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# Deconfounded, quantitative microbiome profiling identifies robust multiple sclerosis markers and clinical covariate associations

Ayla Pauwels[a,b,1], Lindsay Devolder[c,d,1], Gwen Falony[c,d,e], Miguel D'haeseleer[a,b,f], Guy Nagels[b,g,h], Ann Van Remoortel[f], Muriel Derrien[c,d], Marie D'hooghe[a,f,2] and Jeroen Raes[c,d,2]

[a]Neuroprotection and Neuromodulation (NEUR) Research Group, Center for Neurosciences (C4N), Vrije Universiteit Brussel, Brussels, Belgium; [b]Department of Neurology, Universitair Ziekenhuis Brussel (UZ Brussel), Brussels, Belgium; [c]Department of Microbiology, Laboratory of Molecular Bacteriology, Immunology and Transplantation, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium; [d]Center for Microbiology, Laboratory of Bioinformatics and (Eco-)Systems Biology, VIB, Leuven, Belgium; [e]Host-Microbe Interactomics, Wageningen University & Research, Wageningen, Netherlands; [f]Department of Neurology, National Multiple Sclerosis Center, Melsbroek, Belgium; [g]St. Edmund Hall, University of Oxford, Oxford, United Kingdom; [h]AIMS Lab, Center for Neurosciences (C4N), Vrije Universiteit Brussel, Brussels, Belgium

**ABSTRACT**

Despite a wealth of gut microbiota studies in multiple sclerosis (MS), consistent results are lacking. Here, we study confounder effects and use of quantitative microbiome profiling (QMP) in 228 MS patients (103 untreated) and 2860 population controls (Flemish Gut Flora Project (FGFP)). Total bacterial load was lower in relapsing remitting (RR)MS, while strong fecal moisture effects, indicative of longer transit times, in MS vs. FGFP, were driven by primary progressive (PP)MS. Applying cell count and moisture in deconfounded QMP, we identified 21 differentially abundant genera in MS, with a.o. *Lachnobacterium*, *Blautia* enriched, and *Clostridium*, *Bacteroides* depleted. Deconfounded QMP across 10 published studies (1065 patients, 874 controls) did not confirm commonly detected markers (*Akkermansia, Roseburia*), yet lowered *Bacteroides*, and higher *Blautia* and *Methanobrevibacter* emerged as robust MS biomarkers. Lowered butyrate producers (*Butyricicoccus, Butyricimonas*) merit further investigation. Enterotype stratification linked the low cell count *Bacteroides 2* enterotype to low-efficacy DMTs, and the *Prevotella* enterotype to lower disease severity. Serum glial fibrillary acidic protein (GFAP), a disease progression biomarker, was identified as a covariate of gut microbial variation and inversely correlated with *Faecalibacterium* and *Roseburia* abundance in PPMS. Overall, our study provides robust disease markers and emphasizes the importance of QMP and confounder control.

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## Introduction

Multiple sclerosis (MS), a chronic, inflammatory, and degenerative disease affecting the central nervous system, is a leading cause of disability in young adults.[1] Most individuals with MS are diagnosed with a relapsing-remitting form (RRMS), which is characterized by successive periods of relapse and remission. This can evolve into secondary progressive (SP) MS, featuring a steady decline in the relative absence of relapses.[2] About 10–15% of patients experience progression from onset, called primary progressive MS (PPMS). Current phenotyping does not fully capture the clinical heterogeneity in MS, with progression occurring along a continuum from RRMS to SPMS. The disease course and treatment response are largely unpredictable.[3] There is no established prognostic or predictive biomarker for MS, however, candidate

**CONTACT** Jeroen Raes jeroen.raes@kuleuven.be Department of Microbiology, Laboratory of Molecular Bacteriology, Immunology and Transplantation, Rega Institute for Medical Research, KU Leuven, Herestraat 49, 3000,Leuven, Belgium; Marie B. D'hooghe marie.dhooghe@mscenter.be National Multiple Sclerosis Center, Vanheylenstraat 16, 1820, Melsbroek, Steenokkerzeel, Belgium
[1] Shared first authorship.
[2] Shared last authorship.
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blood biomarkers such as neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) have been associated with focal inflammatory activity and disability progression respectively.[4,5] Available disease-modifying therapies (DMTs) target various aspects of the immune system, resulting in reduced relapses and relapse-associated worsening, but without effectively halting progression.[6]

The risk of acquiring MS is assumed to be driven by an interaction between genetic (with over 200 loci associated with MS susceptibility[7]) and environmental factors (e.g., Epstein–Barr Virus, low vitamin D levels, smoking, and obesity).[1] Next to these, the gut microbiome has emerged as a potential contributor to MS risk and progression,[8,9] which was substantiated by animal and human studies.[10] Microbial markers of diagnosis, prognosis, and target of modulation have been gaining increasing interest.[11]

Previous studies investigating the gut microbiota in people with MS consistently report an alteration in gut microbiota. However, the exact MS gut microbiome signature remains to be elucidated,[10] and within-MS microbiome variation further complicates this issue. The importance of appropriate covariate (i.e., associated with the gut microbiota), and confounder (i.e., associated with both the disease of interest and the gut microbiota) control in microbiome (disease) association studies has been stressed in large population-based studies.[12-14] Consequently, while a limited number of covariates (and possible confounders) was considered in early, relatively small scaled (<50 patients)[10] studies, more recent ones started to integrate demographic variables.[15-21] However, most MS studies have not yet integrated microbiome-specific covariates. Intestinal transit time, for example, a critical covariate of gut microbiome composition,[14,22] is still mostly overlooked. Self-reported measures for transit time (i.e., Bristol Stool Scale; BSS) were used by a few studies in pediatric,[23-25] and adult MS patients.[26] Fecal water content (moisture) - another proxy for gut transit time - was employed in only two of the many studies in adult MS.[16,20]

Moreover, despite the advent of quantitative methods, nearly all published MS gut microbiome studies have used relative microbiome profiling (RMP).[27] With great variation in microbial loads between individuals, and potentially even greater variation in the context of disease, RMP can lead to erroneous conclusions.[28] Transformation to quantitative microbiome profiling (QMP) is deemed more appropriate for identifying biologically relevant intergroup differences and associations with clinical data, resulting in lower false-positive and false-negative findings when investigating microbiota-disease associations, and more accurate target identification.[28-30] Finally, low overall microbial cell count can (partially) constitute the microbial signature of disease, like is the case for inflammatory bowel disease (IBD).[28]

In the current study, we performed and compared relative and quantitative microbiome analysis in a clinically well-characterized MS cohort, exploring variation of the gut microbiome within MS and compared with control subjects, while adjusting for microbiome confounding factors, including transit time. We mapped our findings in relation to previous cross-sectional MS microbiome studies and assessed the robustness of previously proposed markers, identifying underlying causes of inconsistencies between studies. We also provide novel insights into gut microbiome associations with clinical MS measures (disability, anxiety, depression and fatigue), and two serum biomarkers of MS, neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP), which have not yet before been associated with gut microbiota metrics.

## Methods

### Study design

#### Current study

We performed a cross-sectional analysis on clinical, blood and microbiome data from patients with relapsing-remitting (RR) MS, primary progressive (PP) MS and secondary progressive (SP) MS, diagnosed according to the 2017 revised McDonald criteria,[31] recruited between December 2018 and January 2021 in the University Hospital Brussels (UZB), Belgium, and the National MS Center (NMSC), Melsbroek, Belgium. Adult individuals with MS could participate if they had no inflammatory bowel disease (IBD) or other chronic gastro-intestinal disease and had not used oral antibiotics three months prior to fecal sample collection. We compared with data from the Flemish Gut Flora Project (FGFP), a large-scale microbiome-focused population cohort from Flanders, Belgium (expanded dataset from.[14,32,33]) The FGFP cohort used in the present study is an expanded version of the first and second round of sampling.[14,33] For

the current study, subjects with MS were excluded from the FGFP dataset, resulting in a control population of 2860 individuals expected to reflect the general population.

### Comparative analysis with previously published studies

Subsequently, we performed a comparative analysis of MS microbiota markers between previously published studies. We summarized the data from all studies reporting on gut microbial differences between adult MS patients ($N > 50$) and controls, based on the following PubMed search: ((multiple sclerosis) AND ("gut" OR "intestinal" OR "gastrointestinal")) AND ("microbiome" OR "microbiota" OR "commensal"), last performed on the 5th of August 2025. We compiled a list of 114 genera ($n = 119$ when complemented with unique taxa identified in the current study), stemming from 10 case-control studies that consisted of at least 50 adult patients with MS. The total number of participants in these studies was 1524 MS subjects, 1266 control subjects. Taxon data were available for 1065 patients and 874 controls (Supplementary Table 1), which we used to visualize compositional differences across studies. Included studies represented different geographic locations in Europe,[16,19-21,34] Asia,[17,35,36] and North America.[15,18,19,37]

### Ethical compliance

Study procedures are compliant with all relevant ethical regulations and align with the Declaration of Helsinki and Belgian privacy laws. The study protocol was approved by the medical ethics committee of the UZB-Brussels University Hospital (approval number B.U.N. 143201836152) and by the local ethics committee of the NMSC. All study participants gave their informed consent.

### General data collection in both the MS and FGFP cohort

Mutual metadata between MS and FGFP consisted of demographic variables (age, BMI, sex), stool parameters (moisture content, calprotectin levels, microbial load), and data describing quality of life (QoL) using the RAND-36 health-related quality of life survey,[38] currently the most widely used QoL questionnaire for measuring health-related quality of life perception in individuals with and without chronic disease. It is comprised of 36 items (scored from 0-100, with higher scores reflecting improved health states) that cover four mental (role limitations caused by emotional health problems; social functioning; emotional well-being; vitality) and four physical (physical functioning; role limitations caused by physical health; body pain; and general health perception) health concepts (RAND 36-Item Health Survey 1.0);[38] Supplementary Table 2).

### Clinical data collection in the MS cohort

Participants with MS were clinically evaluated during a study visit, which consisted of a clinical history taking (time of first symptoms and diagnosis, past and current relapse activity, disease-modifying treatment), followed by a neurologic exam to determine the Expanded Disability Status Scale (EDSS), a measure of overall disability.[39] Based on the EDSS and patients' age, the age-related MS severity score (ARMSS)[40] was determined. In addition, the following functional tests were performed: Nine-Hole Peg Test (9-HPT) to assess manual dexterity,[41] Timed 25-Foot Walk test (T25FW) for short distance walking speed,[42] and the Symbol Digit Modalities Test (SDMT) for information processing speed.[43] Patients were also asked to fill in an online or paper questionnaire, for a broad assessment of their general health status (physical, mental), past and current medication use, lifestyle factors, and bowel and dietary habits. Regarding the latter, participants filled in a validated 32-item semi-quantitative food frequency questionnaire (FFQ) to assess habitual intake of foods and nutrients.[44] Nutrients of the consumed food items were calculated using the Belgian Food Composition Table.[45] The Fatigue Scale for Motor and Cognitive Functions (FSMC) was used to assess fatigue, and the Hospital Anxiety and Depression scale (HADS) for anxiety and depression symptoms.[46,47] Patients also filled in the MS impact scale (MSIS-29) questionnaire, assessing the impact of MS on daily physical and psychological wellbeing.[48] Patients who provided a sample within 4 weeks after relapse onset were defined as during relapse. All other patients were free from relapse activity in the three months before sampling.

### Blood sample collection and analysis in MS cohort

Blood samples were drawn from study participants with MS during the study visit, and sent to an independent, certified clinical laboratory on the same day (*Centrum voor Medische Analyse* (CMA), Belgium), for assessment of specific parameters reflecting glucose metabolism (HbA1c), inflammation and infection (hs-CRP, number of WBCs), hemoglobin status (Hb), liver functioning (glutamate pyruvate transaminase, bilirubin), kidney functioning (glomerular filtration rate, uric acid), cardiovascular health (triglycerides, LDL-cholesterol), and vitamin D levels (Supplementary Table 2). One serum tube was centrifuged at 3500 rpm for 15 minutes at 3–5 °C. The supernatant was transferred to 1 ml cryotubes and stored at −80 °C, and used for analysis of NfL and GFAP. These analyses were performed blinded to clinical data by a board-certified laboratory technician. NfL and GFAP were determined using the Single Molecule Array (Simoa®) HD-1 platform (Simoa® NF-light™ Advantage Kit and GFAP* Discovery Kit, respectively; Quanterix, MA, USA) following the manufacturer's instructions.

### Fecal sample collection in the MS cohort

Fecal collection kits were provided to participants during a clinical visit or sent via regular mail and consisted of a silicone device with pre-formed holes into which fecal material could be transferred. Participants were asked to note down date/time of stool sampling and time since last defecation, and to rate their stool consistency with the Bristol Stool Score. Immediately after sampling, fecal samples were stored at −20 °C in the patients' home freezer. Within one week after sampling, samples were transferred to the NMSC or UZB, for storage at −80 °C. In case of inpatient care at the NMSC, samples were immediately stored at −80 °C. Samples were subsequently transported on dry ice to the Raes Lab, followed by storage at −80 °C until processing.

### Fecal sample analysis

All study samples, both from cases and FGFP controls, were analyzed following identical protocols.[33]

#### Bacterial DNA extraction and sequencing

Fecal DNA was extracted following the protocol described in.[14,49] In summary, DNA was extracted from 150–200mg of the frozen samples using MagAttract PowerMicrobiome DNA/RNA KF kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The V4 region of 16S rRNA genes was amplified using the 515F/806R primer pair and purified using the QIAquick PCR Purification Kit. Sequencing was performed using the Illumina MiSeq platform (MiSeq Reagent Kit v2, Illumina, San Diego, USA). Nucleic acid concentration was subsequently measured with DropQuant, after which the V4 region of the 16S rRNA gene was amplified with 515 F/806 R primers (GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT, respectively), modified to contain Illumina adaptors and dual-index barcodes. PCR amplicons were quantified with Fragment Analyzer (Advanced Analytical Technologies). Sequencing was performed on the Illumina MiSeq platform. As part of the extraction, library preparation, and sequencing workflow, multiple controls were implemented to ensure data quality. Samples were sequenced across several MiSeq runs, with internal negative controls included to detect potential contamination arising during DNA extraction, amplification, and sequencing. Positive controls included duplicate samples from a previous run to assess technical variation, as well as the inclusion of the nonhuman bacterium *Runella slithyformis*, which served to identify barcode crosstalk, since it is not expected in human samples. To further monitor carry-over contamination between runs, different barcode combinations were used in successive sequencing runs. Additionally, all samples—including stool samples, positive controls, and negative controls—were amplified in triplicate using unique barcode combinations, with certain combinations intentionally omitted to detect potential cross-contamination. Amplicon data from the 16S rRNA gene were analyzed following the DADA2 pipeline specifications, with taxonomic assignment of reads (agglomeration at genus-level for FGFP and both genus-level and ASV/species-level for the MS population) performed using the RDP classifier version 2.12. For ASV-level analysis, taxa of

interest that were unclassified at species-level with the RDP classifier, were run through the NCBI nucleotide blast tool to determine potential species identity.

### Relative microbiome profiling

Samples were rarefied to 10,000 reads. Enterotyping (or community typing) was performed with the Dirichlet-multinomial Model (DMM) method in R as previously described[50] on a combined genus-level abundance matrix containing the study and the complete set of Flemish Gut Flora Project (FGFP)[14] reference samples ($N = 2998$) to increase accuracy. Optimal number of clusters ($k = 4$; minimum BIC = 1216179) was determined with the Bayesian Information Criterion (BIC) with calculation of enterotype assignment probability for each sample as previously done.[51] The probability of community-type assignment was $0.97 \pm 0.077$ (mean ± standard deviation). The four identified clusters were named after their enterotype-discriminating predominant taxa,[28] which were Ruminococcaceae (Rum), *Prevotella* (Prev), and *Bacteroides* 1 and 2 (Bact1 and Bact2, respectively).

### Microbial load measurement by flow cytometry

The microbial load (i.e., cell count) of all samples was determined as described by Vandeputte et al.[28] and Vieira-Silva et al.[52]. In brief, frozen (−80 °C) stool aliquots were weighed and dissolved in physiological solution to a total volume of 100 mL (8.5 g/l NaCl; VWR International). Blank samples (1 mL in duplicate) consisted of physiological solution (8.5 g/l NaCl; VWR International). After a dilution step (10,000×), this slurry (2 mL per sample) was filtered with a sterile syringe filter (5 µm pore size; Sartorius Stedium Biotech), as was also done for blanks. One milliliter (two replicates per sample) of either the filtered microbial cell suspension or the blank sample was then stained with SYBR Green (1:100 dilution in dimethylsulfoxide (DMSO), 10,000× concentrate, Thermo Fisher Scientific) and incubated in the dark at 37 °C for 15 minutes. Flow cytometry analysis was performed in duplicate using a C6 Accuri flow cytometer (BD Biosciences) according to a previously published approach.[53] Fluorescence events were monitored using the FL1 533/30 nm and FL > 670 nm optical detectors, with additional collection of forward and sideward-scattered light. With BD Accuri CFlow software, microbial fluorescence events were gated and separated from background events on the FL1/FL3 density plot. A threshold value of 2000 was applied to the FL1 channel. To obtain an accurate microbial load per sample, gated fluorescence events were evaluated on the forward and sideward density plot, and remaining background events were excluded. Instrument and gating settings were kept identical for all samples (fixed staining/gating strategy).[53] Finally, the weight of fecal aliquots was used to transform microbial cell counts into microbial loads per gram of fecal matter.

### Quantitative microbiome profiling

Quantitative microbiome profiling (QMP) was carried out as described by Vandeputte et al.[28] and Vieira-Silva et al.[52]. In short, samples were downsized to an even sampling depth, i.e., the ratio between sampling size (sequencing depth corrected for the number of 16S rRNA gene copies per genus) and microbial load (average total cell count per gram of frozen fecal aliquot). Then, 16S rRNA gene copy numbers were extracted from the rRNA operon copy number database rrnDB).[54] The copy number-corrected sequencing depth per sample was then downsized to the level necessary to match the minimum observed sampling depth in the cohort. Samples with read counts < 150 after rarefaction were excluded from QMP analyses. Rarefied genus abundances were converted to cell count per gram to obtain a QMP matrix. QMP for amplicon sequence variants (ASV) was performed with a similar approach, as described in Tito and colleagues (REF).

### Fecal moisture content

To obtain the fecal moisture content of all samples, frozen (−80 °C), non-homogenized aliquots (0.1–0.2 g) were lyophilized, after which the percentage of lost mass during the freeze-drying process was calculated.

### Fecal calprotectin

Fecal calprotectin levels were determined in frozen (−80 °C) stool aliquots, with weights ranging between 0.075–0.1 g, using the fCAL ELISA kit (Bühlmann) according to the manufacturer's instructions. Based on

the weight of the collected aliquots, fecal calprotectin concentration was converted to µg/g (continuous variable). For categorization of calprotectin values, previously described clinical threshold of inflammation (<50 µg/g: no; 50–200 µg/g low grade; >200 µg/g: inflammation) was used.[51]

### Data analysis and statistical considerations

All analyses were performed using R Statistical Software (version 4.2.0),[55] using packages phyloseq,[56] vegan,[57] DirichletMultinomial,[58] FSA,[59] reshape2,[60] chisq.posthoc.test,[61] dplyr,[62] forcats,[63] lm.beta,[64] ppcor,[65] ggplot2,[66] ggsignif[67] and ggpubr.[68]

Given the large number of non-MS related medication (i.e., non-DMT drugs) and potential co-morbidity data variables ($N > 300$ altogether), these were downsized by the report rate (15%) in the MS study population. For non-MS related medication, commercial names were converted to Anatomical Therapeutic Chemical (ATC) Classification System ATC codes.[69]

To account for the high variability in DMT group sample sizes, DMT was categorized based on efficacy (low-efficacy [interferon-beta, glatiramer acetate, teriflunomide, azathioprine], moderate-efficacy [dimethyl fumarate, fingolimod], high-efficacy [natalizumab, ocrelizumab, alemtuzumab, rituximab, mitoxantrone, cladribine][6,70] and based on administration route (oral [teriflunomide, azathioprine, dimethyl fumarate, fingolimod, cladribine], subcutaneous [glatiramer acetate, interferon-beta], infusion [natalizumab, ocrelizumab, rituximab, alemtuzumab, mitoxantrone]. In terms of obtained microbiota profiles, taxa unclassified at genus level or present in less than 20% of samples were excluded from statistical analyses.

Group differences for continuous variables (both metadata variables and genus-level abundances) were tested with the Wilcoxon Rank-Sum test (two groups; with calculation of effect size as the ratio of the obtained Z-statistic and the square root of the number of observations) or the Kruskal–Wallis $H$ test followed by post-hoc Dunn tests (more than two groups; *dunnTest*, FSA package). For categorical variables (e.g., enterotype), (pairwise) $\chi^2$ tests were used (*chisq.test* function, stats package; *chisq.posthoc.test* function, chisq.posthoc.test package). Correlations between continuous variables (e.g., genus abundances with serum GFAP levels) were performed with non-parametric Spearman correlation tests, with partial/semi-partial correlation tests (*pcor.test* function from the ppcor package) to account for confounding effects. Beta diversity (defined as the difference in global microbiota composition across samples) was visualized through a Principal Coordinate Analysis (PCoA; genus-level Bray–Curtis dissimilarity), with the *adonis2* function (vegan) used for testing for alterations in community structure between FGFP and MS phenotypes. Other gut microbiota covariates were identified using a first distance-based redundancy analysis (dbRDA; vegan's *capscale* function) with their non-redundant contribution to community variation determined via a stepwise dbRDA approach (vegan's *ordiR2step* function).

Partialling out effects of identified confounding variables was achieved using generalized linear models (GLM, *glm* function) and subsequent log-likelihood tests. In these nested GLMs, either (sub) group or enterotype (binomial, link = logit) was used as a response variable, while detected microbiota covariates were used as explanatory variables in the null model. In the alternative model, genus abundance or MS clinical variables of interest were included as an additional explanatory variable. Standardized GLM regression coefficients were determined through the *lm.beta* function from the same-titled package.

Where necessary, multiple testing correction was applied following the Benjamini-Hochberg approach, with a false discovery rate (FDR) of <0.05 considered significant.

## Results

### Description of study cohorts

The newly recruited discovery cohort in this study included 228 patients with MS (179 RRMS, 30 PPMS, and 19 SPMS) (Figure 1A). The FGFP cohort (Flemish Gut Flora Project) included 2860 Belgian population controls.[14,32,33] The FGFP is a cross-sectional cohort from a confined geographic region (Flanders, Belgium), consisting of healthy individuals as well as participants suffering from one or multiple disorders. They underwent exhaustive phenotyping through online questionnaires and health assessments by general medical practitioners. Both the FGFP and MS cohorts were recruited in Flanders, Belgium,

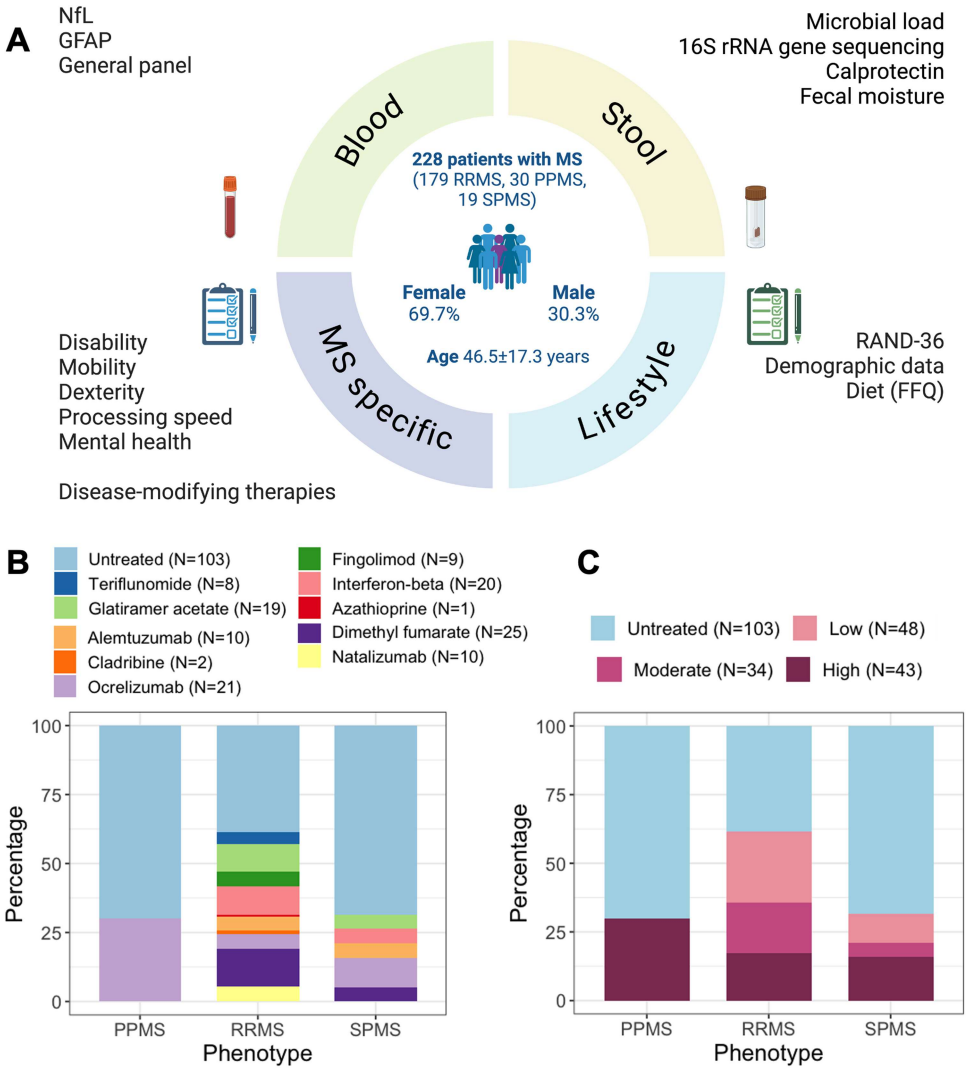


**Figure 1.** (A) MS Study cohort. Figure created with BioRender. (B) Prevalence of disease-modifying therapies (DMT) in MS cohort (C) DMT stratification according to efficacy. Low-efficacy (interferon-beta, glatiramer acetate, teriflunomide, and azathioprine), moderate-efficacy (dimethyl fumarate, fingolimod), high-efficacy (natalizumab, ocrelizumab, alemtuzumab, rituximab, mitoxantrone, and cladribine).

which minimizes biases related to regional variations in lifestyle and dietary habits, and microbiota was analyzed with a similar analytical process.

Table 1 shows clinical phenotypes and values for age, sex, BMI, and fecal moisture content, covering the most established host covariates contributing to gut microbiota composition in the general population,[14] intestinal inflammation, and microbial load. The FGFP control cohort was older than the MS cohort (Wilcoxon rank sum test, $N = 2992$, $Z = 0.11$, FDR = $1.92{\cdot}10^{-9}$). Focusing on the MS phenotypes, we found FGFP controls to be older than the RRMS patients (Dunn test, $N = 2991$, $Z = 7.94$, FDR = $1.17{\cdot}10^{-14}$), and with a lower female-to-male ratio than the RRMS patients (Chi-square test, $N = 2990$, $\chi^2 = 15.50$, FDR = 0.003). The PPMS and SPMS patients were older than the RRMS patients (Dunn test, $N = 209$, $Z = 4.63$, FDR = $1.09{\cdot}10^{-5}$ for PPMS–RRMS; $N = 198$, $Z = 3.91$, FDR = $1.81{\cdot}10^{-4}$ for SPMS–RRMS), and the proportion of male participants was higher in the PPMS than in the RRMS group (Chi square test, $N = 209$, $\chi^2 = 10.38$, FDR = $4.50{\cdot}10^{-3}$). BMI was not different between MS and FGFP, nor between MS phenotypes. Further information on quality of life (RAND-36) and diet was collected. As for the QoL assessment, all eight RAND-36 scores were lower in the MS versus FGFP cohort, reflecting lower QoL in the MS group (Wilcoxon rank sum tests, FDR < 0.05; Supplementary Table 3). Dietary information through a 32-item

**Table 1.** Demographics, fecal moisture content, and fecal cell counts (microbial load) in the FGFP and MS cohort (Wilcoxon Rank Sum tests [2 groups] or Kruskal–Wallis tests with post-hoc Dunn tests [>2 groups] for continuous variables, $\chi^2$ tests for categorical variables). Only significant Dunn test comparisons are shown. The abbreviation "n.s." is noted when none of the comparisons were significant.

| Variable | FGFP ($N = 2860$) | MS ($N = 228$) | $p$-val. FGFP vs. MS | RRMS ($N = 179$) | SPMS ($N = 19$) | PPMS ($N = 30$) | FDR comparisons |
|---|---|---|---|---|---|---|---|
| Age, years RRMS–PPMS: $1.09{\cdot}10^{-5}$ RRMS–SPMS: $1.81{\cdot}10^{-4}$ RRMS–FGFP: $1.17{\cdot}10^{-14}$ | (Median ± IQR) | 54.0 ± 19.0 | 46.5 ± 17.3 | $1.92{\cdot}10^{-9}$ | 43.0 ± 16.0 | 58.0 ± 10.0 | 57.5 ± 11.8 |
| Females, % | 60.6 | 69.7 | $4.00{\cdot}10^{-3}$ | 75.4 | 52.6 | 46.7 | RRMS–PPMS: $4.00{\cdot}10^{-3}$ RRMS–FGFP: $3.00{\cdot}10^{-3}$ |
| BMI, kg/m$^2$ n.s. | (Median ± IQR) | 24.3 ± 5.6 | 24.1 ± 6.6 | 0.80 | 24.3 ± 6.5 | 23.7 ± 7.7 | 23.8 ± 5.9 |
| Moisture, proportion PPMS–FGFP: 0.02 | (Median ± IQR) | 0.73 ± 0.11 | 0.72 ± 0.10 | $4.00{\cdot}10^{-3}$ | 0.72 ± 0.12 | 0.72 ± 0.10 | 0.68 ± 0.072 |
| Microbial load, cells/g RRMS–FGFP: $7.21{\cdot}10^{-8}$ | (Median ± IQR) | $1.10{\cdot}10^{11}$ ± 1-.$16{\cdot}10^{11}$ | $8.80{\cdot}10^{10}$ ± $6.50{\cdot}10^{10}$ | $1.53{\cdot}10^{-9}$ | $8.80{\cdot}10^{10}$ ± $6.60{\cdot}10^{10}$ | $1.00{\cdot}10^{11}$ ± $5.60{\cdot}10^{10}$ | $8.70{\cdot}10^{10}$ ± $6.20{\cdot}10^{10}$ |
| Calprotectin, µg/g n.s. | (Median ± IQR) | 25.50 ± 44.40 | 29.80 ± 57-.00 | n.s. | 29.50 ± 57.10 | 45.70 ± 53.60 | 29.20 ± 36.-20 |

FFQ was solely available for MS subjects, yet no distinctive differences were observed between the MS phenotypes in terms of water, macronutrients (carbohydrates, fat, protein), or fiber intake (Kruskal–Wallis tests, $p > 0.05$; Supplementary Table 3).

Patients were either untreated (45%) or treated with a low-efficacy (21%), moderate-efficacy (15%), or high-efficacy (19%) therapy.[6] Most frequently used treatments were dimethyl fumarate (orally administered), ocrelizumab (intravenous administration), interferon (subcutaneous injection), and glatiramer acetate (subcutaneous injection) (by 25 [11%], 21 [9%], 20 [9%], and 19 [8% of] patients, respectively) (Figures 1B, C, S1A, B).

Twenty-four patients experienced a relapse during sampling. For all other clinical characteristics of the MS cohort, see Table 2 and Supplementary Table 3.

### *MS phenotype and DMTs are associated with fecal moisture, calprotectin*

Stool moisture content (proxy for transit time) was lower in the MS cohort (harder stools) than in the FGFP controls (Wilcoxon rank sum test: $N = 3085$, $Z = -0.05$, $p = 4.00{\cdot}10^{-3}$), largely driven by the PPMS phenotype (Dunn test PPMS–FGFP: $N = 2887$, $Z = -2.93$, FDR = 0.02) (Figure 2A, Table 1, Supplementary Table 3). Microbial load was also lower in MS (Wilcoxon rank sum test: $N = 3088$, $Z = -0.11$, $p = 1.53{\cdot}10^{-9}$), which was mostly driven by RRMS (Dunn test RRMS–FGFP: $N = 3039$, $Z = -5.70$, FDR = $7.21{\cdot}10^{-8}$). While calprotectin (defined as a numerical variable) did not differ between MS and controls, nor between MS phenotypes (Kruskal–Wallis test $N = 2720$, $p = 0.42$), we did find 20 out of 179 RRMS patients to exhibit calprotectin levels above the threshold of 200 µg/g, which is the clinical reference value for gastrointestinal inflammation.[71] These patients did not report symptoms suggestive of IBD. When categorizing calprotectin levels using thresholds[71] of no inflammation (<50 µg/g; FGFP $N = 1791$, MS $N = 150$), low to moderate inflammation (50–200 µg/g; FGFP $N = 573$, MS $N = 58$) and high inflammation (>200 µg/g; FGFP $N = 128$, MS $N = 20$), the proportion of individuals with high inflammation was larger in RRMS (9%) than in FGFP (5%) (Post-hoc chi square test, $N = 2720$, $\chi^2 = 2.49$, FDR = 0.04; Figure 2D). We then examined how DMTs contribute to fecal parameters. Within RRMS, the highest values of calprotectin were seen in patients taking oral DMTs ($N = 45$; dimethyl fumarate, teriflunomide, fingolimod, cladribine, and azathioprine), with this group having higher fecal calprotectin levels than the

**Table 2.** Clinical variables and candidate serum biomarkers in the MS cohort (Kruskal–Wallis tests with post-hoc Dunn tests). Only significant Dunn test comparisons are shown. "n.s." is noted when none of the comparisons was significant.

| Variable | MS ($N = 228$) | RRMS ($N = 179$) | SPMS ($N = 19$) | PPMS ($N = 30$) | FDR comparisons |
|---|---|---|---|---|---|
| Disease duration, years (Median ± IQR) | 13.0 ± 15.0 | 12.0 ± 14.5 | 24.0 ± 14.0 | 10.0 ± 11.5 | RRMS–SPMS: $2.48{\cdot}10^{-5}$<br>SPMS–PPMS: $7.26{\cdot}10^{-5}$ |
| Disability [EDSS] (Median ± IQR) | 3.0 ± 2.0 | 2.5 ± 1.5 | 6.0 ± 2.0 | 6.0 ± 2.5 | RRMS–PPMS: $1.11{\cdot}10^{-10}$<br>RRMS–SPMS: $8.20{\cdot}10^{-11}$ |
| Disability [ARMSS] (Median ± IQR) | 4.6 ± 3.4 | 4.2 ± 3.0 | 7.4 ± 2.9 | 6.9 ± 4.5 | RRMS–PPMS: $9.21{\cdot}10^{-4}$<br>RRMS–SPMS: $1.47{\cdot}10^{-5}$ |
| MS impact (phys.) [MSIS] (Median ± IQR) | 23.0 ± 33.8 | 15.0 ± 29.7 | 54.4 ± 32.2 | 43.7 ± 24.7 | RRMS–PPMS: $2.05{\cdot}10^{-7}$<br>RRMS–SPMS: $1.53{\cdot}10^{-7}$ |
| MS impact (psy.) [MSIS] (Median ± IQR) | 25.0 ± 30.6 | 23.6 ± 30.6 | 34.7 ± 15.3 | 22.2 ± 25.0 | n.s. |
| Mobility [T25FW], sec. (Median ± IQR) | 4.6 ± 2.0 | 4.4 ± 1.2 | 14.1 ± 173.3 | 8.1 ± 24.3 | RRMS–PPMS: $1.03{\cdot}10^{-8}$<br>RRMS–SPMS: $1.40{\cdot}10^{-8}$ |
| Dexterity [9HPT (dom.)], sec. (Median ± IQR) | 19.9 ± 3.8 | 19.5 ± 2.2 | 25.5 ± 11.3 | 23.5 ± 14.6 | RRMS–PPMS: $4.65{\cdot}10^{-6}$<br>RRMS–SPMS: $1.07{\cdot}10^{-4}$ |
| Dexterity [9HPT (non-dom.)], sec. (Median ± IQR) | 20.6 ± 5.5 | 20.0 ± 4.5 | 33.6 ± 16.0 | 24.1 ± 20.6 | RRMS–PPMS: $3.69{\cdot}10^{-4}$<br>RRMS–SPMS: $4.22{\cdot}10^{-7}$<br>SPMS–PPMS: $4.86{\cdot}10^{-2}$ |
| SDMT (Median ± IQR) | 57 ± 15 | 59 ± 14 | 39 ± 31 | 51 ± 16 | RRMS–PPMS: $2.45{\cdot}10^{-4}$<br>RRMS–SPMS: $6.40{\cdot}10^{-6}$ |
| Serum NfL, ng/L (Median ± IQR) | 15.5 ± 9.9 | 13.6 ± 8.0 | 24.0 ± 11.5 | 21.2 ± 11.4 | RRMS–PPMS: $1.62{\cdot}10^{-5}$<br>RRMS–SPMS: $1.41{\cdot}10^{-4}$ |
| Serum GFAP, ng/L (Median ± IQR) | 88.8 ± 61.1 | 81.8 ± 56.6 | 122.8 ± 70.1 | 111.7 ± 83.4 | RRMS–PPMS: $2.0{\cdot}10^{-3}$<br>RRMS–SPMS: $5.0{\cdot}10^{-3}$ |

Abbreviations: 9-HPT = 9-hole peg test, dom = dominant, non-dom = non-dominant, ARMSS = age-related MS severity scale, EDSS = expanded disability status scale, FDR = false discovery ratio, GFAP = glial fibrillary acidic protein, NfL = serum neurofilament light chain, PPMS = primary progressive MS, RRMS = relapsing-remitting MS, SPMS = secondary-progressive MS, T25FW = timed-25 foot walk, sec = seconds.

untreated MS group (Dunn test, $N = 179$, $Z = 2.90$, FDR = 0.02; Figure 2E). Fecal moisture was lower in untreated MS vs. patients on oral DMTs ($N = 170$, $Z = 2.42$, FDR = 0.046; Figure 2F). There was no higher intake of proton pump inhibitors (PPI) or non-steroidal anti-inflammatory drugs (NSAIDs) (medications associated with calprotectin increase) in the oral DMT-treated RRMS group (chi-square test $p > 0.05$). Overall, our analysis revealed that fecal parameters (moisture, cell count, and calprotectin) are associated with disease phenotype and DMTs.

## *Gut microbiome variation in MS relates to both disease phenotype and host factors*

Next, we analyzed gut microbiota from people with MS, using FGFP as reference population. A principal coordinate analysis (PCoA) assessing global gut microbiome variation across the entire dataset showed a significant separation between the total MS population and FGFP (PERMANOVA, Bray–Curtis dissimilarity, based on QMP, $N = 3088$, adjusted $R^2 = 0.008$, $p = 0.001$). Zooming into the MS phenotypes, the distribution of samples differed between RRMS and FGFP along the first PCoA axis (Dunn test RRMS-FGFP: $N = 3039$, $Z = 5.87$, FDR = $2.62.10^{-8}$), while across the second axis, sample distance variation separated RRMS from PPMS $N = 209$, $Z = 2.70$, FDR = 0.04) (Figure 3A). The same analysis on RMP produced similar findings (Figure S2).

Next, we performed a distance-based redundancy analysis on both FGFP and MS cohorts ($N = 2342$ samples with complete metadata; stepwise db-RDA at the genus-level Bray–Curtis dissimilarity (QMP)) to identify covariates driving microbiota variation (Figure 3B). In line with previous studies,[14,22] stool moisture content ($R^2$adj = 2.64%, $p = 0.002$) had the largest contribution, followed by MS phenotype (i.e., RRMS/PPMS/SPMS/FGFP; $R^2$adj = 0.85%, $p = 002$). Other identified microbiota covariates were age ($R^2$adj = 0.33%, $p = 0.002$), sex ($R^2$adj = 0.30%, $p = 002$), general health score, one of the categories of the RAND-36 questionnaire ($R^2$adj = 0.23%, $p = 0.002$), BMI ($R^2$adj = 0.20%, $p = 002$) and fecal calprotectin ($R^2$adj = 0.19%, $p = 0.002$). In total, 4.74% of the variation in the gut microbiota composition could be explained by host covariates (Supplementary Table 4), which is similar in order of magnitude compared to previous studies.[14,72,73]

Additionally, we performed a db-RDA on the MS group separately (Figure 3C, Supplementary Table 5). In a stepwise model, we found that fecal moisture remained the most significant covariate ($N = 215$,

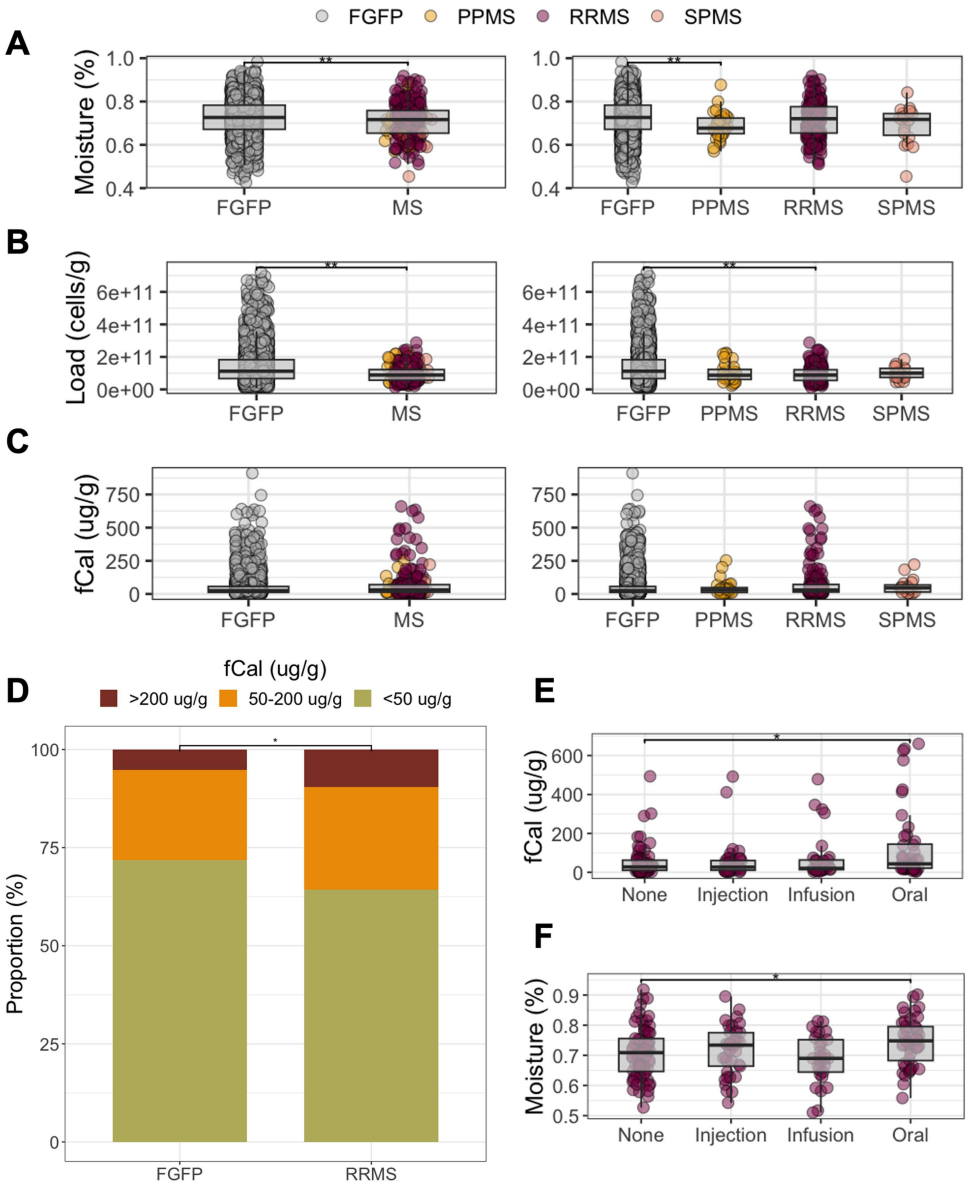


**Figure 2.** Variation of fecal parameters between FGFP controls and MS, and among MS phenotypes. (A) Fecal moisture lower in MS than FGFP ($N = 3085$, $Z = -0.05$, $p = 4.00{\cdot}10^{-3}$), driven by PPMS phenotype ($N = 2887$, $Z = -2.93$, FDR = 0.02). (B) Fecal microbial load lower in MS than FGFP ($N = 3088$, $Z = -0.11$, $p = 1.53.10^{-9}$), driven by RRMS ($N = 3039$, $Z = -5.70$, FDR = $7.21.10^{-8}$). (C) Fecal calprotectin not different in MS vs. FGFP. (D) Fecal calprotectin categorized according to clinical threshold of inflammation (<50 µg/g: no; 50–200 µg/g low grade; >200 µg/g: inflammation) shows high fecal calprotectin more prevalent in RRMS than FGFP ($N = 2720$, $\chi^2 = 2.49$, FDR = 0.04). (E) Fecal calprotectin in MS stratified by DMT administration mode shows higher calprotectin in oral DMT group ($N = 179$, $Z = 2.90$, FDR = 0.022). (F) Fecal moisture in MS stratified by DMT administration mode shows higher moisture in oral DMT group ($N = 170$, $Z = 2.42$, FDR = 0.05). * denotes <0.05, ** denotes <0.01. The box plot body represents the first and third quartiles of the distribution and the median line, with whiskers extending from quartiles to the last data point within 1.5× the interquartile range, and outliers lying beyond.

$R^2$adj = 3.90%, $p = 0.002$), followed by BMI ($R^2$adj = 0.88%, $p = 0.002$), and serum GFAP ($R^2$adj = 0.81, $p = 0.008$). MS phenotype was no longer significant in this analysis, implying that serum GFAP (which is higher in the progressive phenotypes, see Figure 3D; Table 2) might explain a larger amount of gut microbiome variation. Collectively, these results show that host factors contribute to higher variation of the gut microbiota than the disease itself.

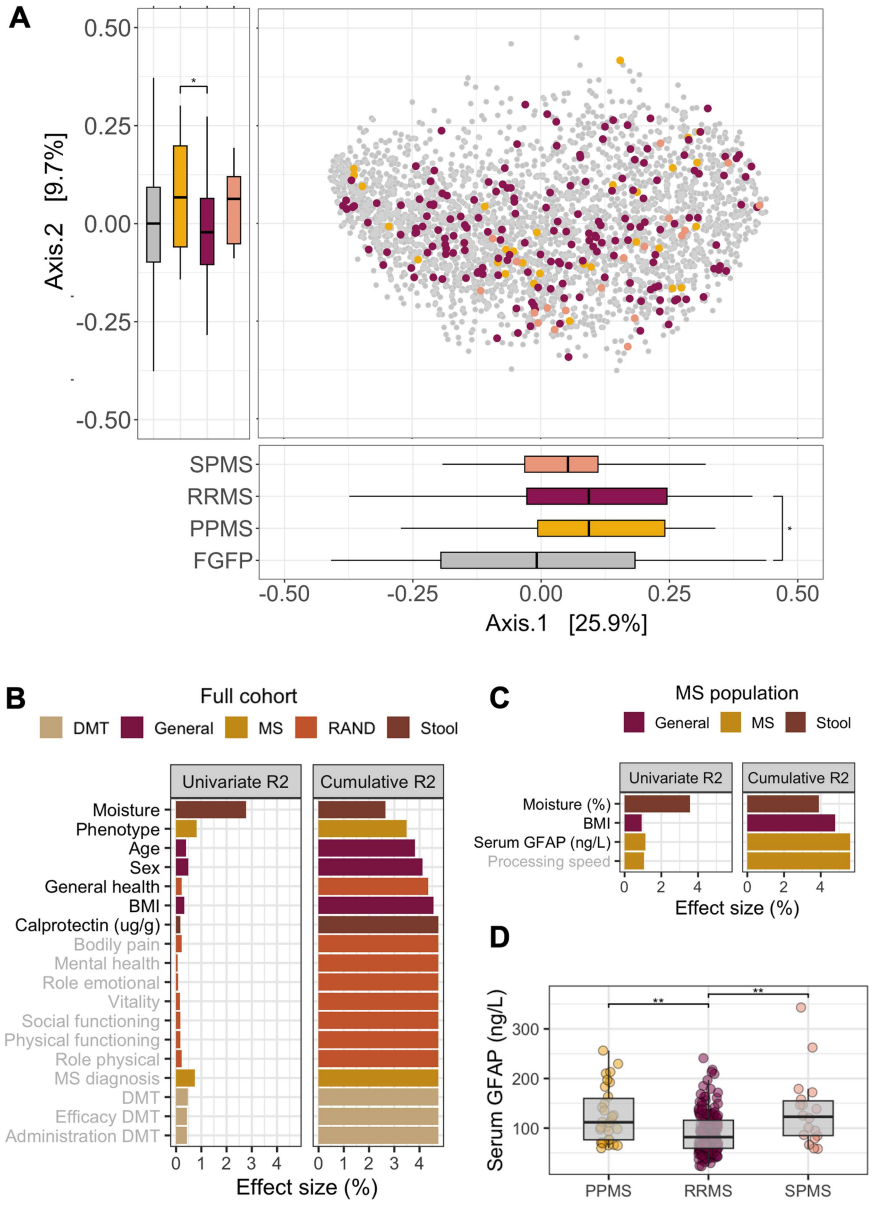


**Figure 3.** (A) Principal coordinate analysis (PcoA) of interindividual differences (Bray–Curtis dissimilarity at the genus level) in quantitative microbiome profiles of the MS cohort and FGFP controls ($N = 2342$). (B) Covariates of microbiota variation by distance-based redundancy analysis (db-RDA), $N = 2342$ samples. Metadata used in a single variable model (light color single R2) or combined in a stepwise model (darker color, cumulative R2). Features that contribute significantly to the stepwise model are listed in black. Total cumulative variation explained $R^2 = 4.74\%$. (C) Covariates of microbiota variation in MS by distance-based redundancy analysis (db-RDA), $N = 215$ samples. Metadata used in a single variable model (light color single R2) or combined in a stepwise model (darker color, cumulative R2). Moisture content, BMI, and serum GFAP contribute significantly to the stepwise model, listed in black, whereas gray labels have nonsignificant contributions in the stepwise model. (D) Serum GFAP levels differ among MS phenotypes (Kruskal–Wallis, $N = 222$, effect size = 18.3, Pval = 0.0001). The box plot body represents the first and third quartiles of the distribution and the median line, with whiskers extending from quartiles to the last data point within 1.5× the interquartile range, and outliers lying beyond.

## *Low-efficacy DMT treatment linked to increased prevalence of Bact2 in MS*

Previous studies have examined the association between DMT and gut microbiota, with conflicting findings.[10] We explored whether ecosystem-level differences can be associated with DMT use and levels of DMT efficacy. Enterotype analysis via the Dirichlet Multinomial Mixtures (DMM) approach[14,28,50] on

MS and FGFP samples identified four clusters, hereafter termed Ruminococcaceae (Rum), *Bacteroides1* (Bact1), *Bacteroides2* (Bact2), and *Prevotella* (Prev), based on probabilistic models for grouping samples according to their similarity in microbiota composition and average genus-level proportional abundance of key genera (Figure 4A, Figure S3). The Bact2 enterotype is of particular interest, as it represents a dysbiotic community type that has been repeatedly associated with disease.[52,72,74] Analysis of enterotype patterns showed a higher Bact2 prevalence (vs. non-Bact2) in MS patients versus FGFP controls (23.7% Bact2 in MS, 16.7% Bact2 in FGFP, Chi squared test, $\chi^2 = 7.3$, $p = 0.005$) (Figure 4B). Bact2 in MS was characterized by lower fecal microbial loads ($N = 228$, Dunn test, Bact1-Bact2 $Z = 4.5$, FDR = $3.23{\cdot}10^{-5}$), while the Rum enterotype was characterized by the lowest stool moisture content ($N = 228$, Bact1-Rum $Z = 3.8$, FDR = $5.80{\cdot}10^{-4}$, Bact2-Rum $Z = 4.4$, FDR = $4.72{\cdot}10^{-5}$, Prev-Rum $Z = 4.68$, FDR = $4.72{\cdot}10^{-5}$), in line with previous observations.[28,52] Fecal calprotectin also differed between enterotypes (Kruskal–Wallis,

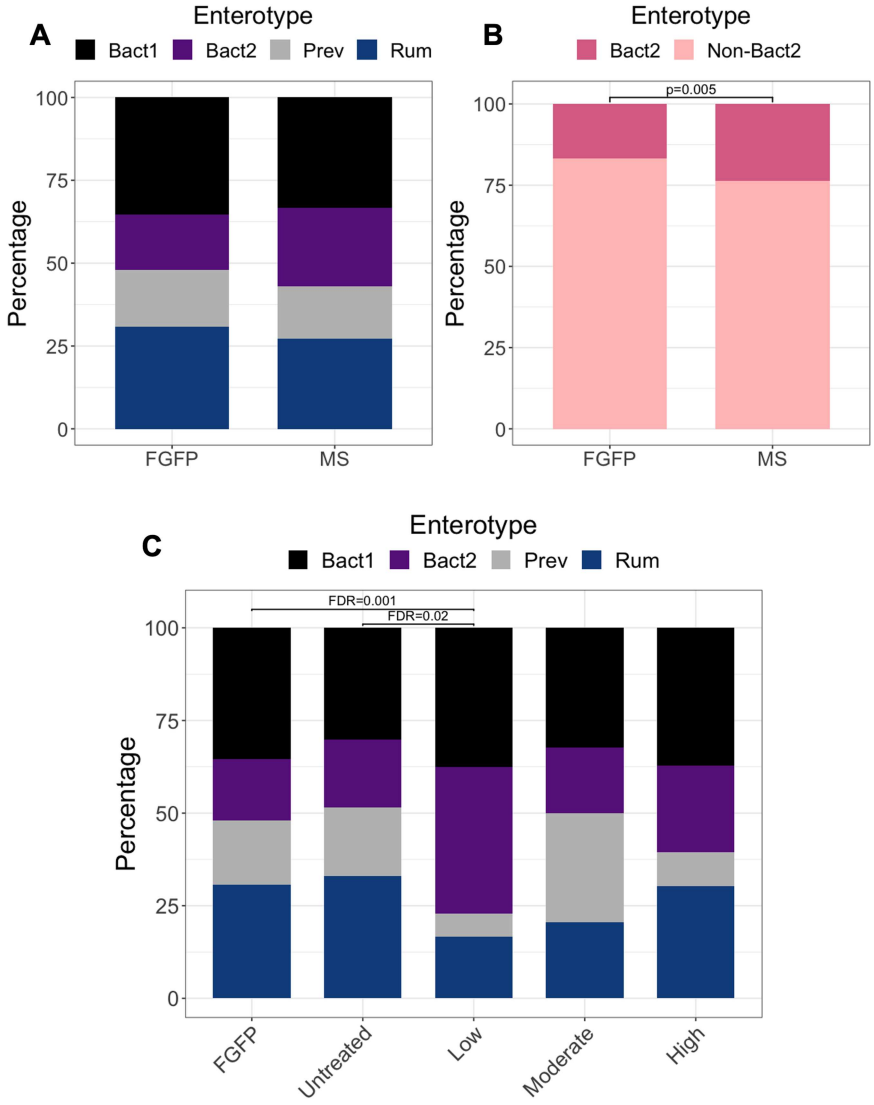


**Figure 4.** Differences in enterotype distribution in FGFP controls ($N = 2860$) and MS subjects ($N = 228$). (A) All enterotypes ($N = 3088$, Chi squared test, $\chi^2 = 7.4$, $p = 0.06$). (B) Enterotypes according to Bact2 ($N = 3088$, Chi squared test, $\chi^2 = 7.3$, $p = 0.005$). (C) FGFP controls ($N = 2860$), and MS patients subcategorized as being untreated ($N = 103$), treated with a low-efficacy DMT ($N = 48$), treated with a moderate-efficacy DMT ($N = 34$), and treated with a high-efficacy DMT ($N = 43$). The proportion of Bact2 was increased in the low-efficacy DMT group when compared with FGFP ($N = 2963$, Chi squared test, $\chi^2 = 21.0$, FDR = $1.1{\cdot}10^{-3}$) and untreated MS patients (Chi squared test, $N = 151$, $\chi^2 = 12.9$, FDR = $2.5{\cdot}10^{-2}$), respectively.

$N = 228$, $p = 0.01$), with Bact2 exhibiting slightly higher calprotectin levels than Rum (Dunn test, Bact2-Rum $Z = 2.5$, FDR = 0.03) (Figure S4, Supplementary Table 6).

Regarding compositional differences among enterotypes, Bact2 was defined by a decrease in *Faecalibacterium* abundance (Dunn tests; Bact1-Bact2, $N = 130$, $Z = 6.79$, FDR = $6.64{\cdot}10^{-11}$; Prev-Bact2, $N = 90$, $Z = 3.68$, FDR = $4.69{\cdot}10^{-4}$) and other producers of SCFAs, such as *Coprococcus* (Bact1-Bact2, $N = 130$, $Z = 5.61$, FDR = $1.22{\cdot}10^{-7}$; Prev-Bact2, $N = 90$, $Z = 3.33$, FDR = $1.71{\cdot}10^{-3}$; Rum-Bact2, $N = 116$, $Z = 4.73$, FDR = $6.61{\cdot}10^{-6}$), *Roseburia* (Bact1-Bact2, $N = 130$, $Z = 4.27$, FDR = $3.89{\cdot}10^{-5}$; Prev-Bact2, $N = 90$, $Z = 2.63$, FDR = $1.27{\cdot}10^{-2}$), and *Gemmiger* (Bact1-Bact2, $N = 130$, $Z = 4.53$, FDR = $3.45{\cdot}10^{-5}$; Prev-Bact2, $N = 90$, $Z = 3.06$, FDR = $4.44{\cdot}10^{-3}$; Rum-Bact2, $N = 116$, $Z = 3.69$, FDR = $6.77{\cdot}10^{-4}$) (Figure S5, Supplementary Table 7).

Looking at the different MS phenotypes, Bact2 percentage was not higher in any of these when compared to FGFP after multiple testing correction (Chi squared test, FDR > 0.05) (Figure S6).

Given the wide variety of DMTs and treatment regimes in the cohort, we next assessed whether certain enterotypes were associated with treatment. While untreated cases did not differ from FGFP controls in terms of enterotype distribution (Chi squared test, $N = 2963$, $\chi^2 = 1.2$, FDR = 0.74), treated patients had a higher Bact2 prevalence than FGFP subjects ($N = 2985$, $\chi^2 = 12.6$, FDR = 0.02) (Figure S7). This pattern seems driven by the low-efficacy DMT group, as these individuals showed higher Bact2 not only in comparison to FGFP controls (Chi squared test, $N = 2908$, $\chi^2 = 21.0$, FDR = $1.1{\cdot}10^{-3}$) but also to untreated MS patients ($N = 151$, $\chi^2 = 12.9$, FDR = $2.5{\cdot}10^{-2}$) (Figure 4C). As for the specific DMTs, the highest Bact2 percentage was found in patients on glatiramer acetate (53% Bact2, $N = 10/19$), followed by teriflunomide (37.5%, $N = 3/8$), interferon-beta (30%, $N = 6/20$), and natalizumab (30%, $N = 3/10$). The only significant comparison was between glatiramer acetate-treated patients and FGFP (Chi-squared test, $N = 2879$, $\chi^2 = 19.0$, FDR = 0.02). Qualitatively, the comparison with untreated patients shows a similar trend, but lacks significance, probably due to small subgroups (Figure S8).

Assessing for potential covariates, the low-efficacy treated patients did not differ from the untreated RRMS in terms of covariates significant in the db-RDA, nor in terms of other clinical or metadata variables (Supplementary Table 8). Overall, this shows that the dysbiotic Bact2 enterotype is overrepresented in MS patients, driven by low-efficacy treated MS patients.

### *Quantitative analysis and deconfounding reveal several associations of bacterial genera with MS, and identify robust markers of MS across multiple studies*

Next, we focused on identifying robust, quantitative microbiome biomarkers for MS. RMP analysis identified 45 genera to be differentially abundant between MS and controls (Wilcoxon rank sum tests, FDR < 0.05; Supplementary Table 9). Applying QMP yielded 31 genera (Wilcoxon rank sum tests; FDR < 0.05; Supplementary Table 10). Twenty-four taxa identified in RMP analysis were retained in QMP, while 21 were unique to RMP and 7 to QMP. The latter category contained *Alistipes, Barnesiella, Butyricicoccus, Holdemanella, Odoribacter, Roseburia* (decreased in QMP MS vs. FGFP), and *Acetanaerobacterium* (increased in MS in QMP MS vs. FGFP) (Figure 5, Supplementary Table 10). Next, we integrated covariates that were associated with gut microbiome variation in our study cohorts, i.e., fecal moisture, age, sex, BMI, calprotectin, and RAND general health score, to identify general MS markers independent of disease-independent physiological factors. This analysis (using QMP) resulted in 21 differentially abundant genera between MS and FGFP controls. Specifically, taxa enriched in MS (GLMs, $N = 2344$, FDR < 0.05 for all) with the strongest effect size were *Lachnobacterium* (std. coef. = 0.38), *Blautia* (std. coef. = 0.29), *Hungatella* (std. coef. = 0.18). Others enriched were *Gemmiger* (std. coef. = 0.03), *Acetanaerobacterium* (std. coef. = 0.03), *and Methanobrevibacter* (std. coef. = 0.02). In an exploratory analysis at higher taxonomic resolution, we found that the most abundant ASVs for each of these genera were assigned to *Lachnobacterium bovis*, *Blautia provencensis*, *Hungatella hathewayi*, *Gemmiger formicilis*, *Methanobrevibacter intestini/Methanobrevibacter smithii*, and *Acetanaerobacterium elongatum* (Supplementary Table 15). By contrast, genera depleted in MS (GLMs, $N = 2344$, FDR < 0.05 for all) consisted of butyrate producers *Butyricicoccus* (std. coef. = 0.09) and *Butyricimonas* (std. coef. = 0.04). Other depleted genera were—ordered from higher to lower effect size—*Clostridium cluster XIVa* (std. coef. = 0.12), *Bacteroides* (std. coef. = 0.09), *Hydrogenoanaerobacterium* (std. coef. = 0.09), *Peptococcus* (std. coef. = 0.07), *Parabacteroides* (std. coef. =

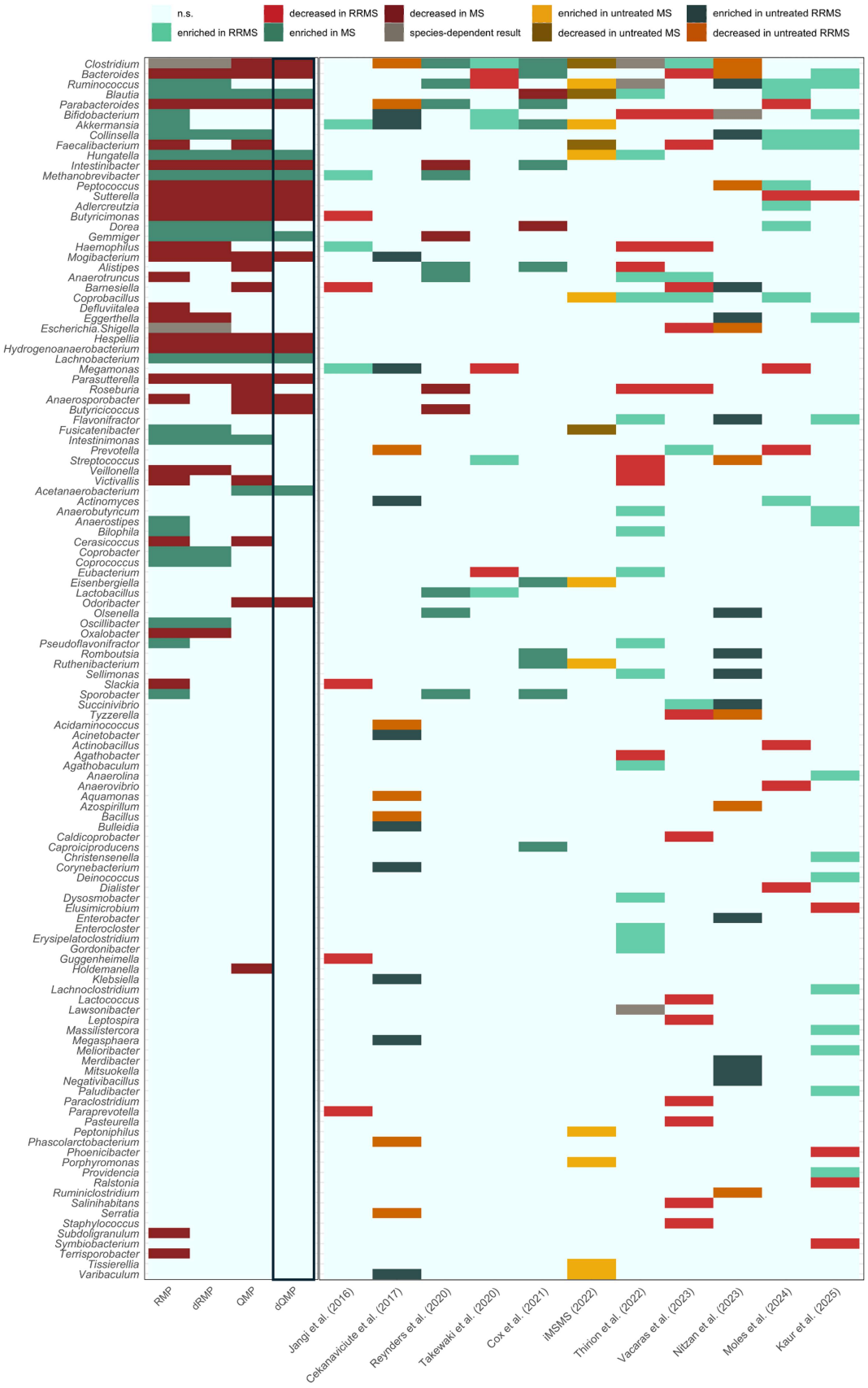


**Figure 5.** Heatmap of differentially abundant genera between MS and healthy controls comparing results from published results of previous cross-sectional studies to current study (RMP not deconfounded—RMP deconfounded for covariates in our dbRDA—QMP not deconfounded - QMP deconfounded for covariates in our dbRDA). Cekanaviciute et al.

(*Caption on next page*)

and Moles et al. did not report adjustment for covariates. Most other studies used demography, anthropometry-related covariates in the differential analysis or used matched populations. Thirion et al. used QMP, all others RMP. Reynders et al. is the only study reporting to correct for fecal moisture content. See Supplementary Table 1. Abbreviations: RMP = relative microbiome profiling. QMP = quantitative microbiome profiling. RRMS = relapsing-remitting multiple sclerosis, PPMS = primary progressive multiple sclerosis.

0.05), *Mogibacterium* (std. coef. = 0.05), *Parasutterella* (std. coef. = 0.04), *Hespellia* (std. coef. = 0.04), *Intestinibacter* (std. coef. = 0.04), *Adlercreutzia* (std. coef. = 0.03), *Sutterella* (std. coef. = 0.03), *Clostridium cluster XIVb* (std. coef. = 0.03), *Anaerosporobacter* (std. coef. = 0.03), and *Odoribacter* (std. coef. = 0.03) (Figure 5, Supplementary Table 10).

We then performed a comprehensive comparative analysis integrating results of this study with 10 previously published mid-to large-scale cross-sectional studies (see methods), encompassing a total of 1065 MS patients ($N = 911$ RRMS/CIS, $N = 154$ PPMS/SPMS), and 874 controls (Figure 5, Supplementary Table 1). We compiled 10 previous studies with our own study results, making a distinction between RMP (relative abundance) and QMP (quantitative abundance using microbial load), with and without adjustment for covariates identified in our db-RDA (Figure 5).

In terms of covariate handling, studies differed by either matching MS and healthy cohorts based on specific covariates (mostly demography)[16,17,20,21,35] or taking covariates into account in the differential analysis.[18,19] All but one study[20] used RMP, and only one[16] reported correction of detected MS-associated taxa for the primary covariate identified in this study (i.e., transit time/fecal moisture content). Results were exceedingly heterogeneous: 40 MS-associated genera were study-specific, meaning that none of these were replicated in either previous literature or the current dataset. Conflicting findings were found for several taxa (e.g., *Alistipes, Bacteroides, Blautia)*, with some studies reporting an increase in abundance, while others observed the inverse.

Findings that were replicated across multiple previous studies included an enrichment of *Akkermansia* in MS (most consistent result with 5 previous reports), as well as a depletion of *Roseburia* (previously reported 3 times) and *Bacteroides* (previously reported 3 times). While our own analyses revealed a higher *Akkermansia* abundance with RMP, this finding was not maintained after correcting for covariates. Specifically, *Akkermansia* showed a negative correlation with moisture (Spearman correlation test, $N = 228$, rho = −0.36, $p = 1.60^{-5}$), indicating that its abundance is increased in individuals with longer transit times, an association that has been established recurrently.[14,22,75] Also, *Akkermansia* abundance was not increased in MS in our QMP analysis. This is in line with the other MS microbiome study that used a QMP approach,[20] where *Akkermansia* was not associated with MS either. Likewise, although *Roseburia* abundance was decreased in MS (vs. controls) using QMP in our cohort, this association failed to reach significance once deconfounded. This finding probably relates to the positive correlation of *Roseburia* abundance with moisture content in MS (Spearman correlation, $N = 228$, rho = 0.22, FDR = 0.04, Supplementary Table 11), as has been shown previously in a healthy population.[76] Several other markers within our study were lost when controlling for covariates, including *Collinsella, Dorea, Faecalibacterium, Alistipes, Barnesiella, Intestinimonas, Victivallis, Cerasicoccus, and Guggenheimella*. These results emphasize the importance of rigorous covariate (and possible confounder) control.

Reversely, some replicating results were further strengthened by this study. Consistent with three out of ten previous reports, we found *Bacteroides* abundance to be decreased in MS, both with RMP and QMP, also after adjusting for covariates. Other taxa that we could corroborate in our deconfounded QMP approach, were increased *Blautia* (twice reported before), and increased *Methanobrevibacter* (twice reported before). These taxa thus constitute the strongest MS biomarkers at this time and would merit further follow-up research. Next to these, some quantitative, covariate-independent markers found in the current cohort, but with less replication evidence, would still merit further investigation, because of a plausible mechanistic interpretation, such as *Butyricicoccus* and *Butyricimonas*.

Taken together, our deconfounded QMP analysis confirmed some previously replicated bacterial markers in MS, but could not confirm others, which might be due to various factors, including study heterogeneity (subjects, analytical), RMP approach, or incomplete confounder control.

### Compositional differences specific to the RRMS phenotype

Next, we focused on MS subtype-specific compositional differences compared to controls. In the absence of significant findings in PPMS ($N = 30$) and SPMS ($N = 19$), possibly due to inadequate statistical power, we excluded these patient groups and focused on RRMS. Several genera were found to be differentially abundant in RRMS ($N = 179$) when compared to controls (QMP after deconfounding) (Supplementary Table 10), while no significant difference in abundance was detected (after deconfounding) when comparing the full MS cohort with the control group.

Specifically, *Collinsella* was more abundant in RRMS than in controls, albeit with a small effect size (GLMs, $N = 2303$, std. coef. = 0.026, FDR = 0.049), consistent with two previous RRMS studies,[34,35] and *Barnesiella* was less prevalent in RRMS ($N = 2303$, std. coef. = −0.025, FDR = 0.035), consistent with two previous studies in RRMS.[15,21] Contrary to two previous studies,[20,35] we found *Flavonifractor* to be less prevalent in RRMS ($N = 2303$, std. coef. = −0.04, FDR = $1.6.10^{-5}$) than in controls. The taxa identified might form RRMS-specific signals of interest to pursue in follow-up studies.

### Associations of gut microbiota composition with MS clinical outcomes and blood markers

We then investigated associations of gut microbial composition with specific MS disability measures (i.e., ARMSS, T25FW, 9-HPT, SDMT), self-reported fatigue, anxiety, and depression (FSMC, HADS), impact of MS (MSIS physical, MSIS psychological), and serum-NfL, -GFAP (Table 2). We deconfounded all findings for identified MS-associated gut microbial covariates (i.e., moisture, BMI, and GFAP).

First, we examined whether there was an association between enterotypes and clinical variables of interest. Patients who harbored the Prev enterotype had lower disability on the ARMSS compared to all other enterotypes, independent of reported covariates (GLMs; Bact2-Prev $N = 86$, std. coef. = −0.46, FDR = $5.0.10^{-4}$; Rum-Prev $N = 92$, std. coef. = −0.41, FDR = $4.0.10^{-3}$; Bact1-Prev $N = 108$, std. coef. = −0.29, FDR = 0.02) (Figure 6A, Supplementary Table 11). Prev-enterotyped patients also showed greater mobility, with the latter defined by a better score on the T25FW test than Bact2- and Rum-enterotyped individuals (GLMs; Bact2-Prev $N = 86$, std. coef. = −0.46, FDR = $5.0.10^{-4}$; Prev-Rum $N = 89$, std. coef. = 0.26, FDR = 0.02) (Figure 6B). Associations between enterotype and the physical MSIS sub score related to differences between Prev and both Bact1 and Bact2 (GLMs; Prev-Bact1 $N = 104$, std. coef. = −0.02, FDR = 0.04; Prev-Bact2 $N$-79, std. coef. = −0.01, FDR = 0.03) (Figure 6C). There were no significant differences between enterotype distribution and 9-HPT, SDMT, fatigue, anxiety, MSIS psychological, or serum NfL levels.

Stratifying by MS phenotype and subsequently repeating abovementioned analyses, the association between ARMSS and enterotype was also detected in the RRMS subpopulation. Specifically, a lower ARMSS was found in Prev when compared to Bact2 (GLM; $N = 70$, std. coef. = −0.43, FDR = $2.0.10^{-3}$) and Bact1 ($N = 93$, std. coef. = −0.31, FDR = 0.02).

As the presence of the Prev enterotype seemed to be associated with lower disability scores, we compared available metadata for differences between enterotypes that might (partially) explain this association. Compared to the other enterotypes, Prev enterotyped patients had a lower BMI (Dunn test; Bact1-Prev, $Z = -4.60$, FDR = $2.50{\cdot}10^{-5}$; Bact2-Prev, $Z = -3.40$, FDR = $2.0.10^{-3}$; Prev-Rum, $Z = 3.03$, FDR = $5.0.10^{-3}$), and, aside from the Rum enterotype, scored higher in terms of self-reported physical functioning (Bact1-Prev, $Z = -2.42$, FDR = 0.03; Bact2-Prev, $Z = -2.67$, FDR = 0.02) and physical health (Bact1-Prev, $Z = -3.17$, FDR = $9.0.10^{-3}$; Bact2-Prev, $Z = -2.84$, FDR = $9.0.10^{-3}$), both as determined with RAND-36. Also, diastolic blood pressure was lower in Prev versus Bact2 ($Z = -2.40$, FDR = 0.05) and vitamin D levels were higher for the Prev enterotype when compared to Bact1 ($Z = 2.74$, FDR = 0.04). Finally, both triglycerides as HbA1c levels were lowered in Prev versus Rum ($Z = 2.73$, FDR = 0.04 and $Z = 3.12$, FDR = 0.01, respectively), lower HbA1c than Rum. Thus, Prev exhibits several differences with the other enterotypes that may be associated with better general and cardiometabolic health.

Next, we checked for associations between the mentioned MS metadata variables and quantitative genus abundances. In the full MS cohort, only correlations likely driven by outliers were detected (*Escherichia/Shigella* abundance correlated with both the 9HPT score (non-dominant hand) and the physical sub score of the MSIS (FDR < 0.05)) (Figure S9). For the RRMS and SPMS phenotypes, we found no significant

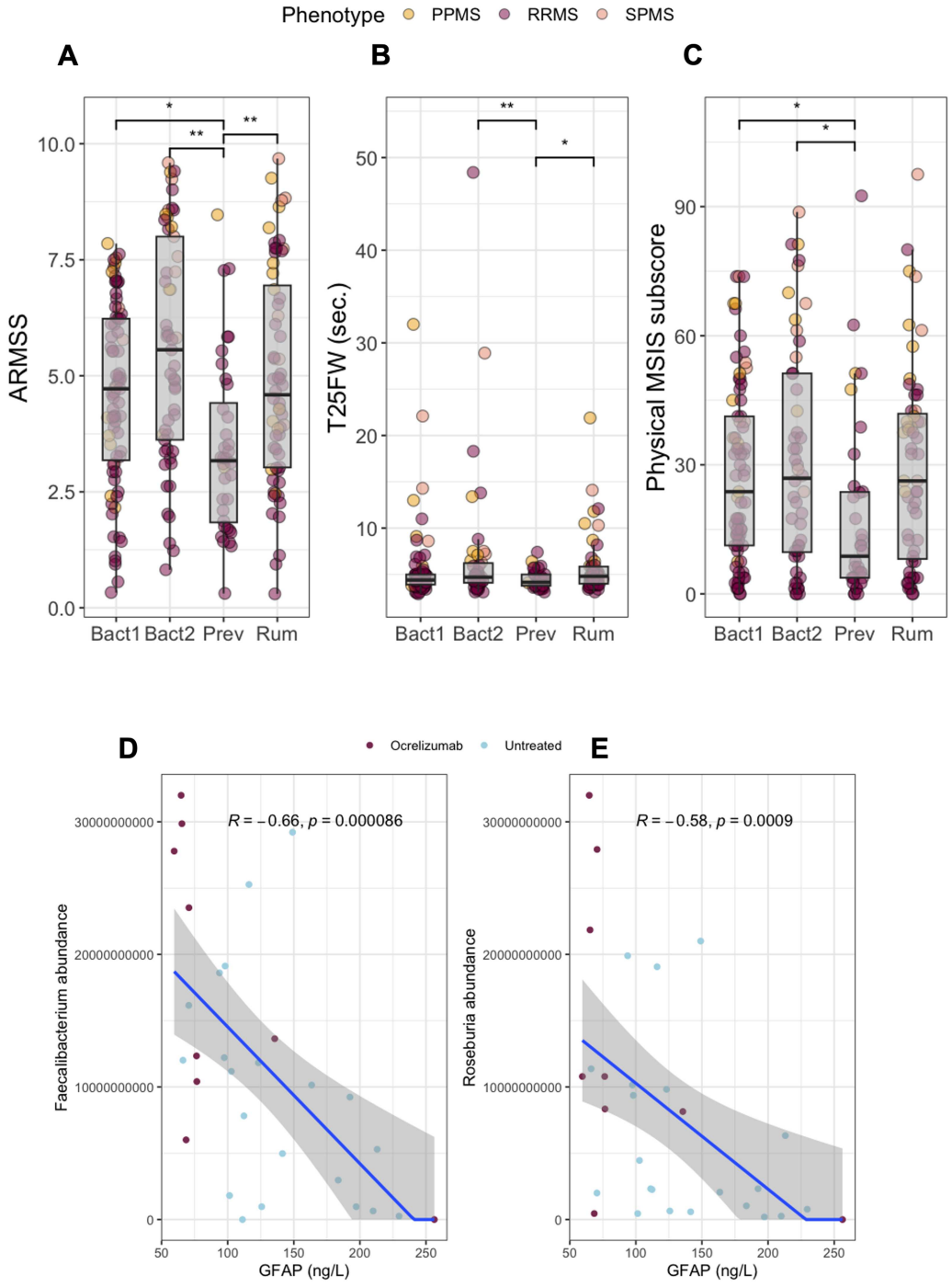


**Figure 6.** Association between clinical and gut microbiome variables. (A) Age-related MS severity score (ARMSS), (B) Timed25-Foot Walk (T25FW), (C) Physical MS Impact subscore (MSIS) in relation to enterotypes in the full MS cohort. * denotes <0.05, ** denotes <0.01. (D and E) Negative correlation of serum-GFAP with *Faecalibacterium* and *Roseburia* abundance in individuals with PPMS, colored by DMT (ocrelizumab treated vs. untreated).

associations (Supplementary Table 13). In the PPMS cohort however, there was a negative correlation between serum GFAP and *Faecalibacterium* (Spearman's $\rho = 0.66$, FDR = 0.01), and *Roseburia* (Spearman's $\rho = 0.58$, FDR = 0.03) (Figure 6D, E), aside from the outlier-driven correlations between *Sutterella* and both GFAP and NfL (FDR < 0.05) (Figure S10, Supplementary Table 13). Serum-GFAP correlated with disease severity as reflected by the ARMSS in the PPMS cohort (rho = 0.39, $p = 0.04$), but not in the RRMS cohort. The correlations between serum GFAP and bacterial taxa were independent of

the covariates identified in the db-RDA, based on partial correlations ($N = 30$ for both, with estimates = -0.66 and −0.61; FDR = $1.0.10^{-4}$ and $5.0.10^{-4}$ for *Faecalibacterium* and *Roseburia,* respectively).

To summarize, the Prev enterotype was associated with lower disability, and two butyrate producers *Faecalibacterium* and *Roseburia* showed an inverse correlation with serum GFAP, a marker for neurodegeneration, in PPMS.

## Discussion

While several cross-sectional studies have compared the gut microbiota composition of people with MS to that of healthy controls, findings have been very inconsistent.[27] This could be related to 1) the large heterogeneity in subjects' disease status and treatment and cohort size, 2) variable gut microbiome technical and analytical approaches across studies, including incomplete covariate (and possible confounder) adjustment, and 3) implementation of relative as opposed to quantitative microbiome analyses. Here, we used a case-control study design ($N = 228$ MS, 2860 HC) to assess the primary covariates of gut microbiome variation in MS, and to study differential taxa abundance between MS and controls in both a relative and quantitative approach, before and after correcting for these covariates. We rebuked as well as confirmed previously proposed markers and described novel ones. We further identified gut microbiome associations with MS-specific clinical features, including a candidate marker of progressive disease.

### *Lower bacterial cell counts in MS emphasize the importance of QMP approaches*

Microbial load has emerged as a critical gut microbiome parameter. Diverse approaches, such as quantitative PCR, flow cytometry, synthetic DNA spike-ins,[77] and recently sequencing-based prediction[78] or host-derived read normalization,[79] are used to assess or predict it. Fecal cell loads were lower in our MS cohort than in the control group, indicating that an overall low bacterial cell count might be part of the MS microbiome signature, albeit less pronounced than in some other diseases such as IBD.[27] This does emphasize, however, the value of implementing cell count to explore the nature of the compositional change and microbiota-disease associations in MS. Of note, a recent study, using a prediction approach to estimate microbial load from sequencing data, found that gut microbiota from MS subjects had higher cell count.[78] Future studies using quantitative approaches are necessary to provide better insight into quantitative differences in MS.

### *Gut transit time is an important confounder of MS gut microbiome variation*

Fecal moisture content, age, sex, BMI, and medication are established as the most important contributors to the gut microbiota variation, both in the general and in non-healthy populations, with moisture being the largest contributor.[14,52,74] In our MS study population, fecal moisture was lower than in the control population, driven by the PPMS phenotype. Also, it was the only covariate with a larger effect size on gut microbiome variation than MS diagnosis and MS phenotype (RR/PP/SPMS), underscoring its importance for covariate control. Neurogenic bowel dysfunction is highly prevalent in MS, with constipation being a common complaint.[80,81] Scintigraphy studies have shown evidence of a slowed colonic transit in MS.[82,83] One study found limited microbial associations with self-reported constipation within the MS population.[26]

Age, sex, BMI, and a general health score (RAND-36) were identified as other covariates of gut microbiome composition, consistent with previous research.[14] In addition, we found fecal calprotectin, a surrogate for gut inflammation, as another contributor not accounted for in previous MS research. This suggests that not only demographic, but also inflammatory markers need to be considered.[74] Underscoring this was the finding of a modestly higher proportion of RRMS patients with calprotectin levels above 200 µg/g in comparison with controls, which could indicate subclinical (in the absence of IBD diagnosis or symptoms) gut inflammation. Fecal calprotectin might confound disease associations with specific taxa, as observed.[74]

### *Bact2 enterotype is increased in low efficacy-treated MS*

In a smaller study (98 people with MS), we previously showed that the low microbial count Bact2 enterotype was enriched in people with MS in comparison with healthy controls, and more in

interferon-treated patients.[16] Our current extended study confirms increased Bact2 in MS, especially in treated MS, and investigates the role of DMTs. When stratifying DMTs according to efficacy,[6] we found that higher Bact2 prevalence was most pronounced in the low efficacy-treated patients (interferon-beta (30% Bact2), glatiramer acetate (53% Bact2), teriflunomide (37.5% Bact2)).

The reason for high Bact2 in low-efficacy treated versus untreated patients is currently unknown. In comparison to the untreated, they did not exhibit higher fecal moisture or calprotectin levels, nor differences in disease severity markers or number of comorbidities. It remains to be elucidated whether increased Bact2 in low-efficacy treated MS is due to the treatment itself or due to other patient characteristics, and whether it puts them at risk for future disease progression, as we previously found indications of higher risk of worsening with a Bact2 enterotype.[8]

Patients on different types of DMT often account for a large proportion of participants in MS studies. Some previous studies investigate cross-sectional associations of DMT with gut microbiome variation, e.g., glatiramer acetate (GA) and dimethyl fumarate (DMF),[84] and IFN.[19] Disentangling the effect of individual DMTs and other disease characteristics is complex and requires large patient groups. Both mechanistic and large prospective studies before and after starting DMT, implementing covariates, are necessary to assess whether and how DMT influences the gut microbiota and disease. To date, very few studies assessed gut microbiome composition changes after starting a DMT (dimethyl fumarate,[85,86] teriflunomide, and interferon beta-1a,[87] ocrelizumab,[88]) and these studies included ≤20 patients per treatment group. Diebold et al. describe a decrease in *Coprococcus euctatus* and *Enterococcus gilvus* and—though not significant—*Akkermansia municiphila* after 3 months of DMF.[86] DMF commonly induces gastrointestinal side-effects like diarrhea and abdominal pain,[89] and, while the study did assess for gastrointestinal side effects via survey, the inclusion of objective measurements of transit time, fecal calprotectin, and other covariates that might be subject to change over time, would be valuable. This is further emphasized by our finding of higher fecal calprotectin in RRMS vs. FGFP, driven by the oral DMT group. In this group, we also found higher moisture levels than in the untreated group, which leads to a hypothesis that gastrointestinal side-effects from oral treatments might be at play.

### *Prevotella enterotype is associated with lower disease severity*

While the *Prevotella* enterotype was not differentially abundant in MS vs. FGFP, we did find lower disability scores (ARMSS, MSIS physical sub-score), higher walking speed (T25FW) in patients with a Prev enterotype in comparison with Rum and Bact2 (and for ARMSS, also Bact1) enterotype. Taken together, this suggests that MS patients with a Prev enterotype might have a milder disease course. Previous reports showed that individuals harboring the Prev enterotype have lower low-density lipoprotein (LDL-) cholesterol levels,[90] suggesting a more favorable cardiovascular risk profile. While LDL levels in our cohort did not differ across enterotypes, Prev-enterotyped patients presented with a lower BMI than others and had a lower diastolic blood pressure than Bact2-enterotyped patients. These findings suggest an overall lower cardiovascular risk profile in Prev-enterotyped MS patients. Whether this observation refers to the reported associations between vascular health and MS outcomes, remains to be illucidated.[91,92]

Alternatively, *Prevotella* species—the main taxa of the so named enterotype—are propionate producers, and the *Prevotella* enterotype is associated with diets supporting production of propionic acid (PA) by the gut microbiota.[93] In EAE, SCFAs such as PA induce an increase of gut-associated regulatory T cells (Tregs) and a reduced systemic immune reaction, resulting in a milder disease course.[94-96] Also, PA levels have been found to be reduced in stool and blood of MS patients,[94,97] with PA supplementation resulting in a Treg increase, a Th17 and Th1 cell decrease in blood, as well as a milder MS disease course in comparison with a non-PA control group matched for age, sex, disease duration, disease severity, and DMT.[94]

### *A deconfounded QMP approach questions the replication of previously proposed MS targets, such as increased Akkermansia*

One of the major challenges in the MS microbiome field is heterogeneity among studies.[27] To contextualize our findings, we compiled results from all previously published case control studies with >50 MS

patients – with one using QMP[20] and one correcting for fecal moisture content[16] – and compared with our RMP and QMP findings before and after deconfounding.

Distinct associations were found for some taxa in function of covariates and/or QMP analysis.

Depletion of ***Bacteroides*** seems one of the most robust and replicated findings, as it was decreased in MS in several previous studies,[17,21,35] as well as in our deconfounded QMP approach. Some research suggests a potential beneficial, anti-inflammatory role for certain strains of the taxonomically diverse *Bacteroides* genus, highlighting its future target potential in immune-mediated diseases like MS. In diet-induced obese mice for example, *Bacteroides uniformis* exerted positive effects on metabolism and immunity.[98] Polysaccharide A (PSA) derived from *Bacteroides fragilis* has been shown to in vitro promote regulatory T cells and dampen inflammatory T-cell activity,[99] which could protect against the development of experimental autoimmune encephalomyelitis (EAE, the experimental mouse model of MS).[100] ***Blautia,*** increased in previous MS studies, was also increased with a high effect size in our deconfounded QMP approach. A recent study - not included in our compilation ($N < 50$) - also identified *Blautia wexlerae* as a major driver of the MS microbial community,[101] and showed that administration of a strain of *B. wexlerae* to EAE mice induced proinflammatory gut microbiota changes and worsened EAE. Literature on *Blautia* is quite divergent, with many studies reporting anti-inflammatory,[102,103] and other pro-inflammatory properties.[104] ***Methanobrevibacter***, found increased in previous studies[15,16] and known to negatively correlate with moisture,[22,105] remained significantly enriched independent of QMP and covariates, however with a relatively low effect size.

Contrary to the abovementioned findings, ***Akkermansia*** abundance—increased in MS across several studies, including our RMP analysis after deconfounding—was not detected as differentially abundant with QMP. Similarly, depletion of ***Roseburia*** in MS did not persist after correcting for covariates (specifically moisture).

Also contrasting with several studies,[27] we did not find *Prevotella* and *Faecalibacterium* lowered in MS. While *Faecalibacterium* was significantly decreased in MS in our RMP and QMP analysis, it disappeared after confounder control. Other butyrate producers, however, i.e., *Butyricicoccus* and *Butyricimonas,* were decreased in the MS cohort in our deconfounded QMP approach. These were not detected in most studies and warrant future mechanistic research. Blood levels of the SCFA butyric acid (BA) have been shown to be decreased in MS patients with higher disability, and to be negatively correlated with the MS severity score.[97] One of the ways in which SCFAs might exert their neuroprotective and anti-inflammatory properties is their ability to modulate glial cells and regulatory T cells.[106]

Specific to RRMS, we found *Collinsella* to be increased (however, with relatively low effect size), like in two previous studies in RRMS.[34,35] Interestingly, *Collinsella* has previously been associated with total-and LDL-cholesterol levels, and thus higher vascular risk, which in turn has been associated with worse MS outcomes.[91,92]

Overall, we do note that confounder-independent markers that are detected using RMP but not QMP might still be useful for diagnostic purposes. However, for mechanistic follow-up we do recommend to focus on only those markers that show quantitative (i.e., QMP) signals.

## *Faecalibacterium and Roseburia as candidate markers for progressive disease*

Though no significant difference in abundance was found between MS and controls, the butyric acid producers *Faecalibacterium* and *Roseburia* showed a moderate negative correlation with serum-GFAP, a serum marker of astrogliosis, a process occurring after central nervous system damage.[5] Serum-GFAP correlates with physical disability in progressive MS and is considered an important candidate marker of disease progression independent of relapses.[4] Yet, this association needs to be examined in larger cohorts with progressive MS, currently a group that is underrepresented in MS microbiome research, due to its naturally lower occurrence.[3]

## *Study limitations*

Limitations of our study include its cross-sectional nature, incompatible data on dietary habits in the control population, and the use of 16S rRNA gene sequencing, which lacks the taxonomic resolution to

gain functional and metabolic insights of the gut microbiome in MS, for which shotgun metagenomic sequencing is preferably applied. However, its strengths are that the cohort contains a relatively large group of clinically well-characterized MS patients with a substantial variety of metadata including clinical characteristics, blood biomarkers, and DMT, and the implementation of study-driven covariate adjustment, combined with a quantitative approach. Collectively, our study reveals novel insight into the gut microbiome in MS, which needs to be followed up to further guide microbiome-based approaches.

## Conclusion

To conclude, our study (re)affirms the importance of QMP methods and implementing adequate deconfounding, including moisture in (MS) microbiome-disease association studies, to identify robust candidates for future studies on gut microbiota biomarkers and treatment targets.

## Disclosure of potential conflicts of interest

J.R., M.D'H., G.F., and L.D. are inventors on the patent application PCT/EP2018/084920 in the name of VIB VZW, Katholieke Universiteit Leuven, KU Leuven R&D, and Vrije Universiteit Brussel covering the microbiome features associated with inflammation described in this article.

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Recruiting and clinical assessment of MS participants were performed by AP, AVR, and MDho. Sampling of MS participants was carried out by AP and AVR. Cleaning of metadata was done by AP and LD. Literature search for comparative analysis was done by AP and MDe. Laboratory experiments, flow cytometry analysis, preprocessing of raw sequencing data, and statistical analyses were carried out by LD.

First version of the manuscript was drafted by AP. Later versions of the manuscript were drafted by AP, LD, MDe, JR, and MDho, with further critical revision by GF, MDha, AVR, and GN. All authors approved the final version for publication.

These authors contributed equally: Ayla Pauwels, Lindsay Devolder.

These authors jointly supervised this work: Jeroen Raes and Marie D'hooghe.

## Author contributions

CRediT: **Ayla Pauwels:** Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing; **Lindsay Devolder:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing; **Gwen Falony:** Conceptualization, Methodology, Supervision, Writing – review & editing; **Miguel D'haeseleer:** Resources, Writing – review & editing; **Guy Nagels:** Resources, Writing – review & editing; **Ann Van Remoortel:** Project administration, Resources, Writing – review & editing; **Muriel Derrien:** Conceptualization, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing; **Marie D'hooghe:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing; **Jeroen Raes:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

## ORCID

Ayla Pauwels 0000-0002-7874-3168
Lindsay Devolder 0000-0002-1945-5166
Miguel D'haeseleer 0000-0003-4378-0089
Guy Nagels 0000-0002-2597-0383
Ann Van Remoortel 0000-0003-1392-2891
Muriel Derrien 0000-0001-8841-9153
Marie D'hooghe 0000-0002-0917-4176
Jeroen Raes 0000-0002-1337-041X

## Data availability statement

Raw amplicon sequencing data and associated limited metadata reported in this study have been deposited in the European Genome-phenome Archive with accession code EGAC00001003263. FGFP 16S rRNA gene sequencing data and metadata can be found under accession code EGAS00001003296. Given the sensitive nature of the data, an anonymized dataset necessary to replicate results will be made available under restricted access through EBI EGA at publication. This dataset will consist of sequencing data and a minimal subset of metadata, hereby ensuring compliance with data protection regulations and ethical considerations, and safeguarding participant/patient privacy.

## Code availability

An open source QMP R-script is available on http://www.raeslab.org/software/QMP and the Github repository (https://github.com/raeslab/QMP).

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