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Epilepsia®

RESEARCH ARTICLE

Medication-resistant epilepsy is associated with a unique gut microbiota signature

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Funding information
Fingerprinting a multiomics biomarker profile in patients with STXBP1-RD,

Abstract

Objective: Dysfunction of the microbiota–gut–brain axis is emerging as a new pathogenic mechanism in epilepsy, potentially impacting on medication response and disease outcome. We investigated the composition of the gut microbiota in a cohort of medication-resistant (MR) and medication-sensitive (MS) pediatric patients with epilepsy.

Methods: Children with epilepsy of genetic and presumed genetic etiologies were evaluated clinically and subgrouped into MR and MS. Age-matched healthy controls (HCs) were also recruited. A food diary was used to evaluate nutritional habits, and the Rome IV questionnaire was used to record gastrointestinal symptoms. The microbiota composition was assessed in stool samples through 16S rRNA. α-Diversity (AD) and β-diversity (BD) were calculated, and differential abundance analysis was performed using linear multivariable models (significance: $p.\text{adj} < .05$).

Results: Forty-one patients (MR:MS = 20:21) with a mean age of 7.2 years (±4.6 SD) and 27 age-matched HCs were recruited. No significant differences in AD were found when comparing patients and HCs. Significant positive correlation was found between AD and age (Chao1 $p.\text{adj} = .0004$, Shannon $p.\text{adj} = .0004$, Simpson $p.\text{adj} = .0028$). BD depicted a different bacterial profile in the epilepsy groups compared to HCs (MS vs. HC: Bray–Curtis $F = 1.783$, $p = .001$; Jaccard $F = 1.24$, $p = .001$; MR vs. HC: Bray–Curtis $F = 2.24$, $p = .001$; Jaccard $F = 1.364$, $p = .001$). At the genus level, the epilepsy groups were characterized by a significant increase in *Hungatella* (MS vs. HC: +4.95 $\log_2$ change; MR vs. HC: +6.72 $\log_2$ change); the *[Eubacterium] siraeum* group changed between the MR and MS subgroups.

Significance: Epileptic patients display unique gut metagenomic signatures compared to HCs. Moreover, a different ratio of the butyrate-producing

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[Eubacterium] siraeum group suggests dissimilarities between patients based on the response to antiseizure medications.

KEYWORDS

epilepsy, gut microbiota signature, microbiota–gut–brain axis, pediatrics

1 | INTRODUCTION

The gut microbiota consists of thousands of bacteria and, to a lesser extent, yeasts, parasites, and viruses, colonizing the human gastrointestinal (GI) tract.[1] This ecosystem plays a crucial role in maintaining immune and metabolic homeostasis and protecting against pathogens. Furthermore, it was shown that dysfunction of intestinal tight junctions can promote the direct passage of substances into the bloodstream, directly impacting neurotransmitter/precursor synthesis and influencing the outcome of neurological and psychiatric disorders.[2,3] This complex network of communication is commonly referred to as the microbiota–gut–brain axis (MGBA). Thanks to the recent advent of next generation sequencing (NGS) and multiomic integration approaches, the MGBA has been consistently studied, particularly in preclinical models of epilepsy, due to the more reproducible condition in which rodents are housed and fed.[4]

In contrast, the human gut microbiota has not yet been completely defined due to the host's individual uniqueness and the high variability of bacterial population and extensive genomic intraspecies variations.[5] Furthermore, exposure to environmental factors and host genetics play a major role in determining microbial diversity between individuals. Starting from intrauterine life, the human gut microbiota is exposed to various "modifying" events (e.g., mode of delivery, breastfeeding, weaning), leading to different and individual microbiome signatures even among healthy individuals.[6] Nevertheless, some studies have attempted to investigate at a taxonomic level the microbiota composition in subjects affected by different neurological/neuropsychiatric disorders (e.g., epilepsy and autism), successfully characterizing different signatures between patients and controls.[3,7] In particular, Türay and colleagues have shown that the gut microbiota profile of children with epilepsy of unknown etiology was characterized by the presence of *Megamonas* and *Coriobacterium*, whereas healthy controls presented different metagenomic signatures.[8] Another study in medication-naïve pediatric epilepsy patients showed an increased abundance in *Akkermansia* spp. and Proteobacteria, and a decreased relative abundance in *Faecalibacterium* spp.[9] Although these studies demonstrate a shift in the gut microbiota composition in pediatric epilepsy, additional research is needed to fully characterize these metagenomic signatures, due to the heterogeneity of the results obtained, the limited sample size, and the different epileptic syndromes investigated.

Key points

- α-Diversity correlates with age in patients with epilepsy.
- In children, *Hungatella* differentiates between epilepsy patients and healthy controls.
- *[Eubacterium] siraeum* group differentiates between MR and MS epilepsy patients.

According to the International League Against Epilepsy (ILAE),[10] the intra- and intersyndromic variability in epileptic phenotypes is reflected in the range of possible etiologies. In approximately 30% of cases, the underlying cause is known or presumed to be genetic.[11] Thanks to NGS, the gap between diagnosed and unsolved cases has been partially filled. New causative genes have emerged and have been associated with diverse electroclinical phenotypes.[12] Moreover, even if a primary epileptogenic focus is identified, genetics should not be ruled out. For example, in tuberous sclerosis complex mutated *TSC1* and *TSC2* provide epileptogenic and systemic features that go beyond the finding of cortical tubers.[11] Consequently, in these cases managing seizures can be troublesome. Medication-resistance is defined as the failure of two "adequately chose, used, and well tolerated" antiseizure medications (ASMs) in reducing seizures.[13] No definite mechanisms have been identified; however, different hypotheses have been adduced, including interneuron and cortical network alterations, as well as polymorphisms in drug-metabolizing enzymes.[14]

Interestingly, the gut microbiota can interact bidirectionally with orally administered medications, which encounter different populations of gut microbes while descending through the GI tract. Whereas on one hand medications and their metabolites can affect the GI microenvironment by modulating bacterial growth and composition, on the other they can be enzymatically transformed by gut resident bacteria, thereby altering their bioavailability, activity, or toxicity.[15] Due to these interactions, it is possible that the gut flora might influence the response to ASMs. A recent study showed changes in the gut microbiota composition of children with focal epilepsy compared to healthy controls.[16] Interestingly, these differences were attenuated posttreatment in the same patients, highlighting a possible interaction between gut microbiota and ASMs. In this study, we investigated gut microbiota differences between medication-resistant and medication-sensitive pediatric epileptic patients with genetic and presumed genetic etiology.

2 | MATERIALS AND METHODS

2.1 | Study population

Pediatric patients with epilepsy due to a definite or presumed genetic condition were recruited at IRCCS Istituto Giannina Gaslini between April 2020 and November 2022. Diagnosis and classification of the epilepsy syndrome was based on the ILAE guidelines.[10] Genetic investigations included array-based comparative genomic hybridization, epilepsy panel, or whole exome sequencing as deemed appropriate as per clinical practice. Clinical data were retrieved through our local database. The inclusion criteria were as follows: (1) age < 18 years, (2) confirmed clinical diagnosis of epilepsy, and (3) written informed consent to participate in the study provided by the patient or by the caregivers/legal guardian. The exclusion criteria (EC) were as follows: (1) age ≥ 18 years, (2) antibiotic treatment started <3 months from recruitment, (3) autoimmune systemic comorbidities or ongoing infections at the time of recruitment, (4) any GI chronic disorder (i.e., Crohn disease), and (5) significant feeding problems (i.e., dysphagia).

Patients were stratified into different subgroups (medication-resistant [MR] and medication-sensitive [MS]) based on their response to ASMs at the time of observation. Patients taking one ASM were taking monotherapy, whereas patients taking two or more ASMs were taking polytherapy. Although these groups differ from the ILAE definition of medication resistance,[13] this grouping was chosen due to challenges in assessing seizure response, tolerability, adequate dosage, and appropriate choice of the ASM for the type of seizures.

The healthy control (HC) group included age-matched neurotypical children with the same EC as above, recruited at the IRCCS Istituto Giannina Gaslini Transfusional Center.

The study was conducted following the Helsinki protocol and approved by our local ethics committee, Comitato Unico Regionale Regione Liguria.

2.2 | Study procedures

A stool sample was collected from each participant at the time of the enrollment by spontaneous or enema defecation using a self-collection stool kit (CeGat). The sampling kit contained a liquid-based DNA preservation solution, and samples were stored at room temperature (20–25°C) until DNA extraction.

At the time of stool sampling, any GI symptom was assessed by referring to the validated Rome IV Diagnostic Questionnaire (Rome Foundation, https://theromefoundation.org), which investigates the frequency and intensity of abdominal pain, constipation, diarrhea, reflux, bloating, dyspepsia, nausea, and vomiting.

All the participants were under free dietary regimens without any nutritional restrictions. The participants and their caretakers were also guided by a nutrition expert in the completion of a semiquantitative, specifically built, dietary questionnaire. Food types were broken down into macronutrient categories: proteins, fats, carbohydrates, and fibers. For fat sources (oil and butter), a reference weight value of 10 g (approximately 1 tablespoon) was considered. A pediatric food atlas (Istituto Scotti Bassani) was shown to the patients and caregivers to more easily assess the most frequently eaten portion sizes. Macronutrient intake was provided per day and converted to frequency per 7 days (daily frequency × 7). When there was uncertainty between two daily frequencies (e.g., 1 or 2 times/day), the mean was calculated.

Descriptive statistical analyses were performed to evaluate demographic and clinical characteristics, with continuous data presented as mean ± SD or median (interquartile range) as appropriate, and ordinal data expressed as number (percentage). GraphPad Prism 7.0 software was used for statistical analysis.

2.3 | DNA extraction and sequencing

Total DNA was extracted using the Zymo BiomicsDNA/Rna Miniprep Kit (Zymo Research), and quality was checked with Qubit dsDNA BR or HS (Thermo Fisher Scientific), as per manufacturer's protocol. Twenty nanograms of DNA was used for library preparation by amplifying the V3–V4 hypervariable regions of the bacterial *16S rRNA* gene using the Zymo Quick 16S NGS library preparation kit (Zymo Research). The final libraries were pooled and sequenced with MiSeq

Sequencing System (Illumina) for 2×250 base paired-end reads (90.71% bases ≥ Q30). Demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (2.20). Reads were filtered for reads containing Ns and low-quality bases and adapter's trimming was performed with Skewer (version 0.2.2).[17] Merging of the reads was performed using R package Divisive Amplicon Denoising Algorithm 2 (DADA2).[18] The quality of the FASTQ files was analyzed with FastQC (version 0.11.5-cegat).[19]

2.4 | Bioinformatic analysis

The quality trimming of the raw 16S amplicon sequencing reads was carried out using fastp[20] with "qualified_quality_phred" set to 20 and "length_required" set to 200. Trimmed reads were imported into Qiime2 (version Amplicon 2023.9).[21] 341F and 806R primer sequences were removed from reads using the Cutadapt plugin.[22] A denoising step including quality control of the sequences through trimming and filtering, removal of chimeras, and paired-end reads merging was carried out using the DADA2 plugin.[18] The phylogenetic tree was constricted using the q2-phylogeny plugin. To increase classification accuracy, the weighted taxonomic classifier was trained.[23] First, RESCRIPt plugin[24] was used to process the SILVA 138.1 SSURef NR99 full-length database.[25] Using the q2-clawback plugin, taxonomic weights were assembled for human stool. Then, the full-length SILVA classifier was retrained using the assembled weights. For taxonomic assignments of the amplicon sequence variants (ASVs) obtained from DADA2, the classify-sklearn option of the q2-feature-classifier plugin[26] was used with the trained weighted classifier. Rooted phylogenetic tree, ASV count table, and taxonomic assignment of ASVs were exported and used in the downstream analysis with R (Table S1).

2.5 | KEGG pathway prediction

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) is a tool used to infer the metabolic potential of the microbial communities in an environment using obtained amplicon sequences. Obtained ASV sequences were exported from Qiime2 and used in PICRUSt2 (version 2.5.2)[27] analysis using default settings to obtain Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog abundances. KEGG pathway counts were normalized by calculating relative abundance percentages, and pathways with at least .001% abundance in at least half of the subjects in any group were kept for downstream differential analysis. Microbiome Multivariable Associations With Linear Models (MaAsLin2) was used for statistical testing using the same settings as in taxonomic analysis.

2.6 | Diversity analysis

The phyloseq (version 1.46.0) package was used for the diversity analysis. A phyloseq object was created using metadata, ASV count and taxonomy tables, and rooted phylogenetic tree.

For α-diversity (AD), rarefying was carried out using the minimum sample size of 13 287, and all indices were calculated by the estimate_richness function. Chao1, Shannon Diversity, and Simpson's Index were used in statistical tests. Chao1 reflects the richness only through the number of species and singletons, whereas the Shannon and Simpson diversity indices take both richness and evenness into account, with the first one being more biased toward dominant bacteria in the community. Generalized linear models (GLMs) with gamma error distribution and log link function were used for all three indices. For each model, one of the AD indices was used as a response variable, and group and covariates (age, sex, carbohydrate, protein, fat, fiber) were used as predictor variables. Two different models were used for comparison of epilepsy group (MR + MS) versus HCs, and comparison of MR versus MS. Probability values obtained from GLMs of three indices of each group comparison were adjusted using false discovery rate. Adjusted *p*-value of .05 was used as a significance threshold (Table S1).

β-Diversity (BD) analysis was conducted using Bray–Curtis and Jaccard dissimilarity indices. Both indices are used to estimate dissimilarities between communities; however, the Bray–Curtis index uses quantitative information, giving more weight to dominant taxa, whereas the Jaccard index uses presence/absence information of taxa and gives equal weight to dominant and rare taxa. BD distances were calculated using not rarefied data. Principle coordinate analysis (PCoA) ordination was performed on each index, and plots were obtained using the first two axes that were sorted in descending order based on the relative eigenvalues, which shows how much of the variation is explained. Each distance matrix was used in permutation-based multivariate analysis of variance (PERMANOVA) to test the significance of the difference of bacterial compositions between groups (MR + MS vs. HC or MR vs. MS). Age, sex, and dietary intakes were used as confounding factors. Significance threshold for *p*-values was set to .05 (Table S1).

2.7 | Differential abundance analysis

Differential abundance (DA) analysis was carried out at the phylum, family, and genus levels (Table S1). First, ASV percentile relative abundances were calculated using the ASV counts in each sample.

All ASVs assigned to any kingdom other than bacteria or assigned to mitochondria were filtered out. For each taxonomic rank of interest, ASV abundances were aggregated, and "unidentified" and (for the family and genus levels only) "uncultured" portions were removed prior to the DA analysis. Taxa with a relative abundance higher than .1 for phylum or .01 for family and genus in at least half of the subjects for each group were kept for DA analysis. MaAsLin2[28] was used to test differential relative abundance of bacteria at different taxonomic levels between groups by adjusting for covariates (i.e., age, carbohydrate, protein, fat, and fibers). Two different models were used for the comparison between the epilepsy and control groups (MR + MS vs. HC; set reference group = HC), and comparison within the epilepsy group (MR vs. MS; set reference group = MS). For each MaAsLin2 model, normalized and filtered data in phylum, family, or genus level were used; no further normalization and transformation were selected. Lastly, "CPLM" was used as an analysis method, and maximum significance was set at .1.

Based on our results, dominance-based analysis was carried out specifically for *Hungatella* (Hung) and the *[Eubacterium] siraeum* group (Eub). First, samples in which neither of the two genera were detected were excluded from the analysis. For the rest of the samples, the relative abundances of the two genera were summed and used as an isolated community. Based on their proportional abundance in that isolated community, a binary score (0 or 1) was assigned as Hung/Eub dominance: if the proportional abundance of Hung was ≥50% in the isolated community, the dominance score was assigned as "1", otherwise "0" was assigned to describe the dominant abundance of Eub. For significance of this dominance score between groups, two GLM functions with family parameter set to "binomial" were used. Group and covariates (age, sex, carbohydrate, protein, fat, and fiber) were used as independent variables. Raw *p*-value of .05 was used as a significance threshold.

Relative abundances of significant genera were centered log-ratio (clr) transformed, after replacing zero values with half of the minimum relative abundance value. Log_2 fold changes were calculated by subtracting log2-transformed median relative abundances in the studied groups. Significance values were adjusted *p*-values obtained from the respective MaAsLin2 model. A heatmap was plotted using the ComplexHeatmap package (version 2.14.0).[29] Correlation plots were obtained using ggplot2 package (version 3.4).

Rooted tree was imported into R, and phyloseq object was created as described above. Significant genera-associated ASVs were divided using the subset_taxa function of the phyloseq package. Trimmed phylogenetic tree was exported using phy_tree of the phyloseq package and write.tree function of the ape (version 5.7.1) package.[30] Genus relative abundance percentiles were clr transformed after replacing zero values with half of minimum abundance value in the genus abundance table. The visualization of trimmed phylogenetic tree encircled by a median clr-transformed genus abundance heatmap was carried out with the iTOL website tool.[31]

3 | RESULTS

3.1 | Demographic and clinical characteristics of the studied cohort

The epilepsy groups (MR + MS) accounted for 41 children (23 females) with a mean age of 7.2 years (±4.6 SD). Twenty-six (63%) patients were born by vaginal delivery, 14 (34%) by cesarean section, and the route of delivery for one child was unknown. Nutrition during infancy was reported as exclusive breast-feeding in 18 cases, mixed (i.e, breast-fed for few months then formula-fed) in seven cases, exclusive formula-feeding in seven cases, and unknown in nine cases (Table 1). The mean ± SD age at weaning was 6 ± 4 months for 18 patients.

The diagnosis was developmental and epileptic encephalopathy in 28 (68%) patients, idiopathic generalized epilepsy in seven (17%) patients, and focal epilepsy in six (15%) patients (Table 1). The MR-to-MS ratio was 20:21. The mean number of concomitant ASMs in the MR group was 3.

For the HCs, 27 children with a mean age of 9.3 years (±4.1 SD) were included in the study (Table S1).

The pairwise Mann–Whitney *U*-test showed no significant difference between the groups in respect to age (MR vs. MS: $p = .57$, MR vs. HC: $p = .19$, MS vs. HC: $p = .06$; Figure S1).

3.2 | Gut microbiota richness increases with age

Taxonomic richness and diversity within each sample was evaluated through AD by focusing on three different indices; Chao1 reflects the richness through the number of species and singletons, whereas the Shannon and Simpson

TABLE 1 Demographic, clinical, and dietary data of the studied cohort.

Patient	Sex	Age at enrollment, years	Sz types (current)	Sz frequency	DD	ID	Epilepsy syndrome/ etiology	ASMs	Other treatments	Rome IV	Proteins[a]	Carbohydrates[a]	Fibers[a]	Fats[a]	Type of delivery	Feeding type/age at weaning	MR status
1	F	11	Tonic Sz with drop	Daily	Y	Y	DEE/idiopathic (WES performed)	CLB, FBM	ALA, FOS, inulin, thiamine	Nonretentive FI	14.5	42	24.5	10.5	Vaginal, eutocic	Artificial/6th month	Y
2	F	17	Focal Sz	Daily	Y	Y	DEE/idiopathic (WES performed)	CLB, VPA, CBD, NTZ	ALA, FOS, inulin, baclofen, trihexyphenidyl	Negative	21	11.5	3.5	20	CS	NA/NA	Y
3	F	7	Focal Sz	Daily	Y	Y	DEE/idiopathic (WES performed)	CLB, VPA, LCM	NA	Negative	12	20.5	5	29	Vaginal, eutocic	BF/6th month	Y
4	F	7	PGTCS	Weekly	Y	Y	DEE/idiopathic (WES performed)	LTG, CBD, DZP	NA	Negative	9	36	3.5	15.5	CS	Mixed/6th month	Y
5	F	5	PGTCS	Daily	Y	Y	DEE/idiopathic (WES performed)	RFM, CZP, VPA, FBM, DZP, CLB	ALA, FOS, inulin	Negative	18	39	7	25	Vaginal, eutocic	NA/NA	Y
6	F	8	PGTCS	Daily	Y	Y	DEE/idiopathic (WES performed)	TPM, CLB, VPA	NA	Negative	17.5	20.5	7	12.5	CS	NA/6th month	Y
7	F	9	PGTCS	SF < 1 year	Y	Y	DEE/idiopathic (WES performed)	VPA, ESX	NA	Negative	19.5	18.5	24.5	7.5	Vaginal, eutocic	Mixed/NA	Y
8	F	4	Focal Sz, SGTCS	Daily	Y	NA	DEE/*TSC2* (c.5238_5255del; p.His1746_Arg1751del)	VPA, CBD, TPM, DZP	NA	Negative	17	27.5	0	15.5	Vaginal, eutocic	Mixed/2nd month	Y
9	F	1	SGTCS, absences	Daily	Y	NA	DEE/idiopathic (WES performed)	LEV, VGB	NA	Functional constipation	10	17	14	7.5	CS	BF/5th month	Y
10	M	12	Myoclonic Sz	Daily	Y	Y	DEE/idiopathic (WES performed)	RFM, CLB, CBD	Melatonin	Negative	16	41.5	7	6	Vaginal, eutocic	BF/4th month	Y
11	F	3	Myoclonic Sz	Daily	Y	NA	DEE/*NEXMIF* (c.1326delTp; p.Asp442Glufs*58)	VPA, CBD	ALA, FOS, inulin	Negative	20.5	25	0	3	CS	Artificial/6th month	Y
12	F	3	Focal Sz	Weekly	N	Y, mild	FE/hypothalamic hamartoma (operated) + idiopathic (WES performed)	CBZ, LEV	NA	Negative	11	36	7	12.5	CS	Mixed/5th month	Y
13	M	4	Absences, focal Sz	Daily	Y	NA	DEE/idiopathic (WES performed)	VPA, CLB, CBD	Melatonin	Nonretentive FI	18	26	17.5	20	Vaginal, eutocic	NA/NA	Y
14	M	4	Epileptic spams	Daily	Y	NA	DEE/*DYNC1H1* (c.10247_10279dup; p.Leu3416_ Asn342dup)	VPA, CBZ, TPM	NA	Functional constipation, aerophagia	9.5	14	19	7.5	CS	Mixed/6th month	Y
15	M	6	Tonic Sz, absences	SF < 1 year	Y	Y	DEE/idiopathic (WES performed)	VPA, TPM	Melatonin	Nonretentive FI	23.5	22.5	7	7.5	Vaginal, eutocic	Mixed/NA	Y
16	M	14	Focal Sz	Monthly	Y	Y	DEE/idiopathic (WES performed)	LTG, CBZ	ALA, FOS, inulin	Negative	25.5	34	8.5	12.5	Vaginal, eutocic	BF/3rd month	Y

(Continues)

TABLE 1 (Continued)

Patient	Sex	Age at enrollment, years	Sz types (current)	Sz frequency	DD	ID	Epilepsy syndrome/ etiology	ASMs	Other treatments	Rome IV	Proteins[a]	Carbohydrates[a]	Fibers[a]	Fats[a]	Type of delivery	Feeding type/age at weaning	MR status
17	M	1	Focal Sz	Daily	Y	NA	DEE/idiopathic (a-CGH performed)	VPA, LEV	NA	Negative	14.5	3	3.5	7.5	Vaginal, eutocic	NA/NA	Y
18	F	15	PGTCS, absences	Daily	Y	Y	DEE/de novo *BRAF* pathogenic variant	VPA, CBD, LCM, LZP	ALA, FOS, inulin	Negative	35	15	7	15	Vaginal, eutocic	Artificial/6th month	Y
19	F	9	Focal Sz	Daily	N	N	FE/idiopathic	LEV, CLB	ALA, FOS, inulin	Negative	21.5	44	4	18	Vaginal, eutocic	NA/NA	Y
20	M	13	Myoclonic Sz	Daily	Y	Y	DEE/idiopathic (WES performed)	ESX, PER, VPA, LEV, CLB, CBD	NA	Negative	46.5	25	7	6	Vaginal, eutocic	BF/NA	Y
21	M	6	PGTCS	SF > 1 year	N	Y	IGE/idiopathic (WES performed)	VPA	NA	Negative	18.5	32.5	1	17	Vaginal, eutocic	BF/6th month	N
22	M	8	Focal Sz	SF > 1 year	N	N	FE/idiopathic (WES performed)	LEV	NA	Negative	20.5	24	7	17	Vaginal, eutocic	BF/5th month	N
23	F	13	Focal Sz	Daily	N	N	FE/idiopathic (epi panel performed)	CBZ	ALA, FOS, inulin	Negative	19.5	22.25	4	10.5	CS	BF/NA	N
24	F	8	Absences	SF > 1 year	N	N	IGE/idiopathic	ESX	NA	Negative	9	24	1.5	7.5	Vaginal, eutocic	BF/6th month	N
25	M	12	Focal Sz	Daily	N	N	FE/idiopathic	CBZ	ALA, FOS, inulin	Negative	9.5	22	17.5	15.5	Vaginal, eutocic	BF/NA	N
26	M	5	Tonic Sz	Yearly	Y	Y	DEE/idiopathic (a-CGH negative)	VPA, DZP	NA	Negative	27.5	35.25	7	15.5	Vaginal, eutocic	BF/6th month	N
27	M	4	Absences, clonic Sz	Daily	Y	NA	DEE/idiopathic (a-CGH performed)	VPA	ALA, FOS, inulin	Negative	31	32.5	5	15.5	Vaginal, eutocic	BF/2nd year	N
28	F	1	Focal Sz	Monthly	Y	NA	DEE/Xp22 deletion (13804Kb) including *CDKL5*	VPA	NA	Negative	17	31.5	3.5	7.5	CS	BF/NA	N
29	F	4	PGTCS	SF > 1 year	N	NA	IGE/idiopathic (a-CGH negative)	CLB	ALA, FOS, inulin	Negative	11.5	25.5	14	25	Vaginal, eutocic	Artificial/NA	N
30	M	6	PGTCS, SE	SF > 1 year	Y	Y	DEE/idiopathic (a-CGH performed)	ESX	NA	Functional constipation	16	27	3	20	CS	NA/NA	N
31	F	12	PGTCS	SF > 1 year	N	N	IGE/idiopathic (a-CGH negative)	VPA	NA	Aerophagia	13.5	19	7	17	CS	NA/NA	N
32	F	0	Focal Sz	SF < 1 year	Y	NA	DEE/idiopathic (WES performed)	LEV	NA	Negative	13.5	24	3	3	Vaginal, eutocic	Artificial/NA	N
33	M	5	Focal Sz	Yearly	N	N	FE/idiopathic (epi panel performed)	VPA	NA	Negative	18.5	61	14	12.5	CS	Artificial/NA	N
34	F	13	PGTCS	Yearly	N	N	IGE/idiopathic (epi panel negative)	ESX	NA	Functional vomiting	20	24	14	7.5	CS	Mixed/NA	N

TABLE 1 (Continued)

Patient	Sex	Age at enrollment, years	Sz types (current)	Sz frequency	DD	ID	Epilepsy syndrome/ etiology	ASMs	Other treatments	Rome IV	Proteins[a]	Carbohydrates[a]	Fibers[a]	Fats[a]	Type of delivery	Feeding type/age at weaning	MR status
35	M	12	Absences, PGTCS	SF > 1 year	Y	Y	DEE/idiopathic (epi panel negative)	VPA, DZP	NA	Functional constipation	19	30	7	10.5	NA	NA/NA	N
36	F	0	Epileptic spams	Daily	Y	NA	DEE/idiopathic (epi panel performed)	LEV	NA	Negative	38	22.5	7	10.5	Vaginal, eutocic	BF/NA	N
37	M	4	FS, PGTCS	Monthly	N	NA	IGE/idiopathic (*SCN1A* and a-CGH negative)	VPA	NA	Negative	21.5	40	7	2.5	CS	BF/NA	N
38	F	2	FS, absences	Monthly	N	NA	IGE/idiopathic (a-CGH performed)	DZP	ALA, FOS, inulin, levothyroxine	Negative	14	38.5	14	15.5	Vaginal, eutocic	BF/6th month	N
39	M	12	Myoclonic Sz	Yearly	N	Y	DEE/idiopathic (WES performed)	LEV	RR	Functional constipation	14.5	46.5	14	7.5	Vaginal, eutocic	BF/NA	N
40	M	3	Focal Sz	Monthly	Y	Y	DEE/idiopathic (a-CGH negative)	CLB	NA	Negative	7	28	0	10.5	Vaginal, eutocic	Artificial/NA	N
41	F	12	Focal Sz	Daily	Y	Y	DEE/idiopathic (a-CGH negative)	LEV	NA	Negative	21.5	39.5	5	6	Vaginal, eutocic	BF/NA	N

Abbreviations: a-CGH, array-based comparative genomic hybridization; ALA, α-lactalbumin; ASM, antiseizure medication; BF, breast-feeding; CBD = cannabidiol; CBZ, carbamazepine; CLB, clobazam; CS, cesarean section; CZP, clonazepam; DD, developmental delay; DEE, developmental and epileptic encephalopathy; DZP, diazepam; epi, epilepsy; ESX, ethosuximide; F, female; FBM, felbamate; FE, focal epilepsy; FI, fecal incontinence; FOS, fruttooligosaccharides; FS, febrile seizures; ID, intellectual disability; IGE, idiopathic generalized epilepsy; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; M, male; MR, medication-resistant; N, no; NA, not available; NTZ, nitrazepam; PGTCS, primarily generalized tonic–clonic seizures; PER, perampanel; RFM, rufinamide; RR, risperidone; SE, status epilepticus; SF, seizure-free; SGTCS, secondarily generalized tonic–clonic seizures; Sz, seizure(s); TPM, topiramate; VGB, vigabatrin; VPA, valproic acid; WES, whole exome sequencing; Y, yes.

[a] For macronutrients, the reference is times eaten per week.

indices take into account both richness and evenness, with the first one being more biased toward dominant bacteria in the community.

All samples ($n=68$) were included for evaluating the differences in the bacterial community composition between the epilepsy groups (MS and MR) and the HC group. Similarly, all the patient samples ($n=41$) were used for comparing the MS versus MR subgroups. We found no significant differences in AD between the groups using the three indices (MR vs. HC: Chao1 p.adj = .75, Shannon p.adj = .799, Simpson p.adj = .898; MS vs. HC: Chao1 p.adj = .898, Shannon p.adj = .898, Simpson p.adj = .799; MS vs. MR: Chao1 p.adj = .668, Shannon p.adj = .456, Simpson p.adj = .586).

Age was the only confounding factor to be significantly correlated with increased bacterial AD in all groups using all the three indices tested (Chao1 p.adj = .0004, Shannon p.adj = .0004, Simpson p.adj = .0028; Figure 1A). No significant association was found with other confounding factors (sex: Chao1 p.adj = .416, Shannon p.adj = .973, Simpson p.adj = .898; carbohydrates: Chao1 p.adj = .275, Shannon p.adj = .159, Simpson p.adj = .168; protein: Chao1 p.adj = .416, Shannon p.adj = .068, Simpson p.adj = .099; fats: Chao1 p.adj = .416, Shannon p.adj = .898, Simpson p.adj = .954; fibers: Chao1 p.adj = .968, Shannon p.adj = .954, Simpson p.adj = .777).

3.3 | BD depicts a different bacterial profile in the epilepsy groups

BD measures intersample species richness and evenness. When comparing the three groups, the pairwise PERMANOVA test showed significant differences based on both dissimilarity indices for MS versus HC (Bray–Curtis $F=1.783$, $p=.001$; Jaccard $F=1.24$, $p=.001$), and MR versus HC (Bray–Curtis $F=2.24$, $p=.001$; Jaccard $F=1.364$, $p=.001$), indicating that epilepsy is associated with a unique gut bacterial profile. Conversely, no significant differences in the intersample bacterial composition were found between the MR and MS subgroups either with the Bray–Curtis ($F=1.241$, $p=.104$) or with the Jaccard index ($F=1.081$, $p=.1$; Figure 1B).

3.4 | Taxonomic analysis reveals a unique bacterial population in the MR subgroup

DA analysis was carried out to investigate differences in the abundance of each taxon at three taxonomic ranks: phylum, family, and genus.

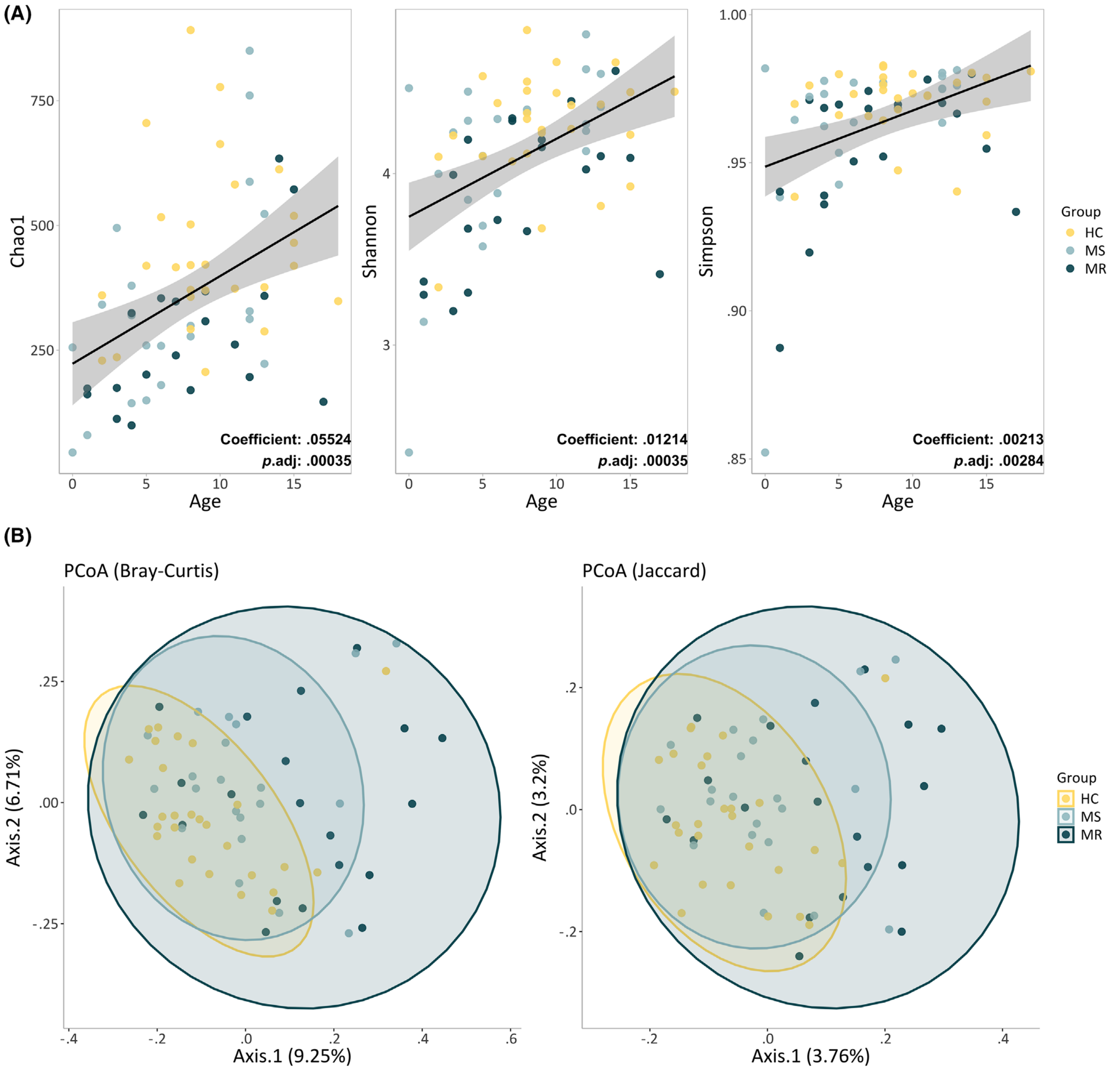
No significant changes between groups were found at the phylum or family level. However, two different genera were obtained from the pairwise comparison of the three groups (Figure 2). Specifically, Hung was more abundant in both the MS and MR groups, compared to HCs (4.95 and 6.72 $\log_2$ fold changes, respectively), whereas Eub further differentiated between the MR and MS groups (−4.87 $\log_2$ changes).

We then constructed a phylogenetic tree showing the ASVs annotated to the statistically significant genera described above (Figure 3). Our results show a similar profile in the MS and HC groups with regard to Eub. On the other hand, the MR group showed lower relative abundance in all the analyzed ASVs. Furthermore, the relative abundance of Hung ASV 1–5 was lowest in the HC group, intermediate in the MS group, and highest in the MR group, suggesting that these populations might play a role in refractory epilepsy.

To explore the relationship between different bacterial populations, we performed the ratio between Bacteroidota and Firmicutes, widely accepted to play an important role in maintaining normal intestinal homeostasis. Our data did not reveal any significant differences between the groups (Figure 4A). At the genus level, we investigated whether epilepsy and medication resistance were associated with a dominance in Hung abundance over Eub. Due to the nature of the data, we were not able to perform a straightforward ratio between Hung and Eub. Instead, we treated Hung and Eub as an isolated community and assigned a binary score based on their dominance. Our results show a significantly increased dominance in Hung in the MR versus HC and in the MS versus HC groups (Figure 4B).

3.5 | Distinctive KEGG pathways identified in MR epilepsy

The gut microbiota produces metabolites that can travel systemically in the circulation and enter the central nervous system (CNS) to regulate brain activity. To gain insight on the functional potential of the populations characterized, we performed metabolic pathway prediction using PICRUSt2. The results revealed an upregulation of D-glutamine and D-glutamate metabolism in MS, compared to MR patients ($p=.03$, p.adj = .358). Furthermore, the MS group showed downregulation of fatty acid biosynthesis compared to the HC group ($p=.046$, p.adj = .62). Lastly, fructose and mannose metabolism was highest in MR patients, compared to MS ($p=.007$, p.adj = .346) and HC ($p=.014$, p.adj = .455; Figure 5).



Comparison	*F*	Pr(>*F*)
MS vs HC	1.782509	.001
MR vs HC	2.240297	.001
MR vs MS	1.241217	.104

Comparison	*F*	Pr(>*F*)
MS vs HC	1.239462	.001
MR vs HC	1.364206	.001
MR vs MS	1.081038	.095

FIGURE 1 α-Diversity (AD) correlates with aging, whereas β-diversity depicts a different bacterial profile in the epilepsy groups compared to healthy controls (HCs). (A) Correlation plots illustrating the associations between three AD indices (Chao1, Shannon Diversity, Simpon's Index) and age. Dots represent individual subjects and are colored based on the group. Black line is the regression line, with a gray area corresponding to the 95% confidence interval. Coefficient and adjusted *p*-values are obtained from respective generalized linear models. (B) Principle coordinate analysis (PCoA) plots of Bray–Curtis and Jaccard dissimilarity indices were obtained using the first two axes. The percentage of total variation explained by each axis is shown next to the axis label. Points represent each individual. Ellipses correspond to 95% confidence intervals for each group. MR, medication-resistant; MS, medication-sensitive. Pr(>F) is the *p* value of the F statistic.

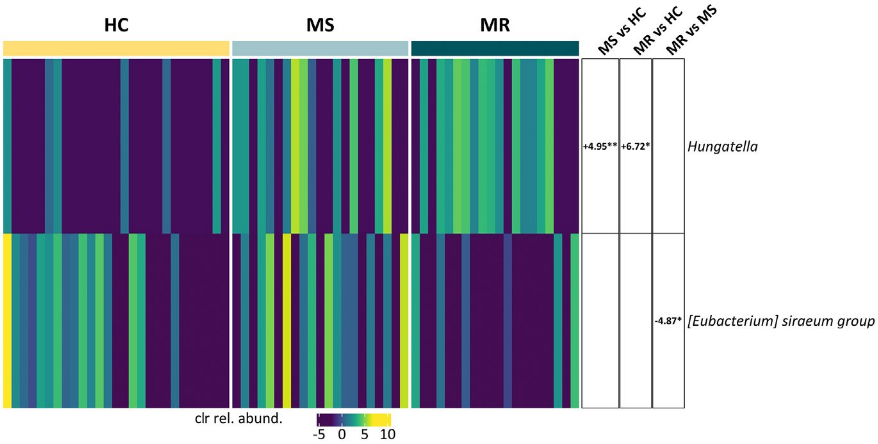


FIGURE 2 Heatmap illustrating the relative abundance distribution of the two significant genera obtained from the differential abundance analysis of the three pairwise comparisons. The relative abundance (rel. abund.) percentiles were transformed into centered log-ratio (clr) and visualized by color gradient. Samples were clustered based on group information. Right annotation columns show the significant changes obtained from each of the three pairwise comparisons, and the $\log_2$ fold change values with the significance stars. *$.01 \le p.\text{adj} < .05$, **$.001 \le p.\text{adj} < .01$. HC, healthy control; MR, medication-resistant; MS, medication-sensitive.

4 | DISCUSSION

MGBA dysfunction is emerging as a new pathogenic mechanism in neurologic disorders. In particular, patients with epilepsy present intestinal dysbiosis, which can modulate disease outcome. Both nutritional habits and drug treatment can alter the gut microbiota composition and affect brain activity, excitotoxicity, and seizures. Here, we investigated gut microbiota populations in pediatric epileptic patients and the role of confounding factors, such as age, sex, and dietary intake, on the resistance to ASMs.

One third of all epilepsy patients suffer from medication-resistant epilepsy, which has been correlated with genetic variation, therapeutic target, drug transporter type, drug pharmacokinetics, and severity of seizures.[13,14] The most common mechanism of action for ASMs is via interaction with ion channels and neurotransmitters to modulate neuronal transmission. Interestingly, new evidence has recently shown that the gut microbiota might also be involved in mediating ASM effects via a bidirectional communication system. Preclinical studies revealed that availability of certain ASM active metabolites can influence gut microbiota growth and composition.[32] In turn, the gut flora can directly affect treatment response by enzymatically transforming the structure of the drug and thereby altering its bioavailability, bioactivity, and toxicity.[15] Furthermore, a recent study found significant differences between the gut microbiota in MS and MR adult epilepsy patients,[33] suggesting a relationship between gut flora composition and medication response status. To our knowledge, this is the first study to investigate this relationship in pediatric patients. Gut microbiota plasticity is highest in children and incrementally diminishes throughout the course of life.[34] Implementing noninvasive interventions in children, such as promoting healthy nutritional habits, might have profound and long-lasting beneficial effects on disease outcome and medication response.

Our results show similar bacterial richness (AD) between the studied groups, with a linear increase correlated with age. This finding is consistent with the literature; starting from early intrauterine life, the human gut microbiota is shaped by maternal and environmental factors.[6] Early reports suggests that around the 3rd year of age, our core microbiota becomes similar to that of adults. However, complete maturation might take longer in some cases,[6] and the gut microbiota is shaped by endogenous and external factors (e.g., diet, physical activity) up to adolescence. Hence, future studies may further subgroup patients into infants (<3 years old), preschool children (3–6 years old), school-aged children (6–12 years old), and lastly teenagers (12–18 years old) to fully uncover the variability of this interesting window for the gut microbiota.

Because AD only takes into consideration the general richness of single samples, but not the types of microbial communities present, two samples may have a very different metagenomic profile, despite having a similar AD. Evaluating the intersample variability (BD), we showed a significant difference between the epilepsy and control groups, revealing that the disease status is associated with gut dysbiosis. Interestingly, there were no statistical differences between MS and MR epilepsy patients, suggesting that medication responsiveness may not be directly associated with a general status of dysbiosis.

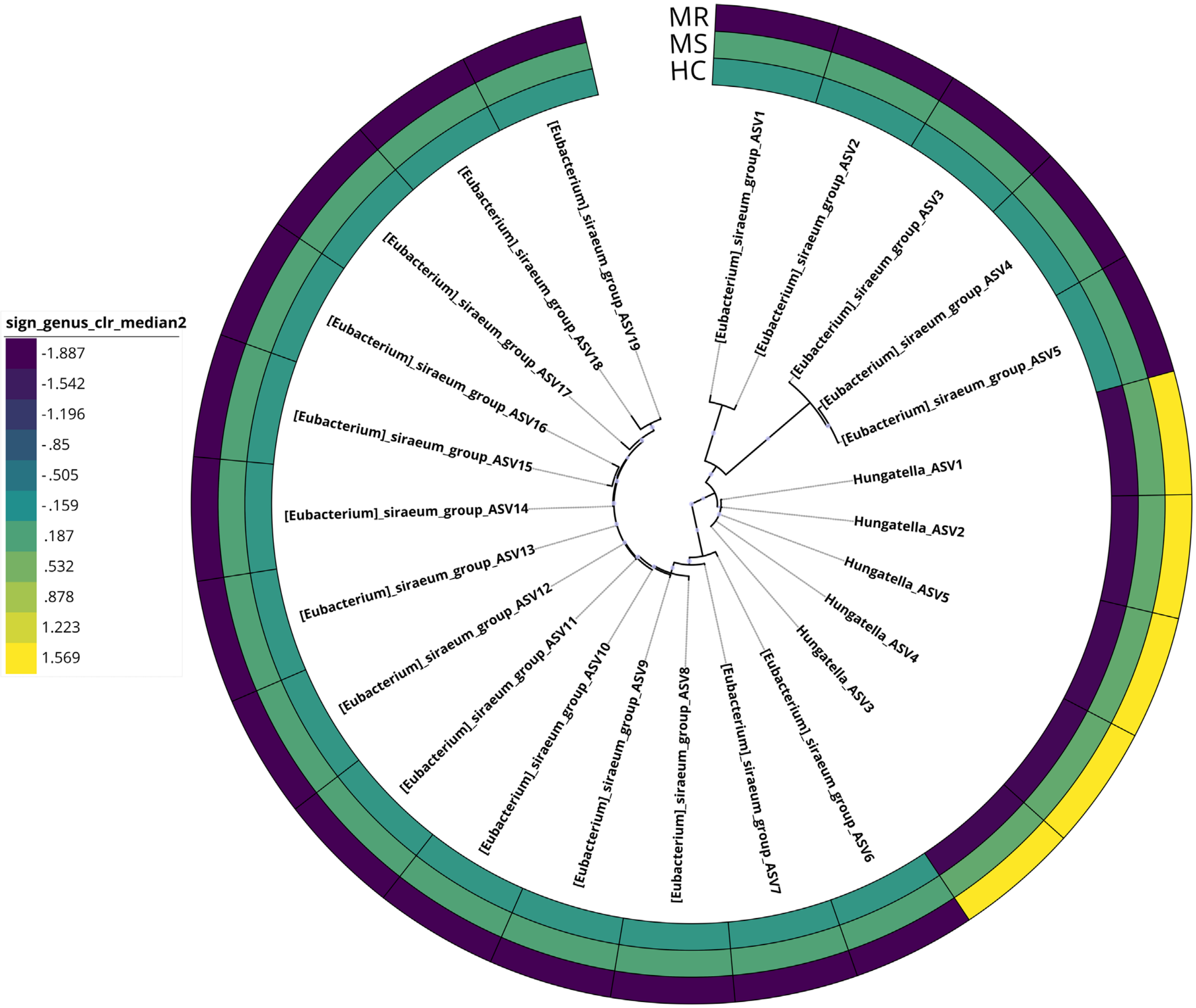


FIGURE 3 Phylogenetic tree showing the amplicon sequence variants (ASVs) annotated to the genera found to significantly change in our population. Rooted tree constructed by alignment of the obtained ASVs was trimmed to include 24 ASVs annotated as *Hungatella* or *[Eubacterium] siraeum* group. Heatmap encircling the phylogenetic tree shows the median of centered log-ratio (clr)-transformed genus relative abundances in each study group. HC, healthy control; MR, medication-resistant; MS, medication-sensitive.

To investigate whether specific bacterial populations were associated with medication responsiveness, we performed taxonomic analysis. No significant changes at the phylum level were found in our populations. However, at the genus level, specific bacterial species were specifically associated with the epilepsy and HC groups, as well as between the MR and MS subgroups. These observations suggest that medication resistance might be associated with minor alterations of specific bacterial populations rather than broader changes, and that fine-tuning of the gut microbiota can have profound changes on health and disease. In particular, we found a significantly higher abundance of Hung in the epilepsy groups (more pronounced in the MR group) compared to the HC group, highlighting an association with the disease. Hung belongs to the Clostridiaceae family and is a gram-positive, anaerobic bacteria known to be highly resistant to antibiotics.[35] Moreover, different strains of this genera are associated with neurodegenerative and progressive neurological disorders, such as multiple sclerosis,[36] suggesting that Hung could play a role in different neurological conditions. A recent study also demonstrated that patients with recurrent major depressive disorder presented a higher abundance of Hung.[37] Whether these changes could anticipate the disease onset or are a direct consequence of the disease remains elusive.

Furthermore, the abundance of Eub was decreased in the MR versus the MS group. The *Eubacterium* spp. are butyrate producers,[38] with the only exception being *E. oxidoreducens*.[39] Butyrate is one of the three main short-chain fatty acids (SCFAs), which can promote health and

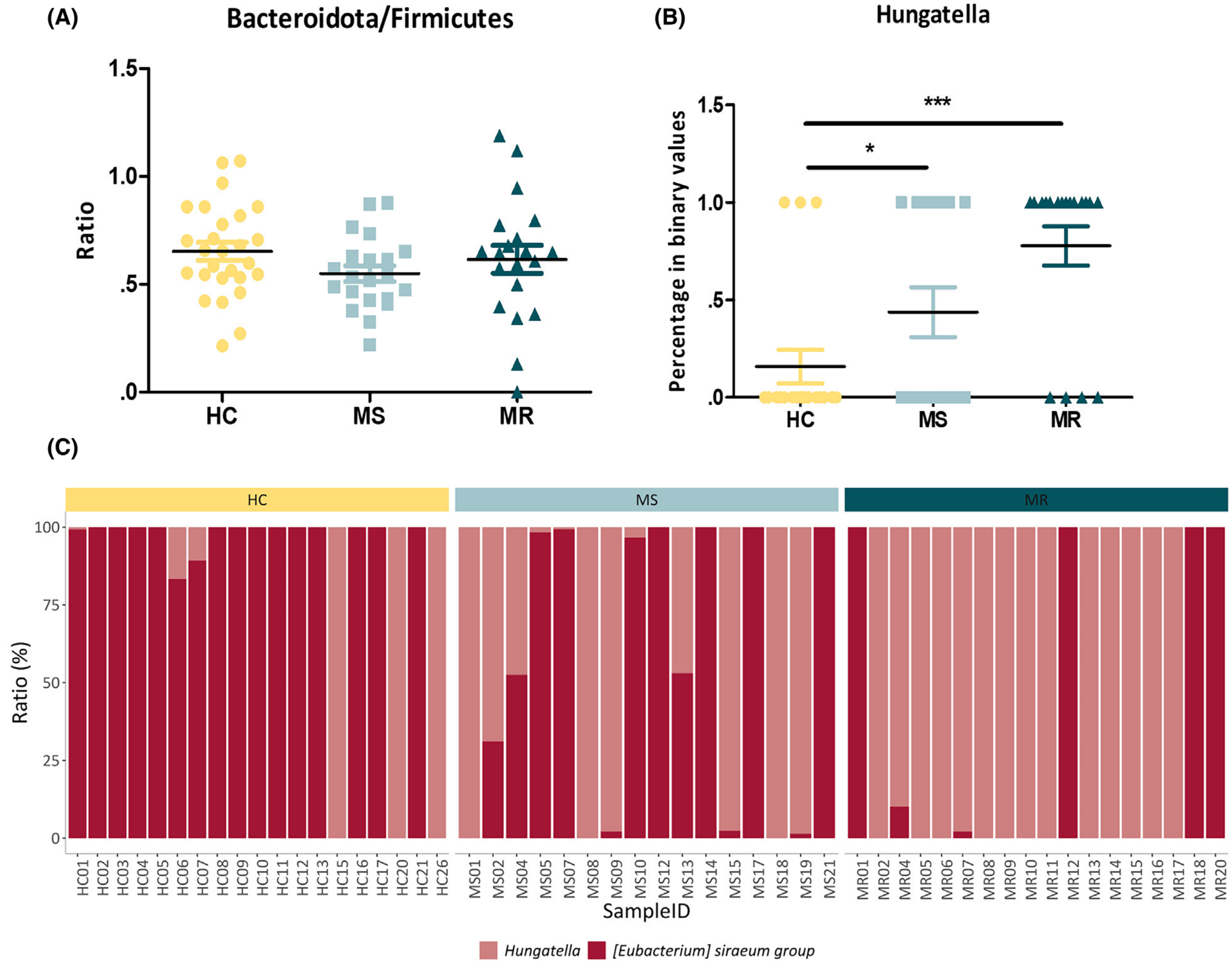


FIGURE 4 Ratio of relevant phyla and statistically significant genera. (A) Scatterplot of the ratio between abundance of Bacteroidota and Firmicutes phyla in the three groups. (B) Scatterplot of *Hungatella* (Hung) dominance over *[Eubacterium] siraeum* group (Eub). (C) Hung/Eub ratio at each sample level in the three groups studied. The relative abundance of Hung and Eub were summed and used as an isolated community. A binary score was assigned based on their proportional abundance: "1" indicated Hung dominance (≥50% abundance) and "0" indicated Eub dominance. *$p \leq .05$, ***$p \leq .0001$. HC, healthy control; MR, medication-resistant; MS, medication-sensitive.

are locally produced by gut bacteria.[38] Once they cross the gut barrier and enter the bloodstream, these compounds can reach the CNS and prevent/reverse neurological conditions.[40] SCFAs exert antiseizure effects by modulating the ratio between inhibitory and excitatory neurotransmitters in the brain.[40] SCFAs also act on a variety of cell types in the CNS to reduce neuroinflammation, increase fatty acid oxidation, promote blood–brain barrier integrity, and regulate cell–cell interactions, mitochondrial function, and gene expression.[41,42] Due to these functions, gut dysbiosis and the concomitant alteration of SCFA levels have been implicated as potential risk factors in many neurological conditions, including epilepsy. Furthermore, *Eubacterium* has also been identified as a producer of γ-aminobutyric acid (GABA), an inhibitory neurotransmitter known to control seizures. GABA has also been proposed as a potential postbiotic mediator for neurological disorders.[43] A recent study has demonstrated that *Eubacterium* was one of the main genera to increase in pediatric patients with medication-resistant epilepsy and positively respond to the ketogenic diet,[44] which in turn is known to increase beneficial species and mediate ASM response in treated individuals.[45] Taken together, these observations suggest that *Eubacterium* could play a role in medication response; however, longitudinal clinical trials are needed to establish causation.

In the gut, different microbial communities coexist in a multifaceted ecosystem engaging in complex interactions.[1] For these reasons, it is important to investigate the proportion of a population in relation to another. The ratio between Bacteroidota and Firmicutes, for instance, is known to play an important role in maintaining normal

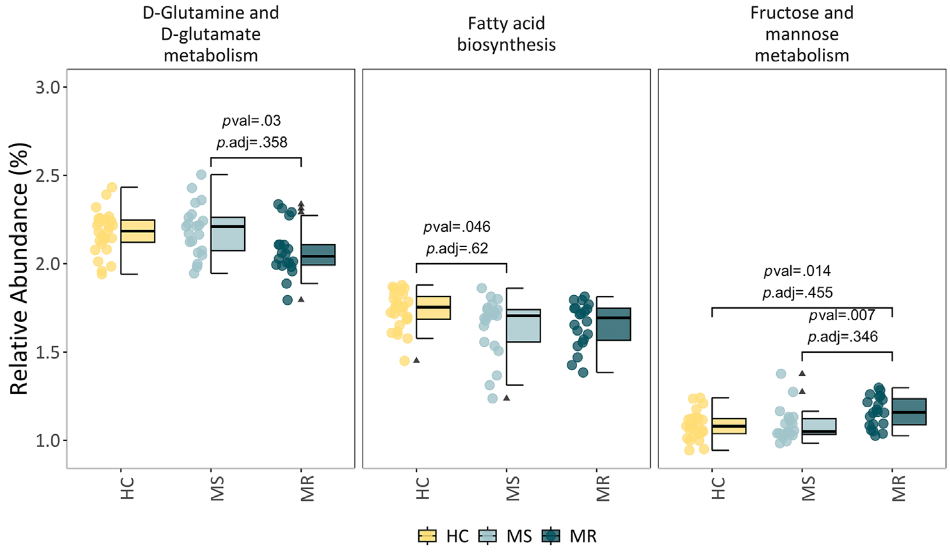


FIGURE 5 Significant changes in Kyoto Encyclopedia of Genes and Genomes pathway relative abundances between groups. Individual samples are represented in dots on left-hand side, whereas the boxplots on the right side depict the median line in black and the interquartile interval with minimum and maximum values. The triangle shapes show outlier data points. Raw and adjusted *p* values are shown for significant changes. HC, healthy control; MR, medication-resistant; MS, medication-sensitive.

intestinal homeostasis and has been associated with different neurological disorders.[1] Our data did not reveal any significant differences between the groups at the phylum level. However, at the genus level we found a dominance in Hung compared to Eub in both epilepsy groups compared to the HCs, highlighting an association between these populations and epilepsy.

A limitation of the current study is the lack of metabolic correlates to our metagenomic findings. To gain insight on the functional potential of the populations characterized, we performed metabolic pathway prediction using PICRUSt2. The results revealed significant differences in D-glutamine and D-glutamate metabolism, fatty acid biosynthesis, and fructose and mannose metabolism, pathways shown to play a role in epilepsy and in medication resistance.[46–48] Interestingly, SCFAs have been shown to modulate lipid, glutamine, and glutamate metabolism,[49] highlighting a potential association between these metabolic pathways and the abundance of SCFA-producers in the gut.

Furthermore, the cross-sectional nature of the study and the choice of monotherapy versus polytherapy as a practical, although potentially imperfect, proxy for medication resistance limit its capability to disentangle the observed association between certain bacterial species and treatment response and establish causality.

Given the potential impact of most ASMs (e.g., valproate, topiramate, cannabidiol, ethosuximide, and clobazam) on the MGBA, future longitudinal studies with pretreatment timepoints could elucidate whether the differences found in this study are independent of treatment-induced changes and can therefore act as true predictive biomarkers of therapeutic response. In our study, there were observable differences in the averages of clinical measurements between the groups, which may suggest potential trends that warrant further investigation. However, our study did not perform differentiation analysis to predict group classifications based on these data.

Modulating the MGBA with pre-/probiotics can also be an effective strategy to improve medication responsiveness in epilepsy patients and should be explored in the future as a therapeutic approach. Lastly, although efforts have been made to try to detangle the possible confounding factors associated with the gut microbiota composition (e.g., age, diet), future studies should go deeper in the analysis, considering more homogenous genetic backgrounds.

Understanding the impact of the MGBA in epilepsy and medication response and exploring how to modulate this axis to improve the clinical outcome is crucial to aid the discovery of predictive biomarkers and develop early intervention plans to enhance medication responsiveness and control frequency and severity of seizures, ultimately ameliorating the quality of life of the affected individuals.

5 | CONCLUSIONS

Epilepsy is a serious health condition, and existing therapies target symptoms, rather than addressing the underlying abnormal biological processes. Epilepsy patients often exhibit gut dysbiosis, but it is still unclear how this dysregulation affects seizure development and the response to

ASMs. There is a need to advance knowledge of epilepsy biology to identify predictive biomarkers and guide the development of novel therapies and early interventions for this multifaceted disease. Our study shows a significantly lower abundance of Eub in medication-resistant epilepsy patients. Interestingly, *Eubacterium hallii* has been recently identified as a candidate for next generation probiotics,[50] with exciting potential for the prevention and treatment of several dysbiosis-associated diseases. Another study shows that ketogenic diet reversed the abundance of epilepsy-associated genera in children with medication-refractory epilepsy.[44]

As dysbiosis is associated with epilepsy and medication resistance, modulating the gut microbiota composition through the use of probiotics and diet could provide an efficacious and noninvasive intervention plan to improve patient symptoms and quality of life. However, additional studies are needed to fully characterize the microbial signatures associated with refractory seizures and establish a causal relationship between dysbiosis and medication responsiveness.

AUTHOR CONTRIBUTIONS

Antonella Riva: Collection of data; drafting. **Eray Sahin:** Data analysis; drafting. **Greta Volpedo:** Drafting. **Noemi Teresa Catania, Isabel Venara, Valentina Biagioli, Ganna Balagura, Elisabetta Amadori, Carmen De Caro, Emanuele Cerulli Irelli, Carlo Di Bonaventura:** Support in collecting the data. **Federico Zara, Osman Ugur Sezerman, Emilio Russo:** Revision of the data. **Pasquale Striano:** Conception of the study; revision and final approval of the manuscript. All authors agree to be accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

None of the authors has any conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the article. The sequencing data were uploaded to BioProject (ID PRJNA1133091) and are publicly available. The scripts used for the data analysis in R can be found in the GitHub repository: https://github.com/eray-sahin/Medication-resistant_epilepsy_is_associated_with_a_unique_gut_microbiota_signature. If additional data are required, they can be requested from the corresponding author.

ETHICS STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. All methods were performed in accordance with the ethical standards as laid down in the Declaration of Helsinki and its later amendments or comparable ethical standards.

PATIENT CONSENT STATEMENT

The present study was approved by the Comitato Unico Regionale Regione Liguria, and patients signed written informed consent (Protcol MOBi-DEE).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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