

DISCOVERY REPORT

Translocation of bacteria from the gut to the brain in mice

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Abstract

Recent advances suggest a correlation between gut dysbiosis and neurological diseases, however, relatively little is known about how gut bacteria impact the brain. Here, we reveal that bacteria can translocate directly from the gut to the brain in small numbers when mice are fed an atherogenic, high-fat diet (Paigen diet) that causes alterations in gut microbiome composition and gut barrier permeability. The bacteria were not found in other systemic sites or the blood, but were detected in the vagus nerve. Right cervical vagotomy reduced bacterial burden in the brain, implicating the vagus nerve as a conduit for bacterial translocation from the gut to the brain. Antibiotic treatment perturbed the composition of the gut microbiome and correspondingly changed the bacteria that localized to the brain in the setting of Paigen diet feeding. To further establish the gut as the origin of bacterial translocation to the brain, we gavaged exogenous *Enterobacter cloacae* into Paigen diet-fed mice, subsequently detecting the *E. cloacae* in the gut and brain. In addition, we monocolonized germ-free mice with *E. cloacae* and only cultured the bacteria from the brains of mice fed Paigen diet, but not those fed standard diet. Localization of bacteria to the brain in Paigen diet-fed mice was reversible with return to normal diet. Bacteria were also detected in the brain of murine models of Alzheimer's, Parkinson's, and autism spectrum disorder fed standard diet. These data reveal a bacterial translocation axis from the gut to the brain, impacted by environmental (diet) and genetic factors, and warrant further investigation to determine if this phenomenon also occurs in humans and to elucidate whether it may play a role in diverse neurological conditions.

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Data availability statement: All data are available in the main text, figures, and the supporting information. All data and materials used in the analysis will be available to any researcher for purposes of reproducing or extending the analysis. The 16S rRNA gene sequencing data have been deposited in NCBI Sequence Read Archive (SRA Accession BioProjects: PRJNA1190946 and PRJNA1219196).

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Abbreviations: ANI, average nucleotide identity; CFU, colony-forming units; CNS, central nervous system; CSF, cerebrospinal fluid; ddPCR, droplet digital PCR; DSS, dextran sulfate sodium; FE, Fisher's exact test; F.I., fluorescence intensity; GF, germ-free; IACUC, Institutional Animal Care and Use Committee; LOD, limit of

Introduction

The gut–brain axis, a bidirectional signaling network between the intestine and the central nervous system (CNS), is crucial to the regulation of host physiology and inflammation [1–4]. Recent evidence has linked the gut–brain axis with neurodegenerative or neurodevelopmental conditions such as Alzheimer's disease, Parkinson's disease, and autism spectrum disorder [1–5]. The gut microbiome has been shown to affect the brain through a variety of pathways, via modulation of the immune system, neuroendocrine system, autonomic nervous system, and secreted metabolites and toxins [1,6–11]. Alterations of gut microbiome composition is sometimes associated with intestinal barrier permeability which may allow the passage of microbes or metabolites out of the intestine and to the intestinal lamina propria and the portal vein [11]. Moreover, several studies have demonstrated the association of a high-fat diet with gut microbiome alterations and increased intestinal permeability [12–14]. However, currently known mechanisms do not explain how the gut microbiome might directly affect the brain to cause pathologies associated with neurological diseases.

Although some studies have reported alterations in the abundance of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroides*, *Ruminococcus*, and *Enterobacteriales* in Alzheimer's disease, there are inconsistencies across studies [15–17]. Such changes in gut microbiome composition have been implicated in the activation of glial cells (i.e., astrocytes and microglia), amyloid beta accumulation, and phosphorylated tau, although there is no direct evidence for these effects [10,18,19]. In Parkinson's disease, changes in gut microbiome composition have been reported to influence intestinal inflammation and gut barrier permeability, as well as the aggregation of α -synuclein in the intestine and CNS [3,20,21]. Moreover, the bacterial family *Prevotellaceae* has been shown to be markedly diminished in patients with Parkinson's disease [22]. However, there is a lack of direct evidence for a causal relationship between the gut microbiome and the disease, and no consistent microbiome-related biomarker has been established for this condition. Further, bacterial metabolites and toxins have been associated with neurodegeneration in Parkinson's disease [23,24], however, the underlying mechanisms are not clear. Similarly, alterations in the many bacterial taxa in the gut microbiome have been implicated in autism spectrum disorder, however, a causal relationship of the gut microbiome as a risk factor for autism spectrum disorder has not been established [25–28]. Taken together, these and other findings do not provide evidence for a direct causal link between the alterations of bacterial composition in the gut microbiome and neurological conditions such as Alzheimer's, Parkinson's, and autism spectrum disorder. Therefore, it is not known if gut bacteria directly impact the brain and pathologies associated with these neurological conditions.

Here, utilizing several murine models of dietary modulation and/or gastrointestinal or neurological disease, we demonstrate that small numbers of bacteria can translocate directly from the gut to the brain via the vagus nerve in conditions of increased intestinal barrier permeability. Translocation of bacteria to the brain induced by atherogenic Paigen diet feeding was abrogated upon return to normal diet, highlighting that the presence of bacteria in the brain can be reversible. Importantly,

detection; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MHB, Mueller Hinton broth; MWU, Mann–Whitney *U* test; PCA, principal component analysis.

bacterial translocation to the brain was also observed in murine models of Alzheimer's, Parkinson's, and autism spectrum disorder, raising the possibility that, if this pathophysiological pathway also occurs in humans, it may contribute to at least some neurological conditions.

Results

Translocation of bacteria from the gut to the brain

We previously studied a functional connection between the gut microbiome and chronic liver disease in the multidrug resistance gene 2 knockout (*Mdr2*^{−/−}) mouse model of cholestatic liver disease [29]. *Mdr2* is a homolog of human biliary transport protein *MDR3*, the absence of which leads to a progressive development of intra-hepatic cholangitis, fibrosis, and cirrhosis in mice, resembling primary sclerosing cholangitis in humans. In *Mdr2*^{−/−} mice, we observed the disruption of bile flow (i.e., cholestasis) and altered composition of gut bacteria in the intestine, including that of *Lactobacillus* [29]. Here, we tested how diet alteration might impact the gut microbiome in this model by feeding *Mdr2*^{−/−} mice an atherogenic, high-fat, Paigen diet. Total bacterial colony-forming units (CFU) were comparable in fecal samples and the ileum of *Mdr2*^{−/−} mice fed regular diet (Control) or Paigen diet (Paigen). However, after 9 days of Paigen diet, *Mdr2*^{−/−} mice exhibited an enrichment of specific bacterial species in the gut such as *Staphylococcus*, *Bacteroides*, and *Akkermansia*, and a reduction of gut commensal lactobacilli in the feces, as compared with mice fed a Control diet (Fig 1A). Similarly, an increased relative abundance of the bacterial families *Staphylococcaceae* (0.5% to 15.4%), *Bacteroidaceae* (0% to 21.8%) and *Akkermansiaceae* (0.16% to 15%), and a decreased abundance of *Lactobacillaceae* (14.6% to 0.8%), were found in fecal samples from mice fed Paigen diet (S1 Fig). The alterations in gut microbiome composition correlated with increased gut barrier permeability in mice fed Paigen diet (Fig 1B). To determine whether the intestinal permeability in these Paigen diet-fed mice might lead to bacterial dissemination out of the intestine, we tested for levels of bacteria in fecal pellets and the ileum, as well as in organs such as the lung, heart, kidney, spleen, brain, and the blood (Figs 1C–1F and S2). While we did not observe bacteria in most systemic organs or the blood (sensitivity <5 CFU, S1 Table), we were surprised to observe small numbers of bacteria in the brains of Paigen diet-fed mice (Fig 1E). Due to the low numbers of bacteria detected in the brains of Paigen diet-fed mice, we used both quantitative (i.e., Mann–Whitney *U* test, MWU) and qualitative (i.e., Fisher's Exact test, FE) analyses to determine statistical significance and a positive association between brains harboring bacteria in samples from the Paigen diet versus Control diet-fed mice (Fig 1E). Interestingly, these bacteria were not present in the meninges or cerebrospinal fluid (CSF), highlighting that this was not meningitis (S2E and S2F Fig). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry identified the bacteria isolated from the brains as *Staphylococcus* species (*Staphylococcus xylosus* and *Staphylococcus sciuri*) and *Enterococcus faecalis*, consistent with the presence of staphylococci and enterococci in the fecal samples and ileum of Paigen diet-fed *Mdr2*^{−/−} mice (Figs 1G–1I and S3). The localization of a low level of bacteria

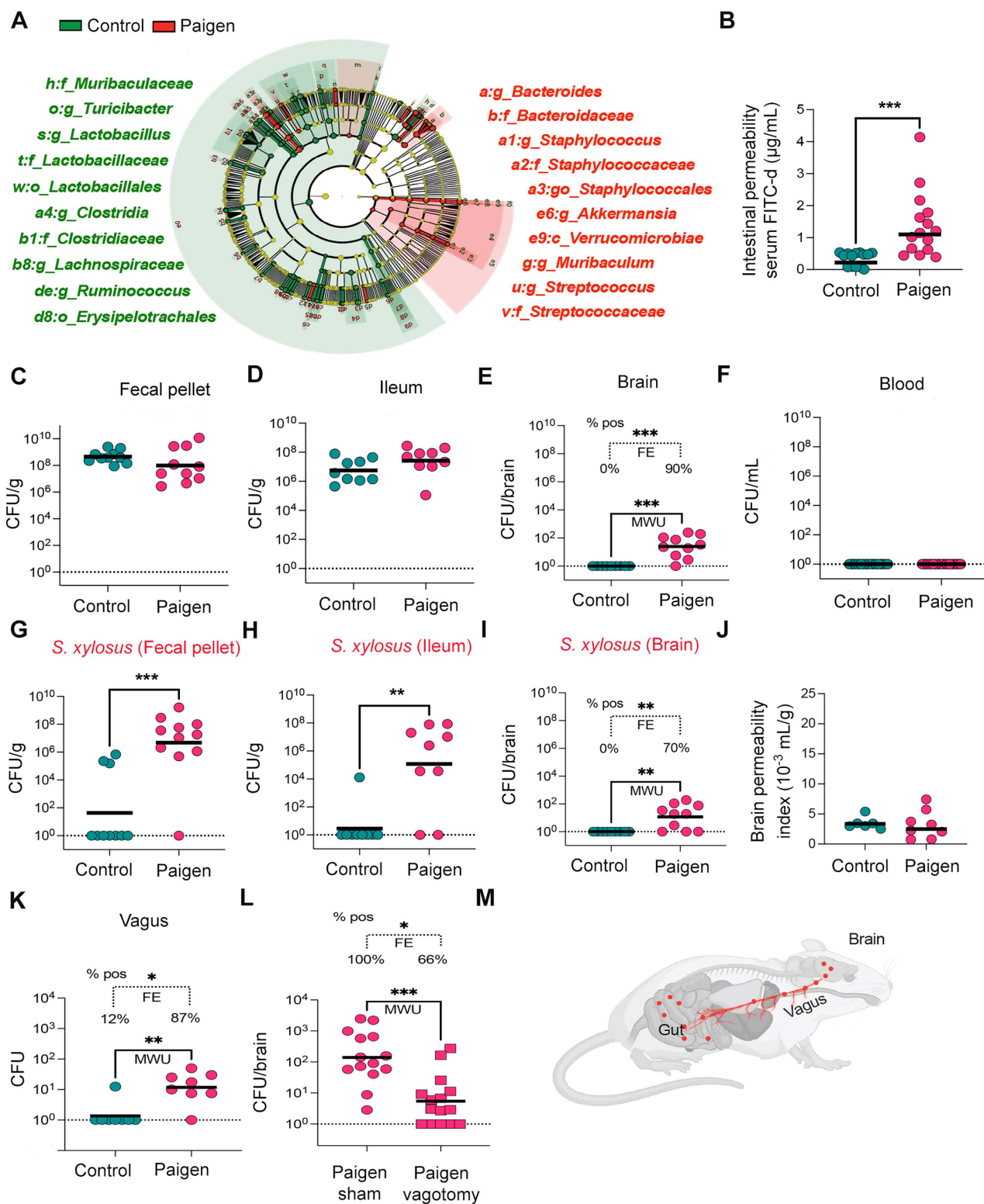


Fig 1. Bacteria translocate from the gut to the brain via vagus nerve. Paigen diet was fed to 12-week-old *Mdr2*^{-/-} mice for 9 days. **(A)** Fecal pellets collected at day 9 were processed for 16S rRNA gene sequencing. Cladogram representation showing the phylogenetic relationship between bacterial families between Control diet (green) and Paigen diet (red) groups. **(B)** Intestinal barrier permeability in Control diet- and Paigen diet-fed mice. **(C–F)**

Total colony-forming units (CFU) of bacteria per gram of (C) fecal pellet and (D) ileum, (E) per brain, and (F) per ml of blood. (G–I) CFU of *Staphylococcus xylosus* per gram of (G) fecal pellet, (H) ileum, and (I) per brain. (J) Brain permeability index of Control diet- and Paigen diet-fed mice. (K) Total CFU of bacteria per ~2 cm of vagus nerve. (L) Total CFU/brain following unilateral right cervical vagotomy of mice. (M) A schematic representation of a bacterial translocation axis to vagus nerve and the brain is demonstrated in Paigen diet-fed mice (created with Biorender.com). Data presented from minimum 2 independent experiments ($n=3-5$ /group). Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated in (E) and (L). Statistical significance was determined by Mann–Whitney U (MWU) test (solid lines) and Fisher Exact (FE) test (dashed lines). * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Line reflects the geometric mean. The raw data for this Figure can be found in [S1](#), [S2](#), and [S3 Data](#) files.

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in the brains of Paigen diet-fed mice was not due to increased blood–brain barrier permeability, which was not observed in these mice or mice fed the Control diet ([Fig 1J](#)). To prevent contamination during tissue processing, we used extremely careful steps in preparation and during sample collection (summarized in [Box 1](#) and [S4 Fig](#)).

Box 1. Steps to prevent contamination during tissue processing

Preparation for tissue collection

- Surface sterilization of the biosafety cabinet using 70% ethanol and UV irradiation
- Sterilization of tools by autoclaving before each harvest

Sample collection

- Donned specific BSL2 lab PPE
- All sample collection performed in Class II biosafety cabinets
- Sterilization of fur/skull with 70% ethanol
- Collection of swabs from each surface during the brain isolation process to check for bacterial contamination (e.g., skin after ethanol spray, skull before and after ethanol spray, and the surface of the brain)
- Dedicated sterilized sets of surgical tools for each organ
- Collection of samples from control mice first, followed by collection from Paigen diet-fed or knockout mice
- Collection of the brain before any other organs from a given mouse
- Storage of all samples in sterile PBS in sterile screw cap tubes
- Confirmation of sterility of the biosafety cabinet surface/environment via use of an open blood agar plate before, during, and after each harvest, which was confirmed to have no bacterial growth after subsequent incubation
- Minimization of the number of mice per harvest to avoid cross-contamination
- Use of filter tips to process samples to avoid cross-contamination

As the vagus nerve connects the gut and the brain, we tested the vagus nerve itself for the presence of bacteria. We isolated small numbers of bacteria, including *S. xylosus*, from the cervical branches of the vagus nerve, but not the spinal cord ([Figs 1K](#) and [S2G–S2I](#)), indicating that the localization of these bacteria to the vagus nerve was specific. Similar to brain samples, both quantitative and qualitative analyses were used to determine statistical significance and a positive association between the vagus samples harboring bacteria from the Paigen diet- versus Control diet-fed mice ([Fig 1K](#)). Low numbers of *S. xylosus* (though not reaching statistical significance) were also detected in the nodose ganglia, the

inferior ganglia of the vagus nerve, in Paigen diet-fed mice (S2H Fig). To test whether the vagus nerve could serve as a route of bacterial translocation to the brain, we next disrupted the right cervical vagus nerve by vagotomy (visual confirmation of vagotomy provided in S5 Fig) [30,31]. Vagotomized and sham-operated mice were subsequently fed Paigen diet. We observed that vagotomized mice fed Paigen diet harbored ~20-fold lower levels of bacteria in the brain than sham controls (further, 5 out of 15 vagotomized mice had no detectable bacteria in the brain) (Fig 1L). Importantly, vagotomy did not alter intestinal barrier permeability or blood–brain barrier permeability, nor the overall bacterial load in the feces or ileum (S5C–S5F Fig). Moreover, gut microbiome composition in vagotomized mice fed Paigen diet closely matched that of sham-operated controls, as demonstrated by principal component analysis (PCA) of 16S rRNA gene sequencing (S5G Fig). Since vagotomy was performed only on the right cervical vagus nerve (bilateral cervical vagotomy would be fatal), the left cervical vagus nerve remained intact and therefore could have served as a route by which bacteria were still able to translocate to the brain (S5 Fig). Indeed, bacteria were cultured from both the right and left cervical vagus nerves in *Mdr2*^{-/-} mice fed Paigen diet (S5A Fig). While we cannot rule out the contribution of other routes, these data indicate that bacterial translocation from the gut to the brain can occur via the vagus nerve in the absence of any observable blood–brain barrier permeability (Fig 1J and 1M).

To further investigate whether bacterial translocation to the brain might occur from the gut, we tested whether bacteria in the brain closely matched those isolated from the gut by whole genome sequencing of bacterial isolates from fecal pellets, the ileum, and the brain of Paigen diet-fed *Mdr2*^{-/-} mice. The results showed that *S. xylosus* isolated from the fecal samples, ileum, and the brain of the same mouse exhibited 99.9982% average nucleotide identity (ANI) (S2 Table). Similarly, *S. sciuri* isolates from these samples exhibited 99.9977% ANI and *E. faecalis* isolates exhibited 99.9989% ANI. These data further demonstrate that the bacteria isolated from the brain match those from the gut. Taken together, these results indicate that dietary modulation can cause alterations to gut microbiome composition and gut barrier permeability, leading to translocation of staphylococci and enterococci to the brain in *Mdr2*^{-/-} mice.

Intestinal microbiome composition dictates which bacteria translocate to the brain

To further explore the link between intestinal bacteria and those that translocate to the brain, we perturbed the microbiome in *Mdr2*^{-/-} mice fed Paigen diet by administration of a cocktail of antibiotics (vancomycin, 500 mg/L; ampicillin 1 g/L; neomycin, 1 g/L; and metronidazole, 1 g/L) in the drinking water. While *Staphylococcus* species were highly enriched in the ileum and fecal pellets of Paigen diet-fed mice, the addition of antibiotic treatment (Abx) instead led to the enrichment of *Paenibacillus cineris* (*P. cineris*) (S6 Fig). Correspondingly, Paigen diet-fed, antibiotic-treated mice rarely harbored *Staphylococcus* sp. in the brain, but rather, *P. cineris* was the predominant bacterium detected in the brains of these mice (4 out of 10 were positive for bacteria). Although *P. cineris* was significantly enriched in the fecal samples and ileum of antibiotic-treated and Paigen diet-fed *Mdr2*^{-/-} mice, due to the modest number of mice with *P. cineris* in the brain (40%), total CFU counts in the brain were not statistically significantly different between mice fed Paigen diet or Control diet. *P. cineris* is an environmental bacterium which has been reported in human sputum [32,33]. It is surprising yet reasonable that we detected it after antibiotic treatment, which significantly altered the gut microbiome. Similar to the previous data with *S. xylosus* in mice fed Paigen diet in the absence of antibiotics, we could not detect *P. cineris* in the blood, other systemic organs, or the spinal cord of mice fed Paigen diet and treated with antibiotics (S6 Fig). In addition, antibiotic treatment did not further exacerbate the intestinal permeability caused by Paigen diet or increase blood–brain barrier permeability (3.5 ± 1.0 versus 3.0 ± 1.4). These data further implicate the gut as the source of brain-localizing bacteria, demonstrating that a perturbation that changes the composition of the microbiome (i.e., diet and antibiotic use) correspondingly changes the bacteria that localize to the brain in the setting of intestinal barrier permeability.

To determine whether bacterial translocation to the brain occurs in mouse backgrounds other than *Mdr2*^{-/-}, we fed wild-type C57BL/6 (B6) mice Paigen diet for 2 weeks. Although total CFUs were moderately decreased in fecal pellets and unchanged in the ileum, Paigen diet led to enrichment of *S. xylosus* and *E. faecalis* in the feces and ileum (Figs 2A, 2B,

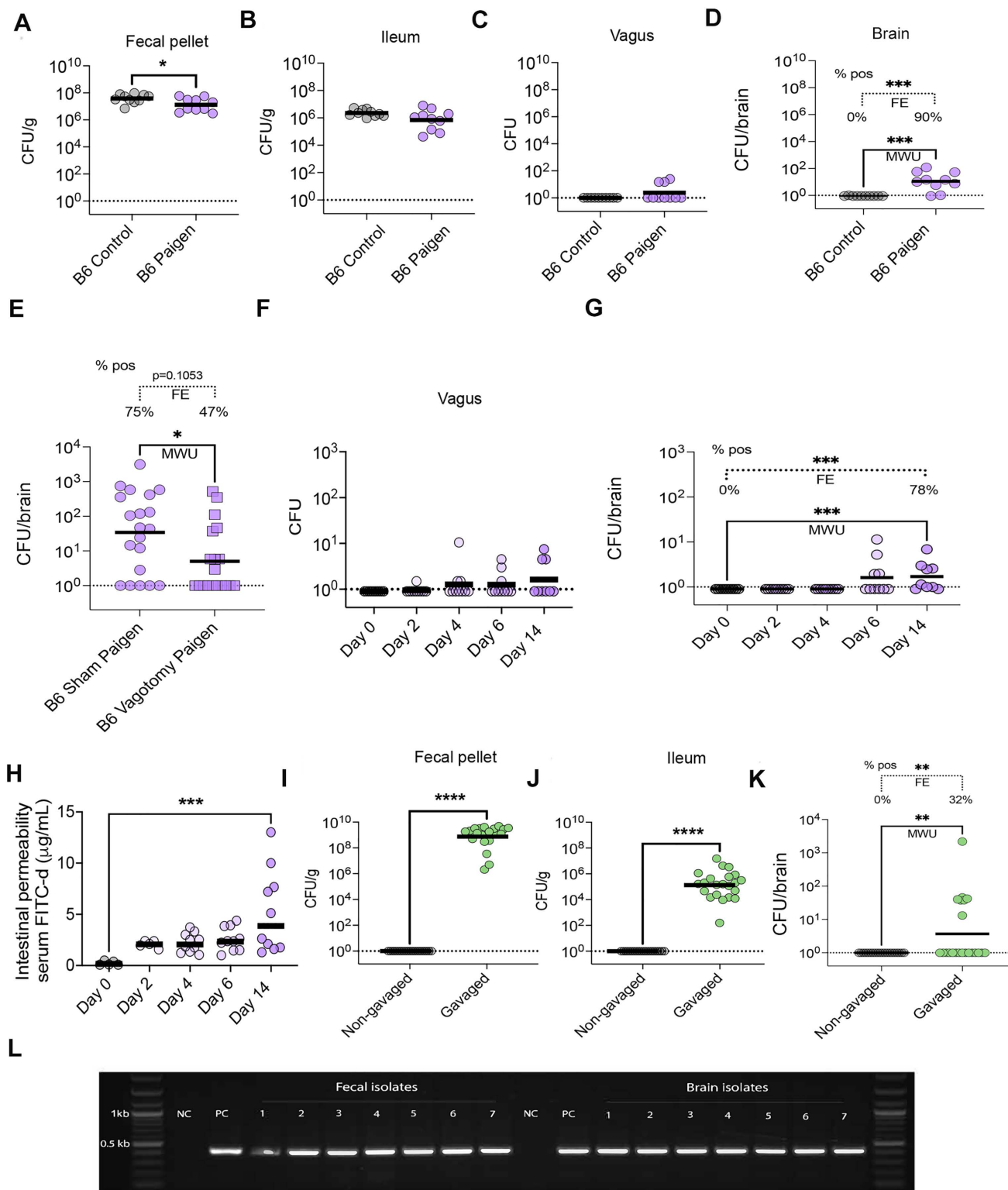


Fig 2. Gut microbiome composition dictates which bacteria translocate to the brain. (A–D) Total CFU of bacteria (A) per gram of fecal pellet, (B) per gram of ileum, (C) per cervical vagus nerve (~2 cm), and (D) per brain of C57BL/6 (B6) mice fed Paigen diet. (E) Total CFU/brain following unilateral right cervical vagotomy of B6 mice fed Paigen diet. (F–H) Kinetics of CFU of bacteria (F) per cervical vagus nerve (~2 cm), (G) per brain, and (H) intestinal

permeability in Paigen diet-fed B6 mice. (I–K) CFU of *E. cloacae* per gram of (I) fecal pellet, (J) ileum, and (K) per brain. (L) Agarose gel image showing bar-coded nucleotide in *E. cloacae* from the fecal and brain isolates after PCR amplification. WT *E. cloacae* were used as negative control (NC), and while *E. cloacae* (JC96) were used as positive control (PC). Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated (D, E, G, K). Statistical significance was determined by Mann–Whitney *U* test (MWU; solid lines) and Fisher Exact (FE; dashed lines) test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Line reflects the geometric mean. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

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and [S7](#)). Similar to *Mdr2*^{-/-} mice, we were unable to detect bacteria in the blood, or blood–brain barrier permeability, in B6 mice fed Paigen diet. Antimicrobials such as CRAMP (the murine ortholog of human LL-37) and lysozyme can be induced in the blood when bacteria are in the blood, but we did not observe any such increases in B6 mice fed Paigen diet ([S8A](#) and [S8B Fig](#)). Although we did not observe bacteria in the blood or most systemic organs, we did detect both *S. xyloso* (statistically significant) and *E. faecalis* (statistically non-significant due to the low percentage of mice with this bacterium in the brain) in the brain of Paigen diet-fed B6 mice ([Figs 2C, 2D, and S7](#)). Taken together (summarized in [Box 2](#)), these data further support our conclusion that bacteria are not present in the blood in mice which do have bacteria in the brain, in the models examined here. Genomic sequencing revealed 99.9989% ANI between *S. xyloso* isolates, and 99.9979% ANI between *E. faecalis* isolates, present in the feces or ileum and those that translocated to the brain of Paigen diet-fed B6 mice ([S2 Table](#)). To test whether the vagus nerve is a route of bacterial translocation to the brain of Paigen diet-fed B6 mice, similar to *Mdr2*^{-/-} mice, we performed right cervical vagotomy. 47% of vagotomized and Paigen diet-fed B6 mice (9 out of 19) harbored bacteria in the brain compared to 75% of sham surgical controls (15 out of 20), and the vagotomized mice that did have bacteria in the brain had on average ~3–4-fold lower levels compared to sham controls ([Fig 2E](#)). These data demonstrate that bacterial translocation to the brain via the vagus nerve is not restricted to *Mdr2*^{-/-} mice fed Paigen diet, but also occurs in wild-type mice of another genetic background (B6) when fed Paigen diet.

Box 2. Lack of detection or evidence of bacteria in the blood

- No detection of bacterial CFUs in the blood of mice harboring bacteria in the brain, despite the high sensitivity of the culturing assay which can detect 1–2 bacterial cells ([S1 Table](#))
- No difference in bacterial load in brain samples collected from mice with or without cardiac perfusion ([S9 Fig](#))
- No increases in blood–brain barrier permeability in mice harboring bacteria in the brain ([Fig 1J](#))
- No detection of bacterial CFUs in the meninges or the CSF ([S2 Fig](#))
- No detection of bacterial CFUs in systemic organs other than the brain ([S2 Fig](#))
- No increase in the levels of the antimicrobials CRAMP or lysozyme (which can be induced when bacteria are present in the blood) in the blood of B6 mice fed Paigen diet, which harbor bacteria in the brain ([S8 Fig](#))

We next investigated the kinetics of bacterial translocation from the gut to the brain during Paigen diet feeding. Interestingly, in B6 mice fed Paigen diet, we detected bacteria in the vagus nerve of one mouse on day 2 (out of 10) and 4 mice on day 4 (out of 10; although not statistically significant), but none in the brains of those mice at the respective timepoints ([Fig 2F and 2G](#)). At day 6, we continued to detect bacteria in the vagus nerve and only then, first detected bacteria in the brain. An increase in gut barrier permeability was observed at days 2, 4, and 6 during Paigen diet feeding ([Fig 2H](#)). The levels of bacteria cultured from the vagus nerve and brain on day 6 were similar to those on day 14 and even on day 56 ([S10 Fig](#)). The bacteria were not detected in the blood, heart, kidney, spleen, lungs, CSF, meninges, or spinal cord on days 4 or 6 in B6 mice fed Paigen diet ([S11 Fig](#)). Although the results were not statistically significant, these temporal data

provide further anecdotal support for the vagus nerve being a conduit for bacterial translocation to the brain since bacteria were only detected in the brain after their prior detection in the vagus nerve.

Importantly, no changes in whole gut transit time or fecal moisture content, parameters of gut function, were observed in B6 mice fed Paigen diet (S12A and S12B Fig). However, consistent with the increased gut barrier permeability observed, histopathological analysis revealed the disruption of the ileal mucosal lining in B6 mice fed Paigen diet (S12C Fig). The disruption of the mucosal lining correlated with a $\sim 2\times$ reduction in the number of goblet cells (which produce mucus) per crypt/villus in B6 mice fed Paigen diet compared to those fed a Control diet (S12D and S12E Fig). These data provide a rationale for why gut barrier permeability is increased in mice fed Paigen diet, since these mice exhibit disruptions to the mucus layer which normally contributes to blocking bacterial translocation out of the intestine.

To elucidate whether we could exogenously alter which bacteria reach the brain, we first disrupted gut bacterial composition by treating B6 mice with a cocktail of antibiotics. We subsequently gavaged *Enterobacter cloacae* (strain JC96 with an engineered, non-natural barcoded nucleotide sequence) which is not observed in the gut microbiome of the B6 mice, into the antibiotic-treated mice and fed them Paigen diet. *E. cloacae* were detected in fecal samples and the ileum on day 5 after gavage (Fig 2I and 2J). On day 8 after gavage, *E. cloacae* were cultured from the brains of 40% of gavaged and Paigen diet-fed B6 mice (Fig 2K). To further establish the gut as the origin of bacterial translocation to the brain, we confirmed the presence of the specific JC96 DNA barcode in the *E. cloacae* isolates from both the fecal pellets and brain samples (Fig 2L).

We subsequently monocolonized germ-free (GF) B6 mice by gavaging *E. cloacae* JC96 with or without Paigen diet feeding. Increased gut barrier permeability was only observed in monocolonized GF mice that were also fed Paigen diet, but not in monocolonized GF mice fed Control diet (Fig 3A). Correspondingly, *E. cloacae* was cultured in the fecal pellets and ileum of monocolonized GF mice fed either Paigen diet or Control diet (Fig 3B and 3C), however, it was only cultured from the vagus nerve and brain of monocolonized GF mice also fed Paigen diet (although statistically insignificant in the vagus due to the low proportion, 25%, of mice with bacteria in this nerve) (Fig 3D and 3E). These data rule out contamination of brain samples with intestinal bacteria during sample collection, since monocolonized GF mice fed Control diet harbored high levels of *E. cloacae* in the gut, yet had no detectable bacteria in the brain. Together, these data highlight that intestinal colonization alone is not sufficient to cause bacterial localization in the brain, but it must be accompanied by gut barrier permeability for bacteria to translocate to the brain. The data further demonstrate that the bacteria isolated from the brain are also present in the gut, consistent with the gut being the source of the bacteria that translocate to the brain.

Detection of bacterial DNA in the brain by PCR

We next attempted to corroborate our bacterial culture data with molecular detection of bacterial DNA in the brain, following the most recent Consensus Statement on guidelines for low-biomass microbiome studies to ensure quality, robustness, and reproducibility (multiple primer sets, different kits for isolation of DNA, and many other controls summarized in Box 3) [34]. We first colonized the gut of B6 mice with *Enterobacter* strain JC96 and fed them Paigen diet. Brains were harvested and microbial DNA was isolated using two independent kits (HostZERO Microbial DNA Kit, Zymo, or QIAamp DNA Microbiome Kit, Qiagen). PCRs specific to the DNA barcode in *Enterobacter* JC96 were performed using distinct primer sets (JCP51 with JCP53, or JCP429 with JCP430). While the presence of bacteria in brain samples was confirmed by our culturing method, we were unable to amplify any DNA products by direct PCR assay of the brain samples (S3 Table, S13A Fig). We therefore determined the limit of detection (LOD) for the PCR methodology. On pure cultures of JC96, the LOD was determined to be $\sim 10^3$ bacterial cells. However, when brain tissue homogenate was spiked with an indicated known number of bacterial CFU, the LOD for the PCR assay was determined to be roughly one log higher, at $\sim 10^4$ bacterial CFU (S4 Table, S13B Fig). Since we only observe low numbers of bacteria in the brain in the models studied here, and this never reaches 10^4 CFU, we therefore reasoned that it may be possible to facilitate detection of the bacteria by standard PCR, if the bacteria in the brain homogenates were first cultured and expanded in Mueller Hinton broth (MHB) for a minimum of 3 hours at 37 °C. When this approach was implemented, the sensitivity of the LOD was

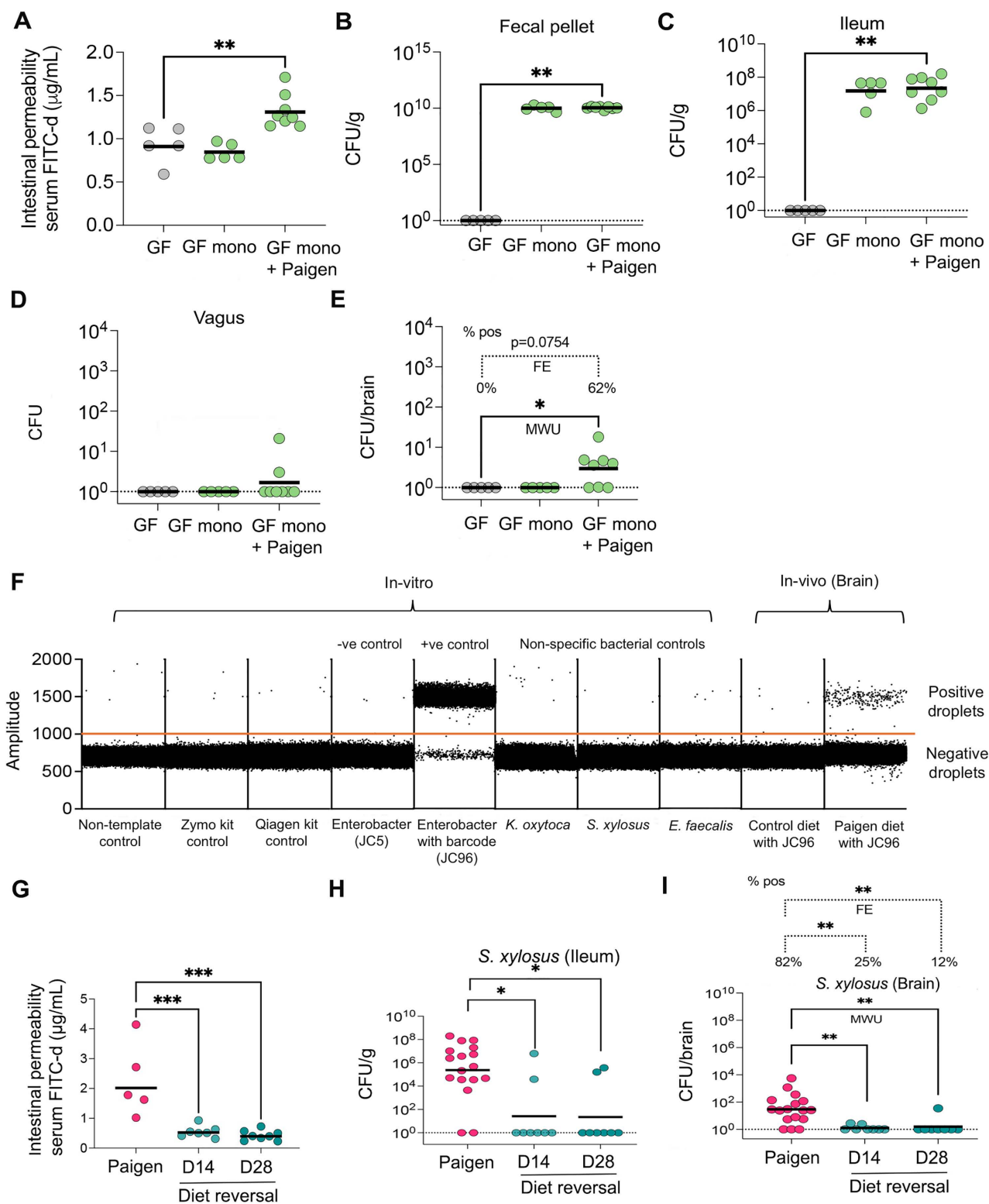


Fig 3. Translocation of monocolonized bacteria to the brain in germ-free mice, detection of bacterial DNA by ddPCR, and diet reversal. (A–E) Germ-free (GF) mice were monocolonized (mono) by gavaging *E. cloacae* with or without Paigen diet feeding, and (A) intestinal permeability and (B–E) bacterial loads (CFU) were determined (B) per gram of fecal pellet, (C) per gram of ileum, (D) per cervical vagus nerve (~2cm) and (E) per brain. (F) *Mdr2*^{-/-} mice were

monocolonized with *E. cloacae* (JC96 strain) and fed Paigen diet. Brains were harvested, and microbial DNA from the brain was isolated using HostZERO Zymo and QIAamp Microbiome Kit. Using primer probe pairs designed for the JC96 barcode, a PCR mix was made using ddPCR supermix for probes (No dUTP) with forward and reverse primers, EcoRI, and DNA template to directly quantitate bacterial DNA. Non-template control (NTC), Zymo kit and Qiagen kit controls, and DNA isolated from *Klebsiella oxytoca* (*K. oxytoca*), *S. xyloso*, *E. faecalis* were used as non-specific controls. DNA isolated from parenteral *Enterobacter* with no barcode (JC5) was used as negative control, while DNA isolated from *Enterobacter* with barcode (JC96) was used as a positive control. For control and experimental groups, DNA isolated from JC96 monocolonized mice fed Control diet and Paigen diet were used. After the PCR, the droplets were read in a QX200 Droplet Digital System by the direct quantification method. The figure was generated using the QuantaSoft Analysis Pro Software. A detailed description of the PCR reaction and primer probes provided in the [Materials and methods](#) section. (G–I) Paigen diet was fed to 12-week-old *Mdr2*^{-/-} mice for 9 days (“Paigen” group), before reversal to conventional diet for 14 (“D14”) or 28 (“D28”) days. (G) Intestinal permeability in Paigen diet-fed mice and diet reversal mice on D14 and D28. (H, I) CFU of *S. xyloso* (H) per gram of ileum and (I) per brain. Data presented from minimum 2 independent experiments ($n=2-3$ /group). Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated in (E) and (I). Statistical significance was determined by Mann–Whitney *U* test (MWU; solid lines) and Fisher Exact (FE; dashed lines) test or one-way analysis of variance, where applicable. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Line reflects the geometric mean. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

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improved by ~100-fold, facilitating the detection of just 10 initial bacteria (prior to culture) by conventional PCR on brain homogenates from Paigen diet-fed mice after culturing and expansion in MHB. Detection of bacteria in the brain by PCR after MHB enrichment from *Mdr2*^{-/-} and B6 mice colonized with JC96 and fed Paigen diet is shown ([S3 Table](#), [S13C](#) and [S13D Fig](#)). Moreover, we used a second set of primers for the detection of *E. cloacae* strain JC96 in the brain of B6 mice using MHB enrichment by standard PCR ([S14 Fig](#)).

Box 3. Detection of low bacterial biomass in the brain by PCR

Steps to prevent contamination

- Aseptic isolation of bacterial DNA donning BSL2 PPE
- Decontamination of work benches, pipettes, plastic wares, instruments, and surfaces using 70% ethanol, followed by 10% bleach
- Usage of filter tips to prevent cross-contamination
- Isolation of microbial DNA from the brains using two different kits (HostZERO Microbial DNA Kit from Zymo and QIAamp DNA Microbiome Kit from Qiagen) to rule out any contamination from the kits
- Amplification of the JC96 barcode using two different primer sets, amplifying two different regions of the genome, to rule out non-specific amplification and/or contamination from PCR reagents

Negative controls

- Inclusion of non-gavaged conventional mice as negative controls for each experiment
- Processing of a control sample of PBS devoid of any DNA during all steps of DNA isolation from each kit
- Use of JC5 (parental strain of JC96, without the DNA barcode) in all experiments to demonstrate the specificity of the PCR reactions for the barcoded DNA
- Running a non-template control in all the PCR reactions using nuclease-free water
- PCR on three different kinds of bacteria (*Klebsiella oxytoca*, *S. xyloso*, and *E. faecalis*) as non-specific controls for the JC96 PCR reactions
- Use of the spleen, devoid of bacterial colonization, as a negative control organ from which to perform microbial DNA isolation
- Sanger sequencing of gel-purified PCR products after barcode amplification to confirm the specificity of the PCR primers

Consistent with the qPCR data which indicating difficulty in detecting low numbers of bacteria, we have not thus far been able to detect bacteria in the brain or vagus nerve by imaging. Therefore, to attempt to detect bacteria in the brain without the need for in vitro expansion, we turned to droplet digital PCR (ddPCR), one of the most sensitive PCR technologies available [35–37]. We employed this approach on brains from *Mdr2*^{-/-} mice (because we observed the highest level of bacterial load in the brain in these mice) gavaged with *Enterobacter* strain JC96 following Abx-treatment and fed Paigen diet. Microbial DNA from the brain was isolated using two different DNA isolation kits. As shown in Fig 3F, ddPCR specifically detected the DNA of barcoded *Enterobacter* JC96. Detection of this barcode in the brain indicated that the detected DNA came specifically from the bacterial strain with which mice were colonized and could not have come from other naturally occurring bacteria in the mice. Importantly, the primers that detected the DNA barcode specific to JC96 did not amplify a PCR product from DNA of the parental JC5 strain which is devoid of the barcode, and similarly did not amplify a product from unrelated negative control strains of *Klebsiella oxytoca*, *Staphylococcus xylosum*, or *Enterococcus faecalis* (Fig 3F). In addition, brains from *Mdr2*^{-/-} mice colonized with JC96 but fed a regular diet, which did not lead to bacterial translocation to the brain, were negative by ddPCR (Fig 3F). These data demonstrate the presence of bacterial DNA in the brain of mice which harbor bacteria as assayed by culturing, corroborating the culture data with molecular detection.

Restoration of conventional diet reverses bacterial localization in the brain

Because mice fed a Paigen diet developed increased intestinal barrier permeability and bacteria translocated to their brains, we tested whether these phenotypes could be reversed upon restoration of a conventional diet. We first fed Paigen diet to *Mdr2*^{-/-} mice for 9 days (“Paigen” group), before subsequently reversing their diet back to the Control diet, and then testing their phenotypes 14 days (D14) and 28 days (D28) later. Restoration to a conventional diet decreased intestinal barrier permeability by 4-fold on D14 and D28 (Fig 3G). In addition, gut microbiome composition on D28 was reversed and closely matched that of Control diet-fed animals (S15 Fig). With only two exceptions, levels of *S. xylosum* were below the detectable limit in both the ileum and brain on D14 (Fig 3H and 3I). Similar to these results from *Mdr2*^{-/-} mice indicating that bacterial localization to the brain can be a reversible process, when B6 mice were fed Paigen diet for two weeks (“Paigen” group) and the diet was subsequently reversed to the Control diet, there was a significant decrease in intestinal barrier permeability on day 7 (D7) and the bacterial loads in the brain on day 21 (D21) and day 28 (D28) (S16A and S16B Fig). These data demonstrate that the localization of bacteria in the brain can be transient as diet reversal led to a reduction of detectable bacteria in the brain.

Bacterial localization in the brain of mouse models of neurologic conditions

Recent evidence implicates the disruption of the gut microbiome and an increase in gut barrier permeability as being associated with neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and neurodevelopmental conditions such as autism spectrum disorder [1–4,38–40]. We employed mouse models for each condition (Alzheimer’s—APP/PS1 WSB.Cg-Tg (APPswe,PSEN1dE9) 85Dbo/How; Parkinson’s—LRRK2 knock-in (B6.Cg-Lrrk2^{tm1.1Hlme}/J; and autism spectrum disorder—BTBR T+ Itpr3^{tf}/J BTBR) to determine if changes in gut microbiome composition and gut barrier permeability might be linked to bacterial translocation to the brain in these mice. APP/PS1 mice exhibited enriched *Ligilactobacillus*, *Streptococcus*, *Clostridium*, *Lachnospiraceae*, *Roseburia*, and *Tyzzereella* in comparison to wild-type B6 mice (Figs 4A and S17), while LRRK2 mice exhibited marked enrichment of *Lactobacillus*, *Eubacterium*, *Staphylococcus*, *Enterorhabdus*, *Coriobacteriia*, and *Flavonifractor* (Figs 4A and S18). BTBR mice showed a broader enrichment of *Firmicutes*, *Lachnospiraceae*, *Corynebacterium*, *Anaeroplasma*, *Monoglobus*, *Fusimonas*, *Murimonas*, *Tyzzereella*, *Erysipelotrichaceae*, *Enterobacter*, and *Robinsoniella* in comparison to B6 mice (Figs 4A and S19). We observed gut barrier permeability to be a common phenotype in these mouse models of Alzheimer’s, Parkinson’s, and autism spectrum disorder (Fig 4B).

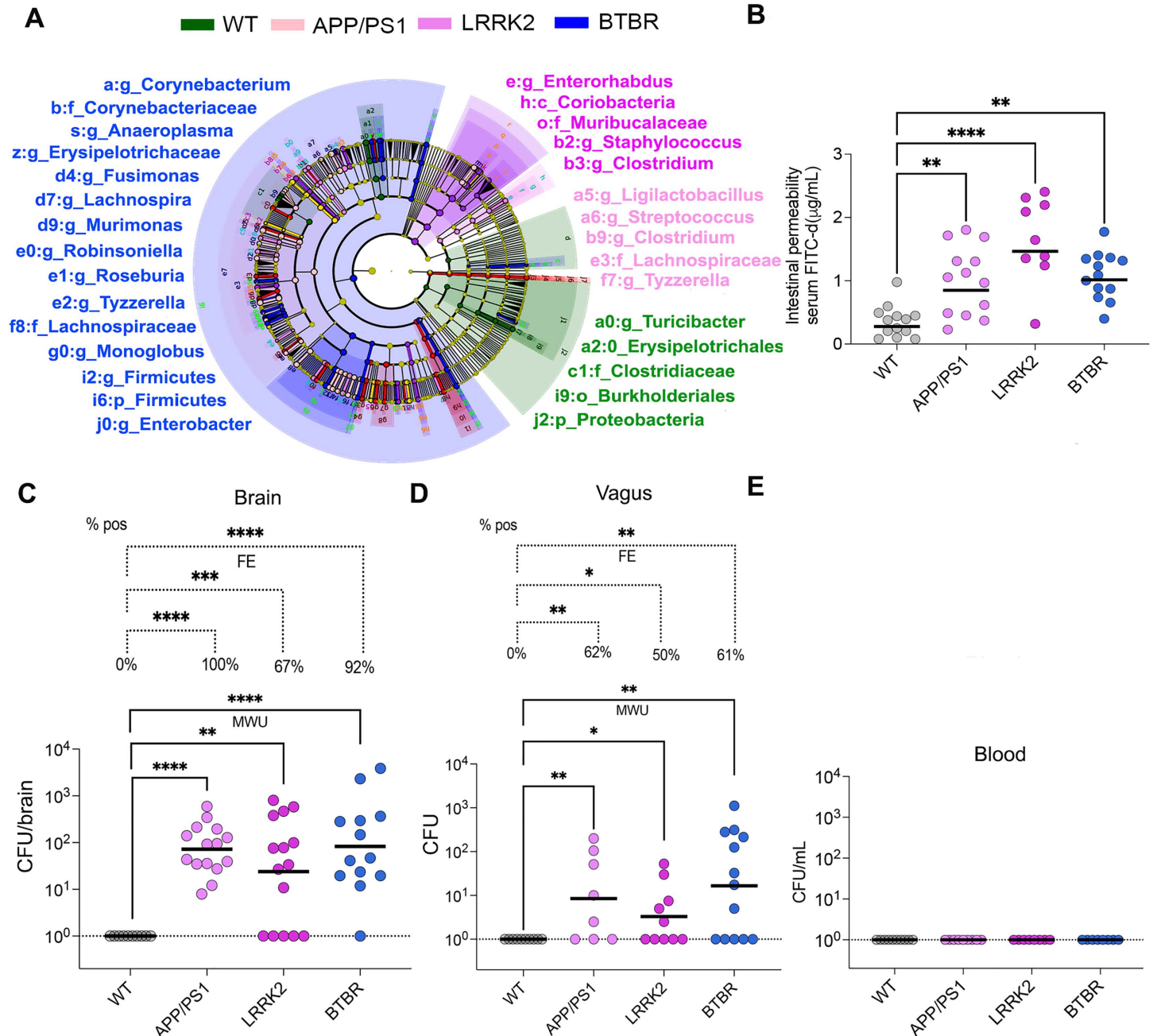


Fig 4. Bacterial translocation to the brains of mouse models of neurologic conditions. (A) Fecal pellets collected from WT, APP/PS1, LRRK2, and BTBR mice from 8- to 15-week-old mice were processed for 16S rRNA gene sequencing. Cladogram representation showing the phylogenetic relationship between bacterial families among WT, APP/PS1, LRRK2, and BTBR mice. (B) Intestinal permeability in WT, APP/PS1, LRRK2, and BTBR mice. (C–E) Total CFU of bacteria (C) per brain, (D) in the vagus nerve (~2 cm), and (E) per mL of blood. Data presented from minimum 2 independent experiments ($n=2-3$ /group). Percentage of mice in each group positive for bacterial CFU in the brain or vagus nerve (% pos) is indicated (C, D). Statistical significance was determined by Mann–Whitney U test (MWU; solid lines) and Fisher Exact test (FE; dashed lines) or one-way analysis of variance, where applicable. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Line reflects the geometric mean. The raw data for this Figure can be found in [S1](#), [S2](#), and [S3 Data](#) files.

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Without any dietary intervention (Control diet), small numbers of bacteria were detected in the brain as well as the vagus nerve in these murine models (Fig 4C and 4D). Importantly, bacteria that were isolated from the brain and the vagus nerve in these models were always also detected in the fecal samples and/or ileum of each respective mouse (S5 and S6 Tables). Furthermore, bacteria were not detected in systemic tissues or the blood (S5 Table, Fig 4E) among these mice, and blood–brain permeability indexes were not elevated compared to WT controls (3.4 ± 1.7 for B6, 3.9 ± 0.2 for APP/PS1, 3.3 ± 2.3 for LRRK2, and 3.6 ± 1.8 for BTBR). These findings provide evidence that alteration of gut microbiome composition and increased gut barrier permeability may lead to bacterial translocation to the brain in at least some mouse models of neurodegenerative and neurodevelopmental disease.

Discussion

Using murine models of gastrointestinal disease, diet modulation, and neurological conditions, we demonstrate that a specific subset of bacteria can translocate from the gut to the brain. The direct link between intestinal bacteria and those isolated in the brain is supported by numerous data; (1) in all models tested, the bacteria isolated from the brain were also detected in fecal or ileal samples, (2) modulation of the composition of the intestinal microbiome with antibiotics, and concomitant with Paigen diet feeding, correspondingly changed the type of bacteria that localized to the brain, (3) intestinal colonization of mice with exogenously delivered bacteria not otherwise detected in the gut microbiome led to isolation of those exogenous bacteria from the brain when mice were fed Paigen diet that increased intestinal barrier permeability, and (4) in all models in which we observed bacteria in the brain, we also observed an increase in intestinal barrier permeability, providing a rationale for how the bacteria could have spread from the intestine.

While we cannot rule out the contribution of other routes, our data suggest that the vagus nerve can serve as an anatomical conduit that facilitates, at least in part if not wholly, this translocation; (1) bacteria were isolated from the cervical vagus nerve, (2) the bacteria in the vagus nerve matched those isolated from the brain and were also detected in the intestinal microbiome, (3) upon Paigen diet feeding in B6 mice, bacteria were detected in the vagus nerve prior to being detected in the brain, and (4) unilateral cervical vagotomized *Mdr2*^{-/-} and B6 mice fed Paigen diet harbored significantly lower levels (~20-fold in *Mdr2*^{-/-} and ~3- to 4-fold in B6 mice, Figs 1L and 2E, respectively) of bacteria in the brain, and ~35% fewer mice in both models harbored any bacteria in the brain, compared to sham controls. To the best of our knowledge, there are no previous studies demonstrating translocation of live bacteria to the brain via the vagus nerve. Some viruses have been shown to migrate to the brain, but in non-natural, exogenous infection systems. The influenza virus has been shown to translocate to the brain when inoculated intranasally in mice, and the HSV-1 virus has been shown to translocate to the brain when injected *trans*-neuronally into the cervical vagus nerve of rats [41,42]. HSV-1 was also isolated from the vagus ganglion of one of 18 human cadavers in a single study [43]. Further, a recent report shows the presence of SARS-CoV-2 RNA in the vagus nerve of deceased patients with COVID-19 [44]. These studies provide highly circumstantial evidence for the presence of viruses in the vagus nerve, but do not show that the vagus nerve is responsible for translocation of pathogens to the brain in natural settings.

We demonstrate that the translocation of bacteria to the brain occurred in the absence of permeability of the blood–brain barrier, no bacteria were detected in the blood, meninges, or CSF, nor was there broader dissemination of the bacteria to other systemic organs. Importantly, as a control, after spiking blood with known dilutions of either *S. xyloso* or *E. cloacae* (S1 Table), the detection limit of these bacteria was 1–2 bacterial cells, demonstrating high sensitivity. These data suggest that if bacteria were present in the blood of *Mdr2*^{-/-} or B6 mice fed Paigen diet, or in the murine models of Alzheimer's, Parkinson's, and autism studied here, the culturing technique we employed would have detected them. Further, it is important to emphasize that we have observed relatively low-level bacterial localization to the brain itself (between 1 and ~1,000 bacterial cells) and no bacteria in the meninges or CSF, highlighting that the phenomenon we describe is distinct from meningitis, which is an acute rather than chronic condition.

To further investigate the connection between intestinal barrier permeability and translocation of bacteria from the gut to the brain, we fed B6 mice increasing concentrations of dextran sulfate sodium (DSS; 1%, 3% or 5%; commonly used to disrupt gut barrier integrity) while on Control diet and in the absence of Paigen diet. Interestingly, we observed increasing levels of intestinal permeability upon DSS treatment ([S20A Fig](#)). While 1% DSS induced a level of intestinal permeability similar to that induced by Paigen diet feeding in the *Mdr2*^{-/-} and B6 models and caused bacterial translocation to the brain similar to those models ([Figs 1E and 2D](#)), this concentration did not cause any bacterial localization to the blood ([S20B Fig](#)). However, B6 mice treated with 5% DSS exhibited a markedly increased level of intestinal permeability, >10-fold higher than mice treated with 3% DSS, and much higher than *Mdr2*^{-/-} or B6 mice fed Paigen diet. Only in mice treated with 5% DSS were we able to recover bacteria from the blood (and clearly demonstrating that our culture system is capable of detecting bacteria in the blood). This robust increase in intestinal permeability in B6 mice treated with 5% DSS was accompanied by a >10-fold increase in the number of bacteria recovered from the brain ([S20E and S21 Fig](#)). These data demonstrate that levels of intestinal permeability that induce bacterial translocation to the brain are far lower than those necessary for bacteria to be recovered from the blood and thus explain why we did not observe bacteremia in the models we have investigated, despite observing bacteria in the brain.

Importantly, in every model/instance in which we observed bacteria in the brain, we also observed increased intestinal permeability above that of baseline control mice, which did not have bacteria in the brain. However, we did observe different levels of bacteria in the brain in different mouse models (especially in B6 and *Mdr2*^{-/-}) which did not necessarily correlate exactly with the level of intestinal permeability, suggesting that increased intestinal permeability is likely not the only factor involved in bacterial localization to the brain or the exact level of bacteria in the brain. There are numerous differences between the experiments with the B6 and *Mdr2*^{-/-} mice that could account for the differences in bacterial load in the brain including: (a) these mice are on different genetic backgrounds (B6 versus FVB/N) and thus there are differences in baseline gut microbiome composition, intestinal barrier integrity, vagal function, the immune system, and the immune response to bacteria, etc. (b) beyond differences in their genetic background, *Mdr2*^{-/-} mice specifically have significant pathology in bile acid secretion into the intestine which can impact both microbiome composition and intestinal physiology. (c) there are differences in timepoints when mice in each model are sampled (B6 at day 14 and *Mdr2*^{-/-} at day 9). Despite these differences, intestinal permeability is associated with bacterial translocation in both these models as well as in models of neurological conditions that are presented in [Fig 4B](#).

We acknowledge that we often detect fewer than 30 CFUs in the brain and vagus samples, which is below the level that provides the greatest quantitative accuracy (30–400 CFUs) when determining colony counts by serial dilution on agar plates for samples with high bacterial load. This may lead to an imprecise quantitative determination of bacterial load in the brain and vagus samples. However, because brain/vagus samples in our study contained relatively few CFU, and were not diluted before plating, any possible variation/inaccuracy in our quantification would likely result in a slight underestimation rather than an overestimation of the number of bacteria present in these samples.

To rule out contamination of the brain during sample preparation, we took numerous precautions and performed many control experiments as follows: (a) the fur over the skull was sprayed with 70% ethanol to sterilize the surface and prevent contamination of the brain from the fur or external environment. We swabbed multiple surfaces during the brain isolation process (skin after ethanol spray, skull before and after ethanol spray, and the surface of the brain) and found no bacterial growth from any of the swabs ([S4 Fig](#)). (b) Brains were harvested and processed first, before intestinal or other samples were collected, to prevent exogenous contamination of the brain samples with intestinal bacteria. (c) No bacteria were detected in the brains of non-gavaged, germ-free mice, indicating that our isolation method did not contaminate brain samples with culturable bacteria. (d) Germ-free mice exogenously gavaged with *Enterobacter* and fed regular diet harbored *Enterobacter* in the intestine but did not have detectable bacteria in the brain. This highlights that intestinal colonization alone is not sufficient to cause bacterial localization in the brain, and thus that contamination of brain samples with intestinal bacteria during sample collection cannot explain the localization of bacteria in the brain. Only when

germ-free mice were gavaged with *Enterobacter* and also fed Paigen diet (which increased intestinal barrier permeability) were *Enterobacter* cultured from the brain. If the bacteria cultured from the brain had been the result of intestinal contamination, they would have been cultured from the brains of germ-free mice gavaged with *Enterobacter* and fed regular diet as well as those fed Paigen diet. (e) Conventional (not germ-free) *Mdr2*^{-/-} and C57BL/6 mice fed regular diet did not harbor bacteria in the brain, although the samples from these mice were processed exactly as those from the same types of mice fed Paigen diet, which harbored bacteria in the brain. If the bacteria in the brain of Paigen diet-fed mice were a result of contamination during sample processing, they would likely have been found in both groups of mice, irrespective of diet. (f) In each mouse model, diet type, and treatment (i.e., antibiotics), while different types of bacteria were isolated from the brain, the bacteria isolated from the brain of a given mouse were also always present in the intestine from that mouse, strongly ruling out the bacteria simply being a result of environmental contamination. g) Genomic sequences of the bacteria isolated from the brain closely matched (~99.99% identity) those from the same species of bacteria in the gut, consistent with the gut being the source of the bacteria in the brain. Additional descriptions of these and other issues have been discussed in the [S1 Text](#).

Paigen diet is an atherogenic diet containing fat (35 kcal%), cholesterol (1.25%), and sodium cholic acid (0.5%), which is different from human diets as well as the western diet, which has been demonstrated to alter intestinal permeability in murine models [12,45,46]. The increased intestinal permeability observed in Paigen diet-fed B6 mice was consistent with the disruption of the mucosal lining (S12C–S12E Fig), suggesting how the bacteria may have translocated to the peripheral vagus nerve.

Importantly, increased intestinal permeability has been reported in patients with Parkinson's disease [47,48]. Serum zonulin levels, linked to intestinal barrier permeability, were found to be upregulated in a cohort of patients with neurodegenerative diseases, including Alzheimer's and Parkinson's [49]. Increased intestinal permeability was found in autistic children [50], as well as increased plasma concentrations of intestinal fatty acid binding protein, a marker for intestinal permeability, which were associated with more severe speech and behavioral defects [51,52]. Bacterial RNA has been detected previously in the postmortem brains of HIV/AIDS and other patients; however, the lack of any negative control patients from whom bacteria were not detected in the brains makes it challenging to interpret these results accurately due to the issue of contamination impacting low-biomass microbiome studies [53]. Presence of bacteria in the brain of post-mortem Alzheimer's disease patients has been suggested by 16S rRNA gene sequencing as well; however, detection of bacterial 16S rRNA gene sequencing reads in control samples as well again poses challenges in accurately interpreting these results [54]. Moreover, some reports have indicated *Borrelia burgdorferi* in postmortem brain biopsies from Alzheimer's patients by both PCR and confocal immunofluorescence microscopy [55,56]. Similar to *Borrelia*, *Chlamydia pneumoniae* has been reported in the brain tissues of Alzheimer's and ALS patients identified using PCR and microscopy [57,58]. It is unclear if these results were due to the presence of live bacteria in the brain before death, and if so, how they would have localized there. Moreover, the reports suggesting the detection of bacteria in the brain of post-mortem human samples are often contradictory as well as being unclear due to the potential for tissue breakdown and bacterial spread after death and prior to tissue collection [55,59–63]. The results presented here provide direct evidence (i) of the presence of small numbers of bacteria within the brain of mice (distinct from meningitis, as no bacteria are detected in the meninges, CSF, or spinal nerve), (ii) delineate intestinal barrier integrity as a critical “gatekeeper” preventing this phenomenon, (iii) highlight intestinal barrier permeability as a cause of bacterial translocation to the brain, (iv) elucidate a pathway by which the bacteria reach the brain (vagus nerve), and (v) identify dietary and genetic causes that facilitate bacterial translocation to the brain. Additionally, we found that the bacteria found in the brain are always a subset of the bacterial species found in the gut (S6 Table). What governs which bacteria translocate to the brain is not clear; it is possible that many types of bacteria can translocate to the brain when intestinal permeability is increased. Certainly, the species of bacteria detected in the brain in this study are influenced by our culturing conditions which would not support the growth and detection of all gut species, as well as the specific models used which each lead to differential enrichment of specific

bacteria in the gut (e.g., *Staphylococcus* species and *Enterococcus* in the Paigen diet feeding models). We cannot, at this point, conclude whether all types of bacteria could translocate to the brain. Additionally, different bacteria may have differing abilities to survive in the brain, which may lead to the detection of some species more frequently than others.

In *Mdr2*^{-/-} and wild-type B6 models of Paigen diet feeding, bacterial localization to the brain was reversible with diet modulation (return to Control diet), demonstrating that this is a dynamic process. We speculate that once intestinal permeability returns to baseline levels, bacteria no longer translocate to the brain. In the setting of diet reversal, the remaining bacteria in the brain likely represent previously translocated bacteria that can persist in the brain for some time before being cleared. Future experiments will be aimed at addressing this question.

Due to low numbers of bacteria, we have not detected bacteria in the brain or vagus nerve by imaging. Further complicating the imaging of bacteria in the vagus nerve, we believe bacterial localization there is transient, and a step in the transit of bacteria from the gut to the brain. We do corroborate our detection of bacteria in the brain via culturing with ddPCR data. Future studies will focus on where the bacteria reside in the brain and which host cells come into contact with translocated bacteria in the brains of these models.

Since we observed bacterial translocation to the brain in models of Alzheimer's, Parkinson's, and autism, further investigation is warranted to determine whether gut-targeted interventions might potentially prevent bacteria from reaching the brain. It will be important to determine if this phenomenon also occurs in humans, as well as performing further studies to understand the mechanisms underlying bacterial translocation to the brain which could vastly change our understanding of neurological and neurodevelopmental conditions.

Materials and methods

Ethics statement

The use of animal models and procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University (Protocol # PROTO-201700372). To minimize co-housing/cage effects and coprophagy on microbiota composition, we minimized housing density (keeping 2–3 mice per cage) and utilized littermate controls. Mouse strains were kept under similar housing conditions and were fed the same rodent chow, unless stated for Paigen Diet. We found no significant differences in the percentage of mice positive for bacteria in the brain in the same group housed in different cages and also in the same group from different experiments. Based on observations and data collected from multiple independent repeats, we consistently detected the same type of bacteria in the brain under the same particular treatment conditions, with similar frequencies of detection, even when different batches of mice were used. These results support the conclusion that cage effects are unlikely to have influenced the findings.

Mouse strains

C57BL/6J (000664), FVB/NJ (001800) (wild-type controls) and *Mdr2*^{-/+} (FVB.129P2-Abcb4^{tm1Bor}/J) mice were obtained from the Jackson Laboratory (ME, USA) and housed in a specific pathogen-free environment, per Emory University, NIH, and IACUC guidelines. *Mdr2*^{-/-} mice, *Mdr2*^{-/+} mice, and control mice were generated by backcrossing littermates until desired true breeding lines were established and confirmed by PCR. BTBR T+ Itpr3^{tf}/J (002282), APP/PS1 WSB.Cg-Tg(APPswe,PSEN1dE9)85Dbo/How (038560), and LRRK2 knockin (B6.Cg-Lrrk2^{tm1.1Hlme}/J (030961) mice were purchased from the Jackson Laboratory and housed in a specific pathogen-free environment per Emory University, NIH, and IACUC guidelines. A standard chow (control; Labdiet 5001) or Paigen's Atherogenic Rodent diet (Research Diets D12336 consisting of fat 35 kcal%, protein 20 kcal%, and carbohydrate 45 kcal% with cholesterol 1.25%, and sodium cholic acid 0.5%. Fat was from soybean oil 5%, cocoa butter 7.5%, coconut oil 3.5%), was fed to *Mdr2*^{-/-} mice (both male and female) (9 days) or B6 mice (14 days). In some experiments, mice were given a cocktail of antibiotics (neomycin sulfate, 1 g/L; ampicillin 1 g/L; vancomycin 500 g/L; and metronidazole 1 g/L) in drinking water, while on Paigen diet.

Intestinal permeability assay

Mice were fasted for 8–10 hours and orally gavaged with 100 μ L of 160 mg/mL FITC-dextran (4 kDa) dissolved in PBS. Blood was collected from mice 4 hours post-gavage by submandibular bleed, and serum was collected after centrifugation. Dilutions of FITC dextran solution ranging from 15.6 ng/mL to 8,000 ng/mL were made for the standard curve. The fluorescence of diluted serum and dilutions for the standard curve was measured in a fluorimeter after excitation of the samples at 488 nm and emission at 521 nm. The amount of FITC-dextran in serum was determined by the standard curve.

Whole gut transit time

To determine whole gut transit time, mice were fasted overnight, followed by removal of water for 1 hour in the morning. The mice were single-housed and gavaged with 100 μ L of 5% carmine red dissolved in 1.5% methyl cellulose. The time between gavage and the time at which the first red fecal pellet was calculated as the whole gut transit time.

Fecal moisture content

For determination of fecal moisture content, fecal pellets were collected, and their weights were noted. This was followed by drying the feces for 24 hours at 95 °C. The difference in the weight of feces before and after drying was considered the moisture content in the feces.

Blood–brain barrier permeability index

The blood–brain barrier permeability index (BPI) was calculated using the FITC tracer as described previously [64]. Briefly, the mice were injected intraperitoneally with 100 μ L of 2 mM FITC dextran (3 kDa) solution in PBS. After 20 min, mice were euthanized, and blood was collected by cardiac puncture, followed by intracardiac perfusion with 20–40 mL of PBS. The brain was collected in 1 mL PBS and homogenized. The homogenate was centrifuged at 15,000g for 20 min, and the fluorescence intensity (F.I.) of FITC in the supernatant, along with F.I. of FITC in serum, was measured using a fluorescence plate reader. The F.I. per gram of the brain was determined by dividing the F.I. of the brain homogenate by the brain weight (normalized brain F.I.). Similarly, F.I. per mL of serum was calculated by dividing the F.I. of the serum by the serum volume used for fluorescence calculation (normalized serum F.I.). The BPI was calculated by dividing the normalized brain F.I. by normalized serum F.I. C57BL/6 mice injected intraperitoneally with 3 mg/kg *Salmonella enterica* LPS was used as a positive control [65]. LPS-injected C57BL/6 mice had a BPI of 16.6 ± 3.6 at 24 hours.

$$\text{Normalized brain fluorescence intensity (F.I.)} = \text{F.I. of the brain homogenate} / \text{brain weight}$$

$$\text{Normalized serum F.I.} = \text{F. I. of the serum} / \text{serum volume (per mL)}$$

$$\text{BPI} = \text{Normalized brain F.I.} / \text{normalized serum F.I.}$$

Blood culture

Mice were euthanized by CO₂ asphyxiation. Blood was collected terminally from the mice by cardiac puncture and put in EDTA/Heparin Blood collection tubes. 100 μ L of the blood was put in 1 mL of BacT/ALERT FA plus aerobic media and incubated for up to 7 days at 37 °C with 5% CO₂ with subculture on blood agar on day 2 and day 7 to culture any bacteria present in the blood. Separately, we assessed the limit of detection (LOD) of the BacT/ALERT FA plus aerobic blood culture media. *Staphylococcus xylosus* and *Enterobacter cloacae* strains were cultured in vitro, diluted, inoculated into

the blood culture media and incubated as above. By measuring the input CFU, we found that these culture conditions can detect as low as 1 CFU of bacteria.

Brain and other organ culture

Mice were euthanized by CO₂ asphyxiation. The brain was dissected first from all mice and collected under aseptic conditions. Following the brain, heart, lungs, kidneys, spleen, and ileum tissues were collected under aseptic conditions. Different sets of tools were used for different organs and each mouse. After each use, the instruments were sterilized by dipping in 70% ethanol and washing them in sterile PBS. The organs were collected in either plastic-capped sterile culture tubes with 1 ml of sterile 1× PBS or in 2 mL omni bead beater tubes with 1.5 mm ceramic beads. The culture tubes were weighed before and after organ collection to calculate the weights of the organs. The organs were homogenized, and the probe of the homogenizer was washed 4 times (2 washes with sterile water, 1 wash with 70% ethanol, and 1 wash with sterile PBS) in between the samples for the plastic-capped culture tubes. Bead-beater tubes were shaken in a Bead Ruptor4 for 20 seconds with speed 5. Depending on the tissues, 150–500 µL of the homogenate was plated on a blood agar plate and incubated at 37 °C with 5% CO₂. The total CFU of bacteria in per gram of tissues (CFU/g) was calculated by counting the number of colonies on the blood agar plate and factoring in the corresponding volume/dilution of homogenate and weight of tissues. We picked one morphologically similar colony of each type, from each sample (e.g., brain, ileum, feces) in each experiment. We identified the colonies by MALDI-TOF mass spectrometry at the Clinical Microbiology Laboratory of Emory University Hospital. The individual species count data are therefore estimates since not every colony on every plate was assessed using this approach.

Vagus nerve and spinal cord culture

Mice were euthanized by CO₂ asphyxiation. The left and right cervical vagus nerves running along the neck (~2 cm in length) were dissected under a dissection microscope aseptically and placed in sterile screw cap tubes with 1.5 mm ceramic beads and 500 µL of PBS. Similarly, the spinal cord was isolated from the trunk aseptically and put in a screw cap tube. The samples were homogenized in a bead homogenizer at a speed of 5 m/s for 20 seconds twice. 200 µL of the homogenate was plated on a blood agar plate and incubated at 37°C with 5% CO₂. The total CFU of bacteria per vagus nerve (~2 cm) or in gram of spinal cord (CFU/g) was calculated by counting the number of colonies on the blood agar plate and factoring in the corresponding volume/dilution of homogenate. Bacterial colonies (one of each morphological type from each sample) isolated from tissue homogenates were identified by MALDI-TOF mass spectrometry at the Clinical Microbiology Laboratory of Emory University Hospital. The individual species count data are therefore estimates since not every colony on every plate was assessed using this approach.

Cervical vagotomy

Mice were anesthetized using 4% isoflurane, and the surgical plane was maintained with 1.5% isoflurane. 1 mg/kg of buprenorphine E.R. was injected subcutaneously as a presurgical analgesic. Mice were placed in the supine position, and the neck area was shaved and cleaned with alcohol and betadine solution. A 2 cm incision was made in the skin in the middle of the neck using a scalpel. Under a dissecting microscope, the connective tissue layer was separated using a pair of forceps to expose mandibular glands. The connective tissue layer was further separated to expose the sternohyoid, sternomastoid, and omohyoid muscles. The sternomastoid muscle of the right side was retracted to visualize the pulsating carotid artery and the right vagus nerve. The right vagus nerve was carefully separated from the carotid sheath, and a portion of the vagus nerve was cut with the help of micro-dissecting scissors. The skin was sutured, and mice were allowed to recover. SHAM surgery was performed, where the right vagus nerve was separated from the carotid sheath but not cut. Of note, bilateral cervical vagotomy was not possible since this would be fatal.

***Enterobacter cloacae* gavage and detection**

Enterobacter cloacae isolate was described previously [66] and was genetically modified for this study by integrating a ~5 kb sequence (bar-coded) in its chromosome (to generate strain JC96). *E. cloacae* from an overnight culture washed in sterile PBS (5×10^7 cfu) was orally gavaged into C57BL/6 mice previously treated with a cocktail of antibiotics. Following gavage, mice were subsequently fed Paigen diet. Colonization of *E. cloacae* was detected in the gut on day 5 after gavage by culturing fecal pellet and the ileum tissue using a standard bacterial culture method. The total CFU of bacteria in per gram of feces or tissues were calculated by counting the number of colonies on the blood agar plate and factoring in the corresponding volume/dilution of homogenate and weight of tissues. Bacterial colonies isolated from feces, ileum, and brain tissue homogenates were identified by MALDI-TOF mass spectrometry at the Clinical Microbiology Laboratory of Emory University Hospital. The presence of bar-coded sequence was confirmed by PCR amplification using oligonucleotides: JCP429: 5'-AGTGGAGAGGGTGAAGGTGA-3' and JCP430: 5'-CAATGTTGTGGCGAATTTTG-3'. These oligonucleotides target an internal region of the bar-coded sequence amplifying 437 bp. Wild-type *E. cloacae* was used for negative control (NC), whereas the genetically modified *E. cloacae* (JC96) was used as positive control (PC).

Monocolonization of mice with *E. cloacae* by oral feeding

B6 mice were treated with a cocktail of antibiotics (Ampicillin, 500 mg/L; Neomycin, 500 mg/L; Metronidazole, 500 mg/L; and Vancomycin, 250 mg/L) for 3 days, and the depletion of microbiota was confirmed by using standard bacterial culture of feces. The mice were fasted for 4 hours prior to feeding a bacterial suspension of *E. cloacae* JC96 containing 10^9 CFU/mL in 2% sucrose solution ad libitum for 15 min. The mice were returned to their cage and continued with ampicillin (500 mg/L in drinking water) treatment to maintain JC96 colonization, as JC96 is ampicillin-resistant. Colonization was confirmed at day 4 after JC96 feeding by bacterial culture of feces. After confirmation of colonization, mice were fed the Paigen diet and follow-up experiments were performed as described above.

Isolation of microbial DNA from the brain

Two different kits (QIAamp DNA Microbiome Kit by Qiagen and HostZERO Microbial DNA Kit from Zymo Research), specialized to isolate microbial DNA from low-biomass samples, were used to isolate microbial DNA from the mouse brain. Both kits use two steps. In the first step, host DNA depletion is performed to remove eukaryotic host DNA by limited permeabilization of the host cells. This is followed by the lysis of prokaryotic cells and the isolation of the prokaryotic DNA. The microbial DNA was isolated following the manufacturer's protocols.

PCR from microbial DNA isolated from the brain

Microbial DNA isolated using the microbiome kits mentioned above was used for PCR. PCR was done for the detection of JC96 DNA using two sets of primers, one targeting the barcode (JCP429, JCP430) and the other targeting a region covering the barcode and *Enterobacter* DNA (JCP51, JCP53) using the GoTaq MasterMix. The following primer sequences were used to detect JC96 from the microbial DNA isolated from the brain.

JCP51: 5' GCCAGTTTCAGCAAGGTTTC 3'

JCP53: 5' GCTCTTCCGCTTCCTCGCTC 3'

JCP429: 5' AGTGGAGAGGGTGAAGGTGA 3'

JCP430: 5' CAATGTTGTGGCGAATTTTG 3'

Initial denaturation was performed at 95 °C for 5' followed by 30 cycles of denaturation at 95 °C for 30", annealing at 52°C (for JCP51, JCP53) or 55 °C (for JCP429, JCP430) for 60", and extension at 72 °C for 90" (for JCP51, JCP53) or 30" (for JCP429, JCP430), followed by final hold at 72 °C for 5'.

For the samples where microbial DNA could not be detected either due to the absence of JC96 DNA or due to lower JC96 DNA levels than the LOD, we used an enrichment strategy. The brain homogenate for those samples was incubated with added MHB overnight at 37 °C, followed by microbial DNA isolation described in the previous step. Uninoculated MHB was included as a negative control.

Droplet digital PCR (ddPCR) for detection of bacterial DNA

Next, we used ddPCR, a more sensitive and high-throughput PCR technique [35–37], to quantify bacterial DNA in the brain without ex vivo enrichment of the bacteria. Briefly, primer probe pairs were designed for the JC96 barcode and a PCR mix was made using ddPCR supermix for probes (No dUTP) following BioRad guideline with forward and reverse primers, probes, EcoRI, and template. A droplet generator was used to divide the PCR mix into droplets using Droplet Generation Oil for Probes from Bio-Rad. The PCR was performed at 95 °C for 10' followed by 40 cycles of denaturation at 94 °C for 30", annealing at 60 °C for 60", and extension at 72 °C for 30", followed by a final hold at 98 °C for 10'. After the PCR, the droplets were read in a QX200 Droplet Digital System by the direct quantification method. The figure was generated using the QuantaSoft Analysis Pro Software.

Forward Primer: 5' GAGGGTGAAGGTGATGCTAC 3'

Reverse Primer: 5' CGGGAAAAGCATTGAACACC 3'

Probe: 5' HEX-CCTGTTCCA ZEN TGGCCAACACTTGTAC 3' IB FQ

Germ-free mouse experiments

The experiments were conducted at the Emory Gnotobiotic Animal Core. Five week old germ-free B6 mice were mono-colonized with *Enterobacter cloacae* JC96. Intestinal colonization was confirmed through fecal samples by culturing and detecting total CFUs in the feces by a standard bacteriological culture as described above, followed by PCR using JCP429 and JCP430 primers and MALDI-TOF identification of the cultured bacteria. After colonization, the mice were fed Paigen diet for two weeks. Mice were harvested aseptically, and gut barrier permeability was assessed along with bacteriological culturing of various samples such as brain, vagus nerve, ileum, heart, spleen, lungs, kidney, meninges, spinal cord, and CSF. Any bacteria detected in the brain were further identified by PCR using JCP429 and JCP430 primers as described above to confirm the presence of the barcode and MALDI-TOF to confirm the species identification.

CRAMP and lysozyme ELISA

The serum was diluted 1:2 for CRAMP (the murine ortholog of human LL37) ELISA (LS Bio, Cat. # LS-F32286) and 1:500 for lysozyme ELISA (Novus Biologicals, Cat. # NBP2-68058), added to the precoated wells and incubated for 90 min at 37 °C, followed by incubation with biotinylated detection antibody for 60 min at 37 °C. After washing the plate, an HRP-streptavidin conjugate was added to the wells and incubated for 30 min at 37 °C. The plates were washed, and the TMB substrate was added. Once blue color was developed, the reaction was stopped by adding the stop solution in the kit, and OD450 was measured in an Agilent BioTek Synergy H1 microplate reader. The concentration of LL37 and lysozyme was calculated in the serum by a standard curve of LL37 and lysozyme proteins provided in the kit.

Alcian Blue Periodic Acidic-Schiff (AB-PAS) staining of the gut

B6 mice were fed either regular or Paigen diet for 14 days and harvested. The small intestine was removed, and the intestinal contents were gently squeezed out of the gut using forceps. The gut tissues were then fixed in 10% formalin solution for 24 hours, followed by preserving them in 70% ethanol. The gut tissues were then dehydrated and embedded in paraffin. 5 µm thin sections were cut in a microtome, and the sections were put on a charged slide. For staining, the slides

were deparaffinized, rehydrated, and then stained with alcian blue and Schiff's reagent to stain acidic and neutral mucins, respectively. The slides were then counterstained with hematoxylin. The slides were then dehydrated and mounted using a xylene-containing mounting medium. The images were acquired in a brightfield microscope.

16S rRNA gene sequencing

Fecal samples were collected from *Mdr2*^{-/-} mice on Control diet or Paigen diet for DNA isolation using Qiagen Power-soil Pro DNA extraction method at Emory Integrated Genomic Core (EIGC) of Emory University School of Medicine. Libraries were prepared using the 16S rRNA gene sequencing library preparation (Illumina workflow). In this workflow, 16S rRNA gene sequencing amplicons were generated using KAPA HiFi HotStart ReadyMix and primers specific to the 16S rRNA gene sequencing (V3-V4) region (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG NGGCWGCAG-3'; 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Purified amplicons were indexed with Illumina Nextera XT Index Primers and purified with Ampure XP beads. 16S rRNA gene sequencing libraries were pooled in equal amounts based on fluorescence quantification. Final library pools were quantitated by quantitative PCR and sequenced on an Illumina MiSeq using MiSeq v3 600 cycle chemistry. For data analysis, paired-end reads were assigned to samples based on their unique barcodes. After trimming of primer sequences by *cutadapt* (v1.9), sequencing reads were merged into tags based on overlapping regions using *FLASH* (v1.2.8). Quality filtering was performed by *fqtrim* (v0.94) with sliding windows to retain sequences having average quality scores ≥ 20 and longer than 100 bp in size. Chimeric sequences were discarded using *Vsearch* (v2.3.4). After dereplication using DADA2 [67], ASV feature sequences were obtained and quantified. Singleton ASVs were excluded from further analysis. Species annotations of ASVs were performed by SILVA (release 132, <https://www.arb-silva.de/documentation/release138/>) [68] and NT-16S. Beta diversity was calculated based on relative abundance of ASVs [69]. *QIIME2* was used for beta diversity analyses, including Chao1, Observed_species, Goods_coverage, Shannon, and Simpson indices. Graphs were generated by *ggplot2* (R package v3.5.2). Differential analysis was performed based on relative abundance values. Linear Discriminant Analysis Effect Size (LEfSe) predictions for bacterial groups showing linear marked enrichment (LDA score) and cladogram representation were generated by using R package v3.5.2 [70]. MWU was used for comparisons between 2 groups. Kruskal–Wallis test was used for comparisons among 3 or more groups. *p* values were corrected for the false discovery rate following Benjamini–Hochberg procedure. A species with *p* < 0.05 was considered significantly differential species. Bioinformatics services and analyses including diversity, taxonomy, and differential analyses for bacterial families were provided by LC Sciences (Houston, Texas).

Genomic sequencing of bacterial isolates

Bacterial isolates were streaked on blood agar plates and processed for DNA extraction, sequencing, and variant calling at SeqCenter (<https://www.seqcenter.com>). Illumina sequencing (200 Mbp; 1.33M reads) or small nanopore combo (minimum 300Mbp long reads, 400Mbp Illumina reads) was performed. One of the three isolates from the same mouse (brain, ileum, or feces) was assembled and compared to the Illumina-generated 2*151 bp paired-end read data from the remaining isolates from the same mouse for variant calling using BreSeq (version 0.38.1) under default settings [71]. The number of predicted mutations (single-nucleotide polymorphism) were counted and ANIs were calculated as described previously [72].

Statistical analysis

Data were analyzed using MWU test or FE test or one-way analysis of variance (ANOVA), where applicable, using Graph Pad Prism software (GraphPad Prism, 10.0.2 (171) version). CFUs from brain or vagus tissue homogenates were analyzed for significance using the MWU and FE. The statistical significance for differential analysis based on relative abundance were determined as described in 16S rRNA gene sequencing.

Supporting information

S1 Fig. Linear discriminant analysis (LDA) and the relative abundance of bacterial families found in feces of *Mdr2*^{-/-} mice fed either Control or Paigen diet. (A) LDA scores representing log changes in bacterial families found in feces of *Mdr2*^{-/-} mice fed either Control or Paigen diet and as determined by 16S rRNA sequencing. (B) Relative abundance of bacterial families found in feces of *Mdr2*^{-/-} mice fed either Control or Paigen diet. Statistical significance was determined by Mann–Whitney test as described in 16S rRNA gene sequencing. **P*<0.05. The raw data for this Figure can be found in [S3](#) and [S4 Data](#) files.

(TIF)

S2 Fig. Colony-forming units (CFU) of bacteria in various organs. (A–G) Total CFU of bacteria per gram of (A) heart, (B) spleen, (C) lungs, (D) kidney, (E) meninges, (F) per mL of cerebral spinal fluid (CSF), and (G) per gram of spinal cord from *Mdr2*^{-/-} mice fed either regular diet (Control) or Paigen diet (Paigen). (H) CFU of bacteria in nodose ganglia. (I) CFU of *S. xylosus* in the vagus nerve. Data are representative of 2 independent experiments. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

(TIF)

S3 Fig. CFU of *S. sciuri* and *E. faecalis* in *Mdr2*^{-/-} mice fed Paigen diet. (A–D) CFU of *S. sciuri* (A) per gram of fecal pellet, (B) per gram of ileum, (C) per cervical vagus nerve (~2 cm), and (D) per brain of *Mdr2*^{-/-} mice fed Paigen diet. (E–H) CFU of *E. faecalis* (E) per gram of fecal pellet, (F) per gram of ileum, (G) per cervical vagus nerve (~2 cm), and (H) per brain of *Mdr2*^{-/-} mice fed Paigen diet. Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated (D, H). Statistical significance for panels D and H was determined by Mann–Whitney Test (solid lines) and Fisher Exact (FE) test (dashed lines). The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

(TIF)

S4 Fig. Steps for brain CFU determination. To rule out contamination during the collection of mouse brain samples, we streaked swabs after each step involved in the dissection of mouse brains on blood agar plates and incubated to check for bacterial growth. Step 1: PBS swab on fur/skin after 70% ethanol spray, Step 2: PBS swab on skull after removing skin, Step 3: PBS swab on skull after 70% ethanol spray, Step 4: PBS swab on brain surface after cutting the skull, and Step 5: Culture brain for detecting CFU of bacteria per gram of brain. No growth was observed on plates from Steps 1–4.

(TIF)

S5 Fig. Vagotomy did not alter intestinal or blood-brain barrier permeability, gut bacterial load, or gut microbiome composition. (A) Total CFU of bacteria detected in the left and right vagus nerve from *Mdr2*^{-/-} mice fed Paigen diet. (B) Steps showing surgical confirmation of vagotomy procedure. (C, D) Intestinal permeability (C) and blood-brain barrier permeability (D) in sham and vagotomized *Mdr2*^{-/-} mice fed Paigen diet. (E, F) Total CFU of bacteria in the fecal pellet (E) and ileum (F). (G) Principal component analysis (PCA) of 16S rRNA gene sequencing showing the gut microbiome composition between sham and vagotomized mice fed Paigen diet. Statistical significance for panels A and C–F was determined by Mann–Whitney Test. **P*<0.05. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

(TIF)

S6 Fig. CFU of bacteria in antibiotic-treated mice. Twelve-week-old *Mdr2*^{-/-} mice were treated with a cocktail of antibiotics during Paigen diet feeding. (A) Intestinal permeability in antibiotic (Abx)-treated mice was determined. (B–D) CFU of *Paenibacillus cineris* (*P. cineris*) (B) per gram of fecal pellet, (C) per gram of ileum, and (D) per brain of Abx-treated mice. (E–L) CFU of *P. cineris* per ml of blood (E), per gram of heart (F), lung (G), spleen (H), kidney (I), per ml of CSF (J), per gram of meninges (K), and spinal cord (L). Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated (D). Statistical significance was determined by Mann–Whitney test

(solid lines) and Fisher Exact (FE) test (dashed lines). $**P<0.01$, $***P<0.001$. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

(TIF)

S7 Fig. CFU of *S. xylosus* and *E. faecalis* in various organs of C57BL/6 mice fed Paigen diet. (A–D) CFU of *S. xylosus* (A) per gram of fecal pellet, (B) per gram of ileum, (C) vagus nerve (~2 cm) and (D) per brain of B6 mice fed Paigen diet. (E–H) CFU of *E. faecalis* (E) per gram of fecal pellet, (F) per gram of ileum, (G) vagus nerve (~2 cm), and (H) per brain of B6 mice fed Paigen diet. Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated (D, H). Statistical significance was determined by Mann–Whitney test (solid lines) and Fisher Exact (FE) test (dashed lines). $*P<0.05$, $**P<0.01$, $***P<0.001$, and $****P<0.0001$. Line reflects the geometric mean. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

(TIF)

S8 Fig. Antimicrobial peptides in sera of B6 mice fed Paigen diet. Paigen diet was fed to 8-week-old B6 mice for 2 weeks. (A) Serum levels of CRAMP (the murine ortholog of human LL-37) and (B) Lysozyme were determined by ELISA. Statistical significance was determined by Mann–Whitney test. $*P<0.05$. Line reflects the geometric mean. The raw data for this Figure can be found in [S1 Data](#).

(TIF)

S9 Fig. Bacterial CFU in the brain with or without blood perfusion. *Mdr2*^{-/-} mice were fed Paigen diet for 9 days and bacterial CFU in the brain was determined in mice with or without cardiac perfusion. Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated. Statistical significance was determined by Mann–Whitney test (solid lines) and Fisher Exact (FE) test (dashed lines). The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

(TIF)

S10 Fig. Intestinal permeability and bacterial load in the brain of B6 mice fed Paigen diet on day 56. (A–C) B6 mice were fed Paigen diet for 56 days and (A) gut permeability, bacterial CFU (B) per brain, and (C) per mL of blood, CSF, or per gram of indicated organs were determined. Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated (B). Statistical significance was determined by the Mann–Whitney test (solid lines) and Fisher Exact (FE) test (dashed lines). $**P<0.01$, $***P<0.001$. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

(TIF)

S11 Fig. Bacterial translocation from the gut to the brain over time in Paigen diet-fed C57BL/6 mice. (A, B) B6 mice were fed Paigen diet and total CFU of bacteria in blood and CSF (CFU/mL), vagus nerve and meninges (CFU), heart, kidney, spleen, lungs, spinal cord and brain (CFU/g) on (A) day 4, and (B) day 6 were determined. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.

(TIF)

S12 Fig. Measurement of gut factors in B6 mice fed Paigen diet. Control diet or Paigen diet was fed to 8-week-old B6 mice for 2 weeks. (A) Whole gut transit time (min) and (B) percent fecal moisture content were evaluated. (C) Alcian Blue Periodic Acid-Schiff (AB-PAS) staining of ileum sections from B6 mice fed Paigen diet. (D, E) Quantification of (D) goblet cells per crypt-villus and (E) the number of breaks in the mucus lining per crypt-villus were evaluated based on AB-PAS staining. Statistical significance was determined by Mann–Whitney test. $*P<0.05$. Line reflects the geometric mean. The raw data for this Figure can be found in [S1 Data](#).

(TIF)

S13 Fig. Determination of limit of detection of *E. cloacae* JC96 strain by standard PCR. (A) Gel showing no detectable bands, showing no amplification by PCR when microbial DNA was isolated using the HostZero Zymo kit

from the brains of mice fed Control or Paigen Diet. **(B)** Determination of the limit of detection of standard PCR for bacterial DNA isolated from the brain by either spiking the brain with a known number of bacteria (left panel), after enriching bacteria by incubating the brain homogenate in MHB overnight (middle panel) or from bacterial culture (right panel). Gel showing amplification of JC96 barcode only in the brain samples of Paigen diet-fed **(C)** *Mdr2*^{-/-} mice and **(D)** B6 mice. The primer set used in the PCR was JCP51 and JCP53. Some of the gel bands appear red due to oversaturation during imaging.
(TIF)

S14 Fig. Detection of *E. cloacae* strain JC96 in the brain of B6 mice using a second set of primers. Gel showing amplification of the JC96 DNA barcode only in the brain samples of Paigen diet-fed B6 mice with a distinct primer set as that used in S13. The primer set used in the PCR was JCP429 and JCP430. Some of the gel bands appear red due to oversaturation during imaging.
(TIF)

S15 Fig. Diet reversal restored gut microbiome composition in *Mdr2*^{-/-} mice closely matching Control group. **(A)** Principal component analysis (PCA) of 16S rRNA gene sequencing showing the gut microbiome composition between Control diet, Paigen diet, and diet reversed mice (those fed Paigen diet and subsequently switched back to Control diet). **(B)** Heat map showing the enrichment of bacterial species between Control diet, Paigen diet, and diet reversed mice. Statistical analysis was performed as described in 16S rRNA gene sequencing [Materials and methods](#) section. The raw data for this Figure can be found in [S3](#) and [S4 Data](#) files.
(TIF)

S16 Fig. Diet reversal in B6 mice reduced intestinal barrier permeability and bacterial load in the brain. **(A)** Intestinal permeability in Paigen diet-fed B6 mice and diet-reversed B6 mice on Day 7 (D7), D14, D21, and D28. **(B)** Bacterial CFU per brain tissue in B6 mice fed Paigen diet following diet reversal quantified on D7, D14, D21, and D28. Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated (B). Statistical significance was determined by Mann–Whitney test (solid lines) and Fisher Exact (FE) test (dashed lines). The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.
(TIF)

S17 Fig. Bacterial families found in fecal samples of APP/PS1 mice. **(A)** Linear discriminant analysis (LDA) score and **(B)** cladogram representation of bacterial families found in fecal samples of APP/PS1 mice compared to WT mice are shown as determined by 16S rRNA sequencing. Statistical analysis was determined by Mann–Whitney test as described in 16S rRNA gene sequencing. The raw data for this Figure can be found in [S3](#) and [S4 Data](#) files.
(TIF)

S18 Fig. Bacterial families found in fecal samples of LRRK2 mice. **(A)** Linear discriminant analysis (LDA) score and **(B)** cladogram representation of bacterial families found in fecal samples of LRRK2 mice compared to WT mice are shown as determined by 16S rRNA sequencing. Statistical analysis was determined by Mann–Whitney test as described in 16S rRNA gene sequencing. The raw data for this Figure can be found in [S3](#) and [S4 Data](#) files.
(TIF)

S19 Fig. Bacterial families found in fecal samples of BTBR mice. **(A)** Linear discriminant analysis (LDA) score and **(B)** cladogram representation of bacterial families found in fecal samples of BTBR mice compared to WT mice are shown as determined by 16S rRNA sequencing. Statistical analysis was determined by Mann–Whitney test as described in 16S rRNA gene sequencing. The raw data for this Figure can be found in [S3](#) and [S4 Data](#) files.
(TIF)

S20 Fig. Detection of bacteria in the brain in dextran sulfate sodium (DSS)-treated mice. (A–E) Different concentrations (1%, 3%, and 5%) of DSS were fed to B6 mice monocolonized with bar-coded *E. cloacae* (JC96), in drinking water for 5 days. (A) Intestinal permeability, and bacterial CFU per mL of blood (B), per gram of fecal pellet (C), per gram of ileum tissue (D), and per brain (E) were determined. Percentage of mice in each group positive for bacterial CFU in the brain (% pos) is indicated (E). Statistical significance was determined by Mann–Whitney (solid lines) and Fisher Exact (FE) test (dashed lines). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. The raw data for this Figure can be found in [S1](#) and [S2 Data](#) files.
(TIF)

S21 Fig. Detection of bacteria in the brain of 5% DSS-treated mice by standard PCR. Different concentrations (1%, 3%, and 5%) of dextran sulfate sodium (DSS) were fed to B6 mice monocolonized with bar-coded *E. cloacae* (JC96), in drinking water for 5 days. Gel showing amplification of JC96 DNA barcode from brain samples of 5% DSS-treated mice by standard PCR without prior incubation to enrich bacteria.
(TIF)

S1 Table. Determination of limit of detection of bacteria in blood.
(DOCX)

S2 Table. Average nucleotide identity (ANI) of bacterial isolates in *Mdr2*^{−/−} and C57BL/6 mice fed Paigen Diet.
(DOCX)

S3 Table. Detection of *E. cloacae* strain JC96 in the brain by culture and PCR approaches.
(DOCX)

S4 Table. Determination of limit of detection of *E. cloacae* strain JC96 by culture and PCR approaches.
(DOCX)

S5 Table. Number of mice where bacteria were detected/total number of experimental mice.
(DOCX)

S6 Table. Presence of bacterial populations in the brain of mouse models of neurological conditions.
(DOCX)

S1 Data. The numerical values for [Figs 1B–1L](#), [2A–2K](#), [3A–3E](#), [3G–3I](#), [4B–4E](#), [S2A–S2I](#), [S3A–S3H](#), [S5A](#), [S5C–S5F](#), [S6A–S6L](#), [S7A–S7H](#), [S8A](#), [S8B](#), [S9](#), [S10A–S10C](#), [S11A](#), [S11B](#), [S12A–S12E](#), [S16A](#), [S16B](#), [S20A–S20E](#), and [S6 Table](#).
(XLSX)

S2 Data. Bacterial colony counts for [Figs 1C–1E](#), [1G–1I](#), [1K–1L](#), [2A–2G](#), [2I–2K](#), [3B–3E](#), [3H](#), [3I](#), [4C–4E](#), [S2H](#), [S5A](#), [S5E](#), [S5F](#), [S6B–S6D](#), [S9](#), [S10B](#), [S16B](#), and [S20C–S20E](#).
(XLSX)

S3 Data. Raw files for Cladogram/LEFSE phylogenetic tree analyses related to [Figs 1A](#), [4A](#), [S15B](#), [S17B](#), [S18B](#), and [S19B](#).
(XLSX)

S4 Data. Metafile with FASTA sequences for [Figs 1A](#), [4A](#), [S15B](#), [S17B](#), [S18B](#), and [S19B](#) analyses. Data has been deposited in NCBI Sequence Read Archive (SRA Accession BioProjects: PRJNA1190946 and PRJNA1219196).
(XLSX)

S1 Text. Frequently Asked questions (FAQs). Additional descriptions of precautions taken to prevent contamination and other issues are discussed in FAQs.
(DOCX)

S1 Raw Images. Raw gel/blot images for [Figs 2](#), [S13](#), [S14](#), and [S21](#).
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References

1. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann Gastroenterol*. 2015;28(2):203–9. PMID: [25830558](#)
2. Golubeva AV, Joyce SA, Moloney G, Burokas A, Sherwin E, Arboleya S, et al. Microbiota-related changes in bile acid & tryptophan metabolism are associated with gastrointestinal dysfunction in a mouse model of autism. *EBioMedicine*. 2017;24:166–78. <https://doi.org/10.1016/j.ebiom.2017.09.020> PMID: [28965876](#)
3. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. *Cell*. 2016;167(6):1469–1480.e12. <https://doi.org/10.1016/j.cell.2016.11.018> PMID: [27912057](#)
4. Gu X, Zhou J, Zhou Y, Wang H, Si N, Ren W, et al. Huanglian Jiedu decoction remodels the periphery microenvironment to inhibit Alzheimer's disease progression based on the "brain-gut" axis through multiple integrated omics. *Alzheimers Res Ther*. 2021;13(1):44. <https://doi.org/10.1186/s13195-021-00779-7> PMID: [33579351](#)
5. Shi F-D, Yong VW. Neuroinflammation across neurological diseases. *Science*. 2025;388(6753):eadx0043. <https://doi.org/10.1126/science.adx0043> PMID: [40536983](#)

6. Ma Q, Xing C, Long W, Wang HY, Liu Q, Wang R-F. Impact of microbiota on central nervous system and neurological diseases: the gut-brain axis. *J Neuroinflammation*. 2019;16(1):53. <https://doi.org/10.1186/s12974-019-1434-3> PMID: 30823925
7. Mou Y, Du Y, Zhou L, Yue J, Hu X, Liu Y, et al. Gut microbiota interact with the brain through systemic chronic inflammation: implications on neuroinflammation, neurodegeneration, and aging. *Front Immunol*. 2022;13:796288. <https://doi.org/10.3389/fimmu.2022.796288> PMID: 35464431
8. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. *Nat Neurosci*. 2017;20(2):145–55. <https://doi.org/10.1038/nn.4476> PMID: 28092661
9. Cryan JF, O'Riordan KJ, Sandhu K, Peterson V, Dinan TG. The gut microbiome in neurological disorders. *Lancet Neurol*. 2020;19(2):179–94. [https://doi.org/10.1016/S1474-4422\(19\)30356-4](https://doi.org/10.1016/S1474-4422(19)30356-4) PMID: 31753762
10. Liu L, Huh JR, Shah K. Microbiota and the gut-brain-axis: implications for new therapeutic design in the CNS. *EBioMedicine*. 2022;77:103908. <https://doi.org/10.1016/j.ebiom.2022.103908> PMID: 35255456
11. Morais LH, Schreiber HL 4th, Mazmanian SK. The gut microbiota-brain axis in behaviour and brain disorders. *Nat Rev Microbiol*. 2021;19(4):241–55. <https://doi.org/10.1038/s41579-020-00460-0> PMID: 33093662
12. Rohr MW, Narasimhulu CA, Rudeski-Rohr TA, Parthasarathy S. Negative effects of a high-fat diet on intestinal permeability: a review. *Adv Nutr*. 2020;11(1):77–91. <https://doi.org/10.1093/advances/nmz061> PMID: 31268137
13. Shi C, Li H, Qu X, Huang L, Kong C, Qin H, et al. High fat diet exacerbates intestinal barrier dysfunction and changes gut microbiota in intestinal-specific ACF7 knockout mice. *Biomed Pharmacother*. 2019;110:537–45. <https://doi.org/10.1016/j.biopha.2018.11.100> PMID: 30530289
14. Johnson AMF, Costanzo A, Gareau MG, Armando AM, Quehenberger O, Jameson JM, et al. High fat diet causes depletion of intestinal eosinophils associated with intestinal permeability. *PLoS One*. 2015;10(4):e0122195. <https://doi.org/10.1371/journal.pone.0122195> PMID: 25837594
15. Zhang H, Chen Y, Wang Z, Xie G, Liu M, Yuan B. Implications of gut microbiota in neurodegenerative diseases. *Front Immunol*. 2022;13:785644.
16. Zhuang Z-Q, Shen L-L, Li W-W, Fu X, Zeng F, Gui L, et al. Gut microbiota is altered in patients with Alzheimer's disease. *J Alzheimers Dis*. 2018;63(4):1337–46. <https://doi.org/10.3233/JAD-180176> PMID: 29758946
17. Vogt NM, Kerby RL, Dill-McFarland KA, Harding SJ, Merluzzi AP, Johnson SC, et al. Gut microbiome alterations in Alzheimer's disease. *Sci Rep*. 2017;7(1):13537. <https://doi.org/10.1038/s41598-017-13601-y> PMID: 29051531
18. Shukla PK, Delotterie DF, Xiao J, Pierre JF, Rao R, McDonald MP, et al. Alterations in the gut-microbial-inflammasome-brain axis in a mouse model of Alzheimer's disease. *Cells*. 2021;10(4):779. <https://doi.org/10.3390/cells10040779> PMID: 33916001
19. Honarpisheh P, Reynolds CR, Blasco Conesa MP, Moruno Manchon JF, Putluri N, Bhattacharjee MB. Dysregulated gut homeostasis observed prior to the accumulation of the brain amyloid-beta in Tg2576 mice. *Int J Mol Sci*. 2020;21(5).
20. Braak H, Rüb U, Gai WP, Del Tredici K. Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J Neural Transm (Vienna)*. 2003;110(5):517–36. <https://doi.org/10.1007/s00702-002-0808-2> PMID: 12721813
21. Stolzenberg E, Berry D, Yang D, Lee EY, Kroemer A, Kaufman S, et al. A role for neuronal alpha-synuclein in gastrointestinal immunity. *J Innate Immun*. 2017;9(5):456–63. <https://doi.org/10.1159/000477990> PMID: 28651250
22. Scheperjans F, Aho V, Pereira PAB, Koskinen K, Paulin L, Pekkonen E, et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov Disord*. 2015;30(3):350–8. <https://doi.org/10.1002/mds.26069> PMID: 25476529
23. Baizabal-Carvallo JF, Alonso-Juarez M. The link between gut dysbiosis and neuroinflammation in Parkinson's disease. *Neuroscience*. 2020;432:160–73. <https://doi.org/10.1016/j.neuroscience.2020.02.030> PMID: 32112917
24. Liang Y, Cui L, Gao J, Zhu M, Zhang Y, Zhang H-L. Gut microbial metabolites in Parkinson's disease: implications of mitochondrial dysfunction in the pathogenesis and treatment. *Mol Neurobiol*. 2021;58(8):3745–58. <https://doi.org/10.1007/s12035-021-02375-0> PMID: 33825149
25. Taniya MA, Chung H-J, Al Mamun A, Alam S, Aziz MA, Emon NU, et al. Role of gut microbiome in autism spectrum disorder and its therapeutic regulation. *Front Cell Infect Microbiol*. 2022;12:915701. <https://doi.org/10.3389/fcimb.2022.915701> PMID: 35937689
26. Peralta-Marzal LN, Rojas-Velazquez D, Rigters D, Prince N, Garssen J, Kraneveld AD, et al. A robust microbiome signature for autism spectrum disorder across different studies using machine learning. *Sci Rep*. 2024;14(1):814. <https://doi.org/10.1038/s41598-023-50601-7> PMID: 38191575
27. Mlynarska E, Barszcz E, Budny E, Gajewska A, Kopec K, Wasiak J. The gut-brain-microbiota connection and its role in autism spectrum disorders. *Nutrients*. 2025;17(7).
28. Mitchell KJ, Dahly DL, Bishop DVM. Conceptual and methodological flaws undermine claims of a link between the gut microbiome and autism. *Neuron*. 2026;114(2):196–211. <https://doi.org/10.1016/j.neuron.2025.10.006> PMID: 41237768
29. Tedesco D, Thapa M, Chin CY, Ge Y, Gong M, Li J, et al. Alterations in intestinal microbiota lead to production of interleukin 17 by intrahepatic $\gamma\delta$ T-cell receptor-positive cells and pathogenesis of cholestatic liver disease. *Gastroenterology*. 2018;154(8):2178–93. <https://doi.org/10.1053/j.gastro.2018.02.019> PMID: 29454797
30. Breit S, Kupferberg A, Rogler G, Hasler G. Vagus nerve as modulator of the brain-gut axis in psychiatric and inflammatory disorders. *Front Psychiatry*. 2018;9:44. <https://doi.org/10.3389/fpsy.2018.00044> PMID: 29593576
31. Chen C, Zhou Y, Wang H, Alam A, Kang SS, Ahn EH, et al. Gut inflammation triggers C/EBP β / δ -secretase-dependent gut-to-brain propagation of A β and Tau fibrils in Alzheimer's disease. *EMBO J*. 2021;40(17):e106320. <https://doi.org/10.15252/embj.2020106320> PMID: 34260075
32. Sáez-Nieto JA, Medina-Pascual MJ, Carrasco G, Garrido N, Fernandez-Torres MA, Villalón P, et al. *Paenibacillus* spp. isolated from human and environmental samples in Spain: detection of 11 new species. *New Microbes New Infect*. 2017;19:19–27. <https://doi.org/10.1016/j.nmni.2017.05.006> PMID: 28702198

33. Leão RS, Pereira RHV, Ferreira AG, Lima AN, Albano RM, Marques EA. First report of *Paenibacillus cineris* from a patient with cystic fibrosis. *Diagn Microbiol Infect Dis*. 2010;66(1):101–3. <https://doi.org/10.1016/j.diagmicrobio.2009.06.011> PMID: [19709843](#)
34. Fierer N, Leung PM, Lappan R, Eisenhofer R, Ricci F, Holland SI, et al. Guidelines for preventing and reporting contamination in low-biomass microbiome studies. *Nat Microbiol*. 2025;10(7):1570–80. <https://doi.org/10.1038/s41564-025-02035-2> PMID: [40542287](#)
35. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem*. 2011;83(22):8604–10. <https://doi.org/10.1021/ac202028g> PMID: [22035192](#)
36. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods*. 2013;10(10):1003–5. <https://doi.org/10.1038/nmeth.2633> PMID: [23995387](#)
37. Taylor SC, Laperriere G, Germain H. Droplet digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable non-sense to publication quality data. *Sci Rep*. 2017;7(1):2409. <https://doi.org/10.1038/s41598-017-02217-x> PMID: [28546538](#)
38. Kowalski K, Mulak A. Brain-gut-microbiota axis in Alzheimer's disease. *J Neurogastroenterol Motil*. 2019;25(1):48–60. <https://doi.org/10.5056/jnm18087> PMID: [30646475](#)
39. Coretti L, Cristiano C, Florio E, Scala G, Lama A, Keller S, et al. Sex-related alterations of gut microbiota composition in the BTBR mouse model of autism spectrum disorder. *Sci Rep*. 2017;7:45356. <https://doi.org/10.1038/srep45356> PMID: [28349974](#)
40. Zhu M, Liu X, Ye Y, Yan X, Cheng Y, Zhao L, et al. Gut microbiota: a novel therapeutic target for Parkinson's disease. *Front Immunol*. 2022;13:937555. <https://doi.org/10.3389/fimmu.2022.937555> PMID: [35812394](#)
41. Matsuda K, Park CH, Sunden Y, Kimura T, Ochiai K, Kida H, et al. The vagus nerve is one route of transneuronal invasion for intranasally inoculated influenza A virus in mice. *Vet Pathol*. 2004;41(2):101–7. <https://doi.org/10.1354/vp.41-2-101> PMID: [15017022](#)
42. Blessing WW, Li YW, Wesselingh SL. Transneuronal transport of herpes simplex virus from the cervical vagus to brain neurons with axonal inputs to central vagal sensory nuclei in the rat. *Neuroscience*. 1991;42(1):261–74. [https://doi.org/10.1016/0306-4522\(91\)90163-j](https://doi.org/10.1016/0306-4522(91)90163-j) PMID: [1650433](#)
43. Warren KG, Brown SM, Wroblewska Z, Gilden D, Koprowski H, Subak-Sharpe J. Isolation of latent herpes simplex virus from the superior cervical and vagus ganglions of human beings. *N Engl J Med*. 1978;298(19):1068–9. <https://doi.org/10.1056/NEJM197805112981907> PMID: [205790](#)
44. Woo MS, Shafiq M, Fitzek A, Dottermusch M, Altmepfen H, Mohammadi B, et al. Vagus nerve inflammation contributes to dysautonomia in COVID-19. *Acta Neuropathol*. 2023;146(3):387–94. <https://doi.org/10.1007/s00401-023-02612-x> PMID: [37452829](#)
45. Lawrie A, Hameed AG, Chamberlain J, Arnold N, Kennerley A, Hopkinson K, et al. Paigen diet-fed apolipoprotein E knockout mice develop severe pulmonary hypertension in an interleukin-1-dependent manner. *Am J Pathol*. 2011;179(4):1693–705. <https://doi.org/10.1016/j.ajpath.2011.06.037> PMID: [21835155](#)
46. Wang Q, He Y, Li X, Zhang T, Liang M, Wang G. *Lactobacillus reuteri* CCFM8631 alleviates hypercholesterolaemia caused by the Paigen atherogenic diet by regulating the gut microbiota. *Nutrients*. 2022;14(6).
47. Forsyth CB, Shannon KM, Kordower JH, Voigt RM, Shaikh M, Jaglin JA, et al. Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. *PLoS One*. 2011;6(12):e28032. <https://doi.org/10.1371/journal.pone.0028032> PMID: [22145021](#)
48. Yang H, Li S, Le W. Intestinal permeability, dysbiosis, inflammation and enteric glia cells: the intestinal etiology of Parkinson's disease. *Aging Dis*. 2022;13(5):1381–90. <https://doi.org/10.14336/AD.2022.01281> PMID: [36186124](#)
49. Loffredo L, Ettore E, Zicari AM, Inghilleri M, Nocella C, Perri L. Oxidative stress and gut-derived lipopolysaccharides in neurodegenerative disease: role of NOX2. *Oxid Med Cell Longev*. 2020;2020:8630275. <https://doi.org/10.1155/2020/8630275>
50. D'Eufemia P, Celli M, Finocchiaro R, Pacifico L, Viozzi L, Zaccagnini M, et al. Abnormal intestinal permeability in children with autism. *Acta Paediatr*. 1996;85(9):1076–9. <https://doi.org/10.1111/j.1651-2227.1996.tb14220.x> PMID: [8888921](#)
51. Teskey G, Anagnostou E, Mankad D, Smile S, Roberts W, Brian J, et al. Intestinal permeability correlates with behavioural severity in very young children with ASD: a preliminary study. *J Neuroimmunol*. 2021;357:577607. <https://doi.org/10.1016/j.jneuroim.2021.577607> PMID: [34044209](#)
52. de Magistris L, Familiari V, Pascotto A, Sapone A, Froli A, Iardino P, et al. Alterations of the intestinal barrier in patients with autism spectrum disorders and in their first-degree relatives. *J Pediatr Gastroenterol Nutr*. 2010;51(4):418–24. <https://doi.org/10.1097/MPG.0b013e3181dcc4a5> PMID: [20683204](#)
53. Branton WG, Ellestad KK, Maingat F, Wheatley BM, Rud E, Warren RL, et al. Brain microbial populations in HIV/AIDS: α-proteobacteria predominate independent of host immune status. *PLoS One*. 2013;8(1):e54673. <https://doi.org/10.1371/journal.pone.0054673> PMID: [23355888](#)
54. Emery DC, Shoemark DK, Batstone TE, Waterfall CM, Coghill JA, Cerajewska TL, et al. 16S rRNA next generation sequencing analysis shows bacteria in Alzheimer's post-mortem brain. *Front Aging Neurosci*. 2017;9:195. <https://doi.org/10.3389/fnagi.2017.00195> PMID: [28676754](#)
55. Senejani AG, Maghsoudlou J, El-Zohiry D, Gaur G, Wawrzyniak K, Caravaglia C, et al. *Borrelia burgdorferi* co-localizing with amyloid markers in Alzheimer's disease brain tissues. *J Alzheimers Dis*. 2022;85(2):889–903. <https://doi.org/10.3233/JAD-215398> PMID: [34897095](#)
56. Gadila SKG, Rosoklija G, Dwork AJ, Fallon BA, Embers ME. Detecting borrelia spirochetes: a case study with validation among autopsy specimens. *Front Neurol*. 2021;12:628045. <https://doi.org/10.3389/fneur.2021.628045> PMID: [34040573](#)
57. Balin BJ, Gérard HC, Arking EJ, Appelt DM, Branigan PJ, Abrams JT, et al. Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain. *Med Microbiol Immunol*. 1998;187(1):23–42. <https://doi.org/10.1007/s004300050071> PMID: [9749980](#)
58. Alonso R, Pisa D, Carrasco L. Searching for bacteria in neural tissue from amyotrophic lateral sclerosis. *Front Neurosci*. 2019;13:171. <https://doi.org/10.3389/fnins.2019.00171> PMID: [30863279](#)

59. Bedarf JR, Beraza N, Khazneh H, Özkurt E, Baker D, Borger V, et al. Much ado about nothing? Off-target amplification can lead to false-positive bacterial brain microbiome detection in healthy and Parkinson's disease individuals. *Microbiome*. 2021;9(1):75. <https://doi.org/10.1186/s40168-021-01012-1> PMID: [33771222](#)
60. Ring RH, Lyons JM. Failure to detect *Chlamydia pneumoniae* in the late-onset Alzheimer's brain. *J Clin Microbiol*. 2000;38(7):2591–4. <https://doi.org/10.1128/JCM.38.7.2591-2594.2000> PMID: [10878049](#)
61. Taylor GS, Vipond IB, Paul ID, Matthews S, Wilcock GK, Caul EO. Failure to correlate *C. pneumoniae* with late onset Alzheimer's disease. *Neurology*. 2002;59(1):142–3. <https://doi.org/10.1212/wnl.59.1.142> PMID: [12105327](#)
62. MacDonald AB. Borrelia in the brains of patients dying with dementia. *JAMA*. 1986;256(16):2195–6. <https://doi.org/10.1001/jama.1986.03380160053011> PMID: [3761515](#)
63. Pisa D, Alonso R, Carrasco L. Parkinson's disease: a comprehensive analysis of fungi and bacteria in brain tissue. *Int J Biol Sci*. 2020;16(7):1135–52. <https://doi.org/10.7150/ijbs.42257> PMID: [32174790](#)
64. Devraj K, Guerit S, Macas J, Reiss Y. An in vivo blood-brain barrier permeability assay in mice using fluorescently labeled tracers. *J Vis Exp*. 2018;2018(132).
65. Banks WA, Gray AM, Erickson MA, Salameh TS, Damodarasamy M, Sheibani N, et al. Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. *J Neuroinflammation*. 2015;12:223. <https://doi.org/10.1186/s12974-015-0434-1> PMID: [26608623](#)
66. Band VI, Crispell EK, Napier BA, Herrera CM, Tharp GK, Vavikolanu K, et al. Antibiotic failure mediated by a resistant subpopulation in *Enterobacter cloacae*. *Nat Microbiol*. 2016;1(6):16053. <https://doi.org/10.1038/nmicrobiol.2016.53> PMID: [27572838](#)
67. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13(7):581–3. <https://doi.org/10.1038/nmeth.3869> PMID: [27214047](#)
68. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2013;41(Database issue):D590–6. <https://doi.org/10.1093/nar/gks1219> PMID: [23193283](#)
69. Li X, Wu P, Zeng X, Lang Q, Lin Y, Huang H, et al. Protocol for correlation analysis of the murine gut microbiome and meta-metabolome using 16S rDNA sequencing and UPLC-MS. *STAR Protoc*. 2022;3(3):101494. <https://doi.org/10.1016/j.xpro.2022.101494> PMID: [35776638](#)
70. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011;12(6):R60. <https://doi.org/10.1186/gb-2011-12-6-r60> PMID: [21702898](#)
71. Deatherage DE, Barrick JE. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods Mol Biol*. 2014;1151:165–88. https://doi.org/10.1007/978-1-4939-0554-6_12 PMID: [24838886](#)
72. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat Commun*. 2018;9(1):5114. <https://doi.org/10.1038/s41467-018-07641-9> PMID: [30504855](#)