

# Immunotherapy-mediated modulation of the gut microbiota in multiple sclerosis and associations with diet and clinical response—the effect of dimethyl fumarate therapy

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## Abstract

**Background:** Accumulating evidence supports a role of the microbiota in health and disease, including in multiple sclerosis (MS). How MS drugs affect the microbiota and whether this is part of their mode of action is yet unknown.

**Objectives:** To assess how dimethyl fumarate (DMF) affects the gut microbiota and whether the microbiota is associated with clinical response or adverse events (AEs) to DMF or diet.

**Design:** An observational cohort study, in which the microbiota from 45 patients with relapsing-remitting MS pre-DMF initiation and following 6 months of DMF therapy, and from 47 matched healthy controls, were compared, and associations with clinical and dietary data assessed.

**Data sources and methods:** Microbial DNA was sequenced and analyzed using MicrobiomeAnalyst. The clinical response was assessed after 1-year DMF therapy based upon evidence of disease activity (relapse,  $\Delta$ EDSS increase  $>1$ , or MRI activity compared to pre-treatment). Dietary data were obtained by food questionnaires.

**Results:** Alterations in relative abundance of several microbes were identified post 6-month DMF therapy compared to pre-treatment, including an increase in Firmicutes, *Lachnospiraceae*, and *Ruminococcaceae*, while reduction in Bacteroidetes and Proteobacteria. Patients who showed disease activity within 1 year from DMF initiation had pre-treatment higher abundance of Proteobacteria, *Flavonifractor*, and *Acidaminococcaceae*, while lower abundance of Firmicutes, *Ruminococcaceae*, *Butyricoccus*, and *Massiliprevotella massiliensis*, compared to patients without disease activity. Patients who discontinued DMF therapy due to AEs had pre-treatment higher abundance of Proteobacteria, Bacteroidetes, *Eggerthella*, and *Lachnoclostridium* and lower abundance of *Ruminococcaceae*, *Megamonas*, and *Holdemanella*, among others. Differentially abundant microbes correlated with intake of several nutrients.

**Conclusion:** DMF immunotherapy is associated with modifications of the microbiota. The microbiota may affect the severity of AEs and the clinical response to DMF, and is potentially modulated by diet. Microbiota-based, personalized treatment approach, integrating pharmacotherapy with dietary components, carries potential to improved clinical outcome.

**Keywords:** clinical response, dimethyl fumarate, disease-modifying therapy, dysbiosis, gastrointestinal adverse events, Mediterranean diet, microbiome, multiple sclerosis, nutrients, therapy

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## Introduction

Multiple sclerosis (MS) is an autoimmune, neurodegenerative disease, characterized by an aberrant immune response targeting the myelin sheath, causing demyelination, axonal, and neuronal loss. MS is a complex, multifactorial disease driven by interactions between genetic and environmental factors, such as smoking, obesity, Epstein-Barr virus infections, Vitamin D deficiency, etc. Growing evidence suggests that the gut microbiome plays a role in diseases such as MS, through the gut-brain axis of interacting substances and neural communication.<sup>1,2</sup> The gut microbiome affects both the development and activity of the immune system, as well as gut permeability and function, production and release of immune-mediating substances such as microbial-associated molecular patterns,<sup>3</sup> short-chain fatty acids (SCFAs), and tryptophan/serotonin,<sup>4</sup> besides additional roles in energy and vitamin production. The microbiota composition of the individual develops during early childhood, influenced by host genetics and environment, but may undergo modifications later on in life, temporarily or stable, due to significant changes of lifestyle, diet, medications, age, or disease.<sup>2,5</sup>

In recent years, several publications have presented differences in the microbiota in patients with relapsing-remitting MS (PwMS) compared to healthy individuals,<sup>4,6–11</sup> however with relatively low overlap between studies, not enabling the definition of a specific MS-microbiome signature. We have recently published a comparison of the microbiota between treatment-naïve PwMS and healthy individuals in the Israeli populations, where we found several microbes at different taxonomic levels to differ between cases and controls.<sup>12</sup> We also showed that the MS-associated microbiota seems in part to be population-specific, and that the MS-differential relative abundance of some microbes is associated with diet, specific nutrient intake, Vitamin D level, age, and gender, suggesting that the microbiota may serve a comprehensive and complex role in MS pathogenesis and pathology. In continuation to the published study, we investigate the effects of various MS disease-modifying therapies (DMTs) on the microbiota. Recent studies suggest that while DMTs seem not to affect the general microbiota composition, they may alter the abundance of specific microbes.<sup>9,13–16</sup> Whether alteration of the microbiota is part of the beneficial immunomodulatory effects of some of the DMTs available for

PwMS, or whether it is a byproduct, with either positive, neutral, or negative effects on the patient's health, is unknown. The current observational, cohort, longitudinal study focused upon the effect of dimethyl fumarate (DMF) therapy on the microbiota, possible associations between microbiota alterations and clinical response, occurrence of adverse events (AEs) leading to drug discontinuation, and association with dietary data.

DMF is an orally administered fumaric acid ester, cleaved in the intestine by esterases to monomethyl fumarate (MMF), the main active metabolite. DMF acts through both neuroprotection, upregulating the nuclear factor (erythroid derived 2)-like 2 (Nrf2) anti-oxidative pathway, and through immunomodulation, by inhibition of the nuclear factor-kappa B pathway, which mediates pro-inflammatory signaling.<sup>17</sup> We and others have previously shown that DMF increases the proportions of anti-inflammatory and regulatory immune cells, while reduces inflammatory cells.<sup>18–21</sup> Transient mild to moderate flushing and gastrointestinal (GI) AEs, such as nausea, vomiting, diarrhea, and abdominal pain, are commonly reported in PwMS treated with DMF, most frequently in the first month of treatment before declining.<sup>22,23</sup> These DMF-associated AEs can be intolerable for some PwMS affecting the adherence to therapy and may lead to drug discontinuation. Some effects of DMF therapy on various microbes in PwMS have been described,<sup>13,16,24–27</sup> however with very low repetition across studies.

## Methods

### Recruitment and sample collection

PwMS ( $N=45$ ) about to initiate DMF therapy, including 33 treatment-naïve PwMS, as well as 47 age- and gender-matched healthy controls (HCs), were recruited at an MS Center in Carmel Medical Center, Israel. The study was conducted under a protocol approved by the Institutional Ethical Review Board (0034-13-CMC), and all participants signed a written informed consent.

Inclusion criteria for PwMS were a diagnosis of relapsing remitting MS according to revised McDonald criteria,<sup>28,29</sup> and being about to initiate DMF therapy. Healthy participants were recruited among PwMS' escorts to the clinic

(mainly unrelated spouses or acquaintances) and hospital's visitors, who had no known relative with MS up to 2nd degree. Inclusion criteria for all participants were age 18–55 years, without antibiotics/probiotics/corticosteroids treatment within the last month, and no irritable bowel disease (IBD), other autoimmune disease, or history of gastric/bowel surgery. The cohorts included fecal samples from 30 PwMS and 43 HC, who were part of the cohorts examined in our recent published study comparing the microbiome of PwMS ( $N=57$ ) and healthy individuals ( $N=43$ ) in the Israeli populations.<sup>12</sup> PwMS initiated oral DMF treatment according to standard regime (starting dose of 120 mg twice a day for 7 days, then maintenance dose of 240 mg twice a day). Fecal samples were collected at recruitment and 6 months after DMF initiation, using a stool preservative tube (Norgen Biotek, Thorold, ON, Canada), frozen immediately upon arrival to the clinic and kept at  $-80^{\circ}$  until DNA extraction. All participants completed a food frequency questionnaires (FFQ) and a Mediterranean Diet Score (MDS) questionnaire, both especially adapted to the Israeli population. The FFQs were analyzed at the Department of Public Health, Faculty of Health Sciences, Ben-Gurion University of the Negev, Israel, for daily intake of energy and 74 various nutrients.<sup>30–32</sup> The MDS is a 17-item Mediterranean Diet adherence screener.<sup>33–35</sup> Demographic and clinical data was obtained, including Vitamin D level at recruitment, body mass index (BMI), level of disability (Expanded Disability Status Scale, EDSS) pre-treatment initiation and during 1-year follow-up, annual relapse rate calculated as mean over the last 2 years prior to recruitment, relapses during 1-year follow-up since DMF initiation, evidence of disease activity on MRI after 1-year follow-up compared to MRI pre-DMF treatment and drug discontinuation due to flushing and GI AEs.

#### *Clinical response definition*

PwMS who discontinued DMF therapy within 1 year due to flushing and GI AEs were defined as patients with intolerable AEs (IAEs). PwMS who continued DMF therapy, despite any GI AEs, were defined as patients without IAE.

Clinical response was assessed after 1-year DMF therapy based upon any evidence of disease activity (relapse, MRI activity, and/or  $\Delta$ EDSS increase  $>1$ ). PwMS were defined as having no evidence

of disease activity (PwNDA) if they had no relapse, no evidence of disease activity on MRI (no worsening compared to previous MRI, e.g., no new or enlarged lesions and no Gadolinium enhancement, according to the summary provided by a Radiologist), and no  $\Delta$ EDSS increase  $>1$ . PwMS who had a relapse within 1 year of initiating DMF, any evidence of disease activity on MRI compared to the previous MRI (pre-DMF) or  $\Delta$ EDSS increase  $>1$  were defined as patients with disease activity (PwDA).

#### *Microbial DNA extraction*

Microbial DNA sequencing was performed as previously described.<sup>12</sup> DNA was extracted using the QIAamp® PowerFecal® Pro DNA kit (Qiagen, Tegelen, The Netherlands), according to manufacturer's instructions. DNA 16s rRNA sequencing was performed at Hy Laboratories Ltd (Rehovot, Israel). The V3V4 region of the 16s rRNA gene was amplified using primers from The Earth Microbiome Project. Access Array primers for Illumina (Fluidigm, San Francisco, CA, USA) were used to add adaptor and index sequences and the reactions cleaned using Kapa Pure beads (Kapa, Roche, Basel, Switzerland). The concentration of each library was measured by Qubit (Life Technologies, Waltham, MA, USA) using the DeNovix dsDNA high sensitivity assay (DeNovix, Wilmington, DE, USA) and samples pooled. The pooled library was sequenced on Illumina Miseq using a Miseq v2 Kit (Illumina, Eindhoven, The Netherlands) to generate  $2 \times 250$  PE reads, at a depth of 100,000 reads/sample. Reads were trimmed for adaptor sequences and quality, paired reads were merged and subjected to operational taxonomic units (OTUs) de novo picking against the SILVA database at  $>97\%$  sequence similarity using the CLC-bio software (Qiagen); 5773 OTUs were identified.

#### *Statistical analysis*

Differences between groups in gender, age, ethnicity, MDS, BMI, proportion of smokers and vegetarians, and the average intake of 75 nutrients between groups were assessed by the Mann–Whitney *U*-test or Kruskal–Wallis test for continuous or ordinal variables and by Fischer's exact test for nominal, categorical variables, using IBM SPSS statistics (v28) (Armonk, NY), at  $p$ -value  $<0.05$ . Analysis of MDS in patients after 6 months of DMF therapy compared to

pre-treatment was assessed by the Wilcoxon paired test or by the Friedman test (MDS groups). Differences in MDS or specific nutrient intake between PwDA and PwNDA or between patients with IAE and patients without IAE were assessed by the Mann–Whitney *U*-test, at  $p$ -value  $< 0.05$ .

Statistical analysis of microbiota data was performed with the MicrobiomeAnalyst web-tool version 2.0 (Xia Lab, McGill University, Montreal, QC, Canada), using the Marker Data Profiling (MDP) Module, according to published protocols.<sup>36–38</sup> OTUs were filtered to include only features with  $\geq 4$  counts in at least 10% of samples. Testing for differences in  $\alpha$ -diversity across groups was assessed using the Shannon alpha index (Kruskal–Wallis test), comparing OTU richness and evenness, while  $\beta$ -diversity was calculated using the Bray–Curtis dissimilarity (PERMANOVA) comparing the similarity and distance between samples, on data normalized by total sum scaling (TSS). Differential relative abundance analysis of individual taxa between PwMS and HC was performed as previously published.<sup>12</sup> Differences in relative abundance of microbes between patients post 6-month DMF therapy compared to pre-treatment, was performed using multiple linear regression with adjustment for subject ID (paired analysis). As a complement, unpaired analyses were performed using DeSeq2 (normalization by Relative Log Expression), adjusted for multiple testing, at false discovery rate (FDR)  $< 0.1$ , and the Linear Discriminant Analysis Effective Size (LEfSe) tool (on TSS normalized data), which identify taxa that best discriminate between groups, at  $p$ -value  $< 0.05$  and linear discriminant analysis (LDA)  $\geq \pm 2$ . The relative tolerant FDR reflects the exploratory nature of the study. Only microbes with significant difference in the paired analysis, or in both two unpaired analyses, were considered differential.

Differences in relative abundance of specific microbes (in pre-treatment samples or in samples after 6-month DMF therapy) between PwNDA and PwDA were calculated using two statistical tools: Linear Regression and DeSeq2, both adjusted for multiple testing at FDR  $< 0.1$ , as well as by LEfSe, at  $p < 0.05$  and LDA  $\geq 2$ . Differences in relative abundance of specific microbes (in pre-treatment samples) between patients with IAE and patients without IAE were calculated by Linear Regression at FDR  $< 0.1$ , by DeSeq2 at

FDR  $< 0.05$  (to reduce the risk of false positive due to small number of IAE cases), and by LEfSe at  $p < 0.05$  and LDA  $\geq 2$ .

Spearman correlations between the relative abundance of the differential microbes, identified in the various analyses, and the dietary data (MDS, Vitamin D, or daily nutrient intake) or disease severity (EDSS), were assessed using IBM SPSS statistics (v28), at  $p$ -value  $< 0.05$  (data normalized by TSS).

### Functional analysis

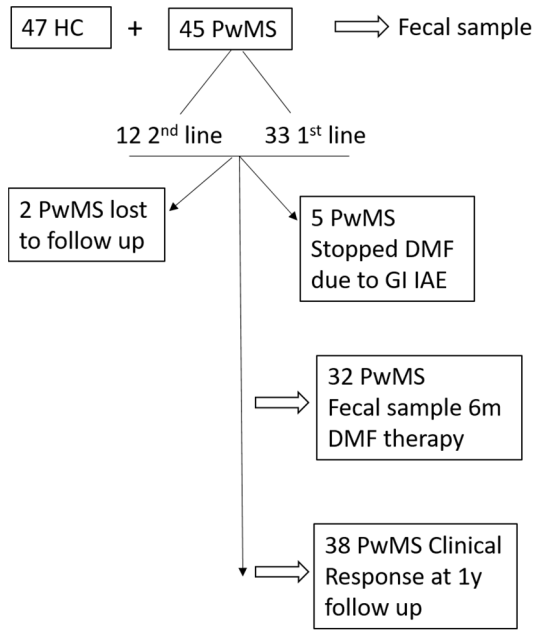
To predict metabolic functions and pathways affected by DMF therapy (from the identified altered microbiota) the Tax4Fun2 package was used,<sup>39</sup> under the MDP Module in the MicrobiomeAnalyst web tool. The tool was applied to all OTUs, filtered for  $\geq 4$  reads in  $\geq 10\%$  samples, scaled by TSS, and then functions predicted by Tax4Fun2 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologies (KOs). The KO abundance table was uploaded into the Shotgun Data Profiling module for differential pathway analysis, enrichment analysis, and metabolic network mapping. KOs were filtered for  $\geq 4$  reads in  $\geq 10\%$  samples and scaled by cumulative sum scaling. Functional metabolic pathways differing between PwMS post 6-month DMF therapy compared to baseline were identified using multiple linear regression adjusted for pairs, at FDR  $< 0.05$ , and the significant KOs mapped to enriched networks at FDR  $< 0.05$ . Similar differential pathway analysis between PwNDA compared to PwDA was performed at FDR  $< 0.05$  and mapped for enrichment at  $p < 0.05$ . LEfSe was performed on KOs to detect metabolic functions that best distinguished between groups at FDR  $< 0.05$  and LDA  $> \pm 0.65$ .

## Results

### Demographic and clinical data of participants

Forty-five PwMS about to initiate DMF therapy, as well as 47 age- and gender-matched HC were recruited to this study (study design presented in Figure 1). Fecal samples were available from all participants at recruitment, and from 32 of the PwMS following 6 months of DMF therapy. Demographic and clinical data are presented in Table 1. PwMS included 33 treatment-naïve





**Figure 1.** Study design.

DMF, dimethyl fumarate; GI IAE, flushing or gastrointestinal intolerable adverse events causing discontinuation of DMF therapy; HC, healthy control; m, months; PwMS, patients with MS; y, year; 1st line, treatment naïve; 2nd line, patients who received at least one prior therapy.

patients (1st line patients), and 12 2nd line patients, with a mean time since last DMT of 26 months. The mean disease duration was 2.4 months, reflecting the considerable majority of 1st line patients, recently diagnosed. There was no significant difference between PwMS and HC in age, gender, BMI, and frequency of smoking or of vegetarians. However, there was a significant difference in ethnicity between the cohorts, with more Jews and less Arabs in the HC cohort ( $p=0.028$ ), yet this was irrelevant for the longitudinal study of PwMS, following DMF initiation. There was no difference in MDS between the cohorts, also not between PwMS before and after 6-month DMF therapy. Five PwMS stopped DMF treatment during follow-up due to IAE, one PwMS due to an insufficient clinical response, and two PwMS were lost to follow-up. Clinical response was available following 1-year treatment for 38 PwMS, of these 27 PwMS (71%) showed no evidence of disease activity during follow-up (PwNDA). Eleven PwMS showed some evidence of disease activity ( $\Delta$ EDSS increase  $>1$ , relapse, and/or evidence of active disease on MRI) (PwDA). Of these, one PwMS discontinued

DMF and shifted DMT  $<1$  year, four PwMS shifted DMT within 12–15 months, while six PwMS continued DMF therapy beyond at least 2 years from treatment initiation, indicating that despite the evidence, the clinical response was regarded sufficient at the time.

#### *Comparison of general microbiota composition*

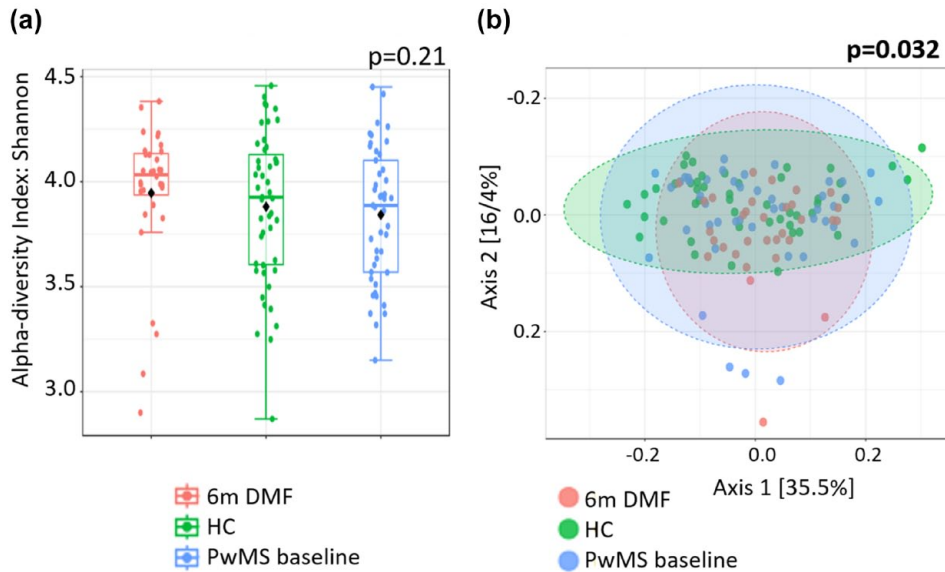
A comparison of the general microbiota composition showed no significant difference in Shannon alpha index diversity between HC, PwMS at baseline, and post 6-month DMF therapy ( $p=0.21$ ) (Figure 2(a)). A pairwise analysis showed also no significant difference between any of the groups (HC vs PwMS:  $p=0.5$ ; PwMS baseline vs 6-month DMF:  $p=0.07$  (trend)). There was a significant difference in Bray–Curtis beta-diversity between the three groups ( $p=0.032$ ) (Figure 2(b)). However, pairwise analysis revealed that this difference was only significant between HC and 6-month DMF patients, while not significant between patients pre- and post-DMF therapy (HC vs 6-month DMF:  $p=0.006$ ; HC vs PwMS:  $p=0.5$ ; PwMS baseline vs 6-month DMF:  $p=0.08$  (trend)).

#### *Comparison of relative abundance of specific microbes*

*Effect of 6-month DMF treatment on the relative abundance of specific microbes.* Microbes with significant alteration in relative abundance after 6-month DMF therapy compared to baseline (pre-treatment) were identified by Multiple Linear Regression adjusted as paired analysis, and complementarily by two unpaired analyses DeSeq2 and LefSe. Twenty-five microbes with differential abundance post 6-month DMF therapy at different taxonomic levels were identified by the paired analysis, including four phyla, four classes, four orders, seven families, and six genera (presented in Table 2, Figure 3). In addition, another phylum, two families, seven genera, and one species were significantly different post DMF therapy in both unpaired analyses (Table 2, Figure S1 (LefSe)). A multifactor subanalysis, adjusted both for subject ID (paired analysis) and for previous treatment (1st or 2nd line patients), is presented in Table S1, and confirmed the significantly differential abundance of the top five genera, seven families, four orders, five classes, and four phyla. This indicates that the effect of previous treatment in the 2nd line patients was

**Table 1.** Demographic and clinical data of participants.

| Study population  | PwMS pre-treatment | PwMS 6-month DMF                               | PwMS 1-year DMF                             | HC         | p-Value PwMS vs HC |
|---|--------------------|--|---|------------|--------------------|
| Age (years)   | 33.8 ± 1.3         |  |   | 38.4 ± 1.7 | 0.06               |
| Female (%)  | 73.3               |  |   | 57.4       | 0.13               |
| BMI (kg/m <sup>2</sup> )  | 25.0 ± 0.8         |  |   | 24.8 ± 0.5 | 0.99               |
| Smoking (%)   | 40.0               |  |   | 25.5       | 0.18               |
| Vegetarian, N (%)   | 1 (2.2)            |  |   | 2 (4.3)    | 1.0                |
| Ethnicity, N (%)  |                    |  |   |            | 0.028              |
| Jewish  | 24 (53.3)          |  |   | 36 (76.6)  |                    |
| Arab  | 21 (46.7)          |  |   | 11 (23.4)  |                    |
| Disease duration (months)   | 2.4 ± 0.7          |  |   |            |                    |
| Treatment naïve, N (%)  | 33 (73.3)          |  |   |            |                    |
| Time since previous DMT (m) (2nd line only) mean ± SE (median)  | 26 ± 10 (14)       |  |   |            |                    |
| Previous DMT (2nd line only), N<br>Fingolimod 1, Glatiramer Acetate 3, Interferon beta 4, Natalizumab 1, Ocrelizumab 1, Teriflunomide 2   |                    |  |   |            |                    |
| Vitamin D (ng/mL)   | 44.0 ± 4.7         |  |   |            |                    |
| MDS   | 7.33 ± 0.3         | 7.7 ± 0.5 (32)<br><i>p</i> = 0.24 <sup>a</sup> |   | 7.49 ± 0.4 | 0.69               |
| MDS, N (%)  |                    |  |   |            | 1.0                |
| Low (≤6)  | 18 (40.0)          | 14 (43.8)                                      |   | 18 (38.3)  |                    |
| Intermediate (7–11)   | 24 (53.3)          | 14 (43.8)                                      |   | 25 (53.2)  |                    |
| High ≥12  | 3 (6.7)            | 4 (12.5)<br><i>p</i> = 0.64 <sup>b</sup>       |   | 4 (8.5)    |                    |
| EDSS mean ± SE<br><i>p</i> -value   | 1.97 ± 0.23        |  | 1.82 ± 0.25<br><i>p</i> = 0.67 <sup>a</sup> |            |                    |
| Annual relapse rate baseline mean ± SE  | 0.48 ± 0.05        |  |   |            |                    |
| Patients with relapse within 1-year DMF treatment, N (%)  |                    |  | 5 (13.2)                                    |            |                    |
| Patients with MRI activity after ~1-year DMF treatment, N (%)   |                    |  | 5 (13.2)                                    |            |                    |
| Patients with ΔEDSS increase >1 after 1-year DMF treatment, N (%)   |                    |  | 5 (13.2)                                    |            |                    |
| PwNDA after 1-year DMF treatment, N (%)   |                    |  | 27 (71.1)                                   |            |                    |
| Patients with IAE, N (%) within 1-year DMF treatment  |                    |  | 5 (11.1)                                    |            |                    |
| <p>Comparison of clinical and demographic data between PwMS (<i>N</i> = 45) pre-treatment, post 6-month and 1-year DMF therapy, and HC (<i>N</i> = 47). Statistical comparison of age, BMI, MDS was assessed using Mann–Whitney <i>U</i>-test; gender, frequency of smoking and vegetarian, ethnicity, MDS groups assessed by Fischer’s exact test.</p> <p><sup>a</sup>Assessed by Wilcoxon’s paired test.</p> <p><sup>b</sup>Assessed by Friedman test.</p> <p>BMI, body mass index; EDSS, Expanded Disability Status Scale; HC, healthy controls; IAE, intolerable adverse events which lead to drug discontinuation; IFN, interferon; MDS, Mediterranean Diet Score; PwMS, patients with MS; PwNDA, PwMS with no evidence of disease activity.</p> |                    |  |   |            |                    |



**Figure 2.** General microbiome comparison. (a) Shannon alpha-diversity and (b) Bray-Curtis beta-diversity at the species level, between HC (healthy control), PwMS (baseline, pre-therapy), and following 6-month DMF treatment (6m DMF).

DMF, dimethyl fumarate; HC, healthy control; PwMS, patients with multiple sclerosis.

minor, likely due to the relatively long period since previous DMT in most cases in our cohort. Microbes with increased abundance after 6-month DMF therapy all belonged to the Firmicutes phylum, the Clostridiales order, and were mostly from the *Lachnospiraceae* family, whereas some taxa belonging to the Selenomonadales or the Lactobacillales orders within the Firmicutes were reduced by DMF therapy. Other microbes reduced by DMF therapy mostly belonged to the Bacteroidetes, the Patenscibacteria, or the Proteobacteria phyla.

*Comparison of microbes differential post 6-month DMF therapy versus pre-treatment and microbes differential between PwMS (pre-treatment) and HC.* As we have recently published data on the comparison of the gut microbiota between HC and treatment-naïve PwMS in the Israeli population,<sup>12</sup> on cohorts which in part overlap with the cohorts of the current study (as described in the section “Methods”), such comparison was not the aim of the current study. However a PwMS/HC analysis was performed (presented in Table S2) to enable a comparison with DMF-altered microbiota (Table 2), revealing only very few overlapping differentially abundant microbes. Some *Lachnospiraceae* genera had lower abundance in PwMS compared to HC, and DMF

therapy enhanced several *Lachnospiraceae* family members. However, *Prevotella* 7 + 9 genera had lower abundance in PwMS compared to HC, and *Prevotella* 7 further decreased post DMF therapy. Likewise, the *Tannerellaceae* family was lower in PwMS than HC, and the abundance further reduced after 6-month DMF therapy.

#### *Associations between microbe abundance (pre-treatment) and clinical response*

In order to identify microbes which may be associated with disease activity, we compared the relative abundance of microbes between patients with or without disease activity during the 1 year follow-up (PwDA vs PwNDA), in baseline samples (Table 3, Figure 4), using three statistical methods. At baseline, two genera, two families, two orders, one class, and three phyla differed between PwNDA and PwDA, according to either DeSeq2 and/or Linear Regression, while LEfSe identified four species, nine genera, and three families that best discriminate between the groups of clinical response. Of these the abundance of the genus *Solobacterium*, a relatively rare bacteria, was significantly higher in PwDA by all three statistical methods, and the abundance of the family *Ruminococcaceae* was higher in PwNDA in two out of three methods, thus presenting the most

**Table 2.** Taxa with differential relative abundance post 6-month DMF therapy compared to pre-treatment.

| Differentially abundant microbes     | Multiple linear regression (paired) |      | LEfSe (unpaired)   |           | DeSeq2 (unpaired)  |      | Highest at |
|--------------------------------------|-------------------------------------|------|--------------------|-----------|--------------------|------|------------|
|                                      | FDR                                 | FC   | FDR                | LDA score | FDR                | FC   |            |
| Species                              |                                     |      |                    |           |                    |      |            |
| Uncultured Bacteroidetes bacterium   | ns                                  |      | 0.089              | −2.7      | 0.005              | 15.7 | Baseline   |
| Genus                                |                                     |      |                    |           |                    |      |            |
| <i>Agathobacter</i>                  | 0.010                               | 2.01 | 0.003              | 4.9       | $5 \times 10^{-5}$ | 0.47 | 6 m DMF    |
| <i>Blautia</i>                       | 0.010                               | 1.88 | 0.003              | 5.3       | 0.008              | 0.56 | 6 m DMF    |
| <i>Butyricimonas</i>                 | 0.087                               | 0.33 | 0.013              | −3.7      | 0.0001             | 3.0  | Baseline   |
| <i>Odoribacter</i>                   | 0.087                               | 0.32 | 0.013              | −3.8      | 0.018              | 2.4  | Baseline   |
| <i>Parabacteroides</i>               | 0.087                               | 0.39 | 0.019              | −4.3      | $6 \times 10^{-6}$ | 3.3  | Baseline   |
| <i>Anaerostipes</i>                  | 0.098                               | 2.08 | 0.029              | 4.2       | 0.044              | 0.51 | 6 m DMF    |
| <i>Alistipes</i>                     | ns                                  |      | 0.045              | −4.8      | $6 \times 10^{-5}$ | 2.7  | Baseline   |
| <i>Ruminococcus gauvreauii</i> group | ns                                  |      | 0.029              | 4.4       | 0.003              | 0.49 | 6 m DMF    |
| <i>Coprococcus 1</i>                 | ns                                  |      | 0.0006             | 4.2       | 0.004              | 0.42 | 6 m DMF    |
| <i>Eubacterium eligens</i> group     | ns                                  |      | 0.027              | 4.6       | 0.008              | 0.45 | 6 m DMF    |
| <i>Dorea</i>                         | ns                                  |      | 0.014              | 4.8       | 0.089              | 0.68 | 6 m DMF    |
| <i>Bacteroides</i>                   | ns                                  |      | 0.068              | −5.6      | 0.022              | 2.0  | Baseline   |
| <i>Prevotella 7</i>                  | ns                                  |      | 0.012              | −3.56     | $6 \times 10^{-6}$ | 73.1 | Baseline   |
| Family                               |                                     |      |                    |           |                    |      |            |
| <i>Lachnospiraceae</i>               | 0.0002                              | 1.65 | $2 \times 10^{-5}$ | 5.9       | $9 \times 10^{-6}$ | 0.39 | 6 m DMF    |
| <i>Marinifilaceae</i>                | 0.021                               | 0.33 | 0.004              | −4.1      | 0.030              | 1.78 | Baseline   |
| <i>Tannerellaceae</i>                | 0.033                               | 0.39 | 0.015              | −4.3      | 0.022              | 1.84 | Baseline   |
| <i>Saccharimonadaceae</i>            | 0.042                               | 0.50 | 0.031              | −2.3      | 0.030              | 3.0  | Baseline   |
| <i>Rikenellaceae</i>                 | 0.042                               | 0.45 | 0.026              | −4.9      | 0.034              | 1.70 | Baseline   |
| <i>Bacteroidaceae</i>                | 0.053                               | 0.44 | 0.036              | −5.6      | ns                 |      | Baseline   |
| <i>Barnesiellaceae</i>               | 0.060                               | 0.31 | 0.043              | −4.1      | ns                 |      | Baseline   |
| <i>Ruminococcaceae</i>               | ns                                  |      | 0.036              | 5.4       | 0.017              | 0.67 | 6 m DMF    |
| <i>Leuconostocaceae</i>              | ns                                  |      | 0.036              | −2.6      | 0.034              | 6.3  | Baseline   |
| Order                                |                                     |      |                    |           |                    |      |            |
| Clostridiales                        | $8 \times 10^{-6}$                  | 1.46 | $9 \times 10^{-7}$ | 6.1       | 0.0002             | 0.51 | 6 m DMF    |

(Continued)



**Table 2.** (Continued)

| Differentially abundant microbes   | Multiple linear regression (paired) |      | LEfSe (unpaired)   |           | DeSeq2 (unpaired)  |      | Highest at |
|--|-------------------------------------|------|--------------------|-----------|--------------------|------|------------|
|  | FDR                                 | FC   | FDR                | LDA score | FDR                | FC   |            |
| Bacteroidales  | $2 \times 10^{-5}$                  | 0.39 | $2 \times 10^{-6}$ | -5.9      | ns                 |      | Baseline   |
| Saccharimonadales  | 0.0014                              | 0.39 | 0.001              | -2.4      | 0.003              | 3.5  | Baseline   |
| Selenomonadales  | 0.026                               | 0.39 | 0.001              | -4.9      | ns                 |      | Baseline   |
| Class  |                                     |      |                    |           |                    |      |            |
| Clostridia   | $6 \times 10^{-6}$                  | 1.4  | $7 \times 10^{-7}$ | 6.1       | $4 \times 10^{-7}$ | 0.39 | 6 m DMF    |
| Bacteroidia  | $1 \times 10^{-5}$                  | 0.39 | $1 \times 10^{-6}$ | -5.9      | ns                 |      | Baseline   |
| Saccharimonadia  | 0.001                               | 0.39 | 0.001              | -2.4      | 0.009              | 3.4  | Baseline   |
| Negativicutes  | 0.019                               | 0.39 | 0.0008             | -4.9      | ns                 |      | Baseline   |
| Phylum   |                                     |      |                    |           |                    |      |            |
| Bacteroidetes  | $8 \times 10^{-6}$                  | 0.39 | $9 \times 10^{-7}$ | -5.9      | 0.040              | 1.49 | Baseline   |
| Firmicutes   | $8 \times 10^{-6}$                  | 1.33 | $9 \times 10^{-7}$ | 6.0       | 0.001              | 0.58 | 6 m DMF    |
| Patescibacteria  | 0.0006                              | 0.39 | 0.0009             | -2.4      | 0.001              | 3.5  | Baseline   |
| Proteobacteria   | 0.03                                | 0.49 | 0.003              | -4.8      | ns                 |      | Baseline   |
| Lentisphaerae  | ns                                  |      | 0.096              | -3.1      | 0.094              | 2.14 | Baseline   |
| Taxa with differential abundance after 6-month DMF therapy compared to baseline (pre-treatment). Multiple Linear Regression (paired analysis, $N=32$ pairs), and unpaired analysis using DeSeq2, and LEfSe ( $N=45+32$ samples), at $FDR < 0.1$ , corrected for multiple testing. Only taxa significant by the paired analysis or by both unpaired analyses are presented.<br>DMF, dimethyl fumarate; FC, fold change; FDR, false discovery rate; LDA, linear discriminant analysis; LEfSe, LDA effect size; m, months; ns, not significant. |                                     |      |                    |           |                    |      |            |

robust results, while other microbes were only differential by one statistical method (Table 3, Figure 4).

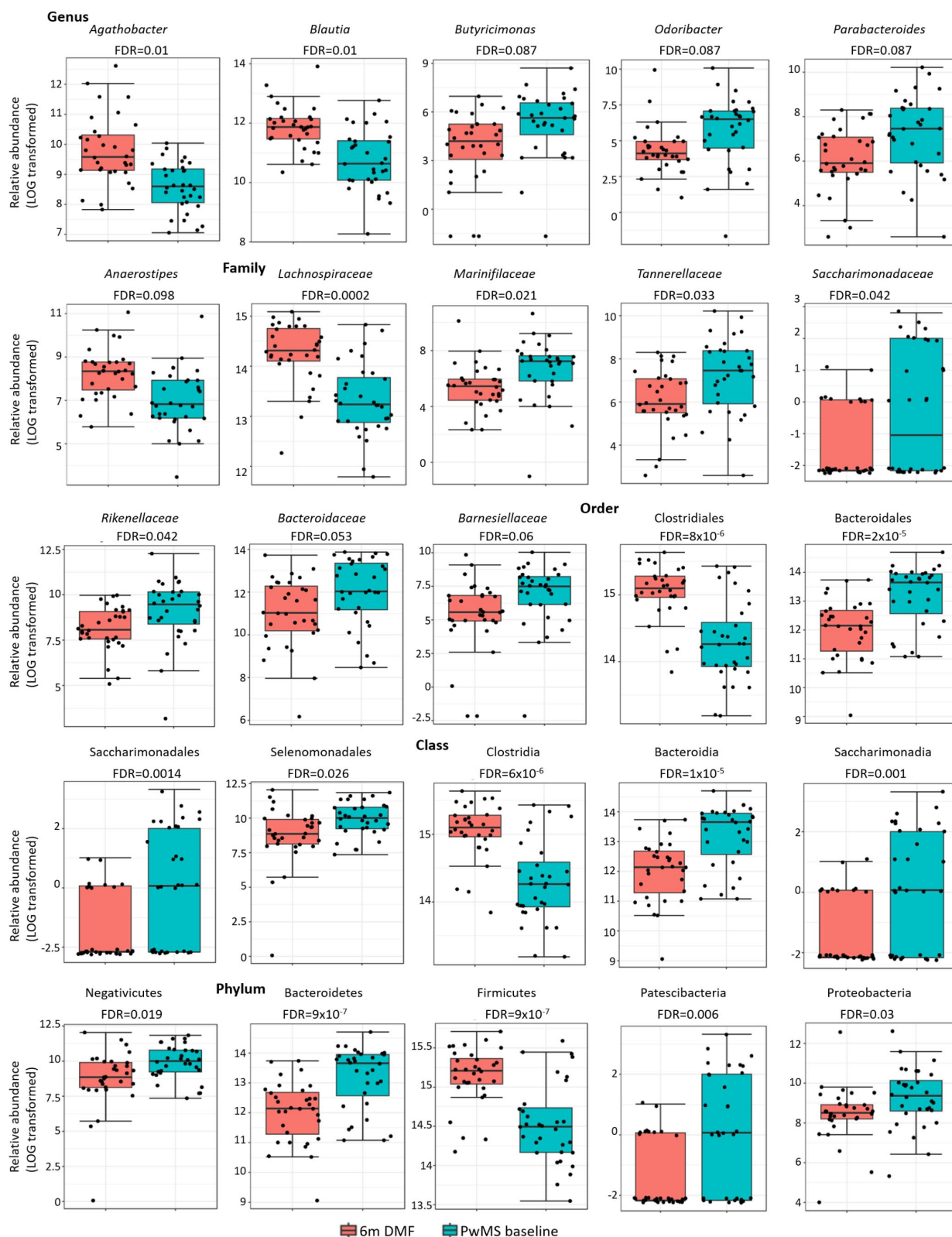
#### *Associations between microbe abundance (post 6-month DMF treatment) and clinical response*

Similar analysis of samples obtained after 6-month DMF therapy (Table 4, Figure 5) revealed three species and six genera with differential relative abundance between PwDA and PwNDA according to DeSeq2 (Linear Regression did not detect any significant results), while LEfSe identified three species, four genera, and two families that best discriminated between PwDA and PwNDA. Of these, the species *Clostridium* sp. K4410.MGS-306 and *Bacteroides uniformis* CL03T12C37, and the genus *Megamonas* were significant by both statistical methods, thus being the most robust

results. There was no overlap between the microbes with differential abundance according to clinical response detected in baseline samples and those detected in samples after 6-month DMF therapy.

#### *Associations between microbe abundance (pre-treatment) and IAE*

Similarly, we compared the relative abundance of microbes in baseline samples from patients with IAE ( $N=5$ ) to patients without IAE ( $N=39$ ) (Tables 5 and S3, Figure 6). With the low number of patients with IAE, the risk of false positive is high, and results are considered with caution. The unspecified species *uncultured rumen bacterium* was significantly lower in patients with IAE by three out of three statistical methods, while two species were significantly higher in IAE



**Figure 3.** Differential abundant taxa in PwMS following 6-month DMF therapy compared to pre-treatment. Representative graphs of taxa with significant difference in relative abundance, as determined by Multiple Linear Regression paired analysis at different taxonomic levels (FDR < 0.1).

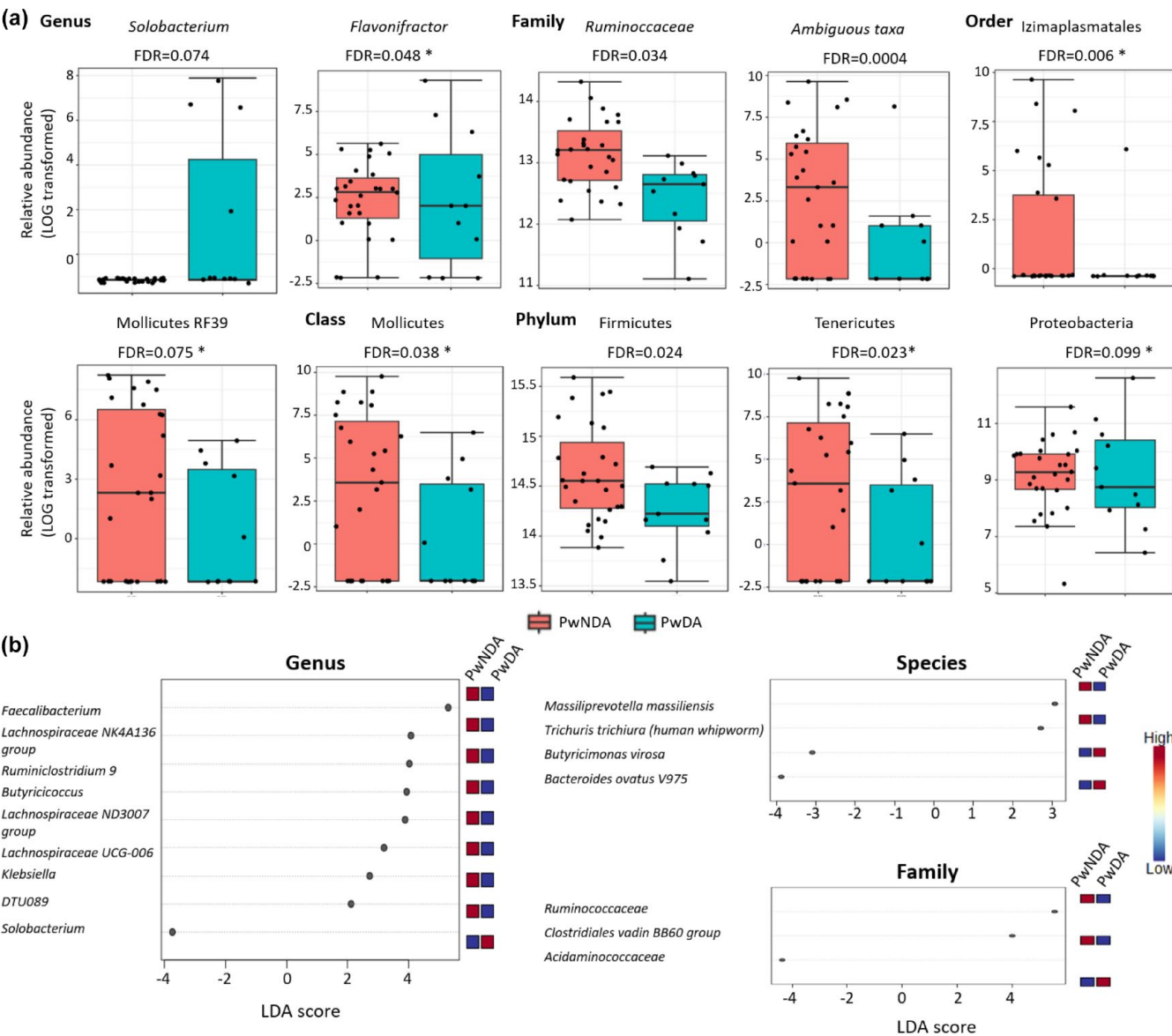
DMF, dimethyl fumarate; FDR, false discovery rate; m, months; PwMS, patients with Multiple Sclerosis.

**Table 3.** Taxa with differential relative abundance between patients with or without evidence of disease activity during 1-year DMF therapy (pre-treatment samples).

| Differentially abundant microbes            | DeSeq2              |                   | Linear regression |      | LEfSe   |           | Highest in |
|---|---------------------|-------------------|-------------------|------|---------|-----------|------------|
|   | FDR                 | FC                | FDR               | FC   | p Value | LDA score |            |
| Species                                     |                     |                   |                   |      |         |           |            |
| <i>Trichuris trichiura</i> (human whipworm) | ns                  |                   | ns                |      | 0.015   | 2.7       | PwNDA      |
| <i>Bacteroides ovatus</i> V975              | ns                  |                   | ns                |      | 0.015   | −3.9      | PwDA       |
| <i>Butyricimonas virosa</i>                 | ns                  |                   | ns                |      | 0.049   | −3.1      | PwDA       |
| <i>Massiliprevotella massiliensis</i>       | ns                  |                   | ns                |      | 0.047   | 3.1       | PwNDA      |
| Genus                                       |                     |                   |                   |      |         |           |            |
| <i>Solobacterium</i>                        | $9 \times 10^{-17}$ | $2.4 \times 10^7$ | 0.074             | 3.5  | 0.001   | −3.7      | PwDA       |
| <i>Flavonifractor</i>                       | 0.048               | 7.5               | ns                |      | ns      |           | PwDA       |
| <i>Faecalibacterium</i>                     | ns                  |                   | ns                |      | 0.003   | 5.3       | PwNDA      |
| <i>Lachnospiraceae</i> NK4A136 group        | ns                  |                   | ns                |      | 0.035   | 4.1       | PwNDA      |
| <i>Lachnospiraceae</i> ND3007 group         | ns                  |                   | ns                |      | 0.044   | 3.9       | PwNDA      |
| <i>Butyricicoccus</i>                       | ns                  |                   | ns                |      | 0.038   | 3.9       | PwNDA      |
| <i>Lachnospiraceae</i> UCG-006              | ns                  |                   | ns                |      | 0.027   | 3.2       | PwNDA      |
| <i>Ruminiclostridium</i> 9                  | ns                  |                   | ns                |      | 0.041   | 4.0       | PwNDA      |
| <i>DTU089</i>                               | ns                  |                   | ns                |      | 0.030   | 2.1       | PwNDA      |
| <i>Klebsiella</i>                           | ns                  |                   | ns                |      | 0.047   | 2.7       | PwNDA      |
| Family                                      |                     |                   |                   |      |         |           |            |
| <i>Ruminococcaceae</i>                      | ns                  |                   | 0.034             | 0.68 | 0.010   | 5.6       | PwNDA      |
| <i>Ambiguous taxa</i>                       | 0.0004              | 0.03              | ns                |      | ns      |           | PwNDA      |
| <i>Clostridiales</i> vadin BB60 group       | ns                  |                   | ns                |      | 0.049   | 4.0       | PwNDA      |
| <i>Acidaminococcaceae</i>                   | ns                  |                   | ns                |      | 0.049   | −4.4      | PwDA       |
| Order                                       |                     |                   |                   |      |         |           |            |
| Izimaplasmatales                            | 0.006               | 0.004             | ns                |      | ns      |           | PwNDA      |
| Mollicutes RF39                             | 0.065               | 0.08              | ns                |      | ns      |           | PwNDA      |
| Class                                       |                     |                   |                   |      |         |           |            |
| Mollicutes                                  | 0.038               | 0.07              | ns                |      | ns      |           | PwNDA      |
| Phylum                                      |                     |                   |                   |      |         |           |            |
| Firmicutes                                  | ns                  |                   | 0.024             | 0.87 | ns      |           | PwNDA      |
| Tenericutes                                 | 0.023               | 0.07              | ns                |      | ns      |           | PwNDA      |
| Proteobacteria                              | 0.099               | 1.9               | ns                |      | ns      |           | PwDA       |

Taxa with significance differential abundance at baseline (pre-DMF therapy) between PwNDA and PwDA ( $N=38$ ). Statistical analysis using DeSeq2, Linear Regression, corrected for multiple testing, at  $FDR < 0.1$ , and LEfSe, at  $p < 0.05$ ,  $LDA > 2$ .

DMF, dimethyl fumarate; FC, fold change; FDR, false discovery rate; LDA, linear discriminant analysis; LEfSe, LDA effect size; ns, not significant; PwDA, PwMS with evidence of disease activity during 1-year follow-up from DMF initiation; PwNDA, PwMS with no evidence of disease activity during 1-year follow-up from DMF initiation.



**Figure 4.** Taxa with differential relative abundance between PwNDA and PwDA in baseline samples (pre-DMF therapy). (a) Representative graphs of taxa with significant difference in relative abundance between PwNDA and PwDA, as determined by Linear Regression, or \* by DeSeq2 (FDR < 0.1). (b) LDA score of differential taxa as determined by LEfSe between PwNDA and PwDA ( $p < 0.05$ ). DMF, dimethyl fumarate; FDR, false discovery rate; LDA, linear discriminant analysis; LEfSe, LDA effect size; PwDA, patients with evidence of disease activity during 1-year follow-up from DMF initiation; PwNDA, patients with no evidence of disease activity during 1-year follow-up from DMF initiation.

patients by both Linear Regression and LEfSe. Five genera were significant by all three statistical methods, namely *Lachnospiraceae* (higher in IAE patients), *Ruminococcaceae* UCG-010, *Ruminococcaceae* NK4A214 group, *Ruminococcaceae* UCG-002, and *Holdemanella* (all lower in IAE patients), while another eight genera were significant by two out of three different methods. The family *Clostridiales vadin BB60 group* was significantly lower in IAE patients by all three methods,

while *Leuconostocaceae* was higher in IAE patients by two out of three methods.

*Associations between abundance of microbes altered by DMF therapy and MDS or clinical data*

Only the *Lachnospiraceae* family, enhanced by DMF therapy, correlated positively with MDS ( $R_s = 0.3$ ,  $p = 0.048$ ). EDSS at baseline correlated

**Table 4.** Taxa with differential relative abundance between patients with or without evidence of disease activity during 1-year DMF therapy (post 6-month DMF samples).

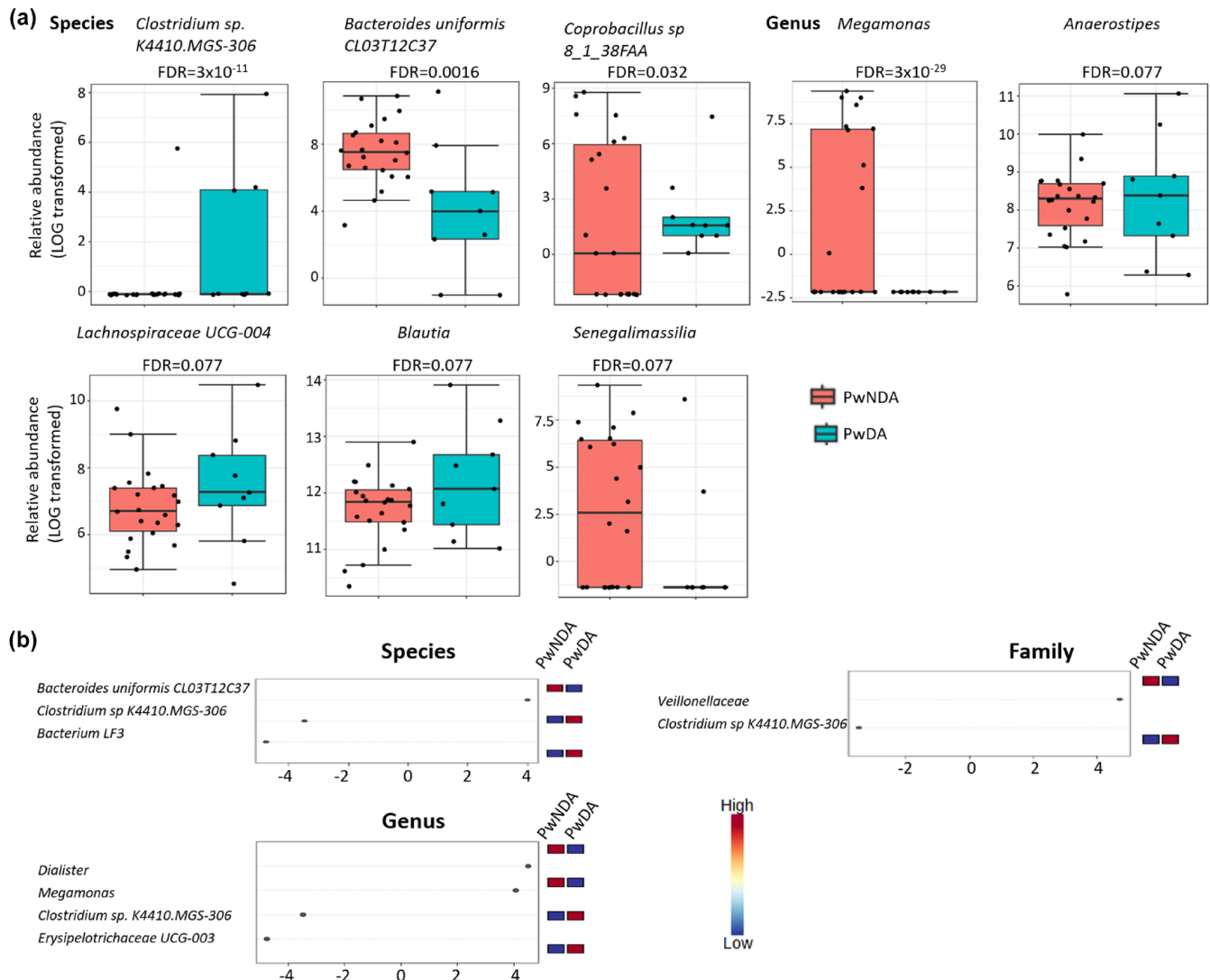
| Differentially abundant microbes   | DeSeq2                |                       | LEfSe   |           | Highest in |
|--|-----------------------|-----------------------|---------|-----------|------------|
|  | FDR                   | FC                    | p Value | LDA score |            |
| Species  |                       |                       |         |           |            |
| <i>Clostridium</i> sp. K4410.MGS-306   | 3 × 10 <sup>-11</sup> | 9.7 × 10 <sup>6</sup> | 0.037   | −3.5      | PwDA       |
| <i>Bacteroides uniformis</i> CL03T12C37  | 0.0016                | 0.132                 | 0.012   | 4.0       | PwNDA      |
| <i>Coprobacillus</i> sp. 8_1_38FAA   | 0.0320                | 0.064                 | ns      |           | PwNDA      |
| <i>Bacterium</i> LF-3  | ns                    |                       | 0.0026  | −4.8      | PwDA       |
| Genus  |                       |                       |         |           |            |
| <i>Megamonas</i>   | 3 × 10 <sup>-29</sup> | 4 × 10 <sup>-8</sup>  | 0.018   | 4.1       | PwNDA      |
| <i>Anaerostipes</i>  | 0.077                 | 3.12                  | ns      |           | PwDA       |
| <i>Lachnospiraceae</i> UCG-004   | 0.077                 | 3.43                  | ns      |           | PwDA       |
| <i>Blautia</i>   | 0.077                 | 2.23                  | ns      |           | PwDA       |
| <i>Senegalimassilia</i>  | 0.077                 | 0.036                 | ns      |           | PwNDA      |
| <i>Catenibacterium</i>   | 0.077                 | 0.0013                | ns      |           | PwNDA      |
| <i>Erysipelotrichaceae</i> UCG-003   | ns                    |                       | 0.0079  | −4.8      | PwDA       |
| <i>Clostridium</i> sp. K4410.MGS-306   | ns                    |                       | 0.037   | −3.5      | PwDA       |
| <i>Dialister</i>   | ns                    |                       | 0.043   | 4.5       | PwNDA      |
| Family   |                       |                       |         |           |            |
| <i>Veillonellaceae</i>   | ns                    |                       | 0.015   | 4.7       | PwNDA      |
| <i>Clostridium</i> sp. K4410.MGS-306   | ns                    |                       | 0.037   | −3.5      | PwDA       |
| Taxa with significance differential abundance between PwNDA and PwDA in samples obtained after 6-month DMF therapy (N=31). Statistical analysis performed using DeSeq2, corrected for multiple testing, at FDR<0.1, and LEfSe, at p<0.05, LDS>2. DMF, dimethyl fumarate; FC, fold change; FDR, false discovery rate; LDA, linear discriminant analysis; LEfSe, LDA effect size; ns, not significant; PwDA, PwMS with evidence of disease activity during 1-year follow-up from DMF initiation; PwNDA, PwMS with no evidence of disease activity during 1-year follow-up from DMF initiation. |                       |                       |         |           |            |

negatively with the abundance of the *Rikenellaceae* family and the *Alistipes* genus (of the same family), the *Barnesiellaceae* and the *Bacteroidaceae* families, and the *Bacteroides* genus, all significantly reduced by DMF therapy ( $R_s = -0.4$ ,  $p = 0.008$ ;  $R_s = -0.43$ ,  $p = 0.004$ ;  $R_s = -0.3$ ,  $p = 0.039$ ;  $R_s = -0.3$ ,  $p = 0.047$ , and  $R_s = -0.3$ ,  $p = 0.047$ , respectively) (Figure 7(a)). Interestingly, the *Barnesiellaceae* and the *Bacteroidaceae* families and the *Bacteroides* genus correlated positively with Vitamin D levels in PwMS at baseline ( $R_s = 0.32$ ,  $p = 0.03$ ;  $R_s = 0.3$ ,  $p = 0.048$ ; and  $R_s = 0.3$ ,  $p = 0.048$ , respectively).

#### Associations between abundance of disease-activity differential microbes and dietary or clinical data

There was no significant difference in MDS between patients with or without evidence of disease activity ( $7.7 \pm 0.7$  and  $7.0 \pm 0.4$ , respectively,  $p = 0.4$ ). The mean alcohol intake was significantly lower in PwDA compared to PwNDA ( $0.17 \text{ gr} \pm 0.2$  and  $1.09 \text{ gr} \pm 0.5$ , respectively,  $p = 0.011$ ), while no other differences in nutrient intake were detected between the two clinical response groups. Several significant correlations were identified between taxa with differential





**Figure 5.** Taxa with differential relative abundance between PwNDA and PwDA in 6-month DMF samples. (a) Representative graphs of taxa with significant difference in relative abundance between PwNDA and PwDA, as determined by DeSeq2 (FDR < 0.1). (b) LDA score of significant differential taxa as determined by LEfSe between PwNDA and PwDA ( $p < 0.05$ ). DMF, dimethyl fumarate; FDR, false discovery rate; LDA, linear discriminant analysis; LEfSe, LDA effect size; PwDA, patients with evidence of disease activity during 1-year follow-up from DMF initiation; PwNDA, patients with no evidence of disease activity during 1-year follow-up from DMF initiation.

abundance between PwDA and PwNDA (from Table 3) and nutrient intake (from Table S4(A)). Of these, the family of *Acidaminococcaceae*, showing highest abundance in PwDA, was the only one that correlated positively with saturated fat and several fatty acids (Figure 7(b)). There were no significant correlations between baseline Vitamin D levels or MDS and abundance of the PwDA/PwNDA differential microbes, but the genus *Lachnospiraceae* UCG-006, highest in PwNDA, correlated positively with EDSS ( $p = 0.025$ ,  $R_s = 0.33$ ) (Figure 7(c)).

#### Associations between abundance of IAE-differential microbes and dietary or clinical data

There was no significant difference in MDS between patients with or without IAE ( $7.8 \pm 1.3$  and  $7.3 \pm 0.3$ , respectively,  $p = 1.0$ ). There was a trend toward a higher intake of unsaturated fatty acids Parinaric and Decosapentaenoid (DPA, omega 3) in patients with IAE compared to patients without IAE ( $0.041 \text{ gr} \pm 0.01$  and  $0.025 \text{ gr} \pm 0.006$ ,  $p = 0.065$ ;  $0.038 \text{ gr} \pm 0.007$  and  $0.027 \text{ gr} \pm 0.004$ ,  $p = 0.053$ , respectively), while other nutrients did not differ between patients

**Table 5.** Taxa with differential relative abundance between patients who discontinue DMF therapy due to IAE and patients without IAE.

| Differentially abundant microbes           | DeSeq2             |                   | Linear Regression |       | LEfSe   |           | Highest at |
|--|--------------------|-------------------|-------------------|-------|---------|-----------|------------|
|  | FDR                | FC                | FDR               | FC    | p Value | LDA score |            |
| Species                                    |                    |                   |                   |       |         |           |            |
| <i>Clostridium orbiscindens</i> 1_3_50AFAA | ns                 |                   | 0.002             | 0.14  | 0.0003  | 2.8       | IAE        |
| <i>Uncultured rumen bacterium</i>          | 0.0007             | 85.5              | 0.077             | 7.67  | 0.006   | −3.1      | No         |
| Genus                                      |                    |                   |                   |       |         |           |            |
| <i>Lachnoclostridium</i>                   | 0.027              | 0.37              | 0.074             | 0.46  | 0.003   | 4.4       | IAE        |
| <i>Romboutsia</i>                          | ns                 |                   | 0.081             | 5.24  | ns      |           | No         |
| <i>Clostridium sensu stricto</i> 1         | ns                 |                   | 0.082             | 9.06  | 0.044   | −4.4      | No         |
| <i>Ruminococcaceae</i> UCG-010             | $9 \times 10^{-6}$ | 222               | 0.084             | 20.39 | 0.005   | −4.1      | No         |
| <i>Ruminococcaceae</i> NK4A214 group       | 0.006              | 14.0              | 0.085             | 21.26 | 0.005   | −4.6      | No         |
| <i>Eggerthella</i>                         | ns                 |                   | 0.092             | 0.09  | 0.006   | 3.5       | IAE        |
| <i>Ruminococcaceae</i> UCG-002             | 0.021              | 5.5               | 0.092             | 10.70 | 0.008   | −4.7      | No         |
| <i>Eubacterium coprostanoligenes</i> group | ns                 |                   | 0.092             | 7.21  | 0.023   | −4.5      | No         |
| <i>Sellimonas</i>                          | ns                 |                   | 0.092             | 0.11  | 0.029   | 3.7       | IAE        |
| <i>Holdemanella</i>                        | $5 \times 10^{-7}$ | 1409              | 0.097             | 89.88 | 0.032   | −4.9      | No         |
| <i>Prevotella</i> 2                        | $3 \times 10^{-8}$ | $1.1 \times 10^7$ | ns                |       | ns      |           | No         |
| <i>Succinivibrio</i>                       | $4 \times 10^{-7}$ | $6.7 \times 10^6$ | ns                |       | ns      |           | No         |
| <i>Ruminococcaceae</i> UCG-005             | 0.0012             | 9.3               | ns                |       | ns      |           | No         |
| <i>Prevotella</i> 7                        | 0.0036             | 0.01              | ns                |       | ns      |           | IAE        |
| <i>Christensenellaceae</i> R-7 group       | 0.006              | 10.0              | na                |       | 0.015   | −4.8      | No         |
| <i>Catenibacterium</i>                     | 0.014              | 933               | ns                |       | ns      |           | No         |
| <i>Oscillibacter</i>                       | 0.021              | 0.26              | ns                |       | ns      |           | IAE        |
| <i>Ruminococcaceae</i> UCG-014             | 0.023              | 22.9              | ns                |       | 0.03    | −4.7      | No         |
| <i>Megamonas</i>                           | 0.037              | 116               | ns                |       | ns      |           | No         |
| <i>Ruminococcaceae</i> UCG-011             | 0.40               | 10.6              | ns                |       | 0.047   | −3.24     | No         |
| <i>Anaerostipes</i>                        | ns                 |                   | ns                |       | 0.021   | 4.6       | IAE        |
| <i>Flavonifractor</i>                      | ns                 |                   | ns                |       | 0.025   | −2.6      | No         |
| <i>Blautia</i>                             | ns                 |                   | ns                |       | 0.037   | 5.2       | IAE        |
| Family                                     |                    |                   |                   |       |         |           |            |
| <i>Leuconostocaceae</i>                    | ns                 |                   | 0.006             | 0.12  | 0.0026  | 3.1       | IAE        |
| <i>Clostridiales</i> vadin BB60 group      | 0.0002             | 75.4              | 0.089             | 11.47 | 0.012   | −4.0      | No         |

(Continued)

Table 5. (Continued)

| Differentially abundant microbes | DeSeq2 |     | Linear Regression |      | LEfSe   |           | Highest at |
|----------------------------------|--------|-----|-------------------|------|---------|-----------|------------|
|                                  | FDR    | FC  | FDR               | FC   | p Value | LDA score |            |
| <i>Christensenellaceae</i>       | 0.030  | 7.1 | ns                |      | 0.013   | -4.8      | No         |
| Order                            |        |     |                   |      |         |           |            |
| Bacteroidales                    | ns     |     | 0.012             | 0.52 | ns      |           | IAE        |
| Phylum                           |        |     |                   |      |         |           |            |
| Bacteroidetes                    | ns     |     | 0.007             | 0.52 | ns      |           | IAE        |
| Firmicutes                       | ns     |     | 0.085             | 1.17 | ns      |           | No         |
| Proteobacteria                   | ns     |     | 0.091             | 0.49 | ns      |           | IAE        |

Taxa with significant differential abundance in baseline samples (pre-DMF therapy) from patients who discontinued DMF therapy due to IAE compared to patients without IAE ( $N=44$ ). Statistical analysis using DeSeq2, at  $FDR < 0.05$ , Linear regression, at  $FDR < 0.1$ , both adjusted for multiple testing, and LEfSe, at  $p < 0.05$ ,  $LDS > 2$ . Only partial of significant differential taxa presented. Full data is presented in Table S3. DMF, dimethyl fumarate; LDA, linear discriminant analysis; LEfSe, LDA effect size; FC, fold change; FDR, false discovery rate; IAE, intolerable adverse events causing discontinuation of DMF therapy; no, patients without IAE; ns, not significant.

with/without IAE. Many significant correlations were identified between microbes with differential abundance between patients with/without IAE (from Table 5 and Table S3) and nutrient intake (from Table S4B). Of these, the *Oscillibacter* genus, highest in IAE patients, was the only one to correlate positively with saturated fat and several saturated fatty acids (Figure 7(d)). *Eggerthella*, highest in patients with IAE, correlated positively with various fatty acids, food energy, carbohydrates, total sugars, and unsaturated fat. Intake of Biotin (Vitamin B7), Vitamin B6, Thiamine (Vitamin B1), or Folate (Vitamin B9) correlated positively with some microbes with highest abundance in patients without IAE such as [*Ruminococcus*] *gnavus* group, *Flavonifractor*, or *Ruminococcus gnavus* CC55 001C. The abundance of the species *Clostridium orbiscindens* 1\_3\_50AFAA and the genus *Eggerthella*, both highest in IAE patients, correlated positively with MDS ( $p=0.022$ ,  $R_s=0.34$  and  $p=0.004$ ,  $R_s=0.43$ , respectively), while the genera *Megamonas* and *Succinivibrio*, both lower in patients with IAE, correlated negatively with MDS ( $p=0.028$ ,  $R_s=-0.33$  and  $p=0.037$ ,  $R_s=-0.31$ , respectively).

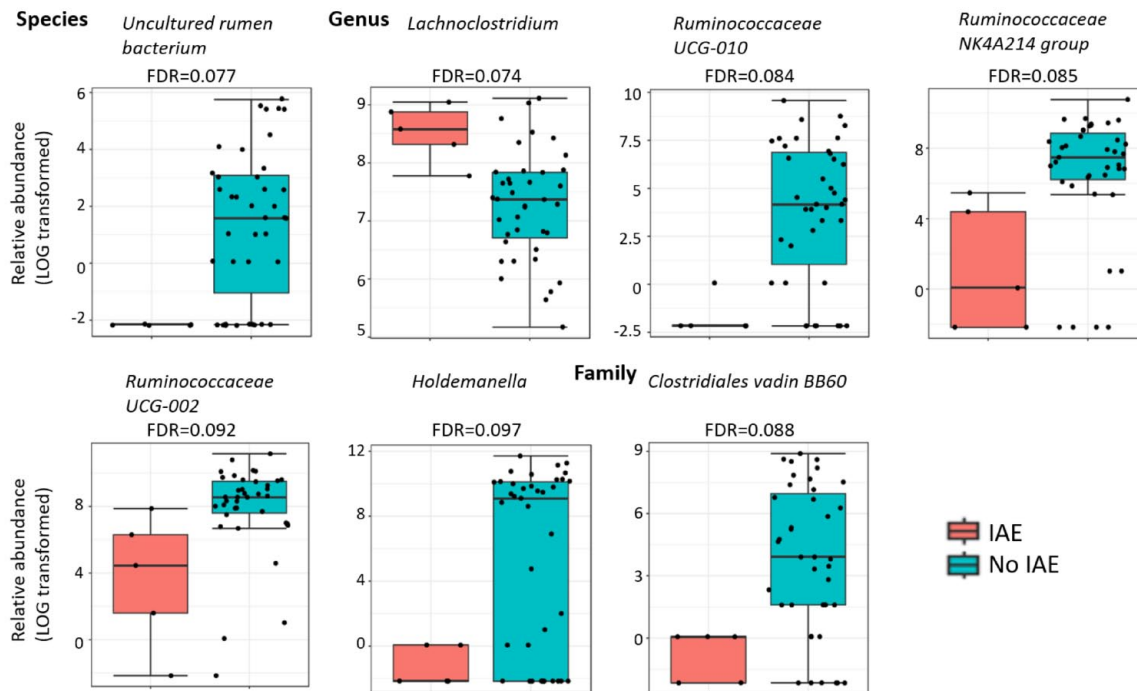
#### Enriched functional pathways

The Tax4Fun2 package, which predicts functional profiles based upon the KEGG database,<sup>37,39</sup> was used to explore functional metabolic

differences in DMF-treated patients compared to pre-treatment, or between PwNDA compared to PwDA, including functional diversity profiling and enrichment analysis. 1079 KO pathways significantly differed post-DMF therapy compared to pre-treatment (Table S5), and these mapped to 8 enriched pathways, at  $FDR < 0.05$ , presented in Table 6(a)). Top enrichment was found in pathways such as Citrate cycle (tricarboxylic acid (TCA) cycle), Lipopolysaccharide biosynthesis, Oxidative phosphorylation, and Biotin metabolism. Two KOs significantly differed between PwNDA and PwDA at  $FDR < 0.05$ , and 89 KOs were significant at  $p < 0.005$  (Table S6), and these 89 KOs mapped to 3 enriched pathways, at  $p < 0.05$  (Table 6(b)), but only included 1 hit each. Puzzling, this included the pathway of drug metabolism-cytochrome P450, although DMF has not been found to interact with hepatic cytochrome P450 (CYP) enzymes.<sup>40</sup>

#### Discussion

With accumulating evidence of a role for the microbiome in human health and disease, including MS, revealing the interactions between microbiota, demographic and clinical factors, DMTs, and disease pathology is highly warranted. Studies from different geographic regions,<sup>4,6-10,41</sup> including ours,<sup>12</sup> have found differential abundance between PwMS and healthy individuals of

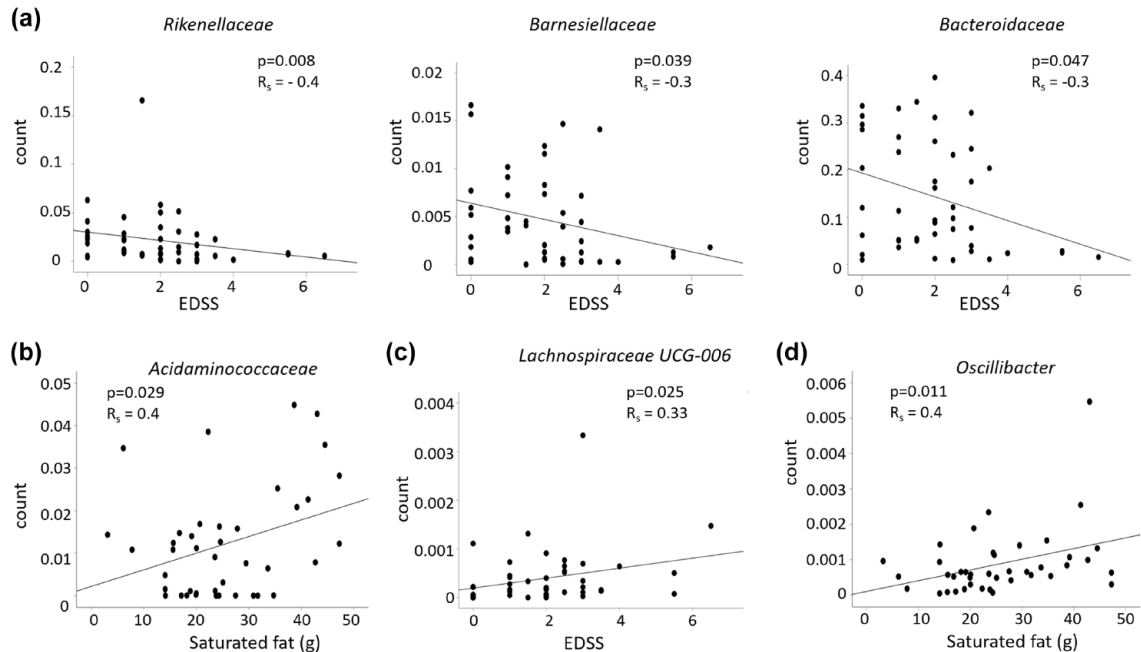


**Figure 6.** Representative figures of taxa with differential relative abundance between patients with IAE and patients without IAE (at baseline). Only taxa significant by three methods are presented (DeSeq2, Linear regression, and LefSe).

LefSe, linear discriminant analysis effect size; FDR, false discovery rate; IAE, intolerable adverse events causing discontinuation of DMF therapy.

various microbes, suggestive of dysbiosis of the microbiota in MS. However, due to the complexity and many confounding factors affecting the microbiota, such as diet, gender, age, medications, disease type and state, comorbidities, geographic location, population, and lifestyle, the overlap between reports is limited, and a worldwide MS-microbiome signature is unavailable. MS drugs may have antimicrobial effects, and their efficacy and tolerability could in part be related to their effects on the microbiota.<sup>25</sup> Studies, mostly cross-sectional, comparing DMT-treated to untreated PwMS, found differences in the abundance of specific microbes, while the general microbiota composition, as determined by  $\alpha$ - or  $\beta$ -diversity, seems not to be affected by treatment,<sup>7,9,13,15,16,25–27,41–44</sup> similarly to our results. One could speculate that the significant difference in  $\beta$ -diversity found between HC and PwMS post DMF-therapy presents both DMF-induced and disease-related differences, while the trend difference between PwMS pre- and post DMF, presents only DMF-induced differences.

There are yet only a few publications on how a specific MS-DMT affects the microbiota, mostly cross-sectional, and with the many confounding factors that may affect the microbiota of an individual, these studies have a high risk of bias, compared to longitudinal studies, making comparison and validation across studies difficult. A recent large study by the iMSMS consortium found that microbial differences detected between untreated PwMS and their healthy house-hold members, were not replicated in treated PwMS, supporting that DMTs alter the microbiota composition.<sup>25</sup> Moreover, the authors found that several taxa altered by DMTs, did not differ between PwMS and HC, similarly to our results. This indicates that DMT-induced alterations do not necessary contribute to restore a healthy microbiota. Whether DMT-induced microbiota alterations are involved in the clinical response to treatment or drug tolerability is a pertinent question. Alterations could be favorable, promoting microbes that induce anti-inflammatory immune cells or intermediates, or could contribute to DMT-associated AEs, which may affect drug



**Figure 7.** Correlations between EDSS or nutrients and abundance of differentially abundant taxa. (a) Differential taxa post 6-month DMF therapy compared to pre-treatment. (b and c) Differential taxa between PwNDA and PwDA. (d) Differential taxa between patients with or without IAE. Spearman correlation were calculated by SPSS on TSS normalized data. DMF, dimethyl fumarate; EDSS, Expanded Disability Status Scale; IAE, intolerable adverse events causing discontinuation of DMF therapy; PwDA, patients with evidence of disease activity during 1-year follow-up from DMF initiation; PwNDA, patients with no evidence of disease activity during 1-year follow-up from DMF initiation;  $R_s$ , Spearman correlation coefficient; TSS, total sum scaling.

adherence. We addressed the effect of 6-month DMF therapy on the microbiota in PwMS in the present study, and investigated associations between microbiota, diet, treatment response, and AEs.

The function of the intestinal barrier, containing the gut mucosal immune system, is important for homeostasis and prevention of diseases, impeding penetrance of microbes and antigenic molecules from the gut.<sup>45</sup> Altered intestinal permeability has been demonstrated in PwMS.<sup>46</sup> Altered intestinal permeability was associated with disease severity in experimental autoimmune encephalomyelitis, and treatment with probiotics or administration of SCFAs improved barrier function, reduced inflammatory immune cells, and promoted anti-inflammatory cytokines.<sup>45,47</sup> The gut barrier contains inter-epithelial tight junction molecules, such as occludins, claudins, junctional adhesion molecules, and zona occludens-1, as well as mucus, fluid, and innate and adaptive immune cells.<sup>45</sup> DMF was shown in vitro to increase the

expression of zona occludens-1 in Experimental Colitis,<sup>48</sup> and to decrease intestinal permeability by strengthening tight junctions, through elevation of SCFA-producing bacteria in a mice model<sup>49</sup>; thus, effects on gut permeability and microbiota could be part of the mechanism of action of DMF. Six recent studies have addressed the effect of DMF therapy on the microbiota composition in MS patients, of these three cross-sectional studies<sup>13,24,25</sup> and three longitudinal studies,<sup>16,26,27</sup> summarized in Table 7. Of these, Diebold et al.<sup>27</sup> also addressed whether DMF-associated changes in the microbiota were associated with lymphopenia, two studies addressed whether such changes were associated with flushing and GI AEs,<sup>16,26</sup> while none have yet addressed associations with clinical response. We found in the present study 36 microbes at different taxonomic levels, with altered relative abundance after 6 months of DMF therapy compared to pre-treatment. We also identified several microbes, whose abundance differed according to evidence of disease activity following 1-year DMF therapy.



**Table 6.** Enriched pathways from differential predicted functional profiles (KOs).

| (a) Enriched Pathways from KOs, differing post 6-month DMF therapy compared to pre-treatment |       |          |      |        | (b) Enriched Pathways from KOs, differing between PwNDA and PwDA |       |          |      |         |
|--|-------|----------|------|--------|--|-------|----------|------|---------|
| Pathway  | Total | Expected | Hits | FDR    | Pathway  | Total | Expected | Hits | p Value |
| Citrate cycle (TCA cycle)  | 54    | 4.78     | 16   | 0.001  | Drug metabolism-cytochrome P450                                  | 7     | 0.002    | 1    | 0.002   |
| LPS biosynthesis   | 44    | 3.9      | 14   | 0.001  | Retinol metabolism   | 9     | 0.003    | 1    | 0.003   |
| Carbon fixation pathways in prokaryotes  | 97    | 8.59     | 22   | 0.0012 | Metabolism of xenobiotics by cytochrome P450                     | 9     | 0.003    | 1    | 0.003   |
| Oxidative phosphorylation  | 112   | 9.92     | 22   | 0.009  |  |       |          |      |         |
| Propanoate metabolism  | 82    | 7.26     | 17   | 0.019  |  |       |          |      |         |
| Biotin metabolism  | 22    | 1.95     | 7    | 0.0496 |  |       |          |      |         |
| Streptomycin biosynthesis  | 17    | 1.51     | 6    | 0.0496 |  |       |          |      |         |
| Glyoxylate and dicarboxylate metabolism  | 85    | 7.53     | 16   | 0.0496 |  |       |          |      |         |

Enriched pathways mapped according to predicted functional profiles (KOs) that differ (a)—post 6-month DMF therapy compared to pre-treatment at FDR < 0.05, or (b)—in PwNDA compared to PwDA, at  $p < 0.05$ .  
DMF, dimethyl fumarate; FDR, false discovery rate; KO, Encyclopedia of Genes and Genomes orthologs (KEGG) orthology; LPS, lipopolysaccharide; PwDA, PwMS with evidence of disease activity during 1-year follow-up from DMF initiation; PwMS, patients with multiple sclerosis; PwNDA, PwMS with no evidence of disease activity during 1-year follow-up from DMF initiation; TCA, tricarboxylic acid.

Moreover, we identified microbes that differed pre-treatment between PwMS who eventually discontinued DMF therapy due to IAE compared to those who continued therapy, despite AEs (without IAE).

One of the beneficial roles of the gut microbiota is the production of SCFAs, mainly butyrate, acetate, and propionate, from digestion of dietary fibers. SCFAs enhance the intestinal barrier integrity, mucus production, protect against gut inflammation, maintain intestinal homeostasis and function, and are also important for immunomodulation, blood brain barrier integrity, and neuroimmune homeostasis.<sup>2,45,50</sup> Butyrate is a highly important SCFA, produced mainly by members of the phylum Firmicutes, such as the families *Lachnospiraceae*, *Lactobacillaceae*, and *Ruminococcaceae*, and serve a role in energy production, gut barrier integrity, and promotion of anti-inflammatory cytokines. In this study, DMF therapy increased the relative abundance of Firmicutes, the order Clostridiales, mainly in members of the *Lachnospiraceae* family, but also of

the *Ruminococcaceae* family, while reduced the abundance of Bacteroidetes, an effect that could promote a beneficial increase in SCFA production. This is supported by the observation of higher abundance of Firmicutes, while lower abundance of Bacteroidetes in PwNDA, compared to PwDA. An increased abundance of Firmicutes and lower abundance of Bacteroidetes following DMF therapy was also reported after 12-weeks DMF treatment in another longitudinal study,<sup>16</sup> while the opposite was reported in a cross-sectional study.<sup>15</sup> Possible explanations for this contradiction include the cross-sectional versus longitudinal study design, technical differences, and variations related to different populations, which we have previously shown affect the microbiota.<sup>12</sup> The detected increased abundance of the *Lachnospiraceae* family and genera within the family following DMF therapy, included an increase in *Blautia*, a main SCFA producer associated with a healthy microbiota, and involved in controlling gut inflammation.<sup>44,51</sup> *Anaerostipes*, another increased genus of this family, was also reported increased after 1-month DMF therapy.<sup>26</sup> Increased

**Table 7.** Summary of studies on effects of DMF therapy on the microbiome.

| Study  | Study design    | Cohort                           | Major findings   |
|--|-----------------|----------------------------------|--|
| Katz Sand et al. (2018) <sup>15</sup>        | Cross-sectional | 75 untreated<br>33 DMF           | No effect on $\alpha$ or $\beta$ diversity<br>Increased: Bacteroidetes, Bacteroidales<br>Reduced: Clostridiales, <i>Lachnospiraceae</i> , <i>Veillonellaceae</i> , Firmicutes, Fusubacteria, Cyanobacteria, Fusobacteriales, Actinomycetales, Campylobacteriales, 9 families, 13 genera.   |
| Storm-Larsen et al. (2019) <sup>16</sup>     | Longitudinal    | 21 DMF<br>(baseline, 2, 12w)     | No effect on $\alpha$ diversity<br>2 weeks: reduction in Actinobacteria, <i>Bifidobacterium</i><br>12 weeks: increase in Firmicutes, <i>Faecalibacterium</i> , and reduction in Bacteroidetes<br>Association with GI: GI-patients had lower <i>Bacteroides</i> (baseline), <i>Dialister</i> (2w)<br>Patients with GI: higher intake of ethanol, proteins (trend), lower intake of carbohydrates. Positive correlation between fiber intake and abundance of Firmicutes and <i>Faecalibacterium</i> .   |
| Cox et al. (2021) <sup>13</sup>              | Cross-sectional | 45 untreated,<br>34 DMF          | No effect on $\beta$ diversity<br>Increased: <i>Roseburia intestinalis</i> .   |
| Diebold et al. (2022) <sup>27</sup>          | Longitudinal    | 20 DMF,<br>(baseline, 12m)       | No effect on $\alpha$ diversity<br>Reduced: <i>Coprococcus eutactus</i> , <i>Enterococcus gilvus</i><br>Increased: <i>Lactobacillus pentosus</i><br>Association with Lymphopenia: presence of <i>Akkermansia muciniphila</i> and absence of <i>Prevotella copri</i> .  |
| Zhou (2022) (iMSMS consortium) <sup>25</sup> | Cross-sectional | 209 untreated<br>86 DMF          | No effect on $\beta$ diversity<br>Reduced: <i>Ruminococcus</i> sp. CAG:177, <i>Coprobacillus</i> sp. D6.   |
| Ferri et al. (2023) <sup>26</sup>            | Longitudinal    | 19 DMF,<br>(baseline, 1, 3, 6 m) | No effect on $\alpha$ diversity<br>Increased: <i>Anaerostipes</i> (1 m), <i>Ruminococcaceae</i> UCG002 (3 m)<br>Reduced: Proteobacteria (1 m), <i>Clostridiaceae</i> , <i>Clostridium sensu stricto</i> 1, <i>Ruminococcaceae</i> NK4A214 group (3 m).<br>Association with GI: increase in <i>Streptococcaceae</i> (1 m), <i>Streptococcus</i> (1 m), <i>Clostridiaceae</i> (3 m), <i>C. sensu stricto</i> 1 (3 m), <i>Blautia</i> (3 m), <i>Subdoligranulum</i> (1 m), <i>Tenericutes</i> (1 m)<br>Reduction in: <i>Bacteroidaceae</i> (1 m), <i>Bacteroides</i> (1 m), <i>Barnesiellaceae</i> (3 m), <i>Barnesiella</i> (3 m), <i>Odoribacter</i> (3 m), Proteobacteria (6 m), <i>Acidaminococcaceae</i> (6 m), <i>Burkholderiaceae</i> (6 m). |
| DMF, dimethyl fumarate; m, months; w, weeks. |                 |                                  |  |

levels of various *Lachnospiraceae* taxa have been reported in several diseases, such as major depressive disorder, Diabetes Type 1 and Metabolic syndrome, while lower levels were found in persons with Crohn's disease or ulcerative colitis,<sup>51</sup> thus emphasizing the complexity of determining disease-associated microbiota, and specific genera or species within a family may serve opposite roles. The *Ruminococcaceae* family, increased by DMF therapy, was highest pre-treatment in PwNDA, and so were members of this family such as *Faecalibacterium*, *Butyricicoccus*, and *Ruminiclostridium*. *Faecalibacterium* was previously

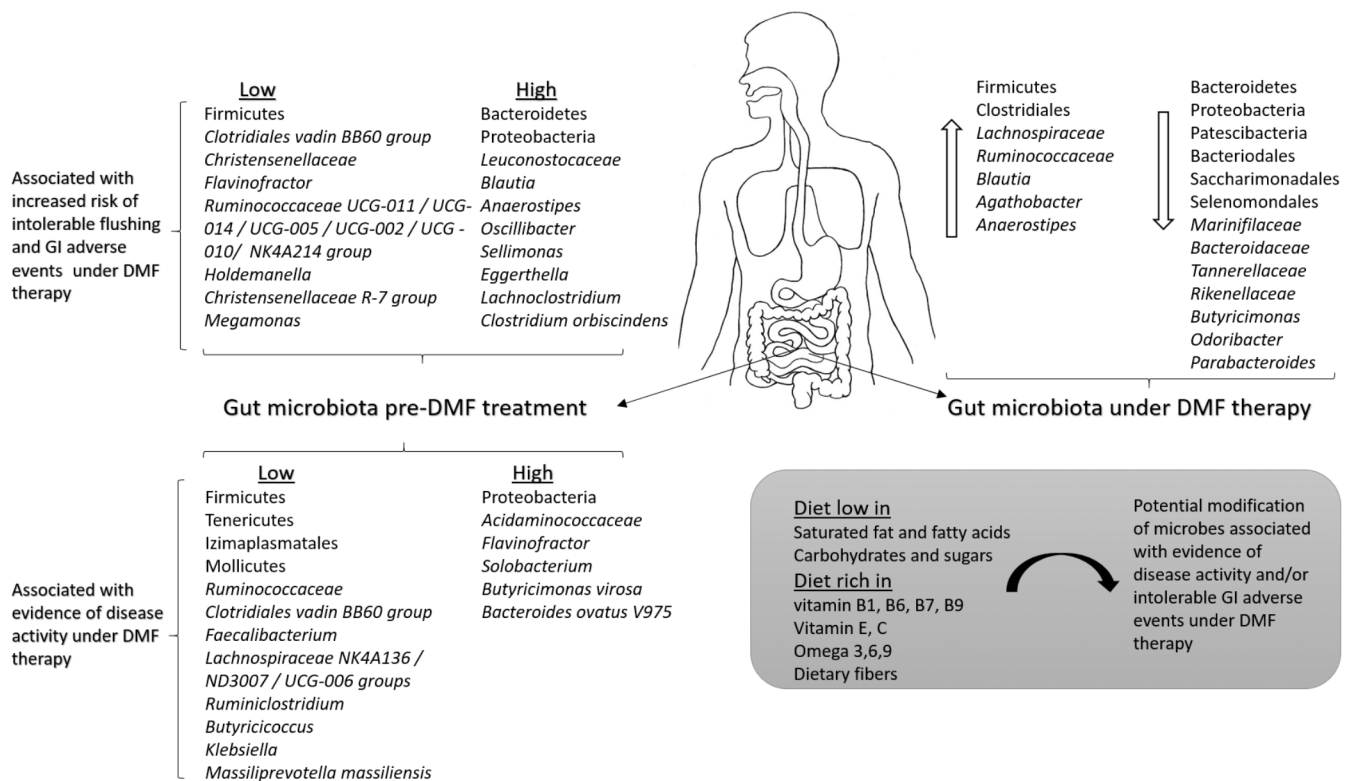
reported to increase after 3-month DMF therapy,<sup>16</sup> and *Butyricicoccus* was reported to be less abundant in PwMS compared to healthy persons,<sup>52</sup> similarly to the current study. The reduction of Proteobacteria following 6-month DMF therapy could also be clinically beneficial, since the abundance of this microbe pre-treatment was highest in PwDA and in patients with IAE. A similar reduction in Proteobacteria after 1 and 3 months of DMF therapy was recently reported.<sup>26</sup> Proteobacteria include several known human pathogens, and dysbiosis of this phylum has been associated with promotion of pro-inflammation

and inflammatory diseases, such as metabolic disorders and IBD,<sup>53,54</sup> and is elevated in PwMS compared to healthy persons.<sup>55</sup> The genus *Prevotella* was reported in several studies to be lower in PwMS compared to healthy persons, including our previous and present study,<sup>6,10,12,56</sup> and was further reduced here by DMF therapy, while not affected by DMF in a cross-sectional study.<sup>15</sup> Increased abundance of specific *Prevotella* species were reported in DMT-treated patients, such as *Prevotella copri* in Interferon- $\beta$ -treated patients,<sup>57</sup> and *Prevotella stercorea* in interferon- $\beta$  or teriflunomide-treated versus untreated patients.<sup>41</sup> Due to taxonomic resolution limitations of 16S sequencing used in the present study,<sup>58</sup> DMF-induced alterations at the species level may have been undetected.

Whether and how the microbiota is related to disease activity or to the clinical response to treatment is yet unknown. Differential microbiota composition was reported in patients with active versus remissive MS,<sup>6</sup> and we have previously found several specific microbes to correlate with disability (EDSS).<sup>12</sup> In pediatric MS, absence of *Fusobacteria*<sup>59</sup> and lower abundance of microbes involved in tryptophan metabolism<sup>60</sup> were associated with increased relapse risk. Moreover, higher abundance of *Butyrivibrio*, *Lachnospiraceae* NKA136 groups, *Ruminococcaceae*, and *Odoribacter* were associated with lower risk, while abundance of a member of Coriobacteriales was associated with higher risk of disease activity in pediatric MS.<sup>61</sup> Similarly, we found higher abundance of *Butyrivibrio*, *Lachnospiraceae* NKA136 groups, and *Ruminococcaceae* in pre-treatment samples of PwNDA. Moreover, the abundance of several genera from the *Ruminococcaceae* or *Lachnospiraceae* families (Firmicutes phylum), as well as of two orders from the Mollicutes class (Tenericutes phylum) was higher in PwNDA, while abundance of two species from the Bacteroidales order was higher in PwDA. Tenericutes was suggested to be associated with negative effects on inflammatory cytokines and protective in some autoimmune diseases,<sup>62</sup> and was elevated by probiotics in a murine model of MS.<sup>63</sup> *Solobacterium*, more abundant in pre-treatment samples of PwDA, has previously been associated with IBD, inflammation, and colorectal cancer.<sup>64</sup> *Massiliprevotella massiliensis*, a *Prevotella* species, was highest in PwNDA, supporting a beneficial abundance, as previously suggested for the *Prevotella* genus.<sup>6,10,12,56</sup> Whether

any of these microbes may predict disease activity or clinical response to treatment requires further validation in large studies. Moreover, the use of complementary assays for assessment of disease activity such as the MS Functional Composite could improve the detection of PwDA and the ability to address also the type and severity of activity. We also found microbes differentiating according to disease activity in samples obtained post 6-month DMF therapy. However, the observed alterations could be a consequence of lowered disease activity following treatment, and not present causality, since these microbes did not differ pre-treatment.

With the relatively common incidence of flushing and GI AEs after DMF initiation, which mostly decreases over time but, in some cases, can lead to drug discontinuation, it is pertinent to study whether specific microbes are associated with higher risk of such IAE. Two publications addressed this question assessing samples either pre-treatment or following DMF therapy.<sup>16,26</sup> In this study, the analysis focused on pre-treatment samples from patients with AEs of a severity that caused discontinuation of DMF therapy. Other PwMS most likely may also have experienced flushing or GI AEs after DMF initiation, either at less severity, faster resolved, or persons may have had higher coping capability. We found that PwMS developing IAE had higher abundance of Bacteroidetes and Bacteroidales pre-treatment, in contrast to a former report.<sup>16</sup> Firmicutes and several genera from the *Ruminococcaceae* family were less abundant in IAE patients, while some genera belonging to the *Lachnospiraceae* family, such as *Lachnoclostridium*, *Anaerostipes*, and *Blautia*, as well as the *Leuconostocaceae* family were more abundant in IAE patients. Lower levels of *Ruminococcaceae* have been reported in people who develop antibiotic-associated diarrhea,<sup>65</sup> indicative that low abundance of members of this family may increase the likelihood of GI symptoms. *Blautia* was transiently increased in MS patients with GI symptoms after 3 months of DMF therapy,<sup>26</sup> and correlated positively with GI symptoms and diarrhea in the general population,<sup>66</sup> thus supporting our results. The genus *Eggerthella*, more abundant in IAE patients, was found to correlate positively with GI symptoms in children with autism spectrum disorder.<sup>67</sup> The genus *Megamonas*, less abundant in IAE patients, was found to be lower in patients with GI symptoms in people undergoing bariatric surgery.<sup>68</sup> The genus *Holdemanella*, less abundant in



**Figure 8.** Graphical summary: Interactions between DMF immunomodulation therapy and the gut microbiota in PwMS—associations with clinical response, drug tolerability, and diet. Graphical figure presenting a summary of results presented in this study. Source: Figure adopted from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. <https://www.niddk.nih.gov/news/media-library/17466>.

IAE patients, has been associated with a healthy state,<sup>69</sup> and the *Holdemanella biformis* species was reported to correlate negatively with symptoms of IBD.<sup>70</sup>

Adherence to a Mediterranean diet did not differ between groups according to disease activity or IAE. The positive correlation between MDS and *Eggerthella* or *C. orbiscindens*, both higher in IAE patients, and the negative correlations between MDS and *Megamonas* or *Succinivibrio*, both lower in IAE patients, do not support that a Mediterranean diet may prevent IAE through alteration of these microbes. However, the *Lachnospiraceae* family, elevated by DMF therapy, correlated positively with MDS, supporting that a Mediterranean diet increases beneficial SCFA producers<sup>71</sup> such as *Lachnospiraceae*.

Several statistical correlations were found between microbes with differential abundance according to disease activity or IAE and the intake of various nutrients, indicating that diet and dietary

recommendations could carry the potential to affect the clinical response or tolerance to DMF, through modification of microbe abundance. Interestingly, *Acidaminococcaceae*, enhanced in PwDA, and *Oscillibacter*, highest in patients with IAE, were the only identified differential microbes that correlated positively with saturated fat and saturated fatty acids. Dietary fibers, fructose, and Vitamin C correlated positively with Bacteroidetes, more abundant in patients with IAE, while correlated negatively with Firmicutes, lower in PwDA and IAE patients. While a high-dietary fiber diet intervention reduced the Firmicutes/Bacteroidetes ratio in persons with rheumatoid arthritis,<sup>72</sup> other studies found that dietary fibers increased the abundance of Firmicutes,<sup>73</sup> and such opposite effects appear to be caused by composition differences between various types of resistant starch in the dietary fiber, such as RS2, RS3, and RS4.<sup>74</sup> Carbohydrates and several unsaturated fatty acids correlated positively with *Eggerthella*, highest in patients with IAE, but also with *Flavonifractor*, lower in patients with IAE,



thus not promoting dietary recommendations. Moreover, intake of several types of Vitamin B (Biotin, Thiamine, Folate, and B6) correlated positively with some microbes that showed higher abundance among patients without IAE, suggesting increased intake might potentially be beneficial in preventing IAE. Further investigation in larger cohorts are necessary to conclude on associations with diet and specific dietary recommendations; however, our observations propose that dietary interventions could have an intriguing potential to affect DMF response and tolerability, through modifications of abundance of specific microbes. Low Vitamin D levels have been associated with increased risk of disease activity in MS.<sup>75</sup> While still debatable whether Vitamin D supplement alters the microbiota,<sup>7,76</sup> we only found a few associations between baseline Vitamin D levels and abundance of microbes modified under DMF therapy, while none with microbes that showed differential proportion according to disease activity or IAE.

The top enriched pathway of predicted functional profiles identified following 6-month DMF therapy was the Citrate cycle (TCA cycle). Predicted enrichment of TCA cycle intermediates following 3-month DMF therapy has also been reported elsewhere.<sup>27</sup> The active metabolite of DMF, MMF, is hydrolyzed within cells to fumaric acid, which is further metabolized through the TCA cycle, and DMF-treated patients have increased blood levels of TCA intermediates and metabolites, such as Succinate, Fumarate, and Malate.<sup>77</sup> Another enriched pathway of predicted functional profiles after DMF therapy was oxidative phosphorylation, interestingly, since DMF has been shown to affect oxidative phosphorylation and mitochondrial function.<sup>78,79</sup> Biotin metabolism pathway was also enriched under DMF therapy. Biotin is a co-factor for four essential carboxylases expressed in oligodendrocytes and neurons, and supplement has therefore been suggested as a therapeutic strategy, although high-dose Biotin supplement in progressive PwMS was unsuccessful.<sup>80</sup> Additional longitudinal replication followed by experimental studies are necessary to assess biological relevance of these predicted functional findings.

A summary of the findings of this study (graphically presented in Figure 8) suggests that a relatively high abundance among PwMS initiating DMF therapy of: Firmicutes (highest in PwNDA, in patients without IAE, and increased by DMF

therapy), *Ruminococcaceae* (highest in PwNDA and increased by DMF therapy), *C. vadin BB60 group* (highest in PwNDA and in patients without IAE), *Megamonas*, and *Catenibacterium* (highest in patients without IAE), is favorable for tolerability and clinical response. In contrast, high abundance of Proteobacteria (highest in PwDA, in patients with IAE, and decreased by DMF therapy), Bacteroidetes, and Bacteroidales (highest in patients with IAE and decreased by DMF therapy), as well as *Leuconostocaceae* (highest in patients with IAE and reduced by DMF therapy), may be unfavorable. The overlap between current publications on microbes altered by DMTs is small. However, there are accumulating indications that treated patients have a beneficial increase in SCFAs-producing microbes, such as members of *Lachnospiraceae* or *Ruminococcaceae*, as shown here following DMF therapy. The functional and clinical impact of therapeutic-induced changes in the abundance of specific microbe communities is for now unclear. Furthermore, while alterations of certain microbes under treatment of a specific DMT may be caused by the mechanism of action of the drug, alterations of microbes observed under the regime of different DMTs may reflect general improvement in disease activity, gut barrier function, and inflammatory state, due to immunomodulatory therapy. The therapeutic potential of these observations raises the question of whether targeting specific microbes can mimic or improve immunomodulatory effects of a DMT, and whether complementary treatment targeting the microbiota, may improve the clinical outcome for the patient, as well as drug tolerability.

The strength of the current study is the longitudinal study design addressing effects of one specific DMT and the inclusion of dietary data and clinical follow-up. The limitations include first the inclusion of both 1st line and 2nd line PwMS in the cohort (although a subanalysis showed this to have minimal effects on results), and, second, the limited sample size, especially of patients with IAE or PwDA. A third limitation is the self-report of dietary data, and the lack of scaling AE levels in patients. The taxonomic resolution limitations of 16S sequencing for identification of microbes below the genera level<sup>58</sup> is another limitation. Lastly, like most available publications in the domain of microbiome and autoimmunity, this study focused upon identifying disease-associated or therapy-induced compositionally effects on the microbiome, while the functional effects and



biological relevance of such associations remain to be evaluated. Following further replication and establishment of robust findings across studies, future studies should proceed to functional experiments, targeting specific microbial communities to evaluate the causative effect on disease activity, drug response, or tolerability.

### Conclusion

This study further clarifies the immunomodulatory role of DMTs, specifically DMF, on the gut microbiota composition, and the potential contribution of microbiota on clinical outcome and drug tolerability. Future goal would be to develop a microbiome-based, personalized treatment approach, integrating pharmacotherapy with dietary components, in synergy, toward improved clinical outcome.

### Declarations

#### *Ethics approval and consent to participate*

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Carmel Medical Center (0034-13-CMC, 26/12/2013). Consent to participate: Written informed consent was obtained from all participants.

#### *Consent for publication*

Not applicable.

#### *Author contributions*

**Elsebeth Staun-Ram:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Visualization; Writing – original draft; Writing – review & editing.

**Anat Volkowich:** Data curation; Investigation; Project administration; Writing – review & editing.

**Ariel Miller:** Conceptualization; Funding acquisition; Investigation; Methodology; Writing – review & editing.

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#### *Competing interests*

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#### *Availability of data and materials*

All relevant data are available within the article or Supplemental Material, or will be made available from the corresponding author upon request from any qualified investigator, under the comply with Institutional Ethical Review Board requirements.

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#### **Supplemental material**

Supplemental material for this article is available online.

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