



OPEN Functional microbiome shifts are associated with neuroinflammatory outcomes under dietary interventions in multiple sclerosis

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Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system. While disease-modifying therapies can reduce relapse rates, their limitations have spurred interest in adjunctive approaches such as fasting and ketogenic diets (FD, KD). In a randomized controlled trial, participants with relapsing–remitting MS followed FD, KD, or a control diet for 9 months, with multi-omic and clinical assessments. Our secondary analysis revealed that KD was associated with distinct gut microbial functional profiles, including enrichment of carbohydrate metabolism modules that co-occurred with lower T2 lesion volume. Stool propionate levels, however, did not correspond to the abundance of the annotated propionate production II module. Key contributors included *Romboutsia timonensis*, *Roseburia intestinalis*, and *Bacteroides thetaiotaomicron*. These microbial changes coincided with diet-associated changes in circulating tryptophan and indole metabolites, suggesting a system-level co-occurrence of changes along the gut–brain axis. We present evidence that KD is associated with coordinated tryptophan-related metabolite changes and species-specific functional differences alongside T2 lesion metrics. These exploratory associations warrant validation and mechanistic investigation.

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Multiple sclerosis (MS) is a chronic immune-mediated disorder characterized by inflammatory demyelination, axonal damage, and plaque formation in the central nervous system (CNS)¹. MS affects approximately 2.8 million individuals worldwide and exhibits a striking female predominance, typically manifesting around the age of 30². While genetic predispositions contribute to susceptibility, environmental factors, including Epstein–Barr virus infection, vitamin D deficiency, smoking, and dietary patterns, influence disease onset and progression³. The pathophysiology involves autoreactive T-cells breaching the blood–brain barrier (BBB) and subsequently infiltrating the CNS, where they lead to myelin destruction⁴. This process disrupts neural signaling, resulting in motor, sensory, and cognitive impairments^{5,6}.

While no cure for MS exists, disease-modifying therapies (DMTs) such as immunomodulators and monoclonal antibodies reduce relapse frequency and lesion formation⁷. However, these treatments often carry significant

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side effects and vary in efficacy depending on patient characteristics and disease progression⁸. This has spurred interest in adjunctive approaches like dietary interventions. In fact, an observational, uncontrolled pilot study of 20 relapsing–remitting MS (RRMS) patients demonstrated that patients following a high-vegetable/low-protein diet for at least 12 months had improved Expanded Disability Status Scale (EDSS) scores and reduced relapse rates compared to those following a Western diet⁹. The high-vegetable/low-protein diet group also showed enhanced anti-inflammatory immune responses. These benefits correlated with higher levels of *Lachnospiraceae*, a bacterial family that produces short-chain fatty acids (SCFAs), suggesting that gut microbiome changes may mediate the dietary effects on MS. While anti-inflammatory diets are generally considered healthy, evidence suggesting a direct impact on MS disease pathology is limited. Nonetheless, emerging research into fasting diets (FD) and ketogenic diets (KD) indicates that these interventions may confer mechanistic benefits relevant to MS^{10,11}.

KDs, which are characterized by low-carbohydrate, high-fat intake, induce the production of ketone bodies such as 3-hydroxybutyric acid (3HB), which has been shown to reduce MS severity in established experimental autoimmune encephalomyelitis (EAE) mouse models^{12,13}. This protective effect occurs through enhanced myelination and reduced axonal degeneration¹⁴. Similarly, intermittent fasting has been shown to inhibit autoimmunity in EAE mice by increasing regulatory T-cell populations, reducing IL-17-producing T-cells, and enhancing gut microbial diversity¹⁵. In translating these findings to the clinical setting, Fitzgerald and colleagues found that both daily and intermittent calorie restriction led to significant weight loss and improved emotional well-being in 31 individuals with RRMS who completed an 8 weeks randomized controlled pilot trial¹⁶. A secondary analysis of this trial showed a reduction in specific subsets of memory T cells, suggesting additional benefits beyond weight loss and emotional well-being¹⁷. A growing body of research suggests that KDs may be beneficial for MS patients when carried out for at least 6 months, indicating their safety and potential to improve fatigue, depression, and quality of life^{18–21}. However, larger, randomized-controlled studies are needed to confirm these results.

SCFAs such as butyric acid and propanoic acid, produced by commensal bacteria fermenting dietary fiber, exhibit anti-inflammatory effects in both the gut and CNS²². SCFAs potentially modulate the gut–brain axis by protecting the BBB, reducing neuroinflammation and oxidative stress, and regulating cell cycle and apoptosis processes²³. In MS patients' feces, however, they are notably depleted²⁴. Addressing this depletion by improving propanoic acid levels in MS patients through supplementation has shown beneficial effects on immunological, neurodegenerative, and clinical outcomes in an uncontrolled study, potentially due to enhanced regulatory T cell differentiation^{25,26}. Tryptophan metabolism, which is in part orchestrated by gut microbes, is similarly disrupted in MS. Pro-inflammatory cytokines divert tryptophan toward the kynurenine pathway, generating neurotoxic quinolinic acid during relapses in MS patients^{27,28}. This conversion is facilitated by indoleamine 2,3-dioxygenase, which can be modulated by SCFAs such as butyric acid²⁹. Conversely, indole derivatives have been reported to decrease neurodegeneration and inflammation in MS model mice, likely upon binding to the aryl hydrocarbon receptor in astrocytes³⁰.

Distinct microbial signatures further underscore the gut–brain axis's role in MS pathogenesis³¹. Fecal microbiota transplants from MS-discordant twins to germ-free mice exacerbate autoimmunity, directly confirming microbiome involvement in disease progression^{32,33}. Furthermore, KDs actively modulate these microbial communities in MS patients through a biphasic response: after initial reduction at 2 weeks, bacterial concentrations progressively increased, exceeding baseline values and reaching healthy control levels by 6 months³⁴. This evidence highlights how dietary strategies may correct metabolite imbalances and microbial dysbiosis to influence MS outcomes. However, the precise effects of dietary interventions on microbial metabolism and metabolic potential in MS remain unclear.

To address the evidence gap, a large-scale, randomized controlled clinical trial has been designed to examine the effects of FD and KD in comparison to a standard healthy control diet (CD) in individuals with RRMS³⁵. In the primary analysis, neither T2 lesion count nor T2 lesion volume on brain MRI differed between intervention groups at 18 months³⁶. This secondary, exploratory analysis investigated diet-associated changes in the gut microbiome, serum metabolome, and microbiome–metabolome relationships, and whether these changes were associated with neuroimmunological outcomes in MS. Due to substantial attrition over the 18-month study period, which reduced statistical power and increased the potential for selection bias from differential dropout, this secondary analysis was restricted to baseline and 9 months follow-up samples. The 9 months time point was selected to retain substantial dietary exposure while preserving a sufficiently large sample for the planned multi-omic analyses.

Results

The nutritional approaches in multiple sclerosis trial

The Nutritional Approaches in Multiple Sclerosis (NAMS) study categorized patients with confirmed diagnosis of RRMS per 2017 McDonald criteria into three distinct dietary intervention groups: a primarily vegetarian Mediterranean diet (CD) following German Society for Nutrition guidelines, the same diet with intermittent fasting and a twice-yearly 7-day Buchinger fast (FD), and a KD restricting carbohydrates to 20–40 g/day while maintaining fat intake at 70–80% of total energy consumption (Fig. 1)^{37,38}. Adherence to the assigned dietary intervention was confirmed for all participants based on the pre-defined compliance criteria, including group session attendance, food records, and ketone measurements where applicable. Participants were eligible if they had stable or no DMT for at least six months, evidence of recent disease activity, and an EDSS score under 4.5, while recent changes in therapy served as key exclusion criteria (Supp. Table 1)³⁵. For this secondary, discovery-oriented analysis, we included 76 per-protocol patients who adhered to the study protocol for 9 months and provided stool and blood samples alongside anthropometric measurements, MRI-based data, and assessments of physical and mental health both at baseline and after 9 months of participation. Stool samples were collected

RRMS Patients

- Stable or no DMT
- Recent disease activity
- EDSS < 4.5

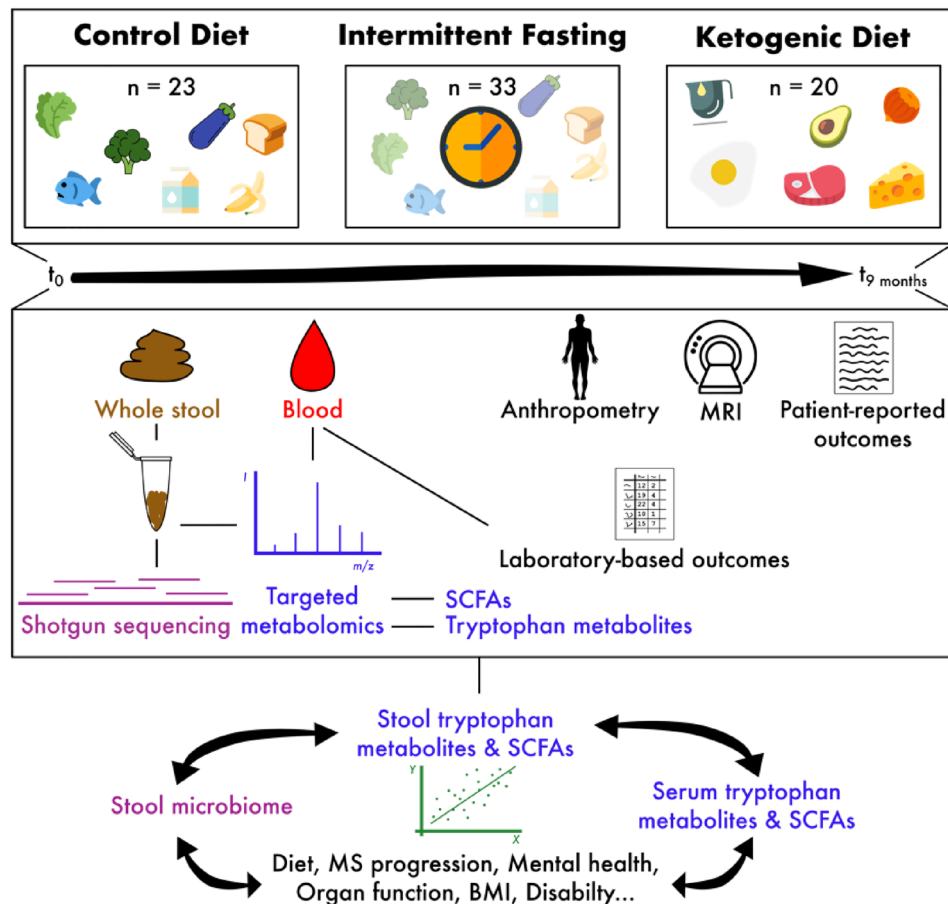


Fig. 1. The NAMS cohort comprised RRMS patients with stable or no DMT treatment, recent disease activity, and an EDSS score below 4.5³⁶. Participants were randomized into three intervention groups: a healthy, predominantly vegetarian Mediterranean control diet, the same diet but with intermittent fasting plus a 7 days fast every six months, and a ketogenic diet. At baseline (t_0) and 9 months after (t_9) the start of the interventions, blood and whole stool samples were collected alongside anthropometric measures, MRI scans, and patient-reported outcomes, including cognition, disability, and mental health assessments. Blood samples were processed for standard clinical markers such as insulin and leptin. Stool samples were homogenized, and aliquots were subjected to shotgun metagenomic sequencing. Blood serum and stool samples were analyzed for SCFA and tryptophan metabolites profiles using two distinct targeted metabolomics approaches.

to assess levels of SCFA and tryptophan metabolites and to characterize the microbial community, while blood samples were used for parallel metabolomic analyses and additional health assessments, such as neurofilament light chain (NfL) concentration (Fig. 1). Baseline demographics showed no differences in age, gender, EDSS scores, T2 lesion load, or DMT usage across groups, though the CD group had shorter disease duration since diagnosis than KD and FD groups ($p = 0.1$, Supp. Table 1)³⁶. Additionally, baseline stool samples from 29 dropout patients were included to analyse dropout bias (Supp. Table 2). These participants withdrew from the study before follow-up and therefore contributed only a single time point.

Diet-associated metabolite changes in relation to baseline metabolite levels

Our measurement of 3HB corresponded to the values measured by the external diagnostic provider Labor Berlin (Fig. S1 A), thereby confirming the reproducibility of the data. No significant baseline differences in stool or serum metabolite levels were detected between patients treated with DMTs and those untreated (Supp. Tables 3 & 4). Acetoacetic acid (AA) and 3HB increased more strongly in the KD group than in the FD and CD groups (estimate_{AA}^{KDvsCD} = 1.61 [0.64, 2.58], estimate_{AA}^{FDvsKD} = -1.34 [-2.24, -0.43], estimate_{3HB}^{KDvsCD} = 1.18 [0.29, 2.06], estimate_{3HB}^{FDvsKD} = -1.31 [-2.13, -0.49], $q_{AA} = 0.001$, Cohen's $f^2_{AA} = 0.27$, $q_{3HB} = 0.001$, Cohen's

$f^2_{3HB} = 0.28$; Figs. S1 B and S2 A). The magnitude of diet-associated changes in AA, 3HB, and HMB also varied according to baseline metabolite levels (Fig. 2). Significant diet-by-baseline interactions were observed for AA, HMB, and 3HB (estimate $_{AA}^{KDvsCD} = -2,611$ [$-3,937, -1,284$], estimate $_{AA}^{KDvsFD} = -2,572$ [$-3,450, -1,695$], estimate $_{AA}^{CDvsFD} = 38.6$ [$-1,248, 1,325$], estimate $_{HMB}^{KDvsCD} = -43.9$ [$-148.4, 60.6$], estimate $_{HMB}^{KDvsFD} = -107.4$ [$-211.6, -3.2$], estimate $_{HMB}^{CDvsFD} = -63.5$ [$-144.8, 17.8$], estimate $_{3HB}^{KDvsCD} = 75.5$ [$-642.5, 793.5$], estimate $_{3HB}^{KDvsFD} = -575.1$ [$-1,248, 97.7$], estimate $_{3HB}^{CDvsFD} = -650.6$ [$-1,273, -27.8$], $q_{AA} = 7 \times 10^{-9}$, $q_{HMB} = 0.046$, $q_{3HB} = 0.046$; Cohen's $f^2_{AA} = 0.72$, Cohen's $f^2_{HMB} = 0.08$, Cohen's $f^2_{3HB} = 0.1$). At one SD above the mean baseline value, no significant between-intervention differences in model-predicted metabolite change were observed for any of these metabolites. At one SD below the mean baseline value of AA, 3HB, and HMB, patients in the KD cohort showed greater elevations than those consuming the FD (estimate $_{AA, -1SD}^{KDvsFD} = -4,832$ [$-6,026, -3,638$], $q_{AA, -1SD}^{KDvsFD} = 8 \times 10^{-14}$, estimate $_{HMB, -1SD}^{KDvsFD} = -85.9$ [$-134.8, -37$], $q_{HMB, -1SD}^{KDvsFD} = 0.018$, estimate $_{3HB, -1SD}^{KDvsFD} = -1,336$ [$-2,164, -507.6$], $q_{3HB, -1SD}^{KDvsFD} = 0.049$). For AA, this pattern was also observable at mean baseline levels and in comparison to the CD group (estimate $_{AA, mean}^{KDvsCD} = -4,908$ [$-6,620, -3,197$], $q_{AA, mean}^{KDvsCD} = 7 \times 10^{-7}$, estimate $_{AA, mean}^{KDvsFD} = -1,926$ [$-2,946, -906.5$], $q_{AA, mean}^{KDvsFD} = 0.007$, estimate $_{AA, mean}^{CDvsFD} = -1,894$ [$-2,822, -965$], $q_{AA, mean}^{CDvsFD} = 0.002$, Fig. 2 B). Measured baseline and 9 months AUCs, absolute AUC changes, and effect sizes for these metabolites, stratified by intervention group, are reported in Supplementary Table 5. Sensitivity analyses using absolute AUC change showed that the influence of outcome scaling differed among metabolites (Supp. Table 6). For AA, the principal KD-associated contrasts were broadly preserved, although the low-baseline KD-CD contrast did not remain significant after

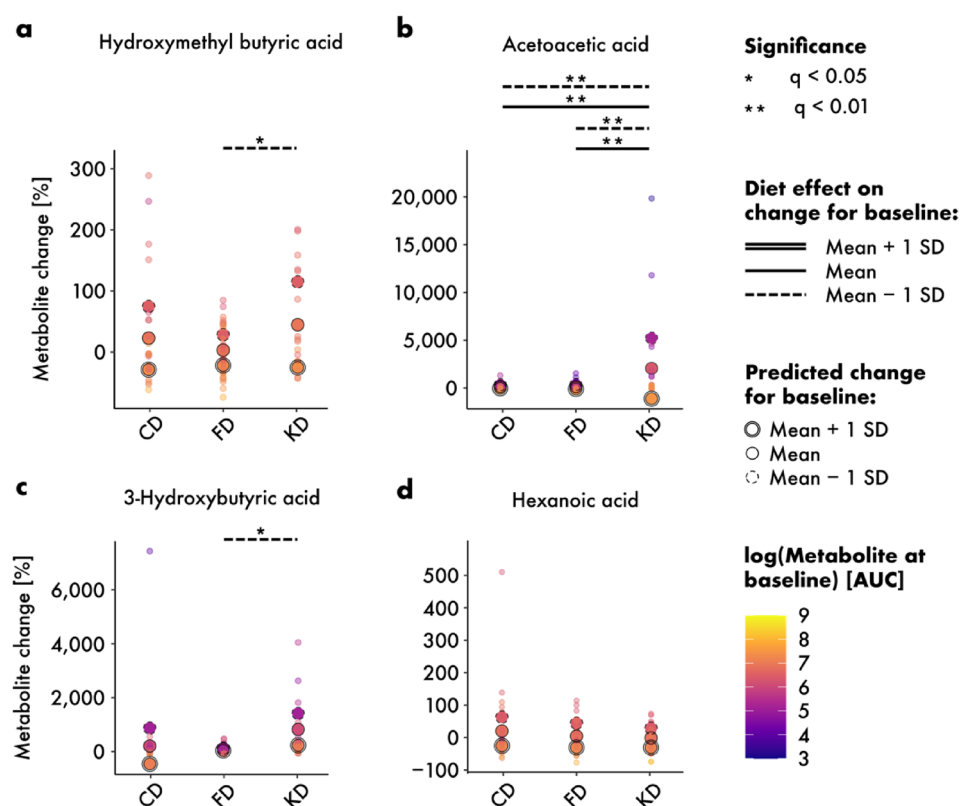


Fig. 2. Serum metabolite changes across baseline levels and dietary interventions. Individual changes from baseline to the 9-month visit are shown for serum HMB, AA, 3HB, and hexanoic acid, together with model-predicted changes at specified baseline metabolite values. Each small dot represents one participant and is positioned on the y-axis according to the change from baseline to 9 months. Dot color indicates the log-transformed baseline value of the corresponding metabolite. For each intervention group, three larger dots indicate the model-predicted change at the mean baseline value and at one standard deviation (SD) below and above the mean. Solid, short-dashed, and double-solid circles denote the mean, mean - 1 SD, and mean + 1 SD, respectively. Horizontal significance bars indicate between-intervention differences in model-predicted change at the corresponding baseline value. Their line styles match those used for the three baseline values. They should not be interpreted as tests of whether the within-group change differs from zero. (a) At one SD below the mean baseline HMB value, the model-predicted change under KD differed significantly from that under FD. (b) At the mean and one SD below the mean baseline AA value, model-predicted changes under KD differed significantly from those under FD and CD. (c) At one SD below the mean baseline 3HB value, the model-predicted change under KD differed significantly from that under FD. (d) No significant between-intervention differences in model-predicted hexanoic acid change were observed at the evaluated baseline values.

FDR correction. For 3HB, absolute-change analyses showed significant KD-associated differences from both CD and FD across all evaluated baseline levels, indicating a robust KD-associated response but less evidence that this response was restricted to participants with low baseline 3HB. For HMB, absolute-change estimates were directionally compatible with the percentage-change analysis, but no corresponding contrast remained significant after FDR correction. Hexanoic acid remained nonsignificant after correction. None of the omnibus diet-by-baseline interaction tests remained significant after FDR correction across the four metabolites when using absolute AUC change as outcome (Supp. Table 6).

Diet-associated changes in circulating tryptophan and indole metabolites

Beyond its role as a precursor to kynurenine and serotonin, tryptophan is also a substrate for pathways yielding indole derivatives and trimethylamine N-oxide (TMAO). Circulating indoles may have both microbial and host-derived contributions. In the KD group, serum tryptophan and kynurenine both decreased, whereas indole-3-acetate (I3A) showed a greater increase than in the other two groups (Fig. 3A, C). These associations were not fully reducible to any individually tested candidate covariate under the confounder-sensitivity framework, although I3A was associated with age at each visit (Fig. S2 A, Supp. Table 1). Importantly, dietary tryptophan levels were not associated with serum tryptophan levels and thus did not confound the result (Fig. S2 B). The kynurenine metabolite 3-hydroxykynurenine did not show dietary group-dependent changes, though its levels tended to remain low after 9 months in the KD group, with a slight increase in the CD group and a slight decrease in the FD group (Fig. 3A). Levels of 5-hydroxytryptophan, a precursor in the serotonin pathway, decreased in both the CD and KD groups, but remained stable in the FD group. However, these differences were not statistically significant (Fig. 3B). Its downstream product, serotonin, did not exhibit group-dependent changes over time. Indole-3-lactate (ILA) and TMAO did not exhibit significant diet-dependent responses (Fig. 3C). In an exploratory post hoc analysis that was not pre-specified, the ratios of I3A and ILA to serum tryptophan increased more strongly in KD than in the other two diets ($q_{I3A} = 0.002$, $q_{ILA} = 0.004$, Fig. S2C). Because a ratio can change through its numerator, denominator, or both, these findings are interpreted as relative shifts in circulating metabolite abundance and do not establish increased indole production or preferential routing of tryptophan into an indole pathway. Baseline levels of tryptophan metabolites did not differ significantly between dietary interventions (Fig. S2 D).

Microbiome diversity remained stable despite dropout bias

Our metagenomic sequencing results revealed that per-protocol KD patients had considerably lower Shannon diversity at baseline compared to per-protocol CD and FD patients (Fig. 4A). Dropout rates for the first 9 months of the study were 32% in the CD, 6% in the FD, and 44% in the KD (Supp. Table 2). Patients dropped out due to various factors, including medication changes, pregnancy, and non-compliance with the dietary protocol³⁶. To assess this dropout bias, we additionally sequenced stool samples from 29 dropout participants who did not complete the trial but for whom complete metadata and samples at baseline were available (Supp. Table 2). Within each intervention group, baseline Shannon diversity did not differ significantly between per-protocol and dropout patients, indicating that alpha diversity was not associated with dropout (Fig. 4A). To estimate the potential effect of this difference on microbial dynamics within each intervention group, we estimated the probable alpha diversity for each dropout patient at 9 months using Multivariate Imputation by Chained Equations (MICE) and calculated the resulting change in diversity. These estimated changes did not differ between dropout and per-protocol patients (Fig. 4B). Similarly, the diets did not lead to a shift in beta diversity ($p_{PERMANOVA} = 0.4$, Fig. 4C). However, KD was associated with reduced relative abundance of *Bifidobacterium* compared to FD and CD ($q_{KDvsFD} = 0.001$, $q_{KDvsCD} = 0.044$, $q_{CDvsFD} = 0.403$, Fig. 4D). All subsequent analyses were conducted exclusively in the per-protocol population, with baseline Shannon diversity included as a potential confounder (Supp. Table 1).

Microbiome functional and compositional contributions to T2 lesion outcomes differed across diets

The same exploratory analytical approach applied to tryptophan pathway metabolites was used to identify gut microbial modules (GMMs) directly associated with the dietary interventions, independent of potential confounding factors³⁹. KD was associated with greater increases in the GMMs glycerol degradation III (MF0062) and propionate production II (MF0094) than the other two diets (Fig. 5A). In contrast, starch degradation (MF0005) and sucrose degradation II (MF0011) were reduced in the KD group compared to the other two groups (Fig. 5A). We then examined whether these changes were associated with T2 lesion count or volume, insulin-like growth factor 1 (IGF1) concentration, NFL concentration, symbol digit modalities test (SDMT) results, or Beck depression inventory II (BDI-II) score, depending on the diet group. T2 lesion count and volume were selected as complementary markers of disease progression, as lesion count alone may remain stable or decrease even when existing lesions enlarge or merge. We constructed a linear mixed model to assess the change in each outcome variable as a function of the three-way interaction between group, a previously identified GMM, and time, while accounting for inter-patient variability. Next, we evaluated whether the inclusion of this three-way interaction significantly improved model performance using KD as a reference and accounted for potential confounders individually. Indeed, changes in starch degradation (MF0005) had a differential association with T2 lesion count that varied between the groups, with increasing abundance of this GMM being correlated with a decrease in T2 lesion count in the FD group specifically (Fig. 5B). Additionally, increasing abundance of the annotated GMM propionate production II (MF0094) was associated with a steeper decrease in T2 lesion volume in KD than in FD and CD. This association does not establish increased propionate production or mediation of a dietary effect (Fig. 5B). We next investigated whether specific microbial species might contribute to this association. To achieve this, we examined which species exhibited associations mirroring the relationship between a GMM

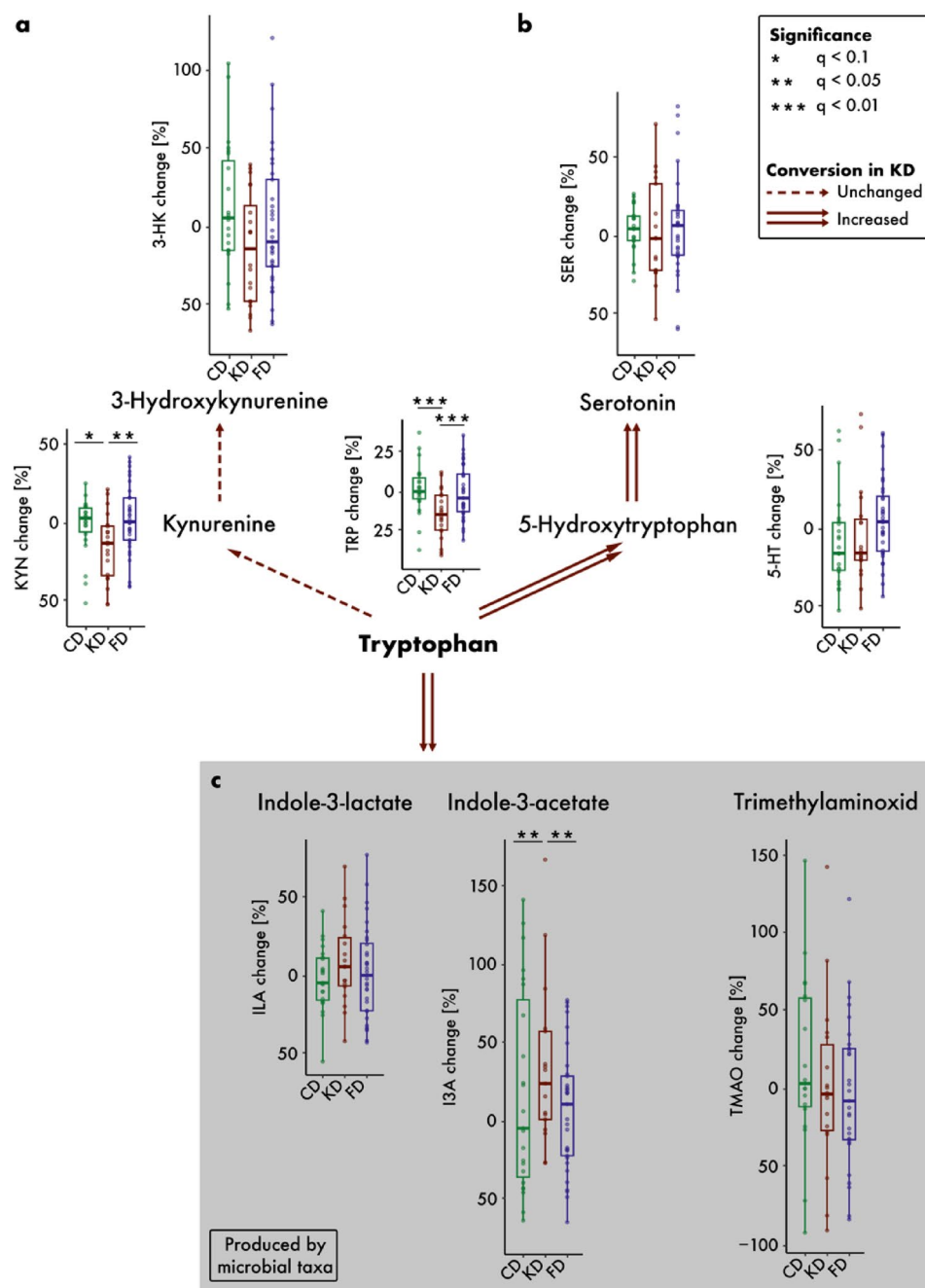


Fig. 3. Tryptophan is metabolized through several pathways, including kynurenine and serotonin pathways and pathways yielding indole derivatives and TMAO. AUC changes from baseline to 9 months are shown on the y-axis (in percent) per patient (dots) and group on the x-axis for tryptophan metabolites. Arrows indicate the metabolic relationships represented schematically. Accompanying changes refer to the measured circulating metabolites and should not be interpreted as direct measurements of pathway activity or metabolic flux. Outliers were removed for visualization only. Significance was determined post hoc using estimated marginal means. **(a)** Kynurenine pathway metabolites mirrored the change pattern observed for tryptophan. **(b)** Serotonin pathway metabolites did not follow the tryptophan trend. 5-hydroxytryptophan levels decreased in both CD and KD, while serotonin levels remained stable over 9 months in all groups. **(c)** Measured indole derivatives and TMAO were not reduced under KD despite lower tryptophan levels, with indole-3-acetate showing a significant increase in KD compared to CD and FD. ILA, indole-3-lactate; I3A, indole-3-acetate; KYN, kynurenine; SER, serotonin; TMAO, trimethylamine N-oxide; TRP, tryptophan; 3-HK, 3-hydroxykynurenine; 5-HT, 5-hydroxytryptophan.

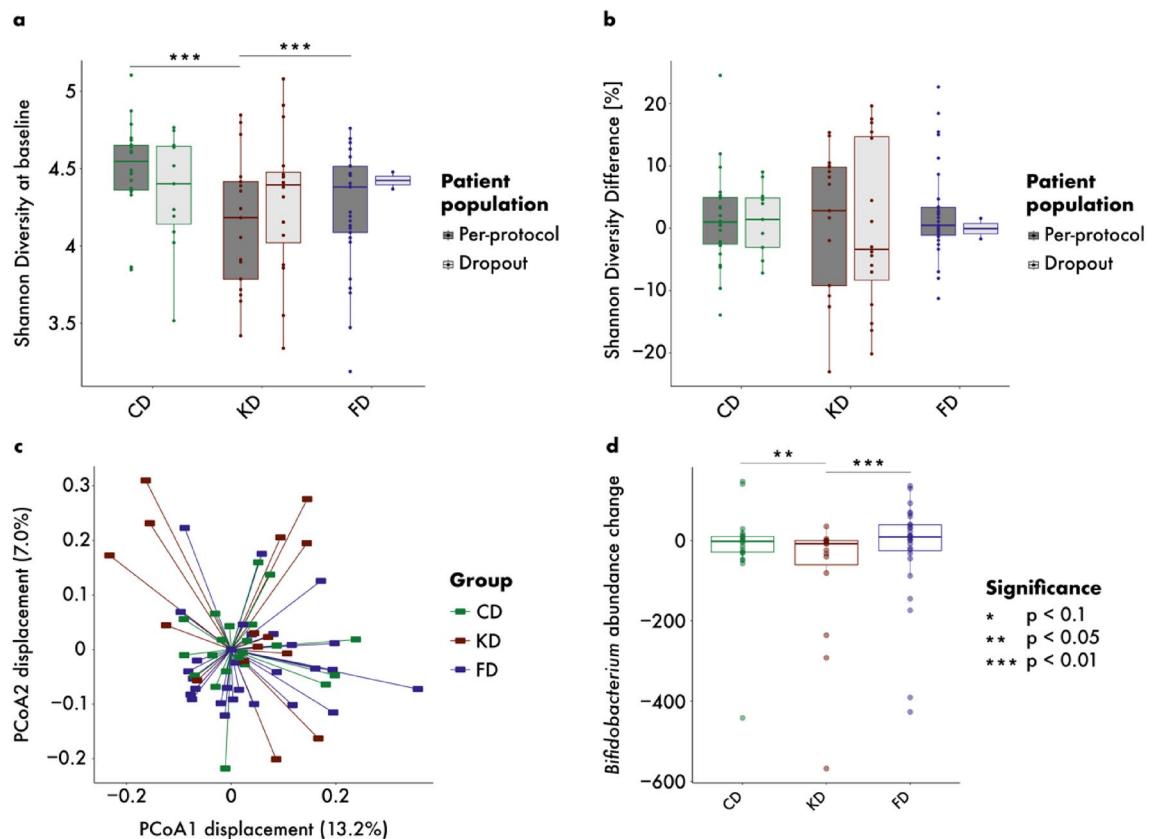


Fig. 4. Microbiome shifts in response to dietary interventions. **(a)** The KD per-protocol population (dark grey boxes) showed significantly lower baseline Shannon diversity compared to both FD and CD per-protocol patients. Dropout patients (light grey boxes) did not differ in their baseline Shannon diversity. Significance was assessed using Dunn's test. **(b)** No significant difference in Shannon diversity changes from baseline to 9 months was observed across dietary interventions in per-protocol and dropout patients ($p=0.4$). Shannon diversity values for dropout patients were imputed using Multivariate Imputation by Chained Equations. **(c)** PCoA based on a Bray–Curtis distance showing no significant beta diversity shift in any diet group. Axes show within-participant displacement from baseline along the original PCoA1 and PCoA2 axes, which explained 13.2% and 7.0% of total compositional variance, respectively. Figure axes are labeled “PCoA1 displacement (13.2%)” and “PCoA2 displacement (7.0%)”. **(d)** *Bifidobacterium* abundance significantly decreased in the KD group, with post-hoc significance assessed using estimated marginal means.

and T2 lesion volume and count. Ten species showed time- and group-dependent relationships with T2 lesion volume and count ($q<0.1$) and were therefore tested for analogous links to group-associated GMMs. Three of these species—*Romboutsia timonensis*, *Roseburia intestinalis*, and *Bacteroides thetaiotaomicron*—exhibited a stronger positive association with propionate production II (MF0094) in KD than in the other two diets (Fig. 5C). Similarly, *Intestinibacter bartlettii* displayed a stronger positive association with starch degradation (MF0005) in KD than in the other two diets and was uniquely associated with decreasing T2 lesion count in the FD group alone (Figs. 5C and S3C). These species' temporal dynamics with both GMMs and outcome metric aligned directionally (Figs. 5 and S3C).

Microbial propanoic acid pathway dynamics varied by diet

Propionate production II (MF0094) comprises only one KEGG orthologue (KO) (K01026, EC:2.8.3.1), which corresponds to propionate CoA-transferase. This enzyme is an integral part of propanoic acid metabolism, primarily transferring CoA from propionyl-CoA to acetate, thereby producing propanoic acid and acetyl-CoA. It has also been shown to transfer CoA to and from other carboxylic acids, such as butyrate, in various taxa⁴⁰. Given this broad enzymatic activity, we examined whether stool levels of acetic acid, butyric acid, and propanoic acid differed among the diet groups, noting that fecal SCFA levels reflect both microbial production and host absorption and may therefore only partially capture microbial metabolic activity⁴¹. Although no statistically significant differences were detected, there was a trend toward lower propanoic acid levels in the KD group and lower butyric acid levels in the FD group (Fig. S4). Next, we tested whether these metabolites reflect the metabolic dynamics of this microbial footprint, independent of the group. Stool acetate, butyrate, and propanoic acid showed no temporal association with propionate production II (MF0094) and lacked group-dependent associations with T2 lesion volume (Fig. 6A, B). However, propanoic acid exhibited group-independent temporal correlations with two of the previously identified species, *R. intestinalis* and *B. thetaiotaomicron*,

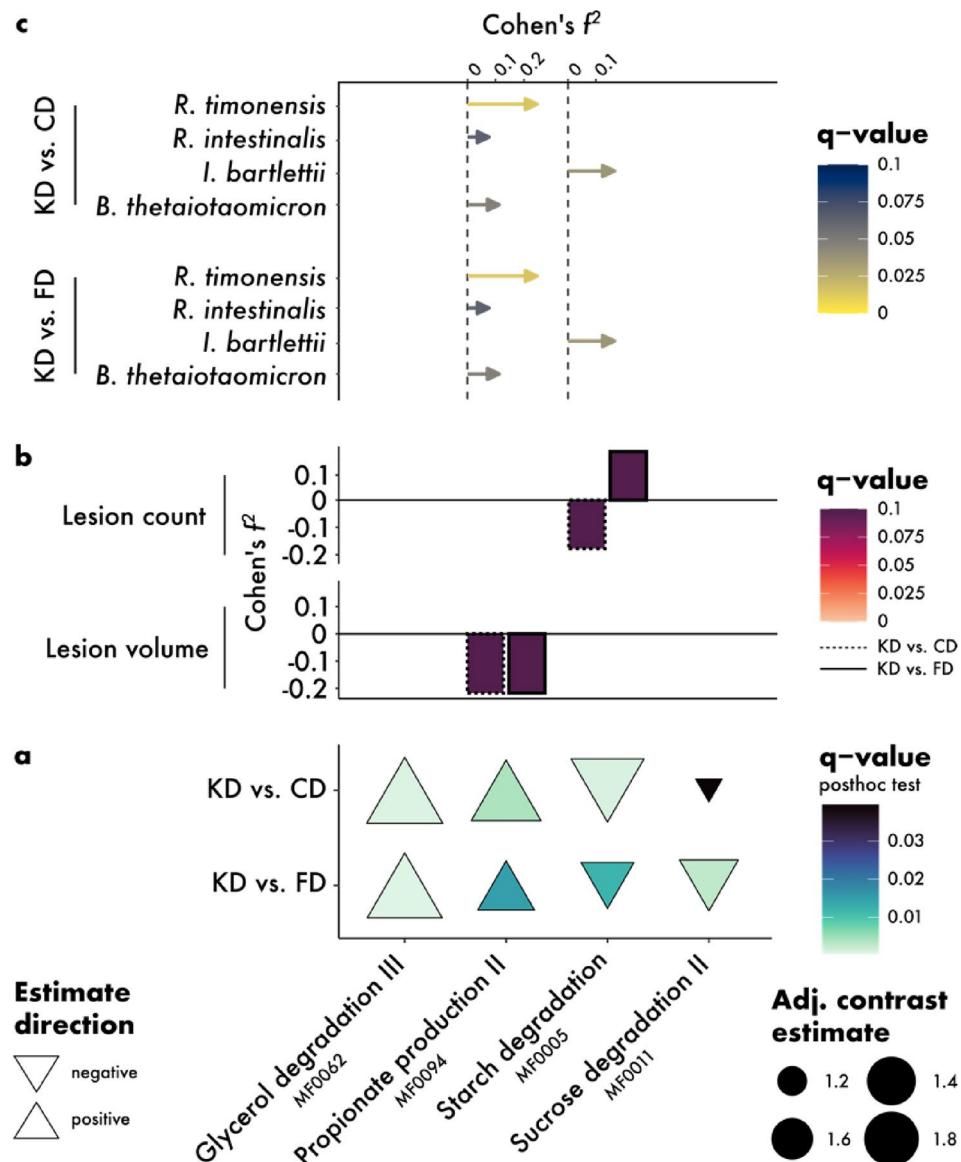


Fig. 5. Gut microbial modules (GMM) link diet to T2 lesion outcomes. **(a)** Post hoc analysis revealed that two GMMs increased and two decreased significantly more under KD compared to the other two diets. The effect size is calculated by dividing contrast estimates by the model's residual standard deviation. **(b)** The association between propionate production II (MF0094) and T2 lesion volume became more negative under KD compared to the other two diets depicted by differently dashed bar outlines. The association between starch degradation (MF0005) and T2 lesion count became more strongly negative in KD versus CD and more strongly positive in KD versus FD. Bars indicate Cohen's f^2 and are filled with the respective q-value. **(c)** Four species that mirrored the GMM-outcome relationship showed stronger positive associations with propionate production II (MF0094) and starch degradation (MF0005) under KD compared to the other two diets. Arrows indicate Cohen's f^2 and have the color of the respective q-value.

showing stronger positive associations with propionate production II (MF0094) across visits in KD compared to CD and FD (Fig. 6C). Acetic acid exhibited this pattern solely with *R. intestinalis* (Fig. 6C). We applied the same analytical framework to investigate whether temporal shifts in tryptophan metabolism within the KD group were linked to propionate production II (MF0094) dynamics. Among the four diet-associated serum tryptophan metabolites, kynurenine and tryptophan demonstrated decreasing levels that were concurrent with rising propionate production II (MF0094), irrespective of intervention group (Fig. 6D). Moreover, the relationship between changing levels of these metabolites and T2 lesion volume changes was stronger and more positive in the KD group compared to CD and FD (Fig. 6E). These metabolites shared no synchronous temporal pattern with any species linked to propionate production II (MF0094) dynamics (Fig. 6F). Furthermore, hypothesis-generating mediation analysis revealed that propionate production II (MF0094) abundance exhibited patterns consistent with mediation of the effect of KD on T2 lesion volume (Fig. S5).

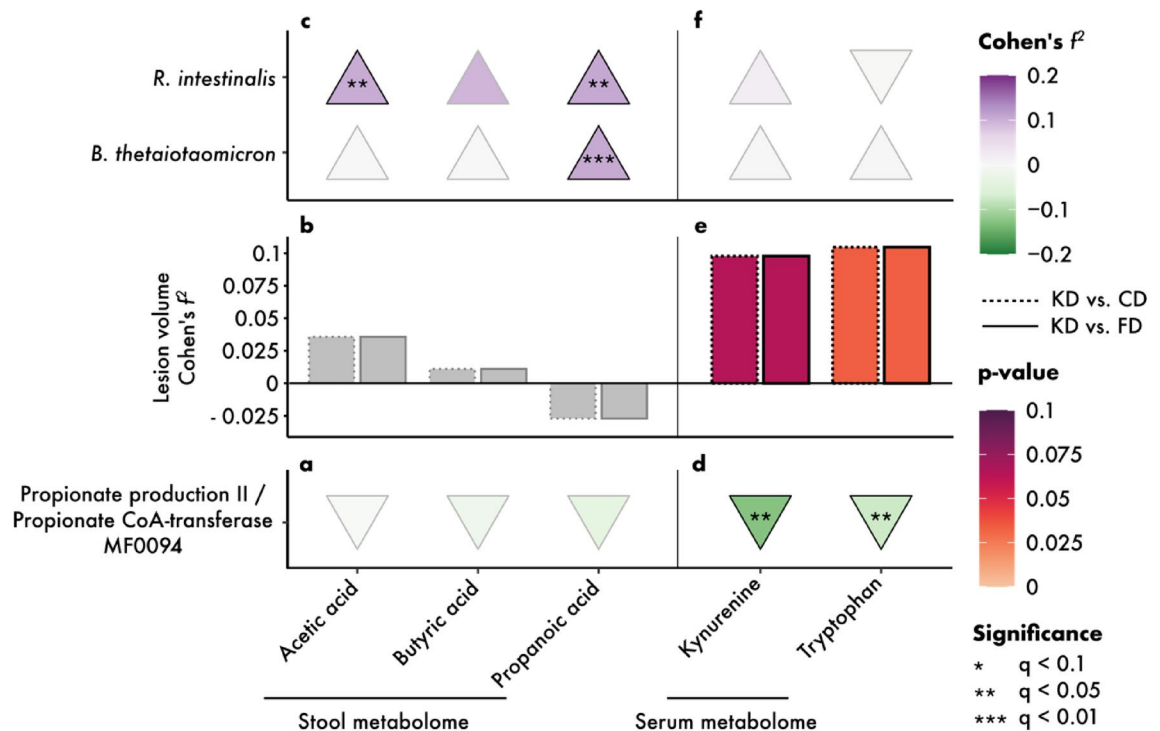


Fig. 6. Metabolome-microbiome associations with MS outcomes. Grey outlines indicate non-significance. (a) Stool metabolites showed no association with propionate production II (MF0094) GMM. (b) Stool metabolites demonstrated no relationship with T2 lesion volume over time that was significantly different between in the diets ($q_{\text{Acetic acid, Propanoic acid}} = 0.6$, $q_{\text{Butyric acid}} = 0.71$). (c) Two T2 lesion volume-related species showed positive correlations with two stool SCFAs across groups. (d) Two group-associated serum metabolites, tryptophan and kynurenine, were negatively associated with propionate production II (MF0094) GMM. (e) Serum tryptophan and kynurenine exhibited a significantly more positive association with T2 lesion volume in KD compared to FD and CD. Bars indicate Cohen's f^2 , are filled with the respective q-value and outlined according to the diet contrast applied. (f) No association was observed between serum tryptophan and kynurenine and species either related to propionate production II (MF0094) GMM or T2 lesion volume.

Discussion

Our findings identify concurrent associations between KD, gut microbial function, and lesion outcomes that warrant further investigation. This is supported by the diet's association with the abundance of four GMMs and the observed negative relationship between propanoic acid production potential GMM and T2 lesion volume in MS, specifically in the KD group. Alongside propionate production II (MF0094), glycerol degradation III (MF0062) was enriched in the KD group. MF0062 includes key enzymes, glycerol dehydratase and 1,3-propanediol dehydrogenase, that mediate the microbial conversion of glycerol to 1,3-propanediol. This process is critical for microbial redox balance, as it regenerates NAD⁺. We hypothesize that, as KD involves increased fat intake, more glycerol is derived from fat metabolism, providing a substrate for microbial fermentation and redox homeostasis. At the same time, the depletion of starch and sucrose degradation modules under KD may reflect carbohydrate scarcity. These shifts collectively suggest that the gut microbiome adapts to the available substrate landscape under KD, with co-occurring changes in metabolic potential to maintain energy production and redox balance and an increased genetic capacity for propanoic acid production^{42,43}. Given that propanoic acid has been implicated in neuroprotective roles in MS, this associative functional shift may be of clinical relevance and warrants further mechanistic investigation²⁶.

Three anaerobic SCFA-producing species, *R. timonensis*, *R. intestinalis*, and *B. thetaiotaomicron*, showed patterns compatible with the diet-dependent association between MF0094 abundance and T2 lesion volume. These co-occurring associations are hypothesis-generating and do not demonstrate that these taxa, MF0094, or propionate mediate an effect of KD on lesion outcomes. Propionate has been proposed to influence systemic and CNS-relevant inflammatory processes through mechanisms including modulation of regulatory and effector T-cell balance, free-fatty-acid-receptor signaling, and histone-deacetylase inhibition^{22,25,26,44–46}. While the present study cannot test the roles of these mechanisms, they provide biologically plausible mechanisms for targeted investigation in future functional studies.

Under FD, periodic nutrient shortages trigger dynamic shifts in the gut microbiome, which may lead to T2 lesion count being a stronger negative correlate of starch-degradation capacity^{47,48}. The strong negative association between *I. bartlettii* and starch degradation (MF0005) under KD, a diet marked by a persistent decline in community-wide starch degradation capacity, potentially highlights *I. bartlettii*'s sensitivity to carbohydrate

availability fluctuations⁴⁹. This pattern also reflects the species' neuroprotective role in high-competition settings that still require some carbohydrate use, potentially through its ability to produce propanoic acid^{50,51}.

Stool metabolomics revealed no correlation between fecal propanoic acid and abundance of the GMM annotated as "propionate production II" (MF0094), and fecal propanoic acid showed a trend toward lower levels under KD^{52,53}. Serum propanoic acid was below the detection limit of the analytical approach used. Fecal SCFA concentrations represent a net residual pool influenced by microbial production, intestinal absorption, gut transit, and host utilization and metabolism and therefore cannot be interpreted as direct measures of microbial SCFA production^{41,54–58}. Accordingly, the absence of a fecal propionate signal does not by itself exclude altered microbial production, but neither does it provide evidence for increased production or host exposure.

MF0094 was observed within a broader set of diet-associated microbial functional changes under KD, including increased glycerol degradation III (MF0062) and decreased starch degradation (MF0005) and sucrose degradation II (MF0011). Similar restructuring of carbohydrate- and fermentation-related microbial functions has been reported in an independent pediatric epilepsy cohort following KD⁵⁹. Across diet groups, MF0094 abundance was also associated with serum tryptophan and kynurenine, indicating that its associations in the present dataset were not restricted to measured SCFAs. These observations provide broader metabolic context for MF0094 but do not establish what biological process its abundance represents.

MF0094 is defined by a single KEGG orthologue, K01026, annotated as propionate CoA-transferase. Its single-orthologue definition reflects the GMM annotation of this metabolic transformation, but changes in K01026 abundance cannot by themselves establish pathway-level propionate-production capacity or metabolic flux. Moreover, the enzyme can mediate CoA transfer involving multiple carboxylic-acid substrates^{40,60}, further limiting a propionate-specific interpretation. We therefore use "propionate production II (MF0094)" as the formal GMM annotation while distinguishing that annotation from biological interpretation. Accordingly, we interpret MF0094 here as an annotated microbial functional feature associated with KD, several microbial and metabolic features, and T2 lesion-volume dynamics, rather than as a direct measure of either propionate production or overall fermentative capacity. Whether these associations reflect propionate-related metabolism, broader SCFA interconversion, or another aspect of the microbial metabolic state cannot be resolved from the present data and requires targeted functional and metabolic validation.

The ketone body 3HB is also a ligand of free fatty acid receptors, demonstrating neuroprotective and neuroregenerative effects comparable to propionate, although likely through different mechanisms and receptor interactions depending on tissue context^{45,61,62}. Many patients on KD were in a ketotic state, as shown by elevated ketone body levels. Percentage-change analyses identified significant diet × baseline interactions for AA, 3HB, and HMB, suggesting that relative metabolic responses may vary with baseline metabolite levels. However, none of the corresponding omnibus interactions were significant after FDR correction when absolute AUC change was used as the outcome. At the same time, substantial KD-associated absolute changes, particularly for AA and 3HB, were reproduced. Thus, the dietary effects themselves are supported across outcome scales, whereas evidence that their magnitude is modified by baseline metabolic state is scale-dependent. It is important to note that absolute AUC changes cannot be compared across different compounds for size of effect, as mass spectrometry is inherently not an absolutely quantitative methodology. The absolute change in concentration could not be reliably determined for all metabolites, and percentage change may exaggerate the effect size in metabolites close to the limit of quantification. Our filtering, background subtraction, and other quality-assurance measures nonetheless ensured the validity of the direction of change observed for each metabolite. These exploratory findings motivate further investigation of baseline metabolic profiles as potential predictors of response to dietary intervention, but do not establish differential clinical benefit from KD. Validation of these compounds requires adjusting methods to increase sensitivity of detection, either by pre-analytical concentration or by use of a more sensitive analytical method.

Previous work indicates that KD can alter circulating tryptophan and kynurenine-pathway metabolites^{63,64}. In our cohort, the kynurenine-to-tryptophan ratio did not differ significantly between groups, dietary tryptophan intake did not differ between groups, and dietary intake was not correlated with serum tryptophan. We additionally observed diet-associated changes in circulating I3A and exploratory post hoc increases in I3A-to-tryptophan and ILA-to-tryptophan ratios under KD. These ratio changes may reflect changes in either term and therefore do not establish preferential routing of tryptophan into indole production. Recent evidence indicates that circulating ILA and indole-3-acetate can have substantial mammalian as well as microbial sources⁶⁵. The measured serum metabolites should therefore not be treated as microbiota-exclusive products. The present findings are consequently interpreted as coordinated changes in circulating tryptophan-related metabolites whose host and microbial contributions and underlying metabolic mechanisms require further study.

Our study presents several shortcomings that we addressed accordingly. The study is a secondary analysis, which inherently offers more flexibility in terms of variable selection and thus more potential bias in analytic and reporting decisions. To mitigate some of the risks of bias associated with multiple testing and discovery-oriented statistical analysis, we transparently documented our variable selection and analytical choices and rigorously applied false discovery rate controlling. The relatively small sample size limits the power to detect subtle changes in microbial community structure and beta diversity, even when taxonomic or functional associations are observed. It also constrains the number of covariates that can be robustly included in multivariable models without overfitting⁶⁶. Our confounder-sensitivity analysis was therefore designed as a scalable screening procedure rather than comprehensive simultaneous multivariable adjustment: it tests whether statistical support for an observed association can be fully accounted for by any individually measured candidate covariate. An association surviving this screen is consequently not demonstrably reducible to any individually tested covariate, but this does not establish absence of confounding. In particular, the approach may miss joint effects of multiple individually weak covariates, interactions that were not explicitly modelled, and residual or unmeasured confounding. Because classification also depends on statistical model support, differences in statistical power

between candidate covariates can affect whether a confounding influence is detected. These limitations constrain the extent to which the reported associations can be considered deconfounded and motivate validation in larger cohorts permitting more comprehensive multivariable modelling.

Gender was considered among the candidate covariates in our analyses, but the sample was predominantly female (78%), limiting assessment of sex- or gender-specific heterogeneity and broader generalizability. Although the proportion of participants without DMT was balanced across diet groups, 15% of KD participants received fingolimod throughout the trial, whereas no participants in the other diet groups received this treatment. Fingolimod has been associated with depletion of *Roseburia faecis*, a propionate-producing taxon, as well as altered levels of microbiome-related metabolites, including amino acids and pyruvate, in serum and feces^{67,68}. DMT use was included among the candidate covariates evaluated in our confounder-sensitivity analyses, but residual effects of specific drug–microbiome interactions cannot be excluded. More generally, although extensive clinical and multi-omic phenotyping allowed us to screen many measured candidate covariates, residual and unmeasured confounding and heterogeneity of intervention responses remain possible. While sensitivity analyses did not reveal a systematic association between dropout and baseline microbial characteristics, these analyses are necessarily limited to observed data, and dropout-related selection bias in estimated intervention effects cannot be excluded.

The NAMS cohort was predominantly Caucasian and recruited in an urban German setting. Generalizability to more ethnically, geographically, and socioeconomically diverse MS populations is therefore uncertain. Population differences relevant to transportability may include diet, environmental and social exposures, healthcare access and treatment patterns, and microbiome composition, all of which may differ across geographic and sociodemographic contexts^{69–72}. Differences in MS-related outcomes have also been reported between racial and ethnic groups⁷³. These factors are particularly relevant to a study of dietary intervention and microbiome–host relationships and may influence the magnitude or nature of the associations observed here. Although ethnicity was considered among the candidate covariates, the small number of participants from other ethnic groups precluded meaningful assessment of population-specific effects and does not establish transportability of the observed associations. Future larger-scale cohorts representing more diverse populations will be required to assess the magnitude and durability of these effects across different MS and microbiome contexts.

Inflammatory state is an additional important potential determinant of both MS disease activity and the microbiome-metabolite relationships examined here. SCFA metabolism and tryptophan-kynurenine/indole pathways participate in immunoregulatory processes, while systemic and intestinal inflammation can themselves alter microbial community structure and host and microbial metabolism^{74,75}. The present dataset included clinical and MRI measures related to disease activity and treatment, including relapse activity, T2 lesion burden, EDSS, NfL, and DMT use, but did not include a dedicated longitudinal panel of systemic or mucosal inflammatory biomarkers. These measures therefore provide complementary information but are not equivalent to direct inflammatory phenotyping. Consequently, we cannot determine to what extent inflammatory state contributed to the observed diet-associated microbiome and metabolite patterns or modified their relationships with clinical outcomes. Future dietary-intervention studies integrating microbiome and metabolome profiling with direct longitudinal inflammatory phenotyping will be required to resolve these bidirectional relationships.

MS disease activity was also evaluated principally through the volume and number of hyperintense lesions on T2-weighted/FLAIR MRI, endpoints with limited pathophysiological specificity. Focal white-matter lesions arise from overlapping inflammatory, demyelinating, and neurodegenerative processes whose relative contributions vary across patients, lesions, and time^{76–78}. New or substantially enlarging T2 lesions can indicate acute focal inflammation, whereas persistent T2/FLAIR abnormalities may subsequently reflect chronic inflammatory, demyelinating, and neurodegenerative processes⁷⁹. Future studies investigating dietary effects on MS activity and severity should therefore incorporate broader inflammatory and imaging phenotyping rather than relying predominantly on T2 lesion volume and number.

Finally, metabolite measurements were analyzed as AUCs rather than validated absolute concentrations, limiting inference about whether the observed differences correspond to physiologically meaningful absolute concentration changes. Similarly, rank-based modelling of microbial features preserves the ordering of observations while discarding quantitative distances between abundance values. Effect estimates from these analyses therefore describe differences on an ordinal abundance scale and cannot be interpreted as fold changes or absolute abundance differences. Ranking was used to reduce sensitivity to highly skewed, sparse distributions and extreme values within the longitudinal and covariate-sensitivity framework. It was not intended to address the compositional nature of microbiome data. Rarefaction likewise addresses unequal sampling depth rather than compositionality. Residual compositional effects therefore remain a limitation of the present microbial analyses, and complementary compositional approaches may yield different quantitative interpretations.

Overall, our findings indicate that the effects of dietary interventions such as KD and FD in MS may be associated with complex, diet-specific shifts in microbial metabolic function and host metabolism, rather than changes in single taxa or SCFA levels alone. To our knowledge, this is the first report in MS of a diet-dependent association between the abundance of the annotated propionate production II module (MF0094) and MRI-derived lesion metrics. MF0094 increased under KD, and higher MF0094 abundance was associated with greater reductions in T2 lesion volume under KD. However, fecal propanoic acid and the other measured SCFAs did not show corresponding positive associations with MF0094. Thus, these data do not establish increased microbial propionate production or a propionate-mediated effect on lesion outcomes. Rather, MF0094 identifies a microbial functional feature associated with both diet and lesion dynamics whose biochemical significance remains unresolved and warrants targeted validation. The efficacy and safety of dietary interventions may further depend on an individual's baseline microbiota composition, metabolic and inflammatory status, and capacity to adapt to nutritional stress. A nuanced understanding of these personalized responses may therefore

inform the refinement of dietary recommendations and the leveraging of gut microbiome function to optimize clinical outcomes.

Methods

Sample acquisition

The NAMS study was registered at ClinicalTrials.gov under NCT03508414 and followed all relevant ethical regulations, including compliance with the Declaration of Helsinki. The ethics committee of Charité—Universitätsmedizin (EA1/200/16) approved the study protocol, and informed consent was obtained from all participants. As described by Bahr et al., the cohort comprised adults with RRMS, an EDSS score below 4.5, and recent disease activity (≥ 1 new MRI lesion or clinical relapse in the previous two years), who were on stable or no DMT for at least six months prior to enrolment (Supp. Table 1)^{35,36}. The original block randomization of participants was done by an external statistician and included stratification according to DMT use, gender, and T2 lesion load. Cerebral MRI scans were acquired at baseline and at 9 months using standardized 3D T1-weighted MPRAGE and 3D FLAIR sequences, with images co-registered to baseline in MNI space. T2-hyperintense lesions were manually segmented by two blinded, experienced raters. T2 lesion count and total lesion volumes were extracted from binary masks using FSL tools. Participants could be discontinued from the study if they withdrew consent, met an exclusion criterion, did not comply with the study protocol, or had a medical reason to stop the intervention. Compliance was defined, for all three groups, as attending at least 8 of the 10 group sessions and all 6 study visits. Food records were used to check compliance with the assigned intervention. Intervention adherence was followed longitudinally through group-session and study-visit attendance, food records, and intervention-specific monitoring. KD participants measured blood ketone levels at home using a portable ketone monitoring device (GlucoMen LX plus) and discussed the results with the study team during scheduled appointments. In the KD group, most blood ketone measurements had to be at least 0.5 mmol/L. In the FD group, participants attended additional meetings during each of the three fasting periods, which allowed the physician and counselor to assess their compliance³⁵. Blood serum in plain tubes and whole stool samples from 76 per-protocol patients were acquired from the NAMS study and stored at -80°C . Additionally, 29 baseline stool samples from dropout patients were acquired. Stool samples were homogenized using an in-house-produced device (Fig. S6). This stool homogenization apparatus comprised a lever with a handlebar that could be affixed to two primary stainless-steel components: a reservoir and a lid. Before use, both components were frozen at -80°C . They maintained a sufficiently low temperature for approximately one hour, facilitating the sequential homogenization of frozen whole stool samples. The stool homogenization process was conducted under a fume hood equipped with a HEPA filter (Waldner, Germany). Each stool sample was retrieved from the freezer and promptly transferred to a sterile Whirl-Pak® Standard Sterilized Sampling Bag (Whirl-Pak, Madison, WI, USA), which was then placed in the reservoir situated on dry ice to ensure continuous cooling. The reservoir was positioned beneath the lid, and a safety pin securing the handlebar to the attached lid was removed. The lid was then lowered onto the sample (Fig. S6). To achieve homogenization, the handlebar was manipulated to move the lid in a vertical motion until the sample was thoroughly pulverized. The pulverized sample was aliquoted, and any remaining material was returned to the original sample container. The apparatus aperture was sanitized with ethanol before processing the next sample. Cooling remained sufficient for up to 1.5 h. After this time, the reservoir and the lid were frozen at -80°C for at least 20 h again.

Metabolomics measurement

Tryptophan metabolites measurement

In this study, 152 serum and 143 fecal samples underwent analysis over 3 batches. Concurrent quantification of 34 tryptophan metabolites and 9 branched-chain amino acids in two separate assays was conducted using a methanol extraction method with a 90% concentration⁸⁰. The mobile phase system consisted of two components: Phase A, comprising 0.2% formic acid in water, and Phase B, consisting of 0.2% formic acid in methanol. Fecal samples were prepared by dissolving them in water to achieve a concentration of 50 mg_{Stool}/mL. For the extraction process, 50 μL of the biological sample was combined with 100 μL of a chilled solution containing 90% methanol, 0.2% formic acid, and 0.02% ascorbic acid, along with internal standards as described elsewhere⁸¹. The extraction protocol involved vortexing the samples for 30 s, followed by centrifugation at 1000 rpm for 10 min at 4°C , and incubation at -20°C for 1 h. Subsequently, the samples were centrifuged again at 12000 rpm for 10 min at 4°C . The analysis was performed using an Agilent 1290 ultra-high performance liquid chromatograph (UHPLC) system coupled to a Thermo Quantiva triple quadrupole mass spectrometer operating in multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved using a Waters XSelect Premier HSS T3 column (2.5 μm , 2.1×150 mm) with a 10 min gradient at around 400 bar, a flow rate of 0.4 mL/min, and an injection volume of 5 μL . The gradient program was as follows: starting at 97% A/3% B and maintaining these conditions for 0.45 min, transitioning to 70% A/30% B at 1.2 min, then to 40% A/60% B by 2.7 min (held until 3.75 min), followed by a transition to 5% A/95% B at 4.5 min (maintained until 6.6 min), and finally returning to the initial conditions at 6.75 min with re-equilibration until 10 min. A calibration curve consisting of a mix of 34 tryptophan standards plus TMA and TMAO was run at 11 concentrations in the matrix background of charcoal-stripped plasma. Level 6 of this mix and a solvent blank were run every 6 samples as an extra quality check. Pooled quality control samples (QCs) were prepared by pooling all of the samples and run at the beginning, end, and every 6 samples during each batch. 6 extracted charcoal-stripped plasma samples were run before 6 extracted water samples at the end of the sequence.

Short-chain fatty acid and ketone body measurement

For the simultaneous quantification of eight SCFAs and two ketone bodies, an analytical method employing 3-nitrophenylhydrazine (3NPH) derivatization was used⁸². Stool aliquots were dissolved in a 50% acetonitrile:water solution to reach a concentration of 33.33 mg_{stool}/mL. The samples were homogenized using a FastPrep, centrifuged, and the supernatant was collected. Sample preparation involved mixing 40 µL of serum or stool supernatant with 20 µL of 200 mM 3NPH, 20 µL of 120 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) containing 6% pyridine, and 10 µL of 50 µM isotope-labeled internal standards. The derivatization reaction was carried out at 40 °C for 45 min, followed by dilution with 410 µL of 10% acetonitrile:water and centrifugation at 6600 × g for 10 min at 20 °C. The supernatants were then transferred to brown glass vials for immediate UPLC-MS/MS analysis. The UPLC-MS/MS system consisted of an Agilent 1290 Infinity II UPLC coupled to a ThermoFisher TSQ Quantiva triple quadrupole mass spectrometer. Chromatographic separation was performed using a Waters ACQUITY BEH C18 column (1.7 µm, 2.1 × 100 mm) maintained at 40 °C with a 5 µL injection volume. The mobile phase comprised water with 0.1% formic acid (Phase A) and acetonitrile with 0.1% formic acid (Phase B) at a flow rate of 0.3 mL/min. An optimized gradient was implemented as follows: 5% B for 5 min, 5–55% B over 12 min, 100% B for 1 min, followed by 2 min at 5% B. Multiple reaction monitoring (MRM) was performed in negative electrospray ionization mode with a spray voltage of 2500 V, an ion transfer tube temperature of 342 °C, and a vaporizer temperature of 300 °C. Quality control samples, as described previously, were also employed in this analysis.

Metabolomics data processing

Data processing per matrix and method utilized *Skyline* software (v. 22.2.0.527, <https://skyline.ms/project/home/software/Skyline/begin.view>) for peak integration and quantification, as well as *R* (v. 4.2.3). Peaks were normalized to corresponding internal standards. Metabolite areas were retained if they were quantifiable or fell between the limit of detection and the lower limit of quantification in at least 60% of samples across all statistical groups. A signal-to-noise filter was applied, requiring signals to exceed at least 3-fold the corresponding peak area observed in the blank sample. Batch correction was performed using pooled QC sample-based robust locally estimated scatterplot smoothing signal correction from the *statTarget* package (v. 1.28.0) with a smoothing parameter set to 0.75^{83,84}. Missing stool tryptophan values (n = 13) were imputed using half minimum value imputation. Compound retention required that the relative standard deviation (RSD) of QCs did not exceed 60% in stool and 30% in serum. QC samples reflect technical variability alone, whereas variation among biological samples captures both technical and true biological differences. To ensure that retained metabolites exhibited biologically meaningful variation rather than analytical noise, only metabolites with a per batch biological sample RSD within samples that was at least 20% larger than the per batch RSD measured in QCs for that batch in all batches were included in the downstream analysis. Processed and corrected peak areas are referred to as AUC throughout the manuscript.

Stool metagenomic sequencing

DNA was extracted using the ZymoBIOMICS-96 MagBead DNA Kit (Zymo Research Europe GmbH, Freiburg, Germany) in the automated system TECAN Fluent® 780 NAP workstation following the manufacturer's instructions with minimal modifications to the lysis step. 100 mg of the homogenized fecal material and 750 µL of lysis buffer were used for mechanical and chemical lysis using a PeQLab Precellys 24 (Bertin Corp., Rockville, MD, USA) for 2 × 15 s at 5500 rpm for a total of three mechanical lysis cycles. The DNA was eluted in 50 µL of ZymoBIOMICS DNase/RNase Free Water. Samples were randomized, and both negative and positive controls were included at each extraction batch. Concentration was measured using Qubit dsDNA Broad Range Kit (Thermo Fisher Scientific, Waltham, USA), revealing 135 samples with sufficient DNA concentration. Samples were stored at -80 °C before shipment to NovoGene for metagenomic sequencing. Shotgun sequencing of 150 bp paired-end reads was performed on the Illumina NovaSeq 6,000.

Metagenomic profiling and diversity imputation

Taxonomic and functional profiling of metagenomic data

Raw metagenomic sequencing data were processed using customized scripts with the *NGLess* framework (v. 1.5)⁸⁵. Quality control and filtering steps removed reads below a minimum quality score of 25 and a read length threshold of 45 bp. Human DNA contamination was filtered by alignment to the GRCh38.p13 genome (masked with ProGenomes3 database parameters, requiring ≥ 45 bp matches and ≥ 90% identity). Taxonomic profiling of bacterial species was performed using *mOTUs* (v. 3)⁸⁶. Functional profiles based on the Kyoto Encyclopedia of Genes and Genomes (KEGG)⁸⁷ were obtained by mapping to the Global Microbial Gene Catalog (GMGC) (v. 1)⁸⁸, binned to KOs, and normalized to fragments per kilobase of gene per million reads mapped (FPKM). This normalization accounts for gene length and total sequencing depth, allowing read counts to be compared across genes and samples on a common scale⁸⁵.

Alpha diversity imputation

MICE with predictive mean matching over 100 iterations was used to impute alpha diversity values for dropout patients at visit three. The *mice* package (v. 3.17) was utilized to identify per-protocol patients within the same intervention group for each dropout patient, ensuring they had a comparable predicted final alpha diversity based on regression modelling⁸⁹. The imputed value was then randomly selected from the final alpha diversity values actually observed in the respective set of per-protocol patients, preserving the original data structure.

Metagenomic sequencing data processing

Our analysis was based only on mOTUs with a prevalence above 50% in one intervention group, or a relative abundance of at least 0.01% to ensure that mOTUs are either widespread or abundant enough to carry a reliable, interpretable biological signal⁹⁰. The filtered table was subsequently utilized to aggregate mOTUs that are part of a higher-level taxon, facilitating the analysis of higher-level taxon abundance. The *omixRpm* package (v. 0.3.3), applying the human gut metabolic modules database by Vieira-Silva et al. and a minimum pathway coverage of 0.3, was used to aggregate KEGG KOs to GMMs^{39,91}. KOs and GMMs were filtered analogously.

Exploratory statistical analysis

Model-based group comparisons and confounder identification

All statistical analyses were performed using R (v. 4.4.2). Given the high-dimensional nature of multi-omics data and the exploratory scope of this study, findings were interpreted with emphasis on effect consistency and biological coherence across features, rather than threshold crossing alone. Baseline differences between diet groups in covariates (BMI, age, gender) were assessed using Kruskal–Wallis tests for continuous variables and Chi-square tests for categorical variables. To evaluate the differences between diet groups regarding the feature development's dependence on its baseline levels, the *interaction* package (v. 1.2.0) was used⁹². Metabolites were analyzed using processed AUCs rather than absolute concentrations, as some data points fell outside the linear range of the calibration curve. Relative within-metabolite responses were therefore expressed as percentage change from baseline. Absolute AUC changes were used only for sensitivity analyses, since AUC values are not comparable across different compounds in terms of effect size. Processed AUCs were log-transformed to satisfy model assumptions by addressing skewed distributions, heteroscedasticity, and multiplicative measurement error. Then, the slope of each group at different values of the logged feature baseline values was calculated using standard values for continuous moderators, specifically its mean and ± 1 SD⁹³. This was evaluated using a model accounting for the interaction between intervention group and baseline feature value on percentage change in the feature, with repeated measurements handled using the *lme4* package (v. 1.1–35.5)⁹⁴. Resulting p-values for AA, 3HB, HMB, and hexanoic acid were FDR-adjusted over all four tested metabolites and all group and interval comparisons. All tryptophan metabolites, which were measured in serum and passed QC, were tested for an association with group: each metabolite's log AUC was linearly modelled with a group-time interaction while accounting for repeated measures. Parameters were estimated via maximum likelihood. The contribution of the group $\times$ time interaction was assessed by likelihood-ratio comparison of nested models with and without the interaction term. The conditional R^2 for each model was calculated using the *MuMIn* package (v. 1.48.4) and used to determine Cohen's f^2 . The same initial association testing was applied individually to candidate covariates, including BMI, age, gender and DMT use, using covariate $\times$ time terms in place of the group $\times$ time interaction (Supp. Material 1). Results were FDR-adjusted across the tested features, with $q < 0.1$ used as the screening threshold in this exploratory multi-omics analysis. Features associated with diet group were subsequently evaluated using a pairwise confounder-sensitivity procedure adapted from Forslund et al. and the longitudinal framework benchmarked by Chen et al.^{95,96}. For each candidate covariate that was also associated with the feature of interest, a repeated-measures model containing both the group $\times$ time and covariate $\times$ time terms was fitted, and the contribution of each interaction was assessed by nested-model comparison as described above. An association was flagged as sensitive to an individual candidate covariate when the covariate $\times$ time term added significant model support ($p < 0.05$) while the group $\times$ time term no longer did ($p > 0.05$). This procedure is intended as a high-dimensional screening test of whether an apparent group-associated longitudinal effect is fully statistically reducible to an individual measured candidate covariate, rather than as simultaneous multivariable adjustment for all potential confounding. Candidate covariates are evaluated separately to keep model complexity approximately constant and to limit instability arising from multicollinearity and increasingly sparse complete-case intersections when large numbers of clinical and demographic variables are considered^{95,96}. Consequently, an association surviving this screen indicates that it was not fully reducible to any individually tested candidate covariate under the specified models. It does not establish the absence of confounding. In particular, the procedure may not detect joint confounding produced only by the combined effects of several individually insufficient covariates, covariate interactions unless explicitly modelled, or residual confounding by unmeasured or insufficiently represented variables. Because classification is based on statistical model support rather than a predefined change in effect estimate, it is also dependent on statistical power and does not quantify confounding magnitude. The same longitudinal and confounder-sensitivity framework was applied to microbial taxonomic features and GMMs, additionally considering microbial alpha diversity and the first two principal-coordinate axes calculated as described above. For these regression analyses, microbial feature abundances were converted to within-feature ranks at the modelling stage. This rank-based representation was chosen to reduce sensitivity to highly skewed and sparse abundance distributions and extreme quantitative values, while retaining an ordinal inferential scale consistent with the non-parametric association tests and effect-size measures used elsewhere in the analytical framework. It was not intended as a compositional normalization of the microbiome data. Ranking preserves the ordering of observations but discards quantitative distances between abundance values. Coefficients and contrasts from these models therefore describe differences on the ranked abundance scale and cannot be interpreted as fold changes or absolute abundance differences. Rank transformation also does not remove the compositional dependence of relative-abundance data. Sequencing-depth normalization and rarefaction, where applied as described above, address unequal sampling effort rather than compositionality. Residual compositional effects therefore remain a limitation of these microbial association analyses. Post hoc tests for significant features were performed by computing estimated marginal means using the *emmeans* package (v. 1.10.5)⁹⁷. For metabolic features, p-values for pairwise comparisons were adjusted using the multivariate t distribution. For ranked metagenomic features, the same framework was applied, but the Dunn procedure was used for p-value adjustment. Effect sizes for model-derived pairwise contrasts were calculated as

standardized mean differences by dividing the estimated contrast by the residual standard deviation of the respective mixed-effects model, using the identity method to preserve the directly estimated between-group difference in longitudinal change. For rank-based microbial models, these standardized effects refer to differences on the ranked/ordinal abundance scale and not to quantitative fold changes in microbial abundance. Baseline-dependent analyses were additionally evaluated using absolute AUC change as a sensitivity analysis to assess the robustness of the observed patterns to outcome scaling. To test whether the effect of dietary intervention on change in metabolite levels from baseline depended on that baseline, we compared, for each metabolite, nested models with and without the diet $\times$ baseline AUC interaction term using a likelihood-ratio test. Resulting p-values were FDR-corrected across the four metabolites tested. To assess whether candidate covariates confounded the interaction, each covariate was added as a fixed effect to the full interaction model. For continuous covariates that changed over the intervention period, both absolute and percentage change were tested as candidate confounders. Sensitivity of the diet $\times$ baseline interaction to each candidate covariate was assessed individually by repeating the nested-model comparison with the covariate included. As above, this procedure tests whether statistical support for the focal interaction is reducible to an individually considered measured covariate and should not be interpreted as comprehensive simultaneous adjustment for all possible confounding.

Alpha and beta diversity assessment

Alpha diversity metrics (Shannon diversity, Chao1 richness, and Simpson's evenness) were calculated using the *microbiome* package (v. 1.28.0) after rarefaction to an even depth of 4000 reads per sample using the *phyloseq* package (v. 1.52, Fig. S3 A). Rarefaction was used specifically for alpha-diversity estimation to standardize sampling effort. The 4000-read threshold was selected from sample-specific rarefaction curves indicating that observed richness was approaching a plateau while retaining the analysis set. To evaluate the sensitivity of diversity estimates to rarefaction, observed richness was compared between raw and rarefied read counts using Spearman correlation (Fig. S3 B). Systematic benchmarking has shown that rarefaction reliably controls for variation in uneven sequencing effort when estimating diversity metrics⁹⁸. The *vegan* package (v. 2.6–8) was used to compute the Bray–Curtis distance for compositional analysis⁹⁹. To evaluate the compositional differences between samples at the genus level, principal coordinate analysis using Euclidean distance and $k=2$ dimensions was conducted. For visualization, patient-specific baselines were used to re-center the ordination space, such that each patient's baseline sample defined their own origin. Displacement vectors from this baseline were then calculated to represent the trajectory of change over time for each individual. A PERMANOVA using 999 permutations constrained within repeated measures was employed to test for group differences.

Assessing diet-dependent predictors of MS outcomes

The diet group-dependent predictive value of a feature for different MS disease outcomes examined in the NAMS study—IGF1 concentration, NfL concentration, T2 lesion volume, T2 lesion count, SDMT, and BDI-II score—was assessed by linearly modelling the outcome variable in response to the three-way interaction between group, time, and feature while accounting for inter-patient variability, thus acknowledging patient differences at baseline. Only the group-time-feature interaction term was tested for significance in a Chi-square test, as described above. KD was used as the reference level. The sign of the estimate of each interaction term for the other diet groups was used to determine the direction of the effect size, Cohen's f^2 , calculated from the conditional R^2 of both models. This resulted in two versions of f^2 , positive or negative, corresponding to the two alternative diet groups. Benjamini–Hochberg adjustment for multiple testing was performed across all tested combinations, and the significance threshold was set to $q < 0.1$. This analysis was conducted in a hypothesis-driven manner for group-associated GMMs ($n_{\text{lesion volume}} = 134$). Covariate-outcome associations were assessed using the same approach as described above for features: the outcome was modeled linearly with a covariate-by-time interaction term while accounting for repeated measures. Covariates significantly associated with an outcome (plus DMT usage, BMI, age, and T2 lesion load) were then tested for confounding. Confounding assessment involved adding each covariate-by-time interaction individually to the full three-way interaction model and testing its influence as described above: a covariate was considered a confounder if the covariate-time interaction significantly improved model fit ($p < 0.05$) while the three-way interaction did not ($p > 0.05$). To identify species related to the GMM-related outcomes, the procedure applying three-way interaction between intervention group, time, and ranked species counts while accounting for inter-patient variability was repeated in a hypothesis-generating manner for all species and these GMM-related outcomes. Species following group-dependent trajectories were identified among those significantly associated with outcomes ($q < 0.1$). For these species, ranked GMM abundances were modeled using three-way interactions between species, time, and group, before adjusting for all GMM-species combinations.

Cross-omics linear modelling

To investigate whether two features from different omics domains develop simultaneously over all diet groups, we linearly modelled one feature using the other (e.g., log abundance of metabolites from microbial features, ranked GMMs from taxa). These models included group and time as fixed effects and random intercepts for repeated measures. A likelihood ratio test (Chi-square) was applied to two nested models to assess the significance of the predictive feature term in the full model. The sign of the estimate of the tested fixed effect feature determined the directionality of f^2 . Benjamini–Hochberg adjustment for multiple testing was performed across all tested combinations. Only samples with results for both omics domains in question were used for multi-omics analysis.

Mediation analysis framework

Mediation analyses were performed using the *mediation* package (v. 4.5.0) in a hypothesis-generating framework¹⁰⁰. These indirect effects were estimated via 10,000 Monte Carlo simulations, with significance determined by 95% bias-corrected confidence intervals. The dataset was filtered to contain only two diet groups at a time (KD vs. CD, KD vs. FD, and CD vs. FD) to account for group-to-group mediation differences. To test whether a group-associated feature mediated the effect of the diet group on another feature or an outcome, two linear mixed-effects models were constructed. One modelled the feature using fixed effects for visit, diet group, and their interaction. The second modelled the other feature or outcome with the same fixed effects and interaction term, adding the previous feature's abundance. The group was used as the treatment variable, and the metabolite or taxon was considered the mediator. To account for repeated measures and baseline variability between individuals, all models applied random intercepts. When tested as potential mediators, metabolite abundances were logged, and taxonomic features were ranked. The effect size was calculated as the absolute value of the average causal mediation effect (ACME) divided by the sum of the absolute values of the ACME and the average direct effect (ADE). Benjamini–Hochberg adjustment for multiple testing was performed across all tested combinations if applicable.

Artificial intelligence use

Use of artificial intelligence (AI) was limited in this manuscript to (i) identify possibly overlooked relevant literature for further reading and reference, (ii) troubleshooting manually written code by helping identify possible causes of bugs or more effective implementations, and (iii) small amounts of text or grammar editing by suggesting more accessible or idiomatic phrasing within a larger manual editing process. Claude Sonnet (Anthropic, v. 4.5) and Perplexity's AI platform were used to assist but never to automate these tasks. All such suggestions were manually assessed and verified by human authors choosing whether and how to integrate each, and all authors take full responsibility for the results.

Data availability

The metagenomics datasets generated and analyzed during the current study are available in the NCBI-SRA repository, PRJNA1428526-Nutritional Approaches in Multiple Sclerosis—NAMS. The metabolomics datasets generated and analyzed during the current study are available in the MetaboLights repository under the accession code MTBLS15689 (<https://www.ebi.ac.uk/metabolights/MTBLS15689>)¹⁰¹.

Code availability

The analysis scripts are permanently stored in the Zenodo repository with the <https://doi.org/10.5281/zenodo.21937282>.

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Author contributions

F.P., J.B., and L.B. planned and conducted the NAMS study. L.M. coordinated timelines and sample logistics. J.K., U.B., and F.G. planned the metabolomics experiments and designed the stool crusher. U.B. and F.G. conducted the metabolomics experiments. V.H.J.D. and F.G. planned and conducted the metagenomic sequencing experiments. F.G. processed and cleaned the metabolomics datasets and metadata. F.G. analyzed the results, visualized the data, discussed results, and wrote the original draft. L.B. and R.R. conducted the study visits. F.P., L.B., M.W., and S.J. guided the interpretation of results. J.K. and S.F. supervised this work. All authors reviewed the manuscript.

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Additional information

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