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Gut microbiome alterations in ICU patients with enteral nutrition-related diarrhea

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Enteral Nutrition-related Diarrhea (END) is an extremely common complication in Intensive Care Unit (ICU) patients. However, it is currently unclear whether the patient's gut microbiota is disturbed. Our study aimed to explore the characteristics of gut microbiota changes in END patients. We divided ICU patients into no-END group ($n=7$) and END group ($n=7$) according to whether they had END, then stool samples were collected separately. The V3-V4 region of stool bacterial 16S rRNA gene was amplified by PCR and sequenced on an Illumina MiSeq PE300 platform. Microbiome data obtained by quality control were analyzed, including microbial community composition, diversity and gene function prediction. The results showed that the dominant gut microbiota in ICU patients who were given total enteral nutrition were Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Verrucomicrobia. Bacterial richness and diversity in END patients were all significantly lower than those in no-END patients. In addition, END caused significant changes in bacterial composition. LEfSe found 34 biomarkers represented by Bacteroidetes and Subdoligranulum in the no-END group as well as 11 biomarkers represented by Enterococcus and Klebsiella in the END group. Finally, through PICRUST function prediction, we found that diarrhea led to abnormal changes in numerous KEGG pathways mainly related to immunity and metabolism. In short, ICU patients with END have severe gut dysbiosis, and our study provides a reliable experimental basis for the patient's microbiota therapy.

KEYWORDS

gut microbiome, enteral nutrition, diarrhea, ICU, 16S rRNA sequencing

Introduction

Intensive care unit (ICU) patients are generally in critical diseases, often accompanied by disorders of consciousness, dysphagia, digestive dysfunction and other states, and are prone to malnutrition (Singer, 2019; Theilla et al., 2021). Enteral nutrition (EN) is a form of oral or tube feeding that provides food and other nutrients to the gastrointestinal tract of people who cannot eat normally (Wischmeyer, 2021). Early nutritional support treatment

helps to correct electrolyte and various nutritional metabolic disorders in patients and also effectively improves the body's resistance, shortens the patient's stay in the ICU and reduces the mortality rate (Singer et al., 2019; De Lazzaro et al., 2022). The 2009 Nutrition Day Global Hospital Survey reports that approximately 10% of hospitalized patients in Europe and Japan receive enteral nutrition, with one-third using it as part of their energy supplement and another two-thirds using it as their sole energy source (Schneider, 2010). EN is the preferred route of feeding, especially when the oral route can no longer meet the needs of patients (Lochs et al., 2006). The European Society of Clinical Nutrition and Metabolism's 2018 nutrition guidelines for critically ill patients strongly recommend early enteral nutrition within 48 h of admission to the hospital for critically ill patients who are unable to eat autonomously (Singer et al., 2019). However, diarrhea is the most common complication of enteral nutrition, with an incidence rate as high as 68% (Zhao et al., 2017; Xie et al., 2021). Surprisingly, the incidence of diarrhea in ICU patients treated with EN is as high as 2–95% (Wiesen et al., 2006; Whelan, 2007). The consequences of enteral nutrition-related diarrhea (END) range from ICU patient discomfort to life-threatening acidosis, increased morbidity and mortality, pressure ulcers and the forced interruption of EN feeding, as well as higher care costs for hospital staff (Adam and Batson, 1997). Unfortunately, when END occurs, some ICU patients experience long-lasting comas due to their critical condition; thus, EN cannot be easily stopped as it is the only source of nutrition. Therefore, it is extremely important to explore the mechanism of END in ICU patients.

The gut microbiome is considered a new functional organ, as it regulates many physiological functions of the host, such as training of immunity, digesting food, regulating gut endocrine function and nerve signals, modifying drug action and metabolism, eliminating toxins and producing numerous compounds that influence the host (Fan and Pedersen, 2021). Undoubtedly, microbiota, nutrients and host cells interact extensively in the gut, and this process is critical for gut homeostasis and host health. Therefore, any dysbiosis can have a negative impact on health, and many diseases are associated with damage to the gut microbiota (Butel, 2014). Studies have found that the concentration and proportion of *Clostridium* and *Bacteroides* are significantly higher in patients with diarrhea (Whelan et al., 2009). Long-term total EN patients have a serious imbalance of the bacterial community; for example, the number of fecal anaerobic bacteria decreases, the number of aerobic bacteria increases, and the cultured strains decrease significantly (Schneider et al., 2000). Other studies have found that the detection rate of *Escherichia coli* in children under 5 years old with acute diarrhea is 7.9% (Zhou et al., 2018). Interestingly, gastrointestinal disorders, such as autism spectrum disorders and diarrhea in children, are closely related to gut microbiota disorders (Krajmalnik-Brown et al., 2015). Fecal bacteria transplantation can improve the gastrointestinal tract and symptoms of autism (Kang et al., 2017). Although, there have been numerous studies on the relationship between diarrhea and the gut microbiota, the

comprehensive and detailed changes in gut microbiota composition and gene function during END in ICU patients are still unknown. Moreover, most previous studies examined the relationship between gut microbiota and diarrhea using traditional identification and culture techniques that are no longer considered optimal for describing the microbiota (Hooda et al., 2012; Braun et al., 2017; Pena-Gonzalez et al., 2019). New molecular biology techniques, especially next-generation sequencing (NGS) technology, such as 16S rRNA gene sequencing and metagenomics, because it can simultaneously detect the dominant species, rare species and some unknown species in the sample quickly and high-throughput, without considering whether they are known and culturable. In addition, microbial community composition, diversity, evolutionary relationships and even gene function can be more accurately studied, and the discovery and classification of new bacterial species can be facilitated. Thus, the emergence of NGS has largely replaced the old methods (Okonkwo et al., 2020).

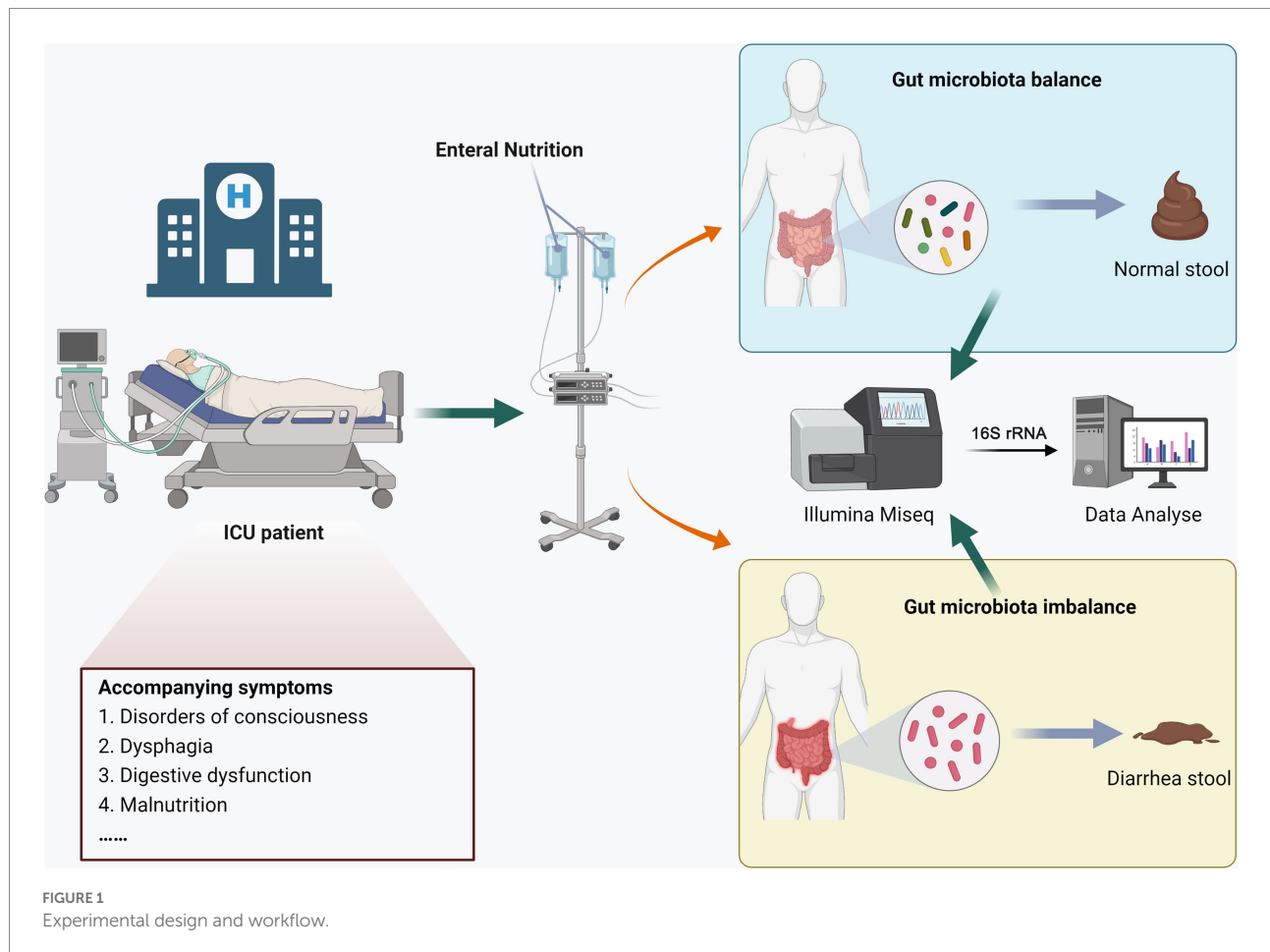
In this study, 16S rRNA gene sequencing was used to detect stool from ICU patients with and without diarrhea receiving total enteral nutrition to compare the gut microbiota changes. Our data show that END patients have distinct gut microbial signatures and significant changes in gene function. These results are important for understanding the roles of these bacteria in the occurrence of END.

Materials and methods

Study subjects and sample collection

This study was approved by the Ethics Review Committee of Henan Provincial People's Hospital, and all participants signed informed consent forms. The process diagram was shown in Figure 1, hospitalized patients were divided into the END group ($n=7$) and the no-END group ($n=7$) according to whether diarrhea occurred during total enteral nutrition treatment in the ICU of our hospital in 2022. The inclusion criteria of the patients were as follows: (1) the type and dose of antibiotics were essentially the same; (2) there was no hypoalbuminemia; (3) there were no potassium preparations, antacids or prokinetic drugs; (4) probiotics or commensal bacteria were not used prior to collection of stool samples; and (5) there was no severe malnutrition, intestinal obstruction or digestive system diseases. At the same time, there were no statistically significant differences in age or sex between the two groups.

As the gold standard for stool collection, the rectal swab method was performed as previously described (Short et al., 2021). Briefly, the patient's anus was first cleaned with soap, water and 70% alcohol. Then, a sterile swab was moistened with normal saline, inserted into the anus 4–5 cm, and rotated gently at the anal sphincter. A sample was taken from the anal crypt, and the swab with the patient's stool was inserted into a sterile tube. Finally, the samples were frozen in liquid nitrogen and immediately stored at -80°C .



DNA extraction and PCR amplification

Gut microbial DNA was extracted from no-END and END samples using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) according to the manufacturer's protocols. The final DNA concentration and purification were determined by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis.

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by a thermocycler PCR system (GeneAmp 9,700, ABI, USA). The PCRs were conducted using the following program: 3 min of denaturation at 95°C; 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C; and a final extension at 72°C for 10 min. PCRs were performed in triplicate in a 20 µl mixture containing 4 µl of 5 × FastPfu Buffer, 2 µl of 2.5 mM dNTPs, 0.8 µl of each primer (5 µM), 0.4 µl of FastPfu Polymerase and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel, further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor[™]-ST (Promega, USA) according to the manufacturer's protocol.

16S rRNA gene sequence analysis

Before analysis, raw fastq sequence files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) the reads were truncated at any site receiving an average quality score ≤ 20 over a 50 bp sliding window; (ii) primers were exactly matched, allowing 2 nucleotide mismatching, and reads containing ambiguous bases were removed; and (iii) sequences whose overlap was longer than 10 bp were merged according to their overlap sequence.

The demultiplexed reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) using UPARSE (version 10.0¹), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm (version 2.2²) in mothur³ against the Silva 132/16s_bacteria⁴ database using a confidence threshold of 70%. Sequencing was performed with an Illumina MiSeq PE300 platform.

1 <http://drive5.com/uparse/>

2 <http://sourceforge.net/projects/rdpclassifier/>

3 <https://www.mothur.org/>

4 <http://www.arb-silva.de>

Statistical analysis

OTU taxonomies (from phylum to species) were determined based on NCBI. For the alpha-diversity analysis, the Ace, Chao index and Shannon and Simpson indices at the similarity level of 97% were calculated to compare the gut bacterial richness and diversity, respectively. For the beta-diversity analysis, analyses were visualized using the R package software. For example, a Venn diagram was implemented using the R package to show unique and shared OTUs. All statistical analyses were conducted using R package software. Differences between populations were analyzed using one-way ANOVA. Linear discriminant analysis (LDA) effect size (LEfSe (Segata et al., 2011)) was used to elucidate the biomarkers in the two groups. Those with an LDA score of 4.0 were considered to be important biomarkers in the no-END and END groups. The metagenomes were predicted from 16S data using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States [PICRUSt (Langille et al., 2013)]. The functional genes were identified from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000). Specifically, PICRUSt is a software package for functional prediction based on 16S amplicon sequencing results. Firstly, the OTU abundance table of microbiota was standardized by PICRUSt (the PICRUSt process stores the KEGG Ortholog (KO) information corresponding to greengene id), that is, the effect of 16S marker gene copy number in the species genome was removed. Then through the greengene id corresponding to each OTU, the KO information and abundance corresponding to the OTU were obtained. Finally, according to the KEGG database, the descriptive information of each pathway was parsed, and the abundance of each functional category was calculated according to the OTU abundance. Furthermore, the G-TEST and Fisher test methods in STAMP software were used to test and visualize the significant differences in the predicted functional pathways by PICRUSt, and the *p* value threshold was 0.000.

Results

END alters the alpha diversity of the gut microbiota

To explore differences in the gut microbiota of healthy people and ICU patients with diarrhea, the Illumina MiSeq platform was used to perform high-throughput sequencing of the v3-v4 region of the bacterial 16S rRNA gene. In total, 593,989 high-quality sequences were obtained from 14 samples, with an average sequence length of 417 in the two groups. These sequences gathered 506,308 (420 types) OTUs with 97% sequence similarity, and each sample contained 31,105 to 40,088 OTUs.

The Ace and Chao indices as well as Shannon and Simpson indices were used to explore the effect of END on the alpha-diversity of the gut microbiota in ICU patients. As shown in Figure 2, the Ace and Chao indices, which represent bacterial

richness in the no-END and END groups, were 199.73 ± 42.78 vs. 113.92 ± 43.40 ($p < 0.01$; Figure 2A) and 200.67 ± 45.22 vs. 105.94 ± 33.73 ($p < 0.001$; Figure 2B), respectively. The bacterial diversity indices (Shannon and Simpson) in the no-END and END groups were 3.53 ± 0.16 vs. 1.33 ± 0.59 ($p < 0.001$; Figure 2C) and 0.06 ± 0.02 vs. 0.44 ± 0.20 ($p < 0.001$; Figure 1D), respectively. These results indicated that compared with no-END patients, the gut microbiota of END patients was disturbed, the richness and diversity of bacteria were all significantly reduced.

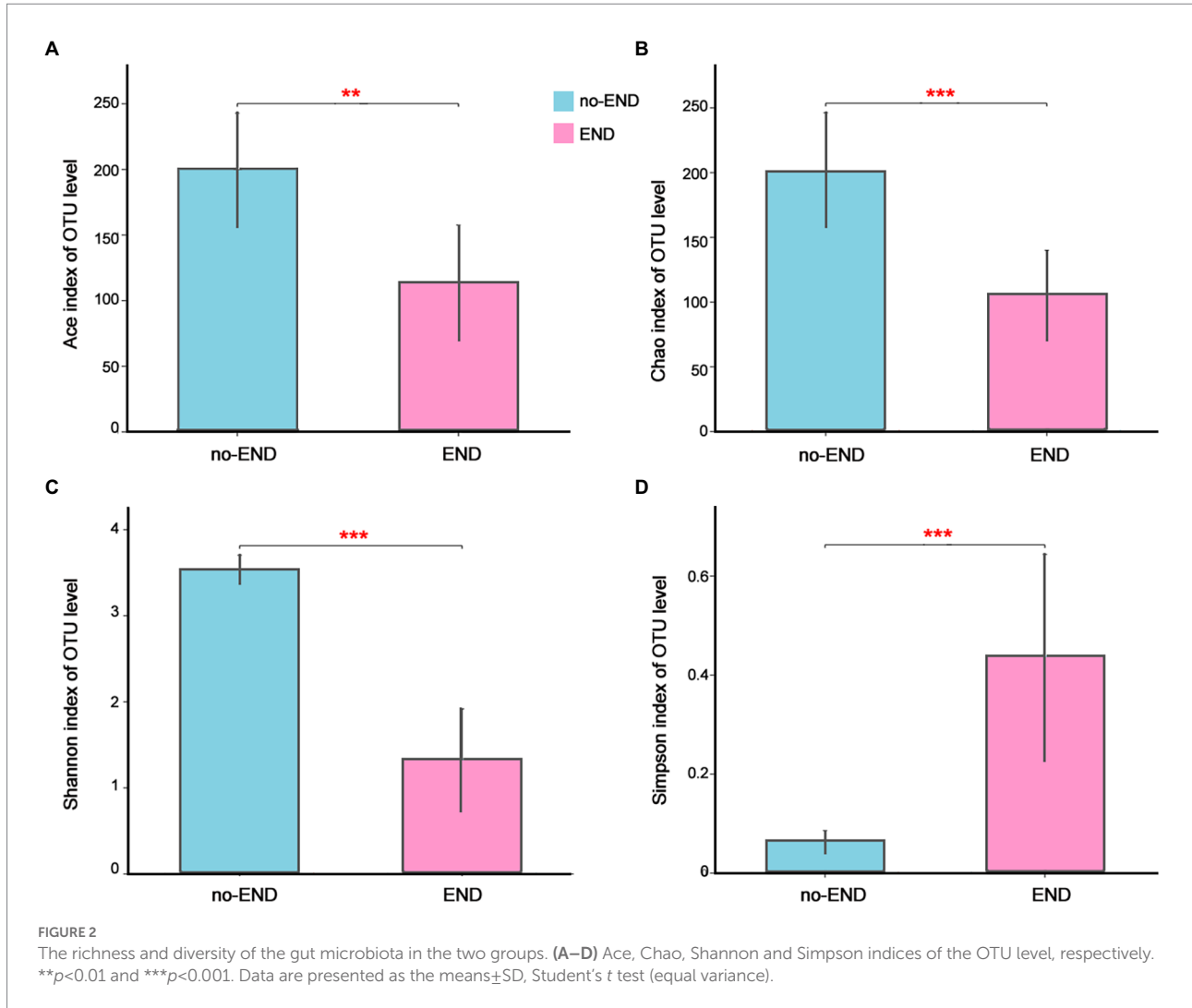
Overall gut microbiota structures

We then investigated the phylum-level bacterial composition of all ICU patient samples, regardless of whether diarrhea occurred. From 16S rRNA gene sequences, OTUs were identified as 13 prokaryotic phyla, and relative abundances of less than 1.00% were classified as "Others." Specifically, the dominant bacterial phyla in all samples were *Firmicutes* (65.68%), *Proteobacteria* (16.13%), *Bacteroidetes* (9.49%), *Actinomycetes* (4.99%), *Verrucobacterium* (3.55%) and others (0.16%; Figure 3A).

To further study the shared and unique gut microbiota of the no-END and END groups, a Venn diagram was constructed to identify the types of OTUs in these two groups (Figure 3B). The results showed that there were 383 and 292 OTUs in the normal group and diarrhea group, respectively. While 225 OTUs (60.71%) were shared by both groups, 128 unique OTUs (30.48%) were present in the no-END group, and 37 unique OTUs (8.81%) newly emerged in the END group. These data strongly confirmed that END caused a significant reduction in gut microbial species, but there were still about 60% OTUs that were not affected by it, indicate the gut microbiota has robust intrinsic stability. However, the restructured host gut microbiota may be the underlying cause of END, and the answer possibly hidden in the remaining 40% of OTUs lost and gained by END patients.

Comparing changes in the bacterial composition of END

The microbial compositions of the no-END and END groups were compared at the genus level. As shown in Figure 4A, the most dominant gut microbiota in the two groups and their relative abundance ratios (Top10) were as follows. No-END group: *Subdoligranulum* (13.22%), *Bifidobacterium* (8.89%), *Blautia* (8.43%), *Bacteroides* (8.52%), *Blautia* (6.17%), *Alistipes* (4.69%), *Christensenellaceae_R-7_group* (4.48%), *Lachnospirillum* (4.27%), *Eubacterium_hallii_group* (3.85%) and *Fusicatenibacter* (3.52%); END group: *Enterococcus* (44.99%), *Klebsiella* (25.90%), other (6.93%), *Akkermansia* (6.71%), *Escherichia-Shigella* (5.02%), *Lachnospirillum* (3.63%), *Sellimonas* (1.60%), *Leuconostoc* (1.26%), *Hungatella* (1.24%) and *Alistipes* (0.71%). The composition of dominant bacteria in the intestine clearly changed after diarrhea. In addition, the clustering tree using the



Bray–Curtis distance algorithm on the left also clearly separated the two sets of samples.

By further comparing the differences in the genus-level gut microbiota between the two groups, we found 15 bacteria that were significantly altered (corrected $p < 0.05$). Specifically, compared with the no-END group, diarrhea led to the massive proliferation of *Enterococcus* and *Klebsiella*, while the other 13 microbiota represented by *Subdoligranulum*, *Bifidobacterium*, and *Blautia* were greatly reduced (Figure 4B).

END causes gut microbiota disturbances

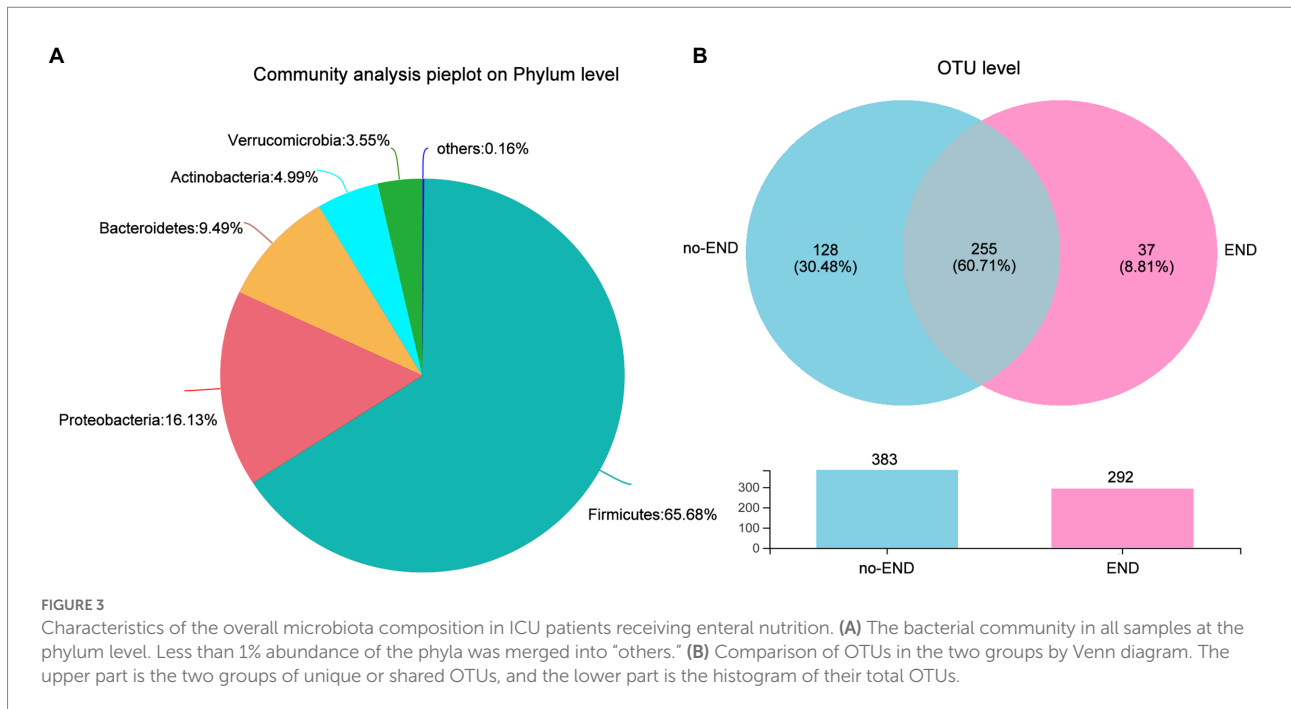
Diarrhea causes numerous changes in the gut microbiota of severely ill patients, leading to a state of ecological imbalance. To determine whether diarrhea affects the abundance of intestinal bacteria at various levels, LefSe (LDA Score > 4.0) was used to analyze the differences in microbial community composition between the two groups at the phylum, class, order, family, and genus levels to indicate the phylogenetic distribution. Compared

with that of the no-END group, the gut microbiota of the END group was disordered (Figure 5A).

The results in Figure 5B clearly show 34 biomarkers in the no-END group and 11 biomarkers in the END group, regardless of their classification level. Specifically, the biomarkers (top 10) of the no-END group were o_Clostridiales, c_Clostridia, f_Lachnospiraceae, f_Ruminococcaceae, o_Bacteroidales, p_Bacteroidetes, c_Bacteroidia, g_Subdoligranulum, c_Actinobacteria and p_Actinobacteria, while c_Bacilli, o_Lactobacillales, g_Enterococcus, f_Enterococcaceae, c_Gammaproteobacteria, p_Proteobacteria, o_Enterobacteriales, f_Enterobacteriaceae, g_Klebsiella and k_norank_d_Bacteria were the biomarkers of the END group (‘p_’, phylum; ‘c_’, class; ‘f_’, family; ‘o_’, order; ‘g_’, genus).

Differences in the gene function of the gut microbiota after END

To infer differentially abundant KEGG pathways associated with END based on the differences in bacterial abundance in the



two groups, PICRUSt and STAMP were used to map microbial genes to KEGG databases.

The results in Figure 6 show that, due to the disorder of the gut microbiota in patients with diarrhea, the bacterial gene function was also significantly different than that in the no-END group. Specifically, by screening KEGG secondary pathways with $p < 0.000$, we identified 24 significantly different pathways. The 7 pathways that were abnormally activated after diarrhea were ascorbate and aldarate metabolism, retinol metabolism, aminobenzoate degradation, Huntington's disease, tryptophan metabolism, the two-component system and the MAPK signaling pathway-fly. In addition, the other 17 pathways represented by Th17-cell differentiation, the IL-17 signaling pathway and necroptosis were significantly inactivated after END. Taken together, the predicted results of these biological functional pathways indicated that pathways mainly related to immunity and metabolism were significantly altered due to the imbalance of gut microbiota after diarrhea. Therefore, we speculate that this may be an important cause of END in ICU patients. However, it should be noted that these results are based on computational predictions, so subsequent experimental verification is essential.

Discussion

The human gut microbiota contains 10^{13} to 10^{14} bacterial cells, which belong to hundreds of different species. The gut microbiota plays an important role in the physical and psychological processes of the human host (Sekirov et al., 2010; Simpson et al., 2021). Therefore, any dysbiosis can have a negative impact on health (Butel, 2014). In healthy individuals, the gut microbiota is mainly

composed of bacteria that have a symbiotic or mutual relationship with the human host. Here, we describe an important result that links diarrhea with enteral nutritional support and gut microbiota. The results showed that ICU patients given enteral nutrition with diarrhea had significantly decreased gut microbial diversity compared with those without diarrhea. A reduction in microbial diversity disrupts intercellular integrity, leading to a leaky gut and increased gut permeability, which in turn leads to gut inflammation (Sharma and Tripathi, 2019). Furthermore, in this study, genus *Enterococcus* and *Klebsiella* were significantly increased when ICU patients presented with END. One study found that critically ill patients often develop intestinal dysbiosis, which is manifested by intestinal overgrowth and the emergence of opportunistic multidrug-resistant pathogens, such as genus *Enterococcus* (Zaborin et al., 2014; McDonald et al., 2016; Ojima et al., 2016). Another study found that *Enterococcus* are present in large numbers in the intestines of diarrheagenic *Escherichia coli* diarrhea patients, which are the main cause of diarrhea in children under 5 years of age worldwide (Gallardo et al., 2020). A North American study found that *Klebsiella* increased nearly 8-fold in small intestinal bacterial overgrowth (SIBO) subjects compared to non-SIBO populations (Leite et al., 2020). Moreover, a Spanish study characterized the intestinal microbiota in the stools of 57 patients with diarrhea from hospital and community-acquired *Clostridium difficile* infection and found that *Klebsiella* was one of the most abundant diarrhea-related bacteria in some patients (Hernandez et al., 2018). These results indicate that genus *Enterococcus* and *Klebsiella* may be pathogenic bacteria of various types of diarrhea, including END, and their excessive proliferation may play an important role in promoting the occurrence of diarrhea.

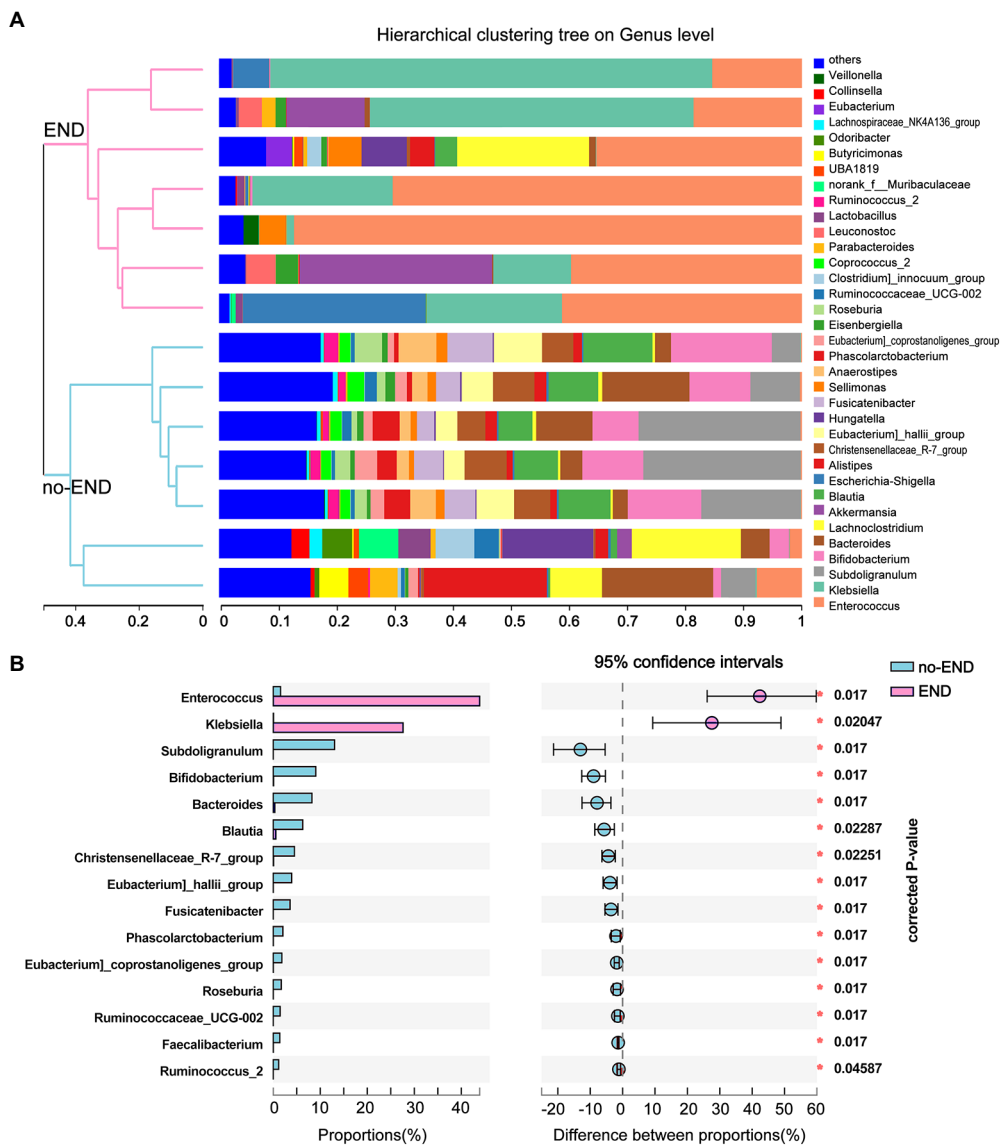
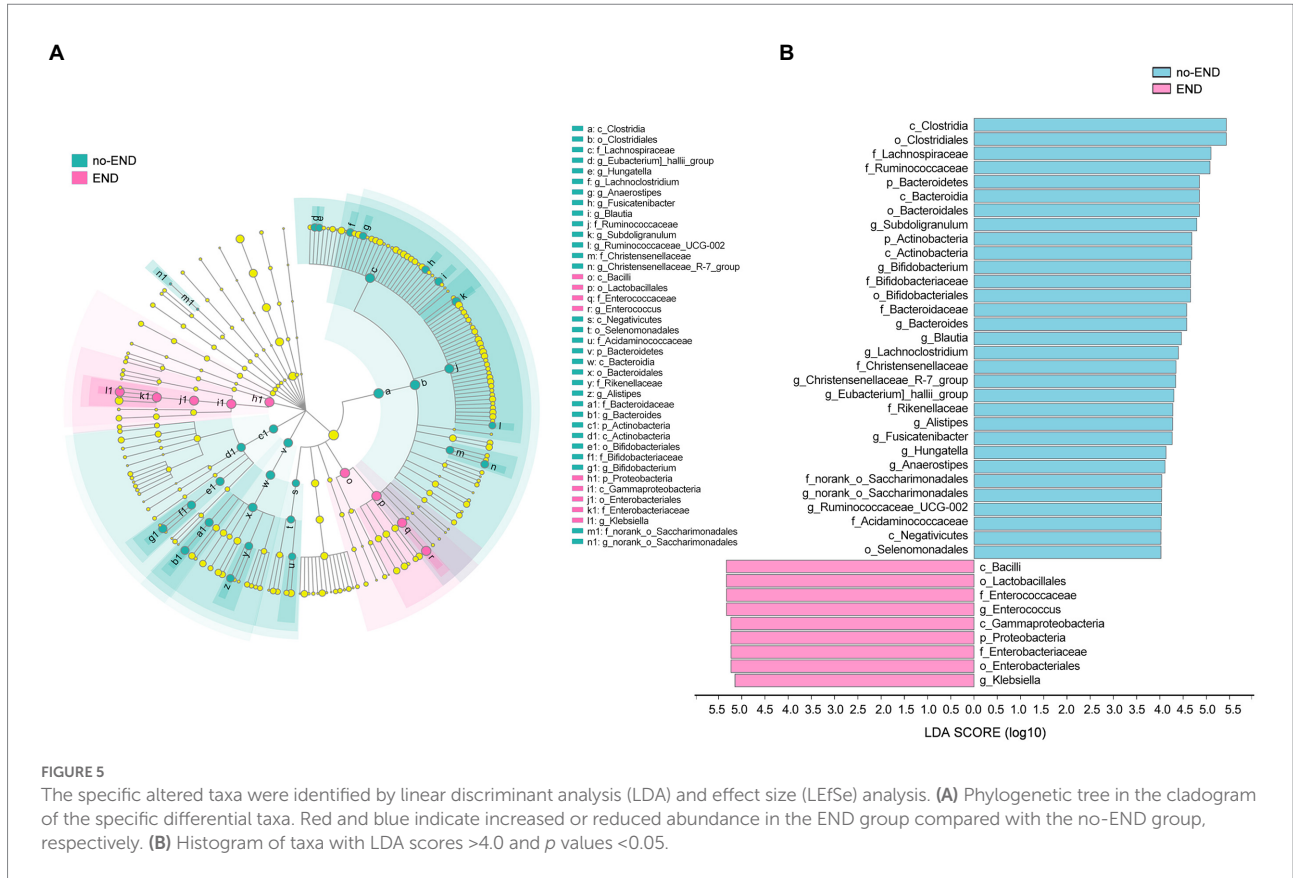


FIGURE 4 Flora distribution and LefSe multilevel species difference. **(A)** Relative composition of genus-level microbiota in the two groups. The legend to the right of the graph indicates the color for each genus of bacterium represented. The low-abundance bacterial group included all bacterial classes with less than 2% total abundance. The hierarchical clustering tree on the left was calculated using the unweighted pair-group method with the arithmetic mean (UPGMA) method, and the relationship between samples was determined by Bray–Curtis distance and the average clustering method. **(B)** 16S rRNA profile comparisons of differentially abundant species between the no-END and END group samples using STAMP analysis at the genus level. The positive difference between proportions denotes a greater abundance in the END group (red), yet the negative difference between proportions shows a greater abundance in the no-END group (blue). The false discovery rate (FDR)-corrected *p* value was calculated based on a two-tailed Wilcoxon rank-sum test. Features with the top 15 (all corrected *p*<0.05) were considered significant and were thus retained.

In contrast, we found that *Subdoligranulum*, *Bifidobacterium*, and *Bacteroides* were significantly reduced in the gut of END patients. One study in diarrhea-predominant irritable bowel syndrome (IBS) patients showed that fecal microbiota in IBS-D featured depleted *Subdoligranulum* (Liu et al., 2020). An animal study found that, compared with rotavirus-mediated newborn calves with diarrhea, the representative genera from *Subdoligranulum* and *Bacteroides* were closely related to healthy calves (Jang et al., 2019). *Bifidobacterium* can protect the intestinal

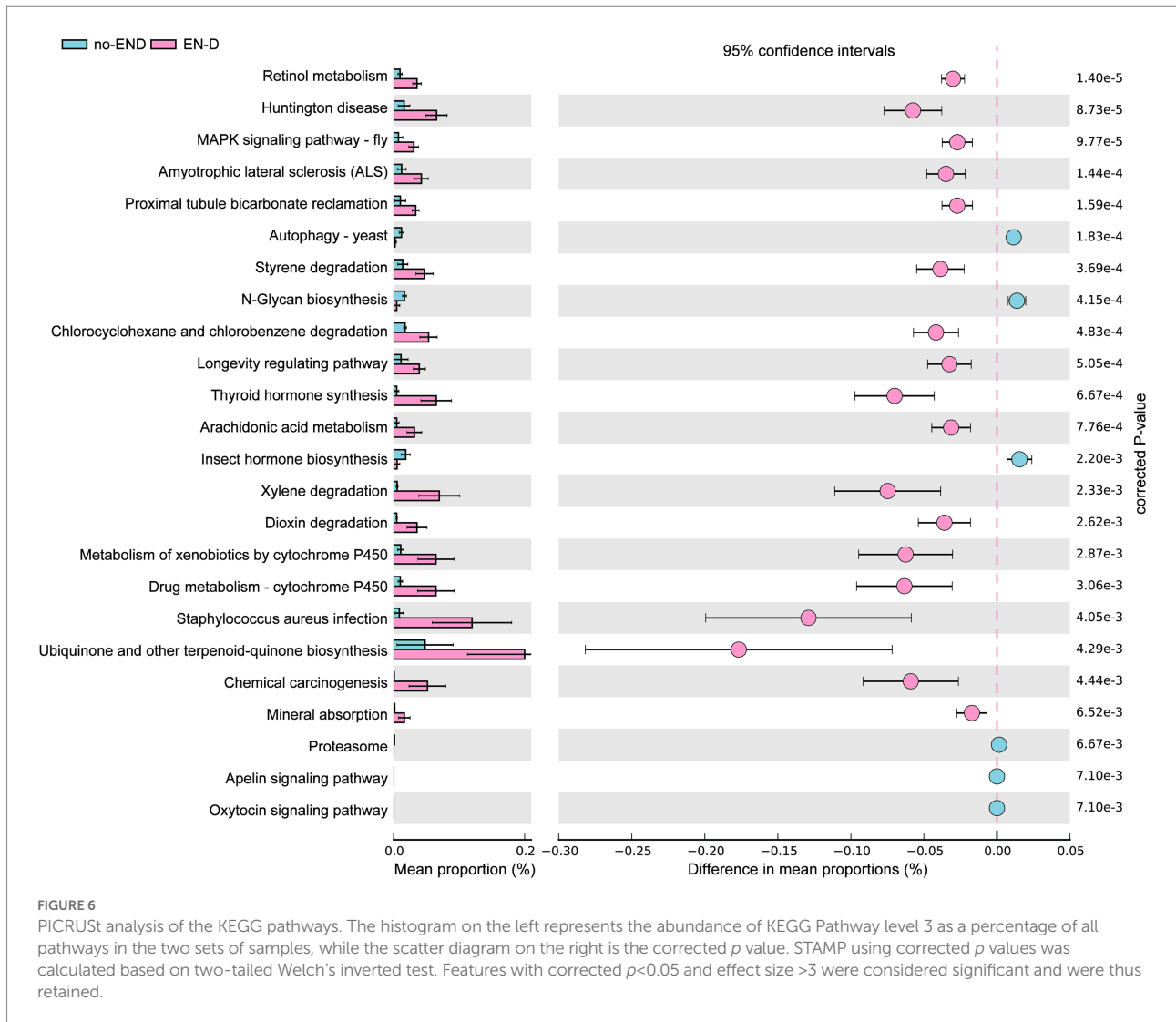
epithelial barrier due to its anti-inflammatory regulatory function (Valdes et al., 2018). According to reports, supplementing *Bifidobacterium* in triple therapy can significantly improve the clearance of *Helicobacter pylori* in the gastrointestinal tract and reduce diarrhea (Sheu et al., 2002), reducing the duration of diarrhea in children with acute diarrhea (Yang et al., 2019) and reducing the symptoms of diarrhea in patients with IBS-D (Skrzydło-Radomska et al., 2020). Another study found that *Bifidobacterium infantis* reduces colonic permeability and



inflammation, decreases interferon-gamma secretion, and maintains the balance of the intestinal system (Ewaschuk et al., 2008). Notably, *Bacteroidetes* are the most important anaerobic bacteria in the gut and are considered candidates for the next generation of probiotics due to their potential role in promoting host health (Wexler, 2007). This shows that *Bacteroides* are human symbiotic bacteria that can play positive roles in human health, and it is a promising therapeutic candidate. Interestingly, our data also showed that *Bacteroidetes* was one of the predominant bacteria in the no-EN samples, while a substantial decrease was observed after diarrhea. In a study of the primate Golden Snub-Nosed Monkey, the most obvious difference between healthy and diarrheal animals was that the *Bacteroides* abundance in diarrheal monkeys was reduced by nearly half (Zhu et al., 2018). These results demonstrated that diarrhea has a microbial component and that dysbiosis contributes to its occurrence. Consequently, the absence of *Subdoligranulum*, *Bifidobacterium*, and *Bacteroides* with this microbial structure could lead to increased susceptibility to END. On the other hand, *Bacteroides fragilis* ZY-312 has been reported to prevent AAD in rats (Zhang et al., 2018). These studies provide more evidence for the important role of *Bacteroidetes* in inhibiting the occurrence of diarrhea.

At present, the usual treatment for patients with diarrhea is probiotics (Li et al., 2021). Probiotics are non-pathogenic live microbial preparations that, when administered in adequate amounts, can alter the host's gut microflora by improving its

intestinal microbial balance, with beneficial effects (Hill et al., 2014). Different health conditions have proven to have beneficial effects with probiotics, such as lactose intolerance, hypercholesterolemia, traveler's diarrhea, acute rotavirus diarrhea, radiotherapy-induced diarrhea, respiratory infections and others (Rondanelli et al., 2017). *Bifidobacterium* is currently one of the main species of probiotics. The probiotics represented by *Bifidobacterium* produce lactic acid, acetic acid, and propionic acid, which lower the intestinal pH and suppress the growth of various pathogenic bacteria, thereby reestablishing the balance of the gut flora. Interestingly, *Bifidobacterium* was also one of the main bacteria significantly reduced in END patients in our study. However, there is convincing evidence from multiple studies supporting the efficacy of probiotics in the treatment of multiple types of diarrhea (Capurso and Koch, 2021; Kambale et al., 2021; Zhang et al., 2021). However, in reality, the efficacy of probiotics for diarrhea in inpatients is not ideal (Allen et al., 2013). This may be either because the daily use of high-dose antibiotics in ICU patients with diarrhea offsets the effect of probiotics or because different types of diarrhea, such as END and AAD, lead to differences in patients' sensitivity to current probiotics. It should also be noted that the effects of probiotics are strain- and dose-specific (Teitelbaum and Walker, 2002). Therefore, we aimed to clarify the type of diarrhea in ICU patients, adjust the types of probiotics, and carry out targeted supplementary interventions. For example, concomitant supplementation and increasing doses



of *Bifidobacterium*, *Subdoligranulum* and *Bacteroides*, which were found to be significantly reduced after END in this study, may have unexpected ameliorating effects on diarrhea symptoms in ICU patients. Moreover, previous studies have shown that the gut microbiome has a close effect on diarrhea through metabolic pathways (Silverman et al., 2017; Rodriguez et al., 2020; Wei et al., 2020). Consistent with previous results, we also found that END mainly caused changes in metabolic pathways, such as inactivation of n-glycan biosynthesis and activation of retinol metabolism, arachidonic acid metabolism and drug metabolism - cytochrome P450 pathways. These results suggest that the gut microbiota may participate in the occurrence of END through these abnormally altered pathways.

Our study has several limitations. First, we analyzed a small number of samples; therefore, our results should be considered as an exploratory approach. However, our data provide valuable information about the importance of gut microbiota in understanding the mechanism of END diarrhea. Second, further longitudinal studies are needed to determine the causal

relationship between the gut microbiome and END. Last, 16S rRNA sequencing technology itself also has some shortcomings, such as the inability to distinguish to the species level. In addition, PICRUSt analysis can only predict known functions of known microorganisms. The above shortcomings need to be further improved by metagenomic sequencing technology. Although these restrictions must be considered when interpreting our results, our data clearly demonstrate that enteral nutrition impairs the gut microbiota and exacerbates the diarrhea response. Thus, we highlight the necessity for timely protective microflora intervention for patients who already are or are about to undergo enteral nutrition treatment to avoid diarrhea.

In summary, these data highlight the comprehensive and detailed characteristics of changes in the gut microbiome at the time of diarrhea in ICU patients receiving EN treatment. By targeting inhibition of abnormal proliferation of *Enterococcus* and *Klebsiella*, and supplementing the loss of *Bifidobacterium*, *Subdoligranulum* and *Bacteroides* may be a novel and effective therapeutic strategy for clinical prevention and treatment of END.

Data availability statement

The data presented in the study are deposited in the NCBI SRA repository, accession number: PRJNA885766, <http://www.ncbi.nlm.nih.gov/bioproject/PRJNA885766>.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Henan Provincial People's Hospital (2022-55). The patients/participants provided their written informed consent to participate in this study.

Author contributions

SZ, LL, and XJ: conceptualization and supervision. WN, SZ, and LL: methodology. HZ, MJ, and SZ: investigation. WN and XJ: analysis, visualization, writing, review, and editing. All authors contributed to the article and approved the submitted version.

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