Breaking Gut: intestinal microbes and their modulation as a therapy in ASD

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Abstract

Autism Spectrum Disorder (ASD) is a complex and heterogeneous group of neurodevelopmental disorders characterized primarily by deficit in social communication and repetitive behaviors. The prevalence of ASD has increased over the past years, with approximately 1 in 100 children now diagnosed with the condition. Beyond neurological alterations, gastrointestinal issues are frequently observed in individuals with ASD, significantly affecting their quality of life. These gastrointestinal abnormalities are often associated with the severity of ASD symptoms, suggesting a bidirectional communication between the gut and brain, commonly known as the gut-brain axis. The crosstalk between the gut and the brain involves neural, immune, endocrine, and metabolic pathways, and alterations in these pathways have been observed in various neurodevelopmental disorders. Further, imbalances in gut microbiome are commonly presented in ASD subjects and associated to ASD manifestations, which suggests the relevance of understanding the underlying processes along the gut-brain axis in ASD. This study aims to characterize the intestinal and brain barrier, neuroinflammation and enteric nervous system activation in a humanized mouse model for ASD, by transplanting fecal samples from autistic children and their neurotypical siblings in BTBR mice. Besides that, one probiotic diet (diet 1) and two symbiotic diets (diet 2 and 3) were tested in order to study diet-induced gut microbiota changes in the ASD-like phenotype in BTBR mice. Microbiota transplantation from ASD children led to changes in both the intestine and brain of BTBR mice. Neuroinflammation was observed in the prefrontal cortex (PFC) along with impairments in the integrity of the intestinal epithelium and blood-brain barrier. Notably, diet 1 could reduce the neuroinflammation in the PFC of ASD-transplanted mice as well as improving the integrity of the gut barrier in the ileum. Additionally, diet 3 was able to improve the integrity of the blood-brain barrier in the hippocampal tissue. Taken together, ASD-associated gut microbiota is linked with neuroinflammation, and pro- and symbiotic diets were able to modulate different processes across the gut-brain axis, which highlights their potential as targeted-therapy for ASD.

Plain Summary

Autism Spectrum Disorder (ASD) is a group of neurodevelopmental disorders characterized by repetitive behavior and difficulties with social interactions. Its prevalence has increased in the last decade, now diagnosing approximately 1 out of 100 children worldwide. In addition to neurological alterations, many individuals with ASD experience digestive issues, which are linked to the severity of their symptoms and compromise their quality of life. This connection suggests a bidirectional communication between the gut and brain, referred to as the 'gut- brain axis'. The communication between these two regions is complex, involving pathways across the nervous, immune, and endocrine systems. Alterations in the gut microbiota, which is the community of microorganism that inhabits in the intestinal tract, are commonly described in ASD subjects and are associated with their symptomatology. This study aims to characterize how changes in the gut microbiota influence the integrity of intestinal and brain barriers, brain inflammation, and enteric nervous system activation. To test this, we transplanted feces from ASD children and their neurotypical siblings into a genetically predisposed mouse model for

ASD (BTBR mice). The study also tested the effects of three different diets–a probiotic diet (diet 1) and two synbiotic diets (diet 2 and diet 3)– on the gut barrier and blood-brain barrier (BBB) integrity, and brain inflammation. The microbiota transplantation from ASD children showed an increase of the inflammatory marker iNOS in the prefrontal cortex (PFC) of ASD-transplanted mice compared to those transplanted with samples from their siblings. Additionally, the expression of different tight junction proteins in the intestinal epithelium and the BBB showed differences between the ASD-transplanted mice and the non-transplanted mice. Regarding dietary interventions, diet 1 showed a reduction of the inflammatory markers iNOS and COX-2 in the PFC of the ASD-transplanted mice, and enhanced the integrity of the gut barrier, compared to the control diet. Moreover, diet 3 improved the integrity of the blood-brain barrier in the hippocampus region. These findings highlight that gut microbiota from ASD subjects contributes to brain inflammation, and dietary interventions show promise as therapies to manage ASD symptoms by modulating processes of the gut-brain axis.

1. Introduction

Autism Spectrum Disorder (ASD) is a complex group of neurodevelopmental disorders primarily characterized by early-appearing social communication deficits and repetitive sensory–motor behaviors (1). The symptoms and degree of severity are different among individuals with ASD, which is why it is described as a 'spectrum' (2,3). Over the past decades, numerous studies have reported a global rise in ASD diagnoses, with estimates of 1 in 100 children now being affected (4). The prevalence is notably higher in males compared to females, with a male-to-female ratio of approximately 4:1 (4,5). The number of the cases can vary among countries but, that may be due to other socio-cultural and socio-economic factors (6). The etiopathogenesis of ASD remains unclear, with several genetic and environmental factors associated to ASD onset like immune system dysfunction, exposure to certain toxins and nutritional imbalances (7). Besides neurological alterations, gastrointestinal dysfunctions, including constipation, diarrhea and abdominal pain, are frequently found in subjects suffering from ASD (8). The high prevalence of intestinal symptoms and their significant correlation with the severity of ASD-related symptoms, suggest a bidirectional communication between the gut and the brain, commonly named as 'gut-brain axis', in ASD (9,10). The complex communication between the gut and the brain involves the autonomic nervous system, and operates through three key pathways - immune, neuronal and endocrine pathways - that in turn overlap and crosstalk between them (11,12).

Accumulative evidence describes that one of the key modulators of the processes along the gut-brain axis is the gut microbiota, which encompasses a vast community of microorganisms, mainly bacteria, that inhabits in the lower part of the gastrointestinal tract (13). It has been described that ASD subjects have an imbalance in the microbiota composition compared to healthy subjects, also known as dysbiosis, with a lower bacterial diversity (14-16). Nevertheless, there is a lack of consistency across the studies, showing different results in regard to differences in microbiota composition (17). As a result, the altered microbiota composition is accompanied by an aberrant metabolite production, which may alter gut barrier integrity (18,19). A higher gut permeability can allow the

passage of bacteria or their harmful components, among other substances, from intestinal lumen to other tissues, which can originate an immune dysregulation and, therefore, provoke an inflammatory cascade (20,21). Increases in several inflammatory cytokines like TNF-α, IL-1β and IL-6, haven been identified repeatedly in ASD studies, proving evidence of peripheral innate activation and dysregulation compared to typically developing children (22). Hence, the cytokine-mediated inflammatory response may breach barriers in the brain, promoting neuroinflammation and affecting brain homeostasis (23). Moreover, immune abnormalities involving excess inflammation have been positively correlated with ASD-associated behavior, including impaired social interactions and non-verbal communication (24,25).

In addition to the contributions of the gut microbiota in ASD development, other studies point at the role of the ENS, a complex network of neurons and enteric glial cells situated throughout the gastrointestinal wall (26). The ENS, often described as the 'second brain', has an important role regulating processes like gut inflammation, and its impairment has been linked to several gastrointestinal diseases (27,28). Enteric glia has been described as a central player in the modulation of the immune response and the regulation of neuroinflammation (26,29). The intricate interplay between the ENS and the immune system is regulated by different effectors, like neurotransmitters and cytokines, and the dysregulation may have an important impact on ASD progression (30). However, further studies are needed to fully understand the implication of the ENS in ASD.

Considering the relevance of intestinal microbes in processes along the gut-brain axis, fecal microbiota transplant from human donors presents a promising exploratory approach to study the effects from the given microbiota. It has been described how the fecal microbiota transplant can induce hallmark autistic behaviors in different mouse models (31,32). This study aims to investigate how human fecal microbiota transplant (hFMT) from ASD children and their neurotypically siblings (SIB) affects to the ASD-like phenotype of BTBR T+Itpr3tf/J (BTBR) mice. This mouse model for ASD exhibits typical ASD characteristics, like repetitive behavior and decreased sociability (33-35). The BTBR mice received a depletion of microbiota followed by a microbiota transplantation with fecal samples from either ASD or SIB children. Analyses investigated intestinal and brain ASD-related problems including gut epithelial permeability and neuroinflammation.

Additionally, diet is known to be a major contributor to modulate gut host microbiota and it has been suggested as a targeted-therapy to ameliorate ASD symptomatology (36). Several studies have shown the importance of dietary composition on controlling or reducing the ASD symptoms (37,38). In fact, probiotic and symbiotic dietary interventions have gained increasing attention as an alternative to restrictive diets (39). There is growing evidence that probiotics, a group of live microorganisms that naturally habit in the gut, can ameliorate gastrointestinal symptoms, and restore gut microbiota impairment, not presenting a risk for the subjects (40,41). Besides that, the synergic combination of probiotics with prebiotics fibers (symbiotics), has also demonstrated a positive modulation of gut microbiota (42). In some cases, the prebiotic fibers might help the viability of probiotic strains, by enhancing their efficacy, and they have been shown an improvement of some of the symptoms in ASD studies (43,44). Also, our aim is to investigate whether different dietary interventions that target gut microbiota can modulate ASD-associated problems. The dietary interventions include a probiotic mix

combining eight different probiotics strains (Vivomixx 450®; diet 1), the same probiotic mix plus galacto-oligosaccharides and fructo-oligosaccharide fibers (Vivomixx® 450 plus GOS/FOS; diet 2) and a single probiotic strain in combination with prebiotic fibers (*L. paracasei* L411 plus GOS/FOS; diet 3).The influence of the different dietary interventions on the ASD-transplanted BTBR mice were assessed in both intestinal and brain regions, by evaluating several immune and structural markers.

2. Materials and methods

2.1. Animals

The animal experiments were approved by the Utrecht University Animal Ethics Committee (DEC approval number: AVD1080020198547) and all experiments were performed in accordance with the governmental guidelines. For the current (or "for this study") study, two different mouse strains were employed, C57BL/6J and BTBR $T^{+}Itpr3^{tf}/J$ (Jackson Laboratories). Upon arrival, 3/4-week-old mice were randomly assigned to an experimental group (8-10 animals per group; detailed in **Table 1**). Animals were housed in groups of 2-3 mice in individually ventilated cages at the animal facility of Utrecht University.

2.2. Microbiota depletion and human fecal microbiota transplantation (hFMT).

After one week of acclimatization period, depletion of the gut microbiota was performed by bowel cleansing according to Le Roy *et al.* (45). Mice underwent human fecal microbial transplantation (hFMT) with fecal samples from either from children with ASD, or their neurotypical siblings (SIB). Prior hFMT, depletion of mouse gut microbiota is needed. To achieve that, 200 µL of polyethylene glycol (PEG) are administered to the mice every 30 minutes in a total of 5 doses via oral gavage, except for the control group that receives water as substitute. Mice were fasting for 2 hours prior to depletion. The hFMT was performed over three consecutive days through an oral gavage of 200 uL of inoculum, being the first administration 6 hours after the last PEG dose. C57BL/6J, included as controls for behavior, were not depleted, did not receive hFTM and received control diet. See **Fig. 1** for the experimental set-up.

2.3. Diets

Mice were fed with different diets after hFMT was performed until the end of the experiment. The formulation of the isocaloric diets was carried out by Danone Nutricia Research (Utrecht, Netherlands) and their production by Ssniff Spezialdiäten (Soest, Germany). All processes were performed in accordance with the American Institute of Nutrition Rodent Diets Guide for Growing and Gestating Mice. For this experiments, two different diets were developed: AIN-93G, as control food, and AIN-93G containing 3% GOS:FOS (9:1) (GOS/FOS), as prebiotic food. The probiotics employed are Vivomixx® 450, which is composed by a mix of eight different strains including *Streptococcus thermophilus* DSM 24731®, three bifidobacterial strains (*B. breve* DSM 24732®, *B. longum* DSM 24736®, *B. infantis* DSM 24737®) and four lactobacilli strains (*L. acidophilus* DSM 24735®, *L. plantarum* DSM 24730®, *L. paracasei* DSM 24733®, *L. delbrueckii subsp.*

bulgaricus DSM 24734®) and a *L. paracasei* L411 strain provided by Nutricia Research. Diets were stored at -20ºC prior to use and both probiotics were administered in the drinking water at 10^9 CFU daily dose. According to these diets, there are four experimental groups: control diet, composed by control food + normal water; Diet 1, composed by control food + water with Vivomixx®; Diet 2, composed by prebiotic food + water with Vivomixx®, Diet 3, composed by prebiotic food + water with *L. paracasei*.

Table 1 | Experimental groups. C57BL/6J did not receive hFMT and received control diet. BTBR mice are divided in groups based on the hFMT (none, from ASD or SIB) and the diet (control diet, diet 1, diet 2 or diet 3).

Mouse strain	hFMT	Diet	N° of animals
C57BL/6J	No	Control	n=9
BTBR $T^{+}Itpr3^{tf}/J$	No	Control	n=8
	ASD	Control	n=10
	SIB	Control	n=10
	ASD	Diet 1	n=10
	SIB	Diet 1	n=10
	ASD	Diet 2	n=10
	SIB	Diet 2	n=10
	ASD	Diet 3	n=10
	SIB	Diet 3	n=10

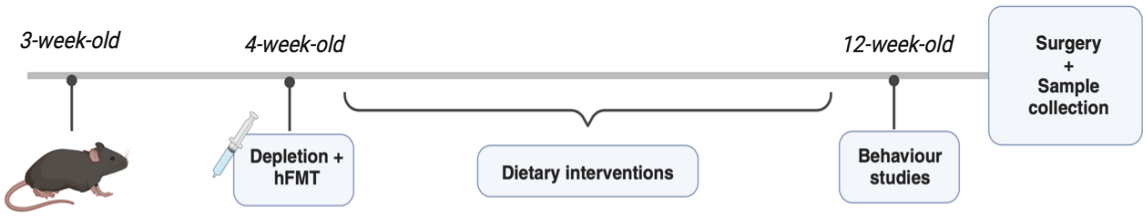


Fig. 1 | Timeline of the mouse experiment. When mice were 4/5-week-old, depletion of gut microbiota followed by hFMT from ASD and SIB children were performed. After that received specific dietary intervention until they were euthanized. When mice were 12/13-week-old, behavioral studies were performed. Mice were sacrificed and samples collected for further analysis at the end of the experiment.

2.4. Tissue collection and preparation

The animals were anesthetized with isoflurane and euthanized. Brain and intestinal samples were collected and stored immediately accordingly. Prefrontal cortex (PFC), hippocampus and distal ileum were snap-frozen and later used for the quantification of different proteins of interest by Western Blot analysis. Also, the proximal colon was collected as Swiss-rolls for immunohistochemical analysis.

For the Swiss rolls, the 5-cm long segments were cut longitudinally along the mesenteric line and rinsed with PBS. Then, the segments were placed with the luminal side facing upward and wrapped to form a Swiss roll. After that, they were fixed in 10% formalin and subsequently embedded in paraffin. 5-µm thick sections were cut using a microtome (Leica Biosystems, Ref. #: 149AUTO00C1). Between three and four complete colon sections were placed in the adhesive slides.

For the tissue homogenization, snap-frozen tissue samples from ileum, prefrontal cortex and hippocampus were weighed, and transferred to Lysing Matrix D 2mL homogenization tubes (MP Biomedicals, Inc., Ref. #: 6913500, Lot #: 170853). PierceTM RIPA buffer (ThermoScientific, Ref. #: 89901, Lot #: XK356177) and protease inhibitor (Roche, Ref. #: 11836170001, Lot #: 66373700) working solution was elaborated in a ratio of 1:200 of protease inhibitor:RIPA. As reference, 500µL of buffer solution was added to every 30mg of tissue. The samples were homogenized using the homogenizer Bertin® Precellys 24 under the following conditions: 6000rpm for 10 seconds three times. After that, samples were centrifuged for 5 minutes at 4°C, 14000rpm. The supernatant was collected and stored at -80°C. The supernatants were collected, and the protein concentration was measured using BCA, following manufacturer instructions.

2.5. Immunohistochemistry

Sections were deparaffinized by serial incubation in xylene and decreasing concentrations of ethanol. Antigen retrieval was performed by incubating the slides in boiling citrate buffer (0.01 M) for 10 min. After letting the slides cool down, they were blocked with 3% BSA/3% normal goat serum/0.1% Tween in PBS for 1 hour at RT. Next, slides were incubated with primary antibody, being rabbit anti-GFAP (1:1000, Dako, Z033), at 4°C overnight. Slides were washed three times with 0.1% Tween in PBS and once with PBS before applying the secondary antibody. Slides were incubated with secondary antibody, goat anti-rabbit AF 594 antibody (1:200, Invitrogen, A11072), for 1 hour at RT, followed by another washing step. Finally, nuclei staining and mounting was performed using ProLong Gold Antifade Mountant with DAPI (Invitrogen, P36931). Digital images were acquired using Leica TCS SP8 confocal microscope with a HCX IRAPO L 25x/0.95 water-immersion lens and GFAP expression level was assessed by measuring the Corrected Total Fluorescence (CTF) for five images per mouse using ImageJ software.

2.6. Western Blot

Firstly, 20 µg of protein were loaded onto 4-20 % gradient precast polyacrylamide gels (Bio-Rad, 5671094) and separated by electrophoresis at 70V for 30min followed by 100V for 60min. After that, the proteins were transferred onto Trans-Blot Turbo Midi PVDF membranes (#1704157; Bio-Rad) by using Trans-Blot Turbo Transfer System for 10 min

(2.5 A, up to 25 V) (#1704150; Bio-Rad). Membranes were blocked with 5% milk powder diluted in PBST (0.1 % Tween 20 in PBS) for 2 hours at RT and incubated overnight at 4°C with the corresponding primary antibody (Table 2).

Table 2 | Primary antibodies used in immunohistochemistry (IHC) and western blotting (WB).

Antibody	Application	Host species	Manufacturer	Reference	Working Dilution
GFAP	IHC	Rabbit	Dako	Z0334	1/1000
iNOS	WB	Rabbit	Cayman	#160107	1/500
COX-2	WB	Rabbit	Invitrogen	#PA3-030A	1/500
ß-Actin	WB	Rabbit	Cell signalling	#4970	1/2000
Occludin	WB	Rabbit	Invitrogen	#40-4700	1/500
Claudin-5	WB	Rabbit	Invitrogen	#34-1600	1/500
ZO-1	WB	Rabbit	Invitrogen	#40-2200	1/500
E-Cadherin	WB	Mouse	BD Biosciences	#610181	1/1000

After washing with PBST three times for 5 minutes, membranes were incubated for 2 hours at RT with peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse secondary antibody (1/3000, Dako P0448 and P0260, respectively). After that, membranes were washed again with PBS-T as mentioned before. Membranes were shortly incubated either with Clarity or Clarity Max ECL Western Blotting Substrates (#1705060 and #1705062 Bio-Rad, respectively). Blots were then developed using ImageQuant RT ECCL Imager and the relative optical densities of the bands were analyzed with image analysis software ImageJ.

2.7. ELISA

PFC tissue samples were processed as described before and the corresponding homogenates were stored at -80 °C until analysis of interleukin-17A (IL-17A) (Invitrogen, #88-7371-77) by ELISA. All steps procedure were performance following manufacturer's instructions. Samples were analyzed in duplicate. Results are shown in Supplementary data.

2.8. Statistical Analysis

All data analysis and statistical tests were conducted using GraphPad Prism 9.3.1 software (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA analysis were performed to study the hFMT and the dietary intervention effects, followed by Tukey's multiple comparisons test, for normally distributed data. For non-normally distributed data, the Kruskal-Wallis test was applied, followed by Dunn's multiple comparisons test. Results are presented as mean ± SEM, with statistical significance defined as $p < 0.05$.

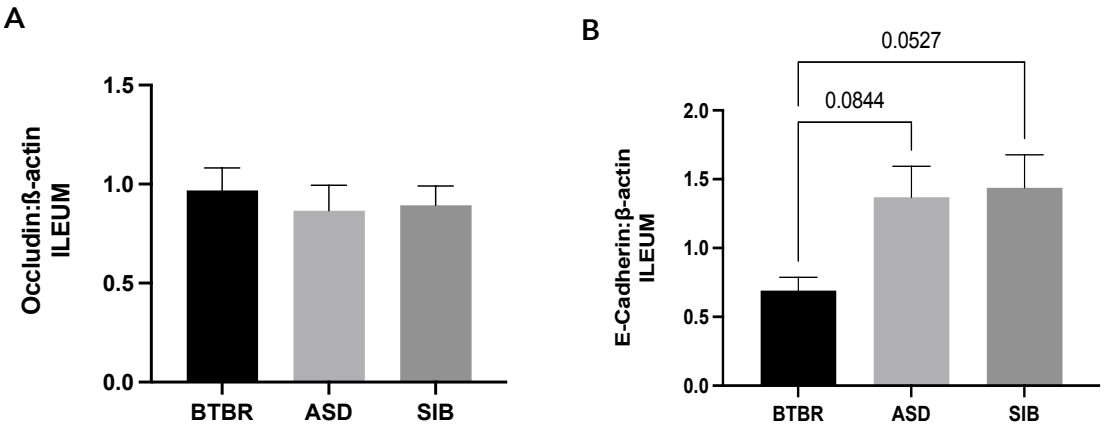
3. Results

3.1. Analysis of gut barrier in the ileum of BTBR showed increased levels of E-cadherin after ASD and SIB hFMT.

The intestinal epithelium is composed by tight junctions and adherens proteins, which main role is 'fence and gate' (46). Different studies have shown a compromised intestinal barrier and deficit of these proteins in ASD subjects, which may allow the leakage of microbial-derived products and other harmful components from the intestinal lumen to surrounding tissues (10,19,21). This inappropriate trafficking can alter immune and metabolic pathways, which may translate into brain alterations (47,48). To evaluate the effect of the microbiota transplant of either ASD or SIB microbes in the distal ileum of BTBR mice, we measured levels of a tight junction protein, occludin, and an adherens protein, E-cadherin, and compared to the non-transplanted BTBR mice. E-cadherin relative levels showed a tendency to be higher in the ASD and SIB groups compared to the non-transplanted BTBR group in the ileum of BTBR mice (**Fig. 3A**). For occludin, no changes were found in the analysis of ileum of BTBR mice (**Fig. 3B**).

3.2. Diet 1 showed a trend elevating the levels of occludin in ileum of ASD-transplanted BTBR mice.

To further explore whether diet-mediated gut microbiota changes have an effect on gut barrier permeability, we also analyzed E-cadherin and occludin in ASD-transplanted BTBR mice fed with different diets, including probiotics with or without prebiotics fibers. E-cadherin did not show any significant differences among the different dietary groups (**Fig. 3C**). Regarding occludin, ASD-transplanted BTBR mice fed with diet 1 presented a trend of higher relative occludin levels compared to ASD-transplanted BTBR fed with control diet (**Fig. 3D**).



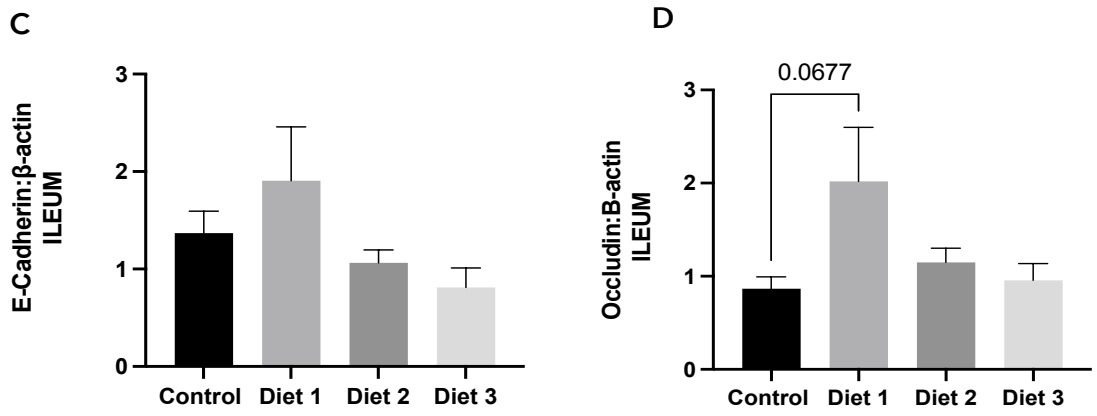


Fig. 3 | Measurement of proteins involved in intestinal barrier integrity in distal ileum of BTBR mice. (A) E-cadherin and (B) occludin relative levels of non-transplanted, ASD-transplanted and SIB-transplanted BTBR mice measured by western blot; (C) E-cadherin and (D) occludin relative levels of ASD-transplanted BTBR mice after different dietary interventions measured by western blot. Protein levels are relative to β-actin. Data represented as the mean ± SEM. n = 8-10.

3.3. Analysis of activated enteric glial cells in the proximal colon of BTBR mice was not affected by hFMT.

Besides maintaining gut homeostasis, enteric glia has also been associated with the modulation of the immune response and the regulation of neuroinflammation (28,20). The enteric glia-immune interactions are gaining substantial interest due to their role in gastrointestinal pathophysiology, particularly relevant in gut-brain disorders (26). Previous studies have shown a stronger activation of the enteric glial cells as a response to microbiota changes, leaky gut or acute inflammation, what occurs during ASD progression, and an increased expression of different markers for the enteric glia in response to gut inflammation (26,28). To measure the activation of the enteric glial cells, the proximal colon tissues were stained with glial fibrillary acidic protein (GFAP), which is a glial marker highly expressed in the enteric glia.

Fig. 4A-C display the representative images of GFAP staining in proximal colon tissue taken by confocal microscopy comparing non-transplanted group, the ASD and the SIB transplanted BTBR mice. To evaluate the microbiota transplant effect in the enteric glia, we measured the corrected total fluorescence (CTF) per 10 consecutive crypts in colon samples (Fig. 4A-C), but statistical analysis did not provide significant difference among the groups (Fig. 4D). Subsequently, we analyzed whether the dietary interventions could modulate the activation of enteric glial cells in the proximal colon of ASD transplanted BTBR mice, but no significant changes were found in quantification (Supplementary Fig. 1).

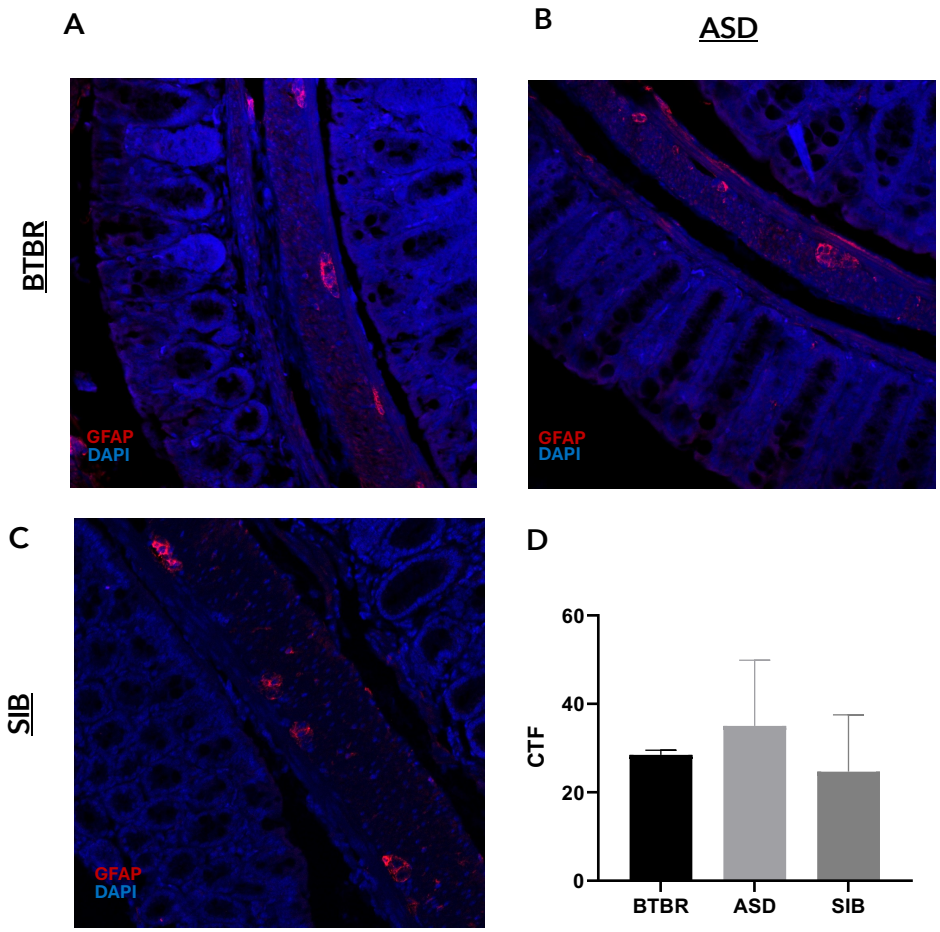


Fig. 4 | Enteric glial cells staining in colonic tissues. Confocal microscopy images of GFAP staining in the colon of (A) non-transplanted BTBR, (B) ASD and (C) SIB transplanted BTBR mice, and (D) analysis of the corrected total fluorescence (CTF) of enteric glial cells when assessing the hFMT effect. The data are represented as mean ± SD, n=6-10.

3.4. Hippocampus of BTBR mice showed a decreased level of claudin-5 after ASD and SIB hFMT, while only PFC of ASD-transplanted BTBR mice exhibited an increased level of ZO-1 compared to non-transplanted BTBR mice.

Brain endothelial cells forming the structure of blood-brain barrier (BBB) are connected by tight junctions and adherens junctions (48). Tight junctions are composed of a complex of transmembrane proteins such claudins, occludin and membrane-associated guanylate kinases, such as ZO-1 (46,48). Neurodevelopmental disorders have been found associated with brain barrier disruption (46,48). Not only the PFC has been widely involved in ASD due its role in the cognition process and sociability, but also hippocampus abnormalities are also associated with spatial reasoning, social interaction and memory (49-51).

To assess the effect of the gut microbiota transplantation on the endothelial permeability, we analyzed the expression of three tight junction proteins, ZO-1, occludin and claudin-5, in the hippocampal and PFC tissue of the non-transplanted BTBR, ASD and SIB-transplanted groups with normal diet. Relative levels of claudin-5 were significantly

lower in the hippocampus of ASD and SIB transplanted BTBR mice compared to the non-transplanted BTBR mice (**Fig. 5A**). No significant changes were found in the analysis of ZO-1 and occludin in the hippocampus (see **Supplementary Fig. 1A-B**). We analyzed relative levels of ZO-1 in the PFC region of the different experimental groups. The analysis revealed a significant higher level of the protein in the SIB group compared to the non-transplant BTBR group, while no significant differences were found when compared to the ASD-transplanted BTBR mice (**Fig. 5B**).

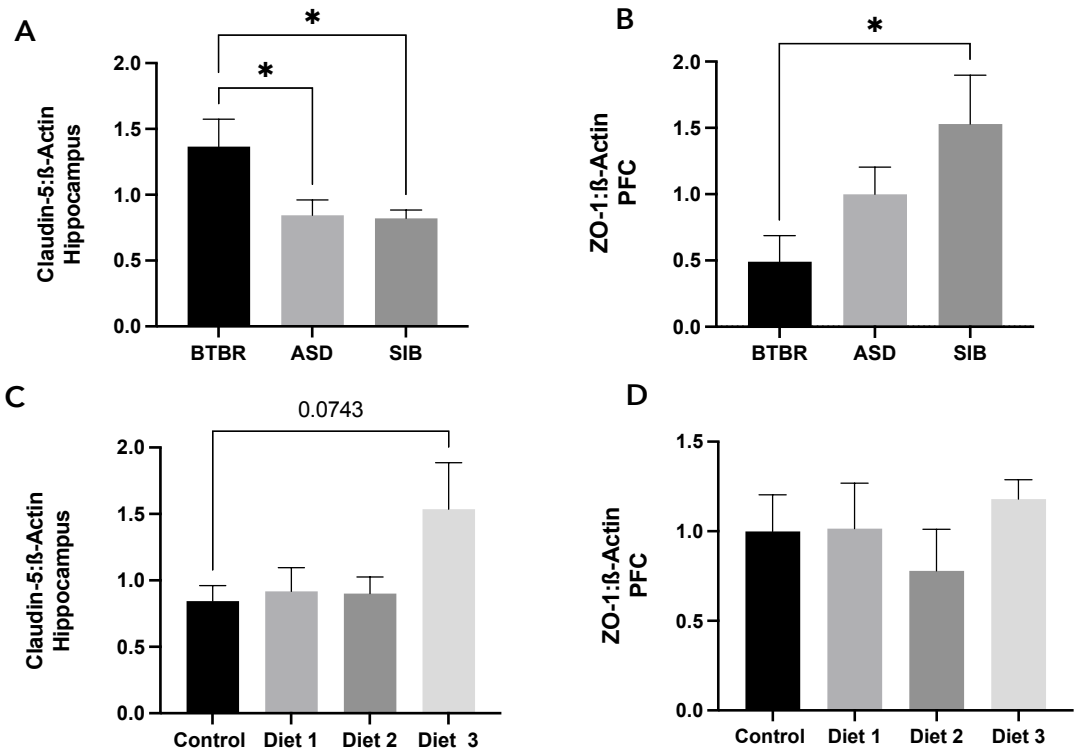


Fig. 5 | Protein barrier levels in the prefrontal cortex and hippocampus. (A) Claudin-5 and (B) ZO-1 relative levels of non-transplanted, ASD-transplanted and SIB-transplanted BTBR mice measured in hippocampus and PFC, respectively; (C) Claudin-5 and (D) ZO-1 relative levels of ASD-transplanted mice across different diets measured in hippocampus and PFC, respectively. Protein levels are relative to β-actin using Western blotting. Data represented as the mean ± SEM. n = 8-10. *$p < 0.05$.

3.5. Diet 3 showed a trend elevating the levels of claudin-5 in the hippocampal tissue of ASD-transplanted BTBR mice.

To further explore whether the dietary interventions affect to the blood-brain barrier permeability, the aforementioned structural proteins were measured in ASD transplanted BTBR mice that received different diets. For hippocampus, a trend can be observed in **Fig. 5C**, where claudin-5 relative levels are higher in ASD transplanted BTBR mice fed with diet 3 compared to the group fed with control diet. Nevertheless, no changes were found in ZO-1 and occludin quantification (see **Supplementary Fig. 2C-D**). Regarding PFC, no significant changes were observed among the groups (**Fig. 5D**).

3.6. Higher levels of inflammatory marker COX-2 were found in the PFC of ASD transplanted mice.

Studies have demonstrated that neuroinflammation plays a significant role in the progression of ASD, and different inflammatory cytokines and proteins are implicated in that process (22,27). In this study, we evaluated the impact of transplanted microbes on neuroinflammation in PFC region. By western blotting, we measured the levels of two key inflammatory proteins – inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) –in the PFC of non-transplanted BTBR, ASD and SIB transplanted BTBR mice. Protein quantification relative proteins to ß-actin showed a significant higher level of COX-2 in the PFC of the ASD group when compared to the SIB group (**Fig. 6A**). No changes were found in iNOS protein quantification (**Fig. 6B**).

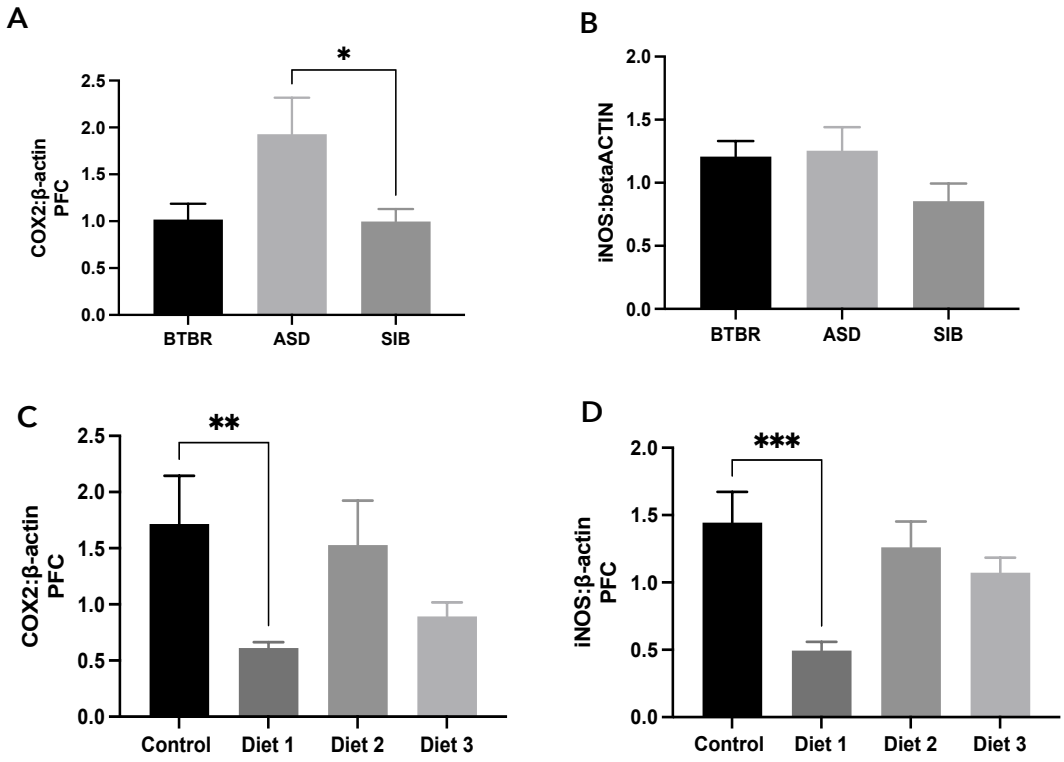


Fig. 6 | Neuroinflammation in the prefrontal cortex PFC. (A) COX-2 and (B) iNOS relative levels in the BTBR, ASD and SIB groups were measured by western blot. Data presented as the mean ± SEM. n = 8-10. *p < 0.05, **p < 0.01, ***p < 0.001.

3.7. Diet 1 showed a reduction of the neuroinflammation within PFC of ASD transplanted mice.

To study whether diet-induced gut microbiota changes might influence neuroinflammation, we analyzed these proteins in the PFC of ASD-transplanted BTBR mice after receiving different diets. Mice fed with diet 1 showed a significant decreased level of both COX-2 ($p < 0.01$) and iNOS ($p < 0.001$) compared to the mice fed with control diet (**Fig. 6C-D**).

4. Discussion

The so-called gut-brain axis, despite of not being anatomically connected, is communicates through different pathways including neural, immune, endocrine and metabolic routes (11,12,27). Recent findings show that the gut-brain axis plays an important role in neurodevelopmental disorders like ASD (52). It has been observed that an impaired gut microbiota composition, as observed in individuals with ASD, can influence on different processes along the gut-brain axis, and therefore, contribute to the ASD-like phenotype (10, 53). To further study the role of gut microbiota and the gut-brain axis in ASD, in this study we investigated the effect of certain microbes from ASD subjects and their neurotypically siblings in a BTBR mouse model for ASD. Diet is a key modulator of host gut microbiota composition, and previous studies suggest that diets including probiotics, prebiotics, and synbiotics may offer therapeutic benefits for individuals with ASD (54). Given that, we were also interested in targeting the gut microbiota as a potential therapeutic strategy, and we tested a prebiotic diet and two symbiotic diets in ASD-transplanted BTBR mice.

4.1. ASD-transplanted microbes' effect in the ASD-like phenotype on BTBR mice.

A key finding of our study is regarding neuroinflammation, where we observed increased levels of the inflammatory marker COX-2 in the PFC of the ASD-transplanted group compared to the SIB group. This increase may point out an association between ASD-associated gut microbes and neuroinflammation, commonly presented in ASD. Moreover, this finding supports previous human and animal studies, where there is disruption of the innate immune system, leading to a pro-inflammatory state in the brain, characterized by increased levels of inflammatory molecules (22,55,56). Among them, IL-17 has also been reported to be involved in the inflammatory response in ASD studies, showing higher levels in peripheral blood and brain tissues (57). Nadeem *et a*l. recently described an overexpression of IL-17A receptor in monocytes from ASD children, with concomitant activation of the NF-kB pathway and increased iNOS expression, which may lead to neuroinflammation (58). We measured the levels of IL-17A in the PFC but our results did not provide any significant difference among the groups (**Supplementary Fig. 3**). While IL-17A is well-known for its role in immune response and pro-inflammatory effects, gaining a deeper understanding of the inflammatory response might include studying IL-17A role in the intestinal region.

Regarding the BBB integrity, we assessed the levels of different tight junction proteins in two brain regions, the PFC and hippocampus. The PFC plays a pivotal role in social behaviour and cognitive processes, and several studies showed an altered PFC activity in individuals with ASD during social tasks (59). Additionally, social interaction, spatial reasoning, and memory are supported by the hippocampus, which has been associated with impaired structure and function in ASD subjects (60). Some ASD studies described a compromised BBB integrity, allowing the passage of harmful molecules or immune-activated complexes into the brain which can lead to neuroinflammation. Promoting a healthy BBB would prevent such inappropriate trafficking, suggesting a possible link between intestinal dysbiosis and brain alterations. Our results show an increase of ZO-1 in the PFC while they show a decrease of claudin-5 in the hippocampal tissue. Claudin-5

is one of the most important tight junction proteins in the regulation of the BBB integrity, and its reduction is associated with an increase in BBB permeability (61). In a recent study, molecular analysis of the components associated with the BBB integrity was conducted in postmortem brain tissue from ASD subjects, showing reduced levels of claudin-5 in cortex and cerebellum but increased levels of claudin-12 in the cortex (46). Similar to our results, this suggests that distinct brain regions might be regulated by different process.

"Leaky gut" is a condition often associated with gastrointestinal disorders, in which the integrity of the intestinal barrier is compromised, allowing harmful substances from the gut lumen to get into the bloodstream (10). We measured the expression of several tight junction and adherens proteins in the gut barrier in order to assess the epithelial permeability. Our results showed an increase of E-cadherin levels in the distal ileum ASD and SIB groups compared to the non-transplanted BTBR group, suggesting that microbiota from ASD and SIB can alter gut epithelial integrity. However, these results differ from existing literature, in which there is a decreased intestinal barrier integrity in ASD subjects that may allow the leakage of different molecules (47, 62-64). Apart from E-cadherin, two prior studies on ASD reported reduced occludin expression in colonic tissues using animal models for ASD (65,66). Since the distal ileum represents a limited section of the gut, different tight junction expression patterns might be found in other regions like the colon, which contains the highest density of gut microbiota.

Enteric glial cells are known to play crucial roles in maintaining homeostasis within the ENS, and also contributing to intestinal functions, immune modulation and neuroinflammation regulation under intestinal disturbances (26). Recently, the enteric glia has gained attention for its possible interactions with the immune system and the gut microbiota, as well as its involvement in the gut-brain axis. Studies have shown that under acute inflammation or changes in the gut permeability, enteric glial cells exhibit an activated state and express higher levels of glial markers like GFAP (26,28). In our study, we measured total fluorescence of GFAP in the proximal colon, but we did not observe significant differences among the groups. Due to the high data variability, the results are inconclusive, indicating that larger sample size is needed to further investigate the role of enteric glial cells in ASD.

Additionally, the previously mentioned immune dysregulation could result from an imbalance of the gut microbiota composition. That gut microbiota dysbiosis may involve an excess of lipopolysaccharide (LPS), a surface molecule on Gram-negative bacteria, which may trigger harmful effects by overactivation of toll-like receptor 4 (TLR-4) in the gut (22). The TLR-4 leads to activation of the NK-kB pathway activation, which is known to play an important role regulating innate inflammatory immune responses and it can induce a higher expression of pro-inflammatory enzymes, such as iNOS and COX-2 (67). This is a possible way by which gut microbiota dysbiosis may contribute to both systemic and brain inflammation. Further, a previous study showed that neuroinflammation is associated with a higher exposure of LPS in a mouse model, where the LPS administration increased the levels of iNOS and COX-2 in both brain and serum (68). ASD subjects are known to exhibit altered microbiota composition and as mention above, compromised BBB integrity, which might derive in a greater leakage of LPS. This could result in stronger TLR-4 activation and subsequent neuroinflammation in the brain, as described in this study.

4.2. Pro- and synbiotic dietary effects in ASD-transplanted BTBR mice.

Recently, targeting gut microbiota composition through dietary interventions, like using pro- and prebiotics, has gained attention as a therapeutic strategy for neurodevelopmental disorders (69). There is evidence of these dietary interventions can locally shift the microbiome towards beneficial bacteria, induce the production of beneficial metabolites and anti-inflammatory cytokines, strengthen the intestinal barrier, and reduce gut and systemic inflammation (70).

Our results revealed significantly decreased levels in the PFC of both inflammatory markers COX-2 and iNOS in the ASD-transplanted BTBR mice fed with diet 1 compared to control diet, suggesting a restorative effect of Vivomixx® on neuroinflammation in the brain of ASD-transplanted BTBR mice. As mentioned above, ASD-associated microbiota is known to be composed by a higher number of bacteria with pro-inflammatory properties, including immune-triggering LPS and abnormal levels of short-chain fatty acids (SCFAs) compared to healthy control children (71) The probiotic strains in Vivomixx® may influence microbiota composition and metabolite production, potentially modifying signaling pathways from the gut microbiota to the brain with neuromodulatory effects. Specifically, the *Lactobacillus* and *Bifidobacterium* strains in Vivomixx® have been shown to modulate gut microbiota and impact the gut-brain axis (72). Our results support previous findings where Vivomixx® was associated with an enhanced inflammatory response, showing a decreased expression of some inflammatory cytokines in the gut, like IL-6 and TNF-α (73). Altogether, these findings suggest that probiotic supplementation may induce gut microbiota changes, which in turn, modulate in immune function and inflammatory responses not only in the gut but also in the brain.

When assessing the BBB integrity, ASD-transplanted mice fed with diet 3 showed increased claudin-5 levels in hippocampus compared to the ASD-transplanted mice fed with control diet. These results suggest an improvement in the integrity of the BBB after dietary intervention with a *L. paracasei* strain in combination with FOS/GOS. On the other hand, when assessing epithelial permeability in the ileum, diet 1 resulted in higher occludin levels compared to control diet, indicating an improvement in gut barrier integrity. As mentioned before, dietary-induced changes in metabolism might play an important role in enhancing the gut barrier permeability. Previous findings also show the positive effect of *L. paracasei* on the intestinal barrier in two mouse models of DSS-induce colitis (74,75). Guo *et al.* reported moderate increase in colonic tight junction proteins, being ZO-1, occludin and claudin-1, after *L. paracasei* supplementation (74). Similarly, Simeoli *et al* found that *a L. paracasei*-based synbiotic therapy (using FOS and arabinogalactan as prebiotics) improved gut barrier integrity, with an increase in ZO-1 and occludin levels (75). Additionally, symbiotic formulations have been previously investigated in mouse models for ASD. In one study using a BTBR mouse model, different dietary interventions including the administration of *Lactobacillus reuteri* RC-14® either alone or with 10% oligofructose-enriched inulin, led to improvements in both intestinal integrity and behavior (44). Another study using a valproic acid mouse model for ASD showed that a synbiotic diet composed by *L. reuteri* and inulin improved social behavior, restored gut epithelium integrity, and attenuated inflammatory responses in the brain (76). Taken together, our results highlight diet as a promising therapeutical strategy for modulating host's gut microbiota, inducing beneficial changes across the gut-brain axis

at immune, intestinal and brain levels. Nevertheless, further research is needed to elucidate the underlying mechanisms of this modulation and to understand how different diets may lead to specific changes.

4.3. Limitations of the study and future perspective.

One of the main limitations of this study is the high data variability within some groups in certain analyses. Variability in the analyses of the immunohistochemical staining of the colonic tissue with a marker for enteric glial cells might be attributed to performing in different batches, potentially causing variations in experimental conditions. Also, fluctuation in laser intensity of the microscope could have impacted the imaging, and in turn, the image analysis accuracy. Technical issues with the Western blot also prevented the analysis of some target proteins as originally planned. Furthermore, the small sample sizes in some groups contributed to the high variability in results. Increasing the number of subjects per group would allow for a more robust analysis and provide sufficient data in the event of technical issues.

It would also be useful to optimize the ELISA process for measuring IL-17A in the brain regions, which could give us more information about the inflammation signaling pathway. Other cytokines could also be studied in the future in the brain area, including IL-8 and IL-10, which have shown abnormal levels in serum of ASD patients (77). Also, recent findings point to the complement protein's role in neurodevelopmental diseases, and it might be interesting to study further their implication in the immune response in ASD subjects (78,79). Another approach for future research could include metabolomic analysis in brain, systemic and gut regions to identify key metabolites involved in signaling pathways relevant for ASD, such as neuroinflammation. Similarly, assessing gut microbiota composition would be very useful as most of the studies published taken together have a lack of consistency. Exploring the connections between gut microbiota composition, metabolite profiles, and cytokine production along the gut-brain axis following dietary interventions may give us a better understanding of how pro- and symbiotic supplementation affects this complex system.

Given the high prevalence of ASD and the incomplete understanding of its etiology, further investigation into its underlying mechanisms is essential for better diagnosis and the discovery of new therapeutic targets. This study shows that microbiota transplant from children diagnosed with ASD can induce changes in the ASD-like phenotype, including neuroinflammation and increased gut barrier permeability. Additionally, a probiotic and a synbiotic diet were able to induce changes in neuroinflammation, as well as in gut and brain barriers, by modulating gut microbiota composition. Interestingly, diet 1 was able to reduce neuroinflammation in PFC of ASD-transplanted BTBR mice compared to the group fed with control diet, showing a significant reduction of the inflammatory markers. Besides that, diet 1 showed an improvement in the gut barrier integrity of distal ileum, with a higher expression of E-cadherin, while diet 3 showed a better integrity of the BBB in the hippocampus, which increased levels of claudin-5, both compared to control diet. Although our results contribute to a better understanding on the brain and intestinal state in ASD, further research, further research needs to be done in order to elucidate the communication within the gut-brain axis and the intrinsic mechanism of the pathways

involved. Diet-induced microbiota changes are associated with reduced neuroinflammation and improved integrity of both intestinal and brain barriers, suggesting the therapeutic potential of gut microbiota-targeted diets for ASD.

5. References

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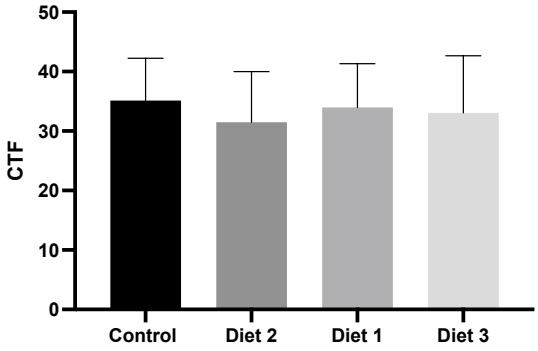
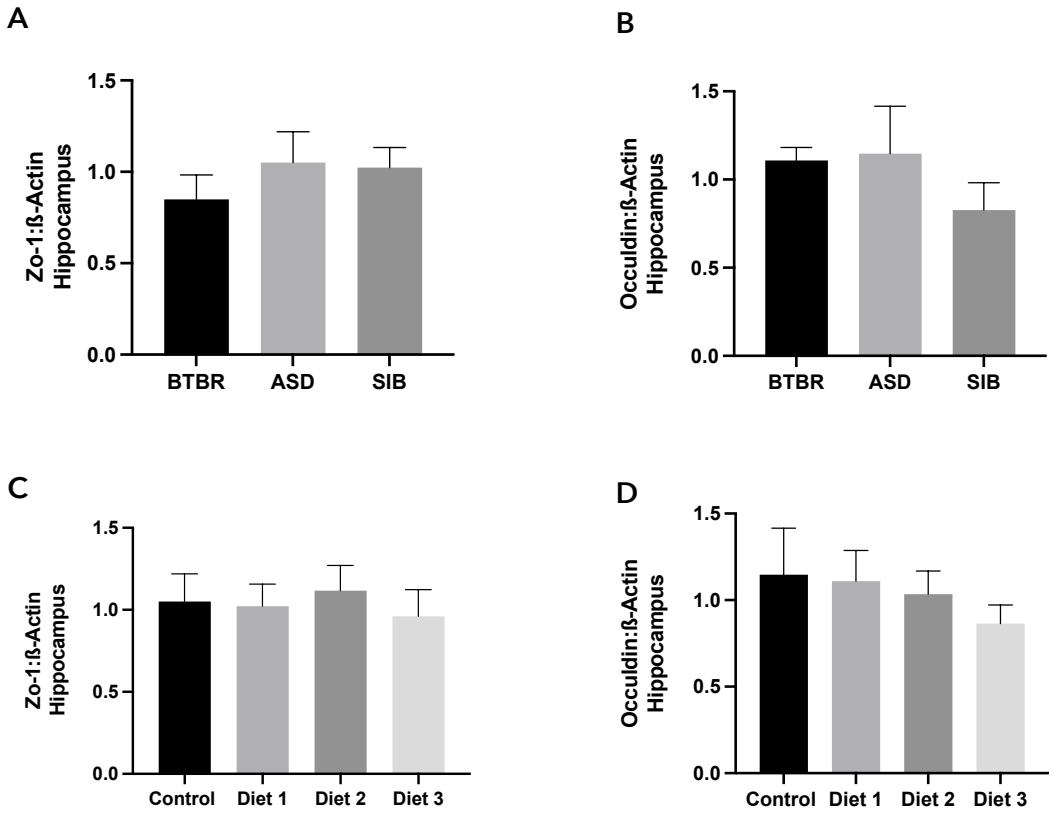
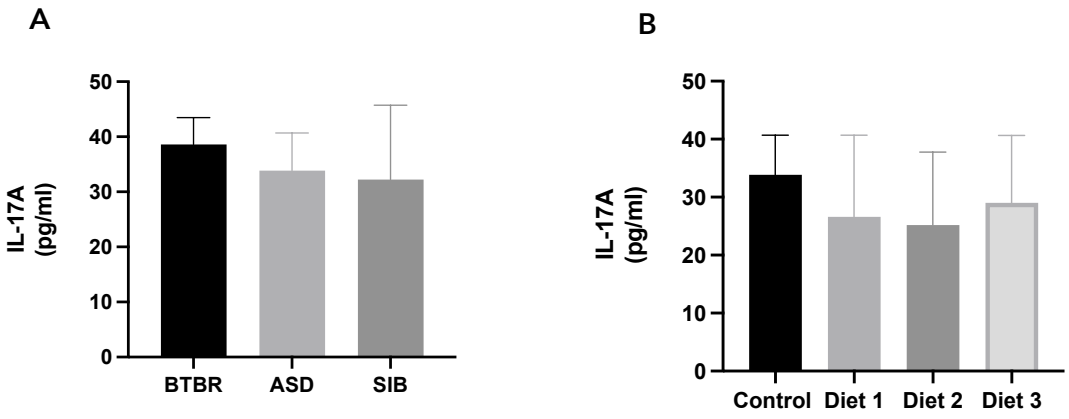
Supplementary Data



Supplementary Fig. 1 | Enteric glial cells staining in colonic tissues. Analysis of the corrected total fluorescence (CTF) of enteric glial cells when assessing different dietary interventions in ASD-transplanted BTBR mice. The data are represented as mean ± SD, n=6-10.



Supplementary Fig. 2 | Protein barrier levels in the prefrontal cortex and hippocampus. (A) ZO-1 and (B) occludin relative levels of BTBR, ASD and SIB mice and (C) ZO-1and (D) occludin relative levels of ASD-transplanted mice across different diets were in hippocampus were measured in hippocampus. Protein levels are relative to β-actin using Western blotting. Data represented as the mean ± SEM. n = 8-10.



Supplementary Fig. 3 | Concentration of IL-17 in PFC (A) Concentration of IL-17A in non-transplanted BTBR mice, ASD-transplanted mice and SIB-transplanted mice and (B) in ASD-transplanted mice fed with different diets were measured in PFC by ELISA. Data represented as the mean ± SEM. n = 8-10.