact of gut microbiota.

In summary, by comparing gut microbiota across different age groups of CFS patients, this study found that disease manifestations and gut microbiota differ across age groups, with *Acinetobacter* and *Erysipelatoclostridium* serving as key taxa distinguishing gut microbiota profiles among old-aged, middle-aged, and young CFS patients. These findings warrant further validation.

Author contributions: XU Yisha contributed to conceptualization, data analysis, and manuscript writing; LI Chunmei was responsible for sample collection; WANG Chengcheng handled subject selection; GUO Wei performed data processing; GAN Yunong conducted statistical analysis; ZHANG Yu managed sample storage and processing; ZHANG Wen performed DNA extraction; YU Congcong supervised quality control.

Conflict of interest: None declared.

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Note: Figure translations are in progress. See original paper for figures.