Fang *et al. Microbial Cell Factories* (2025) 24:64
https://doi.org/10.1186/s12934-025-02684-2

Microbial Cell Factories

RESEARCH Open Access

The comparison of gut microbiota between different types of epilepsy in children

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Abstract

Objective To better understand the variations in gut microbiota in children with different types of epilepsy.

Methods Thirty-seven children with epilepsy were included in the case group, which was further divided into focal (group A, $n=28$) and generalized epilepsy groups (group B, $n=9$) based on the origin and extent of the seizures. The focal epilepsy group was subdivided into the benign childhood epilepsy with centrotemporal spikes (BECT) (group C, $n=9$) and non-BECT groups (group D, $n=19$) based on the appearance of typical centrotemporal spikes or spike-wave complexes on the electroencephalogram (EEG). Additionally, 14 healthy children were selected as the control group (group E, $n=14$).

Results Significant differences were observed in the diversity and composition of gut microbiota between the case and control groups. At the genus level, the abundance of *Megamonas* ($P=0.001$), *Streptococcus* ($P<0.001$), *Romboutsia* ($P=0.001$), *Bacteroides* ($P<0.05$), and *Escherichia/Shigella* ($P<0.05$) was significantly higher in the focal epilepsy group than in the control group (0.027 vs. 0.00009, $P=0.001$; 0.016 vs. 0.002, $P<0.001$; 0.013 vs. 0.002, $P=0.001$; 0.030 vs. 0.002, $P<0.05$, respectively). Additionally, *Escherichia/Shigella* ($P<0.05$) was more abundant in the case group compared to the control group (0.033 vs. 0.002, $P<0.05$). *Bacteroides* ($P<0.05$) was more abundant in the control group than in the case group. *Megamonas* ($P<0.001$) and *Collinsella* ($P<0.05$) were significantly more prevalent in the BECT group than in the control group (0.034 vs. 0.00009, $P<0.001$; 0.014 vs. 0.001, $P<0.05$, respectively). In the non-BECT group, compared to the control group, *Megamonas* ($P=0.013$), *Streptococcus* ($P<0.001$), *Romboutsia* ($P=0.001$), and *Escherichia/Shigella* ($P<0.05$) were found in greater abundance (0.023 vs. 0.00009, $P=0.013$; 0.018 vs. 0.002, $P<0.001$; 0.014 vs. 0.002, $P=0.001$; 0.037 vs. 0.002, $P<0.05$, respectively).

Conclusions Though, there were no statistically significant differences in gut microbiota between the different types of epilepsy, the gut microbiota of children with epilepsy significantly differed from that of healthy controls. The increased abundance of *Escherichia/Shigella* may lead to the worsening of clinical phenotypes and poor prognosis, and it could be a candidate biomarker to identify the focal epilepsy or even non-benign childhood epilepsy with centrotemporal spikes, potentially providing new therapeutic targets for the future.

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Keywords Epilepsy, Gut microbiota, Children, 16SrDNA gene sequencing

Introduction

The incidence of epilepsy in children ranges from 41 per 100,000 to 187 per 100,000, with higher prevalence observed in developing countries. It has been reported that the incidence is highest during the first 12 months of life and gradually decreases to adult levels by the end of the first decade. According to the most recent classification of epilepsy syndromes, only about one-third of children with epilepsy can be assigned to a specific epilepsy syndrome [1–3]. The basic version of the International League Against Epilepsy (ILAE) classification of seizure types categorizes the onset of a seizure as follows: (1) focal, (2) generalized, (3) unknown, or (4) unclassifiable [4]. Focal and generalized seizures also encompass specific types of epilepsy, known as epilepsy syndromes, in which seizure types, electroencephalogram (EEG) findings, and imaging features tend to appear together in the same individual. Examples include infantile spasms, childhood aphasic epilepsy, and benign childhood epilepsy with centrotemporal spikes (BECT). BECT is the most common epilepsy syndrome in children with focal epilepsy, accounting for 14-17% of pediatric epilepsy cases. It has a minimal impact on brain function and is often capable of spontaneous remission [5]. Approximately 30% of patients cannot be effectively treated with clinical medications. This condition is referred to as drug-resistant epilepsy (DRE) [6]. DRE does not now have a uniform or unique definition. It generally refers to a country with insufficient seizure control that renders probably fantastic antiepileptic drugs (AEDs) ineffective at tolerable ranges for 1–2 years and is especially characterized by non-epidemic activities and negative adherence [7]. The pathogenesis of epilepsy is believed to be closely linked to ion channels, neurotransmitter imbalances, genetics, and immunity. However, the exact mechanisms are more complex and not yet fully understood [8]. Moreover, more severe seizures, such as focal secondary bilateral tonic-clonic seizures, may occur following focal seizures. Uncontrolled seizures caused by epilepsy can impair children's growth and development by leading to cognitive deficits and long-term brain dysfunction [9].

Recently, researchers have shown increased interest in gut microbiota. The gut-brain axis, which controls neuronal networks, neuroendocrine, immune, and inflammatory pathways to regulate both intestinal homeostasis and the central nervous system, is a bidirectional communication between the gut and the brain. Advances in sequencing technology have highlighted the crucial regulatory role of the intestinal microbiota in various neurological diseases, such as Parkinson's disease [10], Alzheimer's disease [11], and multiple sclerosis [10], and multiple sclerosis [12]. While people with inflammatory bowel disease are more likely to develop epilepsy, individuals with epilepsy often experience gastrointestinal symptoms [13]. Recent research has linked changes in the gut microbiota to epilepsy. It has been found that there are differences in the gut microbiota between people with epilepsy and healthy individuals. Xie et al. [14] discovered a significant decrease in *Aspergillus* and an increase in *Bacteroides* fragilis in infants with refractory epilepsy compared to healthy children.

Several studies have investigated the gut microbiota of individuals with refractory epilepsy, but none have focused on the gut microbiota of patients with different seizure types of epilepsy, especially those with epileptic syndromes. Therefore, this study aimed to examine the gut microbiota of children with different seizure types of epilepsy and healthy children using high-throughput sequencing technology. Specifically, the study had two main objectives: (1) Compare and analyze the gut microbiota and its differences between different types of epilepsy and healthy children. (2) Compare and analyze the gut microbiota and its differences between different types of epilepsy. (3) Identify new therapeutic targets for epilepsy.

Materials and methods

Study subjects

A total of 37 children with newly diagnosed focal epilepsy, hospitalized in the Neurology Department of Hunan Children's Hospital (Changsha, China) between April 2020 and December 2020, were enrolled as the case group (Fig. 1). The diagnostic criteria for focal epilepsy were based on the ILAE 2017 guidelines [15]. Inclusion criteria were age over two years, electroencephalogram and clinical findings supporting the diagnosis of focal epilepsy, no previous abnormalities on head Magnetic Resonance Imaging, and no family history of epilepsy. Exclusion criteria were the use of specific diets, chronic or acute gastrointestinal disorders, and the use of antibiotics or probiotics within two weeks of either of the two collection time points. The case group was then divided into the focal epilepsy group ($n = 28$) and the generalized epilepsy group ($n = 9$), with the focal epilepsy group further subdivided into the BECT group ($n = 9$) and the non-BECT group ($n = 19$).

Fourteen mentally and physically healthy children of similar ages were included in the control group. To eliminate confounding factors, all subjects were older than three years and had not used antibiotics or intestinal

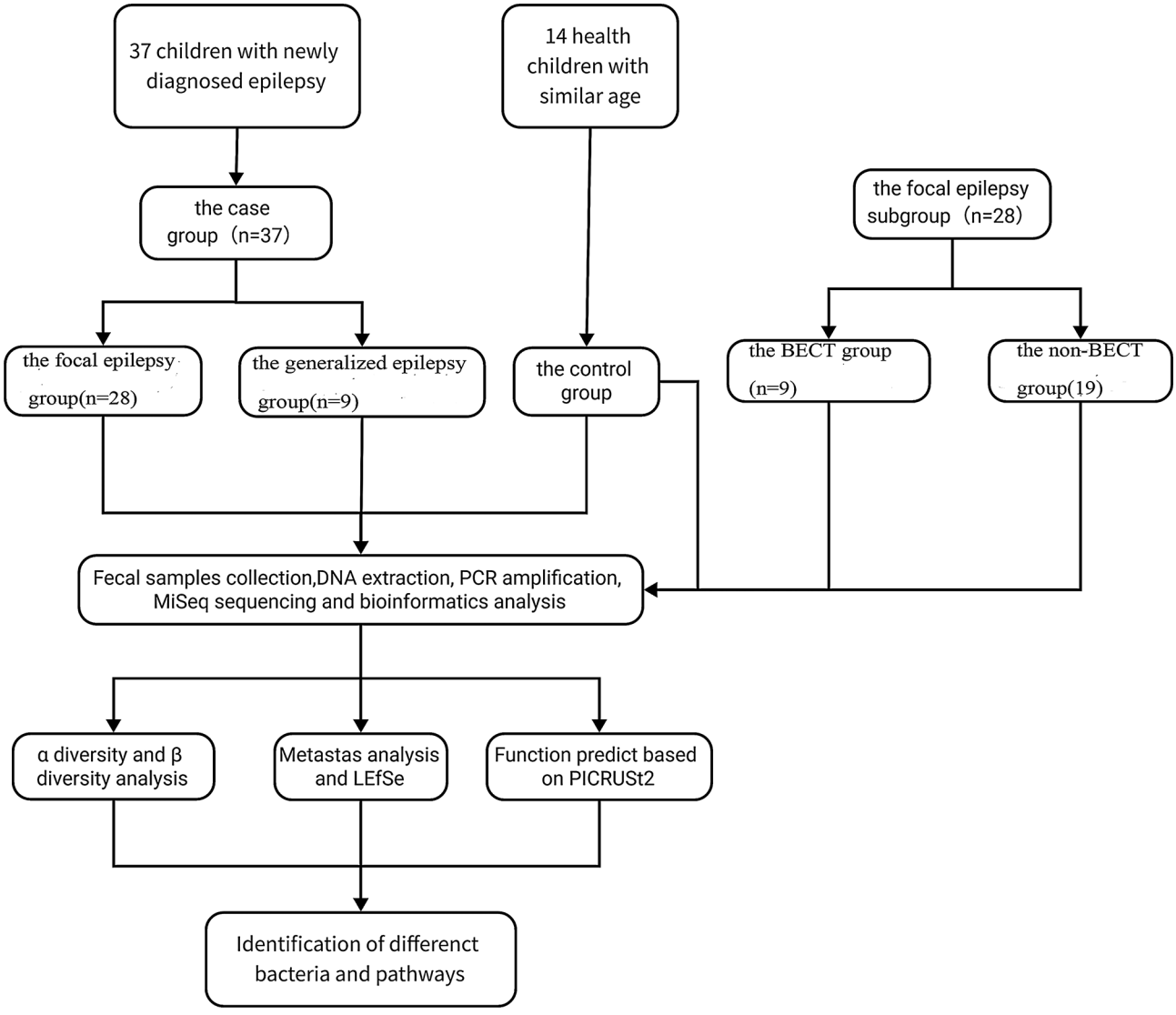

Fig. 1 Flow diagram of study

supplements for at least two weeks. Figure 1 illustrates the recruitment process of all subjects.

The Ethics Committee of Hunan Children's Hospital approved this study (HCHLL-2020-53), and the parents and/or legal guardians of the enrolled children provided informed consent.

Collection of clinical and dietary data

Children's hospitalization records were retrieved from the medical record system. The frequency, type, and duration of seizures were all considered. Further details regarding the study can be found in Changci Zhou et al. [16]. (Supplementary Table 1).

Fecal samples collection

Fecal samples were collected and kept at -80 °C within 30 min.

DNA extraction and high-throughput 16 S rDNA gene sequencing

16S rDNA amplicon sequencing was performed by Genesky Biotechnologies Inc., Shanghai, China (201315). Total genomic DNA was extracted using the FASTDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. The integrity of the genomic DNA was assessed by agarose gel electrophoresis. The concentration and purity of the genomic DNA were measured using a Nanodrop 2000 and a Qubit 3.0 Spectrophotometer. The V4-V5 hypervariable regions of the 16S rDNA gene were amplified using the primers 515F (5'-GTGCCAGCMGCCGCGG-3) and 907R (5'-CCGTCAATTCMTTTRAGTTT-3) [17] and then sequenced on the Illumina NovaSeq 6000 platform (See Table 1).

Table 1 Differences in gut microbiota abundance among focal epilepsy, generalized epilepsy, BECT group, non-BECT group and control groups

Microbiota	Focal epilepsy group	Control group	*P*	q	Generalized epilepsy group	Control group	*P*	q	BECT group	Control group	*P*	q	non-BECT group	Control group	*P*	q
Escherichia/Shigella	0.03	0.002	<0.05	<0.1	0.033	0.002	<0.05	<0.1	0.014	0.002	<0.05	>0.1	0.037	0.002	<0.05	<0.1
Streptococcus	0.016	0.002	<0.001	<0.1	/	/	/	/	/	/	/	/	0.018	0.002	<0.001	<0.1
Megamonas	0.027	0.00009	0.001	<0.1	/	/	/	/	0.034	0.00009	<0.05	<0.1	0.023	0.00009	0.013	<0.1
Romboutsia	0.013	0.002	0.001	<0.1	/	/	/	/	/	/	/	/	0.014	0.002	0.001	<0.1
Bacteroides	0.284	0.433	<0.05	<0.1	/	/	/	/	/	/	/	/	/	/	/	/
Collinsella	/	/	/	/	/	/	/	/	0.014	0.001	<0.05	<0.1	/	/	/	/

The *p*-value represents the statistical significance of the differences between groups, while the *q*-value represents the adjusted value of *p*-values after multiple comparisons

Gut microbial analysis

QIIME2 was used to process the raw read sequences [18], while the cut adapt plugin was employed to trim adapter and primer sequences. The DADA2 plugin was used to assess quality and identify amplicon sequence variants (ASVs) [19]. A pre-trained Naive Bayes classifier, trained on the RDP (version 11.5), was applied to assign taxonomic classifications to ASV representative sequences with a confidence threshold of 0.8. To assess the sufficiency and rationality of the sample size, curve analyses, including the rarefaction curve, Shannon-Wiener curve, and species accumulation curve, were performed. Alpha-diversity was evaluated using richness (Chao1 and ACE) and diversity (Shannon and Simpson). Principal coordinates analysis (PCoA) in beta-diversity, calculated with QIIME2 and visualized using R (Version 4.1.3) was used to evaluate community composition and structure of gut microbiota. Besides, principal component analysis (PCA) based on the genus level of gut microbiota was used to evaluate the value of their contribution to epilepsy. Finally, we analyzed the microbial differences among groups at the phylum and genus levels using Metastats analysis [20] and linear discriminant analysis (LDA) effect size (LEfSe) [21].

Statistical analysis

SPSS was used to analyze the general clinical data of both the cases and controls. The Shapiro-Wilk test was applied to assess whether the data followed a normal distribution. Data are presented as the median and interquartile range [M (P25–P75)], with the Mann-Whitney U test used to compare differences between two groups and the Kruskal-Wallis test used to compare differences among multiple groups. Post-hoc multiple tests were conducted for pairwise comparisons. The Benjamini-Hochberg method was used to adjust the *P*-values (*FDR*) for multiple testing in all multiple comparison analyses [22]. A *P*-value of less than 0.05 was considered statistically significant.

Results

Characteristics of the study sample

There were no significant differences in age, gender, and BMI between the case group and the control group ($P > 0.05$, Supplementary Table 2). A comparison of clinical data between the focal epilepsy group, generalized epilepsy group, and the control group is presented in Supplementary Table 2.

Comparison with the gut microbiota among the focal epilepsy group, generalized epilepsy group, and control group

There were significant differences in alpha diversity among the focal epilepsy group, generalized epilepsy group, and control group ($P<0.05$, Fig. 2a-d). The ACE,

Chao1, Shannon, and Simpson indices in the control group were significantly lower than those in the case group. PCoA revealed a significant divergence in the gut microbiota structure between the case and control groups (Fig. 2e). *Lachnospira* was the primary contributor to the variations in major components at the genus level (Fig. 2f). At the phylum level, the gut microbiota composition in the three groups was primarily dominated by *Firmicutes* and *Bacteroidetes* (Fig. 2g). The relative abundance of *Bacteroidetes* ($P < 0.05$) was significantly lower, while *Firmicutes* ($P < 0.05$) and *Actinobacteria* ($P < 0.05$) were significantly higher in the case group compared to the control group. At the genus level, the relative abundance of *Bacteroides(*$P<0.05$*)* was significantly lower, while *Streptococcus* ($P<0.001$) and *Escherichia/Shigella* ($P<0.05$) were significantly higher in the case group compared to the control group (Fig. 2h). LEfSe among the three groups revealed that *Romboutsia* and *Lactobacillus* were abundant in the gut microbiota of the focal epilepsy group, *Ralstonia* and *Actinomyces* were abundant in the generalized epilepsy group, and *Bacteroides* and *Flavonifractor* were abundant in the control group (Fig. 2i-j).

Comparison with the gut microbiota between the focal epilepsy group and the control group

Alpha diversity in the focal epilepsy group was higher than that in the control group. (Fig. 3a-d). PCoA showed that there was a significant divergence in the gut microbiota structure between the focal epilepsy group and control groups (Fig. 3e).*Megamonas* was the primary contributor to the variations in major components at the genus level (Fig. 3f).According to Metastats analysis, at the phylum level, we found that the relative abundance of *Bacteroidetes* (0.420 vs. 0.562, $P = 0.004$). was significantly lower, while *Firmicutes* (0.511 vs. 0.401, $P = 0.014$) and *Actinobacteria* (0.015 vs. 0.003, $P < 0.001$) were significantly higher in the focal epilepsy group than those in the control group (Fig. 3g). At the genus level, the relative abundance of *Megamonas*, *Streptococcus*, *Romboutsia*, and *Escherichia/Shigella* was significantly higher in the focal epilepsy group than that in the control group (0.027 vs. 0.00009, $P = 0.001$, 0.016 vs. 0.002, $P<0.001$, 0.013 vs. 0.002, $P = 0.001$, 0.030 vs. 0.002, $P<0.05$, respectively) (Fig. 3h). Additionally, LEfSe also discovered *Lactobacillus* and *Streptococcus* were abundant in the focal epilepsy group. *Flavonifractor* and *Hungatella* were abundant in the control group (Fig. 3i-j).

Comparison with the gut microbiota between generalized epilepsy group and the control group

Alpha diversity in generalized epilepsy group was higher than that in the control group (Fig. 4a-d). PCoA showed that there was a significant divergence in the gut microbiota structure between the generalized epilepsy group and control groups (Fig. 4e). *Megamonas* was the primary contributor to the variations in major components at the genus level (Fig. 4f). Metastats analysis revealed that *Bacteroidetes* in the generalized epilepsy group, at the phylum level, decreased significantly than that in the control group (0.331 vs. 0.562, $P = 0.005$). *Firmicutes* and *Actinobacteria* in the generalized epilepsy group increased significantly than those in the control group (0.592 vs. 0.401, $P = 0.012$, 0.017 vs. 0.003, $P = 0.004$, respectively) (Fig. 4g). At the genus level, *Escherichia/Shigella* in the generalized epilepsy group was significantly higher than that in the control group (0.033 vs. 0.002, $P<0.05$) (Fig. 4h). LEfSe revealed that *Actinomyces* and *Ralstonia* were abundant in the generalized epileptic seizure subgroup, and *Bacteroides* and *Faecalibacterium* were abundant in the control group (Fig. 4i-j).

Comparison with the gut microbiota between the focal epilepsy group and generalized epilepsy group

There were no significant differences in alpha diversity and beta diversity between the focal epilepsy group and the generalized epilepsy group. Notably, we were unable to identify any bacteria taxa with significant differences between the focal epilepsy group and the generalized epilepsy group at the phylum and genus levels (Supplementary Fig. 2).

Comparison with the gut microbiota among the BECT group, the non-BECT group and the control group

We discovered that there were significant differences in both alpha diversity and beta diversity among the BECT group, non-BECT group and the control group ($P<0.05$, Fig. 5a-d). PCoA revealed a significant divergence in the gut microbiota structure among the BECT, non-BECT and control groups (Fig. 5e). *Lachnospira* and *Megamonas* were the primary contributors to the variations in major components at the genus level (Fig. 5f). Compared to the BECT and non-BECT groups, the alpha diversity in the control group was significantly lower. At the phylum and genus levels, the relative abundance of *Bacteroidetes* and *Bacteroides* ($P<0.05$) was more abundant in the control group than that in the other two groups. The relative abundance of *Fimicutes* and *Actinobacteria* was more abundant in the BECT group and non-BECT group (Fig. 5g-h). LEfSe also showed *Collinsella* and *Butyricimonas* were abundant in the BECT group, *Lactobacillus* and *Ralstonia* were abundant in the non-BECT group, and *Flavonifractor*, *Hungatella* and *Anaerotruncus* were abundant in the control group (Fig. 5i-j).

Comparison with the gut microbiota between the BECT group and the non-BECT group

There were no significant differences in alpha diversity and beta diversity between the BECT and the non-BECT

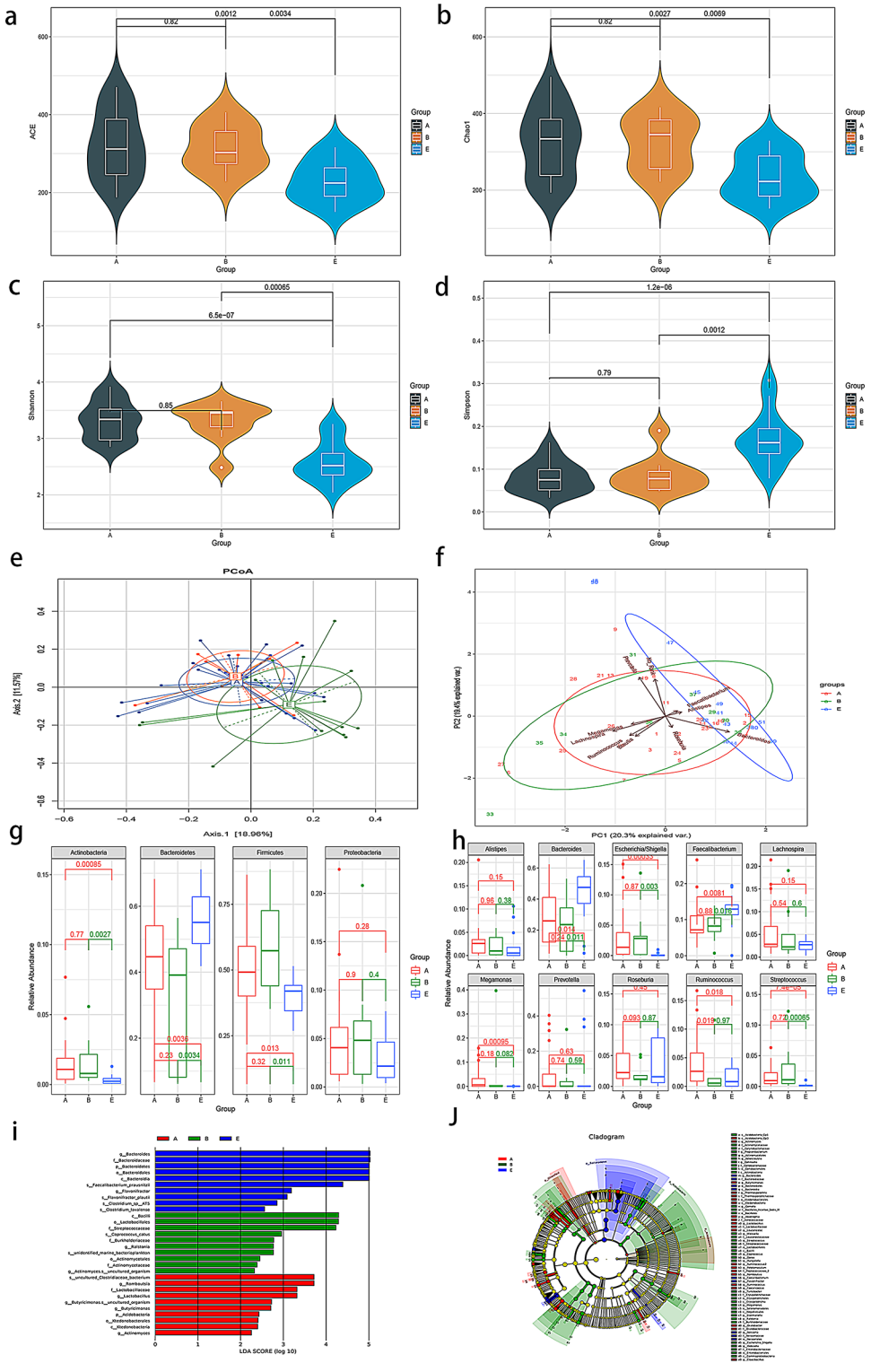

Fig. 2 Comparison of the gut microbiota among the focal epilepsy group, generalized epilepsy group and the control group. (**a-d**) Comparison of the alpha diversity indices among three groups. (**e**) Principal coordinates analysis (PCoA) of beta diversity (based on the Bray–Curtis distance) based on the operational taxonomic unit (OTU) abundance table was performed to evaluate the community composition and structure of the gut microbiota. (**f**) Principal component analysis (PCA) based on the genus level of gut microbiota was used to evaluate the value of their contribution to epilepsy. (**g-h**) Metastats analysis at the phylum(**g**), and genus(**h**) levels among the three groups. (**i-j**) Linear discriminant analysis (LDA)(**g**) value distribution and the cladogram(**j**) of the linear discriminant analysis effect size (LEfSe)among the three groups. (LEfSe is a statistical tool used in microbiota research to identify bacterial taxa or other microbial features that are significantly different between two or more groups, and to estimate their relative effect sizes.) **A**: focal epilepsy group, **B**: generalized epilepsy group E: control group

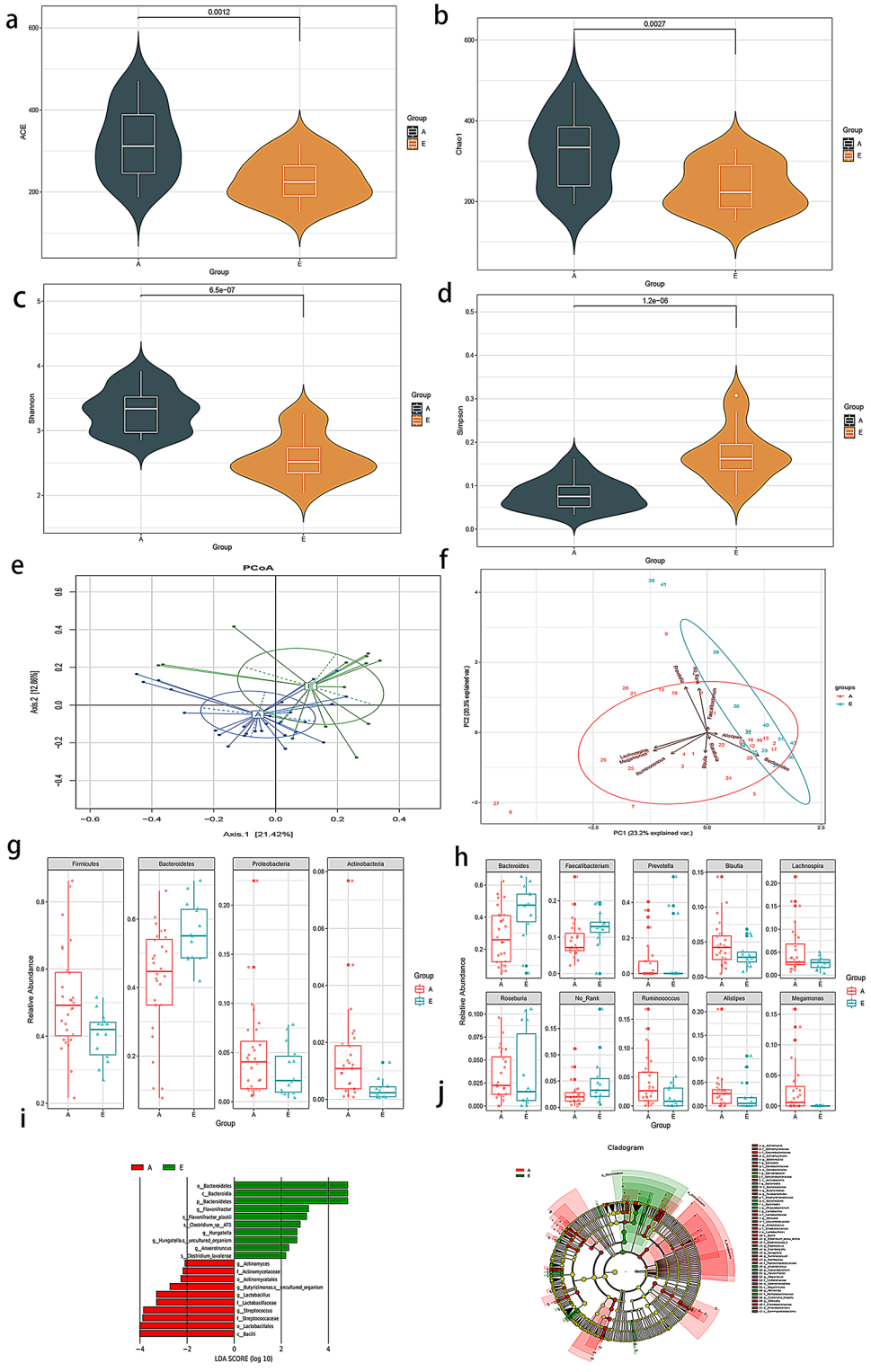

Fig. 3 Comparison of the gut microbiota between the focal epilepsy group and the control group. (**a-d**) Comparison of the alpha diversity indices between two groups. (**e**) Principal coordinates analysis (PCoA) of beta diversity (based on the Bray–Curtis distance) based on the operational taxonomic unit (OTU) abundance table was performed to evaluate the community composition and structure of the gut microbiota. (**f**) Principal component analysis (PCA) based on the genus level of gut microbiota was used to evaluate the value of their contribution to epilepsy. (**g-h**) Metastats analysis at the phylum(**g**), and genus(**h**) levels between the two groups. (**i-j**) Linear discriminant analysis (LDA)(**i**) value distribution and the cladogram(**j**) of the linear discriminant analysis effect size (LEfSe) among the two groups. (LEfSe is a statistical tool used in microbiota research to identify bacterial taxa or other microbial features that are significantly different between two or more groups, and to estimate their relative effect sizes.) **A**: focal epilepsy group, **E**: control group

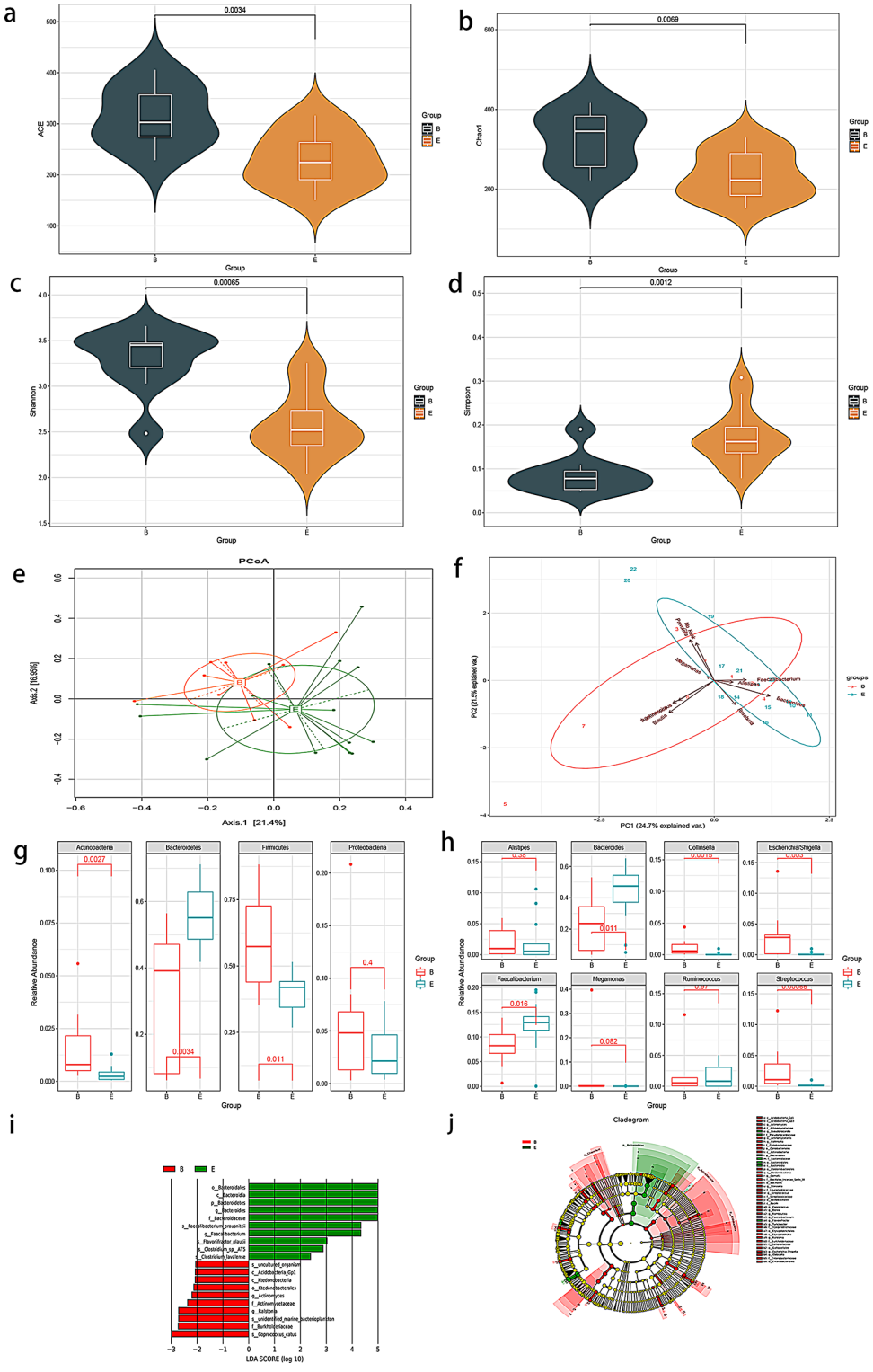

Fig. 4 Comparison of the gut microbiota between the generalized epilepsy group and the control group. (**a-d**) Comparison of the alpha diversity indices between two groups. (**e**) Principal coordinates analysis (PCoA) of beta diversity (based on the Bray–Curtis distance) based on the operational taxonomic unit (OTU) abundance table was performed to evaluate the community composition and structure of the gut microbiota. (**f**) Principal component analysis (PCA) based on the genus level of gut microbiota was used to evaluate the value of their contribution to epilepsy. (**g-h**) Metastats analysis at the phylum(**g**), and genus(**h**) levels between the two groups. (**i-j**) Linear discriminant analysis (LDA)(**i**) value distribution and the cladogram(**j**) of the linear discriminant analysis effect size (LEfSe) among the two groups. (LEfSe is a statistical tool used in microbiota research to identify bacterial taxa or other microbial features that are significantly different between two or more groups, and to estimate their relative effect sizes.) B: generalized epilepsy group, E: control group

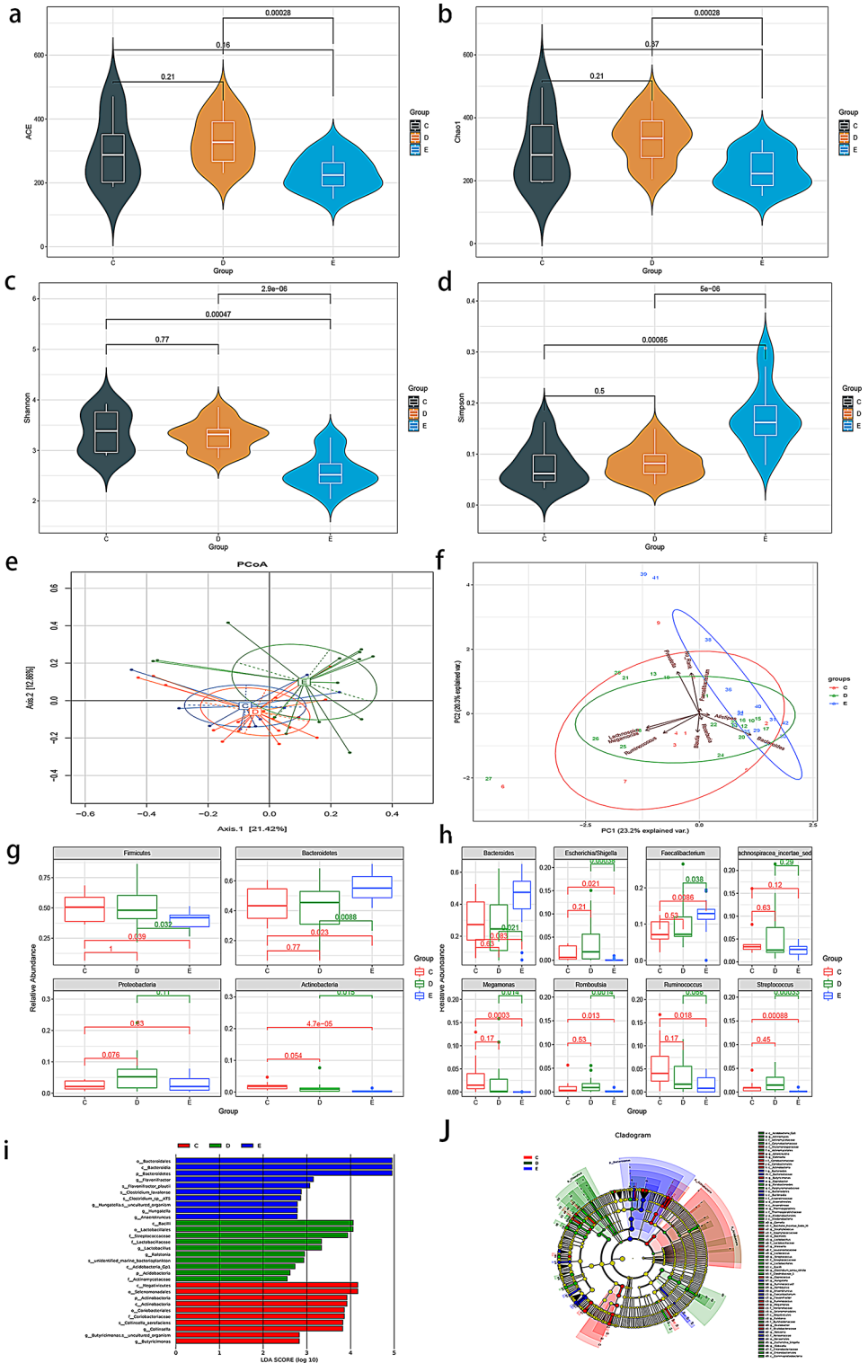

Fig. 5 Comparison of the gut microbiota among the BECT group, non-BECTgroup and the control group. (**a-d**) Comparison of the alpha diversity indices among three groups. (**e**) Principal coordinates analysis (PCoA) of beta diversity (based on the Bray–Curtis distance) based on the operational taxonomic unit (OTU) abundance table was performed to evaluate the community composition and structure of the gut microbiota. (**f**) Principal component analysis (PCA) based on the genus level of gut microbiota was used to evaluate the value of their contribution to epilepsy. (**g-h**) Metastats analysis at the phylum(**g**), and genus(**h**) levels among the three groups. (**i-j**) Linear discriminant analysis (LDA)(**i**) value distribution and the cladogram(**j**) of the linear discriminant analysis effect size (LEfSe) among the three groups. (LEfSe is a statistical tool used in microbiota research to identify bacterial taxa or other microbial features that are significantly different between two or more groups, and to estimate their relative effect sizes.) **C**: BECT group, **D**: non-BECT group **E**: control group

groups. Notably, we were unable to identify any bacteria taxa with significant differences between the BECT and the non-BECT groups at the phylum and genus levels (Supplementary Fig. 3).

Comparison with the gut microbiota between the BECT group and the control group

There was a significant difference in alpha diversity and beta diversity between the BECT group and control group (Fig. 6a-d). PCoA showed that the gut microbiota structure of the BECT group had already significantly diverged from the control group (Fig. 6e). *Ruminococcus* and *Megamonas* were the primary contributors to the variations in major components at the genus level (Fig. 6f). According to Metastats analysis, the relative abundance of *Actinobacteria* in the BECT group was significantly higher than that in the control group at the phylum level (0.020 vs. 0.003, $P<0.001$). At the genus level, the relative abundance of *Megamonas* and *Collinsella* were significantly higher in the BECT group than that in the control group (0.034 vs. 0.00009, $P<0.001$, 0.014 vs. 0.001, $P<0.05$, respectively) (Fig. 6g-h). Also, LEfSe found that *Collinsella* and *Megamonas* were abundant in the BECT group, while *Faecalibacterium* and *Haemophilus* were abundant in the control group (Fig. 6i-j).

Comparison with the gut microbiota between the non-BECT group and the control group

There was a significant difference in alpha diversity and beta diversity between the non-BECT group and control group (Fig. 7a-d). PCoA showed that the gut microbiota structure of the non-BECT group had already significantly diverged from the control group (Fig. 7e). *Bacteroides* were the primary contributor to the variations in major components at the genus level (Fig. 7f).Metastats analysis showed that, compared to the control group *Bacteroidetes* at the phylum level were significantly lower (0.406 vs. 0.562, $P=0.01$), while *Firmicutes* and *Actinobacteria* were significantly higher in the non-BECT group (0.516 vs. 0.401, $P=0.032$, 0.012 vs. 0.003, $P=0.014$, respectively) (Fig. 7g). At the genus level, *Megamonas*, *Streptococcus*, *Romboutsia* and *Escherichia/Shigella* in the non-BECT group were significantly higher than those in the control group (0.023 vs. 0.00009, $P=0.013$, 0.018 vs. 0.002, $P<0.001$, 0.014 vs. 0.002, $P=0.001$, 0.037 vs. 0.002, $P<0.05$, respectively) (Fig. 7h). In addition, LEfSe observed that the non-BECT group's gut microbiota was dominated by *Ralstonia* whereas the control group was dominated by *Hungatella* and *Flavonifractor* (Fig. 7i-j). Interestingly, the difference between *Megamonas* and *Escherichia/Shigella* was significant between both the case and control groups, as well as among the BECT group, non-BECT group, and control group, while *Escherichia/Shigella* did not show a significant difference between the BECT group and control group. Notably, there was a significant higher relative abundance of *Escherichia/Shigella* in the case group, including the non-BECT group, which may serve as a key biomarker for the prognosis and severity of epilepsy.

Discussion

In our study, children with epilepsy exhibited statistically significant differences in gut microbiota diversity compared to healthy controls. The relative abundance of *Actinobacteria*, *Firmicutes*, *Escherichia/Shigella*, and *Megamonas* was elevated, while the relative abundance of *Bacteroidetes* was reduced in both the focal epilepsy group (which includes the BECT and non-BECT subgroups) and the generalized epilepsy group. *E*scherichia/Shigella abundance correlates with epilepsy severity, potentially making it a candidate biomarker.

Our results showed that the alpha diversity was higher not only in the focal epilepsy and the generalized epilepsy groups but also in the BECT and the non-BECT groups. We also found major differences in beta diversity between the focal epilepsy and generalized epilepsy groups and the control group, as well as between the BECT and non-BECT groups compared to the control group. The principal components analysis found that *Megamonas* was primarily responsible for the genus-level variations in between the BECT and non-BECT groups and the control group. Peng and colleagues found that, based on alpha diversity analysis, the gut microbiota abundance in drug-resistant epilepsy patients was higher than that in drug-sensitive epilepsy patients and healthy individuals [23]. Carlson and colleagues revealed that the diversity of gut microbiota was associated with cognitive function, as the higher the alpha diversity was, the worse cognitive performance would be [21]. At the same time, A relationship was found between gut microbiota diversity and cognitive function, with higher alpha diversity being associated with poorer cognitive performance [16]. Therefore, we assumed that the microbial communities in children with focal epilepsy were more diverse and distinct from those in healthy children.

Our study showed that the gut microbiota in all three groups was predominantly composed of *Bacteroidetes* and *Firmicutes* at the phylum level. When compared to the control group, the relative abundance of *Actinobacteria* and *Firmicutes* was increased, while the relative abundance of *Bacteroidetes* was decreased in the focal epilepsy group (including the BECT group, the non-BECT group) and the generalized epilepsy group at the phylum level. Previous research has demonstrated that this composition is characteristic of a healthy gut microbiota. After 12 months, *Bacteroidetes* and *Firmicutes* dominate in both children and adults, which aligns with our findings that the gut microbiota of healthy children and those with

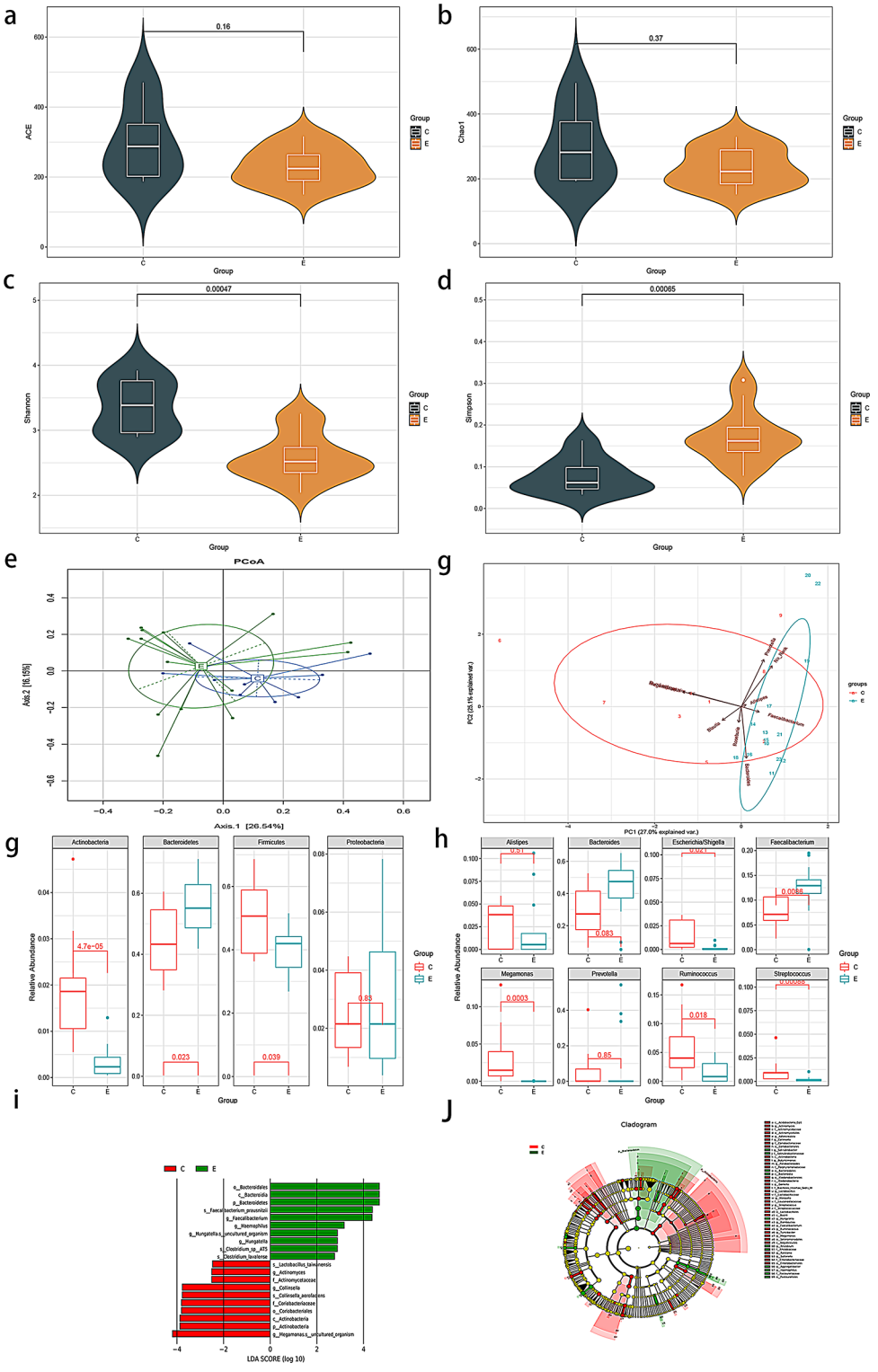

Fig. 6 Comparison of the gut microbiota between the BECT group and the control group. (**a-d**) Comparison of the alpha diversity indices between two groups. (**e**) Principal coordinates analysis (PCoA) of beta diversity (based on the Bray–Curtis distance) based on the operational taxonomic unit (OTU) abundance table was performed to evaluate the community composition and structure of the gut microbiota. (**f**) Principal component analysis (PCA) based on the genus level of gut microbiota was used to evaluate the value of their contribution to epilepsy. (**g-h**) Metastats analysis at the phylum(**g**), and genus(**h**) levels between the two groups. (**i-j**) Linear discriminant analysis (LDA)(**i**) value distribution and the cladogram(**j**) of the linear discriminant analysis effect size (LEfSe) among the two groups. (LEfSe is a statistical tool used in microbiota research to identify bacterial taxa or other microbial features that are significantly different between two or more groups, and to estimate their relative effect sizes.) **C**: BECT group, **E**: control group

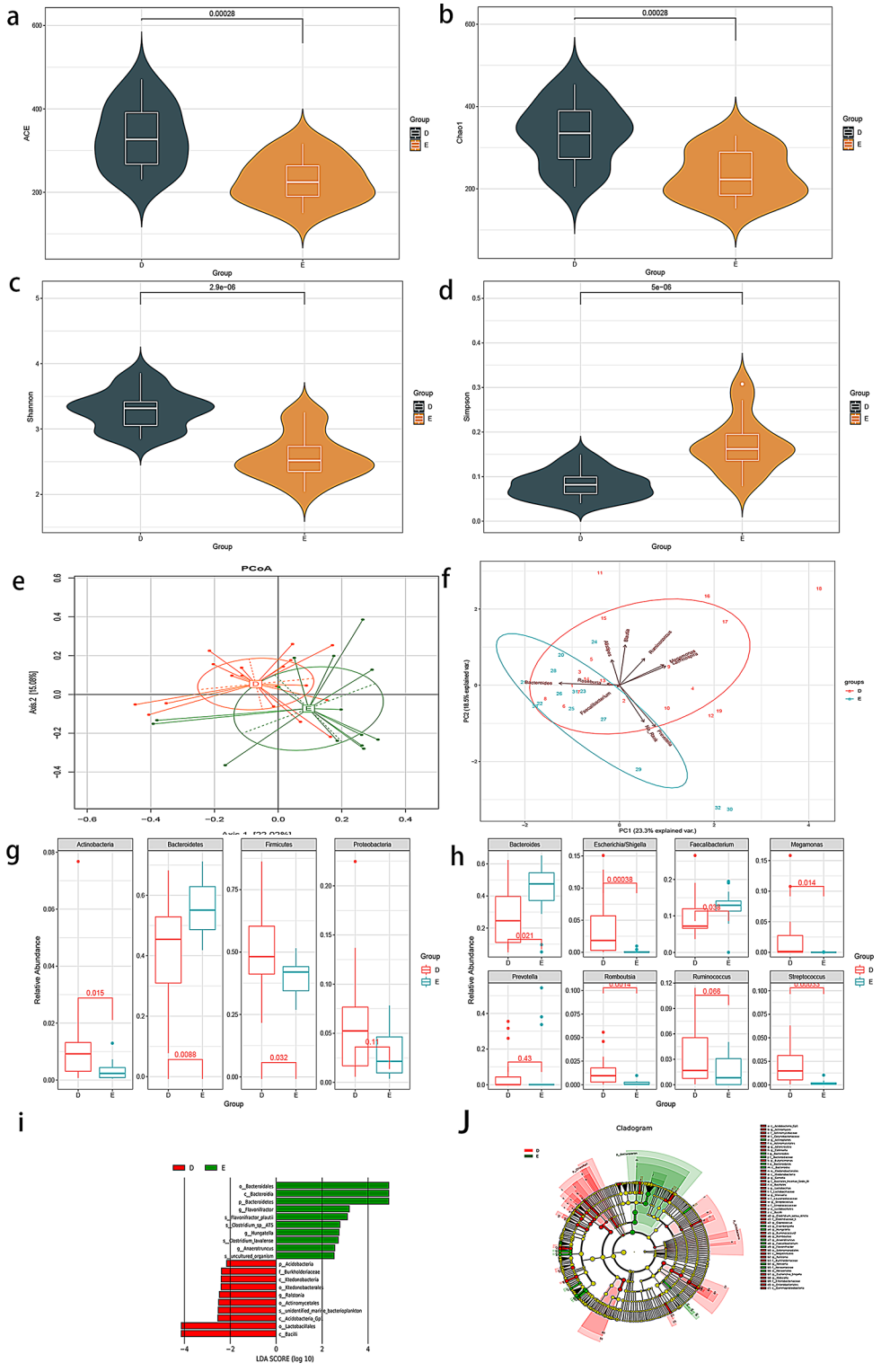

Fig. 7 Comparison of the gut microbiota between the non-BECT group and the control group. (**a-d**) Comparison of the alpha diversity indices between two groups. (**e**) Principal coordinates analysis (PCoA of beta diversity (based on the Bray–Curtis distance) based on the operational taxonomic unit (OTU) abundance table was performed to evaluate the community composition and structure of the gut microbiota. (**f**) Principal component analysis (PCA) based on the genus level of gut microbiota was used to evaluate the value of their contribution to epilepsy. (**g-h**) Metastats analysis at the phylum(**g**), and genus(**h**) levels between the two groups. (**i-j**) Linear discriminant analysis (LDA)(**i**) value distribution and the cladogram(**j**) of the linear discriminant analysis effect size (LEfSe) among the two groups. (LEfSe is a statistical tool used in microbiota research to identify bacterial taxa or other microbial features that are significantly different between two or more groups, and to estimate their relative effect sizes.) **D**: non-BECT group, **E**: control group

focal epilepsy are similar in composition [24], with variations in abundance. The study found that *Bifidobacterium fragilis* improves epilepsy symptoms by modulating the immune system, reducing inflammation, and affecting the release of neurotransmitters [25].

Additionally, at the genus level, *Escherichia/Shigella* were significantly more abundant in patients with poor prognosis (such as those in the focal epilepsy group, generalized epilepsy group, and non-BECT group), whereas no significant differences were observed in the BECT and control groups. Studies have shown that *Escherichia/Shigella* induces pro-inflammatory cytokines through NLRP3 inflammasome-dependent mechanisms [26, 27]. In addition, previous research found a correlation between the levels of IL-1beta, CXCL2, and NLRP3 inflammasomes and the amount of pro-inflammatory *Escherichia/Shigella* in stool, with higher levels observed in patients with cognitive impairment and Alzheimer's amyloidosis compared to the controls [28]. Inflammasomes have been extensively studied to understand how they contribute to the onset of neurological disorders, such as Alzheimer's disease, multiple sclerosis, stroke, and traumatic brain injury [29]. Melatonin may reduce the frequency of epilepsy and seizures [30, 31]. Melatonin has been shown to decrease the occurrence of seizures and epilepsy [32]. Jia's team also observed that after creating an epileptic mouse model and treating it with melatonin, NLRP3, caspase-1, and IL-1β were significantly elevated in untreated epileptic mice compared to the treatment and control groups [33]. These findings suggested that *Escherichia/Shigella* may contribute to the development and progression of epilepsy through inflammatory mechanisms. Previous studies have shown that the abundance of *Escherichia/Shigella* significantly differs before and after treatment [16], suggesting that as the condition improves, the abundance of potential pathogens may decrease accordingly. Therefore, we could conclude that the increase of *Escherichia/Shigella* may play a role in contributing to poor prognosis and the progression of clinical phenotypes, and it could be a candidate biomarker to identify the focal epilepsy or even non-benign childhood epilepsy with centrotemporal spikes.

We also found that focal epilepsy could be associated with changes in the relative abundance of certain other genera, such as an increase in *Streptococcus*, *Collinsella*, *Megamonas*, and *Romboutsia*.

Our results further confirmed that *Streptococcus* was historically more prevalent in both the focal and generalized epilepsy groups. Some studies suggest that the enrichment of *Streptococcus* may elevate the levels of IL-6 and TNF-α [34], which could, in turn, induce epilepsy in children with focal epilepsy by triggering neuroinflammation. This is because IL-6 is linked to epileptic seizures, while TNF-α can increase nervous system excitability by enhancing Ca2 + influx [35, 36].

In addition, the *Megamonas* [37] level in the focal epilepsy group was significantly higher than that in the control group. One study found a significant increase in *Megamonas* in children with autism spectrum disorder [38]. The article about the correlation between infantile spasms and gut microbiota found that, two weeks after treatment for infantile spasms, the *Megamonas* level that in the ineffective group was higher than in the effective treatment group, which was consistent with our article [39].

Interestingly, the *Collinsella* level in the BECT group was significantly higher than that of the control group. *Collinsella* produces short-chain fatty acids (SCFAs). SCFAs can modulate inflammation and reduce the number of inflammatory macrophages and Th 17 cells in the gut, and were once considered important signaling molecules in gut microbiota-brain communication [37, 40]. Previous studies have shown that an enrichment of *Collinsella* might reduce the growth of fermenting bacteria that produce SCFAs [41, 42]. *Collinsella* may potentially promote intestinal permeability by decreasing tight junction protein expression in epithelial cells and increasing IL-17 production. IL-17α is an critical cytokine that can improve macrophages, endothelial cells to produce a large number of inflammatory factors, and promote the occurrence and development of neurological diseases [43]. Related studies have found that IL-17α was higher in cerebrospinal fluid in children with acute seizures [44]. The study on the correlation between infantile spasms and gut microbiota confirmed that the IL-17α levels in infants with spasms were higher than those in the healthy group, and that IL-17α levels decreased after treatment and improvement of the condition [39]. In addition, studies have shown that inter-seizure IL-17α levels have been strongly linked to seizure frequency and severity [45, 46]. Therefore, we could hypothesize that *Collinsella* and *Megamonas* could affect the activation of inflammatory cells by affecting the production of short-chain fatty acids, thus causing changes in intestinal permeability, increase inflammatory factors (IL-17α) into the blood, and finally lead to changes in brain tissue and cells, and promote the progression of epilepsy. We will then test the above hypothesis through animal and cell experiments.

The role of *Romboutsia* in the pathogenesis of epilepsy remains unclear. IN the current study, we found that the severity of the Dravet mouse phenotype, as well as the concentrations of GABA/Glutamate-glutamine cycle factors and glucose levels in hippocampal tissue, were all associated with lower levels of the *Romboutsia* genus [47]. Given the knowledge gap regarding *Romboutsia* as a short-chain fatty acid-producing bacterium and its role in epilepsy, it may be interesting to further investigate its

potential as a disease marker in Dravet syndrome and other epilepsies [47].

There were no significant differences in alpha diversity and gut microbiota between the focal epilepsy and generalized epilepsy groups, which was also the case between the BECT and non-BECT groups. Current studies have not yet identified differences in gut flora among different types of epilepsy, possibly due to limitations in the current level of scientific research. We look forward to further studies in the future.

In summary, the gut-brain axis plays a crucial role in the onset and severity of epilepsy. A combination of probiotics (*Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *and Bifidobacterium infantis*) [48], as well as *Lactobacillus* species (*casei*, *acidophilus*) and *Bifidobacterium bifidum* [49], has shown beneficial effects in an animal model of epilepsy, specifically the pentylenetetrazole-induced kindling model. The researchers found that the levels of *Lactobacillus*, *Roseburia*, and *Lachnospira* were lower in the infantile spasms group (a type of epilepsy) compared to the healthy group. Additionally, the study revealed that the ratio of *Bacteroidota* to *Firmicutes* was higher in epilepsy rats compared to non-epileptic rats. Research has demonstrated that probiotic supplements can significantly reduce both the onset and severity of epilepsy. Specific gut microbiota may serve as a potential therapeutic target for epilepsy in the future, with disease control achievable through the restoration of gut microbiota [50].

Our study also has certain limitations. Firstly, it was a single-center study with a limited sample size, and we used associative approaches rather than causal or experimental methods, which made it difficult to determine the causal relationship between gut microbiota and focal epilepsy. Due to the small number of patients included in this study, it was not possible to perform a statistically robust analysis of confounding factors using multivariate models. However, according to the species accumulation curve of the sample (Supplementary Fig. 1), a total of 51 samples participated in the analysis in this study, and the sample size was sufficient, which could fully reflect the richness of the microbiota. Secondly, the case and control groups were from different families, and we did not control the effects of dietary and regional differences on intestinal flora. Thirdly, the metabolites of the intestinal flora were not measured, so the potential mechanisms between gut microbiota and epilepsy could not be thoroughly analyzed. Finally, children with epilepsy were not followed up for treatment outcomes. However, for the first time, 16 S rDNA high-throughput sequencing and analysis of the gut microbiota in children with focal epilepsy and generalized epilepsy revealed the characteristics and differences in the gut microbiota between these two groups, providing a basis for further research into the potential role of differential gut microbiota distribution in the pathogenesis of epilepsy. This also suggests approaches like fecal microbiota transplantation for the treatment of pediatric epilepsy and offers direction for future research on bacterial metabolites in epilepsy. Although the exact mechanism underlying the amplification of *Escherichia/Shigella* in the gut microbiota of epilepsy patients remains unclear, our findings provide important insights for improving the clinical management of epilepsy. In the future, there can be more collaboration among multiple centers to increase the sample size. We did not fully explore the potential impact of diet on the gut microbiota in this study, which could be a confounding factor in our findings.

Conclusions

The gut microbiota of children with epilepsy differs from that of healthy children. In children with epilepsy, the abundance of *Megamonas* and *Escherichia/Shigella* is increased. Specifically, the abundance of *Escherichia/Shigella* is significantly higher in the poor prognosis groups. This suggests that the increased abundance of *Escherichia/Shigella* may lead to poor prognosis and the worsening of clinical phenotypes, and it could be a candidate biomarker to identify the focal epilepsy or even non-benign childhood epilepsy with centrotemporal spikes, potentially providing new therapeutic targets for the future.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02684-2.

Supplementary Material 1

Acknowledgements

We would like to thank all the children and parents who participated in this study, and we appreciate the support from the data collectors. All authors had access to the study data and reviewed and approved the final manuscript.

Author contributions

SWF, NFH and JQ contributed to the conception and design of the study. Administrative support was provided by LWW, ZHX and JQ. LWW provided the study materials. NFH collected and assembled the data which underwent statistical analysis and interpretation by SWF, JJY, CCZ and JQ.All authors read and approved the final version of manuscript.

Funding

Opening fundings of Hunan Provincial Key Laboratory of Pediatric Orthopedics(2023TP1019), Science and Technology Project of Furong Laboratory(2023SK2111), Hunan Provincial Clinical Medical Research Center for pediatric Limb Deformities. This research was funded by the Science and Technology Department of Hunan Province (2017SK2154).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval
The Ethics Committee of Hunan Children's Hospital approved this study (HCHLL-2020-53), and informed consent was obtained from the parents and/or legal guardians of the enrolled children.

Consent to participate
Written informed consent was obtained from the parents/legal guardians of the subjects.

Competing interests
The authors declare no competing interests.

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Received: 25 June 2024 / Accepted: 21 February 2025
Published online: 14 March 2025

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