Behavioral response to fiber feeding is cohort-dependent and associated with gut microbiota composition in mice

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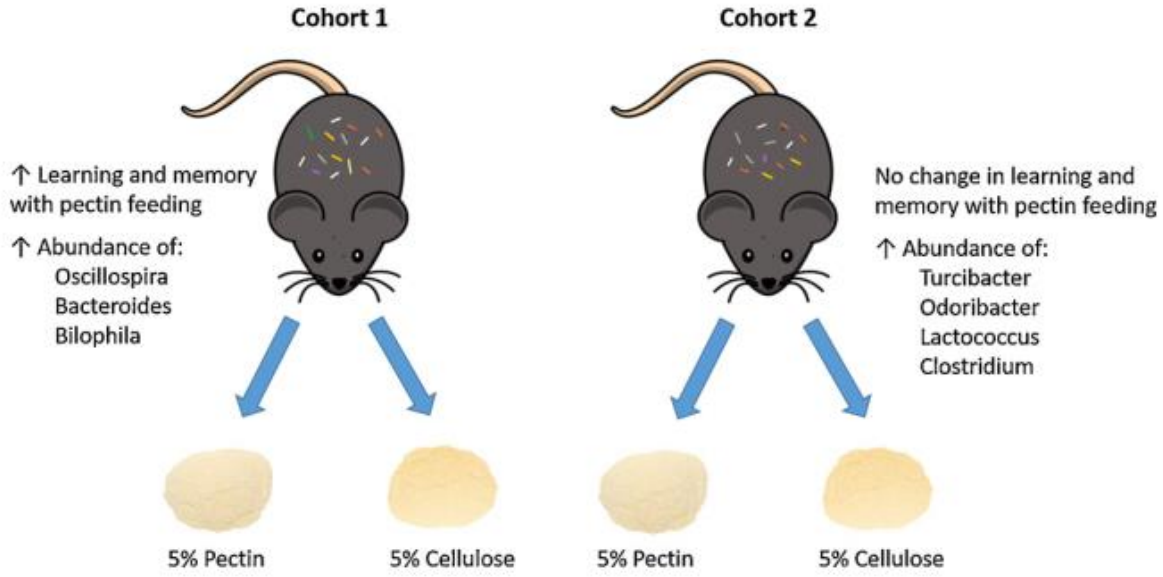
ABSTRACT

Recent data has supported a role for the gut microbiota in improving cognition and shaping behavior. Here, we assessed whether pectin, a soluble, fermentable fiber, could enhance learning and memory in mice. Two cohorts of young male C57Bl/6 J mice, C1 (n = 20) and C2 (n = 20), were obtained from Jackson Laboratory and randomized to semi-purified AIN-93 M diets containing 5% pectin (n = 10) or cellulose (n = 10). After 16 weeks, learning and memory was assessed by Morris Water Maze (MWM) and microbiota composition was analyzed by 16S rRNA sequencing. Despite identical treatment, we observed differences in learning and memory abilities between cohorts, along with distinct microbiotas. In C1, pectin-fed mice spent a higher percentage of time in the target quadrant at the 24-h probe trial of the MWM versus cellulose-fed mice; in C2, no effect of pectin was observed. In both cohorts, UniFrac distance revealed significant differences in gut microbial communities between cellulose-fed and pectin-fed mice. UniFrac analysis also revealed significantly different bacterial communities between cohorts. Further analysis demonstrated that the microbial genera Oscillospira, Bilophila, and Peptostreptococcoceae were more abundant in C1 versus C2, and positively associated with distance from the platform during the 24-h probe test. These data support previous findings that differences in the gut microbiota may play a role in host response to a dietary intervention and could partly explain irreproducibility in psychological and behavioral experiments. Further research is needed to determine if a causal relationship exists.

GRAPHICAL ABSTRACT



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The trillions of microbes inhabiting the gut, collectively called the gut microbiota, affect distant organs within the host and have been implicated in numerous health outcomes. Recent studies have suggested a role for gut microbes in shaping cognitive function and behavior [1]. Dietary changes have been shown to rapidly shift the composition of gut microbial communities [2]. The soluble, fermentable fiber pectin has numerous effects on gut physiology, including delayed gastric emptying [3], reduced glucose absorption [4] and reduced transit time [5]. It also selectively feeds certain species of microbes (e.g. *Eubacterium eligens, Faecalibacterium prausnitzii*) in the large intestine [6,7].

Fermentable fibers such as pectin may be able to influence cognition via the gut-brain axis. Bacterial fermentation end products such as short-chain fatty acids (SCFAs) have been shown to downregulate pro-inflammatory cytokines [8] and increase brain-derived neurotrophic factor expression in the frontal cortex [9]. In addition, mice fed pectin as the sole source of fiber show reduced IL-1β and TNFα expression in the brain in response to endotoxin-induced sickness [10]. A cross-sectional study in children found that intake of pectin was positively associated with increased cognitive control on difficult tasks [11], but whether this occurred via the gut-brain axis is unknown.

In the present study, we assessed learning and memory in two cohorts of young male mice after 16 weeks of pectin or cellulose feeding. We hypothesized that mice fed pectin would have enhanced learning and memory capabilities compared to mice fed only cellulose and that these differences would be related to the composition of the gut microbiota.

Two cohorts of young male, 6-wk-old C57BL/6 J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were purchased in two separate groups of 20 males from Jackson Labs West, and all mice came from room RB03 (Stock #0006644). According to information provided by Jackson Laboratory, mice in this colony are pool weaned; that is, all mice born during any given week are pooled into several large weaning cages. This means that the mice in any given shipment are from multiple mothers, and Jackson Labs does not keep track of specific litters of mice. Animals were raised using a 1:1 blend of Aspen wood chips (PJ Murphy) and Aspen shavings (Northeastern Products Corp.) and fed LabDiet 5K52 (4.3% fiber from cellulose, hemicellulose, and lignin) upon weaning. The first cohort was shipped on October 10, 2014 and arrived at our facility on October 15, 2014, while the second cohort shipped December 12, 2014 and arrived December 17, 2014.

Upon arrival at the University of Illinois, all mice were housed in an AAALAC-accredited, specific pathogen-free facility with identical caging and bedding. After a two-week acclimation period, mice were randomly assigned to a semi-purified 5% pectin (n = 10) or 5% cellulose diet (n = 10) (AIN-93 M, Research Diets, New Brunswick, NJ) for 16 weeks (n = 20 for each experimental cohort). The duration of the feeding period was selected based on previous studies to allow ample time for fiber feeding to induce a significant shift in the gut microbiota. The two diets were tightly controlled and identical in macronutrient composition, total energy, and ingredients, with the exception of cellulose and pectin (and an FD&C red indicator dye) (Table 1). Mice were housed individually so that food and water intake could be recorded weekly. Both cohorts were housed in the same room within the animal facility and interacted with the same animal care attendants. No other animals were in the room at the time of experimentation of either cohort. Extreme care was taken to treat cohorts identically. The University of Illinois at Urbana-Champaign IACUC approved all experiments.

The Morris Water Maze was used to assess visuospatial learning and memory. In this test, mice use visual cues surrounding a circular pool to navigate to a hidden platform, with the water acting as an aversive stimulus. The pool (100 cm diameter) was filled with opaque water (24–26 °C), with a round platform (10 cm diameter) hidden 0.5 cm below the surface in the center of one of the four quadrants. The platform remained in this 'target' quadrant during the acquisition phase of the test. Each mouse completed 4 consecutive trials on each of the 5 days of acquisition, beginning at week 15 on the diet. For each trial, mice were placed in the water in one of three quadrants not containing the platform. Animals were allowed to swim freely for 60 s or until the platform was reached. If mice did not locate the platform in 60 s, they were gently guided to the platform and allowed to remain on it for 10 s. They were then removed from the pool and allowed to rest for 1 min between trials.

On day 5, the platform was removed and all mice completed a 60 s probe trial one hour after the completion of acquisition trials to assess short-term memory for the platform location. A second probe trial was performed on day 6, approximately 24 h after the end of acquisition, to assess long-term memory. The starting quadrant on a given trial and day was determined in a random fashion at the beginning of the experiment, but kept the same for all mice across both cohorts. A video camera suspended over the

Table 1
Ingredient and chemical composition of AIN-93M semi-purified diets.

Diet	**Cellulose**	**Pectin**
Macronutrients	**% by weight**	**% by weight**
Protein	14	14
Carbohydrate	73	73
Fat	4	4
Energy	**3.8 (kcal/gm)**	**3.8 (kcal/gm)**
Ingredients	**(grams)**	**(grams)**
Casein	140	140
L-Cystine	1.8	1.8
Corn Starch	495.69	495.69
Maltodextrin 10	125	125
Sucrose	100	100
Cellulose, BW200	**50**	**0**
Pectin	**0**	**50**
Soybean Oil	40	40
t-Butylhydroquinone	0.008	0.008
Mineral Mix S10022M	35	35
Vitamin Mix V10037	10	10
Choline Bitartate	2.5	2.5
FD&C Red Dye #40	0	0.5

center of the pool was used along with a computerized animal tracking system (Noldus Information Technologies, Netherlands) to calculate average swim speed (cm/s), latency to the platform (s), distance from the platform (cm), and total distance swam (cm). The animal handler was the same individual for both cohorts of mice.

Mice were euthanized via rapid CO2 asphyxiation followed by cervical dislocation one week after behavioral testing. Distal colon contents were collected < 2 cm from the rectum, immediately placed on dry ice, and stored at −80 °C. Bacterial DNA was extracted from colonic digesta samples using the PowerLyzer PowerSoil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA). After quality assurance, the DNA library was constructed using a Fluidigm Access Array system in the Functional Genomics Unit of the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana (UIUC). After library construction, 250 bp of the V4 region of the 16S rRNA gene were amplified per Caporaso et al.[12] and sequenced at the W. M. Keck Center for Biotechnology (UIUC) using an Illumina MiSeq2000 with the use of V3 reagents (Illumina Inc.). High-quality (> 25) sequence data (FASTQ) were analyzed with QIIME 1.9.054. After a quality control step of removing barcodes, primers, and short sequences (< 187 bp), sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 bp, OTUs were classified using closed reference picking with the Greengenes database at 97% similarity. β-diversity (weighted and unweighted UniFrac distances) were computed at an even sampling depth of 10,897 sequences per sample based off of alphadiversity rarefaction curves (data not shown).

Quantitative Insights into Microbial Ecology (QIIME) version 1.9.0 was used for alpha and beta-diversity analysis [12]. Analysis of α-diversity (i.e., Chao1 and Shannon index) and taxonomy (i.e., phyla, OTUs) was performed using a one-way ANOVA or nonparametric Kruskal-Wallis test in the case of unequal variances. Variances were assessed through a Brown-Forsythe equality of variances test. Multiple comparisons of taxonomy were corrected by the Benjamini and Hochberg false discovery rate (FDR) correction factor at $\alpha = 0.05$ [13]. Community structure (β-diversity) of the weighted and unweighted UniFrac distance metrics was generated from QIIME. It was then visualized using EMPeror and analyzed by permutational multivariate analysis of variance to understand differences between groups (PERMANOVA) [14,15]. The α (statistical) was set a priori at 0.05 for all tests of significance.

In cohort 1 (C1), there was a significant time-by-fiber interaction effect for average distance to the platform between the 1 h and 24-hour probe trial ($F_{1,18}$: 10.8, p = 0.004; Fig. 1-A1). This interaction did not hold true for cohort 2 (C2) ($F_{1,18}$: 1.24, p = 0.256; Fig. 1-A2). Additionally, pectin-fed mice in C1 spent a higher percentage of time in target quadrant at the 24-hour probe trial of the MWM compared to cellulose-fed mice (t_{18}: 2.97, p = 0.01; Fig. 1-B1), but no effect of pectin was observed for C2 in the 24-hour probe trial (t_{18}:

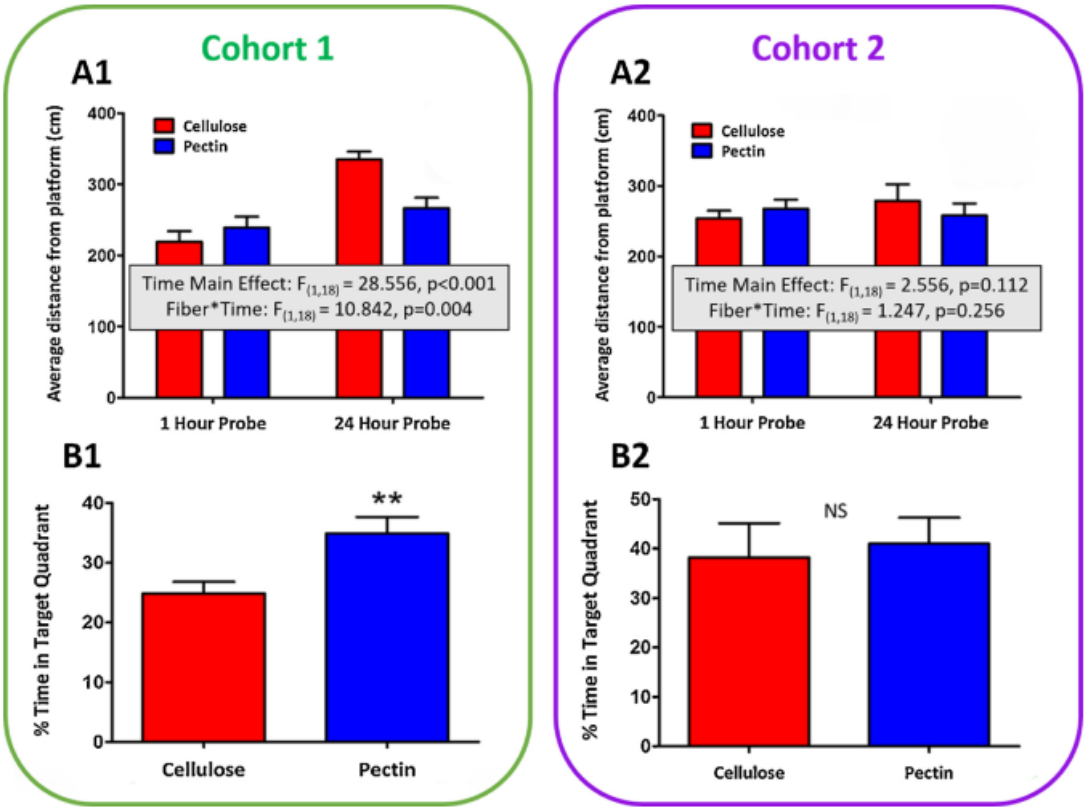


Fig 1. Pectin-fed mice in Cohort 1, but not Cohort 2, show enhanced performance during Morris Water Maze probe trials. (A1 and A2) Average distance from the platform during a probe test 1 h and 24 h after the acquisition phase. (B1 and B2) Percent time spent in the target quadrant at the 24 h probe (first 30 s).

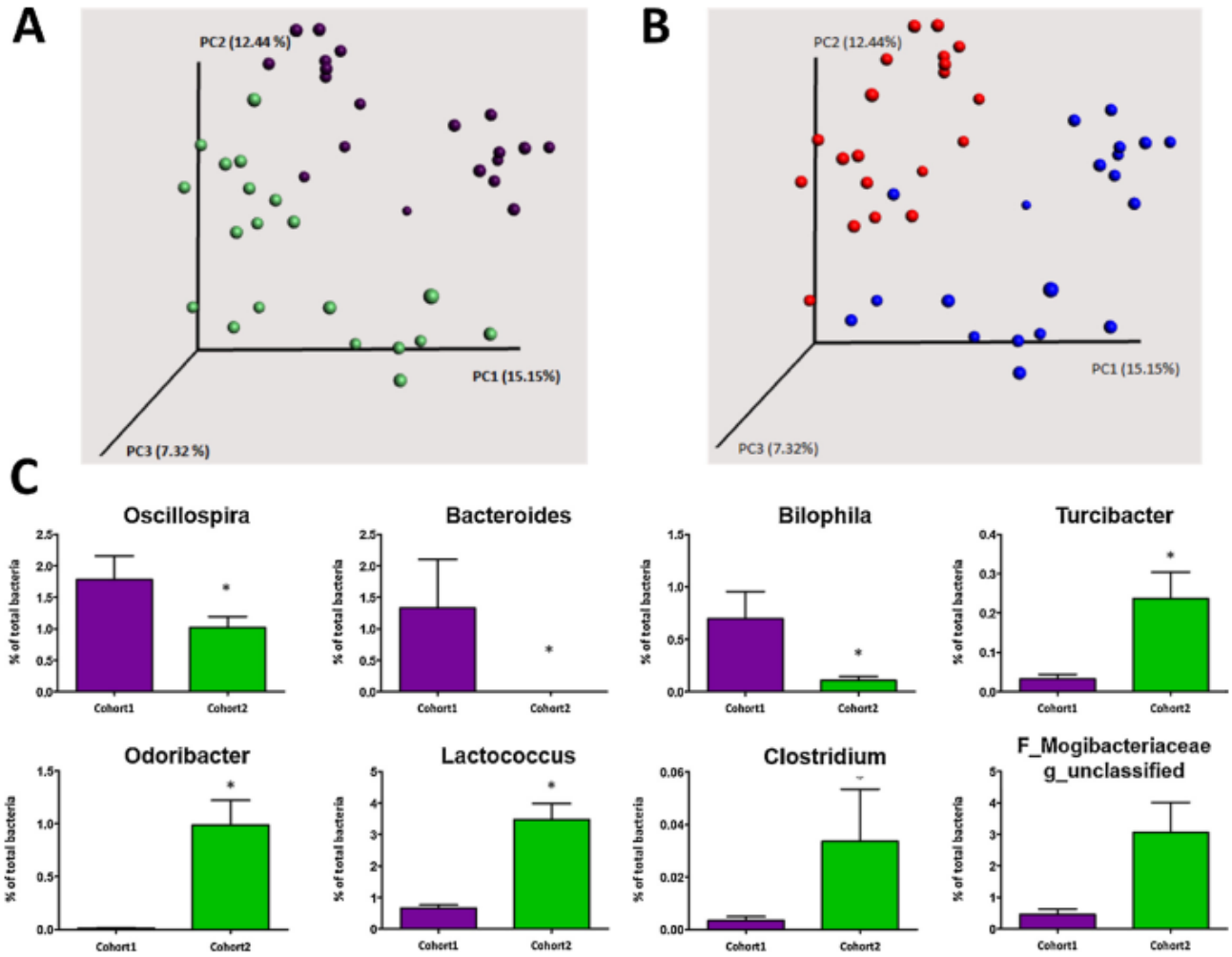


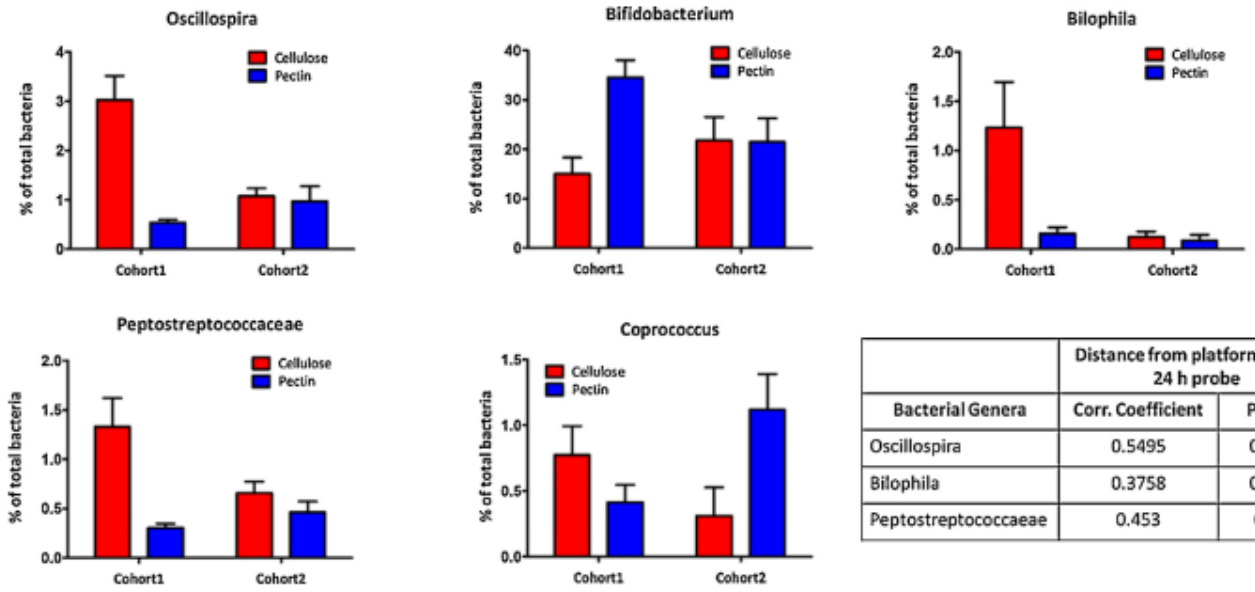
Figure 2. Different cohort and treatment groups had distinct gut microbial compositions. (A/B) A single Principle Coordinates Analysis plot shows significant differences in the microbial composition when visualized by (A) cohort or (B) treatment (Cohort 1 = purple, Cohort 2 = green, Cellulose = red, Pectin = blue). Based on unweighted Unifrac distance metrics of distal colon contents (PERMANOVA $p < 0.01$). Axes represent 'percent data explained' by each coordinate dimension. **(C) Genera of bacteria differentially represented between cohorts, collapsed across treatment groups.** Represented as % of total bacteria. * represents FDR $p < 0.05$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

0.32; p = 0.88; Fig. 1-B2). Thus, despite identical procedures, we observed different behavioral learning outcomes between cohorts.

Following behavioral assessment, mice were sacrificed and contents of the distal colon were collected for bacterial 16S rRNA gene amplification and sequencing. Unweighted UniFrac analysis revealed different bacterial communities between cohorts (PERMANOVA $p < 0.01$) (Fig. 2A), in addition to a significant shift in the community structure of the microbiota by pectin feeding (PERMANOVA $p < 0.01$) (Fig. 2B). Several genera of bacteria differed in abundance between the two cohorts (Fig. 2C). Relative abundance of *Oscillospira*, *Bacteroides*, and *Bilophila* were higher in C1 compared to C2, while relative abundance of *Turcibacter*, *Odoribacter, Lactococcus, Clostridium and Mogibacteriaceae* were higher in C2 compared to C1.

Further analysis revealed that five bacterial genera differed in abundance between pectin-fed mice in the two cohorts (Fig. 3). Pectin fed mice in C1 had reduced abundance of *Oscillospira*, *Bilophila* and *Peptostreptococcaceae* compared to pectin-fed mice in C2. Notably, when collapsed across both groups, all three of these genera were positively associated with distance from the platform area during the 24-hour probe test (Fig. 3, $p < 0.05$). Bifidobacterium and Coprococcus, two genera capable of carbohydrate fermentation, were also differentially abundant in the two pectin-fed groups. Pectin-fed mice in C1 had an increased relative abundance of Bifidobacterium, while pectin-fed mice in C2 had an increased relative abundance of Coprococcus.

In summary, we observed discordant differences in learning and memory after pectin or cellulose feeding in two cohorts of age-matched C57Bl6/J mice. Pectin-fed mice had enhanced long-term learning and memory in C1, but not in C2. These discrepancies in behavioral outcomes may be related to the composition of the gut microbiota, as we also observed differences in gut bacterial composition between the two cohorts across both cellulose and pectin-fed groups. This occurred despite concerted efforts to keep all experimental conditions the same: both cohorts were purchased from the same vendor, fed the same semipurified pectin or cellulose diets, housed in the same room of our specific pathogen-free AAALAC-accredited animal facility,



	Distance from platform during 24 h probe	
Bacterial Genera	Corr. Coefficient	P Value
Oscillospira	0.5495	0.0002
Bilophila	0.3758	0.0169
Peptostreptococcaeae	0.453	0.003

Fig. 3. Five bacterial genera were differentially abundant in pectin-fed mice in C1 and C2. All fiber-by-cohort interactions are significant at FDR $p < 0.05$. Three of these bacterial genera significantly correlated with average distance from the platform during the 24 h probe trial of MWM.

and exposed to the same animal handlers for daily care and a single animal handler for all behavioral testing.

Further analysis of 16-week sequencing data revealed five bacterial genera that were differentially altered between the two cohorts. Three genera of bacteria (*Oscillospira*, *Bilophila*, and *Peptostreptococcaeae*) were higher in relative abundance in C2, and strongly correlated with average distance from platform at the 24-h probe of the MWM. *Oscillospira* is strongly associated with gut permeability and inflammation, while species of the genus *Bilophila* have been found to increase inflammation and accelerate development of colitis [16]. *Peptostreptococcocae* has not been thoroughly studied, but species of this genus tend to be overrepresented in the guts of human colorectal cancer patients. Increased inflammation has been associated with impaired cognition in both mice and humans [17], and pectin has previously been shown to reduce inflammation in the gut and the brain [7,10]. Interestingly, previous studies have found that pectin increases community diversity, but primarily at the level of individual strains and species [18].

While it is possible that the microbial composition of the two cohorts of mice in our study diverged during the feeding period, we think it is more likely that the two cohorts had distinct microbiotas upon arrival to our laboratory, given the age of the mice, our identical treatment of the two cohorts, and the close clustering of individual microbiotas within a given cohort. The composition of the gut microbiota is determined by both host genetic and environmental factors [19–22]. Several studies have sought to determine the source of variability in microbial composition within different strains of genetically inbred mice. One study found that genes, cage, and inter-individual variation contribute approximately 19%, 32%, and 46%, respectively, to the variance in murine gut microbiota composition [20]. Another study found that 26% of this inter-individual variability in the core microbiota could be attributed to cohort [19]. Splitting a litter among different cages at weaning has been shown to cause divergence in microbial profiles after just three weeks [22]. Unfortunately, we did not collect initial or interim fecal samples for 16S rRNA analysis, as this was not our primary outcome for the study. Therefore, we cannot determine the time point at which the microbial communities began to diverge. However, previous studies have shown that preexisting gut microbial communities may modulate an organism's response to dietary fermentable fiber. De Preter et al. found that baseline microbiota activity and initial abundance of *Bifidobacteria* influenced response to a prebiotic intervention in humans [23].

Together, these data provide a possible explanation for the lack of reproducibility in controlled behavioral experiments. Many scientific experiments are found to be difficult or impossible to replicate on subsequent investigation, either by the original researchers themselves or by independent researchers. This is an especially big problem in the field of psychology and behavioral research [24]. A recent large-scale experiment attempted to replicate 100 research findings in psychology and found that only 39 of the published studies could be reproduced [25].

Herein, we presented the distinct behavioral outcomes and microbiotas observed in two cohorts of genetically identical mice received from the same vendor after feeding cellulose or pectin. It is important to note that the standard mouse chow used in most behavioral animal research typically contains cellulose, hemi-cellulose, and lignin for fiber. To our knowledge, there are no studies on the effects of these insoluble fibers on the gut-brain axis. However, the implications of these findings may not be limited to fiber interventions. Differences in the gut microbiota across cohorts may be, at least in part, responsible for the reproducibility crisis in a wide range of behavioral experiments. If this is true, and microbiota differences are present among genetically identical mice from the same vendor at baseline, this might be resolved by housing mice together during an acclimation period or mixing the cage

litter across all groups and cohorts in a study. As mice are coprophagic, the microbiotas of co-housed mice tend to converge over time. Regarding independent research groups attempting to replicate animal experiments of another research group, however, controlling for the influence of the microbiota will be extremely difficult. Further study is needed to determine exactly how much variation exists in the gut microbial composition of genetically identical, commercially available mice, and how this may or may not causally affect the reproducibility and translation of experimental findings in animal experiments. Such studies should use serial sampling techniques to track gut microbial composition over time.

Declarations of interest

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