








## RESEARCH ARTICLE

# Gut microbiome variation is associated to Multiple Sclerosis phenotypic subtypes

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## Abstract

**Objective:** Multiple sclerosis (MS) is a heterogenous, inflammatory disease of the central nervous system. Microbiota alterations in MS versus healthy controls (HC) are observed, but results are inconsistent. We studied diversity, enterotypes, and specific gut microbial taxa variation between MS and HC, and between MS subgroups. **Methods:** Amplicon sequencing of the 16S ribosomal RNA V4 region (Illumina MiSeq) was used to evaluate alpha and beta diversity, enterotypes, and relative taxa abundances on stool samples. MS subgroups were based on phenotype, disease course modifiers, and treatment status. Results were controlled for recently identified confounders of microbiota composition. **Results:** Ninety-eight MS patients and 120 HC were included. Microbial richness was lower in interferon-treated (RRMS\_I,  $N = 24$ ) and untreated relapsing–remitting MS during relapse (RRMS\_R,  $N = 4$ ) when compared to benign (BMS,  $N = 20$ ;  $Z = -3.07$ ,  $P_{\text{corr}} = 0.032$  and  $Z = -2.68$ ,  $P_{\text{corr}} = 0.055$ ) and primary progressive MS (PPMS,  $N = 26$ ;  $Z = -2.39$ ,  $P_{\text{corr}} = 0.062$  and  $Z = -2.26$ ,  $P_{\text{corr}} = 0.071$ ). HC ( $N = 120$ ) and active untreated MS (RRMS\_U,  $N = 24$ ) showed intermediate microbial richness. Enterotypes were associated with clinical subgroups ( $N = 218$ ,  $\chi^2 = 36.10$ ,  $P = 0.002$ ), with *Bacteroides 2* enterotype being more prevalent in RRMS\_I. *Butyricoccus* abundance was lower in PPMS than in RRMS\_U ( $Z = -3.00$ ,  $P_{\text{corr}} = 0.014$ ) and BMS ( $Z = -2.56$ ,  $P_{\text{corr}} = 0.031$ ), lower in RRMS\_I than in BMS ( $Z = -2.50$ ,  $P_{\text{corr}} = 0.034$ ) and RRMS\_U ( $Z = -2.91$ ,  $P_{\text{corr}} = 0.013$ ), and inversely correlated with self-reported physical symptoms ( $\rho = -0.400$ ,  $P_{\text{corr}} = 0.001$ ) and disease severity ( $\rho = -0.223$ ,  $P = 0.027$ ). **Interpretation:** These results emphasize the importance of phenotypic subcategorization in MS-microbiome research, possibly explaining previous result heterogeneity, while showing the potential for specific microbiome-based biomarkers for disease activity and severity.

## Introduction

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS).<sup>1</sup> Patients experience a variety of physical and cognitive symptoms, including bowel dysfunction (>70%).<sup>2</sup> Depending on relapses and/or magnetic resonance imaging (MRI) activity, MS is considered active or nonactive.<sup>3</sup> Relapses are presumably provoked by adaptive immune cells infiltrating the CNS, resulting in focal inflammation and myelin loss.<sup>4</sup> Disability accumulation independent of relapses is suggestive of progression,<sup>3</sup> mainly due to axonal degeneration.<sup>5</sup> Gene–environment interactions are thought to be involved. A modulating role of the gut microbiota has been proposed because of its potential to influence brain development/physiology, regulate immunity,<sup>6,7</sup> and increase the odds of murine CNS-specific autoimmune disease.<sup>8,9</sup>

Relapsing–remitting (RR)MS patients were repeatedly shown to have an altered microbiota composition from healthy controls (HC); however, the identified taxa differed between studies.<sup>9–16</sup> Most studies were small scale and accounted for few confounders.<sup>10,14,16</sup> However, we recently identified >60 covariates affecting microbial composition,<sup>17</sup> among which stool consistency had the largest effect size on primary microbiome markers.<sup>17,18</sup> Although bowel dysfunction is prevalent in MS, this was never controlled for to date.

Controlling for such confounders, we conducted a cross-sectional study in a cohort of MS patients and HC, including MS patients with a presumed high (active RRMS) and low degree of focal CNS inflammation (benign MS (BMS)<sup>19,20</sup>) and nonactive primary progressive (PP)MS). Subgroups were defined by MS phenotype, disease course modifiers, and treatment status. We hypothesized to detect a gut microbial community with higher pro-inflammatory properties in MS subgroups with higher focal CNS inflammatory activity.

## Materials and Methods

### Study population

Patients with MS (2010 McDonald criteria<sup>21</sup>) were recruited in the National MS Center (NMSC), Melsbroek (in- and outpatients) and in the University Hospital, Brussels (outpatients) according to group-specific in- and exclusion criteria (Table 1). Five MS subgroups were pre-defined, including three subgroups with a presumed high degree of focal CNS inflammation: untreated active RRMS (RRMS\_U); untreated RRMS with a relapse at the time of sampling (RRMS\_R); and interferon (IFN) treated RRMS (RRMS\_I). Relapse was defined as new

neurological symptoms lasting  $\geq 24$  h, without fever or other triggers.<sup>22</sup> We selected one class of immune-modulatory drugs (IMD) to obtain a homogenous treatment group. IFN-beta was chosen because injectables are nonaggressive, spare the host's systemic immunity (less bias by a direct immune effect on the gut microbiota as opposed to oral therapy), and limit contact with a hospital environment (less bias compared to regular visits for intravenous therapy). The two subgroups with a presumed low degree of focal CNS inflammation were progressive onset MS without relapses 2 years prior (PPMS) and RRMS with  $\leq 3$  on the Expanded Disability Status Scale (EDSS) after a disease duration of  $\geq 15$  years (BMS).<sup>19,23</sup> We excluded patients with secondary progressive MS, due to the difficulty to separate inflammation from neurodegeneration. HC were recruited among participants' proxies, the HC database of the Metabolic

**Table 1.** In- and exclusion criteria. Untreated groups were untreated for at least 3 months before inclusion, irrespective of any treatment before this interval.

Inclusion criteria	
Untreated active RRMS (RRMS_U)	At least one clinical relapse during 2 years prior to screening, or at least one active, contrast-enhancing lesion on brain MRI in the year prior to screening
EDSS < 7.0	
Untreated active RRMS during relapse (RRMS_R)	Able to perform first data collection prior to corticosteroid therapy
EDSS < 7.0	
Untreated BMS (BMS)	EDSS $\leq 3.0$ at least 15 years after first MS symptoms <sup>19,20</sup>
IFN treated RRMS (RRMS_I)	At least 3 months of stable treatment with interferon beta
EDSS < 7.0	
Non-active PPMS (PPMS)	No clinical relapse within 2 years prior to screening
EDSS < 7.0	
Exclusion criteria for MS patients	
Secondary progressive MS patients	
Disease-modifying treatment other than IFN at screening	
Glatiramer acetate treatment within 3 months prior to screening	
Fingolimod or natalizumab treatment within 6 months prior to screening	
Systemic corticosteroid use within 2 months prior to screening	
Gastrointestinal conditions such as IBD	
Antibiotic use in the 4 weeks prior to screening	
Exclusion criteria for healthy controls	
Systemic corticosteroid use within 2 months prior to screening	
Gastrointestinal conditions such as IBD	
Other diseases of the central nervous system	
Antibiotic use in the 4 weeks prior to screening	
Direct relation to or living with a participant in one of the MS groups	

BMS, benign MS; EDSS, expanded disease status scale; IBD, inflammatory bowel disease; IFN, interferon beta; PPMS, primary progressive MS; RRMS, relapsing–remitting MS.

Department in the University Hospital Brussels, and (para)medical staff in the same geographical regions as MS participants. The HC group was selected to represent age and sex distributions in the whole MS study population. Samples from the Flemish Gut Flora Project (FGFP<sup>17</sup>), an independent, large, and diverse population study on the gut microbiome in Flanders, were added to this HC group and matched for age, sex, BMI, and the Bristol Stool Scale (BSS<sup>24</sup>) against the entire MS population. This allowed to project our results on an independent and representative sample in the same geographical area. The ethics committee of the University Hospital, Brussels, and the local ethics committee of the NMSC, Melsbroek, approved the study (B.U.N. 143201317985), which is in compliance with the principles of the 2013 Declaration of Helsinki. All participants provided written informed consent.

## Study design

During study setup, sample size calculation and power analysis could not be performed because effect size estimates for the gut microbiome were lacking. Following previous recommendations,<sup>25,26</sup> we aimed to include 30 patients per subgroup (and additional FGFP samples for HC). Potential confounders were assessed following FGFP protocols,<sup>17</sup> covering anthropometrics, general health, medication, dietary and bowel habits, lifestyle, fertility, and MS history. Participants noted date and time of stool sampling, time since last defecation, and scored stool consistency (BSS). Participants were further assessed using Age-Related Multiple Sclerosis Severity score (ARMSS,<sup>27</sup> i.e. age-corrected EDSS ranking), Brief-H-Neg Hopelessness scale,<sup>28</sup> Brief Illness Perception Questionnaire (IPQ-K<sup>29</sup>; MS only), three-level EuroQol Five Dimensions' questionnaire (EQ-5D<sup>30</sup>), Fatigue Scale for Motor and Cognitive Functions (FSMC), Hospital Anxiety and Depression Scale (HADS), Hauser Ambulation index (HAI), and Timed 25 Foot Walk Test (T25FW).

The primary objective was to explore whether gut microbiome diversity (alpha and beta), enterotype distribution, and microbial genus abundances were related to MS diagnosis (MS and MS subgroups vs. all HC, respectively) and/or differences in disease course (MS subgroups). Secondary objectives were to determine which genera abundances differed according to IFN treatment (RRMS\_U vs. RRMS\_I), and whether certain genera were associated with disease characteristics and clinical assessments.

## Stool sample collection and processing

Fecal collection kits (including instructions following established protocols<sup>17,18</sup>) were provided during

consultation. Samples were stored at  $-20^{\circ}\text{C}$  immediately after sampling, in the participants' home freezer or at the NMSC, and transferred on dry ice to  $-80^{\circ}\text{C}$  within 48 h. Extraction of microbial DNA from frozen aliquots (150–200 mg) was performed using an adapted Mobio PowerMicrobiome DNA/RNA isolation Kit-based protocol.<sup>17,18</sup> Bacterial DNA was quantified via fluorometry (Qubit, Life Technologies). The hypervariable 4 region (V4) of the 16S rRNA gene was PCR amplified (515F/806R primer set<sup>31</sup>), with Illumina sequencing adaptors and dual-index barcodes to generate dual-barcoded libraries. PCR amplicon quantification was carried out with Fragment Analyzer (Advanced Analytical Technologies) and sequencing on the Illumina MiSeq platform (MiSeq V2 sequencing kit), generating 250 bp paired-end reads. Following demultiplexing of Illumina sequencing data, fastq sequences were merged with FLASH software version (v) 1.2.10.<sup>32</sup> Sequences with a quality score  $<25$  ( $>90\%$  of read length) were excluded from analysis (FASTX-Toolkit v0.0.14, [http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)). Chimeric sequences were removed using the UCHIME algorithm in USEARCH v6.0.307.<sup>33</sup> Taxonomical sequence assignment was performed using the Ribosomal Database Project (RDP) classifier v2.12.<sup>34</sup> Phylum-to-genus matrices were created using custom Perl scripts. Samples were rarefied to 10,000 randomly selected reads.

## Statistical analysis

Data analysis was performed in R (v3.3.1<sup>35</sup>). After normality testing (Shapiro–Wilks test), demographics and clinical data were compared between groups with one-way ANOVA or Wilcoxon rank-sum and Kruskal–Wallis H tests, followed by a post hoc Dunn test (*FSA* R package<sup>36</sup>). Microbiota richness was determined using observed richness ( $S_{\text{obs}}$ ; i.e. total number of genera detected per sample), Pielou's evenness index (i.e. expression of how evenly genera are distributed within one sample), and Simpson's alpha diversity index (i.e. measure of alpha diversity including genus richness and evenness) using *vegan*<sup>37</sup> and *phyloseq*.<sup>38</sup> Group differences were tested with Wilcoxon rank-sum or Kruskal–Wallis tests (with post hoc Dunn test). Beta diversity (i.e. difference in global microbiota composition between samples) was visualized using Principal Coordinate Analysis (PCoA) on genus-level community composition (Bray–Curtis dissimilarity). The *adonis* function (*vegan*) was used to test for differences in community structure between groups, and *betadisper* (*vegan*) to verify whether differences were due to dissimilar dispersions among groups. Variables ( $N = 732$ ) were filtered by report rate in the study population ( $\geq 10\%$ ). Next, when  $r > |0.8|$  using Pearson correlation (*Hmisc*<sup>39</sup>), the variable with the lowest effect size

on genus-level community variation (adjusted R<sup>2</sup>, *vegan's capscale*) was removed. Variables with the highest effect sizes in their category were retained. The 33 remaining metadata variables were used in further analysis. Forward stepwise distance-based redundancy analysis (RDA, *vegan's ordiR2step*) was performed to determine nonredundant microbiome covariates. FGFP individuals were excluded during RDA and correlation analysis with clinical variables, because not all clinical variables used in this study were also assessed in the FGFP cohort. In all other analyses, the FGFP individuals were analyzed alongside the original HC group, whenever the HC group was included. Enterotypes were determined with Dirichlet Multinomial Mixtures (DMM, *DirichletMultinomial* package<sup>40</sup>), using Bayesian Information Criterion (BIC) to calculate the optimal number of clusters.<sup>41</sup> To increase accuracy, enterotyping was performed on a combined genus abundance matrix that included samples of this study and 2999 samples from the FGFP.<sup>17</sup> Clusters were named *Bacteroides* 1 (B1), *Bacteroides* 2 (B2), *Prevotella* (P), and Ruminococcaceae (R) based on enterotype-discriminating predominant taxa.<sup>42</sup> B2 has been characterized by high *Bacteroides* and low *Faecalibacterium* abundances and lowered cell density.<sup>42</sup> Associations between enterotypes and groups were tested using pairwise chi-square tests (*chi.sq.post.hoc*, *fifer* package<sup>43</sup>). Differences in relative genera abundances were tested using Wilcoxon rank-sum and Kruskal–Wallis tests (with post hoc Dunn test). Genus abundance and correlation analysis were restricted to genera with a mean relative abundance  $\geq 0.0001$  (mean  $\geq 1$  in 10,000 reads) and present in  $\geq 20\%$  of samples. Taxa unclassified at genus level were excluded. Where appropriate, analyses were corrected for multiple testing (Benjamini–Hochberg (BH) procedure,<sup>44</sup> FDR) resulting in corrected *P*-values (*P*corr). A false discovery rate (FDR) of  $< 0.1$  was considered statistically significant. Multivariate analysis was performed using nested generalized linear models (GLM, *glm* function). Subgroup comparisons were controlled for stool consistency (BSS), age, sex, and BMI. This was not done for the MS versus HC comparison due to matching at baseline. Adjusted *P*-values (*Padj*) were reported. Standardized regression coefficients were calculated (*lm.beta* function, *QuantPsysc* package<sup>45</sup>). Associations between genera abundances and continuous metadata (with removal of HC for MS-specific scores) were tested using Spearman correlations.

## Results

### Population

From February 2014 to October 2015, 118 MS patients and 30 HC were recruited and delivered stool samples.

**Table 2.** Demographics of the participants included in microbiota analysis. *P*corr = *P*-value corrected for multiple testing (Benjamini–Hochberg, FDR).

	HC total	MS total	<i>P</i>	HC	FGFP	BMS	RRMS_U	RRMS_J	RRMS_R	PPMS	<i>P</i> corr
Number	120	98		22	98	20	24	24	4	26	
Median/mean <sup>4</sup> age (y) (iqr/SD)	49.0 (14.3)	48.0 (13.8)	$> 0.05^1$	54.5 (9.0)	48.0 (14.0)	48.7 (7.5)	44.5 (10.5)	44.0 (7.9)	41.5 (5.1)	51.8 (8.9)	$> 0.001^2$
Female (%)	61.7	60.2	$> 0.05^1$	68.0	60.0	65.0	62.5	75.0	50.0	42.3	$> 0.05^3$
Median BMI (iqr)	23.7 (4.0)	23.6 (4.9)	$> 0.05^1$	24.9 (4.6)	23.5 (4.1)	23.5 (3.4)	23.3 (4.3)	23.9 (5.8)	20.9 (4.6)	24.5 (4.8)	$> 0.05^3$
Median EDSS (iqr)	–	3.0 (3.5)	–	–	–	2.25 (1.0)	2.0 (1.1)	3.0 (2.6)	4.5 (1.9)	6.0 (2.4)	$< 0.001^3$
Median disease duration (y) (iqr)	–	12.5 (12.0)	–	–	–	18.0 (7.0)	6.0 (7.3)	12.5 (12.3)	11.0 (8.3)	8.0 (11.3)	0.001 <sup>3</sup>
Median BSS (iqr)	4 (1.0)	4 (1.0)	$> 0.05^1$	4 (0.8)	4 (1.0)	4 (1.0)	4 (2.0)	4 (2.0)	3 (2.3)	4 (1.0)	–
Inpatient setting or working in a hospital (%) <sup>5</sup>	11 (9.2)	25 (25.5)	–	11 (9.2)	NA	3 (14.3)	4 (16.7)	5 (21.7)	1 (25.0)	12 (46.2)	–

HC, healthy controls; BMS, benign multiple sclerosis; FGFP, Flemish Gut Flora Project; RRMS\_U, untreated active RRMS; RRMS\_J, interferon-beta-treated RRMS; RRMS\_R, RRMS during relapse; PPMS, primary progressive MS; BMI, body mass index; EDSS, expanded disease status scale; BSS, Bristol stool score; iqr, interquartile range; SD, standard deviation; NA, not available.

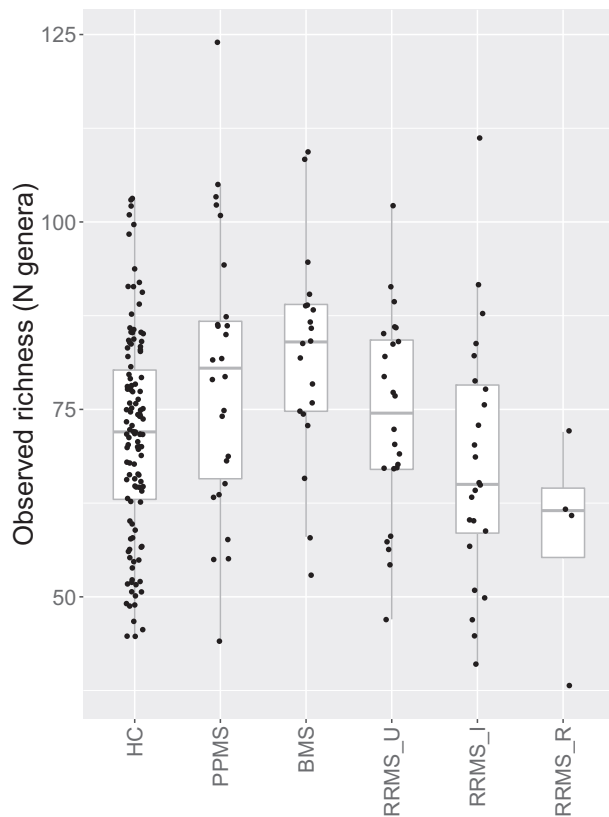
<sup>1</sup>Wilcoxon rank sum test with continuity correction.

<sup>2</sup>One-way ANOVA.

<sup>3</sup>Kruskal–Wallis H test.

<sup>4</sup>Median (interquartile range) and mean (standard deviation) values, depending on results from the Shapiro–Wilk normality test.

<sup>5</sup>No data available for the FGFP controls.



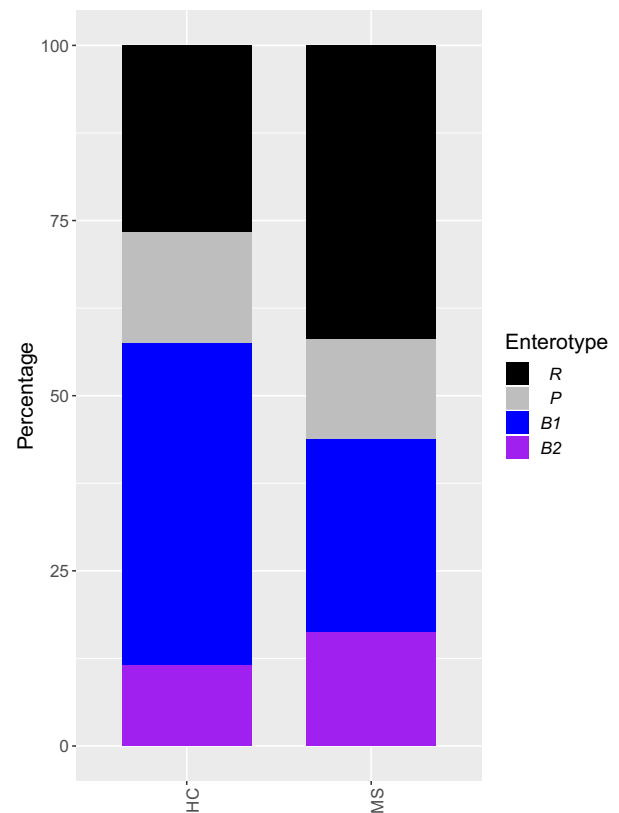
**Figure 1.** Boxplot of observed richness within patients ( $N = 98$ ) and healthy controls ( $N = 120$ , including FGFP samples). Downward trend of observed richness within the relapsing–remitting patient population. The body of the boxplot represents the first and third quartiles of the distribution and median line. The whiskers extend to the last data point within 1.5 times the interquartile range. The outliers lie beyond. Individual data points (dots) may overlap. FGFP, Flemish Gut Flora Project. HC, healthy controls ( $N = 120$ ). PPMS, primary progressive multiple sclerosis ( $N = 26$ ). BMS, benign multiple sclerosis ( $N = 20$ ). RRMS\_U, untreated active RRMS ( $N = 24$ ). RRMS\_I, interferon-beta-treated RRMS ( $N = 24$ ). RRMS\_R, RRMS during a relapse ( $N = 4$ ). Kruskal–Wallis test ( $N = 218$ ,  $\chi^2 = 19.24$ ,  $P_{\text{corr}} = 0.007$ ) with post hoc Dunn tests.

Data from 120 participants (98 MS and 22 HC) was retained for further analysis with 18.9% of samples having insufficient quality-checked reads. Ninety-eight samples from the FGFP were added to the HC pool. No significant differences were found in age, sex, BMI, and BSS between overall MS and HC (Table 2). However, age, EDSS, and disease duration differed among MS subgroups. Higher age and EDSS were seen in PPMS compared to RRMS\_U (Dunn test,  $N = 120$ ,  $Z = 2.74$  and  $4.86$ ,  $P_{\text{corr}} = 0.030$  and  $<0.001$ ) and RRMS\_I ( $Z = 3.16$  and  $3.44$ ,  $P_{\text{corr}} = 0.016$  and  $0.002$ ), and a higher age compared to RRMS\_R ( $Z = 2.36$ ,  $P_{\text{corr}} = 0.061$ ). BMS patients had lower EDSS than PPMS and RRMS\_I

**Table 3.** Differences in observed richness between subgroups. Only significant results are shown. Reported  $P$ -values after Dunn test, with Benjamini–Hochberg corrected  $P$ -values (Kruskal–Wallis test,  $N = 218$ ,  $\chi^2 = 19.24$ ,  $P_{\text{corr}} = 0.007$ ).

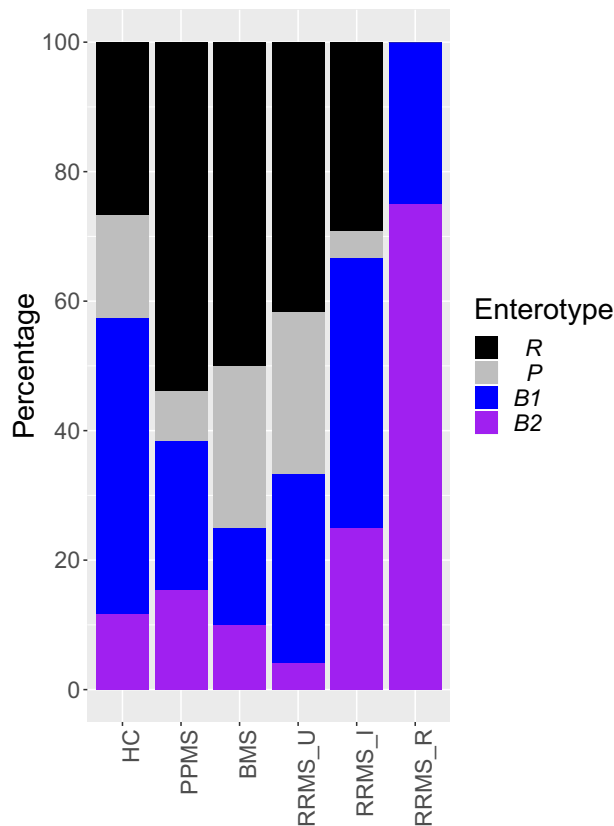
Comparison	$N$	Z-score	$P_{\text{corr}}$
BMS-RRMS_I	218	3.30	0.015
BMS-RRMS_R	218	2.86	0.021
BMS-HC	218	3.01	0.019
PPMS-RRMS_I	218	2.55	0.040
PPMS-RRMS_R	218	2.40	0.049
PPMS-HC	218	-2.09	0.092

HC, healthy controls ( $N = 120$ , including FGFP samples); PPMS, primary progressive multiple sclerosis ( $N = 26$ ); BMS, benign multiple sclerosis ( $N = 20$ ); RRMS\_I, interferon-beta-treated RRMS ( $N = 24$ ); RRMS\_R, RRMS during a relapse ( $N = 4$ );  $P$ , uncorrected  $P$ -value;  $P_{\text{corr}}$ ,  $P$ -value corrected for multiple comparisons with Benjamini–Hochberg, FDR.



**Figure 2.** Bar plot illustrating the distribution of patient and control samples (MS vs. HC) over four enterotypes: *Prevotella* (P,  $N = 33$ ), Ruminococcaceae (R,  $N = 73$ ), *Bacteroides* 1 (B1,  $N = 81$ ), and *Bacteroides* 2 (B2,  $N = 31$ ). FGFP, Flemish Gut Flora Project. HC, healthy controls ( $N = 120$ , including FGFP samples). MS, multiple sclerosis ( $N = 98$ ). Pearson's chi-square test for independence,  $N = 218$ ,  $\chi^2 = 36.10$ ,  $P = 0.002$  with pairwise chi-square tests.

( $Z = -5.56$  and  $2.25$ ,  $P_{\text{corr}} < 0.001$  and  $0.062$ ), and longer disease duration than PPMS and RRMS\_U ( $Z = 2.79$  and  $4.02$ ,  $P_{\text{corr}} = 0.026$  and  $<0.001$ ). RRMS\_I



**Figure 3.** Bar plot illustrating the distribution of patient and control samples (comparison between subgroups) over four enterotypes: *Prevotella* (P,  $N = 33$ ), Ruminococcaceae (R,  $N = 73$ ), *Bacteroides* 1 (B1,  $N = 81$ ), and *Bacteroides* 2 (B2,  $N = 31$ ). FGFP, Flemish Gut Flora Project. HC, healthy controls ( $N = 120$ , including FGFP samples). PPMS, primary progressive multiple sclerosis ( $N = 26$ ). BMS, benign multiple sclerosis ( $N = 20$ ). RRMS\_U, untreated active RRMS ( $N = 24$ ). RRMS\_I, interferon-beta-treated RRMS ( $N = 24$ ). RRMS\_R, RRMS during relapse ( $N = 4$ ). Pearson's chi-square test for independence,  $N = 218$ ,  $\chi^2 = 36.10$ ,  $P = 0.002$  with pairwise chi-square tests.

had longer disease duration than RRMS\_U ( $Z = 2.63$ ,  $P_{\text{corr}} = 0.029$ ).

### Alpha and beta diversity

$S_{\text{obs}}$  varied greatly within our population (Kruskal–Wallis test,  $N = 218$ ,  $\chi^2 = 19.24$ ,  $P_{\text{corr}} = 0.007$ ), with a downward trend from BMS to RRMS\_U, RRMS\_I, and RRMS\_R (Fig. 1) (Table 3). Simpson's alpha diversity index followed a similar trend. A comparison between the original HC group and the FGFP sample is made in Figure S1. There were no significant differences in Pielou's index between subgroups (Kruskal–Wallis test,  $N = 218$ ,  $P_{\text{corr}} > 0.5$ ), nor in  $S_{\text{obs}}$ , Simpson and Pielou indices between overall MS and HC (Wilcoxon rank-sum test,  $N = 218$ ,  $P_{\text{corr}} > 0.5$ ). There were no significant correlations with metadata.

Global microbial composition differed between MS and HC (*adonis* test on beta diversity,  $N = 218$ ,  $R_{2\text{adj}} = 0.004$ ,  $P = 0.027$ ), and between subgroups ( $N = 218$ ,  $R_{2\text{adj}} = 0.016$ ,  $P = 0.003$ ). Excluding HC ( $N = 120$ ), 2.4% of beta diversity variation was explained by allocation to an MS subgroup ( $N = 98$ ,  $R_{2\text{adj}} = 0.024$ ,  $P = 0.012$ ), largely driven by RRMS\_I ( $N = 24$ ). Subcutaneous IFN beta-1-a use (RDA,  $N = 120$ ,  $R_{2\text{adj}} = 0.024$ ,  $P_{\text{corr}} = 0.022$ ), BSS ( $R_{2\text{adj}} = 0.023$ ,  $P_{\text{corr}} = 0.022$ ), subgroup ( $R_{2\text{adj}} = 0.018$ ,  $P_{\text{corr}} = 0.092$ ), weight ( $R_{2\text{adj}} = 0.017$ ,  $P_{\text{corr}} = 0.022$ ), inpatient recruitment ( $R_{2\text{adj}} = 0.014$ ,  $P_{\text{corr}} = 0.056$ ), mobility (25-FWT;  $R_{2\text{adj}} = 0.014$ ,  $P_{\text{corr}} = 0.056$ ), sleep ( $R_{2\text{adj}} = 0.012$ ,  $P_{\text{corr}} = 0.056$ ), hopelessness ( $R_{2\text{adj}} = 0.010$ ,  $P_{\text{corr}} = 0.090$ ), and EQ-5D ( $R_{2\text{adj}} = 0.009$ ,  $P_{\text{corr}} = 0.092$ ) were identified as covariates of microbiome variation. BSS had the highest nonredundant effect size on community variation (2.6%), followed by subcutaneous IFN beta-1-a use, weight, the first hopelessness question, daily amount of sleep, and the fifth EQ-5D question.

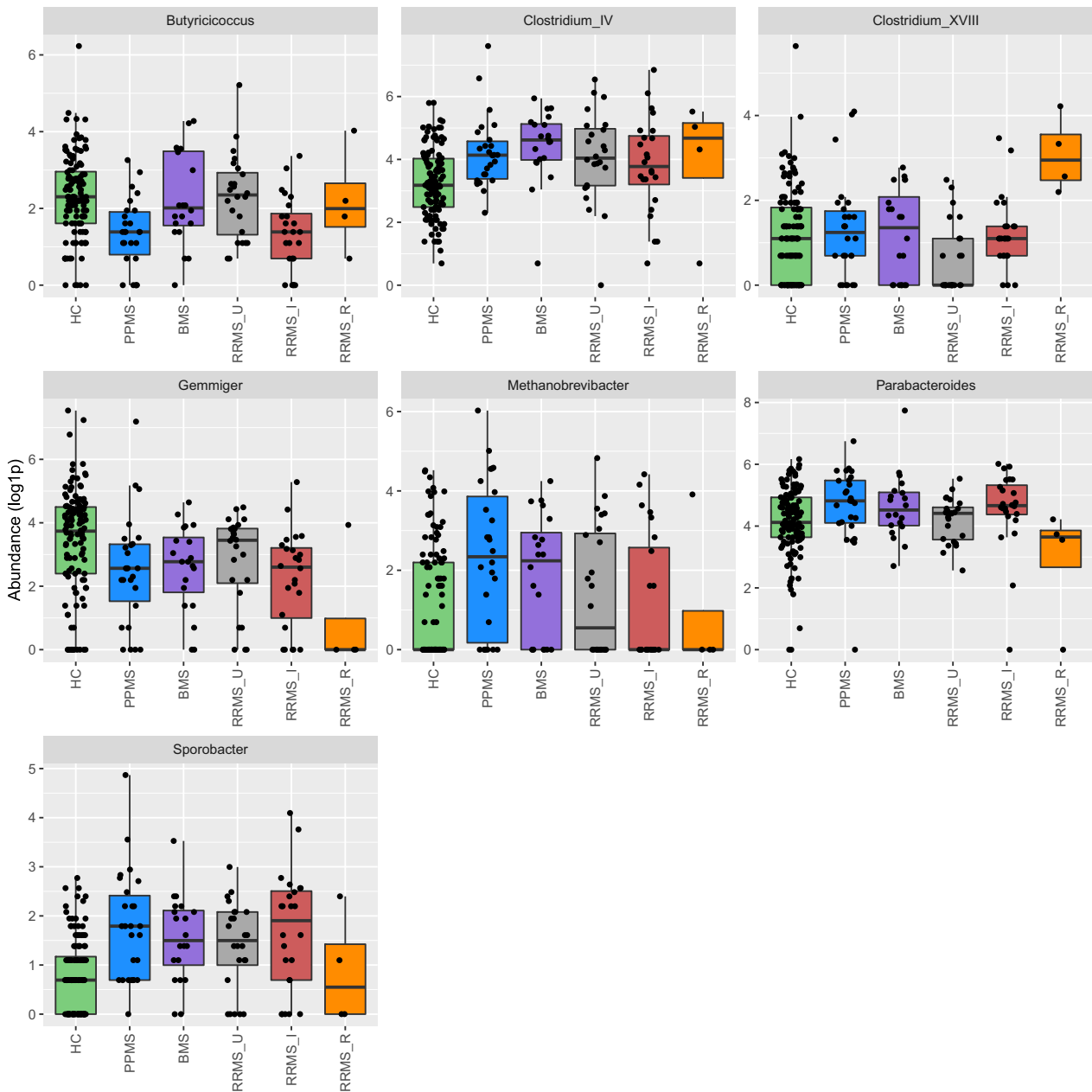
### Enterotypes

Enterotypes were differentially distributed in MS patients versus HC (Pearson's chi-square test for independence,  $N = 218$ ,  $\chi^2 = 8.77$ ,  $P = 0.032$ ), with MS containing the highest proportion of B2 and lowest proportion of B1

**Table 4.** Compositional differences between all MS patients and HC. Only significant results are shown. Reported  $P$ -values after Wilcoxon rank-sum test, with Benjamini–Hochberg corrected  $P$ -values (Wilcoxon rank-sum test,  $N = 218$ ,  $P_{\text{corr}} < 0.1$ ).

Genus	$N$	Effect size	Median MS	Median HC	$P$	$P_{\text{corr}}$
<i>Alistipes</i>	218	-0.18	5.24	4.72	0.007	0.056
<i>Anaerotruncus</i>	218	-0.16	0.69	0	0.016	0.080
<i>Butyrivibrio</i>	218	-0.24	1.7	2.3	<0.001	0.007
<i>Clostridium</i> cluster IV	218	-0.35	4.14	3.18	<0.001	<0.001
<i>Faecalicoccus</i>	218	-0.16	0	0	0.017	0.080
<i>Gemmiger</i>	218	-0.30	2.71	3.73	<0.001	<0.001
<i>Intestinibacter</i>	218	-0.21	0.69	1.79	0.002	0.029
<i>Lactobacillus</i>	218	-0.18	0.69	0	0.009	0.065
<i>Methanobrevibacter</i>	218	-0.20	1.61	0	0.004	0.037
<i>Olsenella</i>	218	-0.19	0.69	0	0.006	0.049
<i>Parabacteroides</i>	218	-0.15	4.56	4.12	0.022	0.097
<i>Roseburia</i>	218	-0.17	6.36	6.71	0.014	0.078
<i>Ruminococcus</i>	218	-0.17	4.86	4.52	0.014	0.078
<i>Sporobacter</i>	218	-0.39	1.61	0.69	<0.001	<0.001

HC, healthy controls ( $N = 120$ , including FGFP samples); MS, multiple sclerosis ( $N = 98$ ); Effect size ( $Z/\sqrt{N}$ ); Median MS, median abundance of genus ( $\log_{10}$  transformed) for MS patients; Median HC, median abundances of genus ( $\log_{10}$  transformed) for HC;  $P$ , uncorrected  $P$ -value;  $P_{\text{corr}}$ ,  $P$ -value corrected for multiple comparisons with Benjamini–Hochberg, FDR.



**Figure 4.** Boxplots illustrating the log<sub>1p</sub> abundances of *Butyricoccus*, *Clostridium* cluster XVIII, *Methanobrevibacter*, *Parabacteroides*, *Clostridium* cluster IV, *Gemmiger*, and *Sporobacter* genera within the population. Abundances were log<sub>1p</sub>-transformed for graphic purposes. Individual data points (dots) may overlap. HC, healthy controls ( $N = 120$ , including FGFP samples). PPMS, primary progressive multiple sclerosis ( $N = 26$ ). BMS, benign multiple sclerosis ( $N = 20$ ). RRMS\_U, untreated active RRMS ( $N = 24$ ). RRMS\_I, interferon-beta-treated RRMS ( $N = 24$ ). RRMS\_R, RRMS during relapse ( $N = 4$ ). Kruskal–Wallis test ( $N = 218$ ,  $P_{corr} < 0.1$ ) with post hoc Dunn tests.

(Fig. 2); and among clinical subgroups ( $N = 218$ ,  $\chi^2 = 36.10$ ,  $P = 0.002$ ; Fig. 3). A comparison between the original HC group and the FGFP sample is made in Figure S2. B2 had a significantly different subgroup distribution compared to R and P (Post hoc pairwise chi-square test,  $N = 218$ ,  $\chi^2 = 12.57$  and  $12.52$ ,  $P_{corr} = 0.047$  for both). Among MS subgroups, most B2-enterotyped

individuals ( $N = 31$ ) either belonged to RRMS\_I (37.5%) or RRMS\_R (18.8%), while P ( $N = 33$ ) and R ( $N = 73$ ) clusters consisted of more BMS (35.7% and 24.3%) and RRMS\_U (42.9% and 24.3%).

Reversely, we found significantly different enterotype distributions between RRMS\_R and RRMS\_U and HC (pairwise chi-square test,  $N = 218$ ,  $\chi^2 = 14.73$  and  $12.47$ ,

**Table 5.** Multivariate analysis with nested generalized linear models taking into account Bristol stool score, age, sex, and BMI. Reported effect sizes and *P*-values after Dunn and chi-squared tests, with *P*-values corrected for multiple comparisons with Benjamini–Hochberg.

Genus	Comparison	<i>N</i>	Z-score Dunn	<i>P</i> corr Dunn	SC	<i>P</i>	<i>P</i> corr
<i>Butyricoccus</i>	PPMS–BMS	46	2.56	0.03	−1.83	0.020	0.030
	PPMS–RRMS_U	50	−2.99	0.01	−2.94	<0.001	0.003
	PPMS–HC	146	4.01	<0.001	2.93	<0.001	<0.001
	RRMS_I–BMS	44	2.50	0.03	−2.35	0.008	0.012
	RRMS_I–RRMS_U	48	−2.91	0.01	−2.54	0.002	0.004
<i>Clostridium</i> cluster IV	RRMS_I–HC	144	−3.86	<0.001	2.62	<0.001	0.002
	BMS–HC	140	4.32	<0.001	−3.26	<0.001	<0.001
	PPMS–HC	146	3.33	0.006	−1.92	0.002	0.005
<i>Clostridium</i> cluster XVIII	RRMS_U–HC	144	−3.02	0.012	−2.36	<0.001	0.002
	RRMS_U–BMS	44	−2.11	0.058	1.86	0.023	0.030
	RRMS_U–PPMS	50	−2.80	0.037	2.01	0.008	0.012
<i>Gemmiger</i>	RRMS_U–RRMS_I	48	−2.32	0.038	1.57	0.023	0.030
	RRMS_U–HC	144	−2.82	0.024	2.20	0.004	0.007
	BMS–HC	140	−2.30	0.081	1.57	0.034	0.041
<i>Methanobrevibacter</i>	PPMS–HC	146	−2.92	0.027	−1.18	0.052	0.057
	RRMS_I–HC	144	−3.39	0.011	2.14	0.002	0.004
	PPMS–RRMS_I	50	2.56	0.078	−1.70	0.045	0.051
<i>Parabacteroides</i>	PPMS–HC	146	−3.65	0.004	−1.80	0.002	0.005
	RRMS_I–RRMS_U	48	2.00	0.098	0.89	0.236	0.236
	RRMS_I–HC	144	2.44	0.004	−1.55	0.064	0.067
<i>Sporobacter</i>	BMS–HC	140	3.31	0.005	−2.29	0.003	0.006
	PPMS–HC	146	4.30	<0.001	−3.64	<0.001	<0.001
	RRMS_I–HC	144	3.75	0.001	−3.80	<0.001	<0.001
	RRMS_U–HC	144	2.99	0.010	−2.21	<0.001	0.002

HC, healthy controls (*N* = 120, including FGFP samples); PPMS, primary progressive multiple sclerosis (*N* = 26); BMS, benign multiple sclerosis (*N* = 20); RRMS\_U, untreated active RRMS (*N* = 24); RRMS\_I, interferon-beta-treated RRMS (*N* = 24); RRMS\_R, RRMS during a relapse (*N* = 4); SC, standard coefficient (beta); *P*, uncorrected *P*-value; *P*corr, *P*-value corrected for multiple comparisons with Benjamini–Hochberg, FDR.

*P*corr = 0.031 and 0.045), between HC and PPMS and BMS (*N* = 218,  $\chi^2 = 8.63$  and 7.83, *P*corr = 0.097 for both), between PPMS and RRMS\_R (*N* = 218,  $\chi^2 = 7.75$ , *P*corr = 0.097), between BMS and RRMS\_I and RRMS\_R (*N* = 218,  $\chi^2 = 8.67$  and 9.96, *P*corr = 0.097 and 0.095), and between RRMS\_U and RRMS\_I (*N* = 218,  $\chi^2 = 8.20$ , *P*corr = 0.097). All but one RRMS\_R patient had a B2 enterotype. The majority of RRMS\_I had B1 (41.67%) or B2 (25%) enterotypes. Graphics showing the mean genera distribution within enterotypes as well as genus level hits per sample per (sub)group are in Figures S3–S10.

### Compositional differences

Fourteen microbial genera were differentially abundant between MS and HC (Wilcoxon rank-sum test, *N* = 218, *P*corr < 0.1) (Table 4). Seven were differentially abundant among subgroups (Kruskal–Wallis test, *N* = 218, *P*corr < 0.1) (Fig. 4). Post hoc Dunn test and nested GLM results are shown in Table 5. The small number of RRMS\_R participants (*N* = 4) did not allow further statistical testing. *Sporobacter*, *Clostridium* cluster IV, and

*Ruminococcus* were more abundant in the original HC group than in the FGFP sample (GLM, *N* = 120, *P*adj < 0.05) (Fig. S11). Median values for the relative abundances of OTU results in MS and HC groups are available in Tables S1 and S2.

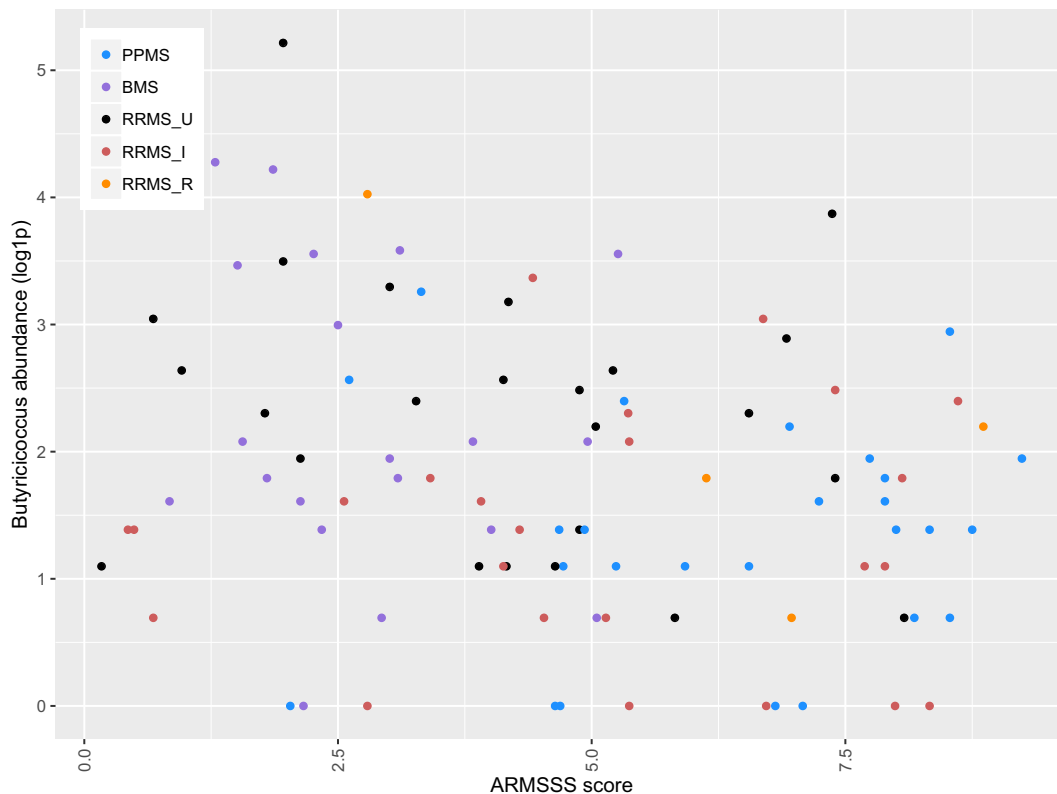
### Correlations with clinical variables

*Butyricoccus* abundance inversely correlated with ARMSS (Spearman correlation test, *N* = 98, rho = −0.223, *P* = 0.027) (Fig. 5), and IPQ-K5 (rho = −0.400, *P* = 0.001) (Fig. 6). One male RRMS\_U participant with the shortest time since diagnosis among RRMS participants had the highest *Butyricoccus* abundance. No correlations were found between genera and other clinical data.

### Discussion

No gut microbial diversity measure significantly differed between MS and HC. However, multiple microbiome readouts differed between MS subgroups, supporting our





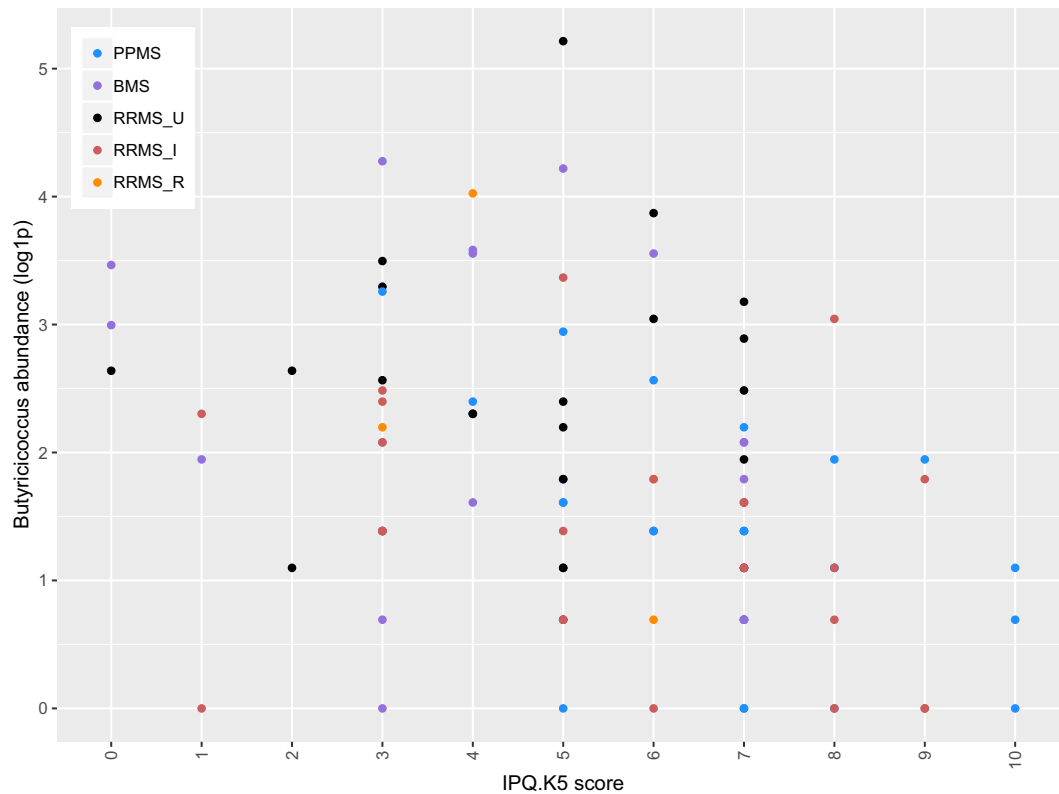
**Figure 5.** Scatterplot illustrating the abundance of *Butyricococcus* (log<sub>1p</sub>-transformed for graphical purposes) according to the ARMSS score of MS patients. ARMSS, age-related multiple sclerosis severity. PPMS, primary progressive multiple sclerosis ( $N = 26$ ). BMS, benign multiple sclerosis ( $N = 20$ ). RRMS\_U, untreated active RRMS ( $N = 24$ ). RRMS\_I, interferon-beta-treated RRMS ( $N = 24$ ). RRMS\_R, RRMS during relapse ( $N = 4$ ). Spearman correlation test,  $N = 98$ ,  $\rho = -0.223$ ,  $P = 0.027$ .

hypothesis of heterogeneity. First,  $S_{\text{obs}}$  was inversely related to the presumed degree of inflammatory disease activity in MS subgroups: highest in BMS and PPMS and lowest in RRMS\_R. The lower  $S_{\text{obs}}$  in RRMS\_I might be explained by the higher amount of CNS inflammation for which treatment was initiated. At the group level, BMS and PPMS patients had a *higher* richness than controls (including the FGFP sample). That  $S_{\text{obs}}$  in HC on average appears to be in between the  $S_{\text{obs}}$  in active (RRMS\_I and RRMS\_R) and nonactive MS groups (PPMS and BMS) is a novel finding,<sup>17,18</sup> which cannot be explained by major confounding factors (e.g. no differences in BSS to account for constipation). What the higher  $S_{\text{obs}}$  in PPMS and BMS patients when compared with HC exactly means, remains uncertain. A recent paper warned against basing health-related conclusions on richness alone, as this could just be indicative of gut ecosystem age (i.e. the time the same ecosystem has been in place depending on the transit time).<sup>46</sup> Additionally, approximately 2.4% of interindividual microbiota compositional variation could be explained by allocation to a subgroup. Previous MS gut microbiota studies mainly focused on RRMS,<sup>9–16</sup> where the lack of a common observed signal is probably

explained by the observed intergroup differences and distinct methodological issues.

Second, a high proportion of MS patients had a B2 enterotype. This was most pronounced in RRMS\_I and RRMS\_R patients, which suggests an association with higher presumed inflammatory activity. However, enterotype distributions of RRMS\_U and BMS were not significantly different, even though they differ in inflammatory disease activity. The relationship between enterotypes and MS phenotypes, and the interplay between IFN and B2 remain to be elucidated. This is the first time that an enterotype is associated to (a specific subgroup of) MS. As this B2 enterotype was associated to Crohn's disease,<sup>42</sup> our results warrant further research on overlapping host-microbiome interactions in immune-mediated inflammatory diseases.

Third, we observed differences in microbial taxa. Results for *Faecalicoccus*,<sup>47</sup> *Methanobrevibacter*,<sup>10,13</sup> *Ruminococcus*,<sup>16</sup> and *Gemmiger*<sup>47</sup> confirm previous findings. While *Alistipes* and *Anaerotruncus* abundances reflect findings in a PPMS mouse model,<sup>48</sup> we are the first to find these differentially abundant in RRMS. Not in line with other studies, *Lactobacillus*,<sup>14</sup> *Parabacteroides*,<sup>14</sup>



**Figure 6.** Scatterplot illustrating the abundance of *Butyricococcus* according to the degree of physical symptoms patients report in the fifth question of the brief illness perception questionnaire (IPQ-K5: 0 = none, 10 = multiple and serious symptoms). The outlier is explained in text. PPMS, primary progressive multiple sclerosis ( $N = 26$ ). BMS, benign multiple sclerosis ( $N = 20$ ). RRMS\_U, untreated active RRMS ( $N = 24$ ). RRMS\_I, interferon-beta-treated RRMS ( $N = 24$ ). RRMS\_R, RRMS during relapse ( $N = 4$ ). Spearman correlation test,  $N = 98$ ,  $\rho = -0.400$ ,  $P_{corr} = 0.001$ .

Sporobacter,<sup>47</sup> and *Clostridium* cluster IV<sup>13,14</sup> results are possibly explained by different population subtypes, since we also included less inflammatory phenotypes, or confounder control. *Olsenella* and *Roseburia* have been described as differentially abundant in other inflammatory conditions.<sup>47,49–51</sup>

*Butyricococcus* abundance was lower in MS than in the total HC group, as confirmed in a previous paper.<sup>47</sup> Between-group analysis suggests an inverse relationship between *Butyricococcus* abundance and presumed CNS inflammation in RRMS. Lower *Butyricococcus* abundance in PPMS might be explained by the less prominent role of focal inflammatory disease activity. *Butyricococcus* is a spore-forming genus from the *Clostridium* cluster IV known to produce short-chain fatty acids,<sup>52</sup> which can initiate anti-inflammatory effects through regulatory T-cell induction.<sup>53</sup> Through this mechanism the gut microbiota might contribute to MS pathogenesis.<sup>54,55</sup> *Butyricococcus* and *Clostridium* cluster XVIII have never before been found differentially abundant within MS.<sup>9–16</sup> While lower abundances of certain *Clostridium* cluster IV species

have been reported, none were of the type known to induce regulatory T-cells.<sup>15</sup> In line with our results, *Butyricococcus pullicaecorum* was shown less abundant in IBD patients than in HC.<sup>50</sup> Lastly, *Butyricococcus* abundance was inversely correlated with age-corrected disease burden and self-reported disability. Given also the fact that *Butyricococcus* was found to be well tolerated for human administration,<sup>56</sup> these results highlight *Butyricococcus* as a promising novel target in MS.

## Strengths

Including MS subgroups with well-defined disease characteristics and disease course modifiers enabled us to evaluate the gut microbiome in terms of higher or lower degree of presumed focal CNS inflammation. Restricting treatment to IFN-beta allowed studying the gut microbiome in a homogenous treatment group. The RRMS\_U group permitted to investigate the gut microbiome in MS without treatment effects. We controlled for the effect of constipation through BSS and other covariates known to affect gut microbial composition.<sup>18</sup>

## Limitations

First, categorization bypasses the natural spectrum of a heterogeneous disease, which may have resulted in errors of classification. Second, without MRI data on subclinical disease activity, the degree of focal inflammation may be underestimated in all groups. Third, despite attempts to increase the size of RRMS\_R ( $N = 4$ ), reported differences require replication in larger, independent study groups. Fourth, it is unclear whether microbial differences between the original HC and FGFP samples reflect natural diversity or sampling bias.

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## Author Contributions

TR performed recruitment and stool sampling in the University Hospital Brussels, aided in statistical analysis, and wrote the body of this manuscript. LD performed data quality checks, laboratory and statistical analyses under the guidance of MVC and JR, and aided in the writing process. MVC performed preprocessing of raw sequencing data for detailed analysis. AVR performed recruitment and stool sampling in the NMSC Melsbroek, and provided organizational support with MD. MD, JDK, GN, MJ, and JR prepared the outline of the study and provided insight.

## Conflict of Interest

LD, BD, and JR have a patent A NEW INFLAMMATION ASSOCIATED, LOW CELL COUNT ENTEROTYPE (EP 17207770.3) issued to VIB VZW, KATHOLIEKE UNIVERSITEIT LEUVEN, K.U.LEUVEN R&D, and VRIJE UNIVERSITEIT BRUSSEL. MVC has a patent PCT/EP2018/084920 pending. MJ and JR have a patent EP 14184535.4—12.09.2014: Biological sampling and storage container, International patent application on 14.09.2015:

PCT/EP2015/070977 issued to VIB, VUB, and LRD. There are no conflicts of interest for the other authors.

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## Supporting Information

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

**Figure S1.** Boxplot of observed richness within the original healthy control samples ( $N = 22$ ) and those from the FGFP ( $N = 98$ ). The body of the boxplot represents the first and third quartiles of the distribution and median line. The whiskers extend to the last data point within 1.5 times the interquartile range. The outliers lie beyond. Individual data points (dots) may overlap. HC\_FGFP, healthy controls from the Flemish Gut Flora Project. HC\_MICR, original healthy control sample.

**Figure S2.** Bar plot illustrating the distribution of four enterotypes within the original healthy control samples and those from the FGFP, *Prevotella* (P), Ruminococcaceae (R), *Bacteroides* 1 (B1), and *Bacteroides* 2 (B2). HC\_FGFP, healthy controls from the Flemish Gut Flora Project. HC\_MICR, original healthy control sample.

**Figure S3.** Barplot showing the distribution of main discriminating genera in enterotypes in the whole study population ( $N = 218$ ). Other genera are combined in “Remainder.” The  $y$ -axis shows the proportional abundance per sample.

**Figure S4.** Barplot showing the distribution of the 61 most prevalent genera in samples from each subject in the healthy control group from the Flemish Gut Flora

Project (HC\_FGFP,  $N = 98$ ). Other genera are combined in “Remainder.” The  $y$ -axis shows the proportional abundance per sample. OTU, operational taxonomic unit.

**Figure S5.** Barplot showing the distribution of the 61 most prevalent genera in samples from each subject in the original healthy control group (HC\_MICR,  $N = 22$ ). Other genera are combined in “Remainder.” The  $y$ -axis shows the proportional abundance per sample. OTU, operational taxonomic unit.

**Figure S6.** Barplot showing the distribution of the 61 most prevalent genera in samples from each subject in the benign MS group (BMS,  $N = 20$ ). Other genera are combined in “Remainder.” The  $y$ -axis shows the proportional abundance per sample. OTU, operational taxonomic unit.

**Figure S7.** Barplot showing the distribution of the 61 most prevalent genera in samples from each subject in the untreated active relapsing–remitting MS group (RRMS\_U,  $N = 24$ ). Other genera are combined in “Remainder.” The  $y$ -axis shows the proportional abundance per sample. OTU, operational taxonomic unit.

**Figure S8.** Barplot showing the distribution of the 61 most prevalent genera in samples from each subject in the active relapsing–remitting MS group treated with interferon beta (RRMS\_I,  $N = 24$ ). Other genera are combined in “Remainder.” The  $y$ -axis shows the proportional abundance per sample. OTU, operational taxonomic unit.

**Figure S9.** Barplot showing the distribution of the 61 most prevalent genera in samples from each subject in the active relapsing–remitting MS group recruited during a relapse (RRMS\_R,  $N = 4$ ). Other genera are combined in “Remainder.” The  $y$ -axis shows the proportional abundance per sample. OTU, operational taxonomic unit.

**Figure S10.** Barplot showing the distribution of the 61 most prevalent genera in samples from each subject in the non-active primary progressive MS group (PPMS,  $N = 26$ ). Other genera are combined in “Remainder.” The  $y$ -axis shows the proportional abundance per sample. OTU, operational taxonomic unit.

**Figure S11.** Boxplots illustrating the  $\log_{10}$  abundances of genera *Bifidobacterium*, *Clostridium* cluster IV, *Clostridium* cluster XIVa, *Fusicatenibacter*, *Romboutsia*, *Ruminococcus*, *Sporobacter*, *Terrisporobacter*, and *Turicibacter* within the healthy control population. Abundances were  $\log_{10}$ -transformed for graphic purposes. Individual data points (dots) may overlap. HC\_FGFP, healthy controls from the Flemish Gut Flora Project. HC\_MICR, original healthy control sample. Kruskal–Wallis test ( $N = 120$ ,  $P_{corr} < 0.1$ ) with post hoc Dunn tests.

**Table S1.** Relative abundance of bacterial genera found per MS subgroup (expressed per 10,000 reads). Taxa unclassified at genus level, genera with a mean relative abundance  $< 1$  in 10,000 reads and present in  $< 20\%$  of all

samples were excluded. Median values are shown. The proportion (%) of samples per group containing the genus is mentioned between brackets.

**Table S2.** Relative abundance of bacterial genera found per healthy control group (expressed per 10,000 reads).

Taxa unclassified at genus level, genera with a mean relative abundance <1 in 10,000 reads and present in <20% of all samples were excluded. Median values are shown. The proportion (%) of samples per group containing the genus is mentioned between brackets.