A.1: Applicant details:

Name & student number:	Youmna Al Halabi 5941172
Affiliation:	Utrecht university, department of pharmacology
Name direct supervisor:	Naika Prince
Name first examiner:	Dr. Paula Perez Pardo
Name second examiner:	Dr. Paul Henricks
Affiliation:	Utrecht university, department of pharmacology

B.1.1: TITLE:

The microbiome and intestinal mucosal immunity: a therapeutic target to alleviate neuroinflammation in autism spectrum disorders

CONTENTS

B.1.2: ABSTRACT:

Autism spectrum disorders (ASD) is a group of conditions that affect neurological development leading to abnormality in communication and social interactions. The microbiota plays a crucial role in modulating mucosal immune cells and the immune link connecting the gut-brain axis. In ASD, there is an imbalance in the dominant T cell subset and inflammatory cytokine levels which has been linked to intestinal dysbiosis. A shift towards the helper T (Th) cell subsets, Th1, Th2 and Th17 over regulatory T cells (Tregs) has been reported. The shift results in an increase in pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines. The disruption in the composition of the microbiota has also been associated with a reduction in the blood brain barrier (BBB) integrity and an altered microglial activation, and localization. Previous studies have highlighted the ability of pro-inflammatory cytokines produced in the gut mucosa to migrate, interact and cross the BBB hence elevating neuroinflammation. This proposal aims to restore cytokine balance and equilibrium among the T cell subsets located in the gut through modulating the microbiota. The involvement of intestinal mucosal immunity in neuroinflammatory processes in the brain makes intestinal immune cells a suitable target in our approach. Prebiotics, probiotics and synbiotics known for their efficacy in promoting anti-inflammatory conditions will be provided to an ASD mouse model which is expected to positively affect mucosal gut inflammation and neuroinflammation. The chosen treatment methodology will be expected to provide a cost-effective option that unlike current medications, is capable of alleviating multiple ASD symptoms.

B.1.3: LAYMAN'S SUMMARY:

Autism spectrum disorders (ASD) are a set of disorders whereby affected individuals showcase behavioral and communication patterns that are different from typically developing individuals. The gut bacteria plays a role in the development and functioning of the immune system. In ASD, there is an alteration in the bacterial composition which displays an effect on immune cells in the gut. Subsequently, there is a dominance of immune cells that increase inflammation in the intestine through the production of pro-inflammatory molecules. Moreover, modification of the gut bacteria in ASD has been linked to heightened inflammation in the body leading to inflammation in the brain and the deterioration of the blood brain barrier that prevents the entry of peripheral immune cells, pathogens and harmful molecules into the brain. In our approach we will attempt to use beneficial bacterial strains and food supplements to modulate the intestinal and neuronal immune system by improving gut bacterial composition. Our proposal will offer a way to resolve multiple ASD symptoms which is a outcome that is not achievable using current medications on the market. There is an increased need for a new approach that targets several ASD manifestations as the prevalence and economic burden associated with ASD are steadily rising.

B.1.4: KEYWORDS & ABBREVIATIONS:

Keywords:

Probiotics, prebiotics, synbiotics, ASD, neuroinflammation.

Abbreviations:

ASD = Autism spectrum disorders.

Short chain fatty acids = SCFAs.

Teffs = Effector T cells.

Th cells = Helper T cells.

Th1 cells = Type 1 helper T cells.

Th2 cells = Type 2 helper T cells.

Th17 cells = Type 17 helper T cells.

Tregs = Regulatory T cells.

Foxp3 = Forkhead box p3.

ROR-γ t = Retinoid-acid receptor-related orphan receptor-γ t.

GATA3 = GATA binding protein 3.

STAT3 = Signal transducer and activator of transcription 3.

T-bet = T-box transcription factor.

β2AR = **β**2 adrenergic receptor.

Occludin = OCLN.

Zonula occludens = ZO.

Interferon-γ = IFN-γ.

Transforming growth factor-**β** = TGF-**β**.

Gut brain axis = GBA.

Blood brain barrier = BBB.

Claudin = CLDN.

Germ free = GF.

Pathogen free = PF.

Aryl hydrocarbon receptor = AHR.

Vascular endothelial growth factor B = VEGF-B.

Bifidobacterium bifidum = B. bifidum.

Bifidobacterium lactis = B. lactis.

Lactobacillus casei = L. casei.

Lactobacillus acidophilus = L. acidophilus.

Galacto-oligosaccharides = GOS.

Fructo-oligosaccharides = FOS.

The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders = DSM-5.

Long chain FOS = lcFOS.

Short chain GOS = scGOS.

Oxygen consumption rate = OCR.

The acidification rate = ECAR.

The complement receptor 3 = CR3.

Health-related quality of life = HRQoL.

B.2.1: RESEARCH TOPIC

B.2.1.1: INSIGHT INTO THE PATHOPHYSIOLOGY OF AUTISM SPECTRUM DISORDERS:

Autism spectrum disorders (ASD) comprise a group of neurodevelopmental conditions whereby affected individuals showcase behavioural dysfunction, an abnormality in communication and language, and an impairment in social skills. Environmental factors as well as genetic predisposition play a significant role in the etiology of ASD (1). Environmental agents such as Immunological signaling proteins, chemicals and food polluting compounds function as regulators of ASD (2). The viral, bacterial, or parasitic stimulation of the maternal immune system during pregnancy can place children at a higher risk for ASD (3, 4). Moreover, the prenatal exposure to certain pesticides including organochlorine insecticides was found to significantly increase the likelihood of being diagnosed with ASD and has been correlated with some of the core characteristics seen in children with the disorders such as mitochondrial dysfunction and neuroinflammation (5).

B.2.1.2: THE MICROBIOTA-MUCOSAL IMMUNE INTERACTION IN ASD:

It is known that a symbiotic connection exists between the microbiota and the immune system of mammals. This connection is mediated using metabolites produced by the gut microbiota mainly, short chain fatty acids (SCFAs), which can affect the fate and function of mucosal immune cells (6). The interaction with the microbiota has been proven to be crucial for a healthy mucosal immunity hence, in ASD, a dysregulated immune response can be partially attributable to a disrupted microbiota.

Aberrations in the functions of effector T cells (Teffs) mainly, type 1 helper T (Th1) cells, type 2 helper T (Th2) cells, type 17 helper T (Th17) cells and regulatory T cells (Tregs) have been reported in ASD. An imbalance in the forkhead box P3 (Foxp3), retinoid-acid receptor-related orphan receptor-γ t (ROR-γ t), GATA binding protein 3 (GATA3), signal transducer and activator of transcription 3 (STAT3) and T-box transcription factor (T-bet) signaling can justify the dysregulation in the function of Th cells in autistic subjects (Table 1) (7). The expression of the proteins which act as regulators of Th1, Th2, Th17 cells and Treg function, were investigated in T cells belonging to children with ASD and their healthier counterparts in a paper by Ahmad et al hence providing insight on the mechanism through which the immune dysregulation in ASD is mediated (Table 1) (7).

Table 1: The T cell subsets that are altered in ASD, their function, transcription factors and levels in ASD. T-box transcription factor = T-bet, GATA binding protein 3 = GATA3, retinoid-acid receptor-related orphan receptor-γ t = ROR-γ t, forkhead box P3 = Foxp3, T-box transcription factor = T-bet, autism spectrum disorders = ASD, type 1 T helper cell = Th1, type 2 T helper cell = Th2, type 17 T helper cell = Th17, regulatory T cells = Tregs.

T cell subset	Function	Transcription factors	Levels in ASD
Th1 cells	Elimination of tumors. Response to bacterial, parasitic and viral infections. Development of hypersensitivity responses (8).	T-bet (7).	Higher in ASD subjects compared to controls (7, 9).
Th2 cells	Response to parasitic infections. Stimulation of tissue repair Mediating type 2 immune responses in allergies (10).	Gata3 (7).	Higher in ASD subjects compared to controls (7, 9).
Th17 cells	Involvement in host autoimmune responses which arise due to bacterial infections (11).	ROR-γ t (7).	Higher in ASD subjects compared to controls (7, 12).
Tregs	Maintenance of self tolerance. Inhibition of immune responses. Inhibition of anti-tumor immunity (13).	Foxp3 (7).	Lower in ASD subjects compared to controls (7, 14).

The imbalance in the presence of Th cells and Tregs in ASD has been speculated to arise as a consequence of the observed alterations in the gut microbiota. One proposed mechanism is that environmental factors can modify the composition of the microbiota and create a shift in the dominant microbial phyla in the gut which subsequently affects mucosal immune cells (15). This concept is supported by the fact that environmental factors such as psychological stressors, food or bacterial toxins can reduce the expression of the mRNA of tight junction proteins including occludin (OCLN) and zonula occludens (ZO)-2 resulting in a reduction in barrier integrity and an increase in intestinal epithelial permeability (16). The altered epithelial permeability enables the penetration of bacterial antigens through the epithelial barrier which eventually evokes resident mucosal immune cells and perturbs the microbiome habitat (17, 18) (Figure 1). Additionally, the relatively low presence of SCFA-producing bacteria and elevation in valeric acid producing bacteria in ASD plays a role in the determination of the

dominant T-lymphocyte lineage (19). Such a shift has a detrimental effect on Tregs as they mainly depend on oxidative metabolism of fatty acids to mediate their suppressive functions (20, 21). In contrast to Tregs, the Th1, Th2 and Th17 cells are not affected by this shift due to their reliance mainly on glycolysis to acquire energy (21). Another notion to consider lies in the fact that the exposure to physiological or psychological stressors may also trigger a surge in adrenaline release (17, 18, 22). Adrenaline can alter the fate of Th1 cells as those cells are the sole expressors of the **β**2 adrenergic receptor (**β**2AR) which can bind adrenaline. The agonism of **β**2AR prevents Th1 cell differentiation indirectly leading to the selection of other T-lymphocyte lineages mainly, Th2 and Th17 cells which explains the predominance of this phenotype in children with ASD (7, 22).

The disparity in Th cells/Tregs ratio and aberrant cytokine profile in autistic individuals can be explained by the proposed theories. In conclusion, the exposure to various environmental factors can decrease intestinal barrier integrity allowing toxins to penetrate the barrier leading to the promotion of the Th cell subsets as well as an increase in the release of pro-inflammatory cytokines including interferon-γ (IFN-γ), interleukin (IL)-13, IL-17 and IL-22 (22-24) (Figure 1). In addition to the increase in pro-inflammatory mediators, the decrease in Treg-released cytokines IL-10 and transforming growth factor-**β** (TGF-**β**) can eventually induce cytokine imbalance (25) (Figure 1).

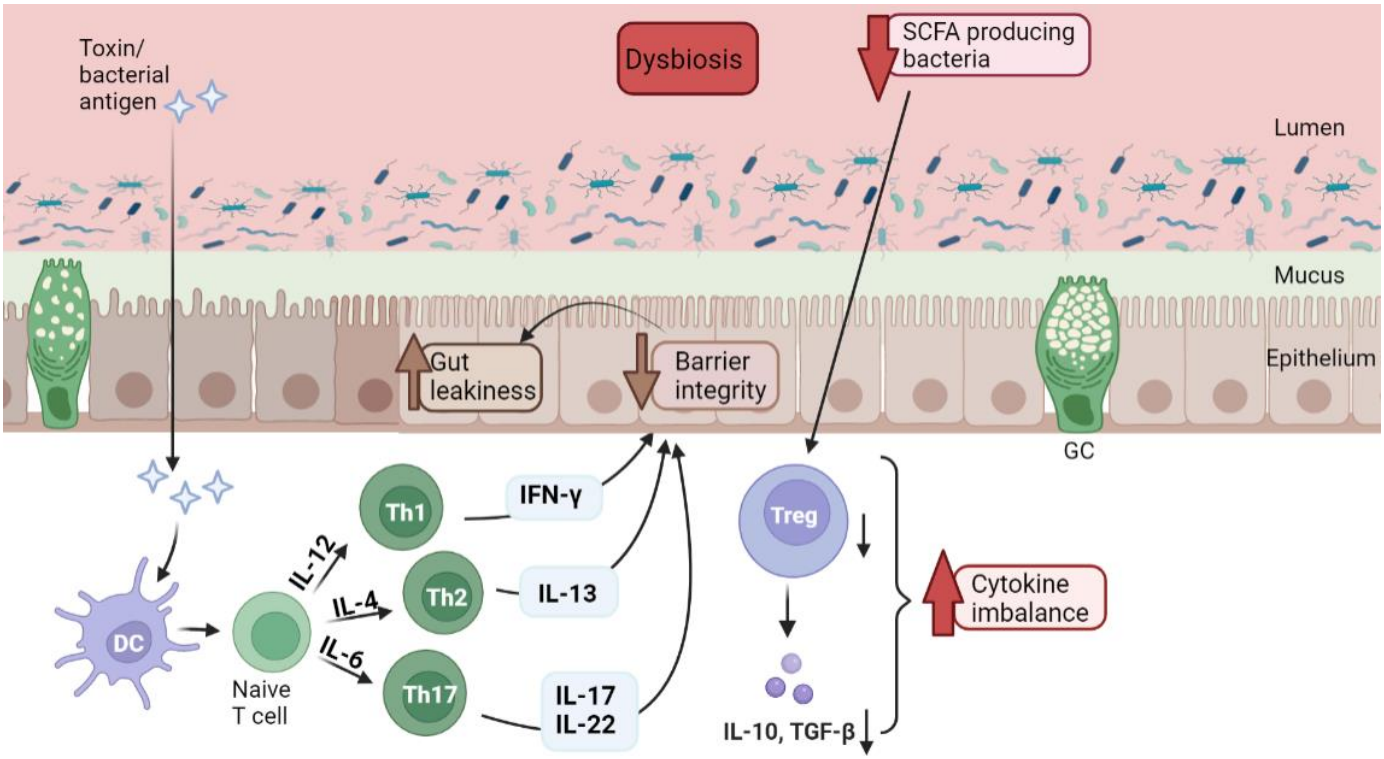

Figure 1: An overview of a possible pathway through which cytokine imbalance is promoted in ASD. Environmental factors (not shown) can induce intestinal barrier damage allowing bacterial toxin penetration. The presentation of bacterial toxins results in a shift in T cell subsets towards the helper T cell subset which leads to a decrease in intestinal barrier integrity and increased gut leakiness. The decrease in short chain fatty acid producing bacteria causes a drop in levels of regulatory T cells and an imbalance in the cytokine production. Goblet cells = GC, dendritic cells = DC, interleukin = IL, helper T cell = Th, regulatory T cell = Treg, short chain fatty acids = SCFA, transforming growth factor-**β** = TGF-**β** , interferon-γ = IFN-γ.

B.2.1.3: THE INFLUENCE OF THE MICROBIOME-IMMUNE SIGNALING ON THE GUT BRAIN AXIS AND NEUROINFLAMMATION IN ASD:

The gut and central nervous system are connected with endocrine, humoral, immune and neural links which permit bidirectional crosstalk hence creating a communication network known as the gut brain axis (GBA) (17). The microbiota is a vital contributor to the GBA and its components, including the gut mucosal immune system (26).

A disrupted microbiota distinguished by altered microbial diversity has been found to play a role in the initiation as well as progression of neuroinflammatory responses in the brain and the subsequent cognitive dysfunction (21, 27). The increase in inflammatory mediators secreted in the intestinal mucosa contribute to neuroinflammation (28, 29). The decrease in the integrity of the blood brain barrier (BBB) and activation of microglia characterize the neuroinflammatory response. The BBB itself is a structure made up of endothelial cells surrounding capillaries providing the brain with blood supply. It is selective of the molecules that are allowed to pass the lipid bilayer therefore preventing pathogens, peripheral immune cells and large molecules from passing through. The permeability and integrity of the BBB are regulated by several proteins which connect the intercellular spaces of the BBB (30) (Table 2). Compromised BBB serum markers as well as the expression of the BBB tight junction proteins, claudin (CLDN)-5, ZO-1 and OCLN were analyzed as shown in table 2 in efforts to fathom the link between microbiota alterations and neuroinflammation (31-33). The concurrent changes in the brain and gut of ASD individuals provide evidence regarding the influence of microbiota on the alterations in the BBB (27, 29).

Table 2: Evidence regarding the influence of the gut microbiota on the expression of tight junction proteins in the blood brain barrier (BBB). Concurrent defects in the gut and BBB as well as serum markers correlated with BBB damage can be detected in ASD. The lack of gut microbiota or gut dysbiosis negatively affects BBB integrity hence providing a link among gut microbiota condition and BBB permeability. Autism spectrum disorders = ASD, blood brain barrier = BBB, claudin = CLDN, occludin = OCLN, zonula occludens = ZO, short chain fatty acids = SCFAs, germ free mice = GF mice, pathogen free mice = PF mice.

Author	Experiment	Year	Outcome	Ref
Fiorentino et al	Tight junction protein expression analysis in ASD *postmortem* brain tissue & intestinal biopsies.	2016	Increase in CLDN-5 expression in ASD cortical and cerebellar tissue. Decrease in OCLN expression in ASD duodenum.	31
Braniste et al	An analysis of BBB tight junction components expression in GF mice with and without PF flora colonization.	2014	GF mice had a decreased expression of CLDN-5 & OCLN compared to PF mice. Increased expression of CLDN-5, ZO-1 & OCLN in the hippocampus, frontal cortex & striatum following GF mice exposure to PF gut microbiota. SCFAs increased OCLN in the hippocampus and cortex of GF mice.	32
Esnafoglu et al	The measurement of serum markers to evaluate BBB integrity in ASD children compared to healthy children.	2017	Children with ASD had significantly higher GFAP serum levels compared to healthy children which correlated with the severity of ASD symptoms.	33

The gut microbiota and the metabolites produced in the gut have an influence on microglial function and the neuropathology in ASD (34-36). The profile of bacterial metabolites found in fecal samples of ASD subjects differs from that of healthy subjects. For example, elevated concentrations of bacterial metabolites such as p-cresol were traced to the ASD-associated bacteria Bacteroidetes and Clostridia (37, 38). The exposure to p-cresol can increase the gene expression of the microglia associated marker CD68 as well as serum values of pro-inflammatory IL-1**β** all of which can worsen the severity of autism (35) (Table 3). CD68 as a marker shows involvement in microglia activation and neuronal loss (39). Additionally, the lack certain metabolites such as kynurenine can lead to microglia activation and can influence inflammation in the central nervous system (CNS). The bacterial metabolite kynurenine has been found to be regulator of the activation of microglia, an effect mediated through its binding to the aryl hydrocarbon receptor (AHR) (40). The precursor of kynurenine, tryptophan, has been reported to be found in low levels in subjects with ASD (41). When tryptophan is sufficiently present and metabolized, its metabolite kynurenine forms a bond with AHR and inhibits the expression of the vascular endothelial growth factor B (VEGF-B) by the microglia hence preventing astrocyte activation as well as promoting an anti-inflammatory effect (40-42). Lastly, bacterial metabolites are capable of influencing the microglia through T cells as many metabolites have been implicated in the maturation and fate of the T-

lymphocytes (28, 43). The major histocompatibility complex class II (MHC-II) is an important microglia surface receptor for microglia-T cell interaction. Based on the work of Pasciuto et al, the absence of MHC-II deemed the microglia unable to obtain an amoeboid morphology and become activated (36) (Table 3).

Table 3: An overview of the involvement of the gut microbiota in the expression of neuroinflammatory markers and microglial processes. Autism spectrum disorders = ASD, central nervous system = CNS, transforming growth factor-α = TGF-α, interleukin-1**β** = IL-1**β**, wild type = WT, major histocompatibility complex II = MHC II, germ free = GF, pathogen free = PF, aryl hydrocarbon receptor = AHR, vascular endothelial growth factor B = VEGF-B.

Author	Experiment	Year	Outcome	Ref
Sun et al	Measuring the expression of neuroinflammatory surface and serum markers following p-Cresol treatment of nephrectomised mice.	2020	Significant increase in IL-1**β** serum values. Increased microglia surface marker CD68 expression in the prefrontal cortex.	35
Pasciuto et al	The influence of the microbiota on T cells was assessed in GF and antibiotic cocktail treated PF mice and WT mice. The direct effect of T cells on microglia development was investigated in the brains of MHC II deficient and WT mice.	2020	Number of brain localized CD4+ T cells in WT mice were higher in comparison to GF and PF mice. Microglia belonging to MHC II deficient mice showed a decreased expression in genes of transcription factors involved in microglial development. Moreover, the depletion of CD4+ T cells inhibited the upregulation of surface microglial maturation markers.	36
Rothha-mmer et al	A mouse model whereby microglia are AHR deficient was created to investigate the effect of the bound AHR on microglia activation and CNS inflammation.	2018	Microglia deficient in AHR promoted a pro-inflammatory environment in the CNS by increasing the expression of VEGF-B. AHR deficient microglia had a reduced expression of TGF-α, a cytokine with anti-inflammatory effects on astrocytes in the CNS. The supplementation of a tryptophan depleted diet in hindered the recovery from CNS-induced symptoms in the mouse model of experimental autoimmune encephalomyelitis (EAE). The supplementation of tryptophan in AHR deficient and control EAE mice alleviated CNS related symptoms.	42

Finally, microglial density and localization was found to be altered in subjects with neuro- developmental and degenerative disorders (44-48). Changes in microglial density were correlated with the microbiota in a study by Erny et al (47). In the study, germ free (GF) mice had defective microglia and a lack of

heterogeneity in their localization which was reflected in the upregulation of survival and proliferation genes including, TGF-**β** and DNA damage inducible transcript 4 (47). In the ASD brain, the highest microglial density was marked in the grey matter of the dorsolateral prefrontal cortex (44) as well as the visual cortex (48).

As explained previously, ASD-related gut microbiota modulation can have its effect on mucosal immunity leading to an increase in pro-inflammatory cytokines and cytokine imbalance. The produced cytokines can migrate to the brain and interact with the BBB or even cross the BBB (49). Pro-inflammatory cytokines have been detected in the ASD brain (50-52) however, there is a lack of evidence on the presence of Th cells in the brain of ASD subjects. High levels of pro-inflammatory cytokines, especially Th1 cell produced IFN-γ, was found to be correlated with an increase in BBB permeability in the cerebellum and cerebrum (53) (Fig. 2). The production of the Th17 cell cytokines, IL-17, has also been proven to lead to remodelling of the BBB through affecting the expression of OCLN, ZO-1 and CLDN-5 (54) (Fig. 2). After crossing the BBB, IL-17 is capable of exacerbating the production of reactive oxygen species by microglia in the CNS (54). Whilst IL-22 activates and instructs microglia to produce tumor necrosis factor- α (TNF-α) in mice (55) (Fig. 2). The activation of resting microglia in the CNS changes their appearance and morphology from ramified to amoeboid as shown in figure 2 (56). The provided evidence links intestinal dysbiosis and gut microbiota alterations to markers of inflammation in the CNS. which are mainly, decreased BBB integrity, lack of heterogeneity in microglial localization and density as well as microglial activation.

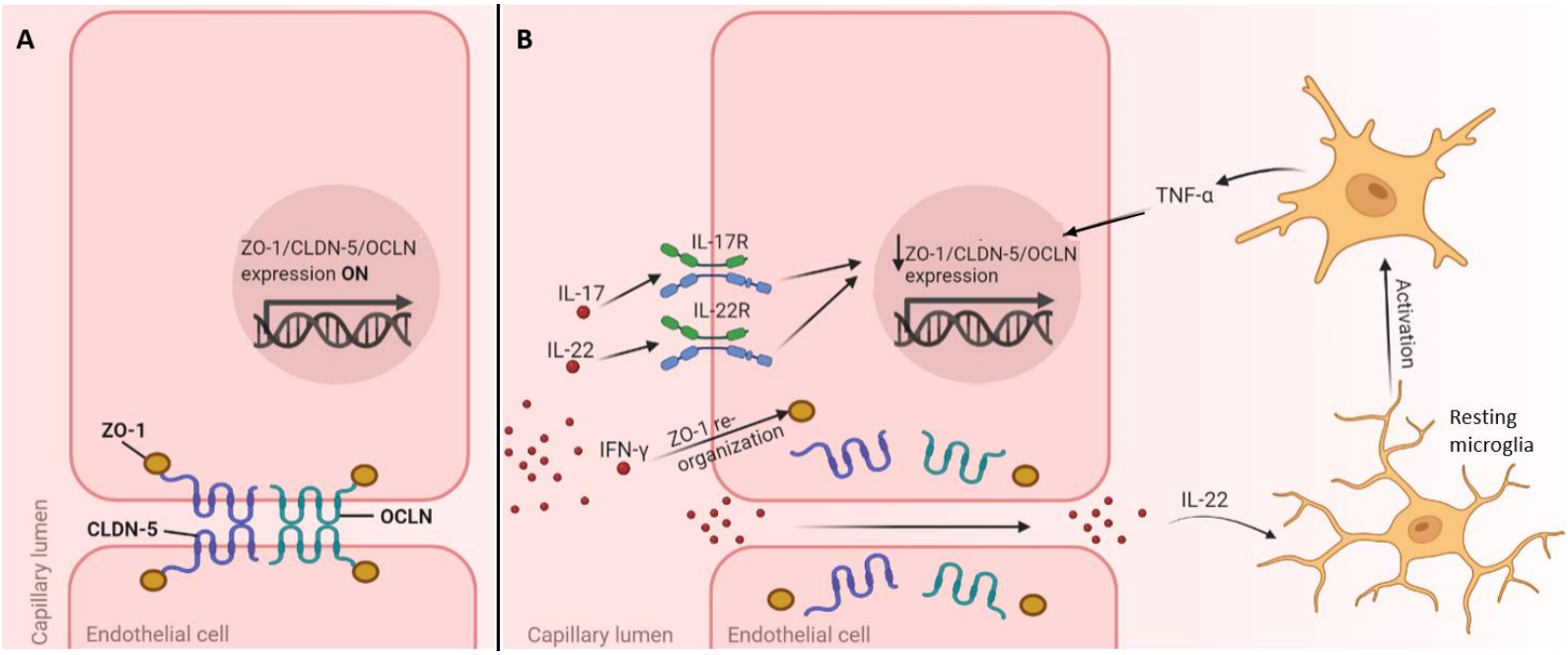

Figure 2: The variation in the permeability and integrity of the blood brain barrier (BBB) under a state of homeostasis (A) and in a pro-inflammatory state (B). Under normal non-inflammatory conditions, the tight junction proteins, ZO-1, CLDN-5 and OCLN maintain the integrity of the BBB (A). The imbalance in cytokine levels and overproduction of pro-inflammatory cytokines in the gut mucosa due to intestinal dysbiosis can affect the BBB negatively (B). The cytokines IL-17, IL-22 and IFN-γ have been implicated in the re-organization and down regulation of tight junction protein expression (B). The increased permeability of the BBB allows cytokines such as IL-22 to enter the CNS and activate resting microglia therefore aggravating the neuroinflammatory response (B). Claudin-5 = CLDN-5, zonula occludens-1 = ZO-1, occluding-5 = OCLN, tight junction = TJ, interleukin-17 = IL-17, interleukin-22 = IL-22, interleukin-17 receptor = IL-17R, interleukin-22 receptor = IL-22R, interferon-γ = IFN-γ, tumor necrosis factor- α = TNF-α.

B.2.1.4: THE USE OF PREBIOTICS AND PROBIOTICS IN MANAGEMENT OF ASD:

The current therapeutic strategies available aim to intervene with a single or a limited number of core symptoms in ASD which highlights its multifactorial nature and the lack of treatments that can alleviate all of ASD symptoms. At present, probiotics and prebiotics are being used to decrease ASD severity through relieving GI disturbances (57, 58). Probiotics can be defined as live bacteria known for their ability to enhance GI microflora and improve immune function. The bacterial strains, Bifidobacterium bifidum (B. bifidum), Bifidobacterium lactis (B. lactis), Lactobacillus casei (L. casei), Lactobacillus acidophilus (L. acidophilus) and other strains were reported to be the most promising for intestinal microbiota modulation (59). Prebiotics are constituted of dietary fibers such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), they are used by the GI microbiota and once degraded, SCFAs arise as by-products of prebiotic metabolism.

The therapeutic potential of prebiotics in disorders whereby microbiota dysfunction is involved in the pathogenesis has been investigated. Zaylaa et al have reported a list of probiotic strains that were most promising in inhibiting or dimming pro-inflammatory immune responses in a TNBS-induced colitis mouse model. L. acidophilus, B. bifidum and Lactobacillus rhamnosus were the most efficient in reducing the production of TNF-α, a pro-inflammatory cytokine that induces apoptosis in intestinal epithelial cells.

The same strains significantly reduced the levels of IL-17 and Th1-inducer, IL-6 in the mouse model as well as increased the expression of the Foxp3 Treg transcription factor in the mice. The results of the study highlighted the protective effects of the strains which was mediated through elevating the Treg response (60). Similarly, data pooled from several studies navigating the influence of prebiotics on metabolic diseases showed an anti-inflammatory response in the gut following FOS intake (61). The effect can be attributed to the ability of prebiotics in attenuating IFN-γ and IL-13 production (62). Lastly, synbiotic mixtures have been gaining popularity as they provide a synergetic effect by improving the growth of beneficial bacterial strains and hindering pathogenic bacteria from proliferating (63). A recent study by Ha et al provided a comparison on the results of probiotic and synbiotic mixture administration in terms of the immune response. The administration of the synbiotic mixture Lactobacillus gasseri and fermented Cudrania tricuspidate extract significantly decreased serum IFN-γ as well as IL-6 and TNF-α mRNA levels in the intestines of IBD mice (64). The potential of probiotics, prebiotics and synbiotics as immunomodulators makes them suitable candidates to treat intestinal mucosal inflammation and restore balance among pro- and anti-inflammatory cytokines in ASD. The suggested treatment methodology will be expected to dim the neuroinflammatory response by restoring immune homeostasis in the gut.

B.2.1.5: HYPOTHESIS & AIM:

Until now, the effect of pre- and probiotic, and synbiotic formula intake on the dysregulated T cell function as well as neuroinflammatory markers in ASD remain unelucidated. This proposal aims to investigate the therapeutic potential of prebiotic, probiotic and synbiotic administration on the intestinal mucosal inflammatory status and neuroinflammation in ASD, on the basis of their relevance in promoting an anti-inflammatory response in the gut as shown previously. The neuronal parameters which will be investigated will include BBB integrity as measured by ZO-1, occludin and CLDN-5 expression and the heterogeneity in microglial localization, and microglial activation which will be assessed by detecting microglial location in the brain as well as microglial morphology change. Moreover, immune parameters including pro- and anti-inflammatory cytokine balance, and T cell subset proportion in the gut mucosa will be analyzed by measuring the expression of T cell specific transcription factors, cytokine levels along with T-cell energy metabolism. It is hypothesized that rebalancing immune homeostasis using prebiotics, probiotics and synbiotics will positively influence neuroinflammatory parameters. Creating a balance among Th cells and Tregs as well as pro- and anti-inflammatory cytokines in the intestinal mucosa will be expected to lead to an increase in tight junction protein expression in the BBB and a shift in microglial activation as measured by their morphology together with their localization/density. Therefore, the research questions to be addressed are: Will the treatment with prebiotics, probiotics or synbiotics change the gut bacteria composition hence also creating a shift in the dominant microbial phyla towards SCFA-producing bacteria? Will the change in the bacterial composition using the mentioned treatment affect T-cell energy metabolism therefore restoring Treg suppressive function, Treg cell population and the balance among the Th cells/Tregs in ASD which will therefore restore cytokine balance? Will the effect on immune parameters be reflected on neuroinflammatory parameters as well hence, will the treatment shift microglial morphology, prevent microglial activation, improve BBB integrity and improve the lack of microglial localization/density in the CNS? The GF and antibiotic treated pathogen free (PF) ASD-induced rodent models will be utilized to answer the defined research questions.

B.2.2: RESEARCH APPROACH:

B.2.2.1: ASD ANIMAL MODEL:

Rationale:

To study the role of the microbiota on immune and neurological alterations in ASD, germ free (GF) and pathogen free (PF) mouse models will be used. Both models will be colonized with gut microbiota belonging to human ASD subjects then utilized to investigate the influence of the microbiome-host interaction and dysbiosis-related manifestations in ASD (26). GF mice do not possess measurable microorganisms whereas PF mice will be devoid of some but not all common microorganisms (65).

Procedure:

A GF C57BL/6 mouse model will be established as described in the paper by Qv et al (26). 3 week old PF C57BL/6 mice will be obtained from the Jackson laboratory (66). The PF mice will be treated with an antibiotic cocktail mainly, natamycin, neomycin, vancomycin and meropenem through oral gavage for a week period in the same doses used by Sun et al. Those antibiotics have been chosen for gut microbiota depletion as they are not absorbed in the intestines (67). Fecal samples will be obtained from ASD children who received diagnosis based on the criteria of the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). The samples will be transplanted to 4 week old mice by oral gavage and 4 groups will be created, GF mice obtaining ASD sample, antibiotic depleted PF mice obtaining ASD sample, and GF mice and antibiotic depleted PF mice that do not receive a transplantation. The progression of ASD will be assessed among the mice that receive the fecal matter transplantation and compared to the mice that that do not receive ASD fecal matter transplantation. In the period before and after the three weeks of the fecal matter transfer, the mice will be subjected to microbiome analysis and behavioral tests as done by Esser et al and Xiao et al, respectively, to assess ASD related changes in the gut microbiota, behavior and social characteristics (68, 69).

B.2.2.2: PREBIOTIC AND PROBIOTIC TREATMENT OF ASD ANIMAL MODELS:

Rationale:

The ability of the prebiotic and probiotic treatment in restoring gut microbiota balance thereby also reducing mucosal gut inflammation and neuroinflammation will be tested. Lactobacillus and Bifidobacterium strains will be utilized for their capability to produce SCFAs, which are metabolic by-products necessary for Tregs functionality (21, 70, 71). It is expected that the strains will demonstrate a potent immunomodulatory effect by decreasing Th cell responses (72). Dietary long chain FOS (lcFOS) and short chain GOS (scGOS) will be supplied to the mice. They will be expected to affect T cell differentiation directly and indirectly by acting as a substrate for probiotics and inducing an increased expression of Foxp3 (73).

Procedure:

After the ASD like mouse models have been established, the mice will be supplied with a probiotic mixture containing Lactobacillus plantarum (DSM 15313), Lactobacillus paracasei (DSM 13434), B. lactis (DSM 18352), B. longum (DSM 20219) (final concentration $1*10^9$ CFU/mL) using an intragastric

feeding tube or a prebiotic mixture of lcFOS and scGOS in pellet form or synbiotic mixture of both pre- and probiotics or a normal diet with no treatment as a control for a period of two weeks. Therefore, 16 groups will be presented. Behavioral tests and microbiome analysis will be conducted mid and after the two week treatment period to keep track of ASD progression. A health check will also be done to ensure that the mice maintain a normal weight and do not suffer from excessive side effects due to the treatment such as severe diarrhea.

B.2.2.3: IMMUNE PARAMETERS EVALUATION:

Rationale:

This step will evaluate whether the treatment with a probiotic, prebiotic or synbiotic mixture results in an alleviation in the immune dysregulation in an ASD mouse model. The metabolic requirements and levels of T cell subsets as well as the cytokine levels will constitute the measured immune parameters after receiving the various treatments. The metabolic requirements and pathways of the different T cell subsets will be measured as those can vary among Th cells and Tregs. As mentioned previously, Tregs showcase elevated fatty acid oxidation and Th cells have a greater reliance on glycolysis as a metabolic pathway making it possible to distinguish among the two subtypes (74). The transcription factors of the Th1, Th2, Th17 cells and Tregs will be used as markers during T cell immunophenotyping (75, 76). Finally, pro- and anti- inflammatory cytokine levels will be assessed which will be reflective of the most dominant T cell phenotype.

Procedure:

Following the treatment period of two weeks, the ex-GF and antibiotic depleted PF ASD mouse models will be sacrificed using intraperitoneal injection of sodium pentobarbital. Serum cytokines will be measured through the use of a multiarray cytokine assay (77) further, the cytokines which will be measured include IL-2, IL-10, IL-13, IL-17, IL-22, TGF-**β** and IFN-γ. T lymphocytes present in the mucosal layer of gut will be isolated using the protocol developed by Qiu et al (78). A portion of the lymphocytes will undergo immunophenotyping and another portion will be cultured to analyze their bioenergetics. Immunophenotyping will be done following the steps described by Malmhall et al.which utilized the expression of the Th cell and Treg transcription factors; T-bet, Gata3, ROR-γ t and Foxp3 (79). A seahorse extracellular flux analyzer will be used to measure the oxygen consumption rate (OCR) along with another parameter, the acidification rate (ECAR), as provided by van der Windt et al (80) which will provide insight into glycolysis as well as oxidative phosphorylation pathways.

B.2.2.4: NEUROINFLAMMATION EVALUATION:

Rationale:

The therapeutic effect of the proposed treatments on improving ASD related neuroinflammatory features including loss of BBB integrity and altered microglial morphology, and microglial cell density in the ASD brain will be assessed. It is speculated that restoring the balance among the T cell subtypes hence also reducing the levels of pro-inflammatory cytokines by improving the composition of the microbiota will mitigate neuroinflammation in ASD. The GF mouse model described in this proposal has been reported to showcase alterations in the BBB integrity which linked to the gut microbiota, a characteristic that made them ideal for mimicking ASD in human subjects. The main alterations were reported to be a

decrease in the expression of ZO-1, OCLN and CLDN-5 tight junction proteins in the BBB located in the striatum of the GF mice in comparison to antibiotic depleted PF mice. Furthermore, the colonization of GF mice with a bacterial strain mix. enhanced BBB integrity and reduced its permeability (32).

Procedure:

The brains of the sacrificed mice will be collected to create coronal sections or lyse the brains. Immunofluorescence will be conducted as described in the protocol by Braniste et al (32) in order to visualize OCLN and CLDN-5 BBB tight junction proteins in the brain sections and compare the differences among the treatment conditions. The sections will also be used to assess microglia density and distribution by immunostaining of the microglia surface marker F4/80 following the steps in the paper by Lawson et al (81). The complement receptor 3 (CR3), a phagocytic receptor used to distinguish activated microglia, will be stained using immunohistochemistry and the area of CR3 will be visualized according to the steps of Young et al to assess the morphology of the microglia in different area of the brain cross sections (82). The brain lysates will be utilized to establish the expression of OCLN, CLDN-5 and ZO-1 using western blot (WesternC chemiluminescence kit, Bio-Rad).

B.2.2.5: WORK PLAN:

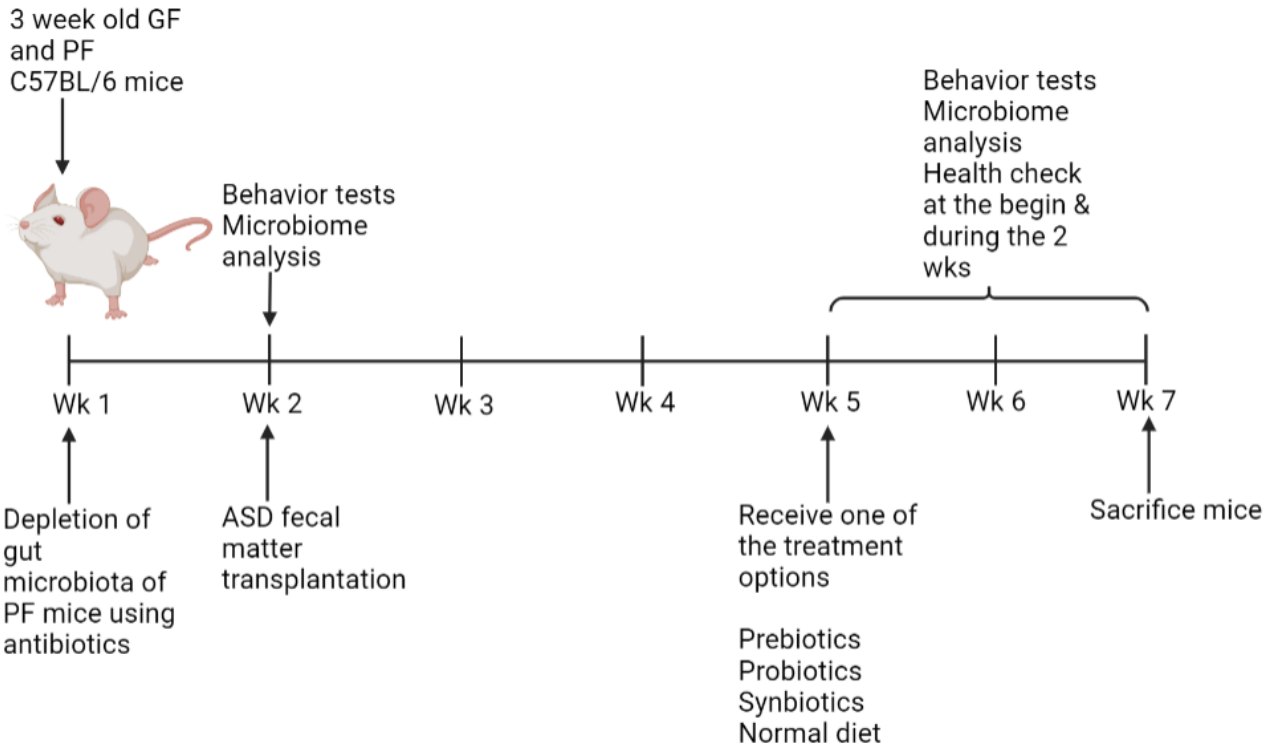

Figure 3: The timeline of germ free and pathogen free C57BL/6 mice preparation and ASD mouse model establishment prior to treatment exposure. Germ free = GF, pathogen free = PF, week = wk, autism spectrum disorder = ASD.

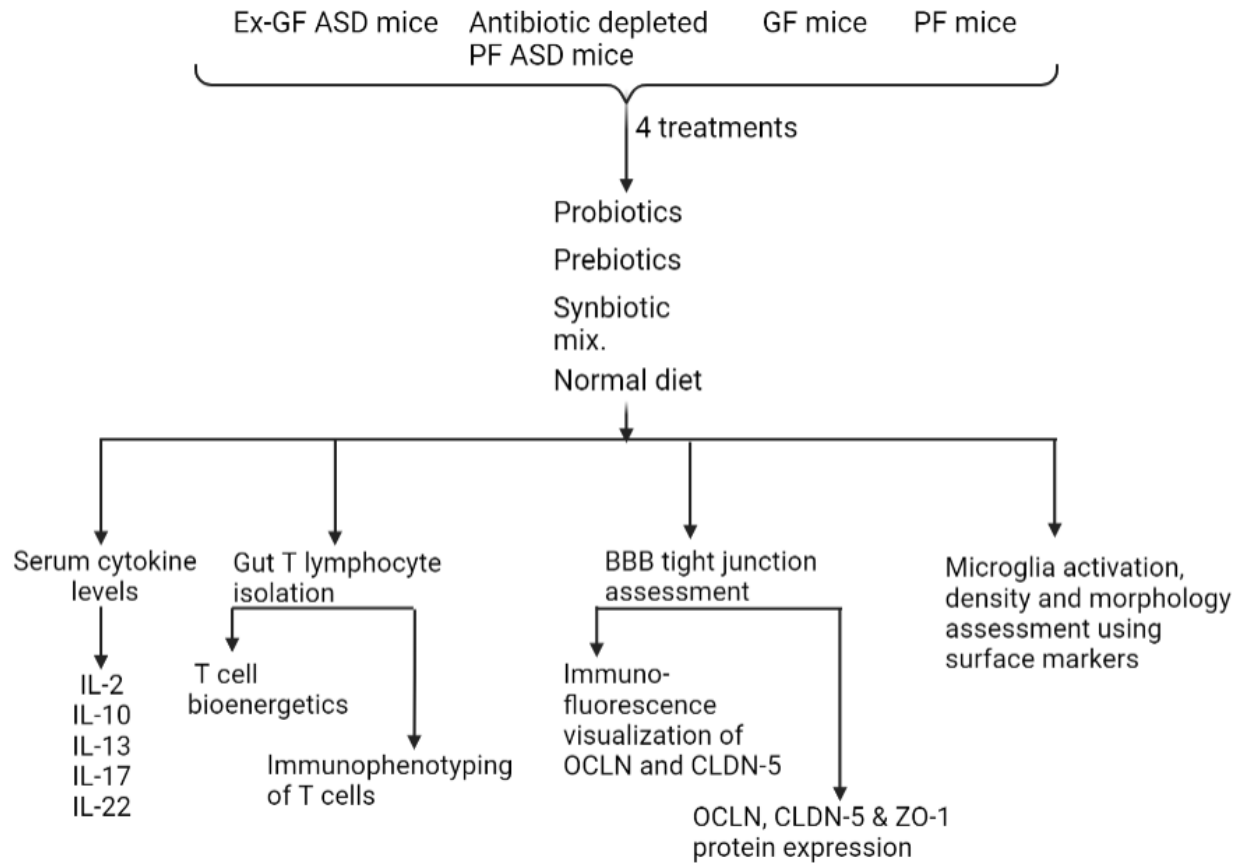

Figure 4: A scheme representing the ASD mouse groups, the treatments used and experiments conducted after the sacrificial of the mice. Interleukin = IL, germ free = GF, pathogen free = PF, autism spectrum disorder = ASD, germ free mice exposed to gut flora of autistic subjects = Ex-GF ASD mice, occludin = OCLN, claudin-5 = CLDN-5, zonula occludens-1 = ZO-1.

B.2.3: FEASIBILITY AND RISK ASSESSMENT:

The current project aims to alleviate neuroinflammation and immune dysfunction in ASD mouse models by targeting the gut microbiota using prebiotics and probiotics. The rationale behind this approach lies in the fact that the gut mucosal immune system is highly affected by the gut microbiota and its composition. Additionally, the imbalance in the T cell subsets and excessive release of pro-inflammatory mediators have been linked to neuroinflammation in ASD. Hence, the goal is to restore homeostasis of the microbiota which is expected to promote an anti-inflammatory mucosal immune response and attenuate neuroinflammation.

The proposed plan makes use of GF and antibiotic treated-PF mice transplanted with fecal matter of ASD individuals. Both models have made it possible to assess the manner through which the microbiota regulate disease. The chosen animal models will allow us to introduce known microbes and test whether certain bacterial strains, in our case ASD-related bacterial strains, play a causative effect in the disease state which is considered a strength in the approach. In terms of feasibility, microbiota depletion using broad spectrum antibiotic is cheaper and easier to achieve. The breeding or maintenance of GF mice is laborious, costly and can require special equipment, and skills (83). Moreover, the development of the immune system of GF mice is affected due to the lack of exposure to microbes during development (84-

87). The issue of proper immune system maturation is not present with antibiotics treated mice however, such treatment regimen may allow for fungus growth (88-90). A factor which makes using GF mice more favorable than antibiotic treated-PF mice is that oral antibiotic intake may disrupt the microbiota of the respiratory tract and permit the development of antibiotic resistance (91-93). Antibiotic intake may cause a decrease in the bacterial load and a shift in the bacterial populations as well as an incomplete clearance of bacteria. Moreover, the depletion of the microbiota might deplete immune cells necessary for antigen presentation such as dendritic cells. Despite some of the drawbacks associated with the mouse models, the GF mouse model remains as the gold standard for microbiota studies and the antibiotic treated-PF mice offer a suitable substitute or a comparable model to the GF mouse model. GF mice will practically serve as the most suitable as they elicit alterations in the BBB associated with the microbiota, which does not require any genetic modifications (83). Lastly, in terms of time, GF and PF mice can be purchased from Charles River Laboratories in order to reduce the amount of time and effort required to create the models. The animals will be raised and treated in the central laboratory animal research facility of Utrecht. Gnotobiotic GF isolators will be purchased and used to house the GF mice.

The feasibility and safety of prebiotic, and probiotic use has to be evaluated prior to the approval of their use on ASD subjects. Previous studies have discussed the ability of prebiotics, probiotics and synbiotics in improving microbiota composition, intestinal discomfort and immune regulation in subjects with disorders characterized by microbiota distortion, such as ASD and IBD (12-16). The administration of a probiotics formula was found to improve core symptoms mainly, GI-related symptoms in autistic children (94). Moreover, probiotics intake has been linked to an increase in the release of anti-inflammatory cytokines, an effect suggested to arise due to the ability of probiotics in competitively inhibiting the binding of pathogens to the intestinal epithelium (95, 96). As for prebiotics, the short-term administration of GOS accompanied by an exclusion diet in a cohort of autisitc children resulted in a significant alteration in the excreted metabolites of the children which reflected the change in the dominant microbial phyla in the gut (97). Finally, the short-term ingestion of a synbiotic mixture by adults with IBD has been reported to significantly reduce the expression of TNF-α a, a cytokine that is crucial for orchestrating and modulating pro-inflammatory immune responses (98). The highlighted studies provide evidence on the efficacy of prebiotics, probiotics and synbiotics in ameliorating manifestations and symptoms associated with gut dysbiosis; however, their safety is still a matter of investigation. The risk associated with receiving probiotic supplements is the highest in individuals with compromised immune function as they may exhibit non-specific immune activation shortly following probiotics administration. In the case of individuals with ASD which suffer from an improper regulation of their immune system, receiving multiple bacterial strains at once may place them at risk of experiencing a heightened immune-stimulating response. The response may vary from person to person and can be specific to the type of probiotic mixture or even strains (99). Additionally, based on the paper of Sorokulova, the toxicity of Bacillus strains is highly dependent on the dose as well as the administration route making the evaluation of the animal studies safety data challenging (100).
The current animal models offer a limited and simplified alternative to testing in humans which elicit more complex interactions in the gut environment (99). When it comes to colonization, there is a lack of data focusing on the persistence or long-term bacterial colonization of the GI in adults following probiotic supplementation. For example, bacterial strains including Lactobacillus casei can showcase a low division rate which can prevent long-term colonization (101). Unlike probiotics, prebiotics supplementation has not been linked to any major risk in disorders encompassing a distorted gut microbiota (102).

B.2.4 (A): SCIENTIFIC IMPACT:

Unlike conventional ASD medications, prebiotics, probiotics and synbiotics are capable of improving GI dysfunction (103, 104). Additionally, some evidence has been provided on the positive impact of bacterial metabolites of non-pathogenic bacteria on ASD social behaviour and repetitive behaviour (105, 106). Such evidence eludes to the assumption that many ASD symptoms can be altered by altering the microbiome and highlights the importance of diversity in the GI microbiome.

In a paper by Yap et al, researchers have argued that subjects with ASD elicit a decreased microbiome diversity due to lacking diversity in their diet (107). This explanation does not acknowledge the role of altered immune function and reformed immune-microbial interactions on the GI microbiome in ASD. In the same paper, the degree of autism and symptoms of the individuals are not communicated which disregards many factors. The first factor is that autism is a spectrum, meaning that not all ASD subjects exhibit similar severity of symptoms, especially behavioural symptoms. Secondly, not all individuals with ASD are selective in their eating habits. In this proposal, we aim to provide evidence regarding the significant influence of gut microbiota constituents on modulating mucosal inflammation and neuroinflammation in ASD. The proposed prebiotics, probiotics and synbiotics are expected to restore a healthy gut microbiota hence also restoring the balance between Th cells and Tregs, and reducing proinflammatory cytokine levels as a result. Probiotics have been reported to modulate immune responses and prevent the aggregation of pathogenic bacteria to the intestinal barrier in IBD (95). Prebiotic intake has been reported to increase the amount of symbiotic bacteria in the colon of an IBD mouse model hence elevating the production of SCFAs. Lastly, synbiotics were found to aid in higher effectivity in the attachment of probiotics to the intestinal wall as well as decrease proinflammatory (108). Cytokine dysregulation can induce neurological changes in the brain of ASD subjects (109) hence, by re-establishing a normal cytokine profile and preventing cytokine imbalance, the treatments are expected to positively influence the BBB integrity, microglial activation and the lack of heterogeneity of microglial density. The experiments in an ASD mouse model are expected to highlight the therapeutic ability of prebiotics, probiotics and symbiotics in restoring mucosal immune homeostasis therefore subsequently reducing neuroinflammation. The results will be anticipated to provide a solid foundation for further research in human ASD subjects.

B.2.4 (B): SOCIETAL AND ECONOMIC IMPACT:

According to data released in 2010, the number of global ASD cases have been estimated to be as high as 52 million cases. A prevalence rate of 7.5 and 7.6 per 1000 has been reported for ASD globally in each of 1990 and 2010, respectively (110). The health-related (HR) quality of life (QoL) or HRQoL, which estimates the influence of health in the mental, physical, social and emotional domains on the QoL, has been utilized to assess the functioning of adults with ASD. Based on a study by Khanna et al, the mental and physical aspects of HRQoL of a group of 291 autistic adults were found to be significantly lower when compared to healthy adults in the united states. Moreover, the increase in the severity of autism was correlated with a decrease in the mental QoL scores (111). A low or poor QoL has also been linked to worse social functioning in adults with ASD (112). Recent reports that summarize population ASD epidemiological data have highlighted the significant increase in ASD prevalence in the general population of developed countries (113-115). An increased prevalence can be attributed to the enhanced diagnostic practices, overall awareness as well as the increased exposure to certain chemicals (115). The

elevation in the number of cases was also coupled to an escalation in the economic burden of ASD. According to the Dutch association for autism, the societal and production loss costs caused by autism in 2015 were estimated at 268 trillion dollars or 236.8 trillion euros (116). The costs were expected to rise significantly within a 10 year period (117). Currently, the approved pharmacological therapies are not capable of targeting several symptoms at a time (118). For example, antipsychotics are widely prescribed to ameliorate behavioural symptoms including irritability, hyperactivity and withdrawal (118, 119). In study by Horlin et al, the costs associated with ASD in terms of diagnosis, treatment and loss of income in a cohort of western Australian children with ASD were calculated. Additionally, the percentage of costs attributed to the treatment of ASD were provided. According to their findings, each family with an ASD child had an estimated annual costs of almost 35 thousand Australian dollars or 22 thousand euros. Pharmacotherapy and the treatments offered to the autistic children made up 8% of the annual declared costs (120). The study offered an insight on the contribution of ASD medication and treatment to the overall annual costs spent on ASD. Considering the limited number of ASD symptoms that current drugs can treat and the costs associated with these drugs, a more affordable therapeutic approach which targets a wider range of symptoms must be investigated.

This project aims to showcase the health benefits and cost effectiveness associated with utilizing prebiotics and probiotics. In terms of their availability, probiotics, especially lactobacillus and bifidobacterial strains, can be found in fermented food such as yoghurt, kefir and tofu (121, 122). FOS can naturally be found in fruits such as bananas while GOS can be obtained from human milk (123, 124). Moreover, some probiotic bacterial strains, prebiotics and synbiotics which have been tested and approved for general use can be found in the form of supplements which makes accessing them easy to the public (125, 126). The findings and outcome of microbiome research for developmental disorders should be communicated more clearly with the public to expand the scale of use as microbiome-targeted interventions (127, 128). The cost effectiveness of the use of probiotics has been previously addressed in a large scale study. Probiotics were found to decrease the health care costs in comparison to conventional pharmacotherapy after treating american subjects with respiratory tract infections (129). Similar studies that discuss the cost-benefit ratio associated with probiotic, prebiotic and synbiotic intake on subjects with ASD are sparse. Regardless, the results of the study can be translated and utilized in different sectors such as that of neurodevelopmental disorders. Most importantly, the results have eluded to the importance of exploration of the cost-effectiveness of nutritional supplements in ASD treatment.

Finally, communicating the manner and duration of prebiotic, probiotic, and synbiotic consumption as found by clinical research is not a light task. However, it is a task that should be taken in order to inform and enhance the public perception on such nutritional interventions that are highly accessible and offer a wide range of benefits for a lower relative cost.

B.2.5: ETHICAL CONSIDERATIONS:

The current proposal requires the use of a large number of mice which may raise some ethical concerns and considerations. The sample size and number of mice required per group will be calculated using the G*power statistical tool. The RIVM policies require the use of animals to be reduced, refined or replaced with robust methods to decrease the need for animal testing. To reduce the number of mice used per experimental group, an in vitro BBB model can be used. The BBB 3D model can be constructed using primary cells and grown in transwells as described by Stone et al (130). An ASD BBB model can be

induced by subjecting the barrier to pro-inflammatory cytokines or bacterial metabolites known to decrease the integrity of the BBB. To assess the effect of T cell subsets on the BBB integrity, serum of ASD rodents can be collected and used in the BBB cell culture. Further, to assess the effect of the prebiotics, probiotics or synbiotics on BBB integrity and immune regulation, the rodents will be treated with one of the three options or supplied with a control diet. Serum will be collected again from the rodents at different points during the treatment period and supplied to the BBB in transwells then BBB integrity will be evaluated using transepithelial electrical resistance and immunological staining of tight junction proteins. Such model does not directly integrate ques from the microbiota however, it may replace the need to test in animal models.

REFERENCES:

1. Yenkoyan K, Grigoryan A, Fereshetyan K, Yepremyan D. Advances in understanding the pathophysiology of autism spectrum disorders. Behavioural brain research. 2017;331:92-101.
2. Banerjee S, Riordan M, Bhat MA. Genetic aspects of autism spectrum disorders: insights from animal models. Frontiers in cellular neuroscience. 2014;8:58.
3. Bilbo SD, Block CL, Bolton JL, Hanamsagar R, Tran PK. Beyond infection - Maternal immune activation by environmental factors, microglial development, and relevance for autism spectrum disorders. Experimental neurology. 2018;299(Pt A):241-51.
4. Spann MN, Sourander A, Surcel H-M, Hinkka-Yli-Salomäki S, Brown AS. Prenatal toxoplasmosis antibody and childhood autism. Autism research : official journal of the International Society for Autism Research. 2017;10(5):769-77.
5. Shelton JF, Hertz-Picciotto I, Pessah IN. Tipping the balance of autism risk: potential mechanisms linking pesticides and autism. Environmental health perspectives. 2012;120(7):944-51.
6. Roussin L, Prince N, Perez-Pardo P, Kraneveld AD, Rabot S, Naudon L. Role of the Gut Microbiota in the Pathophysiology of Autism Spectrum Disorder: Clinical and Preclinical Evidence. Microorganisms. 2020;8(9).
7. Ahmad SF, Zoheir KMA, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, et al. Dysregulation of Th1, Th2, Th17, and T regulatory cell-related transcription factor signaling in children with autism. Molecular neurobiology. 2017;54(6):4390-400.
8. Xu H, Timares L, Elmets CA. 19 - Host Defenses in Skin. In: Rich RR, Fleisher TA, Shearer WT, Schroeder HW, Frew AJ, Weyand CM, editors. Clinical Immunology (Fifth Edition). London: Elsevier; 2019. p. 273-83.e1.
9. Gupta S, Aggarwal S, Rashanravan B, Lee T. Th1- and Th2-like cytokines in CD4+ and CD8+ T cells in autism. Journal of neuroimmunology. 1998;85(1):106-9.
10. Walker JA, McKenzie ANJ. TH2 cell development and function. Nature Reviews Immunology. 2018;18(2):121-33.
11. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity. 2008;28(4):454-67.
12. Al-Ayadhi LY, Mostafa GA. Elevated serum levels of interleukin-17A in children with autism. Journal of Neuroinflammation. 2012;9(1):158.
13. Kondělková K, Vokurková D, Krejsek J, Borská L, Fiala Z, Ctirad A. Regulatory T cells (TREG) and their roles in immune system with respect to immunopathological disorders. Acta medica (Hradec Kralove). 2010;53(2):73-7.
14. Ellul P, Rosenzwajg M, Peyre H, Fourcade G, Mariotti-Ferrandiz E, Trebossen V, et al. Regulatory T lymphocytes/Th17 lymphocytes imbalance in autism spectrum disorders: evidence from a meta-analysis. Molecular autism. 2021;12(1):68.
15. Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain-gut-enteric microbiota axis. Nature reviews Gastroenterology & hepatology. 2009;6(5):306-14.
16. Liu Z, Li N, Neu J. Tight junctions, leaky intestines, and pediatric diseases. Acta Paediatrica. 2005;94(4):386-93.
17. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Annals of gastroenterology. 2015;28(2):203-9.
18. Demaude J, Salvador-Cartier C, Fioramonti J, Ferrier L, Bueno L. Phenotypic changes in colonocytes following acute stress or activation of mast cells in mice: implications for delayed epithelial barrier dysfunction. Gut. 2006;55(5):655-61.
19. Liu S, Li E, Sun Z, Fu D, Duan G, Jiang M, et al. Altered gut microbiota and short chain fatty acids in Chinese children with autism spectrum disorder. Scientific Reports. 2019;9(1):287.
20. Kempkes RWM, Joosten I, Koenen HJPM, He X. Metabolic Pathways Involved in Regulatory T Cell Functionality. Frontiers in Immunology. 2019;10(2839).
21. Peralta-Marzal LN, Prince N, Bajic D, Roussin L, Naudon L, Rabot S, et al. The Impact of Gut Microbiota-Derived Metabolites in Autism Spectrum Disorders. International journal of molecular sciences. 2021;22(18).
22. Powell N, Walker MM, Talley NJ. The mucosal immune system: master regulator of bidirectional gut–brain communications. Nature Reviews Gastroenterology & Hepatology. 2017;14(3):143-59.
23. Smyth D, Phan V, Wang A, McKay DM. Interferon-γ-induced increases in intestinal epithelial macromolecular permeability requires the Src kinase Fyn. Laboratory Investigation. 2011;91(5):764-77.

24. Li M, Oshima T, Ito C, Yamada M, Tomita T, Fukui H, et al. Glutamine Blocks Interleukin-13-Induced Intestinal Epithelial Barrier Dysfunction. Digestion. 2021;102(2):170-9.
25. Komai T, Inoue M, Okamura T, Morita K, Iwasaki Y, Sumitomo S, et al. Transforming Growth Factor-β and Interleukin-10 Synergistically Regulate Humoral Immunity via Modulating Metabolic Signals. Frontiers in Immunology. 2018;9.
26. Qv L, Yang Z, Yao M, Mao S, Li Y, Zhang J, et al. Methods for Establishment and Maintenance of Germ-Free Rat Models. Frontiers in Microbiology. 2020;11(1148).
27. Eissa N, Sadeq A, Sasse A, Sadek B. Role of Neuroinflammation in Autism Spectrum Disorder and the Emergence of Brain Histaminergic System. Lessons Also for BPSD? Frontiers in Pharmacology. 2020;11(886).
28. Gabriel T, Paul S, Berger A, Massoubre C. Anorexia Nervosa and Autism Spectrum Disorders: Future Hopes Linked to Mucosal Immunity. Neuroimmunomodulation. 2019;26(6):265-75.
29. Gładysz D, Krzywdzińska A, Hozyasz KK. Immune Abnormalities in Autism Spectrum Disorder—Could They Hold Promise for Causative Treatment? Molecular neurobiology. 2018;55(8):6387-435.
30. Eshraghi RS, Davies C, Iyengar R, Perez L, Mittal R, Eshraghi AA. Gut-Induced Inflammation during Development May Compromise the Blood-Brain Barrier and Predispose to Autism Spectrum Disorder. Journal of clinical medicine. 2020;10(1).
31. Fiorentino M, Sapone A, Senger S, Camhi SS, Kadzielski SM, Buie TM, et al. Blood-brain barrier and intestinal epithelial barrier alterations in autism spectrum disorders. Molecular autism. 2016;7:49.
32. Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Tóth M, et al. The gut microbiota influences blood-brain barrier permeability in mice. Science translational medicine. 2014;6(263):263ra158.
33. Esnafoglu E, Ayyıldız SN, Cırrık S, Erturk EY, Erdil A, Daglı A, et al. Evaluation of serum Neuron-specific enolase, S100B, myelin basic protein and glial fibrilliary acidic protein as brain specific proteins in children with autism spectrum disorder. International Journal of Developmental Neuroscience. 2017;61:86-91.
34. Choi J, Lee S, Won J, Jin Y, Hong Y, Hur TY, et al. Pathophysiological and neurobehavioral characteristics of a propionic acid-mediated autism-like rat model. PLoS One. 2018;13(2):e0192925.
35. Sun C-Y, Li J-R, Wang Y-Y, Lin S-Y, Ou Y-C, Lin C-J, et al. p-Cresol Sulfate Caused Behavior Disorders and Neurodegeneration in Mice with Unilateral Nephrectomy Involving Oxidative Stress and Neuroinflammation. International journal of molecular sciences. 2020;21(18):6687.
36. Pasciuto E, Burton OT, Roca CP, Lagou V, Rajan WD, Theys T, et al. Microglia Require CD4 T Cells to Complete the Fetal-to-Adult Transition. Cell. 2020;182(3):625-40.e24.
37. Davoli-Ferreira M, Thomson CA, McCoy KD. Microbiota and Microglia Interactions in ASD. Frontiers in Immunology. 2021;12(2024).
38. Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Elevated fecal short chain fatty acid and ammonia concentrations in children with autism spectrum disorder. Digestive diseases and sciences. 2012;57(8):2096-102.
39. Hopperton KE, Mohammad D, Trépanier MO, Giuliano V, Bazinet RP. Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: a systematic review. Molecular Psychiatry. 2018;23(2):177-98.
40. Wekerle H. Brain inflammatory cascade controlled by gut-derived molecules. Nature. 2018;557(7707):642-3.
41. Kałużna-Czaplińska J, Jóźwik-Pruska J, Chirumbolo S, Bjørklund G. Tryptophan status in autism spectrum disorder and the influence of supplementation on its level. Metab Brain Dis. 2017;32(5):1585-93.
42. Rothhammer V, Borucki DM, Tjon EC, Takenaka MC, Chao C-C, Ardura-Fabregat A, et al. Microglial control of astrocytes in response to microbial metabolites. Nature. 2018;557(7707):724-8.
43. Kopec AM, Fiorentino MR, Bilbo SD. Gut-immune-brain dysfunction in Autism: Importance of sex. Brain research. 2018;1693(Pt B):214-7.
44. Morgan JT, Chana G, Pardo CA, Achim C, Semendeferi K, Buckwalter J, et al. Microglial activation and increased microglial density observed in the dorsolateral prefrontal cortex in autism. Biological psychiatry. 2010;68(4):368-76.
45. Sankowski R, Ahmari J, Mezö C, Hrabě de Angelis AL, Fuchs V, Utermöhlen O, et al. Commensal microbiota divergently affect myeloid subsets in the mammalian central nervous system during homeostasis and disease. The EMBO journal.n/a(n/a):e108605.

46. Mossad O, Erny D. The microbiota-microglia axis in central nervous system disorders. Brain pathology (Zurich, Switzerland). 2020;30(6):1159-77.
47. Erny D, Hrabě de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nature neuroscience. 2015;18(7):965-77.
48. Tetreault NA, Hakeem AY, Jiang S, Williams BA, Allman E, Wold BJ, et al. Microglia in the cerebral cortex in autism. Journal of autism and developmental disorders. 2012;42(12):2569-84.
49. Gwak M-G, Chang S-Y. Gut-Brain Connection: Microbiome, Gut Barrier, and Environmental Sensors. Immune Netw. 2021;21(3).
50. Masi A, Glozier N, Dale R, Guastella AJ. The Immune System, Cytokines, and Biomarkers in Autism Spectrum Disorder. Neurosci Bull. 2017;33(2):194-204.
51. Li X, Chauhan A, Sheikh A, Patil S, Chauhan V, Li X-M, et al. Elevated Immune Response in the Brain of Autistic Patients. Journal of neuroimmunology. 2009;207:111-6.
52. Xu N, Li X, Zhong Y. Inflammatory Cytokines: Potential Biomarkers of Immunologic Dysfunction in Autism Spectrum Disorders. Mediators of Inflammation. 2015;2015:531518.
53. Chai Q, He WQ, Zhou M, Lu H, Fu ZF. Enhancement of blood-brain barrier permeability and reduction of tight junction protein expression are modulated by chemokines/cytokines induced by rabies virus infection. J Virol. 2014;88(9):4698-710.
54. Cipollini V, Anrather J, Orzi F, Iadecola C. Th17 and Cognitive Impairment: Possible Mechanisms of Action. Frontiers in Neuroanatomy. 2019;13.
55. Lee D, Jo H, Go C, Jang Y, Chu N, Bae S, et al. The Roles of IL-22 and Its Receptor in the Regulation of Inflammatory Responses in the Brain. International Journal of Molecular Sciences. 2022;23(2):757.
56. Lier J, Streit WJ, Bechmann I. Beyond Activation: Characterizing Microglial Functional Phenotypes. Cells. 2021;10(9):2236.
57. Abdellatif B, McVeigh C, Bendriss G, Chaari A. The Promising Role of Probiotics in Managing the Altered Gut in Autism Spectrum Disorders. International journal of molecular sciences. 2020;21(11).
58. Ng QX, Loke W, Venkatanarayanan N, Lim DY, Soh AYS, Yeo WS. A Systematic Review of the Role of Prebiotics and Probiotics in Autism Spectrum Disorders. Medicina (Kaunas, Lithuania). 2019;55(5).
59. Shi LH, Balakrishnan K, Thiagarajah K, Mohd Ismail NI, Yin OS. Beneficial Properties of Probiotics. Tropical life sciences research. 2016;27(2):73-90.
60. Zaylaa M, Al Kassaa I, Alard J, Peucelle V, Boutillier D, Desramaut J, et al. Probiotics in IBD: Combining in vitro and in vivo models for selecting strains with both anti-inflammatory potential as well as a capacity to restore the gut epithelial barrier. Journal of Functional Foods. 2018;47:304-15.
61. Costa GT, Vasconcelos QDJS, Aragão GF. Fructooligosaccharides on inflammation, immunomodulation, oxidative stress, and gut immune response: a systematic review. Nutrition Reviews. 2021.
62. Tsuda M, Arakawa H, Ishii N, Ubukata C, Michimori M, Noda M, et al. Dietary Fructo-Oligosaccharides Attenuate Early Activation of CD4⁺ T Cells Which Produce both Th1 and Th2 Cytokines in the Intestinal Lymphoid Tissues of a Murine Food Allergy Model. International Archives of Allergy and Immunology. 2017;174(3-4):121-32.
63. Parian AM, Mullin GE, Langhorst J, Brown AC. Chapter 50 - Inflammatory Bowel Disease. In: Rakel D, editor. Integrative Medicine (Fourth Edition): Elsevier; 2018. p. 501-16.e8.
64. Ha J, Oh H, Oh NS, Seo Y, Kang J, Park MH, et al. Anti-Inflammatory Effect of a Peptide Derived from the Synbiotics, Fermented Cudrania tricuspidata with Lactobacillus gasseri, on Inflammatory Bowel Disease. Mediators of Inflammation. 2020;2020:3572809.
65. Dobson GP, Letson HL, Biros E, Morris J. Specific pathogen-free (SPF) animal status as a variable in biomedical research: Have we come full circle? EBioMedicine. 2019;41:42-3.
66. Zhang C, Franklin CL, Ericsson AC. Consideration of Gut Microbiome in Murine Models of Diseases. Microorganisms. 2021;9(5):1062.
67. Sun N, Hu H, Wang F, Li L, Zhu W, Shen Y, et al. Antibiotic-induced microbiome depletion in adult mice disrupts blood-brain barrier and facilitates brain infiltration of monocytes after bone-marrow transplantation. Brain, Behavior, and Immunity. 2021;92:102-14.
68. Xiao L, Yan J, Yang T, Zhu J, Li T, Wei H, et al. Fecal Microbiome Transplantation from Children with Autism Spectrum Disorder Modulates Tryptophan and Serotonergic Synapse Metabolism and Induces Altered Behaviors in Germ-Free Mice. mSystems. 2021;6(2).

69. Esser C, Hochrath K, Teichweyde N, Krutmann J, Chang H-D. Beyond sequencing: fast and easy microbiome profiling by flow cytometry. Archives of Toxicology. 2019;93(9):2703-4.
70. Hati S, Patel M, Mishra BK, Das S. Short-chain fatty acid and vitamin production potentials of Lactobacillus isolated from fermented foods of Khasi Tribes, Meghalaya, India. Annals of Microbiology. 2019;69(11):1191-9.
71. Usta-Gorgun B, Yilmaz-Ersan L. Short-chain fatty acids production by Bifidobacterium species in the presence of salep. Electronic Journal of Biotechnology. 2020;47:29-35.
72. Kim HW, Hong R, Choi EY, Yu K, Kim N, Hyeon JY, et al. A Probiotic Mixture Regulates T Cell Balance and Reduces Atopic Dermatitis Symptoms in Mice. Frontiers in microbiology. 2018;9:2414-.
73. Pujari R, Banerjee G. Impact of prebiotics on immune response: from the bench to the clinic. Immunology & Cell Biology. 2021;99(3):255-73.
74. Cluxton D, Petrasca A, Moran B, Fletcher JM. Differential Regulation of Human Treg and Th17 Cells by Fatty Acid Synthesis and Glycolysis. Frontiers in immunology. 2019;10:115-.
75. Staats J. Immunophenotyping of Human Regulatory T Cells. Methods Mol Biol. 2019;2032:141-77.
76. T cell immunophenotyping Abcam.com2020 [cited 2021. Available from: https://www.abcam.com/primary-antibodies/t-cells-basic-immunophenotyping.
77. Burguillos M. Use of Meso-Scale Discovery™ to Examine Cytokine Content in Microglia Cell Supernatant. Methods Mol Biol. 2013;1041:93-100.
78. Qiu Z, Sheridan BS. Isolating Lymphocytes from the Mouse Small Intestinal Immune System. J Vis Exp. 2018(132).
79. Malmhäll C, Bossios A, Rådinger M, Sjöstrand M, Lu Y, Lundbäck B, et al. Immunophenotyping of circulating T helper cells argues for multiple functions and plasticity of T cells in vivo in humans--possible role in asthma. PLoS One. 2012;7(6):e40012.
80. van der Windt GJW, Chang CH, Pearce EL. Measuring Bioenergetics in T Cells Using a Seahorse Extracellular Flux Analyzer. Curr Protoc Immunol. 2016;113:3.16b.1-3.b.4.
81. Lawson LJ, Perry VH, Dri P, Gordon S. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience. 1990;39(1):151-70.
82. Young KF, Gardner R, Sariana V, Whitman SA, Bartlett MJ, Falk T, et al. Can quantifying morphology and TMEM119 expression distinguish between microglia and infiltrating macrophages after ischemic stroke and reperfusion in male and female mice? Journal of Neuroinflammation. 2021;18(1):58.
83. Kennedy EA, King KY, Baldridge MT. Mouse Microbiota Models: Comparing Germ-Free Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria. Frontiers in Physiology. 2018;9(1534).
84. Liu L, Zhu G. Gut–brain axis and mood disorder. Frontiers in psychiatry. 2018;9:223.
85. Gonzalez-Perez G, Hicks AL, Tekieli TM, Radens CM, Williams BL, Lamousé-Smith ES. Maternal antibiotic treatment impacts development of the neonatal intestinal microbiome and antiviral immunity. The Journal of Immunology. 2016;196(9):3768-79.
86. Deshmukh HS, Liu Y, Menkiti OR, Mei J, Dai N, O'leary CE, et al. The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. Nature medicine. 2014;20(5):524-30.
87. Lamousé-Smith ES, Tzeng A, Starnbach MN. The intestinal flora is required to support antibody responses to systemic immunization in infant and germ free mice. PLoS One. 2011;6(11):e27662.
88. Grasa L, Abecia L, Forcén R, Castro M, de Jalón JAG, Latorre E, et al. Antibiotic-Induced Depletion of Murine Microbiota Induces Mild Inflammation and Changes in Toll-Like Receptor Patterns and Intestinal Motility.
89. Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, et al. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. PLoS One. 2011;6(3):e17996.
90. Zákostelská Z, Málková J, Klimešová K, Rossmann P, Hornová M, Novosádová I, et al. Intestinal microbiota promotes psoriasis-like skin inflammation by enhancing Th17 response. PLoS One. 2016;11(7):e0159539.
91. Brown R, Sequeira R, Clarke T. The microbiota protects against respiratory infection via GM-CSF signaling. Nat Commun 8: 1512. 2017.
92. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity. 2012;37(1):158-70.
93. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proceedings of the National Academy of Sciences. 2011;108(13):5354-9.

94. Santocchi E, Guiducci L, Prosperi M, Calderoni S, Gaggini M, Apicella F, et al. Effects of Probiotic Supplementation on Gastrointestinal, Sensory and Core Symptoms in Autism Spectrum Disorders: A Randomized Controlled Trial. Frontiers in Psychiatry. 2020;11(944).
95. Vanderpool C, Yan F, Polk BD. Mechanisms of probiotic action: Implications for therapeutic applications in inflammatory bowel diseases. Inflammatory Bowel Diseases. 2008;14(11):1585-96.
96. Yan F, Polk DB. Probiotics and Probiotic-Derived Functional Factors—Mechanistic Insights Into Applications for Intestinal Homeostasis. Frontiers in Immunology. 2020;11(1428).
97. Grimaldi R, Gibson GR, Vulevic J, Giallourou N, Castro-Mejía JL, Hansen LH, et al. A prebiotic intervention study in children with autism spectrum disorders (ASDs).
98. Steed H, Macfarlane GT, Blackett KL, Bahrami B, Reynolds N, Walsh SV, et al. Clinical trial: the microbiological and immunological effects of synbiotic consumption – a randomized double-blind placebo-controlled study in active Crohn's disease. Alimentary Pharmacology & Therapeutics. 2010;32(7):872-83.
99. Sanders ME, Akkermans LM, Haller D, Hammerman C, Heimbach J, Hörmannsperger G, et al. Safety assessment of probiotics for human use. Gut microbes. 2010;1(3):164-85.
100. Sorokulova I. Preclinical testing in the development of probiotics: a regulatory perspective with Bacillus strains as an example. Clin Infect Dis. 2008;46 Suppl 2:S92-5; discussion S144-51.
101. Lee YK, Ho PS, Low CS, Arvilommi H, Salminen S. Permanent colonization by Lactobacillus casei is hindered by the low rate of cell division in mouse gut. Appl Environ Microbiol. 2004;70(2):670-4.
102. Armstrong H, Mander I, Zhang Z, Armstrong D, Wine E. Not All Fibers Are Born Equal; Variable Response to Dietary Fiber Subtypes in IBD. Frontiers in Pediatrics. 2021;8(924).
103. Patusco R, Ziegler J. Role of Probiotics in Managing Gastrointestinal Dysfunction in Children with Autism Spectrum Disorder: An Update for Practitioners. Adv Nutr. 2018;9(5):637-50.
104. Tan Q, Orsso CE, Deehan EC, Kung JY, Tun HM, Wine E, et al. Probiotics, prebiotics, synbiotics, and fecal microbiota transplantation in the treatment of behavioral symptoms of autism spectrum disorder: A systematic review. Autism Res. 2021;14(9):1820-36.
105. Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, et al. Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. Cell. 2019;177(6):1600-18.e17.
106. Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, et al. The Microbiota-Gut-Brain Axis. Physiol Rev. 2019;99(4):1877-2013.
107. Yap CX, Henders AK, Alvares GA, Wood DLA, Krause L, Tyson GW, et al. Autism-related dietary preferences mediate autism-gut microbiome associations. Cell. 2021;184(24):5916-31.e17.
108. Akutko K, Stawarski A. Probiotics, Prebiotics and Synbiotics in Inflammatory Bowel Diseases. Journal of Clinical Medicine. 2021;10(11):2466.
109. Goines PE, Ashwood P. Cytokine dysregulation in autism spectrum disorders (ASD): possible role of the environment. Neurotoxicol Teratol. 2013;36:67-81.
110. Baxter AJ, Brugha TS, Erskine HE, Scheurer RW, Vos T, Scott JG. The epidemiology and global burden of autism spectrum disorders. Psychol Med. 2015;45(3):601-13.
111. Khanna R, Jariwala-Parikh K, West-Strum D, Mahabaleshwarkar R. Health-related quality of life and its determinants among adults with autism. Research in Autism Spectrum Disorders. 2014;8(3):157-67.
112. Leader G, Barrett A, Ferrari C, Casburn M, Maher L, Naughton K, et al. Quality of life, gastrointestinal symptoms, sleep problems, social support, and social functioning in adults with autism spectrum disorder. Research in Developmental Disabilities. 2021;112:103915.
113. Data & Statistics on Autism Spectrum Disorder https://www.cdc.gov/ [cited 2022 04-01-2022]. Available from: https://www.cdc.gov/ncbddd/autism/data.html.
114. Lyall K, Croen L, Daniels J, Fallin MD, Ladd-Acosta C, Lee BK, et al. The Changing Epidemiology of Autism Spectrum Disorders. Annu Rev Public Health. 2017;38:81-102.
115. Bölte S, Girdler S, Marschik PB. The contribution of environmental exposure to the etiology of autism spectrum disorder. Cellular and Molecular Life Sciences. 2019;76(7):1275-97.
116. Maatschappelijke kosten voor autisme rijzen de pan uit https://www.autisme.nl/2015 [cited 2022 06-01]. Available from: https://www.autisme.nl/2015/10/07/maatschappelijke-kosten-voor-autisme-rijzen-de-pan-uit/.
117. Rogge N, Janssen J. The Economic Costs of Autism Spectrum Disorder: A Literature Review. J Autism Dev Disord. 2019;49(7):2873-900.

118. LeClerc S, Easley D. Pharmacological therapies for autism spectrum disorder: a review. P T. 2015;40(6):389-97.
119. Shenoy MD, Indla V, Reddy H. Comprehensive Management of Autism: Current Evidence. Indian J Psychol Med. 2017;39(6):727-31.
120. Horlin C, Falkmer M, Parsons R, Albrecht MA, Falkmer T. The Cost of Autism Spectrum Disorders. PLOS ONE. 2014;9(9):e106552.
121. Behera SS, Ray RC, Zdolec N. Lactobacillus plantarum with Functional Properties: An Approach to Increase Safety and Shelf-Life of Fermented Foods. Biomed Res Int. 2018;2018:9361614-.
122. D'Aimmo MR, Mattarelli P, Biavati B, Carlsson NG, Andlid T. The potential of bifidobacteria as a source of natural folate. J Appl Microbiol. 2012;112(5):975-84.
123. Niittynen L, Kajander K, Korpela R. Galacto-oligosaccharides and bowel function. Scand J Food Nutr. 2007;51(2):62-6.
124. Coulston AM, Rock CL, Monsen ER. Preface. In: Coulston AM, Rock CL, Monsen ER, editors. Nutrition in the Prevention and Treatment of Disease. San Diego: Academic Press; 2001. p. xvii-xviii.
125. Pandey KR, Naik SR, Vakil BV. Probiotics, prebiotics and synbiotics- a review. J Food Sci Technol. 2015;52(12):7577-87.
126. Spacova I, Dodiya HB, Happel A-U, Strain C, Vandenheuvel D, Wang X, et al. Future of Probiotics and Prebiotics and the Implications for Early Career Researchers. Front Microbiol. 2020;11:1400-.
127. Cunningham M, Azcarate-Peril MA, Barnard A, Benoit V, Grimaldi R, Guyonnet D, et al. Shaping the Future of Probiotics and Prebiotics. Trends in Microbiology. 2021;29(8):667-85.
128. Shan Y, Segre JA, Chang EB. Responsible stewardship for communicating microbiome research to the press and public. Nature Medicine. 2019;25(6):872-4.
129. Lenoir-Wijnkoop I, Merenstein D, Korchagina D, Broholm C, Sanders ME, Tancredi D. Probiotics Reduce Health Care Cost and Societal Impact of Flu-Like Respiratory Tract Infections in the USA: An Economic Modeling Study. Frontiers in Pharmacology. 2019;10(980).
130. Stone NL, England TJ, O'Sullivan SE. A Novel Transwell Blood Brain Barrier Model Using Primary Human Cells. Frontiers in cellular neuroscience. 2019;13(230).