

Reduced fibre-fermenting capacity of gut microbes in multiple sclerosis may result in prebiotic dietary fibre β -fructan promoting inflammation and CNS damage

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ABSTRACT

Background Some people with multiple sclerosis display changes in their gut microbiota with separate evidence suggesting that symptoms may worsen following a high-fibre diet. We hypothesised that in people with multiple sclerosis whose gut microbiota are less able to efficiently ferment dietary fibres, unfermented β -fructans induce inflammation.

Methods Diet data (n=48 individuals with multiple sclerosis, n=78 unaffected controls) and stool microbiome data (n=31 individuals with multiple sclerosis, n=61 unaffected controls) were previously collected from participants. Daily fibre subtype intakes were calculated and compared with faecal shotgun metagenomic sequencing in paediatric onset multiple sclerosis and unaffected persons. Response to unfermented β -fructans was examined in a germ-free experimental autoimmune encephalomyelitis (EAE) mouse model (unable to ferment fibres). Mice were fed β -fructans or control fibre diet beginning at symptom onset (day 14). EAE scores and weights were recorded daily. Intestinal and central nervous system tissues were collected at two endpoints to examine inflammatory responses and demyelinating lesions.

Results Individuals with paediatric onset multiple sclerosis consumed less β -fructans (2.4 g/day $\pm$ 0.3SD; $p<0.05$) than unaffected participants (3.6 g/day $\pm$ 0.4), which coincided with differences in the gut microbiota including lower fibre fermenting enzymes. Mice exposed to unfermented β -fructans sustained worsened EAE symptoms (day 20–28; $p<0.05$), immune activation in the gut and immune activation plus demyelinating lesions in the spinal cord compared with mice on control diet.

Conclusions The gut microbiota of individuals with paediatric-onset multiple sclerosis showed reduced fibre fermenting properties, and our animal findings suggest that unfermented β -fructans can worsen demyelination and promote gut–brain axis immune activation. Lower β -fructan consumption was observed among participants with paediatric-onset multiple sclerosis. Future longitudinal studies are warranted to confirm the findings uncovered in this manuscript.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Some people with multiple sclerosis display altered gut microbiota, with separate evidence suggesting high-fibre diet worsens symptoms. If gut microbiota do not fully ferment dietary β -fructan fibres, β -fructans can induce inflammation in gut diseases. The impacts of altered gut microbiome on diet–host interactions in multiple sclerosis have not been studied.

WHAT THIS STUDY ADDS

⇒ Persons with multiple sclerosis consume less β -fructan fibres than unaffected participants. Lower β -fructan consumption was associated with differences in the gut microbiota. The presence of unfermented dietary β -fructan fibres induced immune activation in the gut along with immune activation and damage in the central nervous system in a mouse model of multiple sclerosis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Although fibres are typically thought to be beneficial, some dietary fibres may have detrimental effects in select persons with multiple sclerosis who lack fermentative microbial activities. Persons with low fibre-fermenting microbiota who consume high amounts of prebiotic β -fructans may experience worsened multiple sclerosis symptoms. It is possible that low amounts of β -fructans from whole foods may still be tolerated or beneficial, while higher levels of β -fructans may have a negative impact.

INTRODUCTION

Multiple sclerosis is a disease of the central nervous system (CNS) in which neurological disability results from demyelination, inflammation and axonal degeneration.¹ Multiple sclerosis involves interactions between genetic

and environmental factors, including diet and the gut microbiome.² Gut microbiota compositional differences, known as dysbiosis, influence immune dysfunction in multiple sclerosis,^{3,4} but specific aspects of the dysbiosis require further study. Diet has one of the greatest influences on microbiota composition and functions.

Unlike other dietary factors, dietary fibres are not digested by the host; benefits of a high-fibre diet rely on microbial fermentation in the colon, leading to production of beneficial by-products such as short-chain fatty acids (SCFAs).⁵ Studies using the experimental autoimmune encephalomyelitis (EAE) mouse model have demonstrated that SCFAs ameliorate disease course, suppress demyelination and enhance remyelination.^{6,7} In people with multiple sclerosis, SCFA levels are reduced, which has been associated with differences in circulating immune cells and serum cytokine levels.^{8,9} Lower production of SCFAs and loss of fibre-fermenting microbiota in multiple sclerosis^{10–12} suggest that fibre fermentation may be impeded in multiple sclerosis, likely due to altered microbiota.

We previously showed that loss of fibre fermenting capacity in persons with inflammatory bowel disease results specifically in proinflammatory responses to β -fructan fibres, not other tested fibre subtypes; response was driven by β -fructan interactions with toll-like receptor-2 (TLR2) and activation of NOD-containing, LRR-containing and pyrin domain-containing protein 3 (NLRP3).¹³ In this setting, the inflammatory response is driven by an increase in the amount of fibre remaining in the gut due to lack of fermentation and a subsequent decrease in by-products of fermentation, including the anti-inflammatory SCFAs, butyrate and propionate. Interestingly, persons with multiple sclerosis also display changes in TLR2 and loss of fibre-fermenting microbes.^{14–17}

Ketogenic and low-carbohydrate diets have been shown to possibly be beneficial and well-tolerated by people with multiple sclerosis.^{18,19} However, an unintended consequence of such diets may lead to some persons reducing intake of dietary fibre and other essential vitamins and nutrients in attempts to avoid carbohydrates.²⁰ One cross-sectional UK study concluded that people with multiple sclerosis may be sensitive to fibre consumption.¹⁵ Among 2410 people with multiple sclerosis, intake of the recommended daily fibre amount was significantly associated with higher self-care-related problems, pain and problems carrying out usual activities, versus persons consuming a low-fibre diet.

Research increasingly focuses on the role of fibre fermentation in the gut and the subsequent impact on immune-mediated diseases. The benefits of dietary fibres consistently correlate with successful fibre fermentation.²¹ Different microbes ferment different types of dietary fibres;^{5,22} however, whether altered microbiota in multiple sclerosis impacts fermentation of specific fibres is unknown. In this study, we aimed to determine whether the intake of one dietary fibre subtype, β -fructans, was different in paediatric onset multiple sclerosis

versus age-matched unaffected controls and to what extent β -fructan ingestion adversely impacted a murine EAE model of multiple sclerosis lacking fibre fermentation capabilities.

MATERIALS AND METHODS

Participant selection and data sources

Details of recruitment and cohort characteristics were previously published.¹⁷ In the present study, we used published dietary and stool shotgun metagenomics data previously collected from persons ≤ 22 years old enrolled in a Canadian Pediatric Demyelinating Disease Network Study (CPDDN; online supplemental table 1). Paediatric onset multiple sclerosis was defined as those who met McDonald criteria (2017),²³ with symptom onset < 18 years of age. They were treatment-naïve or exposed to interferon- β or glatiramer acetate, at the time of the present substudy. Participants had not taken an antibiotic or corticosteroids within the preceding 30 days. The CPDDN advertised to recruit unaffected youth (controls). Demographics were collected using a questionnaire and other detailed clinical data were obtained using standardised forms, completed by site neurologists and facilitated by a study coordinator.¹⁶ In the current study, we obtained access to diet data and stool shotgun metagenomics sequencing. We started with those participants from the previously published CPDDN cohort who had complete dietary intake data ($n=48$ individuals with multiple sclerosis, $n=78$ unaffected controls), and next evaluated paired microbiota data from those who had both complete dietary data along with paired stool shotgun metagenomics data available ($n=31$ individuals with multiple sclerosis, $n=61$ unaffected controls).

Dietary fibre consumption analysis

Dietary intake was evaluated using the Block Kids Food Screener (NutritionQuest), a validated Food Frequency Questionnaire (FFQ) containing 77 food items to reflect the prior week's diet.²⁴ The questionnaire was completed within 1 week of stool sample collection. Individuals with unreasonably low or high energy intake, that is, < 500 or > 3500 kcal/day, were excluded from the diet data analysis under suspicion of lack of accurate recall. The Canadian Nutrient File online database was applied along with the dietary fibre data set^{13,22} to calculate the daily kcal and the consumption of dietary fibre subtypes including inulin, fructooligosaccharide (FOS), pectin and arabinoxylan (AX). We applied our validated approach to calculate consumption of each of the most abundant dietary fibre subtypes from the collected diet data, as previously.^{13,22} Both g/day and kcal adjusted consumption were calculated (inulin, FOS, pectin and AX).

Stool data processing, gene annotation and phylogenetic profiling

Stool was previously processed using the Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit (D6010) and shotgun

metagenomic was performed using the Illumina NextSeq platform.¹⁷ Here, we assessed previously collected stool shotgun metagenomics data.

Sequences were trimmed with fastq-mcf using a quality score threshold of 20 and allowing a minimal length of trimmed sequences of 100 bp. Trimmed sequences were taxonomically classified with MetaPhlAn3²⁵ using a custom database containing the archaea, bacteria, viruses, plasmid, human and UniVec_Core RefSeq data sets supplemented with the Genome Taxonomy Database. Pseudoalignments were filtered with a confidence threshold of 0.1 (at least 10% of kmers in each query sequence should map to the reference sequence). Taxa accumulating less than 1% of counts in each sample were filtered out to increase the robustness of quantification.

Functional profiling was carried out with HUMAnN3^{26,27} using only end1 of the trimmed sequences described above. Classification was done against the UniRef protein database clustered with a similarity threshold of 90%. Initially, HUMAnN3 identifies species present in the microbiome and then aligns reads against the pangenomes and conducts searches of in-silico translated sequences that were initially unclassified to quantify gene families and metabolic pathways.²⁶

Differential accumulation analysis was performed with the R V.4.2 package DESeq2.²⁸ Count data were transformed using the harmonic mean.

EAE mouse model of multiple sclerosis

Female germ-free C57BL/6 mice aged 7–8 weeks were acclimated to their cages for 1 week and housed with up to five mice per cage. EAE was induced using the Hooke Lab vaccine (MOG_{35–55}/PTX; EK-2110). Detailed procedures were performed according to the manufacturer's instructions. The mice were evaluated blindly every 1–2 days for weight along with using a standard five-point scoring scale (online supplemental table 2) to assess symptoms of EAE such as weakness in limbs, loss of coordination or balance, altered gait or difficulty walking, tail weakness or limpness, weight loss and general lethargy. On symptom onset (day 14), mice were randomly assigned to one of two experimental diets either receiving a diet containing β -fructans as the sole fibre (synergy-1 Beneo 5% β -fructan; n=10 EAE; n=9 control) or a balanced control diet with non-fermentable cellulose as the sole fibre (AIN-76A 5% cellulose; n=11 EAE; n=5 control). Feed was provided ad libitum and body weight was measured every 1–2 days. On day 29, the mice were euthanised and the tissues from the gut (cecum and colon) and CNS (brain and spinal cord) were dissected and either submersed in cold phosphate-buffered saline (PBS) and processed for flow cytometry or single-cell sequencing (scSeq) or were fixed in 4% paraformaldehyde solution before preparation of formalin-fixed paraffin-embedded (FFPE) slides. FFPE CNS and gut tissues from EAE C57BL/6 mice were sectioned at a thickness of 5 μ m, deparaffinised in xylene and rehydrated in ethanol for all staining. Digesta samples were

tested to confirm germ-free status at the end of the study using aerobic and anaerobic culture as well as by PCR.

LFB myelination staining and analysis

Staining was performed using 0.1% Luxol fast blue (LFB; 0.5% glacial acetic acid, in methanol) incubated for 16 hours at 56°C in a water bath. Slides were washed in ethanol followed by deionised water, then placed in a 0.05% sodium carbonate solution for 3 min, followed by a 30 s immersion in 70% ethanol. Slides were counterstained with haematoxylin for 90 s and washed under running deionised water. Subsequently, slides were immersed in 0.3% acetic acid alcohol. Slides were processed using ethanol and xylene and mounted using dibutyl phthalate xylene (DPX) mounting media. Slides were imaged at 40 $\times$ magnification using the Nanozoomer digital slide scanner (Hamamatsu) and images were analysed using NDP.view2 software. LFB was blindly scored manually²⁹ to define the extent of demyelinating lesions by two researchers using Fiji.³⁰

H&E staining and histology

Tissues were stained with haematoxylin for 90 s then differentiated with 0.3% acetic acid alcohol and counterstained with eosin for 10 s. Slides were dehydrated in ethanol and cleared with xylene before mounting using DPX mounting media. Slides were imaged at 40 $\times$ magnification using the Nanozoomer digital slide scanner (Hamamatsu) and images were analysed using NDP.view2 software. Blinded intestinal scoring was conducted independently by two researchers using Fiji. A validated scoring method was used to score (0–4) the intestinal inflammation based on specific schemes for the small intestine (scheme 6) and colon (scheme 4).³¹ Four different sections were scored and averaged for each tissue.

Immunohistochemistry staining and analysis

Citrate antigen retrieval (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) was performed on tissue slides, then blocked with 2% goat serum for 1 hour. Primary antibodies were applied overnight at 4°C in a humidified chamber; CXCR1 (1:10,000, PA5-27184, Thermo Fisher Scientific, Waltham, MA, USA), IL-17A (1: 40, ab79056, Abcam, Cambridge, UK) and CD4 (1:250, 1:500, 14-9766-80, Clone 4SM95, Thermo Fisher Scientific) in 1% bovine serum albumin (BSA). Slides were incubated in 0.3% hydrogen peroxide for 5 min, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Goat-anti-rabbit IgG; ab214880; Abcam for CXCR1 and Goat-anti-rat IgG; ab214882; Abcam for IL-17A and CD4) for 1 hour. Tissues were counter-stained using 3,3'-diaminobenzidine (DAB) and haematoxylin before final rinse steps in ethanol and xylene. Slides were mounted using DPX mounting medium. Stained slides were scanned at 40 $\times$ magnification using the Nanozoomer digital slide scanner (Hamamatsu) and viewed with NDP.view2 software. Cell staining was quantified using Fiji software.³² Colour deconvolution for haematoxylin-DAB was applied

to eliminate background interference. The threshold settings were adjusted to focus on stained areas for CD4 and CXCR1. The watershed tool was used to identify and count specific cells, and the number of particles was quantified.³² The average scores were also calculated and compared between mouse groups.

Single-cell RNA sequencing

Raw FASTQ data is publicly available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive portal under accession number PRJNA1298367. Processed single-cell RNA sequencing (scRNA-seq) data and analysis results are freely available on request.

Following the culling at day 23 (3 days post symptom peak and mid-inflammatory cycle), the intestinal tissue and spinal cord were cut in half longitudinally, and half of each was used for scSeq RNA analysis of three mice consuming a β -fructan diet and in three mice consuming a control diet. Tissue segments were gently cut into approximately 0.5 cm segments to begin manual tissue digestion, followed by chemical digestion (Hanks' Balanced Salt Solution (HBSS) medium, 4% DNase I, 1% Collagenase MA and 0.5% AOF BP Protease). Tissues were digested in chemical digestion buffer at 37°C for 10 min followed by gentle pipetting and repeated chemical digestion to a maximum of four times. Resulting supernatants were passed through 70 μ m cell strainers into 1 mL of cold foetal calf serum (FCS) and 5 mL cold fluorescence activated cell sorting (FACS) buffer (PBS+2% FCS). Cells were pelleted by centrifugation and haemocytometer counting was performed with a desired concentration of 5×10^6 cells/mL. Samples were FACS sorted by the University of Alberta Flow Cytometry Core on a Sony MA900 (100 μ m nozzle) using propidium iodide stain and 50 000 cells were collected per sample. Samples were delivered to the University of Alberta Advanced Cell Exploration Core. The 10x scSeq RNA libraries were prepared using the 10x Genomics Chromium Next GEM Single Cell 3' Gene Expression V.3.1 (Dual Index) and quality control was assessed on a Bioanalyzer. Sequencing was performed by Novogene.

Transcript abundance in scRNA-seq libraries was assessed with Cell Ranger V.8.0.1 (10x Genomics), using as reference the mouse genome GRCm38/mm10 and the corresponding GTF file. Each library was sequenced in triplicate and they were quantified separately. Quantification results were obtained as filtered feature-barcode matrices.

Count matrices generated by Cell Ranger were post-processed using a custom computational pipeline implemented in Python V.3.8. The analysis pipeline employed several bioinformatics tools, including Scanpy V.1.9.3³³ and AnnData.³⁴ Initially, replicates for each sample were merged. For each tissue (cecum and spine cord), control and β -fructan were merged. Merged data sets were filtered to exclude cells where the percentage of mitochondrial RNA surpassed 10% and the number of detected genes was larger than 200 and smaller than 6000. Doublets

were identified and removed using Scrublet.³⁵ Data were normalised to median total counts, logarithmised and feature selection was conducted using the top expressing 2000 genes.

Cell types were identified using CellTypist.³⁶ Cecum and spine cord cells were classified using the models 'Adult_Mouse_Gut.pkl' and 'Mouse_Whole_Brain.pkl', respectively. No model for the spinal cord is available in CellTypist. Data sets were subjected to principal component analysis³⁷ and Leiden graph clustering.³⁸ Uniform Manifold Approximation and Projection (UMAP) embedding was conducted using 'predicted labels' or 'majority voting' for colouring plots and those plots were finally used to curate annotations.

For differential expression analysis, for each tissue, control and β -fructan groups were contrasted using the Wilcoxon rank-sum test as implemented in Scanpy. Genes were considered significantly differentially expressed if they met the following criteria: (1) absolute log₂ fold change >0.25 and (2) adjusted p value <0.05. P values were adjusted for multiple testing using the Benjamini-Hochberg procedure. Differentially expressed genes were visualised using volcano plots, with significant genes highlighted and top-genes labelled. The plots display log₂ fold changes on the x-axis and -log₁₀ (adjusted p-value) on the y-axis.

All the above-described computational analyses were performed using Python V.3.8. The computational pipeline used the following packages: Scanpy, AnnData, Scrublet, SciPy, Pandas, NumPy, Seaborn, Matplotlib, AdjustText, CellTypist and Leidenalg. Analyses were executed in Google Colab with data stored on Google Drive.

Gene Ontology (GO) enrichment analysis was conducted to identify biological processes, molecular functions (MFs) and cellular components associated with differentially expressed genes. Analyses were performed separately for upregulated and downregulated genes using the clusterProfiler package in R.³⁹ Significant differentially expressed genes were partitioned into upregulated (log₂ fold change >0) and downregulated (log₂ fold change <0) sets. The enrichGO function from clusterProfiler was applied to each gene set using the org.Mm.eg.db annotation database with gene symbols as the key type. GO enrichment was performed separately for MF ontology categories with the following parameters: p value adjustment method set to Benjamini-Hochberg, p value cut-off of 0.05 and q value cut-off of 0.05. Results were visualised using dot plots of the top 10 significantly enriched GO terms for both upregulated and downregulated gene sets. The enrichment results, including GO terms, gene counts, p values and adjusted p values, were saved as tab-separated files.

Flow cytometry

Following culling on day 23 (3 days post symptom peak and mid-inflammatory cycle), the intestinal tissue and spinal cord were cut in half longitudinally. Half of each of

the intestine and the spinal cord was used for flow cytometry analysis of three mice who had β -fructan diet and in three mice fed a control diet.

The caecum was excised and cut longitudinally and sectioned into approximately 1 cm segments. Cecum segments were incubated in 10 mL stripping buffer (HBSS+2% foetal bovine serum (FBS), 5 mM EDTA, 15 mM HEPES) at 37°C with shaking for 20 min ($\times 2$), rinsed in 10 mL of ice cold wash buffer (HBSS+2% FBS, 15 mM HEPES), minced with scissors to create a pulp, transferred into 10 mL digestion buffer (RPMI+10% FBS, Penn/Strep, L-glutamine, 1 mM NaPyruvate, β -mercaptoethanol (β -ME), 50 U/mL DNase, 0.5 mg/mL Collagenase IV), incubated at 37°C for 45 min with shaking and filtered through a 70 μ m filter with a cold PBS wash, followed by resuspension and enumeration. Spinal cord tissue was similarly minced and digested. Single cell suspensions (1×10^6) were pelleted in a V-bottom 96 well plate and blocked with Fc-block in FACS buffer (1/100 dilution) for 10 min on ice, followed by staining for surface antigens (online supplemental table 3) with diluted antibodies (1/250 in FACS buffer, 4°C, 30 min). Cells were washed, permeabilised (FoxP3 fix/perm buffer, eBiosciences, Thermo Fisher Scientific) and stained for intracellular antigens according to manufacturers' recommendations. Cells were analysed on an Aurora spectral cytometer. FlowJo (BD, V.10.8) was used to analyse all flow cytometry data.

Statistical analysis

To assess the associations between FOS, paediatric onset multiple sclerosis (case or control), and microbe abundances, analyses were performed in R V.4.2 using the edgeR package. This package allows normalisation using the 'Trimmed Mean of M-values' method and accounts for over-dispersion in the abundance data. Each microbe was included as an outcome variable and predictors included FOS level, paediatric onset multiple sclerosis diagnosis, as well as their interaction. A two-way analysis of variance test was used to assess significant differences among groups in GraphPad Prism V.10 for animal work, while comparison between paediatric onset multiple sclerosis and controls was performed using the analysis of covariance test in SPSS V.26 which was further adjusted for age. All p values were corrected with the Benjamini-Hochberg method and comparisons with corrected p values < 0.05 were considered significant.

RESULTS

Participant summary

Dietary data from 48 paediatric onset multiple sclerosis and 78 unaffected controls were examined from a previously published cohort.¹⁷ Of these participants, only 31 paediatric onset multiple sclerosis participants and 61 unaffected controls had paired stool shotgun metagenomics data available for analysis. Cohort characteristics are shown in online supplemental table 1. All cases had

relapsing–remitting multiple sclerosis. The multiple sclerosis cases and controls were similar in age at diet data collection (average 15.3 and 14.2 years, respectively) and age at stool sample procurement (average 16.5 and 14.9 years, respectively). The average age of multiple sclerosis symptom onset was 15.1 years for the total cohort and 15.7 years for those who had both diet and stool. Females represented 81% of multiple sclerosis cases and 71% of controls for the full cohort and 84% of multiple sclerosis cases and 64% of controls for those with both diet and stool data.

Lower consumption of dietary β -fructan fibres is associated with reduction in microbial metabolites involved in β -fructan fermentation in paediatric onset multiple sclerosis

Individuals with paediatric onset multiple sclerosis consume similar amounts of total fibre based on FFQ reported intake compared with unaffected controls, yet they did consume less β -fructans (FOS and inulin; $p < 0.05$) versus controls (figure 1A). On testing the association of microbiota findings with dietary fibre intake, we found β -fructan fermenting microbes (eg, *Bifidobacterium*) and hydrolysing enzymes (eg, *xyloglucan oligosaccharide beta-galactosidase*, *xylan 1,4-beta-xylosidase*) were lower in paediatric onset multiple sclerosis than in unaffected controls (figure 1B–D). Lower consumption of β -fructan was associated with a higher abundance of *Methanobrevibacter* spp., *Escherichia* spp. and *Desulfovibrio* (figure 2A). Consumption of β -fructan was differentially associated with microbiota composition in unaffected controls compared with paediatric onset multiple sclerosis (figure 2B). Higher β -fructan intake in controls was associated with higher abundance of key fibre-fermenting microbiota, including several *Lactobacillus* spp., while intake of β -fructan in paediatric onset multiple sclerosis was primarily associated with *Enterobacter* spp., *Methanobrevibacter* spp. and *Prevotella* spp.

Unfermented β -fructans worsen CNS demyelination and multiple sclerosis-like symptoms along with both CNS and gastrointestinal immune activation in an EAE mouse model

Direct response to unfermented β -fructans was examined in a germ-free EAE mouse model (unable to ferment fibres; figure 3A). Unfermented β -fructans induced worsened EAE symptoms (day 20–29; $p < 0.05$; figure 3B) and a higher burden of CNS demyelinating lesions compared with EAE on the control cellulose fibre diet (figure 3C). There was no impact of β -fructans on animal weight compared with control diets. Non-EAE control mice showed no response to β -fructans.

As β -fructans are large molecules, they do not pass the intestinal barrier, suggesting that the interactions between β -fructans and the host occur in the gut. We found no significant difference in the caecum (online supplemental figure 1A) and colon (online supplemental figure 1B) histological scores between β -fructan and control diets in either EAE or non-EAE control mice. To examine if there was any impact of β -fructans on known immune markers in the CNS and gut relevant to multiple sclerosis,³ we performed immunohistochemistry using a series of antibodies and uncovered

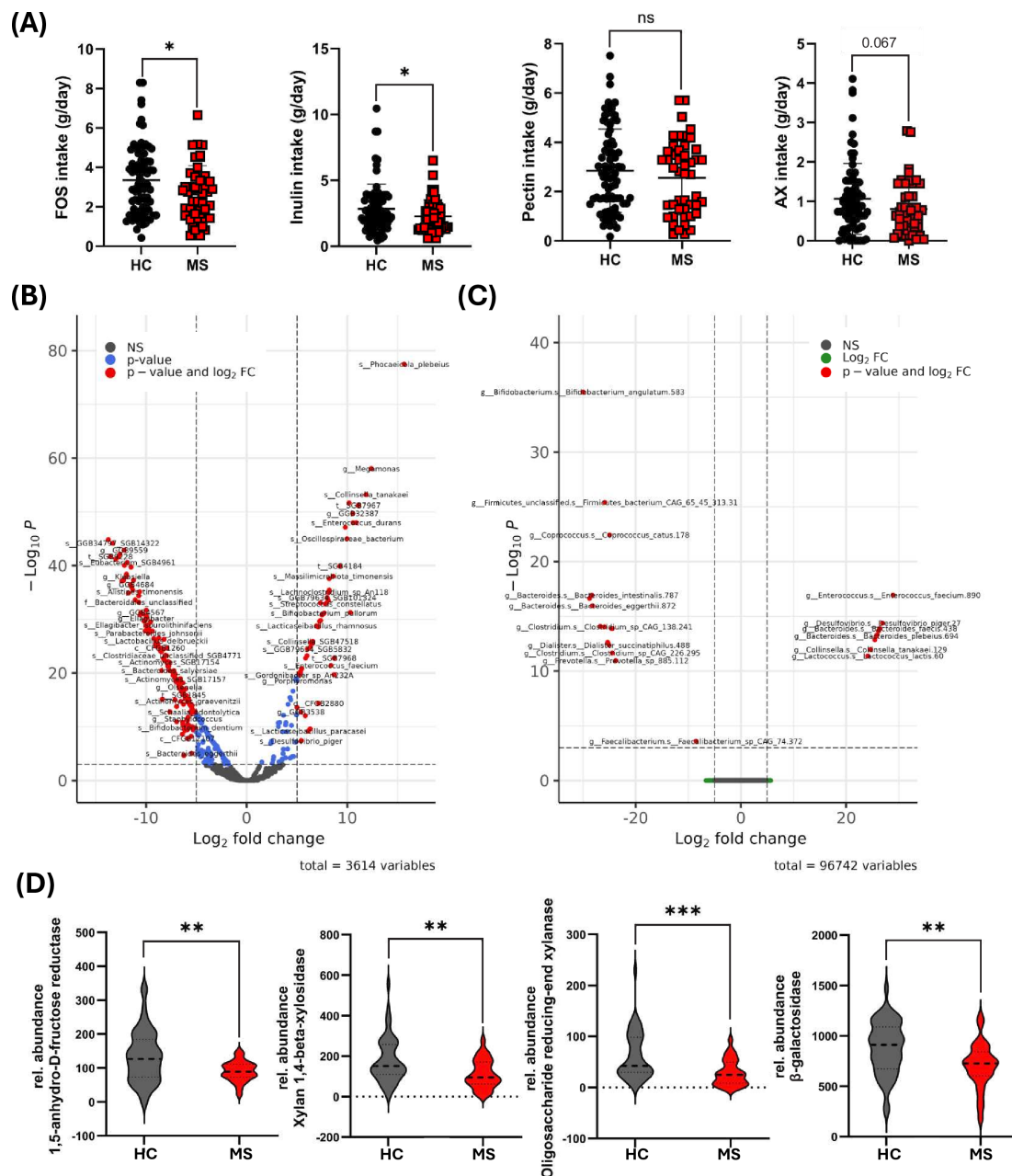


Figure 1 Reduced consumption of dietary β -fructan fibres is associated with reduction in microbial metabolites involved in β -fructan fermentation. (A) Dietary fibre subtypes including β -fructan (FOS, inulin), pectin and AX were assessed using diet data from individuals with paediatric onset MS ($n=48$) and unaffected controls ($n=78$). Microbiota composition and functions were confirmed by shotgun metagenomics in stool collected from a subset of individuals with paediatric onset MS ($n=31$) and control participants ($n=61$) with paired diet and stool data. Shotgun metagenomics data were analysed by (B) MetaPhlAn3 (microbiota composition) and (C) HUMAnN3 (microbiota functions). (D) Microbial metabolites identified by shotgun metagenomics to be significantly altered in MS were presented. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. AX, arabinoxylan; FC, fold change; FOS, fructooligosaccharide; HC, healthy control; MS, multiple sclerosis; ns, not significant.

significantly higher levels of CXCR1 (figure 4A) and CD4 (figure 4B) in CNS, and higher levels of CXCR1 in the colon (figure 4C), but not in cecum (figure 4D).

Consumption of dietary β -fructan fibres in EAE mice is associated with altered immune cell populations and functions in the spinal cord and cecum

We performed flow cytometry and scSeq on tissues collected 3 days postsymptom peak, which is reflective

of the inflammatory phase of EAE. As the initial site of response to dietary β -fructan, the cecum displayed trends towards elevated T helper cell 1 (Th1) cell abundance in β -fructan diet mice compared with control diet mice (figure 5A,B). The top six significantly upregulated gene pathways identified in the cecum of EAE mice consuming β -fructan diet, compared with control diet, included GTPase regulator activity,

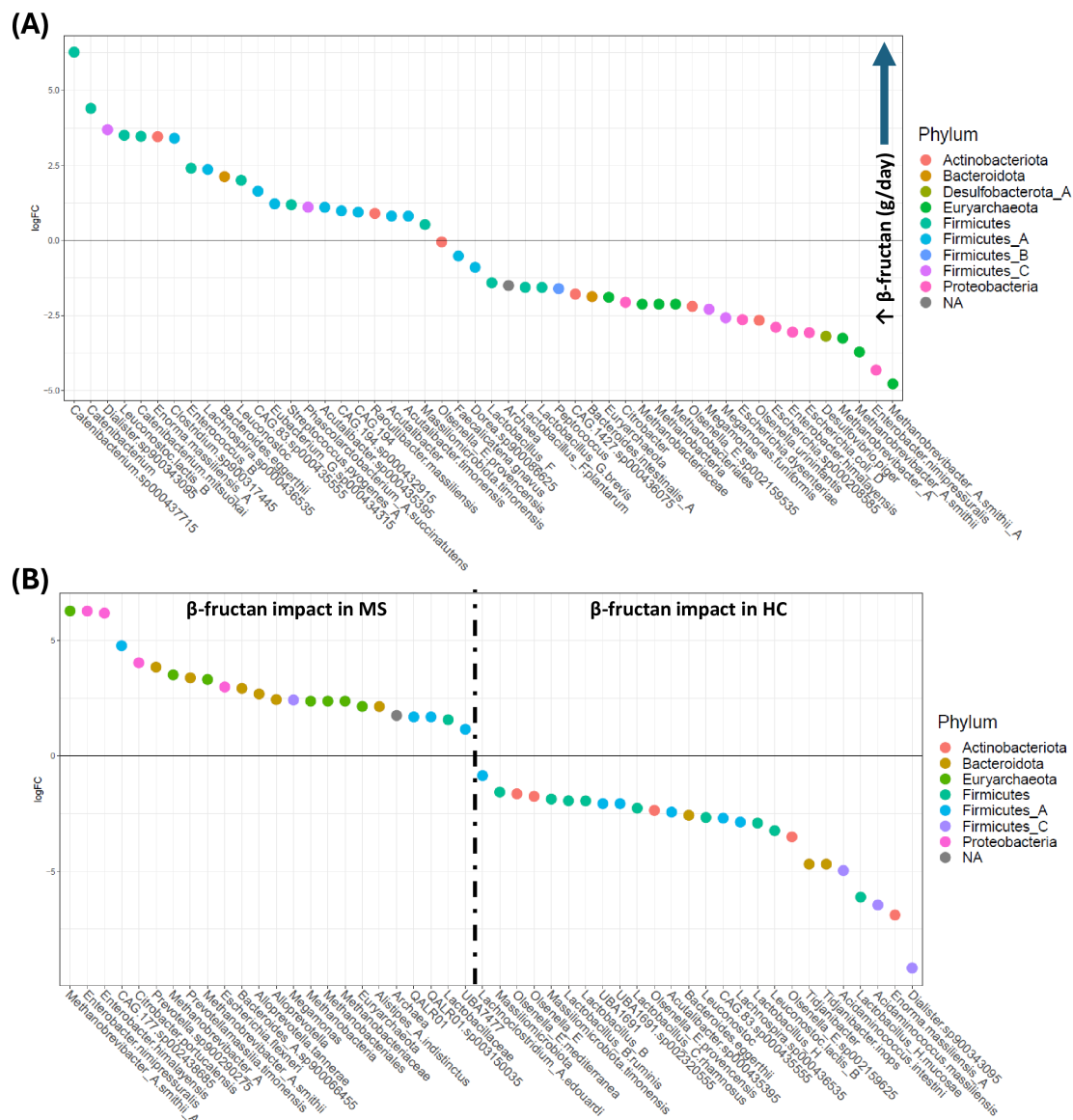


Figure 2 Predictive analysis demonstrated a correlation between consumption of β -fructan and gut microbiota taxonomy. Consumption of dietary β -fructan fibres was correlated with microbiota taxa from shotgun metagenomic analysis of stool samples and was presented as (A) the association of the log₂ FC in microbiota taxa relative abundance with increasing β -fructan consumption (g/day) by one unit; and (B) log₂ FC > 0 indicates that the effect of increased β -fructan consumption (g/day) on microbiota taxa is higher in MS, while log₂ FC < 0 indicates that the effect of increased β -fructan consumption (g/day) on microbiota taxa is higher in HC. FC, fold change; HC, healthy control; MS, multiple sclerosis.

nucleoside-triphosphatase regulator activity, glycosyltransferase activity, hexosyltransferase activity, UDP-glycosyltransferase activity and cytokine binding (figure 5C). The top six downregulated gene pathways included structural constituent of ribosome, ribosomal RNA binding, cadherin binding, messenger RNA 5'-UTR binding, ubiquitin-protein transferase inhibitor activity and ubiquitin-protein transferase regulator activity (figure 5C). In the spinal cord, β -fructan intake in EAE displayed trends towards higher CD45⁺ cells, microglia, CD11c⁺/CD69⁺ cells and CD11c^{hi}/Tbet⁺ cells (figure 6A,B). The top six significantly upregulated gene pathways in the spinal cord of mice consuming the β -fructan diet included primary active transmembrane

transporter activity, active transmembrane transporter activity, oxidoreduction-driven active transmembrane transporter activity, electron transfer activity, NADH dehydrogenase (ubiquinone) activity and NADH dehydrogenase (quinone) activity; the top six downregulated gene pathways included methylated histone binding, methylation-dependent protein binding, modification-dependent protein binding, histone binding, protein transmembrane transporter activity and oxidoreductase activity (figure 6C). Other cell populations measured by flow cytometry did not display any difference in difference between β -fructan and control diets (online supplemental figure 2).

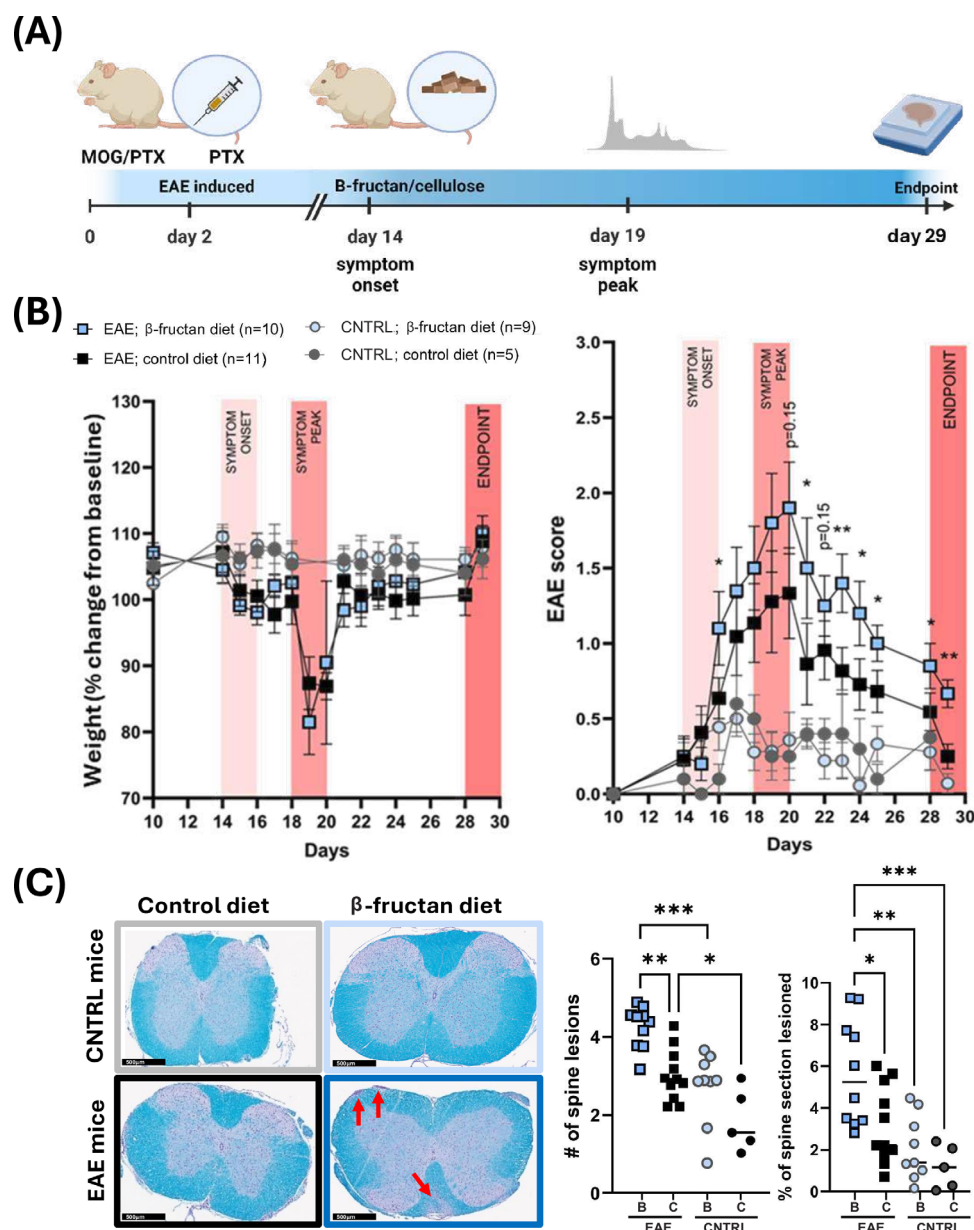


Figure 3 β -fructan diet caused worsened symptom score and increased demyelinating lesions in a germ-free EAE mouse model (unable to ferment fibres). Both C57BL/6 control mice and EAE mice were included in the study. (A) All mice were fed β -fructan diet (n=10 EAE; n=9 control) or control fibre diet (n=11 EAE; n=5 control) beginning at symptom onset (day 14) until 28 days. (B) Animals were weighed and assessed for EAE symptoms daily. (C) At endpoint, spinal cord tissue was collected, paraffin embedded and stained using LFB to identify demyelinating lesions (red arrows). * p <0.05, ** p <0.01, *** p <0.001. B, β -fructan diet; C, control diet; CNTRL, control; EAE, experimental autoimmune encephalomyelitis; LFB, Luxol fast blue; MS, multiple sclerosis.

DISCUSSION

While previous studies in both human cohorts and animal models have suggested that a high-fibre diet is associated with worsened multiple sclerosis,^{15,40} no prior studies have examined mechanisms of fibre–microbiome–host interactions or assessed the impacts of specific fibre subtypes in multiple sclerosis. Consistent with our previous findings in persons with inflammatory bowel disease,¹³ here we found that paediatric onset multiple sclerosis participants consume less β -fructan fibres than unaffected children and adolescents, which coincided with differences in the gut microbiota, including lower fibre fermenting

capacity. In contrast, higher consumption of β -fructans was associated with beneficial microbiota such as *Lactobacillus* spp. in unaffected controls. The same beneficial relationship was not found in paediatric onset multiple sclerosis, which is in line with prior evidence that suggests that the extent to which select dietary fibres impact the host in autoimmunity is highly influenced by an individual's microbiota and differs significantly between healthy and autoimmune disease states.^{41–43} However, the paediatric cohort in the present study involved collection of samples and data at only one time point, which does not allow us to define whether changes in the gut microbiome

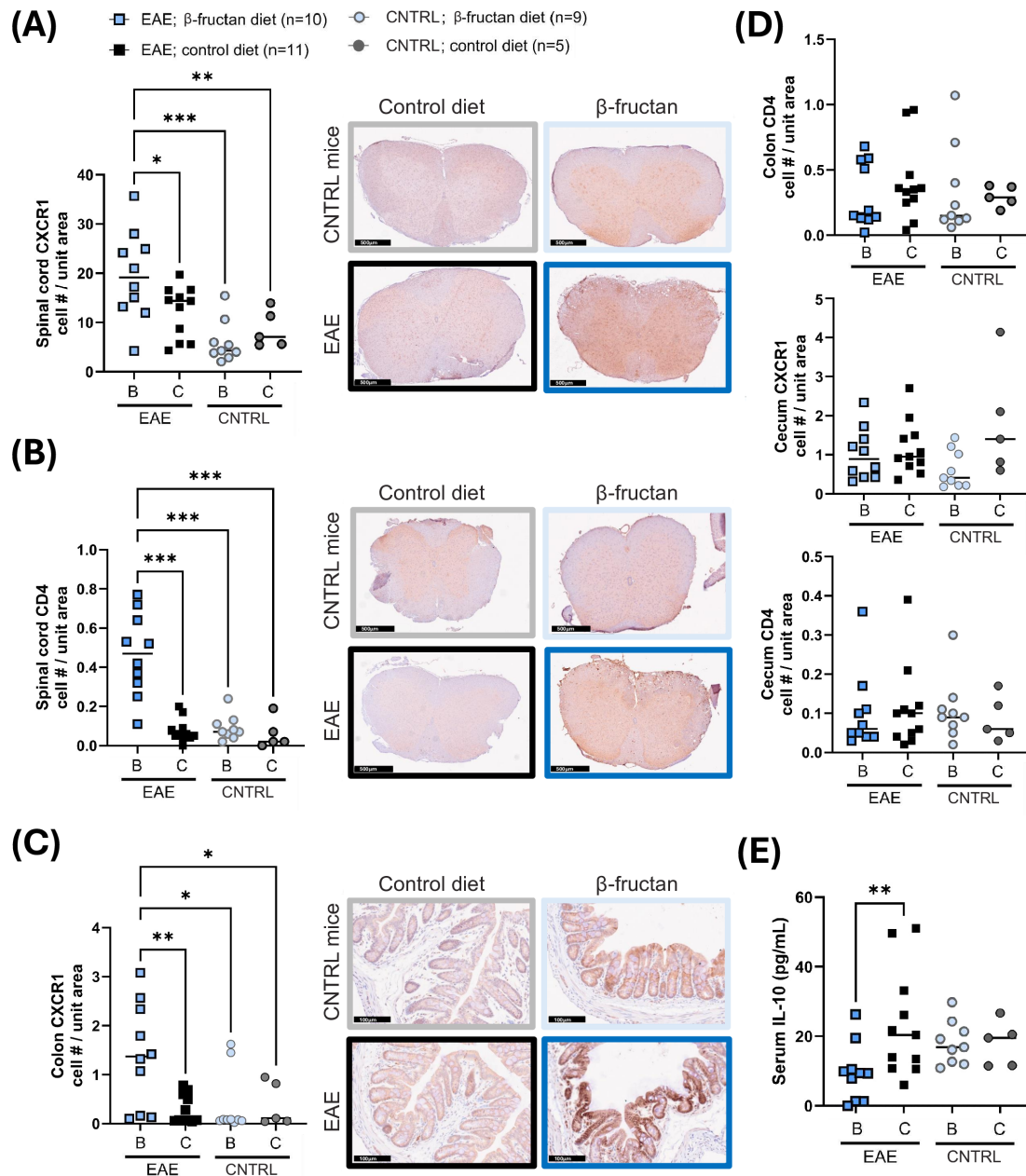
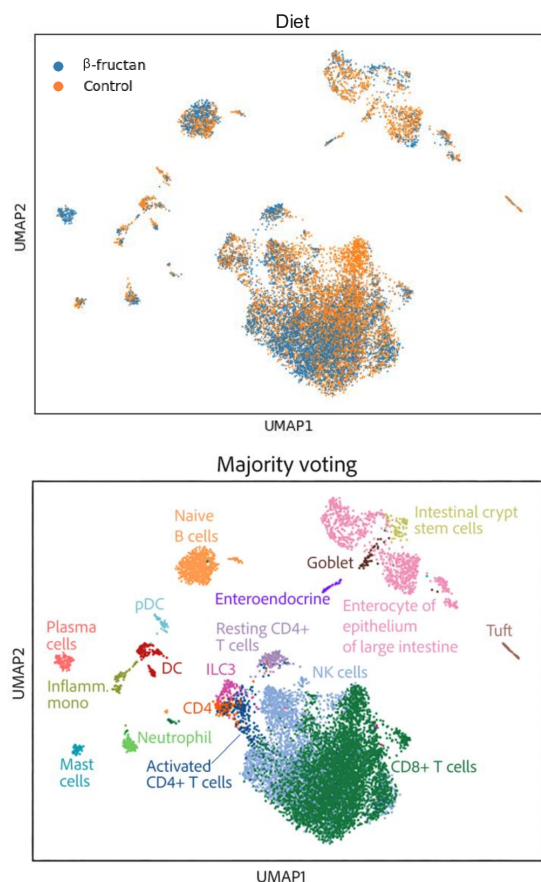


Figure 4 In an EAE mouse model, reduced consumption of dietary β -fructan fibres is associated with reduction in microbial metabolites involved in β -fructan fermentation. All mice were fed a β -fructan diet (n=10 EAE; n=9 control) or control fibre diet (n=11 EAE; n=5 control) beginning at symptom onset (day 14) until 28 days. At endpoint, spinal cord, cecum and colon tissues were collected, paraffin embedded and stained using IHC to identify possible immune changes including (A) CXCR1 in the spinal cord of EAE mice compared with control diet, (B) CD4 in the spinal cord of EAE mice compared with control diet and (C) CXCR1 in the colon of EAE mice compared with control diet, (D) CXCR1 in the cecum and CD4 in the cecum and colon. (E) Multiplex ELISA of mouse serum in EAE mice consuming the β -fructan diet compared with the control diet. *p<0.05, **p<0.01, ***p<0.001. B, β -fructan diet; C, control diet; CNTRL, control; EAE, experimental autoimmune encephalomyelitis; IHC, immunohistochemistry.

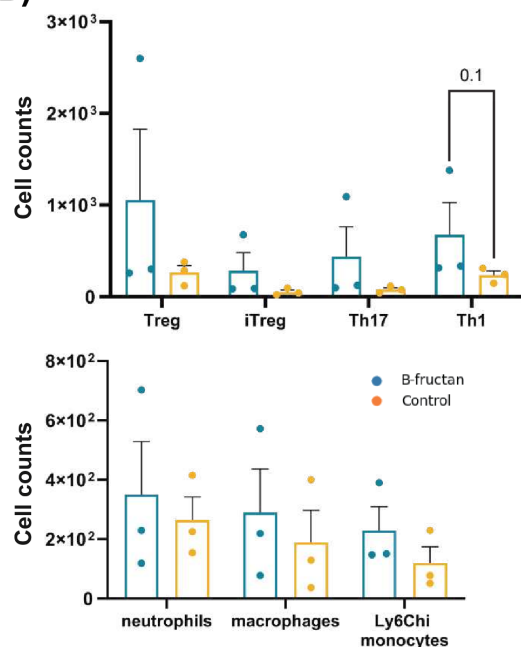
or dietary fibre intake occur first in paediatric onset multiple sclerosis. While microbiota shifts have been identified in our patient cohort,^{41 44} longitudinal studies are needed with experimental approaches such as ex vivo culture of stool microbiota with different dietary fibres to better identify the capacity of microbiota from people with multiple sclerosis to use these molecules.

To overcome these limitations, we used a murine model that offers evidence of why a high-fibre diet may not always be beneficial in multiple sclerosis. We designed this murine experiment based on our prior work in inflammatory bowel disease,^{13 41–43} which showed lower consumption of β -fructans could be due to lower fermentation by gut microbiota, resulting in greater amounts of

(A) Cecum



(B)



(C)

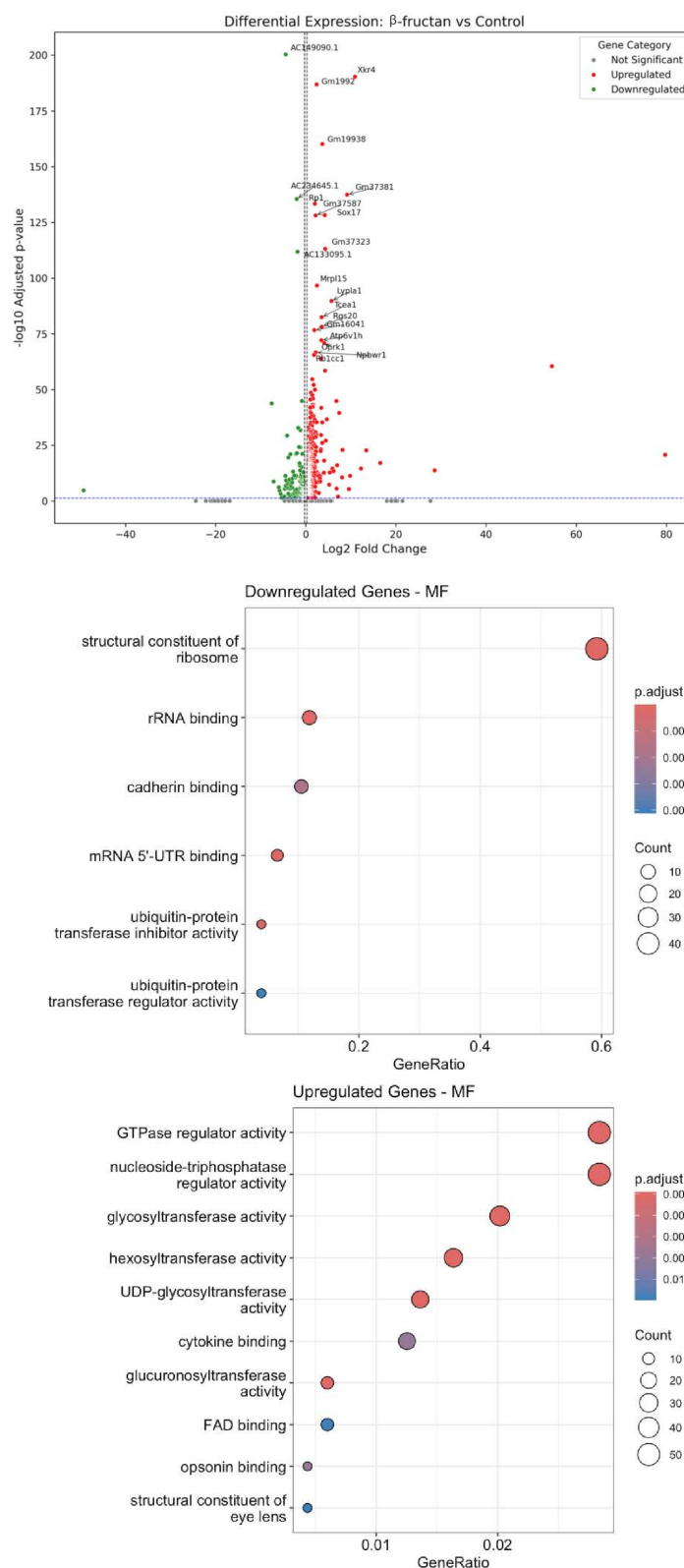
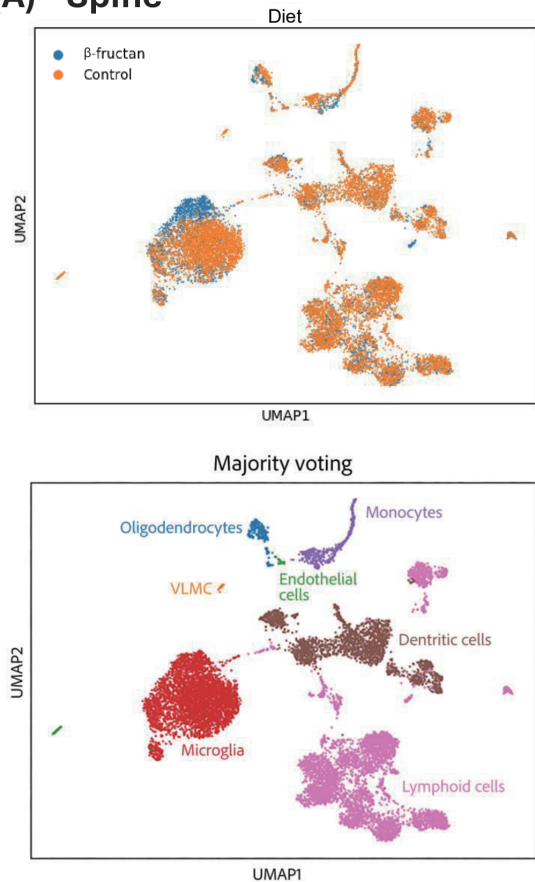
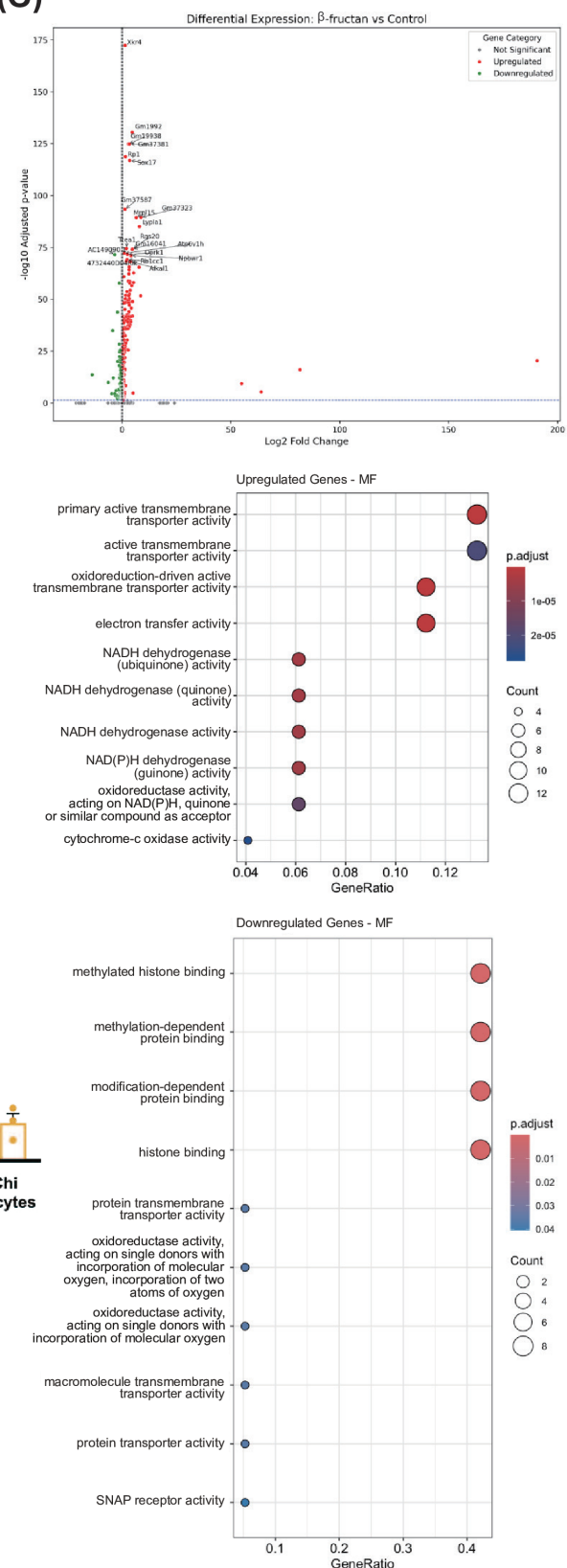


Figure 5 Consumption of dietary β -fructan fibres in EAE mice is associated with altered immune cell populations in the caecum. EAE mice were fed β -fructan diet ($n=3$) or control fibre diet ($n=3$) beginning at symptom onset (day 14) until 23 days. At endpoint cecum tissues were collected for (A) single cell sequencing cell profiles, (B) quantitative flow cytometry of immune cell populations and (C) single-cell RNA sequencing-based differential gene expression and molecular function enrichment analyses. DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; MF, molecular function; mRNA, messenger RNA; NK, natural killer; pDC, plasmacytoid dendritic cell; rRNA, ribosomal RNA; Th1, T helper cell 1; Treg, regulatory T cell; UMAP, Uniform Manifold Approximation and Projection.

(A) Spine



(C)



(B)

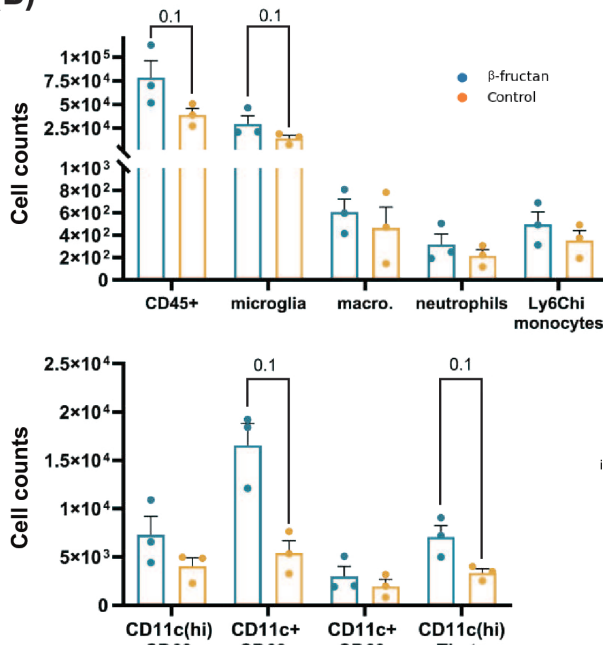


Figure 6 Consumption of dietary β-fructan fibres in EAE mice is associated with altered immune cell populations in the spinal cord. EAE mice were fed β-fructan diet (n=3) or control fibre diet (n=3) beginning at symptom onset (day 14) and maintained until 23 days. At the endpoint, spinal cord tissues were collected for (A) single cell sequencing cell profiles, (B) quantitative flow cytometry of immune cell populations and (C) single-cell RNA sequencing-based differential gene expression and molecular function enrichment analyses. EAE, experimental autoimmune encephalomyelitis; MF, molecular function; UMAP, Uniform Manifold Approximation and Projection; VLMC, vascular leptomeningeal cell.

unfermented β -fructans interacting with host cells of the gut, driving TLR2-NLRP3 activation and inflammatory cytokine secretion in the gut. More recent studies have supported our findings, demonstrating that high levels of dietary β -fructans can induce type-2 inflammation in the gut and lung in mouse models.^{41–43} Here we used a germ-free EAE mouse model to mimic this lower fibre-fermenting capacity. Mice were exposed to diet after EAE symptom onset to examine the impacts of β -fructans in the setting of diagnosed multiple sclerosis where the gut microbiome is known to differ from healthy individuals. In EAE mice exposed to β -fructans, immune activation was seen in the gut and immune activation plus demyelinating lesions in the spinal cord, along with worse EAE symptoms, compared with control diet-fed mice. This indicates a heightened inflammatory response in the CNS, suggesting unfermented β -fructans may induce neuroinflammation in the setting of multiple sclerosis. Neuroinflammation and demyelination in multiple sclerosis likely occur when the immune system mistakenly targets myelin in the CNS; this leads to the breakdown of the blood–brain barrier, allowing immune cells to infiltrate the CNS.^{45 46} Intestinal barrier dysfunction and greater intestinal permeability are common at the onset of EAE, allowing for increased interactions between luminal contents and gut immune cells.⁴⁷ These findings suggest that the impacts of unfermented β -fructans may involve host immune changes reflective of multiple sclerosis, as unfermented β -fructans only induced immune changes and inflammation in EAE mice and not in non-EAE control mice.

Mechanistically, we uncovered that unfermented β -fructan promotes immune activation of pathways with known involvement in the multiple sclerosis gut–brain axis.³ CXCR1 was higher in both the cecum and spinal cord of EAE mice on a β -fructan diet, while CD4 was only significantly higher in the spinal cord. CXCR1⁺ macrophages are known to be involved in gut–brain axis activation in multiple sclerosis and are typically more abundant in patients with multiple sclerosis and EAE mice.^{3 48} CXCR1 knockout mice display reduced sensitivity to EAE.⁴⁸ These data support our findings that β -fructan-induced increase in CXCR1⁺ cells would be associated with worsened EAE outcomes. CXCR1⁺ macrophages also play critical roles in chemoattraction and T cell activation through the production of cytokines that can influence T cell differentiation and function.^{49 50} Multiple sclerosis is mediated by myelin-reactive CD4⁺ Th cells, including primarily Th1 and Th17 lineages.^{51 52} Thus, our results suggest that activation of macrophages in the gut through interactions with unfermented β -fructans may result in inflammatory cytokine signalling that drives infiltration and activation of T cells in the CNS, ultimately promoting demyelination and worsened multiple sclerosis symptoms. This is in line with our findings in inflammatory bowel disease, indicating a key role for macrophages in β -fructan response in the gut.¹³ While

our results suggest that several immune cell populations are likely involved in this response to β -fructans, further research is needed to confirm the complex mechanisms of response as the scRNA-seq and flow cytometry experiments in this study were not well powered and validation studies have not been performed to confirm a mechanistic role of different cell types. Furthermore, the germ-free EAE mice lack gut microbes entirely, which does not adequately reflect the natural microbiome or immune settings of persons with multiple sclerosis. Future studies should aim to use humanised mice colonised with human gut microbiota along with clinical investigations to determine precision responses to different types of dietary fibres, including the β -fructans.

CONCLUSION

The benefits of certain fibres are well studied in select disease settings; however, the benefits of dietary fibres depend on the presence of fibre-fermenting gut microbes.^{5 22} In individuals lacking these microbes, evidence has shown that certain types of fibre (eg, β -fructan) can remain unfermented, triggering inflammation and worsening symptoms.^{13 41 43} These findings, along with the established gut–brain axis,^{3 53} suggest that gut dysbiosis may extend the complex impact of fibres beyond the gut, potentially linking diet, microbiota and autoimmune conditions like multiple sclerosis. Though causality cannot be inferred, findings from this study support initial evidence that if β -fructans are not broken down by gut microbes and an individual continues to consume high amounts of these typically beneficial fibres, they may induce worsened multiple sclerosis symptoms and result in people with multiple sclerosis avoiding foods that make them feel worse. It is possible that low amounts of β -fructan from foods such as garlic and banana may be tolerated or beneficial,⁵⁴ even in those with reduced fibre fermentation capacity, while higher levels of prebiotic β -fructans found in commercial prebiotics, supplements or certain grocery products may have a negative impact until the gut microbiome's fermentation potential is increased. These findings demonstrate that further, more clinically relevant investigations are warranted to fully understand the interaction between specific fibres, microbes and the host in multiple sclerosis and how to support clinical improvements in the gut microbiome and host through precision nutrition approaches in the future.

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Competing interests Authors declare that they have no competing interests of direct relevance to the current study, while several of the authors have held research funding support and associations with research and clinical affiliations, unrelated to the present work. HA is a scientific advisor for Dayhoff Technologies. HT was an advisor for Canada's Drug Agency (2024-25, payment declined; Scientific Advice Program: Invited as a Clinical Expert). BB serves as a consultant to Roche, Novartis, Sanofi and UCB. ABO has served as a consultant to Biogen, Cabaletta, Capstan, GlaxoSmithKline, Immunicon, Merck/EMD Serono, Moderna, Novartis, Roche/Genentech, Sana, Sangamo, Sanofi-Genzyme and Viracta.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval Details of clinical participant recruitment and cohort characteristics were previously published with publicly available data reassessed

in the present study.¹⁶ Human ethics was previously approved and only publicly available data was used in the present study (The SickKids Research Ethics Board #1000017648; University of British Columbia's Clinical Research Ethics Board #H15-03330). Animal ethics was approved by the University of Alberta Research Ethics Office (AUP00004214).

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