ORIGINAL ARTICLE

Gut microbiota analysis in pediatric-onset multiple sclerosis compared to pediatric monophasic demyelinating syndromes and pediatric controls

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Abstract

Background and purpose: Gut microbiota dysbiosis may lead to proinflammatory conditions contributing to multiple sclerosis (MS) etiology. Pediatric-onset MS patients are close to biological disease onset and less exposed to confounders. Therefore, this study investigated gut microbiota composition and functional pathways in pediatric-onset MS, compared to monophasic acquired demyelinating syndromes (mADS) and healthy controls (HCs).

Methods: Pediatric participants were selected from the Dutch national prospective cohort study including ADS patients and HCs <18 years old. Amplicon sequence variants (ASVs) were generated from sequencing the V3/4 regions of the 16S rRNA gene. Functional MetaCyc microbial pathways were predicted based on Enzyme Commission numbers. Gut microbiota composition (alpha/beta diversity and individual microbe abundance at ASV to phylum level) and predicted functional pathways were tested using nonparametric tests, permutational multivariate analysis of variance, and linear regression.

Results: Twenty-six pediatric-onset MS (24 with disease-modifying therapy [DMT]), 25 mADS, and 24 HC subjects were included. Alpha/beta diversity, abundance of individual resident microbes, and microbial functional features were not different between these participant groups. Body mass index (BMI) showed significant differences, with obese children having a lower alpha diversity (Chao1 Index $p = 0.015$, Shannon/Simpson Diversity Index $p = 0.014$/$p = 0.023$), divergent beta diversity ($R^2 = 3.7\%$, $p = 0.013$), and higher abundance of numerous individual resident microbes and functional microbial pathways.

Conclusions: Previous results of gut microbiota composition and predicted functional features could not be validated in this Dutch pediatric-onset MS cohort using a more sensitive 16S pipeline, although it was limited by sample size and DMT use. Notably, several other host-related factors were found to associate with gut microbiota variation, especially BMI.

KEYWORDS
etiology, gastrointestinal microbiome, microbiota, multiple sclerosis, pediatrics

INTRODUCTION

Multiple sclerosis (MS) is a severe and heterogeneous autoimmune disease of the central nervous system (CNS). Although both genetic and environmental factors are found to be involved in MS etiology, exact pathogenesis remains to be elucidated. The gut microbiota fulfil an essential role in the development and regulation of the immune system. Hence, alterations in the balance of gut microbiota composition and/or its functional potential could lead to proinflammatory conditions [1]. Because of the bidirectional interaction between the gut and the CNS, known as the gut–brain axis [2], there has been increasing interest in the role of the gut microbiota in the pathophysiology of MS. Several small case–control studies have been conducted, mainly in adults, and report subtle perturbations to the gut microbiota composition in MS patients [3]. However, findings vary widely across studies, potentially because the gut microbiome composition is known to be associated with a large number of technical but also host-related variables [4, 5]. In children compared to adults, exposure to such confounders is probably limited because of the fewer years lived. Furthermore, specifically for MS, migration studies have shown that certain environmental factors in childhood are essential risk factors for a future diagnosis of MS [6, 7]. Therefore, pediatric-onset MS is a unique and essential group for discovering the key contributors to the complex MS disease, closest to original exposures and biological onset of disease. Accordingly, in the current study we investigated the role of the gut microbiota in pediatric-onset MS, by comparing composition and predicted functional pathways of gut bacteria between pediatric-onset MS, pediatric monophasic acquired demyelinating syndromes (mADS), and pediatric healthy controls (HCs).

MATERIALS AND METHODS

Study participants and definitions

All selected patients and HCs are participants in the Dutch nationwide prospective cohort study for the prediction of outcome in children with a first episode of ADS at <18 years [8]. In the described prospective cohort study, stool samples are collected in patients and related healthy children and adolescents (i.e., mainly family members and/or persons living in the same neighborhood). For the current study, a cross-sectional selection was made of the participants ≤21 years old with collected stool samples, if at final follow-up participants (i) were diagnosed with relapsing–remitting MS or mADS (according to International Pediatric MS Study Group criteria [9]) or (ii) were healthy (specifically without [auto]immune-related disorders, with the exception of asthma, eczema, and allergies).

Demographic, clinical, and laboratory data collected in the described prospective cohort study were used. Ethnicity was determined based on self-report of parental ethnicity and categorized as European or non-European. Body mass index (BMI) was classified as underweight, healthy weight, overweight, or obese, based on age-, sex-, and ethnicity-specific growth charts, including Dutch, Turkish, Moroccan, and Hindu ethnicity [10]. For the participants with another ethnicity, the Dutch growth chart was used.

Stool sample collection

Stool samples were collected in participants without systemic antibiotic use in the 3 months before sampling. These samples were compiled at home using gut microbiome DNA collection kits from OMNIgene•GUT (DNA Genotek, Ottawa, Ontario, Canada) with stabilizing agent included and were sent through regular mail to the Erasmus University Medical Center, Rotterdam, the Netherlands. Together with stool collection, a questionnaire was filled in by the participant or parent for additional details at time of sampling, for example, date of collection, height and weight, used medications (antibiotics, probiotics, hormonal birth control), and date of first menstruation (in girls). Upon arrival at the Erasmus University Medical Center, samples were stored at −80°C.

Gut microbiota profiling

All stool samples of selected participants were analyzed at one time point. Gut microbiota profiling details are described in Supplementary S1. In brief, microbial DNA was extracted from the stool samples and 16S ribosomal RNA gene sequencing of the V3 and V4 variable regions was performed on an Illumina MiSeq sequencer. Reads were processed using the DADA2 package (R v4.1.0) [11] for quality filtering and amplicon sequence variant (ASV) assignment. ASVs were assigned a taxonomy from the SILVA version 138 rRNA database [12]. Final ASV filtering was performed on total abundance (ASV abundance >0.05% of total count) and sample prevalence (ASV present in >1% of samples). Finally, PICRUSt2-predicted functional MetaCyc microbial pathways were determined from obtained 16S rRNA gene sequencing data based on gene families supported by Enzyme Commission (EC) numbers.

Statistical analysis

All statistical analyses were performed in R v4.0.5, with a significance level of $p < 0.05$, using predominantly the Phyloseq [13] and Vegan [14] packages. The samples with missing data in one or more of the variable(s) used in an analysis were excluded for that specific analysis.

Descriptive characteristics were compared between the three participant groups (MS, mADS, and HCs) using chi-squared (or Fisher exact) test and one-way analysis of variance (or Kruskal–Wallis test) for categorical and numerical data, respectively. For specific comparisons of descriptive characteristics between the two patient groups (MS and mADS) chi-squared (or Fisher exact) test and Student t-test (or Wilcoxon rank-sum test) were used.

Gut microbiota composition and predicted functional pathways were first compared between the three participant groups (MS, mADS, and HCs). Subsequently, associations of other covariates with gut microbiota composition and its predicted function were analyzed.

To investigate differences in overall gut microbiota composition, alpha and beta diversity were evaluated. Alpha diversity (microbial variation within a sample) was estimated by the Chao1 Index, a measure of richness (number of species present in a sample), and the Shannon Diversity Index and Simpson Diversity Index, which measure both richness and evenness (relative abundance of different species). Because alpha diversity metrics were not normally distributed, also not after log-transformation, nonparametric tests were used for the comparisons and association analyses with categorical and numerical variables, respectively. Additionally, for the comparisons between the three participant groups, a multiple linear regression model was used to adjust for basic demographic covariates, including age at stool sampling, sex, ethnicity, and BMI. Because only two participants were classified as underweight, the underweight participants were combined with the participants with a healthy weight into one BMI class for all analyses including BMI. When analyzing the association of other covariates with alpha diversity, participant group was also added to the multiple linear model. Beta diversity (microbial variation between samples) Euclidean distance matrices were measured after centered log-ratio (clr) transformation using the Vegan package in R [14]. The association of participant group and other covariates with beta diversity was visualized by principal coordinate analysis and statistically tested using permutation multivariate analysis of variance (PERMANOVA; with 99,999 permutations). Additionally, the above-described basic participant demographics were added to the PERMANOVA for participant group comparisons. In the analyses regarding the associations of other covariates with beta diversity, participant group was added to the PERMANOVA analyses as well.

Finally, the clr-transformed relative abundance of individual microbes and relative abundance of predicted functional pathways were evaluated and compared between the three participant groups by linear regression analyses at several taxonomic levels, including ASV, species, genus, family, order, class, and phylum level, adjusted for above-described basic demographic participant characteristics. Participant group was also added when analyzing other covariates. For all these paralleled multiple analyses a q (false discovery rate multiple testing-corrected p-value) <0.05 was used as significance threshold.

RESULTS

Characteristics of participants with collected stool samples

Stool samples were collected in 75 children who were not exposed to systemic antibiotics within the past 3 months, of whom 26 had MS, 25 had mADS, and 24 were HCs. Table 1 shows the characteristics of participants separated into these three groups. Participants in the MS group were older at time of stool sampling compared to the mADS and HC groups (17.3 vs. 9.2 vs. 10.6 years, respectively; $p < 0.001$). In all groups, there was a slight female preponderance (65%, 64%, and 63%, respectively), not significantly different between groups. In the MS group, a significantly higher BMI was observed compared to the mADS and HC groups (overweight: 38% vs. 14% vs. 0%; $p = 0.010$). Finally, time in mail (TIM), representing time between stool sample collection and receipt in the laboratory, was similar in all groups.

For the two disease groups (MS and mADS) specifically, stool samples were collected at a median disease duration of 30.0 and 19.7 months, respectively, and in the vast majority of patients, last clinical activity (onset or relapse) occurred >3 months prior to stool sampling (92% vs. 96%, respectively). Accordingly, none of the MS patients and only two mADS patients were treated with corticosteroids within the month before sampling (8%). In total, 24 MS patients (92%) used disease-modifying therapy (DMT) at time of stool sampling, of whom 16 (67%) used first-line DMT, including interferon-beta ($n = 7$), glatiramer acetate ($n = 5$), teriflunomide ($n = 3$), and dimethyl fumarate ($n = 1$). The remaining eight patients (33%) were treated with second-line DMT, including natalizumab ($n = 6$) and fingolimod ($n = 2$; Table 1). Of the two patients not using DMT at time of stool sampling, one was treated with interferon-beta until 6 months prior to sampling and one was never exposed to DMT prior to sampling.

Overall gut microbiota diversity between participant groups

All three estimators of alpha diversity (representing the variation within a stool sample) were not significantly different between the MS, mADS, and HC participant groups ($n = 75$; Chao1 Index $p = 0.27$, Shannon Diversity Index $p = 0.84$, and Simpson Diversity Index $p = 0.79$; Figure 1a and Supplementary S2). In the multiple linear regression analysis adjusting for basic demographic characteristics including age at stool sampling, sex, ethnicity, and BMI, only Chao1 Index was significantly different for mADS compared to HCs ($n = 67$, $p = 0.029$). For MS compared to mADS as well as MS compared to HCs, all three alpha diversity measures remained not significantly different. Subsequently, beta diversity (representing the variation between stool samples) examination between the three participant groups showed no significant differences ($n = 75$, $R^2 = 2.95\%$, $p = 0.12$; Figure 1b and Supplementary S2). Again, also after correction for described demographic characteristics, no significant differences were observed ($n = 67$).

Taxa distribution between participant groups

Figure 2 shows the abundance at phylum level, absolute per individual sample (Figure 2a) and relative per participant group

TABLE 1 Characteristics of participants (n = 75) with collected stool samples[a]

General characteristics	MS, n = 26	mADS, n = 25	HCs, n = 24	p[b]
Age at stool sampling, years, median [IQR]	17.3 [15.5–18.6]	9.2 [6.1–11.2]	10.6 [6.7–14.2]	<0.001
Female sex, n (%)	17/26 (65)	16/25 (64)	15/24 (63)	NS
European ethnicity, n (%)	12/26 (46)	17/25 (68)	17/24 (71)	NS
Vaginal birth, n (%)	23/26 (89)	22/24 (92)	20/23 (87)	NS
Breastfed, n (%)[c]	11/15 (73)	9/17 (53)	13/23 (57)	NS
First menstruation prior to stool sampling, n of females (%)	17/17 (100)	3/16 (19)	5/15 (33)	<0.001
BMI at stool sampling, n (%)[d]				**0.010**
Underweight	0/24 (0)	0/21 (0)	2/22 (9)	
Healthy weight	13/24 (54)	16/21 (76)	18/22 (82)	
Overweight	9/24 (38)	3/21 (14)	0/22 (0)	
Obese	2/24 (8)	2/21 (10)	2/22 (9)	
Hormonal birth control use at stool sampling, n of females (%)	9/17 (53)	0/14 (0)	1/15 (7)	0.001
Antibiotics <3 months prior to stool sampling, n (%)[e]	0/26 (0)	0/25 (0)	0/24 (0)	NS
Probiotics <3 days prior to stool sampling, n (%)	3/24 (13)	0/24 (0)	0/21 (0)	NS
Time in mail, days, median [IQR][f]	2.0 [1.0–2.0]	1.0 [1.0–2.0]	2.0 [1.0–2.0]	NS
Disease-specific characteristics	**MS, n = 26**	**mADS, n = 25**		**p[g]**
Age at onset, years, median [IQR]	14.3 [13.3–15.9]	6.2 [5.0–10.0]		<0.001
MOG antibodies, n (%)	1/25 (4)[h]	11/25 (44)		0.003
Disease duration at stool sampling, months, median [IQR]	30.0 [19.3–37.8]	19.7 [6.0–36.6]		NS
Time since last clinical activity at stool sampling[i]				NS
<1 month, n (%)	0/26 (0)	0/25 (0)		
1–3 months, n (%)	2/26 (8)	1/25 (4)		
>3 months, n (%)	24/26 (92)	24/25 (96)		
Corticosteroids <1 month prior to stool sampling, n (%)[e]	0/26 (0)	2/25 (8)		NS
DMT use at stool sampling, n (%)	24/26 (92)	0/25 (0)		<0.001
First line	16/24 (67)[j]			
Second line	8/24 (33)[k]			
Never exposed to DMT prior to stool sampling, n (%)	1/26 (4)	25/25 (100)		<0.001
FU duration, months, median [IQR]	41.7 [27.4–62.2]	35.3 [17.5–52.7]		NS

Abbreviations: BMI, body mass index; DMT, disease-modifying therapy; FU, follow-up; HC, healthy control; IQR, interquartile range; mADS, monophasic acquired demyelinating syndromes; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis.

[a]For every displayed variable, the number of samples without missing data is indicated by the denominator.

[b]Comparison between MS, mADS, and HCs by chi-squared test (or Fisher exact test when applicable) or one-way analysis of variance (or Kruskal–Wallis test when applicable).

[c]Breastfed only, or followed by/combined with bottle-fed.

[d]Age-, sex- and ethnicity-specific categories.

[e]Intravenous or oral.

[f]Time between sampling and arrival at laboratory.

[g]Comparison between MS and mADS by chi-squared test (or Fisher exact test when applicable) or Student t-test (or Wilcoxon rank-sum test when applicable).

[h]This patient tested weak positive for MOG antibodies by cell-based assay, but had a clinical and radiological disease course typical for MS.

[i]Onset attack or relapse.

[j]Including interferon-beta (n = 7), glatiramer acetate (n = 5), teriflunomide (n = 3), and dimethyl fumarate (n = 1).

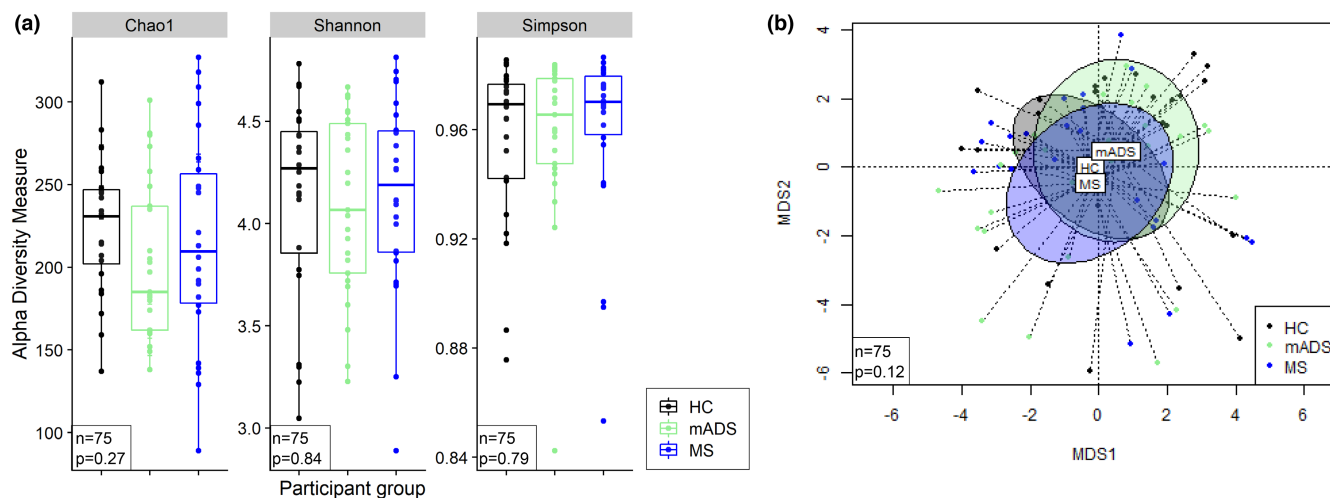[k]Including natalizumab (n = 6) and fingolimod (n = 2).

FIGURE 1 Alpha and beta diversity for multiple sclerosis (MS) versus monophasic acquired demyelinating syndromes (mADS) versus pediatric healthy control (HC) groups. (a) Alpha diversity was estimated by Chao1 Index, which measures richness (number of species), and Shannon Diversity Index and Simpson Diversity Index, which measure both richness and evenness (relative abundance of different species). Differences between participant group were tested statistically for every diversity index separately using Kruskal–Wallis test. (b) Beta diversity distance matrix was visualized by a principal coordinate analysis multidimensional scaling (MDS). Differences between participant groups were tested statistically using permutational multivariate analysis of variance.

(Figure 2b). Minimum sequence depth of all individual samples was 23,583 reads per sample, with a median of 58,244 reads obtained per sample (interquartile range = 48,983–65,751; Figure 2a). In all samples ($n = 75$), a total of 1695 ASVs belonging to 11 phyla were identified. Main phyla across all participant groups were Firmicutes and Bacteroidetes, together accounting for 95% of all phyla. The proportion Firmicutes was highest in the MS group (65%), followed by the mADS group (58%) and HC group (55%). This was observed conversely for the proportion of Bacteroidetes (MS, 29%; mADS, 38%; HC, 39%; Figure 2b). However, these differences were not significant in analysis of the abundance of individual microbes at the phylum level ($n = 67$, $q > 0.05$ for all phyla), nor for this analysis at other taxonomic levels below the phylum level, including class, order, family, genus, species, and single ASV level ($n = 67$; all analyses $q > 0.05$).

Association of covariates with gut microbiota composition

In addition to the described analyses between MS, mADS, and HCs, the association of other covariates with gut microbiota community was tested. Multiple host-related variables showed a significant association with alpha diversity and/or beta diversity, including age at sampling, ethnicity, birth mode (vaginal vs. caesarean section), feeding mode (breastfed vs. bottle-fed only), first menstruation prior to stool sampling, hormonal birth control use at stool sampling, and BMI at stool sampling (Supplementary S2). Importantly, the technical-related variable TIM showed no significant association with alpha or beta diversity.

Of above-described covariates, BMI emerged as the main variable in gut microbiota composition variance. First, a lower alpha diversity was observed in obese participants ($n = 6$) compared to participants who were overweight ($n = 12$) and underweight/ healthy weight ($n = 49$; Chao1 Index $p = 0.015$, Shannon Diversity Index $p = 0.014$, and Simpson Diversity Index $p = 0.023$; Figure 3a). Also corrected for other basic demographics (age at stool sampling, sex, ethnicity, and participant group), alpha diversity remained significantly lower in the obese participants (total $n = 67$; Chao1 Index $p = 0.019$, Shannon Diversity Index $p = 0.002$, and Simpson Diversity Index $p = 0.001$). Second, beta diversity was significantly different for obese participants compared to the other described BMI classes ($n = 67$, $p = 0.013$; Figure 3b), explaining the highest part of total variance in the gut microbiota of all tested variables ($R^2 = 3.7\%$, Supplementary S2). When corrected for the above-described other basic demographics, beta diversity did not remain significantly different between BMI classes ($n = 67$, $R^2 = 3.2\%$, $p = 0.17$). Third, in the analyses of individual microbe abundance, many single ASVs appeared to be more abundant in obese participants compared to participants who were underweight/healthy weight ($n = 67$, $q < 0.05$, corrected for above-described basic demographics; shown in Figure 3c).

Predicted functional microbial pathways

In all samples ($n = 75$), a total of 310 different predicted functional MetaCyc microbial pathways were identified based on EC numbers. The relative abundance of these functional pathways was not different between the three participant groups, adjusted for the described demographics ($n = 67$, $q > 0.05$). Several predicted functional pathways were more or less abundant in obese participants compared to participants who were underweight/healthy weight ($n = 67$, $q < 0.05$, corrected for above-described demographics; Figure 3d).
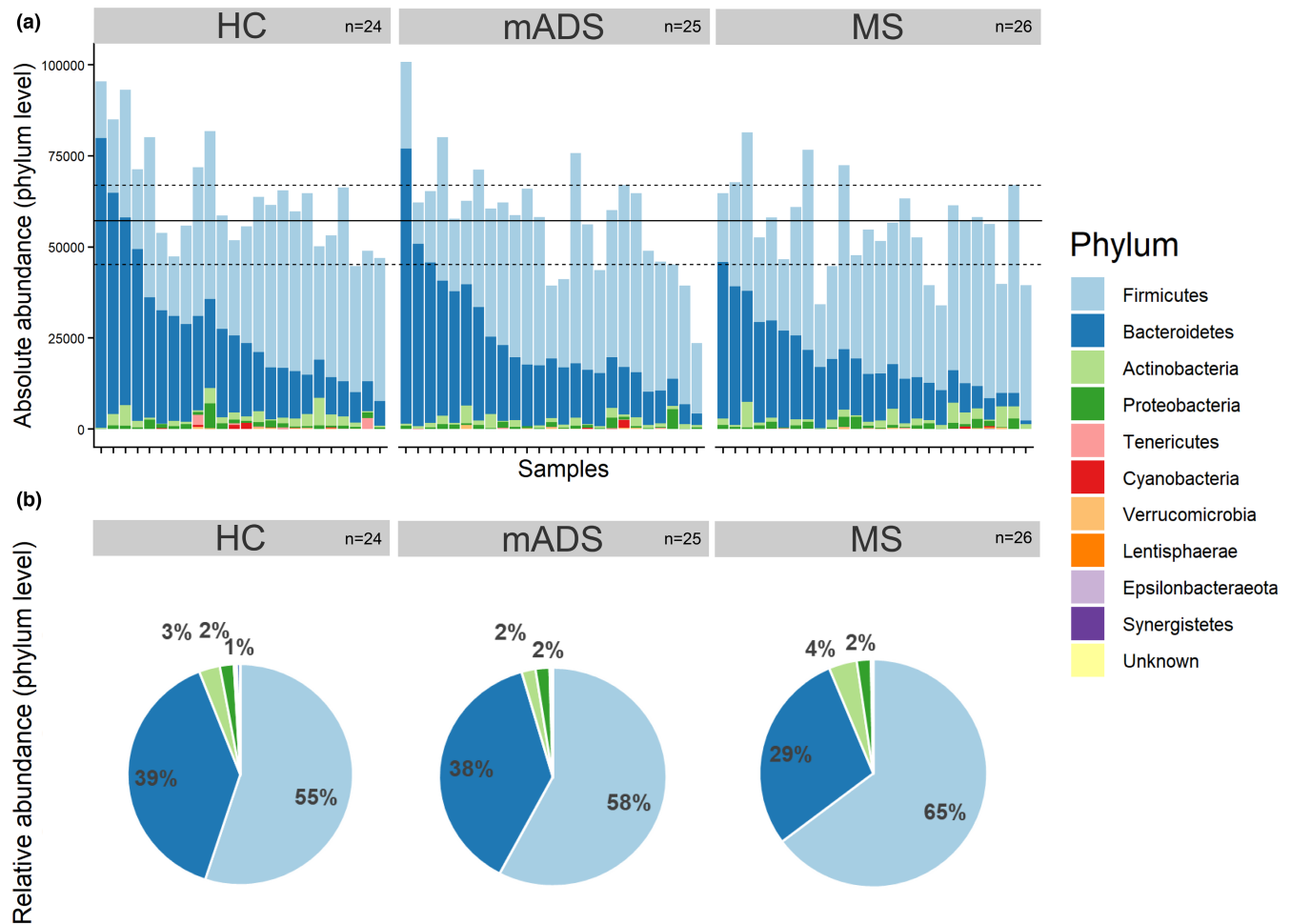
FIGURE 2 Abundance at phylum level, absolute per individual sample and relative per participant group. (a) Absolute abundance shown per individual sample. Line = median number of reads per sample; dashed line = first and third quartile of number of reads per sample. (b) Relative abundance shown per participant group. HC, healthy control; mADS, monophasic acquired demyelinating syndrome; MS, multiple sclerosis.

DISCUSSION

In this pediatric study, no differences were found in composition and functional pathways of gut bacteria between pediatric-onset MS patients, pediatric patients with monophasic demyelinating syndromes, and healthy children, neither in overall bacterial profile expressed by alpha and beta diversity, nor in the relative abundance of individual microbes or relative abundance of predicted functional pathways between these three groups of participants. Although no specific MS disease alterations could be established, multiple host-related factors were found to associate with variation in the gut microbiota, especially BMI.

The overlap in overall gut microbiota community structure between MS patients and other participants is consistent with most previously conducted studies examining alpha and/or beta diversity in adult-onset MS compared to adult HCs [15–20], as well as in pediatric-onset MS compared to pediatric HCs [21–23] and pediatric mADS [22]. In contrast, several previous studies reported differences in individual taxa-level findings [15–17, 19, 20, 24, 25]

and gut microbiota functional potential (based on 16S data) [26] in adult-onset MS and individual taxa-level findings in pediatric-onset MS [21–23], although only with a low concordance of findings across studies [3].

There are several possible explanations for the observed discrepancy between our study and previous studies. First, the applied sequencing techniques and analyzing methods differ. Bioinformatics pipelines are rapidly developing. The currently used highly sensitive DADA2 pipeline clusters the microbial 16S rRNA gene sequences into ASVs based on single nucleotide changes. This new pipeline has less risk of spurious findings compared to the former pipelines used in most of previous studies, which cluster the sequences into operational taxonomic units based on 97% similarity [27]. Mainly due to the explorative nature of most previous studies, several reported significant individual taxa findings did not survive multiple testing [3, 23]. In our study, all individual taxa and predicted functional pathway analyses were corrected for multiple testing using a false discovery rate-corrected p-value (q) of <0.05.
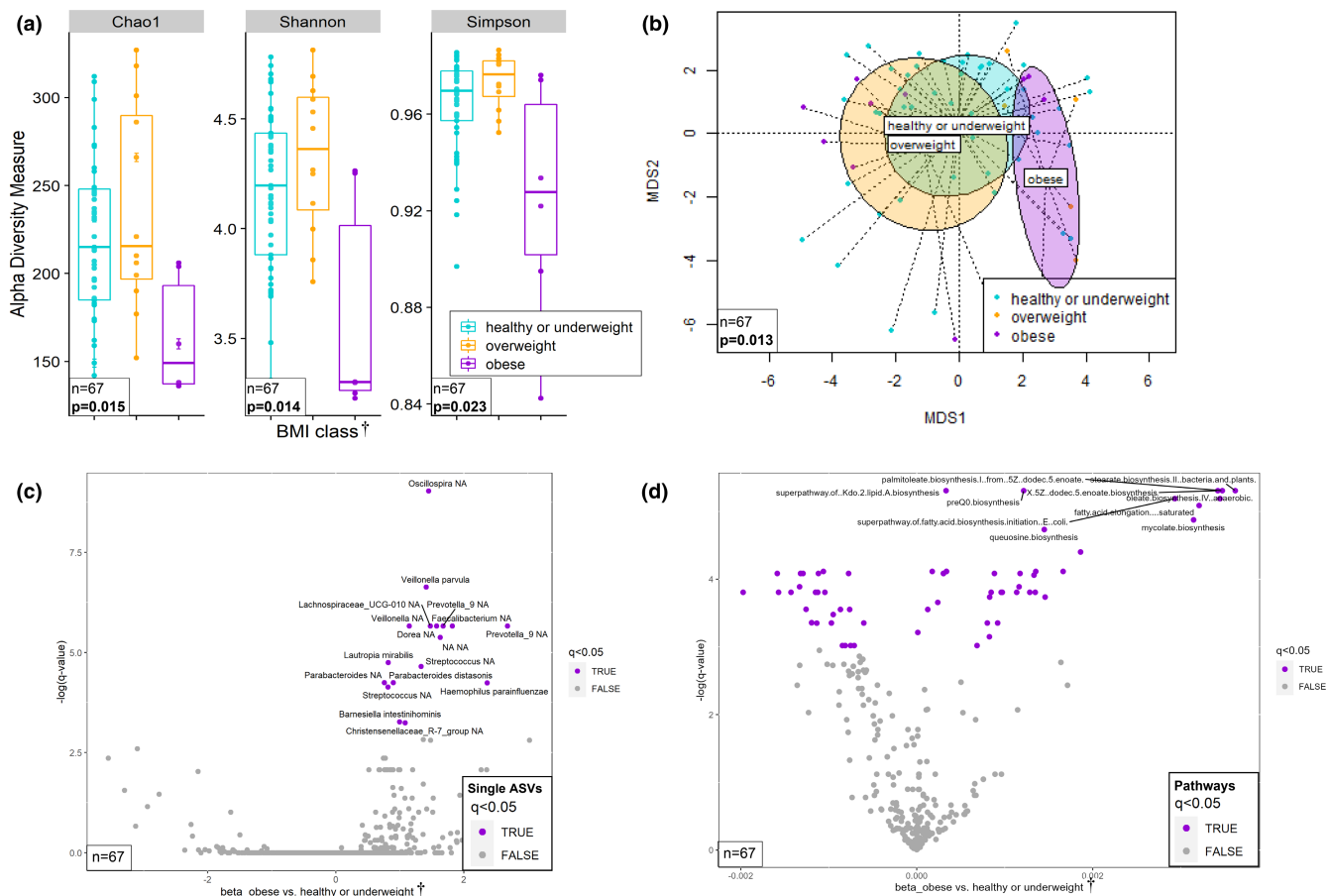
FIGURE 3 Alpha diversity, beta diversity, single microbe, and predicted functional pathway analyses for body mass index (BMI) classes.[†] (a) Alpha diversity was estimated by Chao1 Index, Shannon Diversity Index, and Simpson Diversity Index, which measure both richness (number of species) and evenness (relative abundance of different species). Differences between BMI classes[†] were tested statistically for every diversity index separately using Kruskal–Wallis test. (b) Beta diversity distance matrix was visualized by a principal coordinate analysis multidimensional scaling (MDS). Differences between BMI classes[†] were tested statistically using permutational multivariate analysis of variance. (c) Volcano plot of significantly differential single amplicon sequence variants (ASVs) of obese participants compared to participants who were underweight/healthy weight, corrected for age at stool sampling, sex, ethnicity, and participant group. Significantly different ASVs are shown in purple (q-value [false discovery rate-corrected p-value] < 0.05). (d) Volcano plot of significantly differentially predicted functional pathways of obese participants compared to participants who were underweight/healthy weight, corrected for age at stool sampling, sex, ethnicity, and participant group. Significantly different predicted functional pathways are shown in purple (q < 0.05). MetaCyc labels are shown for the predicted functional pathways with q < 0.01. [†]Age-, sex- and ethnicity-specific classes; NA, not available.

Second, previous studies mainly analyzed adult participants, which could contribute to the observed discrepancy as well. The potential for exposure to influencing factors, such as comorbidities, comedication, and other confounding environmental factors, is higher in adults compared to children. The varying individual taxa-level differences reported in adult-onset MS patients may therefore be due to the longer and broader exposure to such confounders. On top of that, pediatric-onset MS patients presumably have a closer proximity to actual biological start of MS disease and thereby probably have a shorter (sub)clinical disease duration at time of stool sampling. Hence, subtle alterations observed in adult-onset MS patients might also represent differences as a consequence of MS, for example, related to constipation [28], rather than the gut microbiota being causally involved in MS disease pathogenesis.

Third, although none of our MS patients was exposed to systemic corticosteroids, a high proportion (92%) used DMT during sampling, which could have influenced our results. As some previous studies reported subtle differences in individual taxa abundance between MS patients with or without DMT [15, 16, 18, 21–23, 29], DMT exposure might directly or indirectly affect gut microbiota composition. Unfortunately, the limited number of MS patients not exposed to DMT impeded an additional subanalysis regarding DMT in association with gut microbiota composition and microbial pathways.

We found multiple confounders in relation to gut microbiota composition that have been described before, namely, age [5, 19, 28, 30, 31], sex [19, 28, 31], ethnicity [19, 32], birth and feeding mode [33, 34], and BMI [5, 19, 28, 30]. Also, the covariates only applicable to our female participants (menstruation prior to sampling and the use of hormonal birth control) have been indirectly related to the gut

microbiota before via the association with pubertal development [35]. In our study, BMI was found to be the most important factor in relation to gut microbiota variation [19, 30], which is why we added this variable to our models next to the basic variables including age, sex, and ethnicity. BMI has only rarely been taken into account in previous MS gut microbiota studies, but this is interesting as (childhood) obesity is also related to MS [36]. Our study was underpowered to examine MS-specific BMI-related gut microbiota differences, but this would be an important focus for future studies, as this could indicate possible MS treatment or preventive strategies based on the gut microbiota.

Our main limitations are the high exposure to DMT and relatively limited sample size with inclusion of participants with varying ages. First, because of the cross-sectional design of this study, almost all patients were exposed to DMT, whereas preferably stool samples of treatment-naive patients are analyzed. However, future pediatric-onset MS studies with standardized sampling may face the same problem, as in these patients DMT is started as soon as possible after diagnosis due to the high inflammatory activity [37]. Therefore, in this important group of patients representing an early stage of MS, there is often no window to collect samples after corticosteroid treatment following onset of MS symptoms and before start of DMT. Second, as a consequence of the rarity of MS in childhood, our sample size was relatively limited. Although our main analysis still included more than 20 samples per participant group, which is above the generally accepted minimum to detect individual taxa differences [38], our study could have been underpowered to detect slight alterations. Additionally, further subgroup analyses were restricted due to the limited number of included samples. Third, the younger age of participants in the mADS and HC groups compared to the MS group can be explained by known disease characteristics (referring to MS vs. mADS) and the difficulty of stool sample collection in adolescent participants (applying to HCs). We added age at stool sampling to our models to correct for the age variation between participant groups.

In conclusion, previous results of gut microbiota composition and predicted functional pathways could not be validated in this Dutch pediatric-onset MS cohort using the more sensitive 16S DADA2 pipeline, although it was limited by sample size and DMT use. Upcoming metagenomics analyses such as shotgun sequencing and functional metabolic pathway analyses [39] are currently improving and are becoming more affordable. Therefore, these will likely have an important contribution in the near future to better understanding of the complex and functional role of the gut microbiota in MS. Finally, the role of the gut microbiota in relation to BMI and MS deserves further investigation because of the potential treatment target.

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FUNDING INFORMATION

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ETHICS APPROVAL AND PATIENT CONSENT

Study protocol of the Dutch prospective cohort study was approved by the local medical ethical committee of the Erasmus University Medical Center. Within this study, all patients and/or their legal representatives gave written informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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