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The Gut Microbiome in Progressive Multiple Sclerosis

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Abstract

Objective: Investigate the gut microbiome in progressive multiple sclerosis (MS) and how it relates to clinical disease.

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

Results: Microbiota β-diversity differed between MS patients vs. controls but did not differ between RRMS vs. progressive MS or differ based on disease modifying therapies. Disease status had the greatest effect on the microbiome β-diversity, followed by BMI, race, and sex. In both progressive and RRMS, we found increased *Clostridium bolteae, Ruthenibacterium lactatiformans* and *Akkermansia* and decreased *Blautia wexlerae, Dorea formicigenerans*, and *Erysipelotrichaceae CCM*. Unique to progressive MS, we found elevated *Enterobacteriaceae* and *Clostridium g24 FCEY* and decreased *Blautia* and *Agathobaculum*. Several *Clostridium* species

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AUTHOR CONTRIBUTIONS

LMC, HLW, SKT, BIG, RB, TC, and PLD contributed to the conception and design of the study; LMC, SL, BCH, AHM, FD, VW, AS, CW, ST, RC, MCA, and MPT contributed to the acquisition and analysis of data; LMC, SL, CW, AHM, RB, and HLW contributed to drafting the text or preparing the figures.

POTENTIAL CONFLICTS OF INTEREST

Nothing to report.

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 RORγt+ and IL-17 producing γδ T cells.

Interpretation: While 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.

INTRODUCTION

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 the relapsing remitting (RR) to the progressive form (1). Progressive MS is more refractory to therapy, 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 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 T regs (10). Incubating MS microbiota with human PBMCs 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 (PPMS) subjects had elevated *Gemmiger* and *Rumincoccus* and an increase in the family *Verrocomicrobiaceae*, which contains *Akkermansia* (22). A Belgian cohort of 28 PPMS patients had lower *Gemmiger* and *Butyricicoccus* 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).

METHODS

Study subjects and clinical metadata.

Microbiome study subjects were recruited from the CLIMB study conducted at the MS center at the Brigham & Women's Hospital. Subjects were selected based on a diagnosis of RRMS or progressive MS, and willingness to participate in a microbiome study, and meeting the inclusion/exclusion criteria (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 multiple sclerosis included a diagnosis of MS according to the latest McDonald's Criteria. Disease subtypes were further classified as relapsing remitting MS 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 brain (27) and deep gray matter (28) 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), 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 Harvard Biopolymers facility, and sequence analysis

was performed in QIIME2. Samples were sequenced on two 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 was performed using DADA2 for each run, then paired samples were merged. Any sample with less than 1000 reads was then removed from analysis. For taxonomic assignment, sequences from EZ-biocloud database formatted for QIIME and released May 2018 was used as a reference set (30). The RDP classifier was trained against the V4 region of this database, then was used to identify sequences. Significant differences in alpha-diversity was performed by the non-parametric Kruskal Wallis test, differences in beta-diversity was performed by PERMANOVA, with correction for multiple comparison testing, and assessment of contributors to microbiome variation was determined 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 the alpha 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) tool (32) 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 7 10-fold serial dilution in pre-reduced anaerobically sterilized (PRAS) saline and 100 μL of the 10^{-4} through 10^{-7} dilutions were plated on minimal mucin media (Anaerobe Systems). 8-10 colonies were isolated in pure culture per microbiota donor after incubation of 3-7 days. Isolates were frozen in Brucella broth (Difco) plus 15% glycerol, and the near full length 16S rRNA gene was amplified using the 8F and 1510R primers according to previous methods (33). After PCR, 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 (NCBI). Finally, phylogenetic tree of isolates and references 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 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 μL) or vehicle control of BHI+mucin broth were delivered to 9-week-old female C57BL6J mice (n = 10-14/group) by oral gavage 3 times a week beginning 2.2 weeks prior to disease induction and receiving bacteria treatment up to 2 weeks post disease induction. Experimental autoimmune encephalomyelitis was induced by injecting 150 μg of myelin oligodendrocyte glycoprotein (MOG) and Complete Freund’s Adjuvant (CFA) subcutaneously and

administering a peritoneal injection of 200 ng 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's test and Dunn's correction for multiple comparisons. Animals were housed in a biosafety level 2 facility (BSL2) using autoclaved cages and aseptic handling procedures and kept under a 12-hour light/dark cycle. All animal experiments were conducted according to an IACUC 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. 10 days post disease induction, splenocytes were isolated for flow cytometric analyses. Red blood cells were lysed with ACK, and dead cells were stained with fixable viability dye Aqua Zombie (1:1000 diluted in PBS; Biolegend). Surface markers were stained for 25 min at 4°C in FACs buffer (Mg^{2+} and Ca^{2+} free HBSS with 2% FCS, 0.4% EDTA [0.5 M] and 2.5% HEPES [1M]), cells were fixed in Cytoperm/Cytofix (eBioscience), permeabilized with Perm/Wash Buffer (eBiosciences), and then stained for intracellular markers. Extracellular antibodies used were: PerCp-Cy5.5-anti-TCRβ (H57-597; 1:800; Biolegend), BV785-anti-CD4 (RM4-5; 1:400; Biolegend), BV711-anti-CD8a (53-6.7; 1:400; BD), and APC-anti-TCDγδ (GL-3,1:100, ThermoFisher). To measure T cell transcription factors, intracellular antibodies used were FITC-anti-Foxp3 (FJK-16s; 1:100; ThermoFisher), and BV421-anti-RORγT (Q31-378, 1:100, BD). For intracellular cytokine staining, cells were first stimulated for 4 h with PMA (phorbol 12-myristate 13-aceate; 50 ng ml^{-1}; Sigma-Aldrich) and ionomycin (1 μM; Sigma-Aldrich) and a protein-transport inhibitor containing monensin (1 μg 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; ThermoFisher), FITC-anti-IL-10 (Jes5-16E3; 1:100; BioLegend), and PE-anti-GM-CSF (MP1-22E9, 1:100, ThermoFisher). Flow-cytometric acquisition was performed on a Fortessa (BD Biosciences) by using DIVA software (BD Biosciences) and data were analyzed with FlowJo software versions 10.1 (TreeStar Inc). Cells were gated on lymphocytes, single cells, live cells, then T cell subsets were divided into TCRβ+CD4+, TCRβ+CD8+, or TCRβ-TCRγδ+ cell populations, and then transcription factors (FoxP3 and RORγT) and cytokines (IL-10, GM-CSF, IL-17, and INFγ) were measured.

RESULTS

Patients with progressive MS have alterations in intestinal microbiota composition.

We analyzed the microbiota in 40 healthy controls, 199 RRMS patients, and 44 progressive patients by sequencing the V4 region of the microbial 16S rRNA gene. Examining alpha-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 healthy controls (Fig 1A). Examining β-diversity, we found that overall microbiota community structure differed between both progressive and relapsing patients vs. healthy controls but did not differ between progressive and RRMS (Fig 1B,C). At the phylum

through genus (Fig 2AB) and at species levels (Fig 2C), we found that progressive patients had unique changes compared to RRMS and healthy controls including an increase in *Enterobacteriaceae, Ruminococcaceae FJ366134* and *Clostridaceae g24 FCEY* and a decrease in *Dorea longicatena, Anaerococcus vaginalis*, and *Blautia faecis*. Progressive MS patients shared microbiota alterations with RRMS patients compared to healthy controls, 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) and was originally isolated from an autistic patient (36) and has been reported to be elevated in neuromyelitis optica (37). In addition to increased abundance, *C. bolteae* had a higher prevalence in progressive MS (50% of subjects) compared to healthy controls (12% of subjects). There were two bacteria that were altered in all three 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, BMI, sex, race, and ethnicity on microbiome β-diversity. For this analysis, we restricted the dataset to subjects with 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 vs. HC while adjusting for these variables. As shown in Figure 3C, we found that 14 bacteria were increased and 2 decreased in both progressive and RRMS vs. healthy controls, with the largest increases in *Romboutsia timonensis* and unclassified *Bacteroides*, and the largest decreases in *Blautia wexlerae* and *Dorea formicigenerans* (Fig 3D). RRMS had unique differences vs. HC in our adjusted model, including decreased *Erysipelotrichaceae CCMM* and *Ruminococcaceae PAC000672* and increased *Bacteroides uniformis* and *Pseudoflavonifractor PAC001038* (Fig 3E). Progressive MS had unique changes vs. HC with the largest increases in *Akkermansia muciniphila* and *Streptococcus* and the largest decreases in *Blautia unclassified* and *Agathobaculum DQ795333*. Many of the bacteria we identified in our adjusted model (Fig 3) were similar to the bacteria we identified with LEfSe (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 (Fig 3A-B).

The 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 beta-diversity in patients receiving four 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 beta-diversity, whereas all MS treatment subgroups differed from healthy controls ($p < 0.05$, PERMANOVA) (Fig 4A-B). These data suggest that disease status has a greater effect on the microbiota than

treatment. While there were no changes in beta-diversity, we found changes in specific bacteria that were linked to therapy. Although for each DMT, we found fewer bacteria that differed vs. untreated MS (orange bars) than differed vs. HC (blue bars bars) (Fig 4B). As shown in Fig 4C-D, many of the bacteria that differed between untreated-MS vs. HC also differed between treated-MS vs. HC including an increase in *Clostridium 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 vs. both untreated MS and HC (Fig 4E-F). Specifically, we found that anti-CD20 increased *Faecalibacterium prauznitzii*, and DMF increased *Roseburia intestinalis*, two 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 *Ruthenibacterium lactatiformans* in all DMTs vs. HC, suggesting that treatment may partially contribute to the increased *Ruthenibacterium lactatiformans* we identified in Fig 2-3.

Identification of bacteria associated with disability.

To determine whether bacteria we identified in progressive and RRMS were associated with disability, we examined the relationship between the microbiota and the expanded disability status score (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 inulinovorans* 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 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.

Identification of bacteria associated with MRI measures of disease.

Atrophy is more severe in progressive vs. 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 neurogenesis (44), myelination (45) 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 and RRMS patients in our CLIMB longitudinal cohort study for which we obtained quantitative measures of brain T2 lesions and both whole brain and regional deep gray matter volumes from 3T MRI. Progressive patients had higher T2 lesion volume and lower normalized whole brain, total deep gray, and thalamic volumes (Fig 6A). Because brain volume negatively correlated with age (Fig 6B), 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. nexile*, and *Clostridium g24* FCEY (Fig 6C), consistent with our EDSS data (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, Akkermansia muciniphila, Erysipelotrichaceae CCMM* (Fig 6C), consistent with our observation that some of these bacteria were associated with lower disability (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 vs 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 six metrics (“lower extremity motor function” through “satisfaction”), higher scores indicate higher quality of life, and for the last five metrics (“emotional dyscontrol” through “fatigue”), higher scores indicate lower quality of life (Fig 7A). Because there were no differences in fatigue, anxiety and depression in our progressive vs. RRMS subjects (Fig 7A), we analyzed a combined cohort of progressive and RRMS patients. We found that *Enterobacteriaceae, C. bolteae* and other *Clostridia* positively correlated with fatigue suggesting a potential detrimental role whereas *Erysipelotrichaceae CCMM, Romboutsia timonensis*, and *Lachnospiraceae PAC00194* negatively correlated with fatigue, depression, and anxiety, suggesting a potential beneficial role (Fig 7C).

MS-associated *Akkermansia* ameliorates EAE.

As discussed above, we found that *Akkermansia* was negatively correlated with EDSS and MRI burden of disease (Fig 5, 6) suggesting that *Akkermansia* has a beneficial role in MS. Thus, we investigated whether MS-associated *Akkermansia* had a beneficial effect in the MS model of EAE. We identified progressive and RRMS patients with high *Akkermansia* and were able to distinguish three 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 *Akkermansia muciniphila* and strain RRMS1- BWH-H3 was 98.7% identical to the Type strain, indicating a new sub-strain of *A. muciniphila*. Strain Prog1- BWH-I7 was only 96.5% identical, indicating a new species of *Akkermansia*. To test the role 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 (Fig 8B). As a control, we used *Bacteroides cellulosilyticus*, a commensal gut microbe that was not altered in MS. In addition, we measured immune responses in an independent experiment 10 days post-immunization and found that *Akkermansia* strain BWH-H3 reduced RORγT positive γδ T cells and IL-17 producing γδ T cells. No effect was observed in FoxP3, RORγt, IL-10,

IFNγ, 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 CNS autoimmunity.

DISCUSSION

While the microbiome plays a clear role in relapsing-remitting MS, there are few studies that have characterized the microbiome in progressive MS and connected changes in the progressive microbiome to clinical disease. Approximately half of the microbiota changes we found were unique in progressive MS vs. HC compared to RRMS vs. HC. We identified two bacteria that were altered in both types of MS, but more prominent in progressive MS. *Erysipelotrichaceae CCMM* was decreased in RRMS vs. HC and decreased even further in progressive MS vs. both RRMS and HC. Consistent with a potentially beneficial role, *Erysipelotrichaceae CCMM* was associated and 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 two recently described bacteria, *Faecalibacillus intestinalis* and *Faecalibacillus faecis* (49). Little is known about the functions of these bacteria in neurologic or autoimmune disease. *Ruthenibacterium lactatiformans* was increased in RRMS vs. HC and increased even further in progressive MS vs. both RRMS and HC. *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 vs. HC, higher in progressive vs. RRMS, and positively correlated with EDSS (51, 52). Elevated CSF lactate has been found in RRMS patients and was associated with long-term disease progression (53).

We identified six bacteria that were specifically elevated in progressive MS vs. 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 *Clostridium bolteae*, which we found to be elevated in both RR and progressive MS and associated with disability and fatigue. *Enterobacteriaceae* is a family of Gram-negative facultative anaerobes which encompasses many important gut bacteria, which include *E. 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 while 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 two bacteria that were uniquely decreased in progressive MS, *Agathobaculum DQ795333* and *Blautia* unclassified. 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* 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, *Blautia luti* and *Blautia wexlerae* (which we found decreased in both RR 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, *Blautia unclassified* had the strongest association with 9 out 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 and RRMS patients vs. healthy controls, including depletion of *Erysipelotrichaceae CCMM and Blautia wexlerae (discussed above)*, and *Dorea 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 and RRMS patients, including *Clostridium bolteae, C. nexile*, and *C. scindens*, which have all been reported to be elevated in new-onset, treatment naive 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 and RRMS patients. At the species level, *Akkermansia muciniphila* was significantly increased in progressive MS ($p = 0.02$ MaAsLin), while 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 anti-seizure effects of a ketogenic diet (70) and improves disease in an animal model of ALS (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 and RRMS patients, colonized animals with these strains prior to EAE induction and found that MS-derived *Akkermansia* ameliorated EAE. *Akkermansia muciniphila strain BWH-H3* had the strongest protective effect, which was associated with decrease in RORγT+ and IL-17 producing γδ T cells. There are large populations of γδ T cells in the intestinal mucosa which respond rapidly to the microbiota (72). This microbiota-γδ T cell interaction could be relevant in MS as γδ T cells traffic to the CNS and produce high levels of IL-17 in EAE, and IL-17 producing γδ T 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 NOD progressive EAE model (74). Humans co-evolved 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, healthy controls, 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 1). 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 over 60 years of age (77). In order 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 *Verrocomicrobiaceae* (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 *Butyricicoccus* (23) and 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). While there is debate on whether bacterial neurotransmitters cross the blood brain barrier, it has been shown that administering gamma amino butyric 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 disease modifying therapies, 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 disease modifying therapies 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 vs progressive MS. In our cohort, we had 19-47 RRMS subjects per treatment group and only 2-6 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 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 healthy controls and some of these immune changes persisted after discontinuation of the probiotic (15). We have now identified new bacterial species that may find usefulness 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.

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DATA AVAILABILITY

The microbiota 16S rRNA sequence data has been submitted to the National Center for Biotechnology Information (NCBI) Short Read Archives (SRA) 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.

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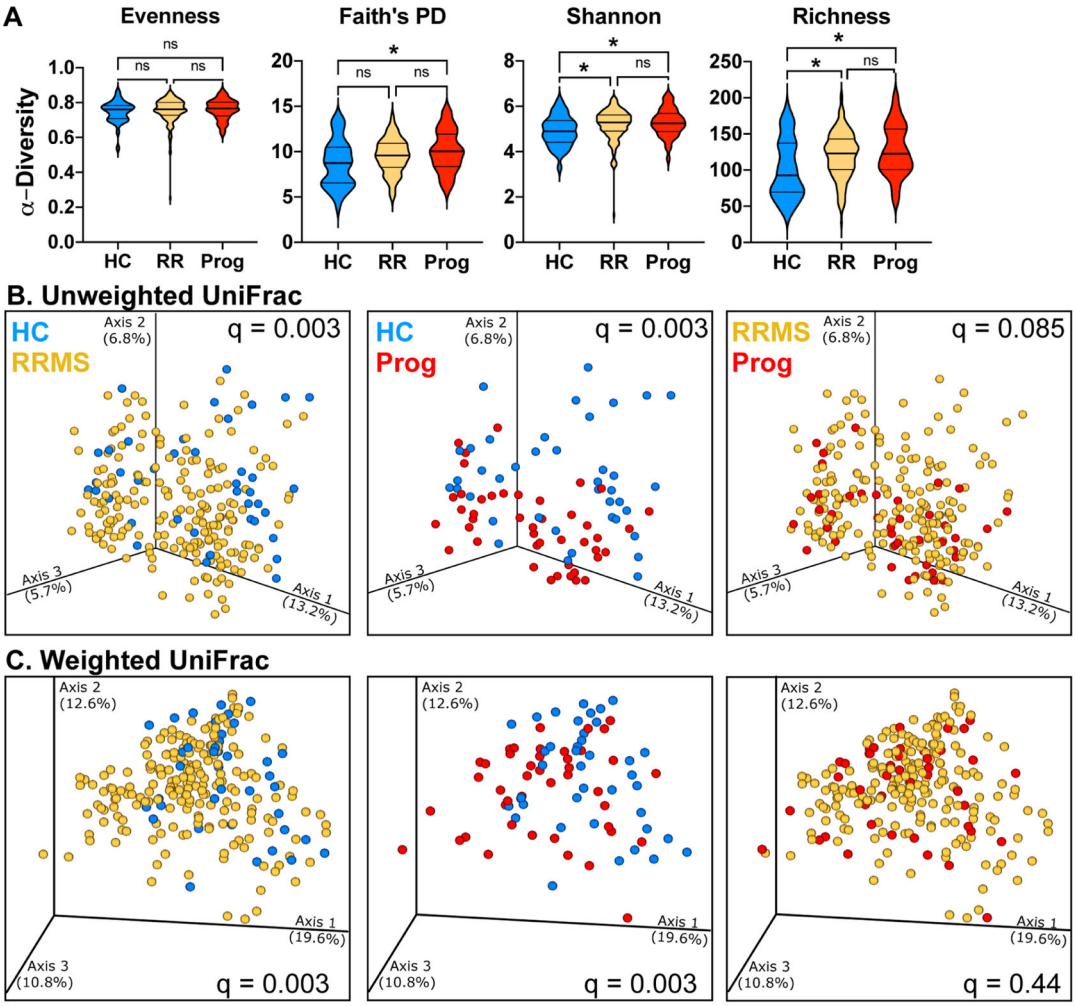

Figure 1. Microbiota α- and β-diversity in relapsing and progressive MS.

A) Alpha 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 (n = 40), RRMS (n = 199), and progressive MS (n = 44). * $p < 0.05$ Kruskal-Wallis. **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, 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, healthy control (blue), RRMS (yellow), or progressive MS (red).

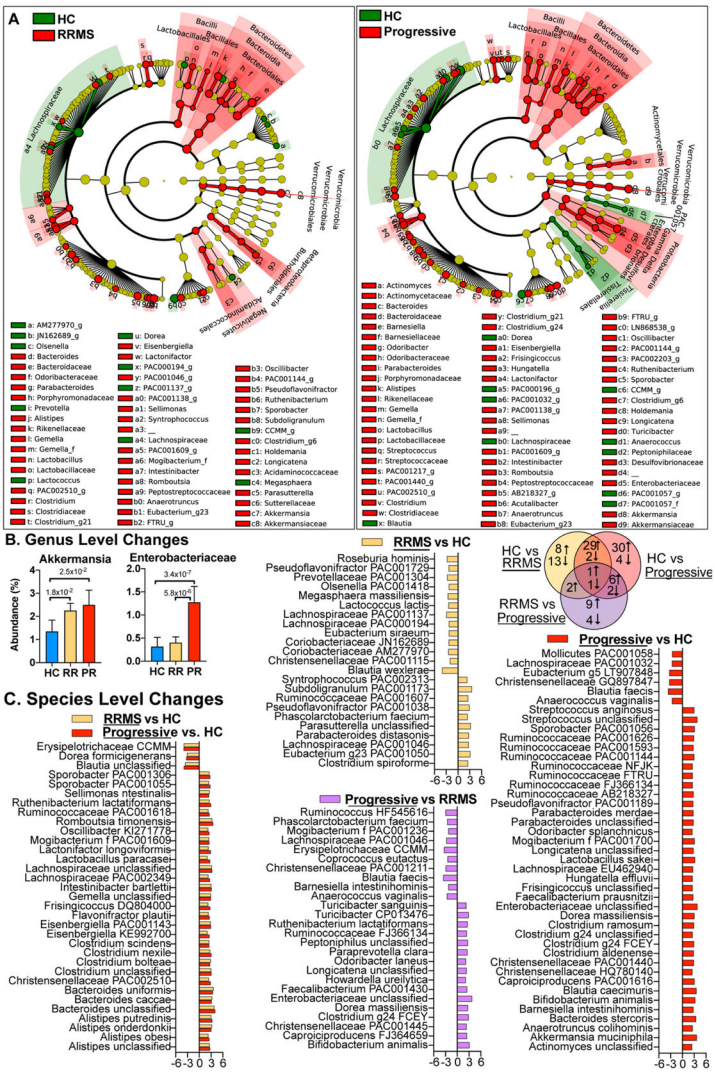

Figure 2. Compositional differences in the microbiota of progressive and relapsing MS.

Microbiota was sequenced in healthy controls (HC, n=40), RRMS (n = 199), and Progressive 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 indicates a taxon present but not differentially abundant. 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)** 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.

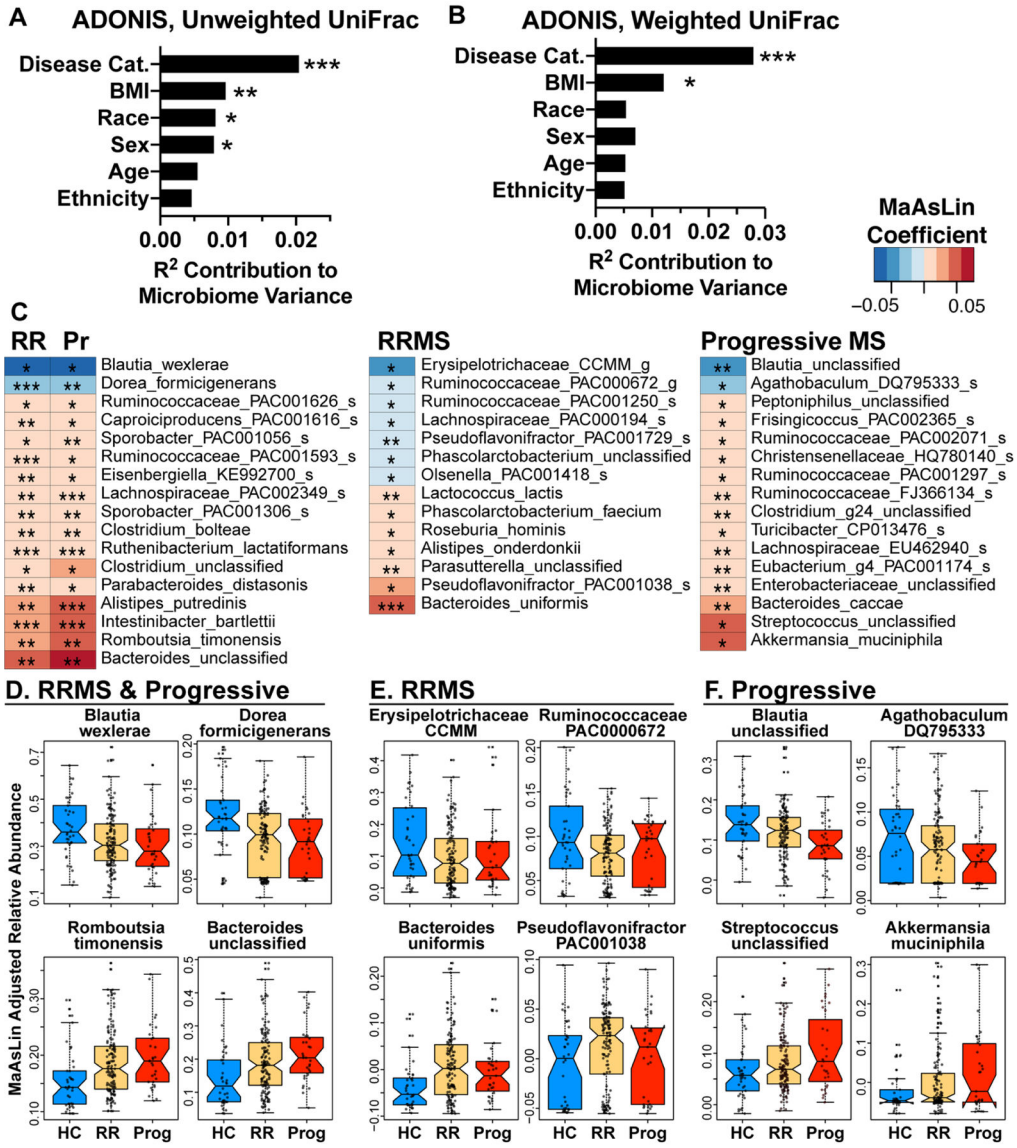

Figure 3. Microbiota differences adjusted for host variables.

A-B) Effect of host factors on microbiome beta-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 BMI, (n = 38 HC, n = 135 RRMS, n = 31 progressive MS) **C)** Microbiota altered in RR or progressive MS vs. healthy control, MaAsLin, adjusted for age, BMI, sex, race, and ethnicity. **D-F)** Abundance of the two most decreased taxa and two most increased taxa in both RRMS and progressive MS vs. HC **(D)**, unique to RRMS **(E)**, or unique to progressive MS **(F)**. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

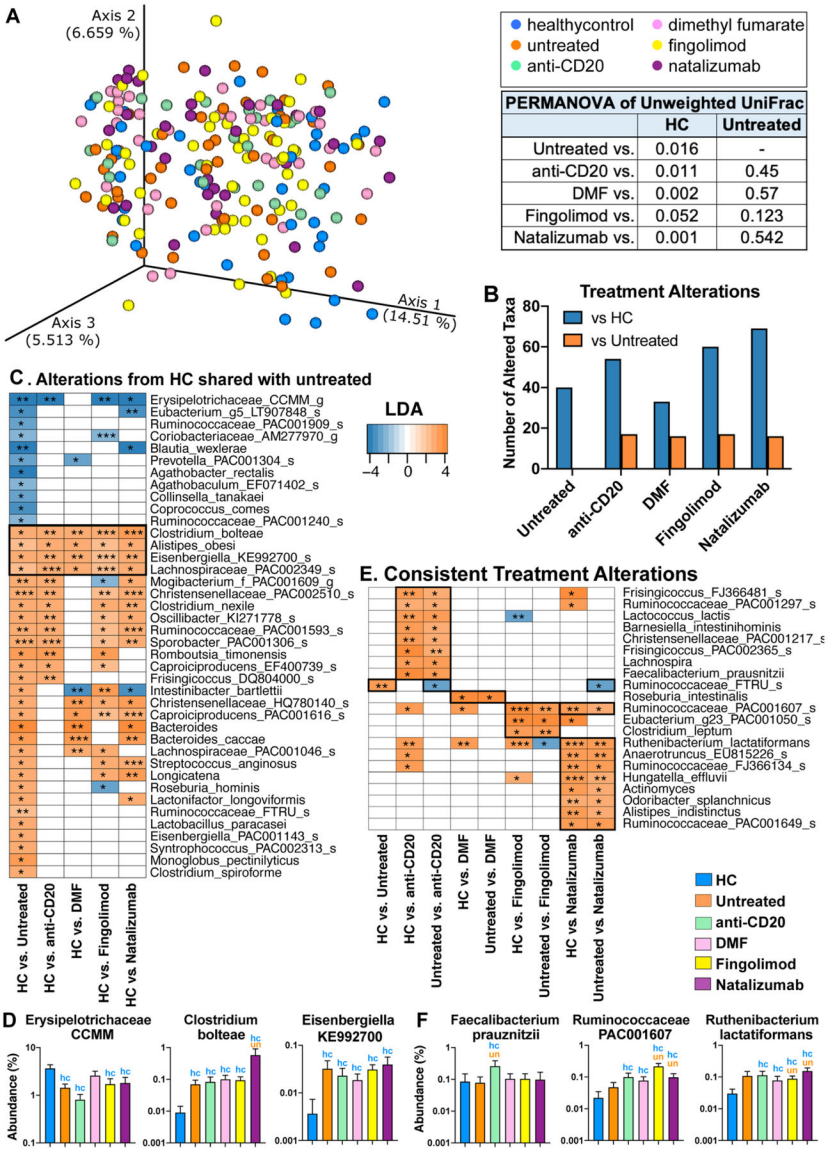

Figure 4. The effect of treatment on the MS microbiota.

A) PCoA of unweighted UniFrac distances of RRMS and progressive subjects not on treatment (n = 33) or treated with anti-CD20 (n = 25), dimethyl fumarate (n = 33), fingolimod (n = 57), or natalizumab (n = 36), or healthy controls (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 DMT group vs. healthy controls (blue bar) or vs. untreated MS (orange bar). **C)** Linear discriminant analysis (LDA) effect size of bacteria altered in untreated MS vs. HC, and whether those bacteria are similarly altered in each DMT group. **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

vs. HC and DMT vs. untreated MS. **F)** Representative bacteria consistently altered by treatment compared to both healthy control and untreated MS patients.

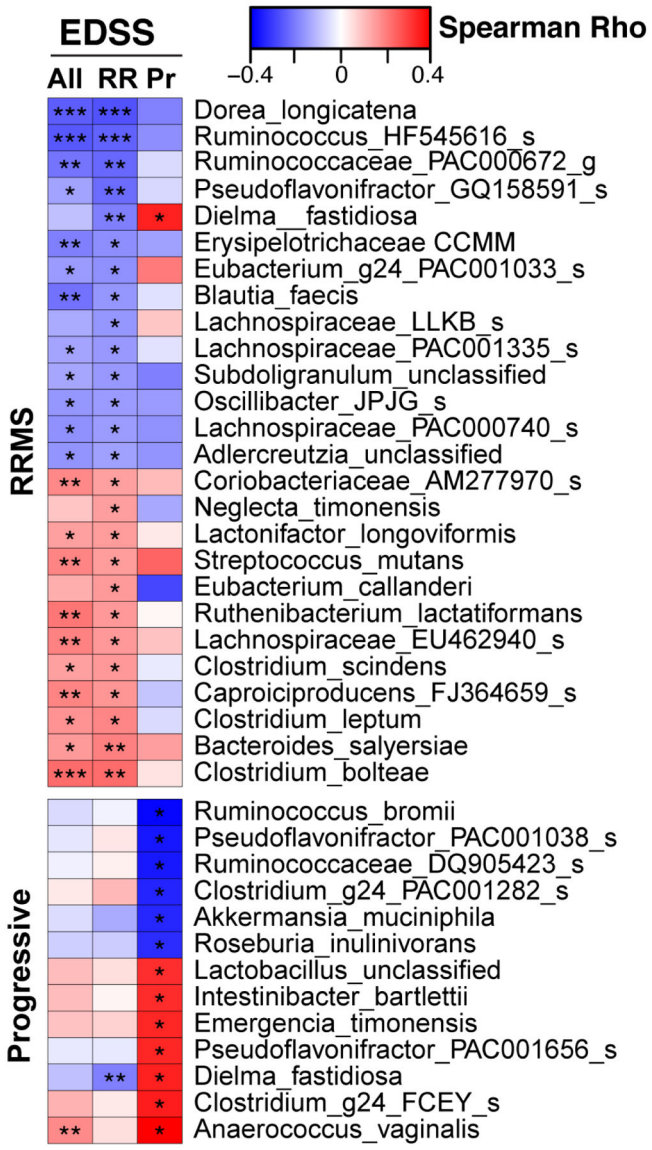

Figure 5. Microbiota associated with disability.

Microbiota correlations with EDSS scores show unique relationships in RRMS (n = 198) and progressive MS (n = 43), Spearman correlations, adjusted for age. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

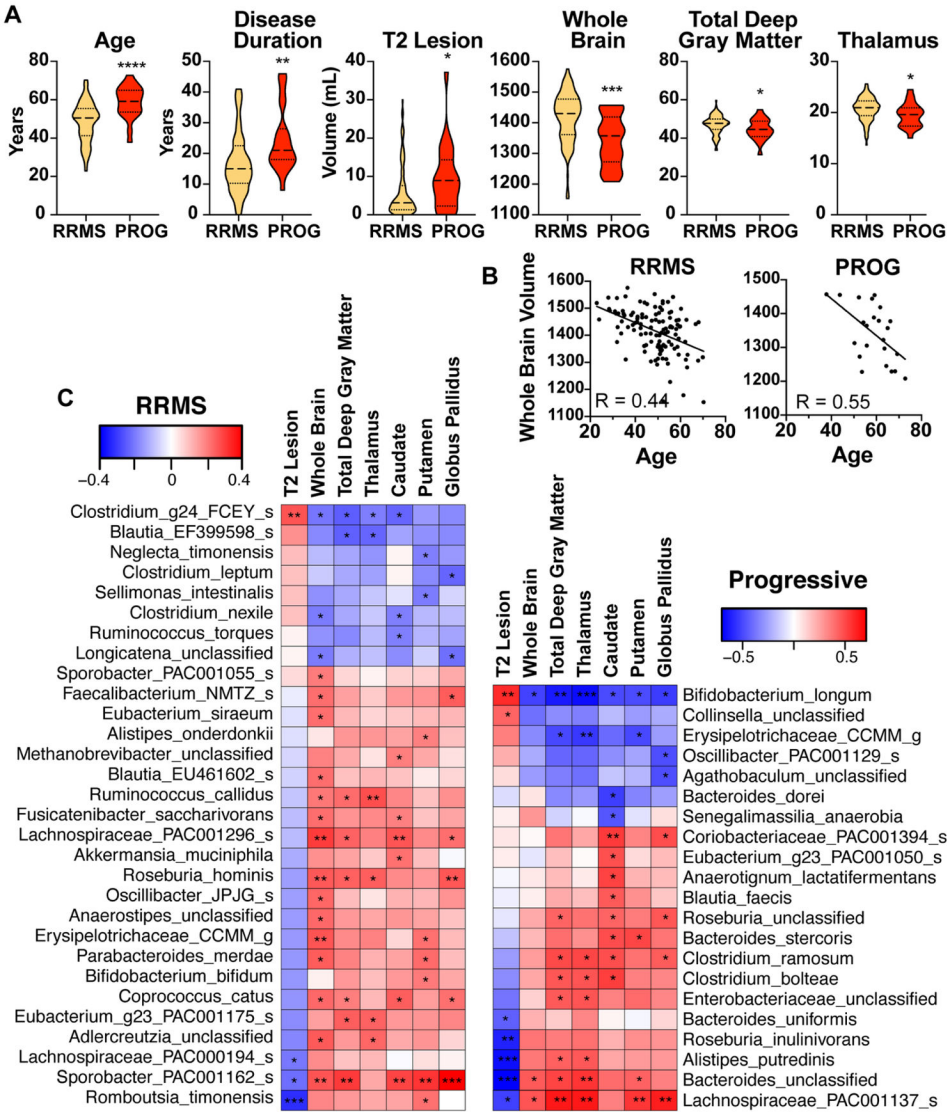

Figure 6. Associations between the microbiota and MRI brain measurements in progressive MS.

A) Age, disease duration, and brain 3T MRI measurements in progressive MS (n = 23) and 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 correlation adjusted for age. * p < 0.05, ** p < 0.01, *** p < 0.001.

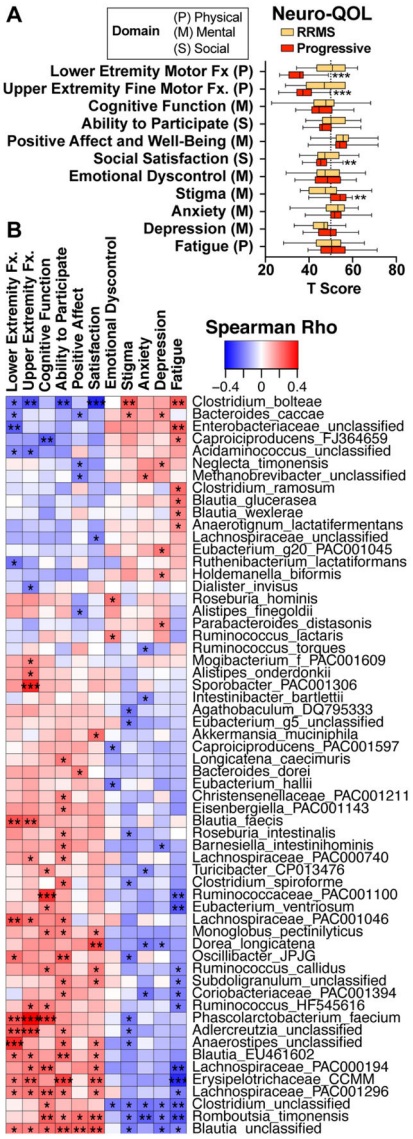

Figure 7. Microbiota associations with quality of life.

A) Quality of life measurements in 95 relapsing (RRMS) and 27 progressive patients were assessed using the NeuroQOL questionnaire across three domains: physical, mental, and social. Departure from the population norm (T score = 50) in RRMS and progressive patients. **B)** Microbiota correlations with quality of life, Spearman correlation adjusted for age. * p<0.05, **<0.01, ***< 0.001.

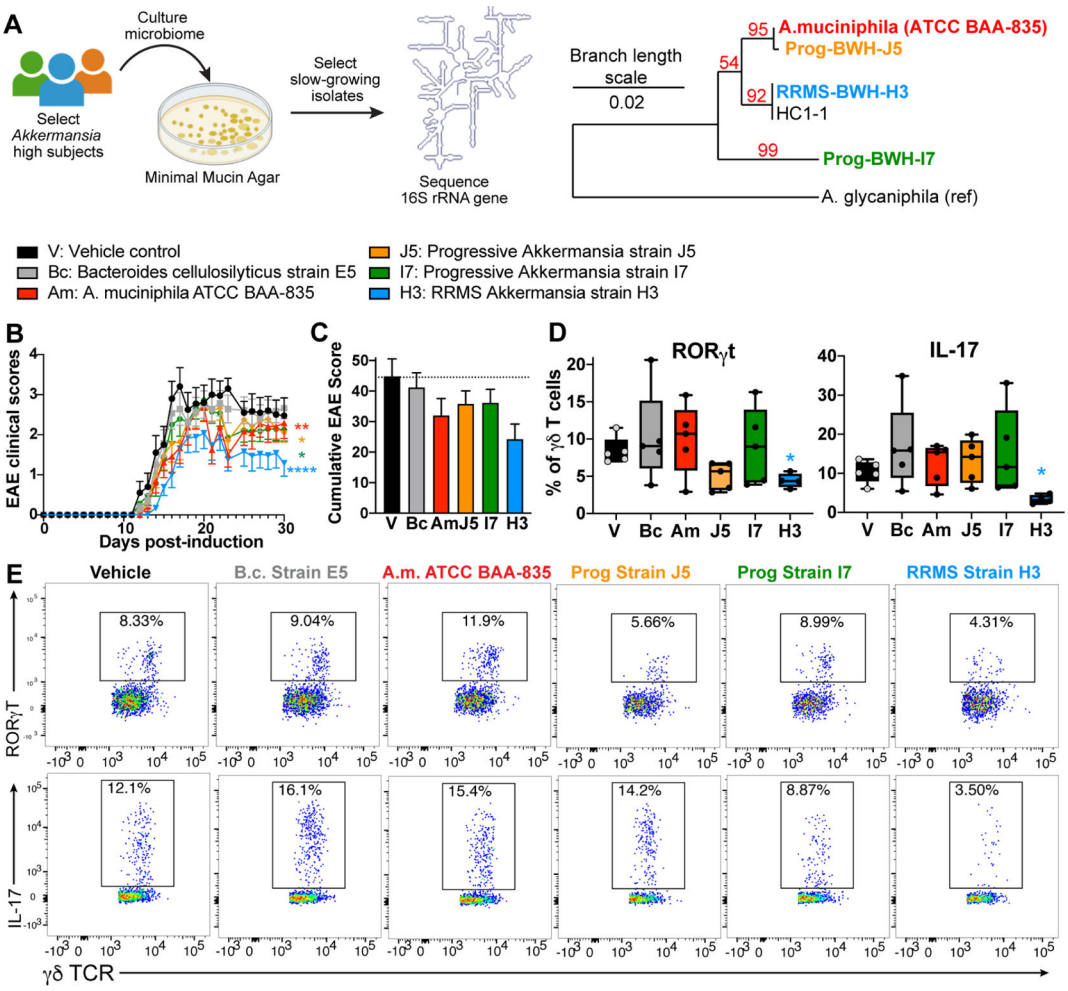

Figure 8. MS-associated *Akkermansia* ameliorates EAE.

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. Phylogenetic tree constructed from near full length 16S rRNA sequences shows three phylotypes of *Akkermansia* isolated from HC and MS patients. **B)** Akkermansia isolated from RRMS and progressive 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. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ Freidman's test with Dunn's correction for multiple comparisons. **C)** Cumulative EAE scores. **D-E)** Mice were colonized with *B. cellulosilyticus, Akkermansia muciniphila* Type strain, and three MS-derived Akkermansia strains, n = 5 per group. EAE was induced, and immunologic responses were measured 10 days later. **D)** Levels of RORγT+ γδ T cells in unstimulated splenocytes and levels of IL-17 production in PMA/ionomycin stimulated splenocytes. * $p < 0.05$, one-way ANOVA. **E)** Representative FACs plots of RORγT and IL-17 production from splenic γδ-T cells.

Table 1:

Study Subject Demographics

	HC	RRMS	Prog
Number of Subjects	40	199	44
Number Female Subjects (%)	28 (70.0%)	152 (76.4%)	31 (70.4%)
Age (years) ± St. dev.	45.4 ± 9.2	49.3 ± 9.5	57.8 ± 7.6
Body Mass Index ± St. dev.	28 ± 7.9	27.1 ± 6.3	28.4 ± 6.5
Race			
White	38	188	42
Black	1	7	1
More than one race	1	3	1
Unknown, not reported	0	1	0
Ethnicity			
Not Hispanic or Latino	39	193	43
Hispanic or Latino	1	6	1
Disease Duration ± St. dev.	NA	16.3 ± 10	24 ± 10.9
EDSS ± St. dev.	NA	1.8 ± 1.2	5 ± 1.8
Treatment			
Untreated	NA	40	5
Fingolimod	NA	47	2
Dimethyl fumarate	NA	29	5
Natalizumab	NA	28	3
anti-CD20	NA	19	6
Interferon-beta	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
More than one DMT	NA	1	3
Recently Off Treatment	NA	4	2