

Gut Microbiome in Progressive Multiple Sclerosis

Laura M. Cox, PhD ,¹ Amir Hadi Maghzi, MD,¹ Shirong Liu, MD, PhD,¹ Stephanie K. Tankou, MD, PhD,² Fyonn H. Dhang, BS,¹ Valerie Willocq, BS,³ Anya Song, BS,¹ Caroline Wasén, PhD ,¹ Shahamat Tauhid, MD,¹ Renxin Chu, PhD,¹ Mark C. Anderson, MD,¹ Philip L. De Jager, MD, PhD ,⁴ Mariann Polgar-Turcsanyi, MBA,¹ Brian C. Healy, PhD,⁵ Bonnie I. Glanz, PhD,¹ Rohit Bakshi, MD ,¹ Tanuja Chitnis, MD ,¹ and Howard L. Weiner, MD¹

Objective: This study was undertaken to investigate the gut microbiome in progressive multiple sclerosis (MS) and how it relates to clinical disease.

Methods: We sequenced the microbiota from healthy controls and relapsing–remitting MS (RRMS) and progressive MS patients and correlated the levels of bacteria with clinical features of disease, including Expanded Disability Status Scale (EDSS), quality of life, and brain magnetic resonance imaging lesions/atrophy. We colonized mice with MS-derived *Akkermansia* and induced experimental autoimmune encephalomyelitis (EAE).

Results: Microbiota β-diversity differed between MS patients and controls but did not differ between RRMS and progressive MS or differ based on disease-modifying therapies. Disease status had the greatest effect on the microbiome β-diversity, followed by body mass index, race, and sex. In both progressive MS and RRMS, we found increased *Clostridium bolteae*, *Ruthenibacterium lactatiformans*, and *Akkermansia* and decreased *Blautia wexlerae*, *Dorea formicigenerans*, and *Erysipelotrichaceae CCMM*. Unique to progressive MS, we found elevated *Enterobacteriaceae* and *Clostridium g24 FCEY* and decreased *Blautia* and *Agathobaculum*. Several *Clostridium* species were associated with higher EDSS and fatigue scores. Contrary to the view that elevated *Akkermansia* in MS has a detrimental role, we found that *Akkermansia* was linked to lower disability, suggesting a beneficial role. Consistent with this, we found that *Akkermansia* isolated from MS patients ameliorated EAE, which was linked to a reduction in ROR γ t+ and IL-17-producing $\gamma\delta$ T cells.

Interpretation: Whereas some microbiota alterations are shared in relapsing and progressive MS, we identified unique bacteria associated with progressive MS and clinical measures of disease. Furthermore, elevated *Akkermansia* in MS may be a compensatory beneficial response in the MS microbiome.

ANN NEUROL 2021;89:1195–1211

Multiple sclerosis (MS) is an autoimmune disease triggered by environmental factors that act on a genetically susceptible host. One of the most poorly understood aspects of MS is the biology associated with the transition from relapsing–remitting MS (RRMS) to the progressive form.¹ Progressive MS is more refractory to therapy and is associated with greater physical disability and with greater impairment of quality of life, including fatigue and depression. Thus, there is

a critical need to understand factors associated with progressive MS and how to modify them.

The microbiome encompasses trillions of organisms that can affect neurologic disease by modulating immune cells that traffic from the gut to the brain, secreting neuroactive metabolites, altering endocrine signaling pathways, and triggering afferent neurons.² Studies in animal models demonstrate that the gut microbiota can affect

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.26084

Received Oct 5, 2020, and in revised form Apr 12, 2021. Accepted for publication Apr 12, 2021.

Address correspondence to Dr Weiner, 60 Fenwood Road, Boston, MA 02115. E-mail: hweiner@rics.bwh.harvard.edu

From the ¹Ann Romney Center for Neurologic Diseases, Harvard Medical School, Brigham and Women's Hospital, Boston, MA; ²Mount Sinai Health System, New York, NY; ³Department of Neurology, Harvard Medical School, Harvard University Wyss Institute for Biologically Inspired Engineering, Boston, MA; ⁴Department of Neurology, Columbia University Medical Center, New York, NY; and ⁵Department of Neurology, Biostatistics Center, Massachusetts General Hospital, Brigham and Women's Hospital, Boston, MA

neuroinflammation. Germ-free and antibiotic-treated mice are resistant to both induced and spontaneous experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis.^{3–6} Administering bacteria that promote T regulatory cells, including polysaccharide A-positive *Bacteroides fragilis*, can ameliorate EAE,⁷ whereas administering bacteria that induce Th17 cells can worsen EAE. In addition, bacteria may act together through biomimicry and inflammation to induce spinal cord inflammation.⁸ Colonizing spontaneous EAE mice with microbiota from RRMS patients worsens disease^{9,10} and is linked with decreased IL-10-producing Tregs.¹⁰ Incubating MS microbiota with human peripheral blood mononuclear cells induced proinflammatory responses in vitro,⁹ suggesting that the MS microbiota both lack beneficial microbes that regulate autoimmunity and have an overabundance of proinflammatory bacteria.

Several laboratories, including ours, have reported microbiome alterations in RRMS,^{9–19} including increases in *Akkermansia* and decreases in butyrate-producing bacteria. Microbiota alterations in MS may be primary drivers of the disease or may instead reflect alterations secondary to the disease process. We found that the host can shape the microbiome through the secretion of microRNAs.²⁰ Furthermore, we found that MS patients and mice at peak EAE have elevated intestinal miR-30d, which increases the levels of *Akkermansia* and ameliorates EAE.²⁰ This suggests that elevated *Akkermansia* may result from positive host selection, rather than contributing to MS. Studies have found low *Prevotella* in RRMS patients, and administering human-derived *Prevotella histicola* improved EAE,²¹ suggesting that increasing depleted bacteria can lessen neuroinflammation. We found that administering a probiotic to RRMS patients reversed the inflammatory phenotype of peripheral monocytes, and that immune changes were durable months after the probiotic was stopped.¹⁵ Taken together, these studies demonstrate that the microbiota plays an important role in MS and that modulating the microbiota has therapeutic potential.

To date, there have been few studies of the microbiome in progressive MS. A Russian cohort of 15 primary progressive MS (PPMS) subjects had elevated *Gemmiger* and *Ruminococcus* and an increase in the family *Verrucomicrobiaceae*, which contains *Akkermansia*.²² A Belgian cohort of 28 PPMS patients had lower *Gemmiger* and *Butyrivibacter* and higher *Methanobrevibacter*, *Sporobacter*, and *Clostridium* cluster IV in PPMS.²³ A Japanese cohort of 15 SPMS subjects had elevated *Clostridium* and decreased butyrate producers.²⁴ We investigated the microbiome in progressive MS, compared it to subjects

with relapsing disease, and identified microbial taxa that correlated with disability, quality of life, and brain magnetic resonance imaging (MRI) measures (lesions and atrophy).

Materials and Methods

Study Subjects and Clinical Metadata

Microbiome study subjects were recruited from the CLIMB study conducted at the MS center at the Brigham and Women's Hospital. Subjects were selected based on a diagnosis of RRMS or progressive MS, willingness to participate in a microbiome study, and meeting the inclusion/exclusion criteria (see below). The protocols for this study received prior approvals from all institutional review boards, and informed consent was obtained from each subject. Study subjects collected a stool sample at home, then shipped samples overnight on icepacks to the laboratory, and samples were frozen at -80°C upon receipt. Inclusion criteria for subjects with MS included a diagnosis of MS according to the latest McDonald criteria. Disease subtypes were further classified as RRMS or progressive MS, which included both primary and secondary progressive. Exclusion criteria included pregnancy, history of gastrointestinal surgery, intestinal bowel disease, and antibiotics within the past 3 months. Quality of life was assessed in 95 RRMS and 27 progressive MS patients with the validated Neuro-QOL questionnaire.²⁵ Patients in our CLIMB study undergo yearly 3T MRI scans using a consistent high-resolution protocol.²⁶ We employed automated pipelines to quantify brain T2 lesion volume,²⁶ and normalized whole brain²⁷ and deep gray matter²⁸ volumes. Healthy subjects from the PhenoGenetic Project,²⁹ a resource of individuals who are self-reported to be free of chronic infectious and inflammatory diseases and recallable by demographic or genotypic feature for biosampling, were approached to provide a stool sample. Collection and processing procedures were identical to the ones used for the MS patients.

Microbiome Analysis

DNA was extracted using the DNAeasy PowerLyzer Microbiome DNA extraction kit (QIAGEN, Hilden, Germany), and the V4 region of the 16S rRNA gene was amplified using barcoded primers developed by the Earth Microbiome Project and as previously described.¹⁵ Paired-end reads were sequenced on the MiSeq at a Harvard Biopolymers facility, and sequence analysis was performed in QIIME2. Samples were sequenced on 2 MiSeq runs, with 173 samples sequenced on both runs, 57 samples sequenced only on the first run, and 52 samples only sequenced on the second run. Denoising and quality filtering of data were performed using DADA2 for each run, then paired samples were merged. Any sample with less than 1,000 reads was then removed from analysis. For taxonomic assignment, sequences from the EZ-biocloud database formatted for QIIME and released May 2018 were used as a reference set.³⁰ The ribosomal database project (RDP) classifier was trained against the V4 region of this database, then was used to identify sequences. Testing for significant differences in α -diversity was performed

by the nonparametric Kruskal-Wallis test, differences in β -diversity were investigated by PERMANOVA, with correction for multiple comparison testing, and contributors to microbiome variation were assessed by the ADONIS test. After relative abundance was calculated, species that had less than 10% prevalence in any group (HC, RRMS, progressive) were removed from differential testing and correlation analysis. Compositional differences were determined using linear discriminant analysis effect size (LEfSe) with α set at 0.05, and the effect size set at greater than 2.³¹ To adjust these findings for other factors that may affect the microbiome, the microbiome multivariable associations with linear models (MaAsLin) tool³² was used to identify compositional differences while adjusting for age, BMI, sex, race, and ethnicity. To identify bacteria linked with EDSS, MRI measurements, and quality of life, Spearman correlations were performed in R and were adjusted for age using R package ppcor.

Isolation and Identification of MS-Derived Akkermansia

Stool samples from 6 individuals with detectable *Akkermansia* via 16S V4 rRNA sequencing underwent seven 10-fold serial dilution in prereduced anaerobically sterilized saline, and 100 μ l of the 10⁻⁴ through 10⁻⁷ dilutions was plated on minimal mucin media (Anaerobe Systems, Morgan Hill, CA). Eight to 10 colonies were isolated in pure culture per microbiota donor after incubation of 3 to 7 days. Isolates were frozen in Brucella broth (Difco Laboratories, Detroit, MI) plus 15% glycerol, and the nearly full-length 16S rRNA gene was amplified using the 8F and 1510R primers according to previous methods.³³ After polymerase chain reaction, primers were removed by ExoSapIT, then sequenced by Sanger Sequencing at the Dana Farber Sequencing Core. Forward and reverse sequences were then quality trimmed and joined using UGENE software. Identification and percent identity were then performed using batch BLAST, National Centers for Biotechnology Information. Finally, a phylogenetic tree of isolates and reference sequences was constructed using Phylogeny.fr.

Effect of MS-Derived Akkermansia on EAE

MS-derived *Akkermansia* strains BWH-J5, BWH-I7, and BWH-H3, as well as the Type strain of *Akkermansia muciniphila*, were grown in brain heart infusion (BHI) + mucin broth as previously described.³⁴ *Bacteroides cellulosilyticus* strain BWH-E5, which is not altered in RR or progressive MS, was administered as a control bacterium. Live bacteria (OD600 0.32, 200 μ l) or vehicle control of BHI + mucin broth was delivered to 9-week-old female C57BL6J mice ($n = 10$ –14/group) by oral gavage 3 times per week beginning 2.2 weeks prior to disease induction; bacteria treatment was received up to 2 weeks after disease induction. EAE was induced by injecting 150 μ g of myelin oligodendrocyte glycoprotein (MOG) and complete Freund adjuvant subcutaneously and administering a peritoneal injection of 200ng of pertussis toxin on the same day, and 48 hours later, and disability scores were determined by standard scoring criteria as previously described.³⁴ Differences between the groups were determined by Friedman test and Dunn correction for multiple

comparisons. Animals were housed in a biosafety level 2 facility using autoclaved cages and aseptic handling procedures and kept under a 12-hour light/dark cycle. All animal experiments were conducted according to an institutional animal care and use committee-approved protocol.

Effect of MS-Derived Akkermansia on T Cells in EAE

For immunologic analysis, a second cohort of 9-week-old female C57BL6J mice ($n = 5$ per group) received the same microbiota strains, dosing, and immunization as described above. Ten days after disease induction, splenocytes were isolated for flow cytometric analyses. Red blood cells were lysed with ammonium-chloride-potassium (ACK) lysis buffer, and dead cells were stained with the fixable viability dye Aqua Zombie (1:1,000 diluted in phosphate-buffered saline; BioLegend, San Diego, CA). Surface markers were stained for 25 minutes at 4°C in fluorescence-activated cell sorting buffer (Mg^{2+} - and Ca^{2+} -free Hank balanced salt solution with 2% fetal calf serum, 0.4% ethylenediaminetetraacetic acid [0.5 M], and 2.5% hydroxyethylpipерazine ethane sulfonic acid [1 M]), cells were fixed in CytoPerm/Cytofix (eBioscience, San Diego, CA), permeabilized with Perm/Wash Buffer (eBioscience), and then stained for intracellular markers. Extracellular antibodies used were PerCP-Cy5.5-anti-TCR β (H57-597, 1:800, BioLegend), BV785-anti-CD4 (RM4-5, 1:400, BioLegend), BV711-anti-CD8a (53-6.7, 1:400; BD Biosciences, Franklin Lakes, NJ), and APC-anti-TCD $\gamma\delta$ (GL-3, 1:100; Thermo Fisher Scientific, Waltham, MA). To measure T cell transcription factors, intracellular antibodies used were FITC-anti-Foxp3 (FJK-16s, 1:100; Thermo Fisher Scientific) and BV421-anti-ROR γ T (Q31-378, 1:100, BD Biosciences). For intracellular cytokine staining, cells were first stimulated for 4 hours with phorbol 12-myristate 13-acetate (50ng ml⁻¹; Sigma-Aldrich, St Louis, MO) and ionomycin (1 μ M, Sigma-Aldrich) and a protein-transport inhibitor containing monensin (1 μ g ml⁻¹, GolgiStop, BD Biosciences) before detection by staining with antibodies. Intracellular antibodies used were BV421-anti-IFN- γ (XMG1.2, 1:400, BioLegend), PE-Cy7-anti-IL-17A (eBio17B7, 1:400, Thermo Fisher Scientific), FITC-anti-IL-10 (Jes5-16E3, 1:100, BioLegend), and PE-anti-GM-CSF (MP1-22E9, 1:100, Thermo Fisher Scientific). Flow cytometric acquisition was performed on a Fortessa device (BD Biosciences) using DIVA software (BD Biosciences), and data were analyzed with FlowJo software version 10.1 (Tree Star, Ashland, OR). Cells were gated on lymphocytes, single cells, and live cells, then T cell subsets were divided into TCR β +CD4+, TCR β +CD8+, or TCR β -TCR $\gamma\delta$ + cell populations, and then transcription factors (FoxP3 and ROR γ T) and cytokines (IL-10, GM-CSF, IL-17, and INF γ) were measured.

Results

Patients with Progressive MS Have Alterations in Intestinal Microbiota Composition

We analyzed the microbiota in 40 healthy controls (HCs), 199 RRMS patients, and 44 progressive MS patients by

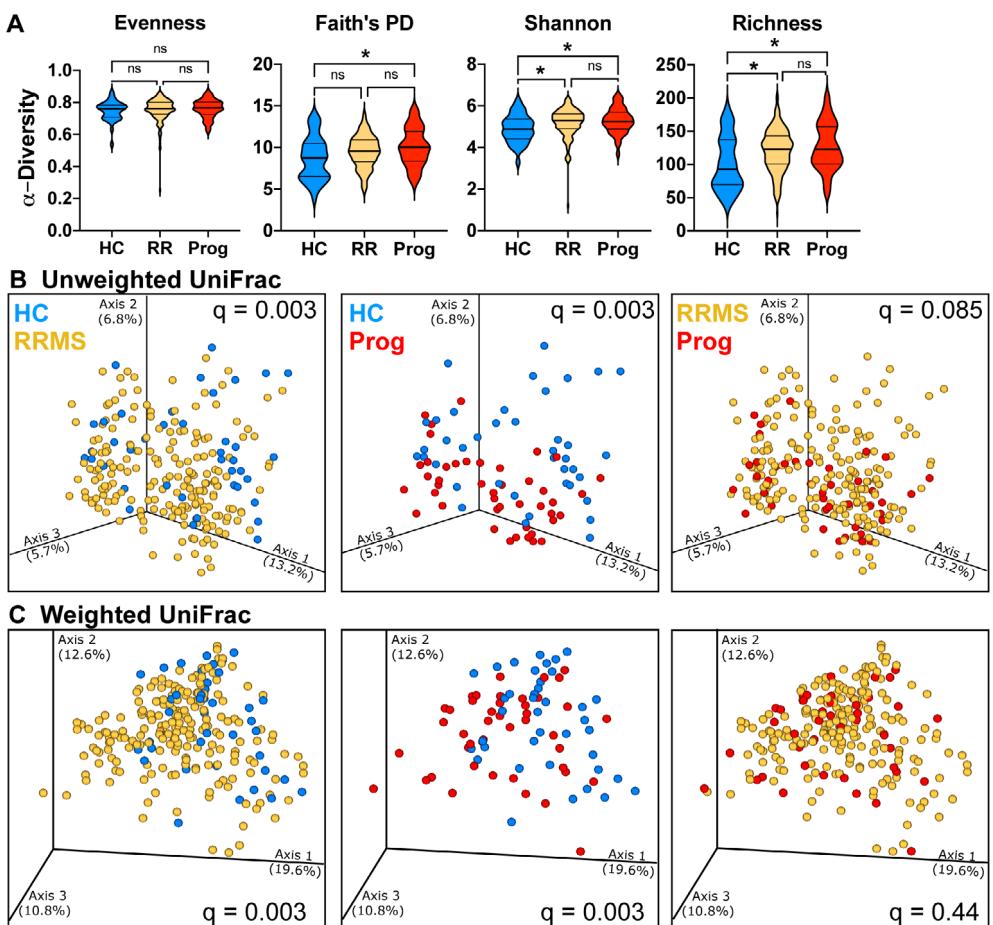


FIGURE 1: Microbiota α - and β -diversity in relapsing and progressive multiple sclerosis (MS). (A) α -Diversity metrics for evenness, Faith's phylogenetic diversity, Shannon diversity, and richness (number of features) were calculated at an average sampling depth of 5,000 reads per sample in healthy controls (HC; n = 40), relapsing-remitting (RR) MS (n = 199), and progressive (Prog) MS (n = 44). * $p < 0.05$, Kruskal-Wallis. ns = not significant. (B, C) Principal coordinate analysis of intestinal microbiota samples based on unweighted (B) and weighted (C) UniFrac distances show significantly different clustering between HC and RRMS, and between HC and progressive MS, but not between RRMS and progressive MS. q = PERMANOVA p values adjusted for false discovery rate. Each dot represents the microbiota from one individual: HC, blue; RRMS, yellow; progressive MS, red.

sequencing the V4 region of the microbial 16S rRNA gene. Examining α -diversity, we found no changes in evenness, but found slightly elevated phylogenetic diversity, Shannon diversity, and richness in both RRMS and progressive MS compared to HCs (Fig 1A). Examining β -diversity, we found that overall microbiota community structure differed between progressive MS patients and HCs and between relapsing patients and HCs, but did not differ between progressive MS and RRMS (see Fig 1B, C). At the phylum through genus (Fig 2A, B) and species levels (see Fig 2C), we found that progressive MS patients had unique changes compared to RRMS and HCs, including an increase in *Enterobacteriaceae*, *Ruminococcaceae* FJ366134, and *Clostridiaceae* g24 FCEY and a decrease in *Dorea longicatena*, *Anaerococcus vaginalis*, and *Blautia faecis*. Progressive MS patients shared microbiota alterations with RRMS patients compared to HCs, including an increase in *Akkermansia* at the genus level and *Clostridium bolteae* at the species level and a decrease

in *Dorea formicigenerans* and unclassified *Blautia* at the species level. *C bolteae* is associated with the induction of Th17 cells,³⁵ was originally isolated from an autistic patient,³⁶ and has been reported to be elevated in neuro-myelitis optica.³⁷ In addition to increased abundance, *C bolteae* had a higher prevalence in progressive MS (50% of subjects) compared to HCs (12% of subjects). There were 2 bacteria that were altered in all 3 comparisons, including a reduction in the not yet cultured *Erysipelotrichaceae* CCMM and an increase in recently discovered *Ruthenibacterium lactatiformans*, which was one of the most significantly elevated bacteria in both RRMS and progressive MS.

Microbiota Changes Adjusted for Host Factors

Because host factors can affect the microbiome, we measured the effect disease status, age, body mass index (BMI), sex, race, and ethnicity on microbiome β -diversity. For this analysis, we restricted the dataset to subjects with

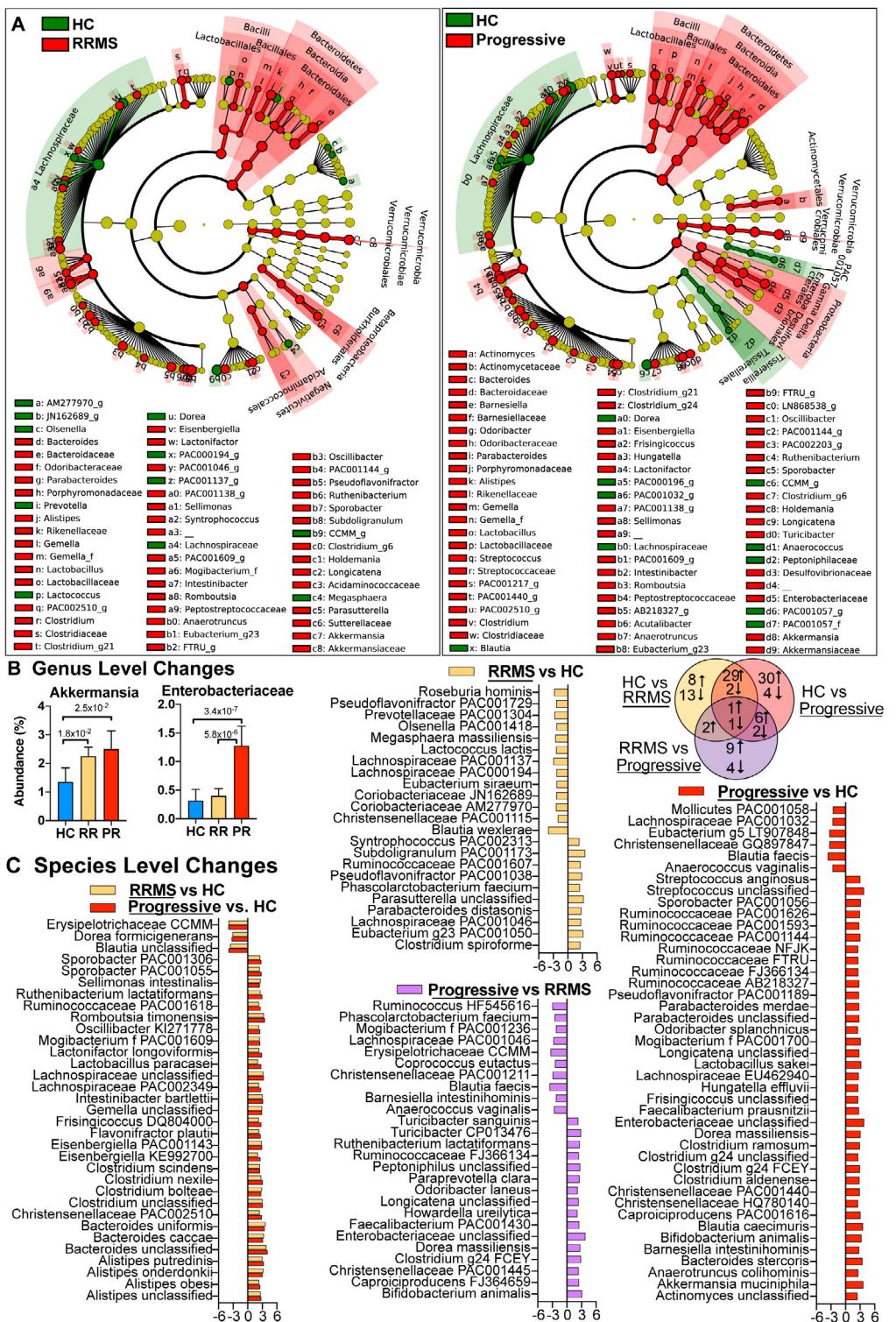


FIGURE 2: Compositional differences in the microbiota of progressive and relapsing multiple sclerosis (MS). Microbiota was sequenced in healthy controls (HC; n = 40), relapsing-remitting (RR) MS (n = 199), and progressive (PR) MS (n = 44) subjects. (A) Differences are visualized on a cladogram, which shows all changes at the phylum level (inner dots, outer wedge label) through genus level (outer dots, labeled with small letters for abbreviation). Red (MS) or green (HC) circles indicate increased levels in corresponding groups; yellow circles indicate a taxon is present but not differentially abundant. The size of the dot corresponds to the overall abundance of that taxon in the microbiome. (B) The relative abundance of selected microbiota altered in progressive MS. (C) Linear discriminant analysis (LDA) effect size of significantly altered bacteria at the lowest classifiable levels and Venn diagram showing the number of bacteria increased or decreased in each comparison. Positive LDA effect size = up in the underlined group.

a reported BMI, race, and ethnicity (HC = 38, RRMS = 135, progressive MS = 31 subjects). We found that disease status had the largest effect on microbiome composition ($p = 0.001$), followed by BMI, race, and sex, whereas age and ethnicity did not have a significant effect. We then used the MaAsLin test to identify bacteria in MS

versus HC while adjusting for these variables. As shown in Figure 3C, we found that 14 bacteria were increased and 2 decreased in both progressive MS and RRMS versus HCs, with the largest increases in *Romboutsia timonensis* and unclassified *Bacteroides*, and the largest decreases in *Blautia wexlerae* and *D formicigenerans* (see Fig 3). RRMS

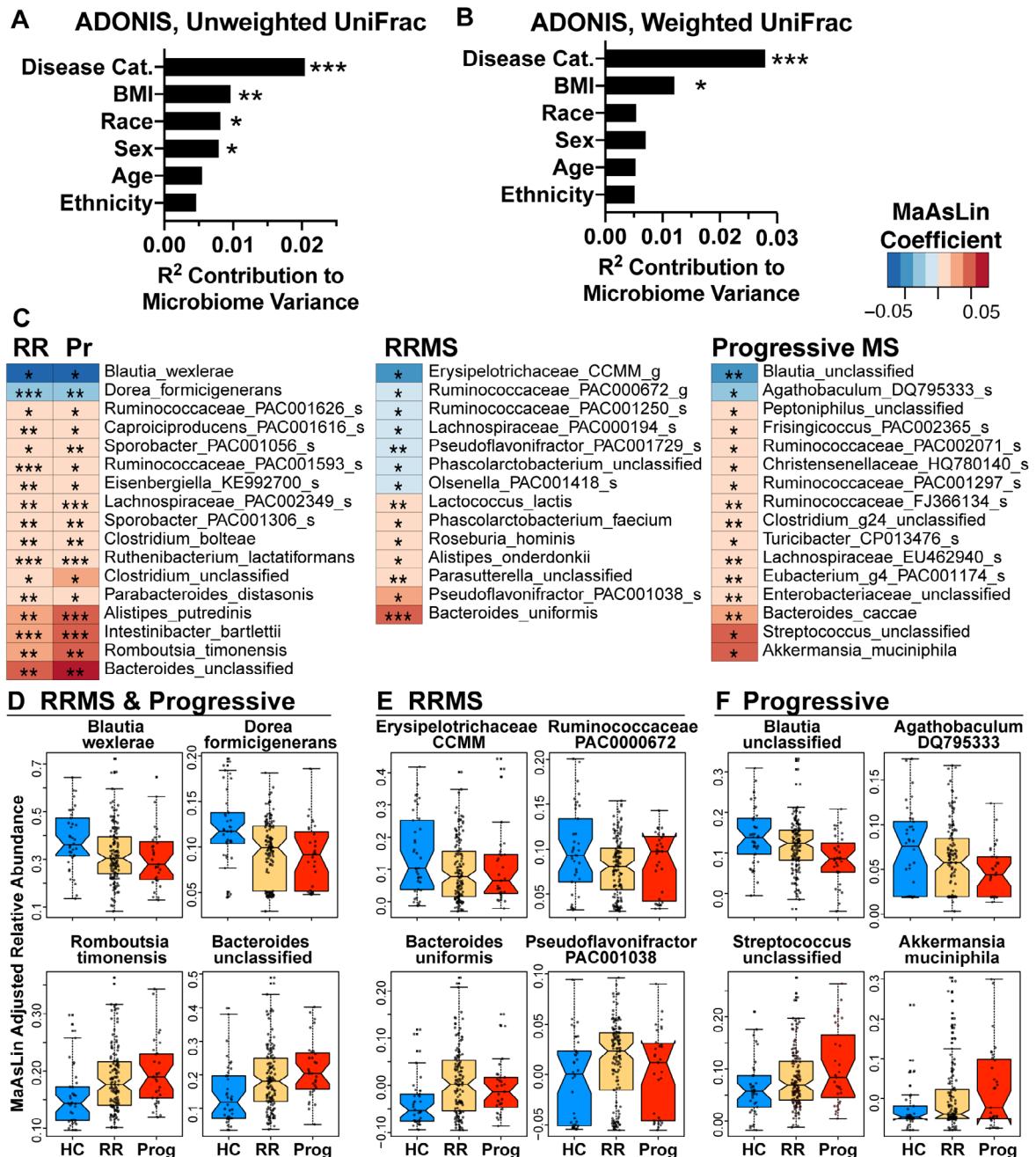


FIGURE 3: Microbiota differences adjusted for host variables. (A, B) Effect of host factors on microbiome β -diversity was measured using the ADONIS test of unweighted and weighted UniFrac distances. Analysis was restricted to subjects with complete demographic information and a recorded body mass index (BMI; n = 38 healthy controls [HC], n = 135 relapsing-remitting [RR] multiple sclerosis [MS], n = 31 progressive MS). (C) Microbiota altered in RR or progressive (Pr) MS versus HCs, MaAsLin, adjusted for age, BMI, sex, race, and ethnicity. (D–F) Abundance of the 2 most decreased taxa and 2 most increased taxa in both RRMS and progressive (Prog) MS versus HCs (D), unique to RRMS (E), or unique to progressive MS (F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

had unique differences versus HC in our adjusted model, including decreased *Erysipelotrichaceae* CCMM and *Ruminococcaceae* PAC000672 and increased *Bacteroides*

uniformis and *Pseudoflavonifractor* PAC001038. Progressive MS had unique changes versus HC, with the largest increases in *A muciniphila* and *Streptococcus* and the

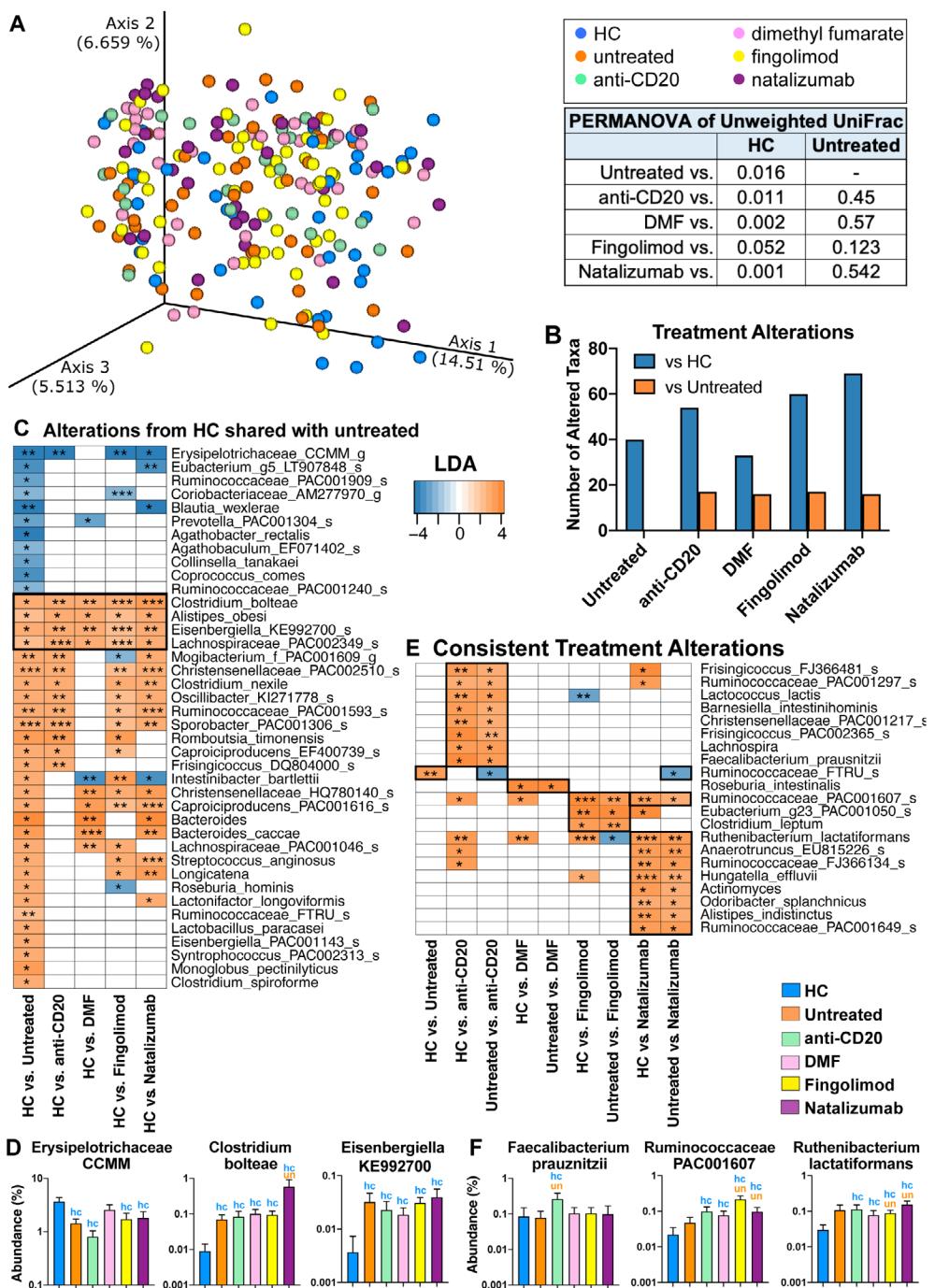


FIGURE 4: The effect of treatment on the multiple sclerosis (MS) microbiota. (A) Principal coordinate analysis of unweighted UniFrac distances of relapsing-remitting MS and progressive MS subjects not on treatment ($n = 33$) or treated with anti-CD20 ($n = 25$), dimethyl fumarate (DMF; $n = 33$), fingolimod ($n = 57$), or natalizumab ($n = 36$), or healthy controls (HC; $n = 40$). PERMANOVA test for clustering reveals differences between healthy controls and MS patients on treatment, but not between untreated MS patients and those on therapy. (B) Number of taxa altered comparing disease-modifying therapy (DMT) group versus healthy controls (blue bars) or versus untreated MS (orange bars). (C) Linear discriminant analysis (LDA) effect size of bacteria altered in untreated MS versus HC, and whether those bacteria are similarly altered in each DMT group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) Representative bacteria consistently altered in MS, regardless of treatment status. Blue hc = significantly different from healthy controls, orange un = significantly different from untreated MS patients, $p < 0.05$, LDA > 2 LEfSe. (E) Bacteria consistently altered by DMT versus HC and DMT versus untreated MS. (F) Representative bacteria consistently altered by treatment compared to both HC and untreated MS patients.

largest decreases in unclassified *Blautia* and *Agathobaculum DQ795333*. Many of the bacteria we identified in our adjusted model were similar to the bacteria we identified with LEfSe (see Fig 2). This is consistent with our finding that disease status had the greatest effect on microbiota composition compared to the other demographic variables we examined (see Fig 3A, B).

Effect of Disease-Modifying Therapy on the Microbiota

To determine the extent to which disease-modifying therapy (DMT) affected the microbiome, we examined changes in β -diversity in patients receiving 4 commonly used treatments at our center, including anti-CD20 (rituximab and ocrelizumab), dimethyl fumarate, fingolimod, and natalizumab. We found that the overall microbiota composition in subjects on DMTs did not differ from untreated MS patients in β -diversity, whereas all MS treatment subgroups differed from HCs ($p < 0.05$, PERMANOVA; Fig 4A, B). These data suggest that disease status has a greater effect on the microbiota than treatment. Whereas there were no changes in β -diversity, we found changes in specific bacteria that were linked to therapy. For each DMT, we found fewer bacteria that differed versus untreated MS (orange bars) than differed versus HC (blue bars in Fig 4B). As shown in Figures 4C and D, many of the bacteria that differed between untreated MS and HCs also differed between treated MS and HC, including an increase in *C bolteae*, *Eisenbergiella KE992700*, *Alistipes obesi*, and *Lachnospiraceae PAC002349* and a decrease in *Erysipelotrichaceae CCMM*. For each treatment, we found unique bacteria modulated by the DMT versus both untreated MS and HCs. Specifically, we found that anti-CD20 increased *Faecalibacterium prausnitzii*, and DMF increased *Roseburia intestinalis*, 2 butyrate producers reported to be reduced in MS.^{13,17,18} In addition, we found that fingolimod and natalizumab increased *Ruminococcaceae PAC001607*. Finally, we found increased *R lactatiformans* in all DMTs versus HCs, suggesting that treatment may partially contribute to the increased *R lactatiformans* we identified in Figures 2 and 3.

Identification of Bacteria Associated with Disability

To determine whether bacteria we identified in progressive MS and RRMS were associated with disability, we examined the relationship between the microbiota and the Expanded Disability Status Scale (EDSS), adjusting for age (Fig 5). Because progressive MS patients have greater disability than RRMS patients, we examined the

relationship between the microbiome and EDSS for each disease category separately as well as for the two categories together. In both progressive and relapsing MS, we found several *Clostridium* species that were associated with worse EDSS, including *Clostridium g24 FCEY* (closely related to *C bolteae*) in progressive MS and *C bolteae*, *C leptum*, and *C scindens* in RRMS. Butyrate producers had negative correlations with EDSS, including *Ruminococcaceae HF545616* in RRMS, and *Ruminococcus bromii* and *Roseburia inulinivorans* in progressive MS. This is consistent with a potential beneficial role for butyrate producing bacteria in MS.³⁸ Unexpectedly, we found that *Akkermansia* was negatively correlated with EDSS in progressive MS patients. Thus, contrary to the view that *Akkermansia* has a detrimental role in MS, our findings raise the possibility that elevated *Akkermansia* could represent a compensatory microbiome response to the disease.

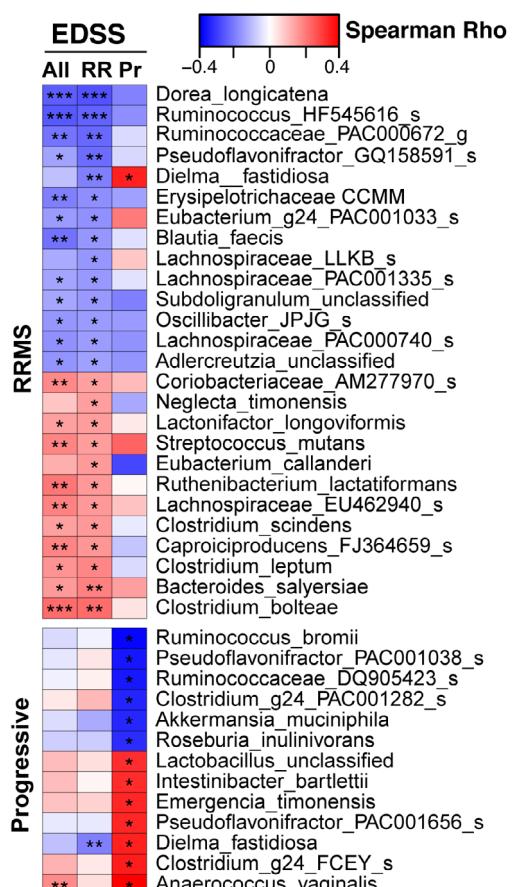


FIGURE 5: Microbiota associated with disability. Microbiota correlations with Expanded Disability Status Scale (EDSS) scores show unique relationships in relapsing-remitting (RR) multiple sclerosis (MS; $n = 198$) and progressive (Pr) MS ($n = 43$). Spearman correlations are adjusted for age. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Identification of Bacteria Associated with MRI Measures of Disease

Atrophy is more severe in progressive MS versus RRMS,^{39,40} particularly in gray matter areas.^{39,41} Furthermore, brain volume loss in the first year of the disease is a strong predictor of future neurologic impairment.^{42,43} The microbiome can alter neurogenesis⁴⁴ and

myelination⁴⁵ and influence inflammation in the brain,⁴⁶ but its relationship to MRI metrics in MS is unknown. To determine whether there were associations between the gut microbiome and MRI measures of disease severity, we identified a cohort of progressive MS and RRMS patients in our CLIMB longitudinal cohort study for which we obtained quantitative measures of brain T2 lesions and

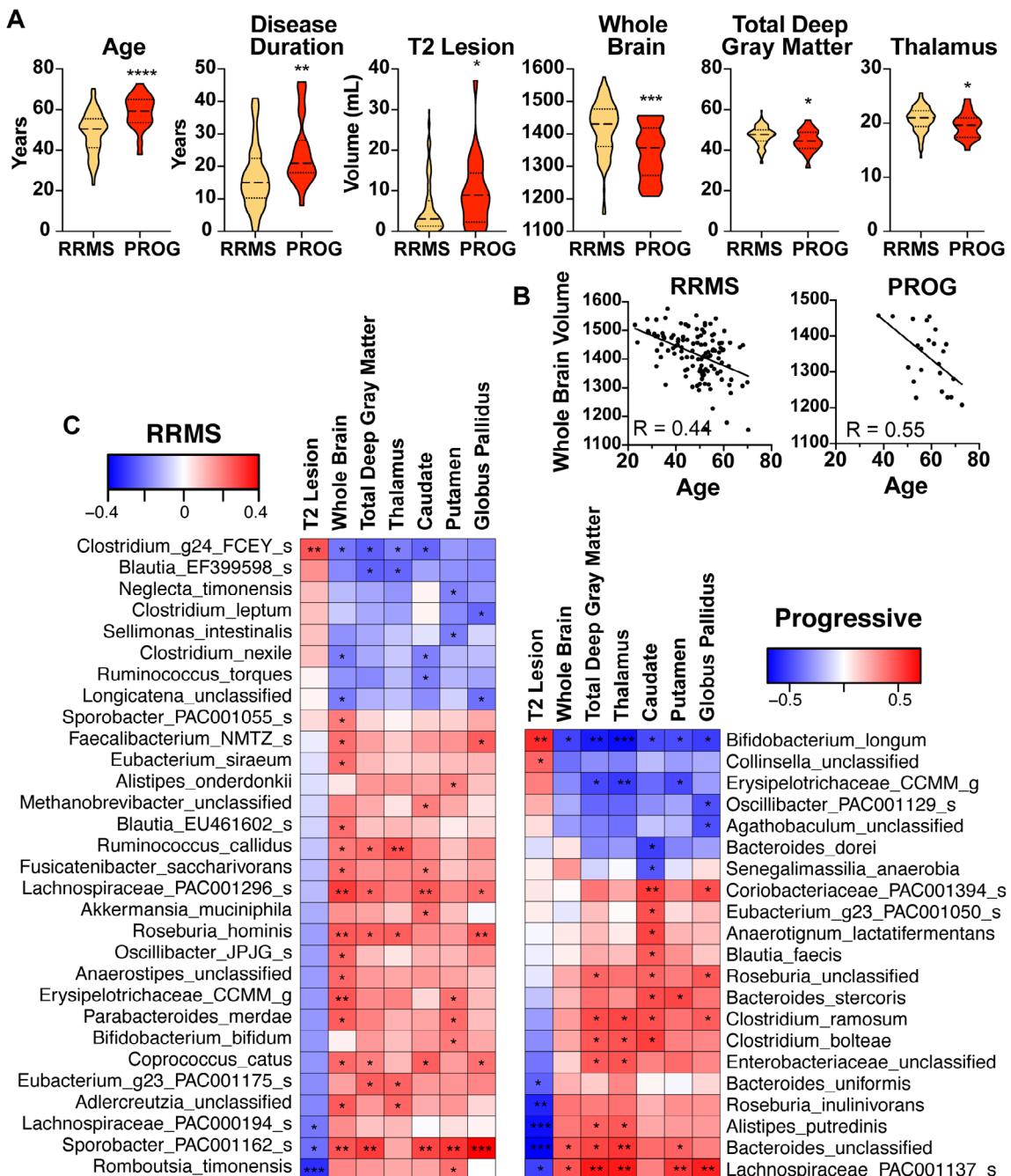


FIGURE 6: Associations between the microbiota and magnetic resonance imaging (MRI) brain measurements in progressive (PROG) multiple sclerosis (MS). (A) Age, disease duration, and brain 3T MRI measurements in progressive MS (n = 23) and relapsing-remitting MS (RRMS; n = 116) patients, t test. (B) Brain volume negatively correlated with age, linear regression, $p < 0.001$, $R = 0.44$ and 0.55 for RRMS and progressive MS, respectively. (C) Bacteria that correlate with lesion volume (upper section) and brain volume (lower section) in RRMS and progressive MS. Spearman correlations are adjusted for age. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

both whole brain and regional deep gray matter volumes from 3T MRI. Progressive MS patients had higher T2 lesion volume and lower normalized whole brain, total deep gray, and thalamic volumes (Fig 6). Because brain volume negatively correlated with age, we adjusted our MRI microbiota analysis for age. In RRMS, we found several *Clostridium* species associated with increased T2 lesion volume and decreased brain volume, including *C. leptum*, *C. nixile*, and *Clostridium g24 FCEY*, consistent with our EDSS data (see Fig 5), suggesting a detrimental role. In RRMS, several bacteria had a negative correlation with T2 lesion volume and positive correlation with brain volume, including *Sporobacter PAC00162*, *A. muciniphila*, and *Erysipelotrichaceae CCMM* (see Fig 6C), consistent with our observation that some of these bacteria were associated with lower disability (see Fig 5). However, in progressive MS, we found opposite relationships between the microbiota and MRI measures. For example, *Erysipelotrichaceae CCMM* had a negative correlation with brain volume, and several *Clostridium* species positively correlated with increased brain volume. This may reflect different biologic processes in RRMS versus progressive MS.

Identification of Bacteria Associated with Quality of Life

Quality of life is an important global indicator of MS disease status and is routinely measured as part of our CLIMB longitudinal cohort study.^{47,48} We asked whether there was an association between the gut microbiota and quality of life using the validated NeuroQOL questionnaire, which measures 3 major domains of quality of life: physical, mental, and social. For the first 6 metrics (“lower extremity motor function” through “satisfaction”), higher scores indicate higher quality of life, and for the last 5 metrics (“emotional dyscontrol” through “fatigue”), higher scores indicate lower quality of life (Fig 7). Because there were no differences in fatigue, anxiety, and depression in our progressive MS versus RRMS subjects, we analyzed a combined cohort of progressive MS and RRMS patients. We found that *Enterobacteriaceae*, *C. bolteae*, and other *Clostridia* positively correlated with fatigue, suggesting a potential detrimental role, whereas *Erysipelotrichaceae CCMM*, *R. timonensis*, and *Lachnospiraceae PAC00194* negatively correlated with fatigue, depression, and anxiety, suggesting a potential beneficial role.

MS-Associated Akkermansia Ameliorates EAE

As discussed above, we found that *Akkermansia* was negatively correlated with EDSS and MRI burden of disease (see Figs 5 and 6), suggesting that *Akkermansia* has a beneficial role in MS. Thus, we investigated whether

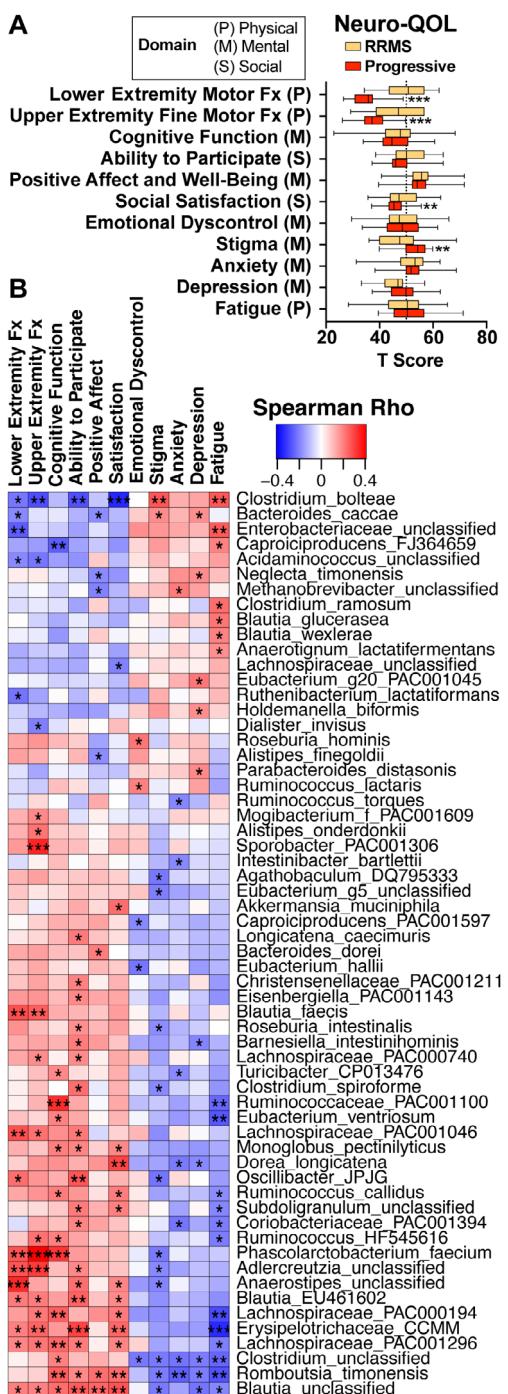


FIGURE 7: Microbiota associations with quality of life. (A) Quality of life measurements in 95 relapsing-remitting multiple sclerosis (RRMS) and 27 progressive MS patients were assessed using the NeuroQOL questionnaire across 3 domains: physical, mental, and social. Departure from the population norm (T score = 50) in RRMS and progressive MS patients. (B) Microbiota correlations with quality of life. Spearman correlations are adjusted for age. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Fx = function.

MS-associated *Akkermansia* had a beneficial effect in the MS model of EAE. We identified progressive MS and RRMS patients with high *Akkermansia* and were able to

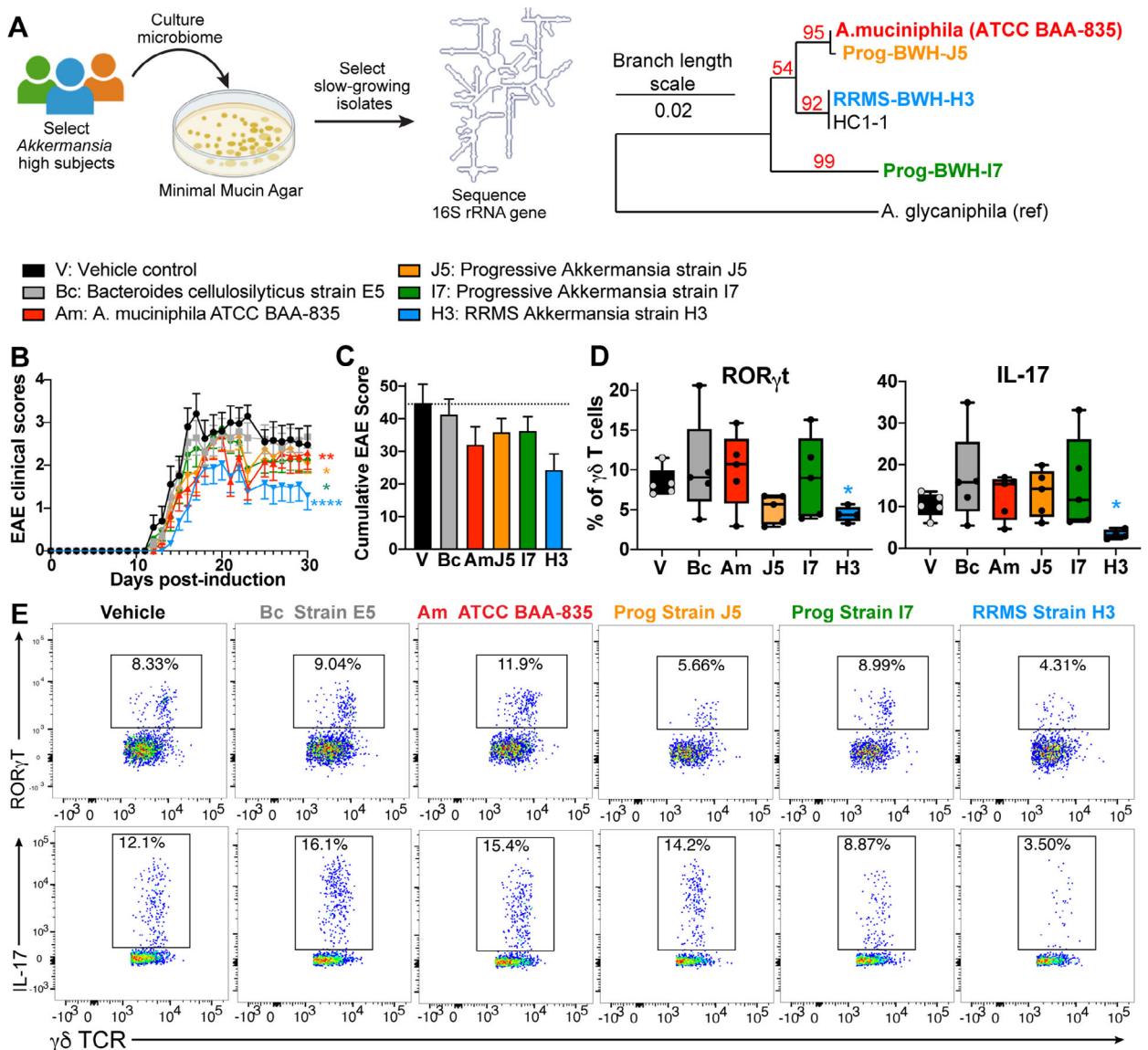


FIGURE 8: Multiple sclerosis (MS)-associated Akkermansia ameliorates experimental autoimmune encephalomyelitis (EAE). (A) Stool samples from MS patients with high levels of Akkermansia, corresponding to 3 different V4 16S rRNA sequences, were plated on minimal mucin agar, and slow-growing strains were isolated and identified by 16S rRNA Sanger sequencing. A phylogenetic tree constructed from nearly full-length 16S rRNA sequences shows 3 phylotypes of Akkermansia isolated from healthy controls and MS patients. (B) Akkermansia isolated from relapsing-remitting MS (RRMS) and progressive (Prog) MS subjects reduce EAE score in the C57/MOG model, whereas the control bacteria *Bacteroides cellulosilyticus* (Bc) does not, n = 10–14 mice per group. *p < 0.05, **p < 0.01, ****p < 0.0001, Friedman test with Dunn correction for multiple comparisons. (C) Cumulative EAE scores. (D, E) Mice were colonized with *B. cellulosilyticus*, *Akkermansia muciniphila* Type strain, and 3 MS-derived Akkermansia strains, n = 5 per group. EAE was induced, and immunologic responses were measured 10 days later. (D) Levels of ROR γ T+ $\gamma\delta$ T cells in unstimulated splenocytes and levels of IL-17 production in phorbol 12-myristate 13-acetate/ionomycin-stimulated splenocytes. *p < 0.05, 1-way analysis of variance. (E) Representative fluorescently-activated cell sorting plots of ROR γ T and IL-17 production from splenic $\gamma\delta$ -T cells.

distinguish 3 subtypes based on 16S rRNA V4 sequences (Fig 8A). We then isolated bacteria on minimal mucin media from 6 subjects, identified bacterial strains using Sanger sequencing, and recovered *Akkermansia* isolates from 4 of our subjects (HC1, RRMS1, Prog1, Prog2). The strain Prog2-BWH-J5 was nearly identical (99.7%) to the Type strain of *A. muciniphila*, and strain RRMS1-BWH-H3 was 98.7% identical to the Type strain,

indicating a new substrate of *A. muciniphila*. Strain Prog1-BWH-I7 was only 96.5% identical, indicating a new species of *Akkermansia*. To test the role of MS-derived *Akkermansia* in disease, we colonized C57/BL6 mice with *Akkermansia* strains BWH-J5, BWH-H3, and BWH-I7 and found that they all lowered disease in the MOG/C57 model of EAE, with the strongest protective effect from strain H3 (see Fig 8B). As a control, we used *B. cellulosilyticus*, a commensal

TABLE. Study Subject Demographics

Characteristic	HCs	RRMS	Prog
Subjects, n	40	199	44
Female subjects, n (%)	28 (70.0%)	152 (76.4%)	31 (70.4%)
Age, yr ± SD	45.4 ± 9.2	49.3 ± 9.5	57.8 ± 7.6
Body mass index ± SD	28 ± 7.9	27.1 ± 6.3	28.4 ± 6.5
Race, n			
White	38	188	42
Black	1	7	1
>1 race	1	3	1
Unknown, not reported	0	1	0
Ethnicity, n			
Not Hispanic or Latino	39	193	43
Hispanic or Latino	1	6	1
Disease duration, yr ± SD	NA	16.3 ± 10	24 ± 10.9
EDSS ± SD	NA	1.8 ± 1.2	5 ± 1.8
Treatment, n			
Untreated	NA	40	5
Fingolimod	NA	47	2
Dimethyl fumarate	NA	29	5
Natalizumab	NA	28	3
Anti-CD20	NA	19	6
Interferon-β	NA	14	5
Glatiramer acetate	NA	12	3
Mycophenolate mofetil	NA	1	4
Methylprednisolone	NA	3	1
Teriflunomide	NA	0	3
Methotrexate	NA	1	0
Other	NA	0	2
>1 DMT	NA	1	3
Recently off treatment	NA	4	2

DMT = disease-modifying therapy; EDSS = Expanded Disability Status Scale; HC = healthy control; MS = multiple sclerosis; NA = not available; Prog = progressive MS; RRMS = relapsing-remitting MS; SD = standard deviation.

gut microbe that was not altered in MS. In addition, we measured immune responses in an independent experiment 10 days postimmunization and found that *Akkermansia* strain BWH-H3 reduced ROR γ T+ $\gamma\delta$ T cells and IL-17-producing $\gamma\delta$ T cells. No effect was observed in

FoxP3, ROR γ t, IL-10, IFN γ , or IL-17 expression in CD4 or CD8 T cells (not shown). These findings support a beneficial role for *Akkermansia* in MS and suggest that there may be strain-specific effects on central nervous system (CNS) autoimmunity.

Discussion

Although the microbiome plays a clear role in RRMS, there are few studies that have characterized the microbiome in progressive MS and connected changes in the progressive MS microbiome to clinical disease. Approximately half of the microbiota changes we found were unique in progressive MS versus HC compared to RRMS versus HC. We identified 2 bacteria that were altered in both types of MS, but more prominent in progressive MS. *Erysipelotrichaceae CCMM* was decreased in RRMS versus HC and decreased even further in progressive MS versus both RRMS and HC. Consistent with a potentially beneficial role, *Erysipelotrichaceae CCMM* was associated with increased motor and cognitive function and decreased EDSS and fatigue. The sequence of the V4 region of the 16S rRNA from *Erysipelotrichaceae CCMM* in our study was identical to 2 recently described bacteria, *Faecalibacillus intestinalis* and *Faecalibacillus faecis*.⁴⁹ Little is known about the functions of these bacteria in neurologic or autoimmune disease. *R. lactatiformans* was increased in RRMS versus HC and increased even further in progressive MS versus both RRMS and HCs. *R. lactatiformans* was associated with increased EDSS and decreased lower extremity motor function, consistent with a potentially detrimental role. *R. lactatiformans* is a lactate-producing member of the *Ruminococcaceae* family,⁵⁰ and lactate is hypothesized to contribute to disease progression in MS by contributing to mitochondria dysfunction.^{51,52} Serum lactate has been reported to be higher in MS versus HC, higher in progressive MS versus RRMS, and positively correlated with EDSS.^{51,52} Elevated cerebrospinal fluid lactate has been found in RRMS patients and was associated with long-term disease progression.⁵³

We identified 6 bacteria that were specifically elevated in progressive MS versus both HC and RRMS, including *Enterobacteriaceae*, *Bifidobacterium animalis*, *Clostridium g24 FCEY*, *Dorea massiliensis*, *Longicatena*, and *Ruminococcaceae FJ366134*. Two bacteria were uniquely decreased in progressive MS, including *Lachnospiraceae PAC001046* and *Phascolarctobacterium faecium*. Adjusting for age, sex, race, ethnicity, and BMI, we confirmed that *Enterobacteriaceae*, *Clostridium g24 FCEY*, and *Ruminococcaceae FJ366134* were uniquely elevated in progressive MS. We also found that *Clostridium g24 FCEY* was associated with greater disability, and *Enterobacteriaceae* was associated with fatigue. *Clostridium g24 FCEY* is a not-yet-cultivated member of the *Lachnospiraceae* family, and is closely related to *C. bolteae*, which we found to be elevated in both RRMS and progressive MS and associated with disability and fatigue. *Enterobacteriaceae* is a family of Gram-negative facultative anaerobes that encompasses many important gut bacteria, which include *Escherichia coli*, *Shigella*, and

Salmonella. The *Enterobacteriaceae* family is difficult to speciate based on 16S sequencing alone, because *E. coli* and *Shigella* have identical 16S rRNA sequences. Some species may be commensal, whereas others are pathogenic.^{54,55} Several *Enterobacteriaceae* strains can attach to the intestinal mucosa and stimulate immune responses.⁵⁶ Thus, additional studies using shotgun metagenomics are needed to identify these bacteria at the species level and to determine whether progressive MS patients have an enrichment in other bacteria with the property of adhering to the intestinal mucosa.

Adjusting for age, we identified 2 bacteria that were uniquely decreased in progressive MS, *Agathobaculum DQ795333* and unclassified *Blautia*. The sequence from *Agathobaculum DQ795333* was 99.6% similar to *Agathobaculum butyriciproducens*, a recently discovered butyrate-producing bacteria in the *Ruminococcaceae* family.⁵⁷ The gut microbiota can lessen inflammatory disease by producing butyrate and inducing T regulatory cells, and several studies have reported decreased butyrate producers in MS.^{58,59} The sequence from unclassified *Blautia* was 99.6% similar to *Blautia luti*, an acetate- and succinate-producing member of the *Lachnospiraceae* family. *Blautia* species play important roles in carbohydrate fermentation, which supports cross-feeding networks in the microbiome, and *Blautia* species have been proposed as bacteria with high potential for use as next-generation probiotics.⁶⁰ Of note, *B. luti* and *B. wexlerae* (which we found decreased in both RRMS and progressive MS) were reported to be depleted in children with obesity and insulin resistance and had negative correlations with markers of inflammation in the stool.⁶¹ Furthermore, secreted products from *B. luti* and *B. wexlerae* can exert an anti-inflammatory effect on peripheral blood mononuclear cells.⁶¹ In our study, unclassified *Blautia* had the strongest association, with 9 of 11 quality of life parameters, including a positive correlation with motor function, cognitive function, and affect, and a negative correlation with fatigue and depression.

We identified alterations in the microbiota that were similar in both progressive MS and RRMS patients versus HCs, including depletion of *Erysipelotrichaceae CCMM* and *B. wexlerae* (discussed above), and *D. formicigenerans*. *D. formicigenerans* is a member of the *Lachnospiraceae* family and produces abundant amounts of formic acid.⁶² Studies have shown that administering formic acid to pigs increases levels of beneficial microbes and suppresses pathogenic members of the *Enterobacteriaceae* family (enterotoxigenic *E. coli* and *Salmonella*).^{63,64} We found several *Clostridium* species were elevated in both progressive MS and RRMS patients, including *C. bolteae*, *C. nexile*, and *C.*

scindens, which have all been reported to be elevated in new onset, treatment-naïve MS patients.¹⁷ Adjusting for confounders including BMI, age, race, ethnicity, and sex, we confirmed that *C bolteae* was elevated in both groups. *C bolteae* was originally isolated from an autistic child and may induce Th17 cells by direct attachment to the mucosa.³⁵ It has been shown that gut microorganisms may act together via molecular mimicry and induction of Th17 cells to worsen spinal cord inflammation in the EAE model,⁸ and the bacteria that we identified may contribute to disease through multiple mechanisms. We have also found that *C bolteae* is elevated in patients with neuromyelitis optica spectrum disorders and shares protein sequence homology with aquaporin 4,³⁷ suggesting that it may also act by molecular mimicry.

We observed elevated *Akkermansia* at the genus level in both progressive MS and RRMS patients. At the species level, *A muciniphila* was significantly increased in progressive MS ($p = 0.02$, MaAsLin), whereas there was only a trend of increased *A muciniphila* in our RRMS subjects ($p = 0.10$, MaAsLin). This could reflect additional strain and species level variation in *Akkermansia* in RRMS, which we were able to identify through use of a new taxonomic reference database from EzBioCloud.³⁰ We and others have previously observed elevated levels of *Akkermansia* in RRMS,^{9–11,18} and elevated *Akkermansia* in MS is also observed in a recent meta-analysis of microbiota alterations in autoimmunity.⁶⁵ Of note, *Akkermansia* has been reported to have a beneficial role in multiple diseases.^{66,67} *Akkermansia* improves metabolism in obese and diabetic mice,^{66,68} improves cancer check point immunotherapy,⁶⁹ is associated with the antiseizure effects of a ketogenic diet,⁷⁰ and improves disease in an animal model of amyotrophic lateral sclerosis.⁶⁷ Furthermore, the anti-inflammatory properties of *Akkermansia* can be strain-specific,⁷¹ warranting further study of *Akkermansia* strains in neurologic and inflammatory diseases.

We found that *Akkermansia* negatively correlated with disability and T2 lesion volume, and positively correlated with brain volume. This was unexpected, given that it has been assumed that elevated *Akkermansia* in MS is detrimental.^{9,11} To directly test the in vivo properties of *Akkermansia*, we isolated *Akkermansia* from progressive MS and RRMS patients, colonized animals with these strains prior to EAE induction, and found that MS-derived *Akkermansia* ameliorated EAE. *A muciniphila* strain BWH-H3 had the strongest protective effect, which was associated with decrease in ROR γ T+ and IL-17-producing $\gamma\delta$ T cells. There are large populations of $\gamma\delta$ T cells in the intestinal mucosa, which respond rapidly to the microbiota.⁷² This microbiota- $\gamma\delta$ T cell interaction could be relevant in MS, as $\gamma\delta$ T cells traffic to the CNS and produce high levels of IL-17 in EAE, and

IL-17-producing $\gamma\delta$ T cells are elevated in the blood of MS patients.⁷³ Consistent with our findings in the C57 model, investigators found that mice with higher levels of *Akkermansia* had less progression in the non-obese diabetic (NOD) progressive EAE model.⁷⁴ Humans coevolved with the gut microbiota and developed the production of microRNAs to selectively enhance the growth of specific bacteria.²⁰ We previously found that MS patients and mice at peak EAE had increased levels of the microRNA miR-30d in the gut, which increases *Akkermansia* levels.³⁴ Taken together, our findings suggest that elevated *Akkermansia* may be a beneficial compensatory microbiome response in MS.

Alterations in the microbiota may reflect differences in patient populations, rather than be drivers of disease. In our study, HC, RRMS, and progressive MS subjects were well-matched for BMI, sex, gender, race, and ethnicity. MS disproportionately affects women and Whites of European descent,^{75,76} which is reflected in the study population that we recruited from our center (Table). Progressive MS patients were on average older than RRMS patients, consistent with the observation that age is one of the greatest risk factors for progressive MS.⁴³ The microbiome composition in adults is relatively stable and similar between young adults (20–40 years of age) and middle-aged (40–60 years) adults, but can differ in adults older than 60 years.⁷⁷ To address this, we adjusted microbiota correlations with EDSS, MRI, and quality of life for age. We determined the contribution of demographic factors to microbiome variation, and found that after disease status, BMI had the largest contribution to variation in the microbiome. Race and sex affected β -diversity based on unweighted UniFrac distances but did not affect β -diversity based on weighted UniFrac distances. We found no contribution of age or ethnicity to microbiome variation in our cohort.

Several of our findings were found in other MS studies of progressive MS.¹⁷ A Russian cohort of 15 PPMS subjects also reported elevated *Verrucomicrobiaceae* (the family that contains *Akkermansia*),²² Consistent with our observation of decreased butyrate-producing bacteria in progressive MS (*Agathobaculum*), a Belgian cohort of 28 PPMS patients reported lower *Butyrivibacter*²³ and a Japanese cohort of 15 SPMS subjects reported decreased butyrate producers.²⁴ Furthermore, the Japanese cohort reported elevated *Clostridium* species, similar to our results in progressive MS.²⁴ Our cohort of 44 progressive MS subjects is the largest progressive MS population studied to date. Future longitudinal investigations will further clarify the relationship between the microbiome and disease progression over time.

We observed a correlation between the microbiome and quality of life. The mechanism for this association is not

clear, but it may be related to the microbial production of neurotransmitters.^{78,79} Although there is debate on whether bacterial neurotransmitters cross the blood–brain barrier, it has been shown that administering γ -aminobutyric acid (GABA)-producing *Lactobacillus* reduces anxiety and depression in animal models,⁷⁸ and GABA-producing *Bacteroides* correlates with functional MRI connectivity in patients with major depressive disorder.⁷⁹ Self-reported fatigue is a risk factor for the transition to progressive MS.⁸⁰ Our study identifies bacteria that are associated with fatigue and other measurements of quality of life, which may provide an avenue to affect this through the microbiome.

We had previously found that DMTs, including glatiramer acetate and interferon, normalized some of the MS-associated changes in specific bacteria,¹¹ and others have reported on MS treatment effects in MS.⁸¹ However, in this larger study, we found that disease status had a much greater effect on the microbiome than therapy. This suggests that currently used DMTs primarily act on immune mechanisms of the disease rather than through the gut. A limitation of our study is that we examined changes in microbiota in a cross-sectional study. Thus, interindividual variability may mask treatment-induced changes. Further work comparing the microbiota before and after therapy could help better define the effect of DMTs on the gut microbiota. In addition, there may be a differential effect of therapy on the microbiome in RRMS versus progressive MS. In our cohort, we had 19 to 47 RRMS subjects per treatment group and only 2 to 6 progressive MS subjects per treatment group. In a separate analysis of only RRMS subjects, the majority of taxonomic changes were similar (not shown). Because we had small numbers of progressive MS subjects in each subject group, we could not analyze them separately.

In summary, we have shown that there are unique changes in the microbiome in progressive MS, and that other features of the MS microbiota were observed in both relapsing and progressive disease. Importantly, we found correlations between the microbiome and both clinical and MRI measures of disease, supporting a role for the microbiome in the disease process. We experimentally validated our finding of a correlation between *Akkermansia* and less disease in MS by transferring MS-derived *Akkermansia* into EAE mice and demonstrating a beneficial effect. This finding can serve as a framework to test additional candidates that we identified in our study. Furthermore, comparative genomic analysis may be an approach that could identify strain-specific factors in *Akkermansia* that confer protection in MS. A major question is whether microbiota manipulation is a viable therapeutic avenue to treat MS. We previously reported that a probiotic containing *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* strains promoted anti-inflammatory immunity in RRMS

patients and HCs, and some of these immune changes persisted after discontinuation of the probiotic.¹⁵ We have now identified new bacterial species that may be found useful for the treatment of progressive MS, including *Akkermansia*. Although it is now accepted that the microbiome plays an important biologic role in MS, it must be emphasized that the mechanisms by which the microbiome affects MS have not been well defined, and many confounding factors exist. Nonetheless, our findings support the possibility that microbiota manipulation may one day be used as a treatment for MS and identify unique microbiota changes in progressive disease.

Acknowledgments

This work was supported by NIH grant R01NS087226 from the National Institute of Neurologic Disorders and Stroke (NINDS), the NextGen Collaborative Grant from the Brigham Research Institute, and the Water Cove Charitable Foundation. L.M.C. was supported by the Nancy Davis Race to Erase MS Young Investigator Award. A.H.M. was supported by a clinician-scientist development award from the National MS Society.

Author Contributions

L.M.C., H.L.W., S.K.T., B.I.G., R.B., T.C., and P.L.D.J. contributed to the conception and design of the study; L.M.C., S.L., B.C.H., A.H.M., F.H.D., V.W., A.S., C.W., S.T., R.C., M.C.A., and M.P.-T. contributed to the acquisition and analysis of data; L.M.C., S.L., C.W., A.H.M., R.B., and H.L.W. contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

Nothing to report.

Data Availability

The microbiota 16S rRNA sequence data have been submitted to the National Center for Biotechnology Information Short Read Archives under Bioproject accession number PRJNA721421, which is publicly available. The deidentified metadata, including diagnosis, age, treatment, race, ethnicity, BMI, sex, and EDSS, have been included along with the sequencing data. EDSS is recorded under the column heading host_phenotype.

References

1. Baecher-Allan C, Kaskow BJ, Weiner HL. Multiple sclerosis: mechanisms and immunotherapy. *Neuron* 2018;97:742–768.
2. Cox LM, Weiner HL. Microbiota signaling pathways that influence neurologic disease. *Neurotherapeutics* 2018;15:135–145.

3. Berer K, Mues M, Koutrolos M, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 2011;479:538–541.
4. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 2011;108:4615–4622.
5. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, et al. Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. *J Immunol* 2009;183:6041–6050.
6. Yokote H, Miyake S, Croxford JL, et al. NKT cell-dependent amelioration of a mouse model of multiple sclerosis by altering gut flora. *Am J Pathol* 2010;173:1714–1723.
7. Ochoa-Reparaz J, Mielcarz DW, Wang Y, et al. A polysaccharide from the human commensal *Bacteroides fragilis* protects against CNS demyelinating disease. *Mucosal Immunol* 2010;3:487–495.
8. Miyauchi E, Kim SW, Suda W, et al. Gut microorganisms act together to exacerbate inflammation in spinal cords. *Nature* 2020;585:102–106.
9. Cekanaviciute E, Yoo BB, Runia TF, et al. Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. *Proc Natl Acad Sci U S A* 2017;114:10713–10718.
10. Berer K, Gerdes LA, Cekanaviciute E, et al. Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc Natl Acad Sci U S A* 2017;114:10719–10724.
11. Jang S, Gandhi R, Cox LM, et al. Alterations of the human gut microbiome in multiple sclerosis. *Nat Commun* 2016;7:12015.
12. Tremlett H, Fadrosh DW, Faruqi AA, et al. Associations between the gut microbiota and host immune markers in pediatric multiple sclerosis and controls. *BMC Neurol* 2016;16:182.
13. Miyake S, Kim S, Suda W, et al. Dysbiosis in the gut microbiota of patients with multiple sclerosis, with a striking depletion of species belonging to clostridia XIVa and IV clusters. *PLoS One* 2015;10:e0137429.
14. Chen J, Chia N, Kalari KR, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep* 2016;6:28484.
15. Tankou SK, Regev K, Healy BC, et al. A probiotic modulates the microbiome and immunity in multiple sclerosis. *Ann Neurol* 2018;83:1147–1161.
16. Cosorich I, Dalla-Costa G, Sorini C, et al. High frequency of intestinal TH17 cells correlates with microbiota alterations and disease activity in multiple sclerosis. *Sci Adv* 2017;3:e1700492.
17. Ventura RE, Izumi T, Battaglia T, et al. Gut microbiome of treatment-naïve MS patients of different ethnicities early in disease course. *Sci Rep* 2019;9:16396.
18. Cantarel BL, Wabant E, Chehoud C, et al. Gut microbiota in multiple sclerosis: possible influence of immunomodulators. *J Investig Med* 2015;63:729–734.
19. Tremlett H, Fadrosh D, Faruqi A, et al. Gut microbiota in early pediatric multiple sclerosis: a case–control study. *Eur J Neurol* 2016;23:1308–1321.
20. Liu S, da Cunha AP, Rezende RM, et al. The host shapes the gut microbiota via fecal microRNA. *Cell Host Microbe* 2016;19:32–43.
21. Mangalam A, Shahi SK, Luckey D, et al. Human gut-derived commensal bacteria suppress CNS inflammatory and demyelinating disease. *Cell Rep*. 2017;20(6):1269–1277. <http://doi.org/10.1016/j.celrep.2017.07.031>.
22. Kozhieva M, Naumova N, Alikina T, et al. Primary progressive multiple sclerosis in a Russian cohort: relationship with gut bacterial diversity. *BMC Microbiol* 2019;19:309.
23. Reynders T, Devolder L, Valles-Colomer M, et al. Gut microbiome variation is associated to multiple sclerosis phenotypic subtypes. *Ann Clin Transl Neurol* 2020;7:406–419.
24. Takewaki D, Suda W, Sato W, et al. Alterations of the gut ecological and functional microenvironment in different stages of multiple sclerosis. *Proc Natl Acad Sci U S A* 2020;117:22402–22412.
25. Celli D, Lai JS, Nowinski CJ, et al. Neuro-QOL: brief measures of health-related quality of life for clinical research in neurology. *Neurology* 2012;78:1860–1867.
26. Meier DS, Guttmann CRG, Tummala S, et al. Dual-sensitivity multiple sclerosis lesion and CSF segmentation for multichannel 3T brain MRI. *J Neuroimaging* 2018;28:36–47.
27. Chu R, Tauhid S, Glanz BI, et al. Whole brain volume measured from 1.5T versus 3T MRI in healthy subjects and patients with multiple sclerosis. *J Neuroimaging* 2016;26:62–67.
28. Chu R, Hurwitz S, Tauhid S, Bakshi R. Automated segmentation of cerebral deep gray matter from MRI scans: effect of field strength on sensitivity and reliability. *BMC Neurol* 2017;17:172.
29. Raj T, Rothamel K, Mostafavi S, et al. Polarization of the effects of autoimmune and neurodegenerative risk alleles in leukocytes. *Science* 2014;344:519–523.
30. Yoon SH, Ha SM, Kwon S, et al. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.
31. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12:R60.
32. Morgan XC, Tickle TL, Sokol H, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol* 2012;13(9):R79. <http://doi.org/10.1186/gb-2012-13-9-r79>.
33. Cox LM, Sohn J, Tyrrell KL, et al. Description of two novel members of the family Erysipelotrichaceae: *ileibacterium valens* gen. Nov., sp. nov. and *Dubosiella newyorkensis*, gen. Nov., sp. nov., from the murine intestine, and emendation to the description of *Faecalibaculum rodentium*. *Int J Syst Evol Microbiol* 2017;67:1247–1254.
34. Liu S, Rezende RM, Moreira TG, et al. Oral administration of miR-30d from feces of MS patients suppresses MS-like symptoms in mice by expanding *Akkermansia muciniphila*. *Cell Host Microbe* 2019;26:779–794.
35. Atarashi K, Tanoue T, Ando M, et al. Th17 cell induction by adhesion of microbes to intestinal epithelial cells. *Cell* 2015;163:367–380.
36. Song Y, Liu C, Molitoris DR, et al. *Clostridium bolteae* sp. nov., isolated from human sources. *Syst Appl Microbiol* 2003;26:84–89.
37. Pandit L, Cox LM, Malli C, et al. *Clostridium bolteae* is elevated in neuromyelitis optica spectrum disorder in India and shares sequence similarity with AQP4. *Neurol Neuroimmunol Neuroinflamm* 2021;8:e907.
38. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett* 2009;294:1–8.
39. Bakshi R, Thompson AJ, Rocca MA, et al. MRI in multiple sclerosis: current status and future prospects. *Lancet Neurol* 2008;7:615–625.
40. Holland CM, Charil A, Csapo I, et al. The relationship between normal cerebral perfusion patterns and white matter lesion distribution in 1,249 patients with multiple sclerosis. *J Neuroimaging* 2012;22:129–136.
41. Pirko I, Lucchinetti CF, Sriram S, Bakshi R. Gray matter involvement in multiple sclerosis. *Neurology* 2007;68:634–642.
42. Andrávizou A, Dardiotis E, Artemiadis A, et al. Brain atrophy in multiple sclerosis: mechanisms, clinical relevance and treatment options. *Auto Immun Highlights* 2019;10:7.
43. Miller DH, Lublin FD, Sormani MP, et al. Brain atrophy and disability worsening in primary progressive multiple sclerosis: insights from the INFORMS study. *Ann Clin Transl Neurol* 2018;5:346–356.

44. Mohle L, Mattei D, Heimesaat MM, et al. Ly6C(hi) monocytes provide a link between antibiotic-induced changes in gut microbiota and adult hippocampal neurogenesis. *Cell Rep* 2016;15:1945–1956.
45. Hoban AE, Stilling RM, Ryan FJ, et al. Regulation of prefrontal cortex myelination by the microbiota. *Transl Psychiatry* 2016;6:e774.
46. Rothhammer V, Mascanfroni ID, Bunse L, et al. Type I interferons and microbial metabolites of tryptophan modulate astrocyte activity and central nervous system inflammation via the aryl hydrocarbon receptor. *Nat Med* 2016;22:586–597.
47. Glanz BI, Healy BC, Rintell DJ, et al. The association between cognitive impairment and quality of life in patients with early multiple sclerosis. *J Neurol Sci* 2010;290:75–79.
48. Glanz BI, Degano IR, Rintell DJ, et al. Work productivity in relapsing multiple sclerosis: associations with disability, depression, fatigue, anxiety, cognition, and health-related quality of life. *Value Health* 2012;15:1029–1035.
49. Seo B, Jeon K, Baek I, et al. Faecalibacillus intestinalis gen. Nov., sp. nov. and Faecalibacillus faecis sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2019;69:2120–2128.
50. Shkoporov AN, Chaplin AV, Shcherbakova VA, et al. Ruthenibacterium lactatiformans gen. Nov., sp. nov., an anaerobic, lactate-producing member of the family Ruminococcaceae isolated from human faeces. *Int J Syst Evol Microbiol* 2016;66:3041–3049.
51. Amorini AM, Nociti V, Petzold A, et al. Serum lactate as a novel potential biomarker in multiple sclerosis. *Biochim Biophys Acta* 1842;2014:1137–1143.
52. Esmael A, Talaat M, Egila H, Eltoukhy K. Mitochondrial dysfunction and serum lactate as a biomarker for the progression and disability in MS and its correlation with the radiological findings. *Neurol Res*. 2021;1–9. <http://doi.org/10.1080/01616412.2021.1893567>.
53. Albanese M, Zagaglia S, Landi D, et al. Cerebrospinal fluid lactate is associated with multiple sclerosis disease progression. *J Neuroinflamm* 2016;13:36.
54. Parsot C, Sansonetti PJ. The virulence plasmid of *Shigellae*: an archipelago of pathogenicity islands? Pathogenicity islands and other mobile virulence elements. In: Kaper JB, Hacker J, eds. *Pathogenicity Islands and Other Mobile Virulence Elements*. Washington, DC: American Society for Microbiology; 1999;151–165. <https://doi.org/10.1128/9781555818173.ch8>.
55. Kaper JB, Mellies JL, Nataro JP. Pathogenicity islands and other mobile genetic elements of diarrheagenic *Escherichia coli*. In: Kaper J, Jorg H, eds. *Pathogenicity Islands and other mobile virulence elements*. Washington, DC: American Society for Microbiology; 1999;33–58. <https://doi.org/10.1128/9781555818173.ch3>.
56. Kim M, Galan C, Hill AA, et al. Critical role for the microbiota in CX (3)CR1(+) intestinal mononuclear phagocyte regulation of intestinal T cell responses. *Immunity* 2018;49:151–163.e5.
57. Ahn S, Jin T-E, Chang D-H, et al. Agathobaculum butyriciproducens gen. Nov. & sp. nov., a strict anaerobic, butyrate-producing gut bacterium isolated from human faeces and reclassification of *Eubacterium desmolans* as *Agathobaculum desmolans* comb. nov. *Int J Syst Evol Microbiol* 2016;66:3656–3661.
58. Mirza A, Forbes JD, Zhu F, et al. The multiple sclerosis gut microbiota: a systematic review. *Mult Scler Relat Disord* 2020;37:101427.
59. Noto D, Miyake S. Gut dysbiosis and multiple sclerosis. *Clin Immunol*. 2020;108380. <http://doi.org/10.1016/j.clim.2020.108380>.
60. Liu X, Mao B, Gu J, et al. Blautia—a new functional genus with potential probiotic properties? *Gut Microbes* 2021;13:1–21.
61. Benitez-Paez A, Gomez Del Pugar EM, Lopez-Almela I, et al. Depletion of Blautia species in the microbiota of obese children relates to intestinal inflammation and metabolic phenotype worsening. *mSystems* 2020;5:e00857–e00819.
62. Taras D, Simmering R, Collins MD, et al. Reclassification of *Eubacterium formicigerans* Holdeman and Moore 1974 as *Dorea formicigerans* gen. Nov., comb. nov., and description of *Dorea longicatena* sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2002;52:423–428.
63. Ren C, Wang Y, Lin X, et al. A combination of formic acid and mono-laurin attenuates enterotoxigenic *Escherichia coli* induced intestinal inflammation in piglets by inhibiting the NF- κ B/MAPK pathways with modulation of gut microbiota. *J Agric Food Chem* 2020;68:4155–4165.
64. Luise D, Correa F, Bosi P, Trevisi P. A review of the effect of formic acid and its salts on the gastrointestinal microbiota and performance of pigs. *Animals (Basel)* 2020;10:887.
65. Volkova A, Ruggles KV. Predictive metagenomic analysis of autoimmune disease identifies robust autoimmunity and disease specific microbial signatures. *Front Microbiol* 2021;12:621310.
66. Cani PD, de Vos WM. Next-generation beneficial microbes: the case of *Akkermansia muciniphila*. *Front Microbiol* 2017;8:1765.
67. Blacher E, Bashiardes S, Shapiro H, et al. Potential roles of gut microbiome and metabolites in modulating ALS in mice. *Nature* 2019;572:474–480.
68. Plovier H, Everard A, Druart C, et al. A purified membrane protein from *Akkermansia muciniphila* or the pasteurized bacterium improves metabolism in obese and diabetic mice. *Nat Med* 2017;23:107–113.
69. Routy B, Le Chatelier E, Derosa L, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* 2018;359:91–97.
70. Olson CA, Vuong HE, Yano JM, et al. The gut microbiota mediates the anti-seizure effects of the ketogenic diet. *Cell* 2018;174:497.
71. Zhai R, Xue X, Zhang L, et al. Strain-specific anti-inflammatory properties of two *Akkermansia muciniphila* strains on chronic colitis in mice. *Front Cell Infect Microbiol* 2019;9:239.
72. Duan J, Chung H, Troy E, Kasper DL. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. *Cell Host Microbe* 2010;7:140–150.
73. Wo J, Zhang F, Li Z, et al. The role of gamma-delta T cells in diseases of the central nervous system. *Front Immunol* 2020;11:580304.
74. Colpitts SL, Kasper EJ, Keever A, et al. A bidirectional association between the gut microbiota and CNS disease in a biphasic murine model of multiple sclerosis. *Gut Microbes* 2017;8:561–573.
75. Amezcua L, McCauley JL. Race and ethnicity on MS presentation and disease course. *Mult Scler* 2020;26:561–567.
76. Harbo HF, Gold R, Tintore M. Sex and gender issues in multiple sclerosis. *Ther Adv Neurol Disord* 2013;6:237–248.
77. Ghosh TS, Das M, Jeffery IB, O'Toole PW. Adjusting for age improves identification of gut microbiome alterations in multiple diseases. *Elife* 2020;9:e50240.
78. Bravo JA, Forsythe P, Chew MV, et al. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A* 2011;108:16050–16055.
79. Strandwitz P, Kim KH, Terekhova D, et al. GABA-modulating bacteria of the human gut microbiota. *Nat Microbiol* 2019;4:396–403.
80. Vaughn C, Kavak K, Bushra A, et al. Self-reported fatigue and lower limb problems predictive of conversion to secondary progressive multiple sclerosis in an aging sample of patients (S10.004). *Neurology* 2017;88(16 suppl):S10.004.
81. Sand IK, Zhu Y, Ntranos A, et al. Disease-modifying therapies alter gut microbial composition in MS. *Neurol Neuroimmunol Neuroinflamm* 2018;6:e517.