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Prebiotics Supplementation In Patients With Drug-Resistant Epilepsy: A Pilot Italian Study

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Introduction

Growing evidence shows that the gut microbiota has the ability to influence and modulate essential functions for host homeostasis, including metabolism, cardiovascular functions, as well as the immune/inflammatory processes[1]. Moreover, the gut microbiota may act on neural development, neuroinflammation, activation of stress response, neurotransmission, and on behaviors such as sociability and anxiety. At the same time, brain influences the composition of the gut microbiota and modulates the gastrointestinal (GI) tract. This close bidirectional communication between the brain and the gut is yet known as the microbiota-gut brain axis (MGBA)[2].

Nowadays, multiple interventions directly acting on this gut microbiota are available and either probiotics, prebiotics, symbiotic, diet, or fecal microbiota transplantation may be used as a supplementary treatment in a wide range of neurodevelopmental disorders including epilepsy. Particularly, up to 25-30% of patients do not respond to common anti-seizure medications (ASMs) being defined as drug-resistant epilepsy (DRE)[3]. In this context regulating brain activity through gut microbiota-driven approaches may prevent the use of more invasive treatments such as the vagal nerve stimulation (VNS) or epilepsy surgery[4].

The microbiota-gut brain axis

The gut microbiota is a highly dynamic and complex system which counts approximately 10^{14} cells of 1000 different species[5], having ~150 times more genetic material than the human genome[6]. The human gut microbiota mainly consists of bacteria, however also viruses, fungi, archaea, and parasites can reside in the GI tract[7]. Overall, the bacterial composition of the gut microbiota is mainly comprised of five phyla of which about 90% are *Firmicutes* and

Bacteroidetes, whereas the remaining are *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*[8].

Nowadays knowledge points toward the ability of the brain to produce substances and/or send signals which may influence the composition of the gut microbiota and modulate the GI tract, by regulating motility, secretion, absorption, and blood flow. Meanwhile, the gut produces a wide range of neurotrophic substances, including short chain fatty acids (SCFAs) (e.g. acetate, propionate, and butyrate), neurotransmitters (e.g. γ-aminobutyric acid (GABA), serotonin, and acetylcholine) and amino acids (e.g. tryptophan (TRP), tyrosine, and phenylalanine)[9,10,11], that may in turn impact on brain functions and behaviors[2,12]. For instance, the gut microbiota can potentially influence the central nervous system (CNS) through various pathways including the endocrine, vagus nerve-dependent and immune signaling, as well as the direct action of microbial products (the so called metabolites) which can act as signaling molecules in the brain[13,14,15,16,17,18].

Neural communication is mainly conducted through the enteric nervous system (ENS), one of the three branches of the autonomic nervous system (ANS). While the ENS interacts with the CNS via neurotransmitters such as adrenaline, noradrenaline, and acetylcholine[19]; on the other hand, the intestinal microbiota regulates the electrophysiological thresholds of the ENS' neurons[20] and a strong support derives from the evidence that germ-free (GF) mice show decreased excitability of myenteric sensory neurons as compared to normal mice[21]. Commensal intestinal microbiota are necessary for normal excitability of gut sensory neurons and thus provide a potential mechanism for the transfer of information between the gut and the nervous system[21]. Moreover, the gut microbiota may indirectly control neurotransmitter synthesis by stimulating the enteroendocrine and neuroendocrine cells[22,23], modifying the available

precursors of neuroactive chemicals, and even impacting on the expression of neurotransmitter-related genes at a transcriptional level[24]. Finally, the gut bacteria play a crucial role in the initial colonization and homeostasis of glial cells in the intestinal mucosa[25].

The immune system is another way through which the gut microbiota and the brain may communicate[26,27]. As a matter of fact, the gut houses the gut-associated lymphoid tissues (GALT) which protect the body from microbial invasion via the gut. A variety of gut and GALT immune cells, such as T cells, macrophages, and dendritic cells (DCs) can cross the blood-brain barrier (BBB) and affect neuronal and glia functioning in the brain[28]. Additionally, the systemic circulating immune factors (e.g., cytokines and chemokines) can influence the brain via the vagus nerve and circumventricular organs[29]. Once in the brain, the pro-inflammatory cytokines can trigger further neuro-inflammation in the nervous system, thereby causing increased permeability of the BBB[30]. Moreover, cytokines directly act by altering the concentrations of several neurotransmitters in the brain, including serotonin, dopamine, and glutamate[31]. Non-inflammatory cytokines also serve as mediators for intestinal microbes to regulate brain functioning[19].

Throughout life, several exogenous and/or endogenous factors can impact on the gut microbiota composition, possibly leading to a condition known as dysbiosis. Some growing evidence shows that dysbiosis could be causally linked to a wide range of GI, systemic, as well as neurological diseases[7,12]. For example, antibiotic exposure in the neonatal period has shown to induce a reduction in the levels of plasma granulocyte colony stimulating factor (G-CSF) in mice models[32]. In turn, the G-CSF can stimulate neurogenesis in the brain by crossing the BBB and hence providing a potential therapeutic agent for normal brain development[19].

In early life, cesarean section, antibiotics exposure, diet, as well as other environmental factors or habits may distort the establishment of a normal-well-composed microbiome, consequently adversely affecting health throughout one's lifespan[33,22]. Indeed, the development of our core microbiota occurs in parallel with the growth, maturation, and sprouting of neurons in the young brain[34], and unlike common thought yet the bacterial presence has been demonstrated in the intrauterine environment, strongly suggesting its influence on brain development even before birth[35,36,37,38,39]. Thus, disruption of these elements could eventually alter developmental trajectories, leading to the onset of neurodevelopmental and other brain disorders later in life[40,41,42]. Moreover, in animal studies, showed that both pre- and post-natal periods are highly critical developmental windows ultimately influencing behavior during adulthood and accounting for a large proportion of the autism spectrum disorder (ASD) cases[43]. As a matter of fact, maternal exposure to different nutrients (e.g., high fat diet (HFD)) or drugs (e.g., valproic acid (VPA)) during pregnancy has been associated with ASD[44,45].

As alterations of the intestinal microbiota can impact on brain development, yet the "correction" of dysbiosis could eventually restore events in some extent. With this in mind, Saunders and colleagues did investigate whether the manipulation of the gut microbiota at early developmental stages could prevent the negative effects induced by maternal infection with a mouse-adapted influenza virus on behavior models of cognition and expression of the serotonin 5-HT2A receptor in the mouse frontal cortex[46].

Nevertheless, to correct dysbiosis and obtain a beneficial effect on neurodevelopment it is essential to exploit a narrow window of time. In Buffington et al. the study[47] the microbiota of GF mice was manipulated by transplanting fecal microbiota from adult maternal regular diet (MRD) and maternal high-fat diet (MHFD) offspring. As expected, no changes in behavior were

obtained in mice transplanted from MHFD (as they too had dysbiosis). In contrast, mice transplanted from MRD exhibited normal social behavior. However, this positive effect was obtained only in GF mice who received fecal microbiota at weaning (4-weeks-old), not in those who received it at 8-weeks-old. These data reveal a neurodevelopmental window during which microbial reconstitution effectively improves social behavior[47].

Probiotics, prebiotics, postbiotics

Probiotics are defined by the World Health Organization (WHO) as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". These microorganisms have been shown to exert a wide range of effects on the host[48], ranging from the modulation of the immune system and the immune response[49], up to the creation of a healthy gut environment through the modulation of the gut microbiota. Indeed, the intake of beneficial bacteria can regulate the gut microbiota composition, promoting the establishment of a favorable microbial state which in turn may promote the maintenance of beneficial microorganisms[50,51]. Studies support the role of probiotics in modulating the MGBA and improving brain behaviors, but the beneficial effects are divergent and strongly depend on the strain used[52,53].

As an alternative, or in combination with probiotics, also *prebiotics* can be used to modulate the gut microbiota and the MGBA[7]. A *prebiotic* is defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as "a substrate that is selectively utilized by host micro-organisms and confers a health benefit". Hence it could include either soluble fermentable fibers, non-digestible oligosaccharides (NDOs), or human milk oligosaccharides (HMOs)[7]. Since fibers' bonds cannot be broken down by digestive enzymes, soluble fermentable fibers go over relatively intact into the large intestine. Here, they are fermented by commensal bacteria to produce large quantities of acetate, propionate, and butyrate[54]; these

compounds having respectively two, three, and four carbon atoms, are SCFAs, largely produced by microbial fermentation of complex polysaccharides in the colon.

Consequently, SCFAs are a group of *postbiotics* defined as "molecules released by bacteria and other microorganisms that, when administered in adequate amounts, confer health benefits to the host"[55]. In other words, *postbiotics* are the active compounds that are generated by *probiotics* when they ferment the *prebiotic* substrate. It has been shown that a variety of polysaccharides can improve brain functioning after oral, systemic, and/or localized administration either in *in-vitro*, animals, and humans studies[56]. Furthermore, the supplementation with a mixed polysaccharide product (Ambrotose® complex) could significantly improve both cognitive function and mood in healthy middle-aged adults[57]. *Prebiotics* act on the MGBA through two main mechanisms of action: they can stimulate the growth of gut bacteria that produce neuroactive metabolites; on the other hand, they can directly influence signaling molecules in the brain[53].

Probiotics, *prebiotics* and *postbiotics* are strictly interrelated and can affect the MGBA. *Probiotics* can directly produce neurotransmitters or stimulate host cells to synthesize these neurochemicals, hence, they may be used as delivery vehicles for neuroactive compounds[10]. Different bacterial strains may synthesize different neurotransmitters: GABA is secreted by certain strains of *Lactobacillus* and *Bifidobacterium*; conversely, norepinephrine is mainly produced by *Saccharomyces*, *Bacillus*, and *Escherichia*. Serotonin by *Enterococcus*, *Streptococcus*, *Escherichia*, and *Candida*; dopamine by *Bacillus* and *Serratia*; acetylcholine by *Bacillus* and few lactic acid bacteria (LAB) strains; glutamate by various coryneform and LAB strains[9,10,58,59,60]. Several studies have demonstrated that mice and/or rats fed with *probiotics* showed altered neurotransmitter composition and/or changes in their target receptor throughout

different brain regions. Moreover, a recent study[61] has proved direct evidence of *probiotics* in modulating neurotransmitter release through the use of magnetic resonance spectroscopy (MRS). Particularly, mice treated with *Lactobacillus rhamnosus* (JB-1) showed increased levels of glutamate, N-acetyl aspartate, and GABA in the brain, indicating that the *probiotic* could regulate brain activity via metabolic pathways and further suggesting the possibility for a clinical translation into the clinical practice. Likewise, other studies have demonstrated that the ingestion of *L. rhamnosus* (JB-1) could regulate stress-induced behavior and alter GABA mRNA expression in mice[62,63]. In 2008, Desbonnet et al.[64] proved that the administration of *Bifidobacterium infantis* could reduce dopamine and serotonin metabolites in the frontal cortex of rats, although without any discernible change in rats' behavior. Later, in 2010, Desbonnet and colleagues[22] demonstrated that the treatment with *B. infantis* could normalize the immune response, reinstate the basal noradrenaline concentration, and also reverse the behavioral deficits. In a further study[65] *Bifidobacterium longum* str. NCC3001 repressed anxiety-like behavior and normalized brain-derived neurotrophic factor expression in the hippocampus of mice with mild to moderate colitis.

SCFAs have been shown to affect the host through multiple mechanisms including the regulation of histone acetylation and methylation[66,67], the secretion of various hormones (e.g., glucagon-like peptide 1 (GLP-1) and peptide YY (PYY)) and neurochemicals (e.g., serotonin)[68,69], and the induction of vagus nerve signalling[70,71]. Furthermore, SCFAs can directly interact with the nervous system by activating sympathetic neurons and affecting behavior and neural signaling across the BBB[72,73,74]. Finally, several studies[75,76,77] have demonstrated that the treatment with SCFAs can restore intestinal permeability, an effective barrier against the risk of bacterial translocation.

Butyrate is one of the most important SCFAs and has been implied in several host physiological functions, including energy homeostasis, obesity, immune system regulation, cancer, and brain functioning[78,79,80,81]. In addition, butyrate directly affects serotonin and gut's hormones release in the enteric nervous system, thereby stimulating the vagus nerve and the endocrine signaling; butyrate also stimulates the hypothalamus-pituitary-adrenal (HPA) axis[18,82]. Alternatively, when artificially administered at high concentrations (>100 mg/kg), butyrate acts as a potent drug with well-established, versatile systemic functions[67]. Indeed, butyrate is also widely used as an experimental pharmacological compound, and recently, has found place in neuroscience research[78,83]. Some of these effects are probably due to the histone deacetylase (HDAC) inhibition, which in turn may inhibit nuclear factor κB (NF-κB) activation in the large intestine[84]. Moreover, butyrate relieves HDAC inhibition of Foxp3, thus it promotes the generation of regulatory T (Treg) cells[85,86], which suppress inflammation.

Current literature positively views the effect of increased production of butyrate and other SCFAs. However, based on the low peripheral concentrations of butyrate and the specific location of transporters and receptors, it appears unlikely that butyrate will enter the brain in sufficient quantities to directly exert its effect (e.g., receptor binding, HDAC inhibition, to become a feasible energy source). This is also unlikely during a high-fiber diet[67].

Other microbiota regulators

The ketogenic diet (KD) is another way in which the MGBA could be regulated. The KD is a high-fat and low-carbohydrate diet inducing ketone bodies production. The KD initiation represents a major shift in macronutrient composition for most patients; this shift is likely to impact the gut microbiota substantially[7]. The extensive study by Olson et al.[87] provides hard evidence for the impact of the KD on the gut microbiota. The KD is a well-established, non-

pharmacologic treatment used since 1920s in children with drug refractory epilepsy[88,89]; its specific anticonvulsant mechanism of action remains not fully elucidated, but recent studies have proposed several potential mechanisms. KD induces ketosis, and ketones are used as an alternative energy substrate for ATP production in the cells of the body, including the brain. This metabolic shift induces many biochemical, metabolic, and hormonal changes that may contribute to decrease neuronal excitability and reduce the number of seizures[3] .

The evidence of the effect of the KD on DRE is multiple. Two randomized controlled prospective studies evaluated the efficacy of KD in medically refractory epilepsy in children: the responder rate was 38% and 50% respectively for the two trials[90,91]. Recent study has investigated changes in the gut microbiota in patients with epilepsy during KD[92]. In the study by Lindefeldt et al.[92] after 3 months on KD intervention, it was revealed, through whole metagenomic sequencing, that the relative abundance of fibre-consuming bacteria, such as *Bifidobacterium*, was considerably lowered in patients, suggesting the role of the microbiota in seizure susceptibility and the potential anti-seizure efficacy in KD treatment. In a study performed by Zhang et al.[93] after 6 months of KD treatment in children with epilepsy, 2 patients were seizure free, 3 had ≥90% seizure reduction, 5 had a reduction of 50–89%, and 10 had <50% reduction; all 10 effective patients had an improvement in EEG, while non-responders showed no obvious change. In this pilot exploratory study, the most important finding was that the composition of gut bacteria differed significantly after KD compared with untreated patients. This phenomenon was also seen between different efficacy patients. A study by Xie et al.[94] reported that KD had a significant effect on imbalanced gut microbiota in children with refractory epilepsy and found that *Proteobacteria* decreased dramatically, while *Bacteroides*

increased significantly after KD. Some evidence shows that the gut microbiota was modified after KD in a murine model of autism spectrum disorder[95].

Fecal microbiota transplantation (FMT) is a treatment indicated for recurrent *Clostridium difficile* infections and inflammatory bowel diseases, that modifies substantially the gut microbiota composition and potentially corrects its alterations[96]. Very few studies have been performed to test FMT efficacy in neuropsychiatric disorders[97]. Some studies suggested a beneficial effect of FMT on epilepsy, Tourette syndrome, and diabetic neuropathy, but evidence was restricted to case reports and limited numbers of animal studies[98]. Medel-Matus et al.[99] demonstrated that stress-induced kindling epileptogenesis could be transferred from stressed rats to naïve (sham-stressed) Sprague-Dawley rats via FMTs, while the proepileptic effect of chronic stress was counteracted in stressed rats through FMTs from naïve rats.

In one case-report, a 22-year-old patient with Crohn's disease and a 17-year history of seizures received a FMT to treat Crohn's[100]. Before FMT, she experienced frequent seizures when not using sodium valproate treatment. During the 20 months follow-up the patient was reported to be seizure-free despite discontinuing antiepileptic drug treatment. Furthermore, the Crohn's disease activity index improved. Based on this case, a registered interventional study (NCT02889627) with a single group assignment is ongoing with FMT in patients with epilepsy, but no results are yet available.

In an open-label clinical trial[101,102], 18 children with ASD and GI symptoms received daily FMT for 7-8 weeks by mixing standardized human gut microbiota. GI and behavioral ASD symptoms ameliorated, and this improvement persisted until 2 years after the treatment. Furthermore, there was a correlation between ASD symptoms and GI symptoms. However, this was an open-label

study without a placebo group in a heterogenous group of 18 participants, in which 12 changed their medication, diet, or nutritional supplements during the study.

An abstract[103] reporting an open-label, randomized and waitlist-controlled trial, showed improvements of ASD symptoms and changes in GI symptoms 2 months after two FMTs in 24 ASD-children compared to 24 control ASD-children. However, improvements of ASD symptoms were temporary. Seven FMT-patients reported adverse events, such as nausea, fever, and allergy, but these were all mild and transient. There was no placebo-group and there was lack of information on α- and β-diversity of the gut microbiota, pre-treatment, and amount of donor feces. Potential benefits of FMT should be carefully weighed against the potential risks, and future studies should focus primarily on safety, with effectiveness of FMT as a secondary endpoint. Preliminary literature suggests that FMT may be a promising treatment option for several neurological disorders, but the evidence is limited. The neurological disorder with the most evidence on efficacy of a healthy donor FMT is ASD[98].

Gut microbiota in neuropsychiatric disorders

Some clinical studies show that patients affected by neuropsychiatric disorders, such as autism spectrum disorders and epilepsy, as well as schizophrenia, major depressive disorder, Alzheimer's and Parkinson's diseases, and multiple sclerosis could have alteration in gut microbiota composition and MGBA. However, it is currently unclear whether the alterations are directly related and proportional to the severity of these disorders[104,105]. Furthermore, the prevalence of GI symptoms, (e.g. constipation, diarrhea, and abdominal pain) as common comorbidities in many neurological diseases[106,107,108,109,110,111] suggests a possible link between gut microbiota and the brain, in addition to the possibility that the microbiota is involved in the pathophysiology of the disease.

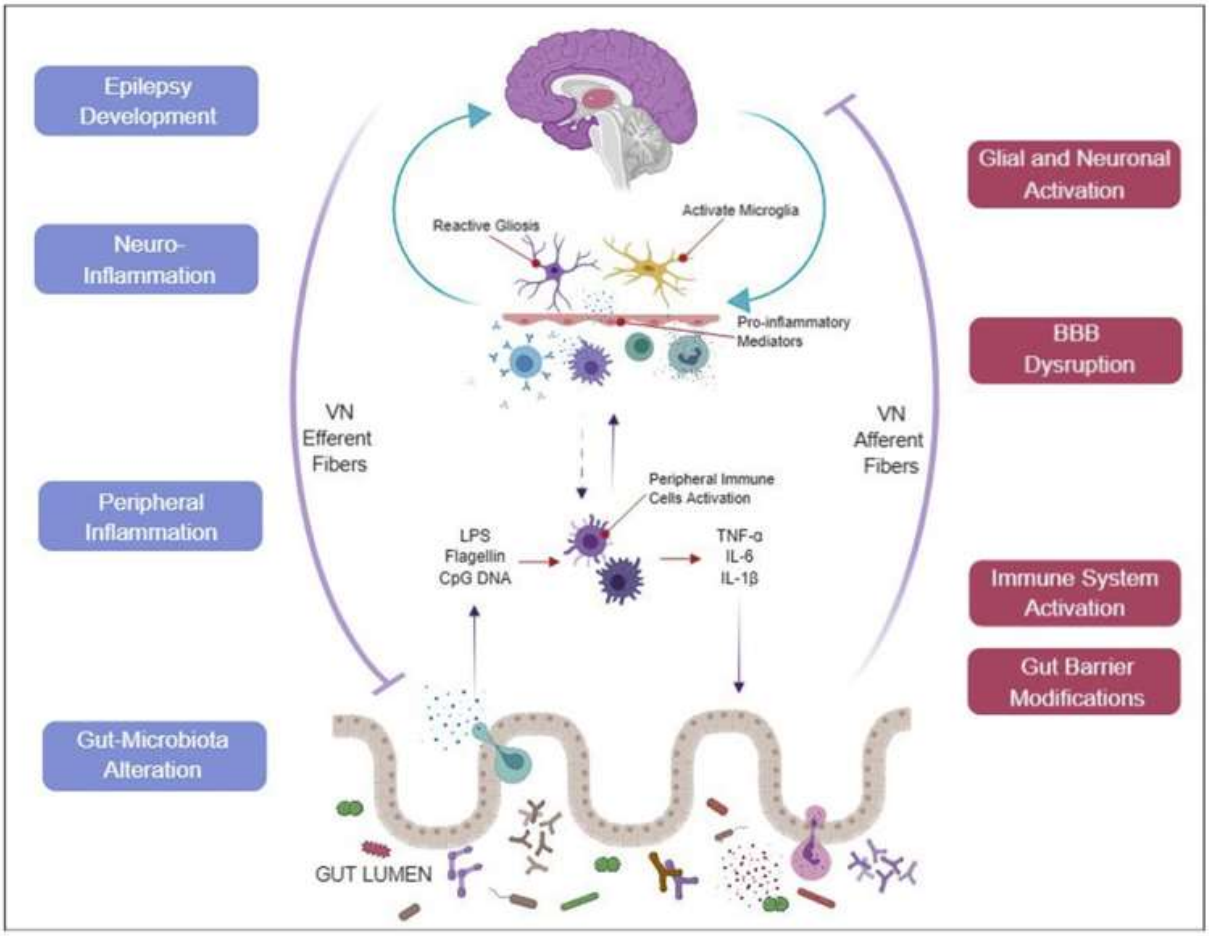
Gut microbiota in epilepsy

Epilepsy is a heterogeneous group of neurological diseases affecting approximately 70 million people worldwide[112]. Epilepsy is defined as the predisposition for an individual to have recurrent and spontaneous seizures[113]. In advanced countries, the incidence is around 40–70 per 100,000/year, whereas in less advanced countries, it is higher, approximately 100–190 per 100,000/year[114]. In addition to being a very frequent pathology, 25-30% of patients do not respond to proper antiepileptic drugs and therefore have a DRE[114].

There is some evidence that the gut microbiota structure in epileptic infants differs from that in healthy infants[94]. It may be possible that dysbiosis is more relevant in certain subtypes of

Figure 1: Microbiota gut brain axis in epileptic patient

De Caro et al. Neuroscience and Behavioral Review 107 (2019) 750-764



epilepsy. Moreover, Peng et al. found out differences between the gut microbiota composition of the patients with drug sensitive epilepsy and patients with DRE[115]. In drug-resistant patients (n = 42), there was a relative abundance of rare bacteria mainly belonging to the phylum *Firmicutes* compared to drug sensitive patients (n = 49); *Bifidobacteria* and *Lactobacilli* were associated with less than four seizures per year in both patient groups. Various studies[92,94,115] reported increased abundance of the phyla *Firmicutes* relative to *Bacteroidetes* in subjects with refractory epilepsy, and maybe some bacteria of the phylum *Firmicutes* could alter neurotransmitter levels[115].

If a dysbiosis can be confirmed, microbiota-targeted strategies may be developed as alternative treatments for epilepsy. These might aim to re-establish a healthy microbial community using prebiotics, probiotics, or FMTs from healthy donor[3].

Gut microbiota in autism spectrum disorders

Kelly et al.[116] found out that the composition of the gut microbiota in children with ASD differs from that of neuro-typical individuals. Moreover, the GI disorders, in particular diarrhea, bloating and constipation, are the most frequently symptoms associated with behavioral and emotional symptoms in children with ASD[117]. Almost half of them present GI dysfunction[118]. Finally, a dysbiosis and an alteration of the stability and composition of the gut microbiota were found in children with ASD compared to healthy controls aged-matched[108]. All that leads to hypothesize that alteration of the gut microbiota may play a role in the pathogenesis and maintenance of ASD. Therefore, according to several studies, the improvement of behaviors in ASD may be achievable through the regulation of the gut microbiota. However, further studies still need to be performed to confirm this possibility[7].

Preclinical studies

To support the efficacy of treatment with probiotics and prebiotics in autism and epilepsy, I searched for studies that used this therapeutic strategy. In recent years, some studies have been conducted. Although the topic is of great interest, to date there are only few studies on the role of probiotics and prebiotics in neurodevelopmental disorders. The following are the most representative studies that can give a global view of current knowledge on the subject.

Preclinical studies on epilepsy

Three studies[119,120,121] were selected relating to the use of probiotics/prebiotics in mouse models of epilepsy. The studies reported in **Table 1** are preclinical studies, which analyzed the effects of supplementation with probiotics and/or prebiotics in animals.

1. *Effects of Probiotic Consumption on Absence Seizures*[119]

   This study analyzed the effects of probiotics supplementation on absence seizures in Genetic Absence Epilepsy Rats from Strasbourg (GAERS) rat model. Nine animals were divided into 2 groups: probiotic-fed group (n=4) and control-fed group (n=5). Several studies displayed that the probiotics administration could alter neurotransmitters expression in different brain areas. Particularly, *L. rhamnosus* could affect GABA mRNA expression. For this, it was hypothesized that the alteration of GABA receptors after taking probiotics could influence the occurrence of absence seizures. To quantify this effect, cumulative duration, and number of spike-and-wave discharges (SWDs) of GAERS were measured. After 1-month period of probiotic consumption, EEG recordings of every animal were monitored for 3 consecutive days over 3-hour period between 9 am and noon. Each recording was divided into 20-minute periods. Cumulative duration and number of SWDs were calculated for each period and for each individual animal. Afterwards, mean cumulative duration and

Table 1: Preclinical use of pre- and probiotics. Animal models of epilepsy

Study	Sample size	Study population	Drugs	End points	Conclusions
Akkol et al.[119]	9	Rats	Sachet: 2g of mixture of probiotic, vitamins, and fiber dissolved in 500-mL bottle of drinking water. Bottles were replaced twice a week	EEG recordings	Probiotic consumption had no effect on duration or number of SWDs of GAERS after 1-month feeding period
Bagheri et al.[120]	40	Rats	Mixture of L. rhamnosus, L. reuteri, and B. infantis. 1 ml solution/day (a total of 3 × CFU~10^9) of probiotic mixture for 3 weeks.	Sz severity; spatial learning and memory (Morris water maze test)	Probiotic supplementation reduces sz severity and partially improved the spatial learning and memory in the kindled rats.
Tahmasebi [121]	128	Rats	Mixture of L. casei, L. acidophilus, B. bifidum. 1 ml solution/day (a total of CFU ~10^{13}) of probiotic mixture for 6 weeks. NS: 400 mg/kg/day for 2 weeks	Anticonvulsant effect; spatial learning and memory (Morris water maze test)	Probiotic and NS supplementation have some protection against sz and sz-induced cognitive impairment.

Sz: Seizure
SWCs: Spike and wave discharges
GAERS: Genetic Absence Epilepsy Rats from Strasbourg
PTZ: Pentylenetetrazol
CFU: Colony-forming unit
NS: Nigella Sativa

number of SWDs for the 2 groups were calculated. No statistically significant difference was found between groups in either comparison. Although no significant differences in SWDs were found in the two groups, it was not possible to assert that there were no effects on neurotransmitters or receptor expression levels. This because immunohistochemical evaluation was not performed.

2. *Effect of probiotic supplementation on seizure activity and cognitive performance in PTZ-induced chemical kindling*[120]

This study analyzed 40 2-month-old male Wistar rats. In these rats, chronic epileptic seizures were induced by the administration of a subconvulsive dose of pentylenetetrazol (PTZ 35mg/kg). The chemical kindling induced by PTZ, a $GABA_A$ receptor antagonist, is an indistinguishable model of clinically resistant epilepsy. The animals were divided into 5 groups of 8 individuals each. The groups were as follows:

Group 1: normal control group (CON), received carrier of probiotic

Group 2: kindled rats; received carrier of probiotic (PTZ)

Group 3: kindled rats; received probiotic 24 days before kindling (PRO+PTZ)

Group 4: kindled rats; received probiotic during kindling (PTZ+PRO)

Group 5: kindled rats; positive control group received 150mg/kg valproic acid (VPA)

PTZ administration was once every other day for 24 days. The intensity of the convulsions was registered according to modified Racine's scale (stage 0-5). Three consequent stage 5 seizure after PTZ injection is considered as full kindle state. Probiotic supplementation included a mixture of three bacteria: *Lactobacillus reuteri, L. rhamnosus, and B. infantis* (CFU~10^9 for each). The probiotic-treated animals received 1 ml solution/day (a total of 3×CFU~10^9) of probiotic mixture via intragastric gavage. The control rats received 1ml carrier of the probiotics. The treatment lasted for 3 weeks. The Morris water maze test was

used to evaluate changes in learning and memory. This test involves a first acquisition phase during which learning is evaluated, followed by a probe trial phase which evaluates memory. In the acquisition phase, the time elapsed (escape latency) and the distance traveled to locate the platform were measured as learning scores. Spatial retention in the probe trial was measured concerning the duration of time spent and the distance traveled in the memorized region of the water maze. On the last day of treatment, mice were sacrificed, and their brains removed for study. A biochemical evaluation of brain contents of GABA, nitric oxide (NO), malondealdehyde (MDA), and total antioxidant capacity (TAC) was performed. The concentration of GABA was measured using the enzyme-linked immunosorbent assay (ELISA) kit. NO metabolites were measured by colorimetric method. Concentration of MDA was evaluated using the thiobarbituric acid reactive substance method. Ferric reducing ability of plasma (FRAP) assay was used for measuring TAC of blood. The groups tested showed different seizure activity. In the PTZ group the score of seizure was gradually increased over the experiment. The seizure severity in the PRO + PTZ showed a steady manner throughout the chronic chemical kindling. This group showed a significant difference with the PTZ group. Valproate efficiently protected the animals against the effect of PTZ where the VAP group rats showed the least level of the kindling compared with the other kindled rats. Therefore, the probiotic administration decreases the level of epileptic activity. The incidence of full kindling (stage 5) in the tested groups was also considered. Fifty percent (4 out of 8) of animals in the PTZ group exhibited the score of 5 within 3 consecutive scorings. None of the kindled rats in the VAP and PTZ + PRO groups (only 1 rat) showed the score of 5 over the experiment. The Fisher's exact probability test displayed a significant difference between the PTZ group compared with the VAP and PTZ + PRO groups. Concerning the full kindling phase, no statistical difference was evident between the

animals in the VAP and both probiotic-treated groups. These findings indicate that the probiotic supplementation substantially reduces the seizure severity. As for the Morris water maze test, the analysis indicated significant differences between the groups. The PTZ + PRO rats required less time to learn location of the hidden platform than did the PTZ animals. In comparison with the PTZ rats both probiotic treated groups traveled less distance to find the hidden platform. The PTZ + PRO rats significantly overcame the VPA and PRO + PTZ groups in the time elapsed in the target quadrant. Concerning the distance passed in the correct quadrant the PTZ + PRO animals showed to be superior to all testing groups. Regarding the concentration of GABA in brain tissue, there are no variations between the CON and PTZ groups. Treatment with probiotics in the PTZ + PRO group significantly increased concentrations compared to the other groups. MDA levels increased in the PTZ group compared to the control group. In the groups treated with probiotics the concentration is highly reduced compared to the PTZ group, but also compared to the CON group. The concentration of NO is unchanged between CON and PTZ groups, while it is significantly reduced in the groups treated with probiotics and in the VAP group. The chemical kindling had no substantial effect of the TAC level of the brain. There was a slight decrease in the VPA group compared with the CON one. Whereas simultaneous kindling and probiotic treatment highly elevated the brain concentration of TAC in the PTZ + PRO rats compared with the other testing groups, the pretreatment with the probiotic mixture in the PRO + PTZ was ineffective on the antioxidant index. In conclusion, the probiotic bacteria substantially reduce seizure severity. The oral bacteriotherapy also partly improved the spatial learning and memory in the kindled rats. Moreover, the probiotic treatment reasonably increases the GABA activity and improves the antioxidant/oxidant balance in the kindled rats.

3. *Probiotics and Nigella sativa extract supplementation improved behavioral and electrophysiological effects of PTZ-induced chemical kindling in rats*[121]

128 Wistar rats were taken for this study, randomly divided into 8 groups: control group (saline), kindled group (PTZ + saline), kindled and probiotic treated group (PTZ + PRO), kindled and Nigella sativa (NS) treated group (PTZ + NS), kindled and probiotics + NS treated group (PTZ + PRO + NS), control non kindled probiotic treated group (PRO), control non kindled NS treated group (NS), and control non kindled probiotic + NS treated group (PRO + NS). To prepare the NS extract, 100g of powdered seeds of NS were solved in ethanol (70%). The extract was then dissolved in saline and administered via intragastric gavage at dosage of 400mg/kg/day. The probiotic supplements were a mixture of three bacteria consisting of *Lactobacillus casei*, *Lactobacillus acidophilus and Bifidobacterium bifidum*. The probiotic-treated animals received 1 ml solution/day (a total of CFU ~10^{13}) of probiotic mixture for 6 weeks and the NS-treated animals received 400 mg/kg/day for 2 weeks before starting PTZ kindling (for a total of 10 weeks of probiotics and 6 weeks of nigella treatment). PTZ kindling was induced by the administration of subconvulsive doses of PTZ (37.5 mg/kg). PTZ was injected intraperitoneally once every other day for 28 days. The convulsive behavior was monitored for 20 min. The intensity of the convulsions was registered according to modified Racine's scale (stage 0-5). The recording parameters were as follows: seizure stage, latency to the onset of stage 2 and stage 5 seizures, and stage 5 duration. The Morris water maze test was used to examinate changes in spatial learning and memory of the rats. In the first part of the experiment, the anticonvulsant effect of probiotics and NS was evaluated, alone and in co-administration. In addition, the effect of supplementation on spatial learning and memory in the PTZ-induced kindling model was evaluated. Regarding the anticonvulsant effect, supplementation with probiotics showed a

delay in the development of kindling, a lower stage of seizure intensity, and an increase in stage 2 latency. Treatment with NS reduced the rate of kindling development and increased stage 5 latency. When given together, they resulted in suppression of kindling development, an increase in stage 2 and 5 latencies, and a significant reduction in stage 5 duration. Analysis showed that the escape latency and the distance traveled to find platform were increased in fully kindling animals compared to control group. Moreover, the PTZ kindled group displayed less preferences toward the target quadrant by spending less time and travelling shorter distances compared to control group. Latency to find hidden place was reduced in probiotic + PTZ. NS and PRO + NS supplementation reduced the escape latency, moreover, the NS-treated group spent significantly more time in the platform quadrant compared with kindled animals. In control non-kindled rats, NS, probiotics, and coadministration of probiotics and NS significantly decreased escape latency and distance traveled to find the platform as compared with the control group. In the probe trial test, data showed that the probiotics + NS group spent significantly more time and traveled more distance in the platform quadrant compared with control animals. The second part of the study was focused on the electrophysiological study. To explore the synaptic mechanisms underlying the memory impairment in PTZ-kindled rats, the field population spike (PS) before long term potentiation (LTP) induction was analyzed in the dentate gyrus region in vivo. Data showed that PTZ kindling had no significant effects on PS amplitude compared with control animals. Supplementation of probiotics and NS in PTZ-kindled animals significantly increased the PS amplitude in comparison with PTZ + saline group, suggesting that probiotics and NS administration enhance the synaptic strength in PTZ-kindled rats. In addition, PTZ kindling had no significant effect on field excitatory postsynaptic potential (fEPSP). Post hoc analysis with Tukey's revealed that LTP was significantly reduced in PTZ

+ probiotics, PTZ + NS, and PTZ + probiotics + NS groups. One-way analysis of fEPSP slope potentiation showed the same change in fEPSP slope and PS amplitude. Analysis of the field PS before LTP induction in the control non-kindled rats showed that supplementation of NS and NS + probiotics significantly increased the PS amplitude in control non-kindled animal. A one-way ANOVA revealed that the potentiation of the PS amplitude was significantly increased in NS and NS + probiotics treatment animals. In conclusion, PTZ kindling causes significant spatial learning and memory impairment in Morris water maze; coadministration of NS extract and probiotics, in addition to inhibiting kindling development, can prevent the changes in synaptic plasticity and learning and memory in rats.

Preclinical studies on ASD

Table 2 shows preclinical studies[47,62,122,123,124,125] performed on animal models of autism. Currently, there are more studies on supplementation with probiotics and/or probiotics in animal models of autism than in animal models of epilepsy. However, even in this area the evidence is still very limited.

1. *Microbiota Modulate Behavioral and Physiological Abnormalities Associated with Neurodevelopmental Disorders*[122]

This study explored several aspects in maternal immune activation (MIA) mouse model, which are known to exhibit characteristics of the ASD. At first, the study displayed the presence of GI barrier defects in MIA mouse model. Deficit in intestinal barrier integrity was reflected in an increased translocation of FITC-dextran across the intestinal epithelium, into the circulation. The loss of integrity was found in the 3-weeks-old MIA offspring, indicating that the abnormality developed during early life. In addition to the increased

Table 2: Preclinical use of pre- and probiotics. Animal models of autism

Study	Sample size	Study population	Drugs	End points	Conclusions
Hsiao et al.[122]		Mice	*B. fragilis* every other day for 6 days at weaning	PPI, OF, MB, SIT, and AUV	Human commensal bacterium can improve ASD-related GI deficits and behavioral abnormalities in mice
Bravo et al.[62]	36	Mice	*L. rhamnosus*	OF, SIH, EPM, FC, and FST	*L. rhamnosus* can affect behavioral and physiological responses, modulate the GABAergic system in mice, have beneficial effects on depression and anxiety and therapeutic potential in modulating brain and behavior
Buffington et al.[47]	30	Mice	*L. reuteri* into drinking water of MHFD offspring at weaning for 4 weeks	RSIT, SNT	*L. reuteri* improves sociability and preference for social novelty in MHFD offspring
Tabouy et al.[123]	31	Shank3 KO mice	10^9 bacteria of *L. reuteri* in a volume of 200 µl of PBS	SIT	Treatment of Shank3 KO mice with *L. reuteri* induced an attenuation of unsocial behavior specifically in male Shank3 mice, and a decrease in repetitive behaviors in both male and female Shank3 KO mice.
Sgritta et al.[124]		Mice	*L. reuteri* ~1x10^8 CFUs/mouse/day	TCST, RSIT, OF	*L. reuteri* rescues social deficits in several ASD mouse models. *L. reuteri* reverses social deficits via the vagus nerve. OXTR inhibition prevents *L. reuteri* effects on social behavior and VTA plasticity
Sunand et al.[125]	56	Rats	*L. Plantarum, L. Casei, L. Acidophilus, L. Bulgaricus*, inulin, sodium valproate.	MWM, SIT, OF	The daily supplementation of *Lactobacillus* strains reverses autistic deficits

MHFD: Maternal high-fat diet
PPI: Prepulse inhibition
OF: Open field
MB: Marble burying
SIT: Social interaction test
AUV: Adult ultrasonic vocalization
SIH: Stress-induced hyperthermia
EPM: Elevated plus maze
FC: Fear conditioning
FST: Forced swim test
RSIT: Reciprocal social interaction test
SNT: Social novelty tests
TCST: Three chamber social test
MWM: Morris water maze
OXTR: Oxytocin receptors
VTA: Ventral tegmental area
ASD: Autism spectrum disorder

intestinal permeability, an abnormal intestinal cytokine profile was found. Subsequently, to evaluated whether MIA induces gut microbiota alteration, fecal flora of samples from adult MIA or control offspring was studied by 16S rRNA gene sequencing. MIA leads to intestinal microbiota dysbiosis, driven mainly by alterations in specific operational taxonomic units (OTUs) of the bacterial classes *Clostridia* and *Bacteroidia*. After the assessment of the characteristics of the MIA mice, the effects of supplementation with *B. fragilis* were evaluated. It was considered whether the supplementation with *B. fragilis* could impact on MIA-associated GI abnormalities. The mice were treated with *B. fragilis* at weaning and were tested for GI abnormalities at 8 weeks of age. *B. fragilis* improved intestinal permeability, corrected alterations in tight junctions, and re-established MIA-associated increases in colon interleukin (IL) 6 mRNA and protein levels. Following improvement of the GI barrier defects, the effects of *B. fragilis* in MIA offspring on the intestinal microbiota were assessed. Result suggests that, although treatment of MIA offspring with *B. fragilis* may not lead to persistent colonization, this probiotic corrects the relative abundance of specific groups of related microbes of the *Lachnospiraceae* family as well as unclassified *Bacteriodales*. Furthermore, treatment of MIA offspring with *B. fragilis* ameliorates particular MIA-associated alterations in the commensal microbiota. After, was tested whether *B. fragilis* treatment impacts anxiety-like, sensorimotor, repetitive, communicative, and social behavior in offspring. MIA and control offspring were behaviorally tested at 6 weeks of age for prepulse inhibition (PPI), open field exploration, marble burying, three-chamber social test, and adult ultrasonic vocalizations. Oral treatment with *B. fragilis* improved many aspects of behavior. *B. fragilis* improves sensorimotor gating in MIA offspring. *B. fragilis*-treated MIA offspring do not exhibit anxiety-like behavior in the open field and exhibit decreased levels of stereotyped marble burying and restored

communicative behavior. Although *B. fragilis*-treated MIA offspring exhibit improved communicative, repetitive, anxiety-like, and sensorimotor behavior, they retain deficits in sociability and social preference. In this study gas chromatography/liquid chromatography with mass spectrometry (GC/LC-MS)-based metabolomic profiling was used to identify MIA-associated changes in serum metabolites. 322 metabolites were assessed and MIA leads to statistically significant alterations in 8% of all serum metabolites detected. Serum levels of 4-ethylphenylsulfate (4EPS), p-cresol, indolepyruvate, serotonin was increased in MIA offspring. Additionally, serum levels of glycolate, imidazole propionate, and N-acetylserine were altered. All these metabolites were influenced by the composition of the gut microbiota and were normalized with *B. fragilis* treatment. Finally, it was evaluated whether the increase in serum 4EPS was sufficient to cause any ASD-related behavioral abnormalities in naive mice. Thus, the mice were treated with 4EPS potassium salt or with the vehicle every day from 3 to 6 weeks of age. The data showed that elevated systemic levels of a metabolite regulated by gut microbes causes anxiety-like behavior, suggesting that molecular connections between the gut and the brain may be associated with specific symptoms relevant to ASD and other neurodevelopmental disorders.

2. *Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve*[62]

The study was conducted on 36 adult male BALB/c mice. It aimed to evaluate the effects of *L. rhamnosus* on the central expression of GABA receptors, on the reduction of stress-induced plasma corticosterone levels, on anxiety- and depression-related behavior. Furthermore, the elimination of these effects in vagotomized mice was evaluated. The effects found in *L. rhamnosus*-fed mice (n = 16) were compared with those obtained in broth-fed mice (n = 20) as a control group. To test the behavior were adopted: open field

test, stress induced hyperthermia (SIH) test, elevated plus maze (EPM) test, fear conditioning and forced swim test (FST). Plasma corticosterone concentration was determined with a Correlate-EIA enzyme immunoassay kit. Chronic administration of *L. rhamnosus* determined a no significant decrease of SIH. On the EPM, *L. rhamnosus* showed an anxiolytic effect. On FST *L. rhamnosus*-fed animals spent less time immobile compared with broth-fed mice. On fear-related behavior *L. rhamnosus* had significant effects only on day 2 (memory testing), while there were no significant differences in day 1 and 3 (memory extinction). Stress-induced corticosterone was measured in plasma 30 min after FST. Stress-induced levels of corticosterone are significantly lower in *L. rhamnosus*-fed mice compared with broth fed control animals. The administration of *L. rhamnosus* also acts on GABA receptors expression. *L. rhamnosus* determined an increase of $GABA_{A\alpha2}$ mRNA levels in some brain areas (i.e., dentate gyrus) and a reduction in others (i.e., cingulate cortex 1, prelimbic and infralimbic cortical areas, basolateral amygdala, and central amygdala). However, no differences in $GABA_{A\alpha2}$ mRNA were found in cornus ammonis area 3 and 1 neuronal layer of the hippocampus of *L. rhamnosus* compared with broth-fed mice. Furthermore, the study displayed that, in the case of vagotomy, all the effects induced by the intake of *L. rhamnosus* are eliminated. Vagotomy interferes with the anxiolytic effect of taking *L. rhamnosus* and this is found in behavioral tests. The expression of GABA receptors induced by *L. rhamnosus* is also modified.

3. *Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring*[47]

In this study, the first aim was to assess how diet-induced maternal obesity influenced the neurodevelopment of the offspring. To do this, the female mice were fed either regular diet or high-fat diet for 8 weeks. Since maternal obesity affects neurodevelopmental disorders,

including autism, social behavior in MRD and MHFD offspring were studied. Compared to MRD offspring, MHFD offspring had fewer reciprocal interaction. Furthermore, using the three-chambers test, MHFD offspring showed impaired sociability and no preference for social novelty. In addition to causing social deficits, maternal obesity alters the gut microbiota. Sequencing of the 16S rRNA gene was used to assess bacterial composition and community structure in offspring feces. This demonstrated in the MRD and MHFD offspring that the microbiota was dominated by Bacteroidetes and Firmicutes. While bacterial diversity computed based on weighted UniFrac distances (the assessment of community structure by considering abundance of operational taxonomic units [OTUs]) did not differ significantly between the offspring from either diet group, unweighted analyses of UniFrac distances (assessment of community structure by considering only OTU presence/absence) revealed a marked difference between the structures of the bacterial communities. Furthermore, the diversity of the microbiota in the MHFD offspring was reduced compared to the MRD microbiota. Since mice are coprophages, if they are co-housed, they will eat the feces of the other specimens, transferring the gut microbiota between them. The idea was of co-housed one MHFD offspring with three MRD offspring to examined whether this could prevent social deficits in MHFD offspring. Fecal samples were collected, and social behavior was analyzed. The results showed that after cohabitation the MHFD offspring exhibited normal reciprocal social interaction, normal sociability, and a preference for social novelty. Furthermore, there has also been a shift in the bacterial phylogenetic profile. This supports the idea that the microbiota of MHFD offspring is deficient in one or more beneficial bacteria necessary for normal social behavior. However, if a lack of certain bacteria causes abnormal behavior, then the same problem should also exist in GF mice. There are studies that have confirmed this hypothesis[126]. To confirm the causal role of the

microbiota, fecal microbiota from adult MRD and MHFD offspring was transplanted into 4-week and 8-week-old GF mice. There were no positive results in mice transplanted with microbiota from MHFD, while, in those transplanted with microbiota from MRD offspring, there was a normalization of social behavior, but only in 4 weeks of age. These results confirmed the role of the microbiota in social behavior, but also highlighted the window period within which microbial reconstitution effectively improved social behavior. However, while co-housing of MHFD with MRD offspring restores social behavior, it has failed to save the marble burial. As a result, GF mice also showed increased marble burial, and fecal microbial transplants from MRD (or MHFD) offspring into GF mice failed to reverse the repetitive behavior. This suggests that repetitive behaviors are not affected by changes in the microbiota. Metagenomic shotgun sequencing of fecal samples from both MHFD and MRD offspring was performed to investigate which bacterial species absent in the MHFD microbial community cause social behavioral deficits. Among the various reduced species in MHFD offspring microbiota, *L. reuteri* was the most reduced *L. reuteri* plays an important role in promoting oxytocin levels, which plays an essential role in social behavior. For this it was decided to supplement MHFD offspring at weaning with *L. reuteri* for 4 weeks. After this period, the behavior was evaluated and the results showed an improvement in sociability and preference for social novelty, however there were no effects on anxiety. *L. reuteri* is responsible for oxytocin levels. Fewer oxytocin immunoreactive neurons in MHFD offspring compared to MRD offspring were found. However, in *L. reuteri*-treated MHFD offspring, the number of oxytocin-expressing cells was higher than in control-treated MHFD offspring. Previous studies have shown that there are areas such as the ventral tegmental area and the nucleus accumbens that respond to rewarding stimuli and are involved in social behaviors. Furthermore, social stimulation can be a rewarding

stimulus and trigger synaptic potentiation in ventral tegmental area (VTA) DA neurons in birds. With these premises, has been verified whether direct social interaction evokes LTP of synaptic inputs to VTA DA neurons. The electrophysiological study found that MRD offspring spent significantly more time interacting with a stranger than a familiar mouse, but MHFD offspring did not. Thus, social interaction induces a long-lasting increase in the activity of the dopaminergic reward system of MRD, but not in MHFD, offspring. Following the results obtained with the administration of *L. reuteri* Buffington et al. wondered whether direct administration of oxytocin could also reverse the behavioral and electrophysiological deficits characteristic of MHFD offspring. To evaluate this hypothesis, intranasal oxytocin was administered to MHFD offspring, and the reciprocal social interactions were measured after 30 minutes. Although either oxytocin alone or social interaction alone failed to rescue social interaction induced LTP in the VTA, the combination of social interaction and oxytocin treatment restored LTP in the VTA of MHFD. Accordingly, oxytocin treatment improved reciprocal social interaction, as well as sociability and the preference for social novelty. Thus, oxytocin administration rescues social behavior and related neural adaptations in the VTA of MHFD offspring.

4. *Dysbiosis of microbiome and probiotic treatment in a genetic model of autism spectrum disorders*[123]

This study was based on recent evidence relating to the impact of the microbiota on behavior and its possible dysregulation in neurodevelopmental conditions. Thus, it was studied whether the gene Shank3, associated with neurodevelopmental disorder, could influence gut microbiota, and whether probiotics, by modifying the microbiota, could be a therapeutic strategy. To assess the microbiota, DNA from fecal samples of 31 Shank3 KO mice e di 27 wild type mice was extracted and 16S rRNA gene sequencing was used. A decrease in the

relative abundance of *Lactobacillus* in Shank3 KO mice compared to wild type (WT) mice was found. The relative abundance of several other members of the microbiome were also decreased in Shank3 KO mice. At the genus level, *Coprococcus, Bacteroides, Acetobacter, Turicibacter,* and *Prevotella* were also decreased in Shank3 KO mice. In contrast, only the family *Veillonellaceae* and genus *Veillonella* were increased in the Shank3 KO mice. Real Time PCR analysis was performed on the genera *Lactobacillus, Prevotella,* and *Veillonella* to validate the sequencing analysis. These three species were the most abundant among the dysregulated microbiota; furthermore, *Lactobacillus* and *Prevotella* have previously been implicated in autism or social behavior. All three species were reduced in female Shank KO mice, while in males *Lactobacillus* and *Prevotella* were reduced and *Veillonella* was increased. Previous studies suggest a correlation between *Lactobacillus*, autism-related behaviors, and GABAergic function. Thus, to evaluate a possible correlation with *Lactobacillus* levels, GABA receptor expression at hippocampal level was studied in Shank3 KO mice and in WT mice. GABRA1, GABRA2, and GABRB1 levels all decreased significantly in the Shank3 KO hippocampus. Specifically, the abundance of *L. reuteri* correlated significantly with expression of each of the three GABA receptor subunits. Since gut microbiota could influence behavior also through regulation of the immune system, Plasma levels of 6 key immune markers were tested in Shank3 KO mice. The results showed an alteration of all the markers studied. Subsequently, the effects10^9 bacteria of *L. reuteri* of treatment with 10^9 bacteria of *L. reuteri* on Shank3 KO mice were assessed. Mice were treated with probiotics or PBS for 3 weeks by biweekly gavage. Fecal *L. reuteri* levels increased after supplementation. To evaluate the behavior, were adopted: the three-chamber test to evaluate the social interact, the marble burying test, for repetitive and perseverative behaviors, the open field and the elevated plus maze test, to evaluate anxiety-like behavior.

The treated mice showed in the three-chambered social interaction task less time spent in the empty zone and more time investigating the stranger compartment. *L. reuteri* treatment induced a partial attenuation of the unsocial behavior of male Shank3 KO mice, while it did not produce this effect in females. *L. reuteri* treated mice also buried significantly fewer marbles in the marble burying test. In contrast, *L. reuteri* had no effect on anxiety-related behaviors in mice, as determined in the open field and elevated plus maze tests. Furthermore, no effects on social behaviors, marble burying, or anxiety-like behaviors in wild type mice were found. Treatment with *L. reuteri* also acted on GABA receptors expression. Treated Shank3 KO male showed an increase in hippocampal expression GABRA2 and GABRB1, and a modest decrease in hippocampal GABRA1, in addition to an increase in all three genes in the prefrontal cortex. Female Shank3 KO mice treated displayed an increase in GABRA1, GABRA2, and GABRB1 gene expression in the hippocampus, and an in increase in GABRA1 and GABRA2 expression in the prefrontal cortex. To determine if gene expression changes translate to changes in protein levels, western blot analysis of GABRA1 was performed. This confirmed increases in GABA receptor levels in most experimental groups. Considering the role of *L. reuteri* in oxytocin signaling, the oxytocin gene expression in the hypothalamus of shank3 KO mice treated with *L. reuteri* was tested. Male Shank3 KO mice displayed an increased hypothalamic expression of oxytocin after treatment. In contrast, *L. reuteri* induced a decrease in oxytocin gene expression in female KO mice. These results may partly explain the differential behavioral effects of *L. reuteri* between male and female mice regarding social behavior. Finally, to test if *L. reuteri* can induce changes in the immune response, levels of plasma immune markers in Shank3 KO male mice after *L. reuteri* administration were tested. *L. reuteri* administration increased all tested markers, except for the reduction of IL-17a plasma levels.

5. *Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder*[124]

This study analyzed microbiota-mediated changes in social behavior in different mouse models of autism. Shank3B$^{-/-}$ mice, VPA mouse model, and BTBR T+ Itpr3tf/J (BTBR) inbred mouse were used. The first objective was to compare the intestinal microbiota of Shank3B$^{-/-}$ mice with that of WT littermates through the 16S rRNA gene sequencing on fecal samples. Bacterial composition, computed based on weighted UniFrac distances or Shannon diversity index, was not significantly altered in Shank3B$^{-/-}$ mice compared to WT littermates. By contrast, the bacterial diversity measured by unweighted UniFrac analysis, revealed a significant difference in the phylogenetic profile of the microbial communities between genotypes. Shank3B$^{-/-}$ mice specifically have lower levels of *L. reuteri* compared to their WT littermates. To examine whether reduced *L. reuteri* levels in the gut of Shank 3B$^{-/-}$ mice could account for their social behavioral deficits, Shank3B$^{-/-}$ mice were treated with either vehicle or *L. reuteri*, added daily in drinking water for 4 weeks. Social behaviors were first examined in the three-chamber sociability and social novelty tests. To test sociability, the time that the experimental mouse spent interacting with either a stranger mouse or an empty wired cup was assessed. WT mice displayed normal sociability, by contrast, Shank3B$^{-/-}$ mice showed no preference for the stranger mouse over the empty cup, indicating impaired sociability. In addition, as in WT controls, Shank3B$^{-/-}$ mice displayed normal preference for social novelty. Remarkably, treatment with *L. reuteri* rescued sociability in Shank3B$^{-/-}$ mice. To further support these findings, reciprocal social interaction was assessed in Shank3B$^{-/-}$ mice and WT mice. Shank3B$^{-/-}$ mice interacted significantly less than WT littermates, and treatment with *L. reuteri* reversed the social deficits in the mutant mice. It is noteworthy that Shank3B$^{-/-}$ mice are hypo-active, a

condition that was not improved by *L. reuteri*. Thus, treatment with *L. reuteri* selectively reverses the ASD-like social deficits in Shank3B$^{-/-}$ mice. Later, they wandered whether the VPA mouse model of ASD is also characterized by alterations in their microbial ecology. Changes in the composition of the microbiota of VPA-treated mice were found, but *L. reuteri* levels were not reduced in these mice. Although *L. reuteri* levels were not altered, it was questioned whether supplementation with *L. reuteri* could improve social deficits in the offspring of VPA mice as a promoter of oxytocin levels. Interestingly, treatment with *L. reuteri* ameliorates the social deficits in VPA mice. These data demonstrate that, like in the MHFD model, *L. reuteri* also corrects the social deficits in another environmental model of ASD (VPA) with alterations in the gut microbiome. As well as the previous mice models, BTBR inbred mouse line exhibits the core ASD symptoms, including abnormal social behavior. This model is considered an idiopathic model of ASD. An altered microbiota was also found in these mice, in particular a specific reduction of *L. reuteri* was found. Therefore, also in this case, the effect on social behavior of supplementation with *L. reuteri* in these mice was evaluated. Indeed, treatment with *L. reuteri* improved the social deficits in the three chamber and reciprocal social interaction tasks in the BTBR mice. Altogether, treatment with *L. reuteri* selectively reverses the ASD-like social deficits in genetic, environmental, and idiopathic models of ASD. To understand through which mechanism *L. reuteri* induces an improvement in social behavior, the intestinal microbiota of Shank3B$^{-/-}$ mice treated with *L. reuteri* and treated with vehicle was analyzed with 16S rRNA gene sequencing. *L reuteri* does not significantly alter the microbiota. Thus, it was hypothesized that the effect depended on *L. reuteri* interaction with other members of the microbial community. To test this hypothesis, the GF mice were monocolonized with *L. reuteri* at weaning and their behavior was evaluated after 8 weeks. GF mice showed social deficits

compared to conventionally colonized mice. Remarkably, monocolonization with *L. reuteri* was sufficient to reverse the social deficits in GF mice, supporting the notion that *L. reuteri* acts solo, rescuing social behavior in the absence of other members of the community. Another aspect considered is intestinal permeability, which is often associated with ASD. Thus, Shank3B$^{-/-}$ mice were tested for intestinal permeability by administering fluorescein isothiocyanate-dextran (FITC-dextran) by oral gavage and measuring its concentration in the serum. Compared to control littermates, Shank3B$^{-/-}$ mice showed no changes in gut permeability. Accordingly, the expression of key tight junction proteins was not altered in Shank3B$^{-/-}$ mice. Later, it was examined whether the vagus nerve is a communication channel for *L. reuteri* between the intestine and the brain. Bilateral vagotomy was performed in mice to assess this. What we have seen is that *L. reuteri* is capable of rescue social behavior in sham-operated, but not in vagotomized Shank3B$^{-/-}$ mice. This showed that *L. reuteri* reversed the social deficits in Shank3B$^{-/-}$ mice in a vagus nerve-dependent manner. At this point it was evaluated whether the administration of oxytocin could directly Rescues Social Behavioral Deficits in Genetic, Environmental, and Idiopathic Models of ASD without resorting to supplementation with *L. reuteri*. Given that *L. reuteri* increases oxytocin levels, this hypothesis has been evaluated. As expected, intranasal oxytocin reversed the social deficits in Shank3B$^{-/-}$ mice. Moreover, oxytocin improved the social behaviors that are deficient in VPA, BTBR mice, and partially in GF mice, all models in which *L. reuteri* effectively rescues their social deficits. Interestingly, like *L. reuteri* treatment, oxytocin had no effect on the hypoactivity behavior in the Shank3B$^{-/-}$ mice. Together, these data suggest that oxytocinergic signaling is involved in the mechanism of action by which *L. reuteri* selectively restores social behavior in several ASD mouse models. Finally, a further role of *L. reuteri* was demonstrated. *L. reuteri* restores social interaction-induced synaptic

potentiation in ventral tegmental area of Shank3B$^{-/-}$ mice. However, this effect does not exist in mice lacking the oxytocin receptor in dopaminergic neurons.

6. *Supplementation of Lactobacillus Probiotic Strains Supports Gut Brain-Axis and Defends Autistic Deficits Occurred by Valproic Acid-Induced Prenatal Model of Autism*[125]

Pregnant rats were taken to conduct this study. On an embryonic day (ED) 12 VPA at a dosage 400 mg/kg was administrated. The administration of VPA has been used to induce autism. After birth, on the 8th postnatal day (PND) the puppies were divided into 7 groups of 8 individuals each. Depending on the group, the mice received a different supplementation:

Group 1: vehicle treated group (inulin 3mg, p.o daily)

Group 2: autistic group (VPA 400mg/kg, i.p)

Group 3: VPA + *L. plantarum* (not less than (NLT) 1 billion CFU/ml, p.o)

Group 4: VPA + *L. casei* (NLT 1 billion CFU/ml, p.o)

Group 5: VPA + *L. acidophilus* (NLT 1 billion CFU/ml, p.o)

Group 6: VPA + *L. bulgaricus* (NLT 1 billion CFU/ml, p.o)

Group 7: VPA + multilactobacillus strains (NLT 1 billion CFU/ml, p.o)

The treatment duration for this study was PND 08-50 with daily supplementation of probiotic strains NLT 1 billion CFU/ml. After this period, the mice were sacrificed. To analyze the behavior various domains were tested at different PNDs. Negative geotropism was tested on PND 7-10. There was an increase in time taken to re-orient in autistic group compared to vehicle group. Treatment with *Lactobacillus* strains decreased the time to taken re-orient with the autistic group. Eye-opening was observed daily every day after birth PND 12-16. Autistic group present a delayed eye-opening compared to vehicle group. Treatment

with *Lactobacillus* strains improved the result in eye-opening. Swimming performance was assessed on PNDs 22, 24 and 26. Autistic group showed a worse swimming performance compared to vehicle group. Treatment with *Lactobacillus* strains showed an improvement in performance. T-maze test was performed on PND 29-31to evaluate the repetitive/restricted behavior. There was a low alteration score reported in the autistic group when compared with the vehicle group. Treatment with *Lactobacillus* strains showed that alteration score was significantly improved when compared with an autistic group. Morris water waze was performed on PND 48-50 to assess deficits in memory. In autistic group there was poor cognition with the identification of hidden platform compared to vehicle group. Treatment with *Lactobacillus* strains showed that latency to identify the hidden platform was significantly increased than autistic subjects. Social interaction was performed on PND 36-40, and various parameters were analyzed: allogroming, anogenital inspections, pinning's, play behavior, social exploration. Autistic group showed a lower level of social interaction compared to vehicle group. Treatment with *Lactobacillus* strains improved all social interaction activities compared with an autistic group. The exploratory behavior was evaluated by open-field habituation task method on PND 46-48. There was a decrease in exploration and nature of behavior in autistic rats when compared with the vehicle group. Treatment with *Lactobacillus* strains improved their stereotype behavior compared to autistic group. In addition, biochemical parameters were investigated. The Acetylcholinesterase (AchE) activity was measured in brain tissue by the reaction of thiocholine with dithiobis nitrobenzoate ions. AchE activity increased in autistic mice, but supplementation with *Lactobacillus* strains attenuated this increase. Brain-derived neurotrophic factor (BDNF), serotonin, IL-6 and tumor necrosis factor α (TNFα) was measured in blood and brain sample by ELISA method. Elevated BDNF levels were found

in the autistic group compared to the vehicle group. Autistic mice showed hyperserotonemia relative to the vehicle group. Treatment with *Lactobacillus* strains reduced serotonin activity. The prenatal induction of autism caused an increase in TNFα, and IL-6 levels compared to the vehicle group. Supplementation with *Lactobacillus* strains significantly attenuated the increase in TNFα and IL-6 levels. On PND 50 rats were sacrificed and their brains were studied by histopathological analysis. This study proved, daily supplementation of *Lactobacillus* strains has reversed autistic deficits and improved immune functions might because of gut and brain symbiotic relationship.

Clinical studies

After selecting and analyzing the preclinical studies, the same procedure was done on clinical studies. Also in this case, the studies found are few; 2 studies[127,128] on epilepsy and 9 studies[129,130,131,132,133,134,135,136,137] on ASD were selected. The disparity of available material is considerable.

Clinical studies on epilepsy

Studies that investigated probiotic supplementation in epileptic subjects are shown in **Table 3**.

1. *The beneficial effect of probiotics as a supplementary treatment in drug-resistant epilepsy: a pilot study*[127]

They enrolled 45 subjects with DRE, aged> 18 years, with at least one epileptic episode per month and on antiepileptic therapy at the time of the study. The main objective of the study was to evaluate the role of probiotics in controlling the number of seizures and improving the quality of life (QoL) in patients with DRE. The secondary endpoints were the change in markers of inflammation and immune activity, safety, and tolerability. To evaluate the

Table 3: Clinical use of probiotics in epilepsy

Study	Population	Sample Size	Age Range	Pathology	Period Of Therapy	Product Composition	Endpoints	Scale For Endpoints	Results	Adverse Events
Gómez-Eguílaz et al.[127]	Adolescents and young adults	45	≥18 years	DRE	4 months	Mixture: *L. acidophilus, L. plantarum, L. casei, L. helveticus, L. brevis, B. lactis, S. salivarius*. Sachets: 2×10^{11} live bacteria. 2 sachets per day.	Reduction in number of sz. Improvement in QoL. Change in markers of inflammation and immune activity. Safety and tolerability.	Questionnaires for number of sz. QOLIE-10 Spanish version. Serum levels of IL-6, sCD14, GABA.	28,9% displayed >50% reduction in number of seizures. Significant improvement in QoL. Decrease of IL-6 and sCD14, increase of GABA	2 diarrhea grade 1; 1 respiratory disease (probably unrelated to probiotics)
Yeom et al.[128]	Neonates	228	Neonates (>34 gestational weeks)	Rotavirus infection, Sz	Immediately after birth	Probiotics	Rotavirus as a risk factor of neonatal sz Probiotics as a protective factor for sz	Rotavirus ELISA on stool sample. MRI in patients with sz to evaluate WMI pattern.	Rotavirus is a risk factors for sz only in patients without probiotics administration	—

DRE: drug-resistant epilepsy
Sz: seizure
QoL: quality of life
QOLIE-10: quality of life in epilepsy-10
MRI: magnetic resonance imaging
WMI: white matter injury
sCD14: soluble CD14
GABA: γ-aminobutyric acid
IL-6: interleukin 6

secondary endpoints, it was decided to measure the blood levels of IL-6, soluble CD14 (sCD14), and GABA. Before starting the administration of the probiotic, the subjects were evaluated for 3 months. The supplementation period lasted 4 months and was followed by another 4 months of follow up. During all phases of the study, patients completed questionnaires relating to the number of seizures, therapeutic adherence (anti-epileptic drug and probiotics) and quality of life. QoL was assessed through the Spanish version of quality of life in epilepsy-10 (QOLIE-10). The supplementation was a mixture containing: *L. acidophilus, L. plantarum, L. casei, L. helveticus, L. brevis, Bifidobacterium lactis, Streptococcus salivarius subsp. Thermophilus*. Each sachet contained $2x10^{11}$ live bacteria and was administered twice daily. The therapeutic goal was to achieve a reduction of seizures> 50% compared to the baseline period. Of the initial 45 patients only 43 completed the study, however all results were calculated based on the initial 45 patients. The reduction target of> 50% was achieved in 28.9% of patients (n = 13). Of these, 10 subjects maintained a lower number of seizures even in the following 4 months of follow up. In patients in whom probiotics were effective there was also an improvement in QoL both during and after supplementation. As for the serum levels of IL-6 these decreased during supplementation, but re-increased after discontinuation, therefore no significant differences were observed. The reduction in sCD14 levels was also not statistically significant. Conversely, GABA levels increased during supplementation, returning to baseline after discontinuation of probiotics. Clinical and biochemical analysis has shown that probiotics are safe. Only 3 patients experienced adverse events. Two subjects had self-limited grade 1 diarrhea, while another subject had respiratory disease probably not related to probiotics, but to a viral infection. However, the consequences of longer therapy are not yet known.

2. Neonatal seizures and white matter injury: Role of rotavirus infection and probiotics[128]

The population analyzed in this study is very different from that selected in the previous one. Infants with gestational age> 34 weeks admitted to the neonatal intensive care unit for more than 3 days were selected. Of these, 34 were diagnosed with seizures. Subjects with other obvious causes of seizures were excluded from the study. After excluding those who did not meet the inclusion criteria, 228 infants continued the study, including 22 with seizures. Fecal samples were collected at day 3 and 7 of life in the children born and at the day of admission of the children born. They were tested using a rotavirus ELISA and the test was repeated weekly until discharge and whenever patients had symptoms of rotavirus infection. Thanks to this evaluation, the infants were divided into two groups: rotavirus-positive (n = 78) and rotavirus-negative (n = 150). Infants with seizures underwent brain magnetic resonance imaging (MRI) within 5 days of symptom onset. White matter injury pattern was observed in 9 patients. Comparing the 2 groups it was found that seizures and diarrhea were more frequent in the Rotavirus-positive group. Rotavirus infection increased the risk of neonatal seizures. Probiotic delivery at birth was also less common in the Rotavirus-positive group. It was noted that probiotic administration immediately after birth significantly decreased the risk for seizures. A stratified analysis according to probiotic administration immediately after birth showed that rotavirus infection was a significant risk factor only in patients without probiotic medication. All patients with white matter injury (WMI) were treated with antiepileptic drugs. Rotavirus was detected in the stool specimens of all these patients. On comparing between patients with and without the WMI pattern during neonatal seizures, rotavirus infection and seizure onset between day 4 and 6 of life were characteristic of neonatal seizures with WMI pattern. No probiotics were administered immediately after birth in the subgroup with WMI pattern. Overall, this study showed that

rotavirus is an independent risk factor of neonatal seizures, but probiotic administration at birth might decrease the risk of rotavirus-associated seizure.

Clinical studies on ASD

As for the studies conducted on subjects affected by ASD, these are more numerous, but still too few to provide solid evidence. **Table 4** shows the studies considered.

1. *A double-blind, placebo-controlled, crossover-designed probiotic feeding study in children diagnosed with autistic spectrum disorders*[129]

62 children with ASD (3 females and 59 males), aged between 4 and 15 years, were initially recruited. Of these, only 17 subjects completed the 12-week study. The study included: first feeding period, first washout period, second feeding period, and second washout period. Each period lasted 3 weeks, with an overall study duration of 12 weeks. The subjects were divided into two groups: in the first the subjects received placebo in the first feeding period and probiotic during the second feeding period; vice versa in group 2. Probiotic supplementation consisted of capsules each containing $4.5x10^{10}$ CFUs of *L. plantarum* WCFS1. Changes in the fecal microbiota from the beginning to the end of the study were evaluated using fluorescent in situ hybridization (FISH). Parents were asked to keep a diary to monitor changes in GI function and symptoms. It was evaluated: fecal number and consistency, presence and intensity of abdominal pain, intestinal swelling, and flatulence. To assess the behavioral symptoms related to autism, parents completed the Development Behavior Checklist (DBC) - primary care version before the start of the study and at the end of each feeding and washout period. The DBC can be scored at three levels. The first is the Total Behavior Problem Score (TBPS), which gives an overall measure of behavioral/emotional disturbance. The second level is that of the subscale scores which

Table 4: Clinical use of pre- and probiotics in ASD

Study	Population	Sample Size	Age Range	Pathology	Treatment period	Product Composition	Endpoints	Scales For Endpoints	Results	Adverse Events
Parracho et al.[129]	Children and adolescents	62	4-16y	ASD	3+3 weeks	*L. plantarum* ($4.5x10^{10}$ cfu/capsule)	Bacterial population level GI function (No. and consistency of fecal samples) GI symptoms Behavior score	FISH Diary DBC-primary care version TBPS	> Lab158 and < Erec482 No significant differences in number of bowel movements, in GI symptoms, and in TBPS. Higher percentage of formed stool samples	Skin rash, diarrhea, weight lose
Kaluzna-Czaplinske et al.[130]	Children	22	4-10y	ASD	2 months	*L. acidophilus* ($5x10^{9}$cfu/g) twice daily	Level of DA and DA/LA ratio after probiotic Autistic behavior	Capillary GC/MS in urine Qtn	Level of DA and DA/LA ratio decrease after probiotic therapy. Improvement in ability of concentration and carrying put orders.	–
Tomova et al.[131]	Children	10 ASD 9 not ASD siblings 10 HC	ASD 2-9y Siblings 5-17 y HC-11 y	ASD	4 months	*Lactobacillus, Bifidobacterium* and *Streptococcus* x3 a day	Changes in fecal microbiota Correlation between GI disorders	Stool RT-PCR and genomic Qtn ADI score and CARS score	Probiotic normalized *Bacteroidetes/Firmicutes* ratio, *Desulfovibrio* and *Bifidobacteriu*	–

							and autism severity		*m* Correlation GI disorders-ASD severity (only on ADI)	
Grossi et al.[132]	Child	1	12y	ASD	4 weeks	*Bifidobacteria* ($9x10^{10}$cfu/g), *Lactobacilli* ($8x10^{10}$ cfu/g), *Streptococci* ($20x10^{10}$cfu/g)	GI symptoms and autistic core symptoms	ADOS-2A (SA and RRB domains)	Probiotic reduced severity of GI symptoms and improved Autistic core symptoms	–
Shaaban et al.[133]	Children	30 ASD 30 HC	5-9y	ASD	3 months	*L. acidophilus, L. rhamnosus and B. longum* $100x10^{6}$cfu/g	Fecal microbial composition Autism severity GI symptoms Anthropometric measures	Stool RT-PCR ATEC 6-GSI BMI	Increase of *Lactobacillus* and *Bifidobacterium* Decrease severity of ASD Improvement in 6-GSI Decrease in the body weight (in overweight)	Diarrhea, bloating, abdominal cramps and skin rash mild and transient

Grimaldi et al.[134]	Children	30	4-11y	ASD	6 weeks	B-GOS®	Impact of ED and prebiotic intervention	GI symptoms and sleep diary, anxiety and ASD behaviors qtn, FISH analysis, 16S rRNA	ED: <abdominal pain and bowel movement, <*Bifidobacterium* and *Veillonellaceae* >*Faecalibacterium* and *Bacteroides*. B-GOS®: improved anti-social behavior, >*Lachnospiraceae*	–
Sanctuary et al.[135]	Children	11	2-11y	ASD and GI symptoms	12 weeks	BCP, *B. infantis*	Tolerability GI and autistic symptoms	CHARGE GIH survey, BSS, QPGS-RIII, ABC, RBS-R, ABAS-II	Reduction in frequency of GI symptoms and of aberrant behaviors BCP±*B.infantis* well tolerated	Gassiness, stomachache, sleep disorder, lethargy
Liu et al.[136]	Children and adolescents	80	7-15y	ASD	4 weeks	*L. plantarum* ($3x10^{10}$ cfu/capsule)	Changes in autistic behaviors	ABC and CBCL qtn, SRS score, SNAP-IV and CGI-I	Improvement in opposition/defiance behaviors and in total score of SNAP-IV	–

Arnold et al.[137]	Children	13	3-12y	ASD, anxiety and GI symptoms	8+8 weeks	*Lactobacilli*, *Bifidobacteria* and *Streptococcus*	ASD symptoms GI symptoms Microbiota composition	PedsQL GI module, PRAS-ASD, parent-selected target symptoms 16S rDNA AS	No significant improvement for PedsQL and PRAS-ASD. Improvement of the 1st target symptom selected No significant alteration of microbiota	Infections

ASD: Autism spectrum disorder
GI: Gastrointestinal
CFU: Colony-Forming Unit
No.: Number
Qtn: Questionnaire
HC: Healthy control
GC/MS: gas chromatography/mass spectrometry
RT-PCR: Real time polymerase chain reaction
FISH: Fluorescent In Situ Hybridization
ED: Exclusion diet
BMI: Body mass index
DA: D-arabinitol
LA: L-arabinitol
Lab158: lactobacilli and enterococci group
Erec484: Clostridium cluster XIVa
B-GOS®: Bimuno® galactooligosaccharide
BCP: Bovine colostrum product
B.: Bifidobacterium
L.: Lactobacillus
ADI: Autism Diagnostic Interview
PRAS-ASD: Parent-Rated Anxiety Scale for Autism Spectrum Disorder
CARS: Childhood Autism Rating Scale
DBC: Developmental Behavior Checklist
TBPS: Total Behavior Problem Score
6-GSI: 6 Item Gastrointestinal Severity Index
ADOS-2A: Autism Diagnostic Observation Schedule-Second Edition
SA: Social Affect
RRB: Restricted Repetitive Behaviors
ATEC: Autism Treatment Evaluation Checklist
CHARGE GIH: CHARGE Gastrointestinal History
BSS: Bristol Stool Scale
QPGS-RIII: Questionnaire on Pediatric Gastrointestinal Symptoms- Rome III
ABC: Autism Behavior Checklist
CBCL: Child Behavior CheckList
RBS-R: Repetitive Behavior Scale-Revised
ABAS-II: Adaptive Behavior Assessment System-II edition
SRS: Social Responsiveness Scale
SNAP-IV: Swanson, Nolan, and Pelham-IV-Taiwan version
CGI-I: Clinical Global Impression-Improvement
PedsQL: Pediatric Quality of Life

measure disturbance in five dimensions (disruptive/antisocial behavior, self-absorbed behavior, communication, anxiety problems and social-relating problems). The third level is for scoring of individual items. Probiotic supplementation significantly increased the number of *Lactobacilli/Enterococci* and reduced the Erac482 count in the fecal microbiota of ASD children compared to placebo. The number of bowel movements did not vary significantly and for GI symptoms there were also no notable differences between probiotics and placebo. However, supplementation with probiotics resulted in a higher percentage of stool samples formed than placebo. There were no significant differences in TBPS between the two feeding periods, however, there was a reduction in the score compared to the baseline both during the placebo period and during the probiotic period. No significant differences were observed in the five subscales between probiotics and placebo, although the baseline median score was higher than that of probiotic feeding. Only the disruptive/antisocial behavior subscale started from an average score higher than the clinical cut-off and dropped below following the administration of both probiotic and placebo; all the others showed a reduction without falling below the cut-off. During the study 3 subjects dropped out due to adverse effects. One had a skin rash three days after starting the administration of the probiotic. The second had diarrhea during the probiotic period, and the third lost 1.2 kg while taking the probiotic. The proportion of subjects who dropped out of the study, especially during the baseline period, was very high and greatly influenced the statistical power of the study.

2. *The level of arabinitol in autistic children after probiotic therapy*[130]

The aim of the study was to find out whether there are significant differences between D-arabinitol (DA) and ratio DA/L-arabinitol (LA) in urine of autistic children before and after

probiotic treatment. 22 children aged 4-10 with autism were recruited. All participants tended to present GI symptoms such as abdominal pain, constipation, and diarrhea. All subjects received capsules containing $5x10^5$ CFUs/g of *L. acidophilus* twice a day for 2 months. To evaluate the GI symptoms, the characteristics of the diet and the intake of antibiotics (in the last year), questionnaires submitted to the parents were used. For quantification and monitoring of DA and DA/LA ratio in urine capillary GC/MS was used. DA levels were significantly higher in urine prior to probiotic supplementation. In addition, the DA / LA ratio was significantly reduced after treatment. The supplementation with probiotics has also led to a significant improvement in the ability to concentrate and complete orders.

3. Gastrointestinal microbiota in children with autism in Slovakia[131]

The aim of this study was to elucidate changes in fecal microbiota in children with autism and determine its role in the development of associated GI disorders and possibly other manifestations of autism. 10 children with autism were studied aged 2-9 years, 9 siblings aged 5-17 years and 10 healthy children aged 2-11 years. Of the 10 children with autism, only 9 underwent probiotic therapy. The GI condition was assessed through a parental questionary. The supplementation consisted of one capsule administered orally 3 times a day for 4 months. Each capsule contained 3 species of *Lactobacillus* (60%), 2 species of *Bifidobacteria* (25%) and one species of *Streptococcus* (15%). To analyze the fecal microbiota, stool samples were taken, and the DNA of the bacterial species present was extracted. Real time PCR was also performed to investigate the predominant genus of intestinal microflora or the specific bacterial species that are hypothesized to participate in the pathogenesis of autism. Moreover, on the stool samples the levels of TNFα were

measured using the sensitive ELISA. Finally, venous blood samples were taken to measure plasma levels of oxytocin, testosterone and dehydroepiandrosterone sulfate (DHEA-S) using the ELISA method. The results of the study compared on the one hand the data of autistic subjects with those of siblings and healthy controls, on the other hand the data of the participants before and after supplementation with probiotics. Prior to initiating therapy, GI dysfunction was significantly higher in autistic subjects and their siblings than in healthy controls. However, no correlation was demonstrated between Childhood Autism Rating Scale (CARS) assessment and GI manifestations. It was noted that children with severe GI symptoms had lower *Clostridia* and *Desulfovibrio* counts and a lower *Bacteroidetes/Firmicutes* ratio than children with milder GI symptoms. There was also higher *Clostridia* and *Desulfovibrio* count and lower *Bacteroidetes/Firmicutes* ratio in children with severe autism compared to children with mild autism. The change in the *Bacteroidetes/Firmicutes* ratio had only a negative correlation with the severity of autism as assessed with the Autism Diagnostic Interview (ADI) score. On the contrary, there is a positive correlation with the relative quantity of *Desulfovibrio* and the severity of the manifestations of autism; this is mainly due to the very strong correlation between *Desulfovibrio* and ADI restrictive/repetitive behavior subscale. However, no correlation was found between the severity of autism assessed with the CARS score and the fecal microbiota. After supplementation with probiotics, the number of *Firmicutes* decreased and the *Bacteroidetes/Firmicutes* ratio increased to the level of healthy subjects. *Bifidobacterium* counts dropped and the absolute amount of *Lactobacillus* also decreased to a level not significantly different from that in healthy subjects. On the other hand, the relative amount of *Lactobacillus* has doubled after the probiotic. The siblings group also showed changes in the fecal microbiota after supplementation with probiotics. Fecal TNFα levels increased in

autistic subjects and their siblings compared to healthy controls and autistic subjects after the probiotic. In fact, probiotics reduced the levels of TNFα in the stool of autistic children. However, this difference was not significant. A strong correlation between TNFα levels and GI symptoms and a tendency towards correlation between TNFα levels and autism severity were found. Regarding the levels of plasma hormones, lower oxytocin levels in autistic patients and their siblings than in healthy controls were found. There appeared to be a positive correlation between oxytocin levels and the severity of autism. There were lower DHEA-S levels in autistic children compared to siblings and healthy controls. There was a trend towards correlation between the reduction of DHEA-S and the reduction of *Bacteroidetes/Firmicutes* ratio in autistic subjects. Testosterone levels not significantly differed between groups, but there was a positive correlation between testosterone levels and the severity of autism.

4. *Unexpected improvement in core autism spectrum disorder symptoms after long-term treatment with probiotics*[132]

This study is a case report of a 12-year-old boy diagnosed with ASD, severe cognitive impairment and celiac disease. Following a diagnosis of irritable bowel, the subject began treatment with probiotics. The supplementation consisted of a mixture containing $9x10^{10}$ CFUs of *Bifidobacteria* (*B. breve, B. longum, B. infantis*), $8x10^{10}$ *Lactobacilli* (*L. acidophilus, L. plantarum, L. paracasei, L. bulgaricus, L. delbrueckii subsp.*), and $20x10^{10}$ *Streptococci* (S. *Thermophilus, S. salivarius subsp*.). Already after a few weeks the subject showed a reduction in the severity of abdominal symptoms, so the supplementation was continued from February to May 2014. In addition to the improvement in GI symptoms, an improvement in symptoms and manifestations of the ASD. For this reason, it was evaluated whether probiotic therapy could also have effects on the core symptoms of ASD (social

interaction, communication, and/or restricted, repetitive, and stereotyped patterns of behavior and interest), using Autism Diagnostic Observation Schedule - Second Edition (ADOS-2). ADOS-2 examines behavior in two domains: social affect (SA), and restricted repetitive behavior (RRB). The ADOS-2 assessment was repeated 6 times during the study: twice during the baseline assessment, twice during the treatment assessment and twice during the post-treatment assessment. The treatment led to a reduction in abdominal symptoms as expected, but also to an improvement in the core symptoms of autism. The score of the SA domain went from 20 to 18 after two months of treatment with a further reduction of one point in the following two months. The score then remained stable at 17 during the 10-month follow-up.

5. *The role of probiotics in children with autism spectrum disorder: A prospective, open-label study*[133]

30 subjects with autism were enrolled and given supplementation with probiotics for 3 months. Before starting the supplementation, the fecal microbiota was analyzed by real-time PCR. The data obtained were compared with those found in 30 healthy controls and it was found that in subjects with autism the levels of Bifidobacteria were significantly lower. Furthermore, the fecal microbiota of autistic patients before and after therapy was compared. As regards the assessment of the severity of ASD symptoms, the 4 sub-scales of the Autism Treatment Evaluation Checklist (ATEC) score (speech/language communication, sociability, sensory/cognitive awareness, and health/physical /behavior) were assessed individually before and after the months of probiotic supplementation. GI symptoms were assessed before and after therapy using a modified version of the gastrointestinal severity index (6-GSI), which includes constipation, diarrhea, fecal consistency, fecal odor, flatulence, and abdominal pain. The anthropometric measures of all study participants were

also evaluated, and the data showed that 60% of the subjects were overweight, while the other 40% were within the normal range. The supplementation consisted of a daily dose of 5g of powder dissolved in water. Each gram of powder contained $100x10^6$ CFUs of the three probiotic strains: *L. acidophilus*, *L. rhamnosus* and *B. longum*. After 3 months of supplementation there was a significant increase in *Bifidobacteria and Lactobacillus* in stool PCR of autistic children. The total ATEC score decreased, indicating a reduction in the severity of autism symptoms. Improvement was recorded in all 4 ATEC categories. There was also an improvement in the total 6-GSI with a significant reduction in the score of constipation, flatulence, and abdominal pain. Following supplementation, body weight decreased in initially overweight patients. In addition to all this, a negative correlation was found between the increase in Bifidobacteria in the intestinal microbiota and the reduction in the constipation score. Side effects were rare, mild, and transient. Side effects were diarrhea (one patient), bloating (two patients), abdominal cramps (two patients) and skin rash (one patient). Overall, there were beneficial effects both in behavior and in GI manifestations linked to autism. However, this study has important limitations: small sample, unblinding, lack of a placebo control group.

6. A prebiotic intervention study in children with autism spectrum disorders (ASDs)[134]

In this study, the impact of an exclusion diet and the administration for 6 weeks of Bimuno galacto-oligosaccharides (B-GOS®) in 30 autistic children was evaluated. Initially 41 subjects aged 4-11 years were enrolled. However, 11 children left the study during the baseline. The remaining 30 were divided into 2 groups (A and B) according to whether their diet was non-restrictive (n=18) or exclusion diet (n=12). Then within these groups they were randomly assigned to the two feeding groups: Group 1 received placebo, group 2 the

probiotic. The supplementation period was 6 weeks. 4 participants left the study before the end, but only 1 left it due to adverse events. This one had experienced severe diarrhea and abdominal pain after two days of treatment. In total, only 26 participants completed the 10-week study. A daily questionnaire was used to assess symptoms and GI function. The Bristol Stool scale was used to assess fecal consistency. To evaluate the effectiveness of the treatment, parents were asked to complete the ATEC. The autism spectrum quotient (AQ) was used to evaluate the symptoms of autism. The empathy and systemizing quotient (EQ-SQ) was used to assess the children's ability to understand and process emotions and thoughts. Finally, the Spence's Children Anxiety Scale-Parent version (SCAS-P) was used to study the level of anxiety. In addition, a 5-day sleep diary was compiled during the baseline and during the last week of treatment to evaluate the effect of B-GOS® on any sleep disorders. Stool and urine samples were collected weekly. During the analysis of the results, the samples taken at baseline (weeks 1 and 2), after treatment (weeks 6, 7, 8) and during the follow-up (weeks 9 and 10) were then considered. H-NMR was used for the metabolic analysis of urine. The bacterial composition was studied through FISH analysis. Furthermore, microbiota composition was evaluated via 16S rRNA gene amplicon sequencing. The exclusion diet has a strong impact on GI problems, associated with lower abdominal pain and bowel movement scores. The study showed that GI symptoms generally tended to subside after B-GOS®. A sleep benefit was also found in 23% of participants after taking B-GOS®: sleep duration longer than 1 hour and fewer problems with falling asleep. Improvements were found in behavioral scores relating to anxiety and symptoms related to autism after B-GOS® in patients on an exclusion diet. The results of the AQ questionnaire social skill scale reflect an improvement in antisocial behavior from ATEC questionnaire on both exclusion diet and treatment. FISH analysis was performed on fecal samples at

baseline, during treatment and in follow up. Beyond an increase in *Bifidobacterium*, no significant differences were found between treatments. At baseline there was a significant difference in the intestinal bacterial population between the two groups of participants depending on the type of diet. *Bacteroides* spp., *Rikenellaceae*, *Roseburia* spp, *F. prausnitzii* and *Clostridiaceae* were present in higher proportion in the exclusion diet group, whereas *Eggerthella lenta*, *Bifidobacterium* spp., B. *fragilis*, *Akkermansia muciphila*, *Streptococcus anginosus*, *Lactocloboccus* spp. were present in higher relative abundance in the unrestricted diet. Additionally, *Bifidobacteria* were found in lower abundances in the exclusion diet group compared to the unrestricted diet group. Also, a reduction in the *Veillonellaceae* family was observed. The different composition was also evaluated after the treatment. B-GOS® is positively associated with bacterial populations in ASD children on a non-restrictive diet. Furthermore, in these subjects B-GOS® increased diversity in microbiota composition although not significantly. Significant results were obtained in ASD children on the exclusion diet undergoing B-GOS® compared with the placebo group. Different bacterial populations were found between placebo and B.GOS. Notably, *B. adolescentis* and *B. longum* were abundant within *Bifidobacterium* spp., with *B. longum* being significantly predominant in children with ASD on the exclusion diet compared to placebo. No significant metabolic differences were observed when comparing the urine spectra of children with ASD on the two different diets at baseline. On the contrary, the comparison of the fecal profiles of children on an exclusion diet and an unrestricted diet revealed distinct metabolic perturbations at baseline. Depending on the diet, the intestinal bacterial population changes, and as the bacterial population varies, there will be metabolic variations. In subjects on the exclusion diet, correlations of the bacterial flora with glycerol and propionate, valine, leucine and isoleucine, lysine and alanine have been identified. While, in subjects on an

unrestricted diet, correlations of bacterial flora with lactate, tyrosine, isoleucine, leucine, phenylalanine and valine, glucose and 2-hydroxy-2-methylbutyrate have been identified. Intervention with B-GOS® led to significant alterations in the urinary spectra profiles of children with ASD following unrestricted diets, indicating that B-GOS® supplementation contributed to the metabolic variation. Urinary spectra of autistic volunteers receiving B-GOS® contain greater amounts of creatinine, creatine, dimethylglycine (DMG), dimethylalanine (DMA), carnitine, citrate, adipate, and trimethylamine-N-oxide (TMAO) than autistic children taking placebo. Furthermore, B-GOS® supplementation appeared to reduce the amount of phenylacetylglycine, phenylalanine and β-hydroxybutyrate. Metabolic changes were also observed in fecal samples after B-GOS® intervention. Ethanol, DMG and SCFA (butyrate, valerate) were positively correlated with the intake of B-GOS®. Increased butyrate production was also detected in children with ASD after excluding diets; however, these changes were not significant. Furthermore, lower amino acid and lactate levels were detected in the B-GOS® group, compared to placebo. Overall, the study showed a significant impact of the diet on the gut microbiota. An exclusion diet is associated with increased amino acid excretion and potential nutrient malabsorption problems, which is why it should be reconsidered as a first-choice intervention. Instead, a combination approach between prebiotics and exclusion diet results in a significant improvement in antisocial behavior, suggesting that such a strategy may be more relevant for the improvement of these aspects.

7. *Pilot study of probiotic/colostrum supplementation on gut function in children with autism and gastrointestinal symptoms*[135]

The objective of this study was to assess tolerability of a probiotic (*B. infantis*) in combination with a bovine colostrum product (BCP) as a source of prebiotic

oligosaccharides and to evaluate GI symptoms, microbiome, and immune factors in children with ASD and GI co-morbidities. 11 children aged 2-11 years were selected and were randomly assigned to receive either the BCP only or the combination (BCP + *B. infantis*) during the first or second arm of the study. After the first 5-week arm, participants underwent a 2-week wash out period in which no treatment was received, followed by the second 5-week arm. The colostrum powder dose administered was 0.15 g/lb body weight per day, while the probiotic dose administered was 20 billion CFU per day. Parents were asked to complete a dosing diary to assess compliance as well as a daily stool log, contained a 7-point Bristol Stool Scale, to keep track of their child's bowel movement frequency and consistency. Parents were also asked to complete GI symptom questionnaires, including the CHARGE Gastrointestinal History (GIH) survey and the Questionnaire on Pediatric Gastrointestinal Symptoms-Rome III Version (QPGS-RIII), and validated questionnaires relevant to behavior, including the Aberrant Behavior Checklist (ABC), the Repetitive Behavior Scale-Revised (RBS-R), and the Adaptive Behavior Assessment System–Second Edition (ABAS-II). Blood, urine, and stool specimen collection were also performed at each visit. Of the 11 patients who started the study, only 9 completed it and only 8 were included in the final analysis. In general, both the BCP only and the combination treatment (BCP + *B. infantis*) were well tolerated with no participants needing to withdraw due to adverse events. However, parents of 3 participants reported that their children did not like the taste of the supplement, although all were compliant with study protocol and were able to complete the study. The most reported side effects included increased gassiness (n = 2), stomachache (n = 1), sleep disturbances (n = 1) and lethargy (n = 1) on the BCP only arm and weight gain (n = 2), increased gassiness (n = 2) and hives (n = 1) on the combination treatment arm. There was no carry over effect for changes in microbial taxa, stool frequency,

stool consistency, frequency of GI symptoms and ABC scores, such that the outcomes were similar if the participants received the BCP only treatment first compared to receiving the combination treatment first. Only one outcome measure was shown to have a significant order effect. Frequency of diarrhea, based on the GIH survey, showed a mean increase in symptoms on the combination arm if they received BCP only first and a mean decrease in scores on the combination arm if they received the combination treatment first that was statistically significant. However, there was no difference based on order for this outcome measure on the BCP only treatment. There was improvement in GI symptoms in both groups, but greater in the combined treatment arm. The percentage of stools of normal consistency also increased after the combination treatment. There was a reduction in the frequency of pain associated with bowel movements in both groups. There was also a reduction in the frequency of diarrhea in the BCP only group. There was also an increase in appetite and consumption of new foods in both groups, but greater on BCP only treatment. No differences in adaptive behaviors were observed based on the ABAS-II questionnaire or repetitive behaviors based on the RBS-R. A significant reduction in certain aberrant behaviors was found based on the ABC questionnaire data during the BCP only treatment. In the BCP only group, there was a significant reduction in irritability, stereotypy, hyperactivity, and total scores, along with a trend toward significant reduction in lethargy. The combination treatment demonstrated a significant reduction only in lethargy. Analysis of the fecal composition revealed a general lack of global changes to the fecal microbiome with either treatment. The microbiome of half of the participants did not change enterotype throughout the study. No global changes in fecal, urinary or serum metabolite profiles based on treatment were observe. However, several participants displayed high initial fecal ethanol and methanol concentrations. Both ethanol and methanol levels were significantly reduced

after treatment. There were no significant differences between treatments in terms of initial ethanol and methanol levels or change in ethanol and methanol levels. There were no significant changes observed for any of the urine or serum metabolites. Overall, there was a reduction in intracellular expression of certain cytokines by both CD4+ and CD8+ T cells. In stimulated cells, the frequency of CD4+ /IL-13+ T cells was significantly lower after combination treatment. There was also a significant reduction in the frequency of CD8+ /TNFα+ T cells with the BCP only treatment. No significant differences in the frequency of CD4+ or CD8+ T cells expressing interferon-γ (IFNγ), IL-17, or IL-6 were found.

8. *Effects of Lactobacillus plantarum PS128 on Children with Autism Spectrum Disorder in Taiwan: A Randomized, Double-Blind, Placebo-Controlled Trial*[136]

In this study, males aged 7-15 years with ASD were recruited. 80 patients were involved, divided into two groups with a 1: 1 ratio. One group would have received the probiotic, the other group the placebo. The first group was given probiotic capsules each containing $3x10^{10}$ CFUs of *L. plantarum* with microcrystalline cellulose as the carrier. The second group received as placebo capsules containing only cellulose microcrystalline. The primary outcomes of this study were changes in the Autism Behavior Checklist-Taiwan version (ABC-T) questionnaire, the SRS scores, and the Child Behavior Checklist (CBCL) questionnaire. The secondary outcomes were improvement in the Chinese version of the Swanson, Nolan, and Pelham-IV (SNAP-IV) assessment, the Clinical Global Impression-Improvement (CGI-I) and the Clinical Global Impression-Severity (CGI-S). The CGI-I and CGI-S forms were completed at baseline and after 4 weeks of supplementation by physicians. Parents completed ABC-T, CBCL, SRS, and SNAP-IV questionnaires. Only 71 subjects completed the study (36 probiotic, 35 placebo). 3 subjects dropped out of the study because of prescribed antibiotics. 5 subjects withdrew their informed consent. One subject

did not complete the Autism Diagnostic Interview-Revised (ADI-R). No adverse events were reported in any of the participants. The CGI-S score was like the baseline in both groups studied. The CGI-S score showed only minimal improvement for both groups. There were no differences between the two groups in total ABC-T score and subscales score either at baseline or after 4 weeks. Also, in the total score and in the subscale scores of SRS there were no differences between the two groups either at baseline or at week 4. Same thing in the total CBCL scores, however the consumption of probiotic for 4 weeks led to a reduction in the anxiety score, in rule-breaking behavior. The children in the placebo group showed a reduction in scores for problems related to outsourcing over a period of 4 weeks. The SNAP-IV score was also similar in the two groups both at baseline and after 4 weeks. However, there was a reduction in the total score, in hyperactivity and impulsivity, in opposition and challenge within 4 weeks in the probiotic group. In conclusion, *L. plantarum* can improve some symptoms of autism, primarily those associated with disruptive and rule-breaking behaviors and hyperactivity/impulsivity. Furthermore, it seems that the effectiveness of probiotics is age dependent, with better effects in the youngest than in the older ones.

9. *Probiotics for Gastrointestinal Symptoms and Quality of Life in Autism: A Placebo-Controlled Pilot Trial*[137]

In this study, 13 children aged 3-12 years with ASD, anxiety, and GI symptoms were randomized into a probiotic crossover trial of 8 weeks each on probiotic mix and placebo separated by a 3-week washout. Among the inclusion criteria there was the diagnosis of ASD confirmed by ADI-R or ADOS and the presence of GI symptoms for at least 2 months. Treatment consisted of probiotic mix made up of four strains of *Lactobacilli* (L. *casei, L. plantarum, L. acidophilus, and L. delbrueckii subsp. bulgaricus*), three strains of *Bifidobacteria* (*B. longum, B. infantis, and B. breve*), one strain of *S. thermophiles*, and

starch. All measures were done at baseline, week 4, week 8 (end of first condition), week 11, week 15, and week 19 (end of second condition). The primary outcome measure was the GI Module of the Pediatric Quality of Life (PedsQL). The main measure of emotional stability/anxiety was the 25-item single-factor Parent-Rated Anxiety Scale for Autism Spectrum Disorder (PRAS-ASD). An important secondary measure was Target Symptom Rating for which parents are asked to name the two problems of most concern to them at baseline. The ABC evaluates irritability, social withdrawal, stereotypes, hyperactive, inappropriate speech. SRS measures the severity of autism spectrum symptoms. Children's Sleep Habits Questionnaire was a secondary outcome measure. The Parenting Stress Index (PSI) Short Form was completed at baseline and end of each 8-week to evaluate the degree of stress in the parent/child relationship. Analysis for 16S rDNA amplicon sequences was done. Stools were collected at baseline, end of each condition, and end of washout. There were no serious adverse effects. However, there were four infections while on probiotics and none while on placebo which the physician did not consider attributable to study treatment. 13 patients started the study, but 3 dropped out. Over the 19-weck study period, each outcome measure showed improvement over baseline, with the probiotic phase showing more improvement than the placebo phase, but the difference for PedsQL and PRAS-ASD did not meet statistical significance. Regarding the symptoms selected by the parents on the first target symptom participants showed more improvement while on probiotics over placebo, but second target symptom effect was not significant. Fecal microbiota characterization was performed in the 10 children who completed the crossover study. No specific treatment-associated shift was evident in either α-diversity or family level composition bacterial species that could be attributed to probiotic administration; that is, properties did not significantly alter microbiome community complexity or composition in

the stool. It was found that the relative abundance of *Lactobacillus* correlated significantly with the PedsQL score. The probiotic mix is a safe treatment in children with ASD and GI symptoms, but efficacy for quality of life is unproven.

Background

The role of α-lactalbumin, FOS and inulin in epilepsy

In the '90s we saw the reversal of the role of serotonin in epilepsy: mistakenly believed to be proconvulsant, it was found to be anticonvulsant[138]. Since serotonin is synthesized starting from an essential amino acid, tryptophan (TRP), which competes with other Large Neutral Amino Acids (LNAAs) both for cerebral uptake and intestinal absorption, dietary strategies aimed at increasing both plasma TRP and its cerebral uptake are useful. Confirming the role of serotonin, a reduced ability to obtain TRP from the diet (nutritional vulnerability)[139] has been reported in epileptic patients, which may also depend on its altered metabolism with a greater production of indole catabolites, which perform reinforcing actions on the intestinal membrane. A reduction of 1/3 of the brain serotonin synthesis rate was estimated in epileptic patients compared to controls[140]. Oral assumptions of the α-lactalbumin (ALAC) serum protein were found to be able to increase the plasma TRP/LNAAs ratio[141], but, in presence of an altered metabolism of TRP, this increase does not automatically correspond to an increase in its cerebral uptake, and therefore to an increase in brain synthesis of serotonin. The microbiota controls the metabolism of TRP and an altered microbiota has been reported in epileptic patients[142]. Therefore, restoring a correct microbiota allows not only to increase plasma TRP, but also to restore its correct metabolism and its adequate cerebral uptake. Thanks to its specific action on the digestive system, ALAC was found to be able of both modifying the microbiota and increasing the speed of synthesis of brain serotonin by up to 5 times[143]. Moreover, ALAC showed protecting properties in mouse models of epileptogenesis, reducing spontaneous seizures development[144]. In addition, ALAC was found to increase neuropeptide Y (NPY)[145], which has been defined as a potent endogenous anticonvulsant. Properly nourishing the cells of the digestive system

facilitates their response to the stimulation of ALAC. The specific nutrients of these cells are SCFAs which also perform an anti-inflammatory action as they are inhibitors of HDAC. A key nutrient and maximum inhibitor of HDAC is butyric acid[146]. Butyric acid and other SCFAs are also required by the fermentation of indigestible fibers (e.g., FOS and inulin) by colon bacteria. After having gathered evidence about the hypothetical use of prebiotics in patients with epilepsy, and after having noticed the utility of ALAC, FOS and inulin in improving the ability to obtain TRP from the diet, and consequently to produce serotonin, we decided to select drug-resistant epileptic patients and treat them with for 3 months.

Aim of the study

We conducted a pilot, prospective, open-label, multicentre study to evaluate the efficacy of a prebiotic mixture as adjunctive treatment in children and adults with drug-resistant epilepsy (DRE).

Patients and Methods

Patients

Patients with DRE of different aetiologies and aged > 3 years were recruited between August 2020 and May 2021 from 7 Italian Epilepsy Centres: IRCCS Istituto Giannina Gaslini, Genoa; A.O.U. Policlinico Federico II, Naples; A.O.U. Pisana, Pisa; Ospedale Martini, Turin; Ospedale Santa

Figure 2: Italian epilepsy centres

Maria della Misericordia, Perugia; Ospedale Fatebenefratelli, Milan; Ospedale Policlinico Umberto I, Rome.

Written informed consent was provided by patients or their parents/caregivers. The study was conducted following the Good Clinical Practice guidelines and approved by our local EC.

Methods

Eligible patients underwent a "screening visit" and then received the prebiotic mixture (sachets of 3g ALAC, FOS and inulin + tablets of 580mg of sodium butyrate) twice a day for 3 months. Clinical and treatment data were collected at the enrollment and at follow-up visits during the study. Particular attention was paid to the following outcome measures:

- Mean number of total seizures during the treatment period
- Number of seizure-free days
- Improvement of intestinal symptoms and fecal consistency, assessed throughout the validated ROMA 4 questionnaire and the Bristol Stool Test (BST), respectively
- Changes in the perceived quality of life (QoL) (i.e., attention, social interaction, and sleep rhythm/quality)
- Modification of the intestinal microbiota

Stool samples were collected both at the enrollment and at the end of the 3 months treatment period using the MyMicrobiota stool samples kit. Following collection samples underwent a 16S rRNA gene microbial profiling, aiming at evaluating the genomic profile of the bacteria present in the intestine. Statistical analysis was then performed to define whether the bacterial genera are in excess or in defect as compared to controls. In the intestine of healthy adults, the phylum *Bacteroidetes* corresponds to approximately 45-55% of all the species, the phylum Firmicutes to 40-50%, while the remaining are mainly Proteobacteria and Actinobacteria. The

study of the microbiota using the MyMicrobiota analisys highlights the biodiversity level using the Observed OTUs (Operational Taxonomic Units) index, represented by a curve: the X axis shows the number of analyzed sequences, the Y axis shows the number of Observed OTUs calculated by pooling sequences with 100% identity, excluding chimeric OTUs and single-sequence OTUs in order to exclude false positives. The higher the value reached by the curve, the greater the number of bacterial groups contained in the sample. The relationship between bacterial content classified as phylum Firmicutes and Phylum Bacteroidetes is an index of the well-being of the intestinal microbiota[147]. From the ratio it is possible to deduce the type of dysbiosis: fermentative (ratio higher than normal) or putrefactive (ratio lower than normal).

Assessment of effectiveness

A seizure diary (**Supplementary Figure 1**) was provided to patients' parents/caregivers in order to strictly monitor the changes in the number of seizures throughout the study.

Seizure frequency was provided per week since the previous visit and efficacy outcome were assessed at months 1, 2, and 3. According to other published studies[148,149,150] weekly seizure frequency was converted to frequency per 28 days (weekly frequency × 4). Percentage change in seizure frequency for each patient was calculated as ([seizure frequency per 28 days] − [seizure frequency at baseline]) / [seizure frequency at baseline] × 100. Median percentage changes in seizure frequency were calculated due to interpatient variability.

Questionnaires on QoL (**Supplementary Figure 2**), food diaries (**Supplementary Figure 4**), and the BST (**Supplementary Figure 3**) were also provided to assess the other outcome measures during the study period.

Results

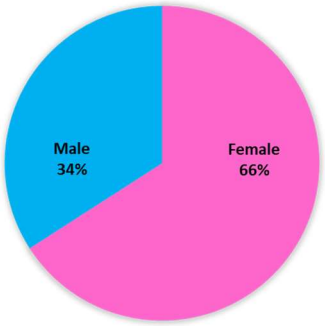
Demographic features

A total of 45 (29 female and 16 male) patients were eligible. Mean age at randomization was 15.37 ± 9.11 years (age range, 3-57 years).

Figure 3: Demographic features

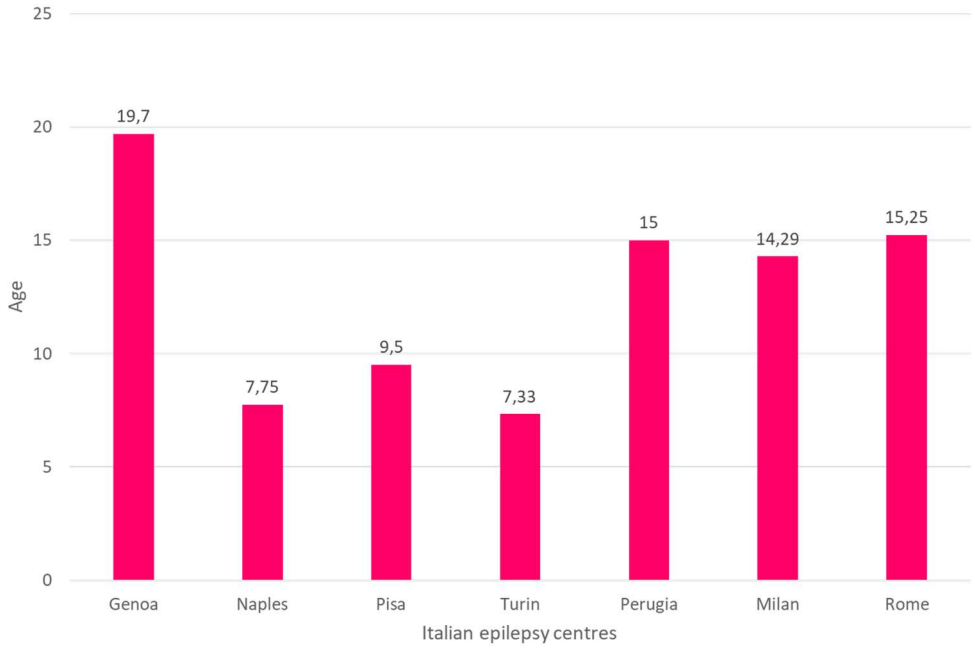
Centres	Numbers of patients	Male/Female ratio	Mean age
Genoa	20	1:4	19.7 ± 10.40
Naples	4	4:0	7.75 ± 2.17
Pisa	4	1:1	9.5 ± 4.39
Turin	5	1:4	7.33 ± 3.51
Perugia	1	1:0	15
Milan	7	4:3	14.29 ± 2.69
Rome	4	0:4	15.5 ± 7.22

Figure 4: Male/Female ratio



There was a significant difference in the mean age of the patients enrolled in the different centers that partecipated in the study (**Figure 3**, **Figure 5**). The greatest difference was found between the centre of Genoa and those of Naples, Pisa, and Milan.

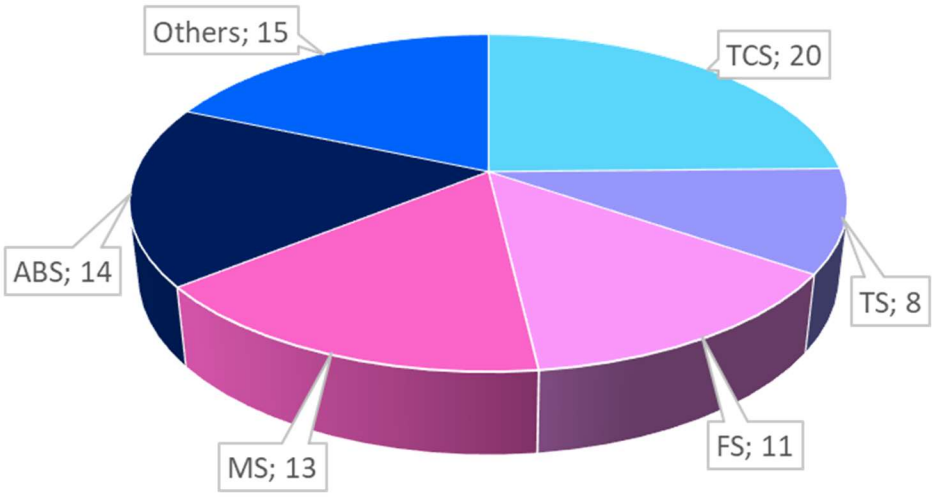
Figure 5: Mean age at randomization



Clinical features of the studied population

Patients presented heterogenous types of seizures including tonic-clonic (44.44%), typical and atypical absences (31.11%), myoclonic (28.89%), focal (24.44%), tonic (17.78%), atonic seizures (15.56%). Infantile and tonic spasms were present in 4.44% of patients each. Dyscognitive seizures were present in 6.67% of patients and visual hallucination (occipital seizures) in 2.22% of patients. Electrical status epilepticus in sleep (ESES) was present in 4.44% of patients. Each patient presented one or more types of seizures.

Figure 6: Seizure Types



According to the highly heterogeneous selection *criteria* 13 (28.89%) patients were affected by genetic generalized epilepsy (GGE): 9 (69.23%) not specified, 2 (15.38%) Janz syndrome, 1 (7.69%) juvenile absence epilepsy, 1 (7.69%) myoclonic absence epilepsy, and.

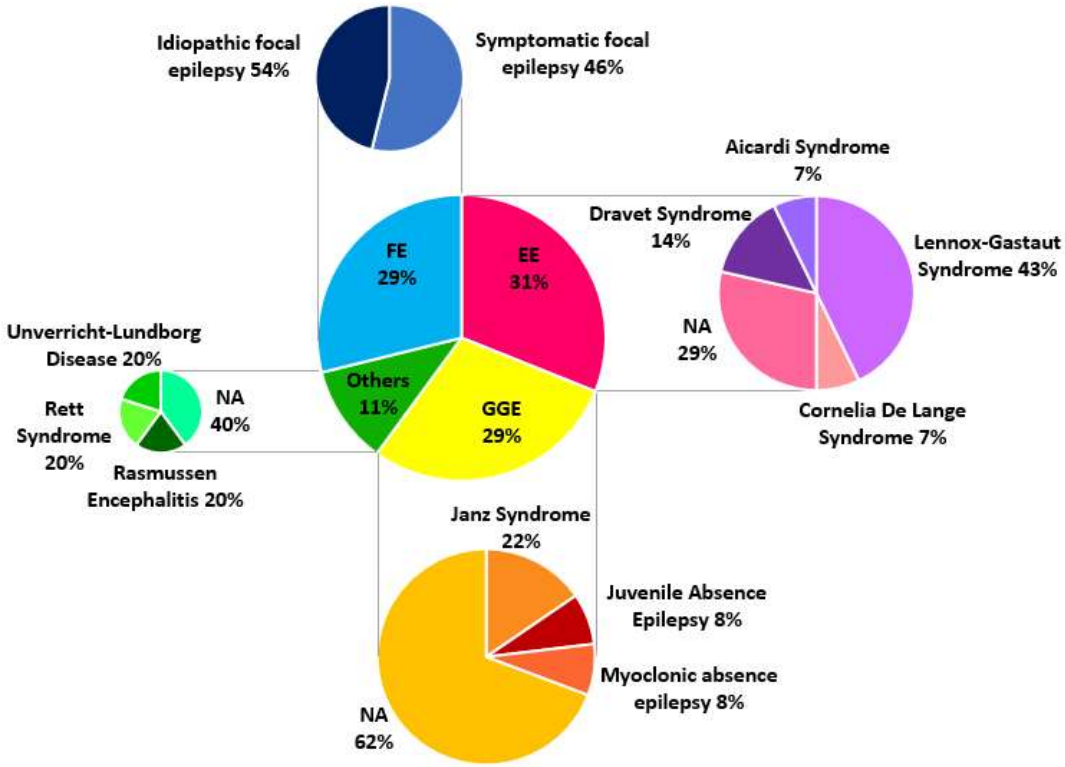
Thirteen (26.67%) patients showed focal epilepsy: 7 (53.85%) idiopathic focal epilepsy and 6 (46.15%) symptomatic focal epilepsy.

Fourteen (31.11%) patients were affected by epileptic encephalopathy (EE): 6 (42.86%) Lennox-Gastaut syndrome, 4 (28.57%) not specified, 2 (14.26%) Dravet syndrome, 1 (7.14%) Aicardi syndrome, and 1 (7.14%) Cornelia De Lange syndrome.

Five (11.11%) patients presented other types of epilepsy including 1 (20%) patient each with Unverricht-Lundborg Disease, Rett Syndrome, and Rasmussen encephalitis. Two (40%) had not specified types of epilepsy.

Figure 7: Seizures aetiology

EE: Epileptic Encephalopathy
GGE: Genetic Generalized Epilepsy
FE: Focal Epilepsy
NA: Not Available



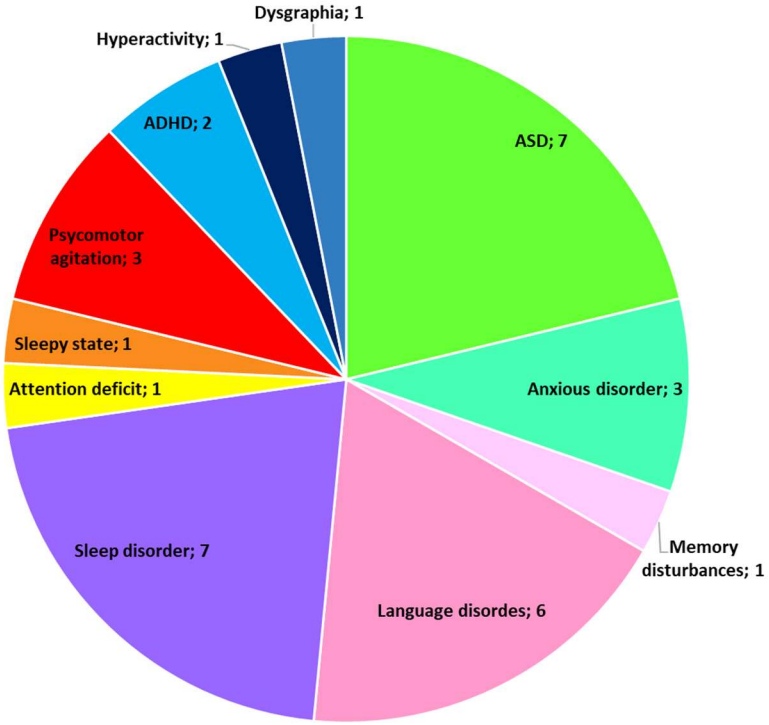
The neurological examination was performed in 39 (86.67%) patients. Eighteen (46.15%) patients had a normal neurological examination, 21 (53.85%) showed abnormalities, including: diffuse hypotonia (38.10%), hemiparesis (14.29%), tremors (9.52%), gait disturbances (9.52%), clumsiness (9.52%), dystonic and dyskinetic movements (9.52%), spastic-dystonic paraparesis

(9.52%), spastic tetraparesis (9.52%), , hypertonia (9.52%), dysarthria (4.76%), myoclonus (4.76%), ataxia (4.76%), paresis in a limb (4.76%), hyperexcitable osteotendinous reflexes and tendency to clonus (4.76%), stereotypies (4.76%), and adiadokokinesia (4.76%).

Among the 45 patients enrolled, 28 (62.22%) had neuropsychiatric comorbidities. ASD was present in 25% of the patients, while sleep disturbances and language disorders were found in 7

Figure 8: Neuropsychiatric comorbidities

ASD: Autism Spectrum Disorder
ADHD: Attention Deficit Hyperactivity Disorder



(25%) and 6 (22.2%) patients, respectively. Anxious disorders (10.71%), psychomotor agitation (10.71%), ADHD (7.14%), hyperactivity (3.57%), attention deficit (3.57%), memory disturbances (3.57%), sleepy state (3.57%), and dysgraphia (3.57%) were the remaining neuropsychiatric comorbidities.

Intellectual disability (ID) was present in 27 (60%) patients and absent in 13 (28.89%) patients, while in the remaining 5 (11.11%) patients it was not determined.

Twenty-three (51.11%) patients had developmental delay (DD), while 17 (37.78%) had normal development. In 5 (11.11%) patients the rate of DD was not determined.

Furthermore, 11 (24.44%) patients had other comorbidities: dysmorphic features (36.36%), gastroesophageal reflux disease (27.27%), headache (18.18%), anemia (9.09%), recurrent respiratory infections (9.09%), scoliosis (9.09%), and weight <3rd percentile (9.09%).

Imaging and EEG findings

In 31 (68.89%) patients an MRI was performed. It was normal in 17 (54.84%) patients, while abnormalities were found in 14 (45.16%) patients. The abnormal findings included: cortical malformation (21.43%); extensive hypo-ischemic damage (14.29); microcephaly (21.43%); outcomes of encephalitis (7.14%); cortical and cerebellar atrophy (7.14%); cerebellar hemispheres asymmetry (right and worm hypoplasia) (7.14%); atrophic corpus callosum and reduced brainstem (7.14%); cystic encephalomalacia (14.29%); bilateral lesions of the thalamus, basal ganglia and to the corticospinal tract of the midbrain and medulla (7.14%); Rasmussen encephalitis (7.14%); dysmorphic signs (7.14%); hydromielia C2-C7 (7.14%).

The EEG pattern of 42 (93.33%) patients was evaluated, while that of 3 (6.67%) patients was not available. Epileptiform anomalies were found in all EEG and included: generalized spike-waves (50%); focal spike-waves (9.52%); diffuse epileptiform abnormalities over the left hemisphere with contralateral diffusion (9.52%); generalized epileptiform abnormalities (7.14%); right or left temporal epileptiform abnormalities (7.14%); diffuse/focal slow spike and spike-wave discharges (4.76%); occipital epileptiform abnormalities (4.76%); focal

epileptiform abnormalities (2.38%); left frontal-temporal slow waves and spikes (2.38%); bilateral frontal epileptiform abnormalities (2.38%); bilateral central-parietal epileptiform abnormalities (2.38%); bilateral Rolandic epileptiform abnormalities (2.38%); slow cortical activity (2.38%).

Genetic investigations

Of 45 patients, 24 (53.33%) performed a genetic evaluation: 20 (83.33%) performed NGS, 8 (33.33%) performed Array-CGH.

For 8 (40%) patients the NGS is still ongoing. In 11 (55%) patients, pathogenetic variants were found in the following genes: *SCN1A* gene encoding the alpha subunit of the Nav1.1 sodium channel, *LIS1* gene encoding the subunit of the complex platelet activating factor acetyl hydrolase 1B, *SMCA1* gene encoding the structural maintenance of chromosomes protein 1A, *CSTB* gene encoding the Cystatin-B, *NALCN* gene encoding the voltage-independent nonselective cation channel, *CHD4* gene encoding the chromodomain helicase DNA binding protein 4, *SPTAN1* gene encoding the spectrin alpha non-erythrocytic 1, *TSC2* gene encoding the tuberin, *CDKL5* gene encoding the cyclin-dependent kinase-like 5, *CHD2* gene encoding the chromodomain helicase DNA binding protein 2, *SHANK3* gene encoding the protein SH3 and multiple ankyrin repeat domains 3, and *NHLRC1* gene encoding NHL repeat containing E3 ubiquitin protein ligase 1.

In 1 (5%) patient the whole-exome study (WES) did not reveal any causative mutation.

Finally, a variant of uncertain significance (VOUS)in the *DEPDC5* gene, encoding DEP Domain Containing 5, was found in 1 (5%) patient.

Array-CGH did not show alterations in 5 (62.5%) patients, while in 3 (37.5%) patients chromosomal alterations were found: interstitial deletion of chromosome 16 (33%), duplication/inversion 15q11 (33 %), chromosomal rearrangement (33%).

Compliance to therapy and seizures outcome

Three (6.67%) patients suspended treatment with prebiotic mixture due to inefficacy. Particularly, 1 patient withdrew after 1 month, and 2 patients withdrew after an unspecified period.

2 (4,44%) patients experienced problem taking the tablet and the caregiver had to break it.

Comparison between mean monthly total seizures at randomization and at 3-months of follow-up resulted in a p=.185. Comparison between specific seizure types at randomization and at the end of the treatment resulted in the following: tonic-clonic seizures p=.102; tonic seizures p = .529; focal seizures p=.771; myoclonic seizures p=.528; absences p=.225; others type of seizure p=.022.

Figure 9: Comparison between mean monthly seizures at randomization and at 3 months of follow-up

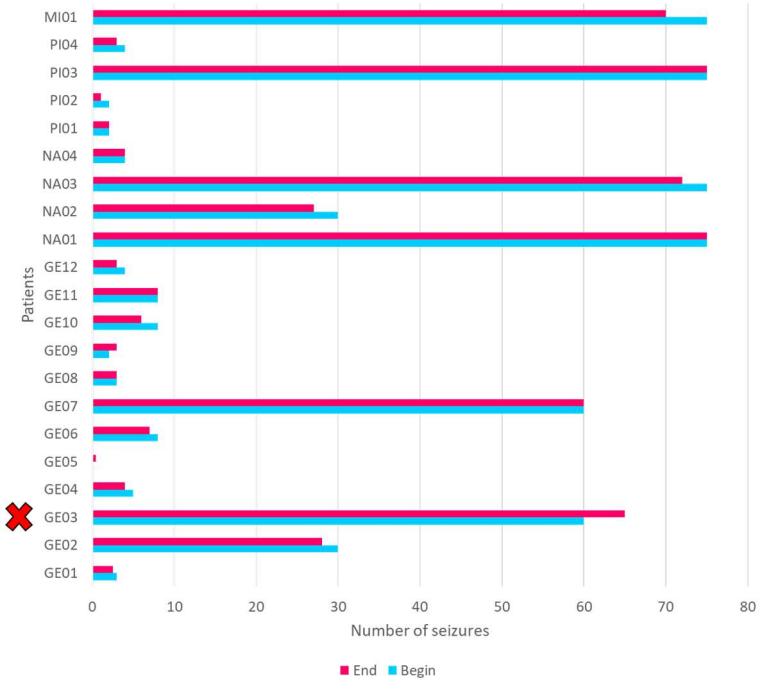


Figure 9 compares the number of monthly seizures before and after prebiotic mixture therapy of patients who have completed the 3-month follow-up. The red X indicates a patient who was excluded from the study due to inadequate treatment intake.

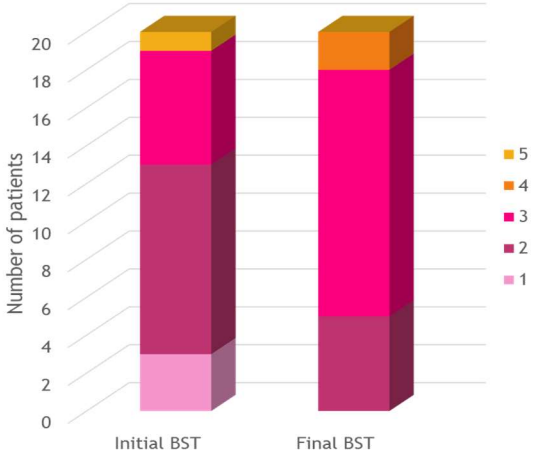
We also evaluated whether there was an improvement in mean monthly seizures in subjects who presented an improvement in BST. This resulted in a p=.055.

Basing on the questionnaires on the perceived QoL, 2 (10%) caregivers reported improvements: 1 (50%) patient experienced improved attention and partecipation; 1 (50%) patient experienced greater social interaction.

Intestinal function assessment

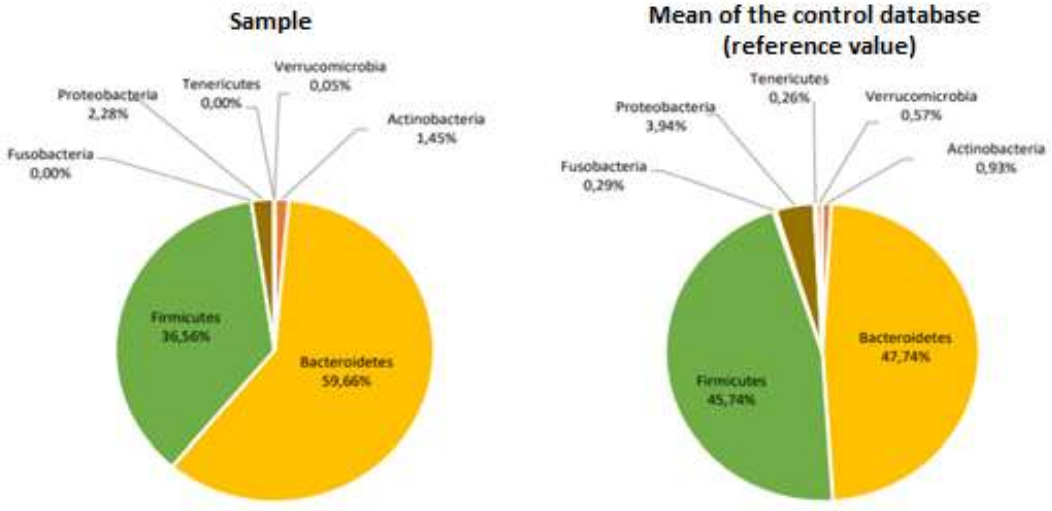
Caregivers reported improvements in intestinal functions. A value of p=.017 resulted from the comparison between BST scores at randomization and at the end of the study (**Figure 10**). However, there were improvements already after 1 month of treatment.

Figure 10: Comparison between BST scores at randomization and at the end of the study



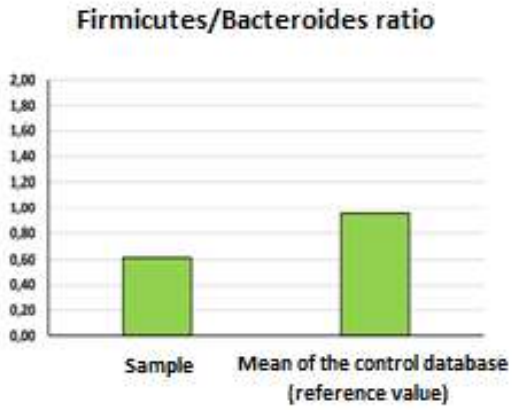
Significant changes were found in the composition of the gut microbiota. **Figure 11** shows the composition of a patient's (GE04) microbiota after 3 months of therapy compared to the mean

Figure 11: Composition of a patient's microbiota after 3 months of therapy compared to control database



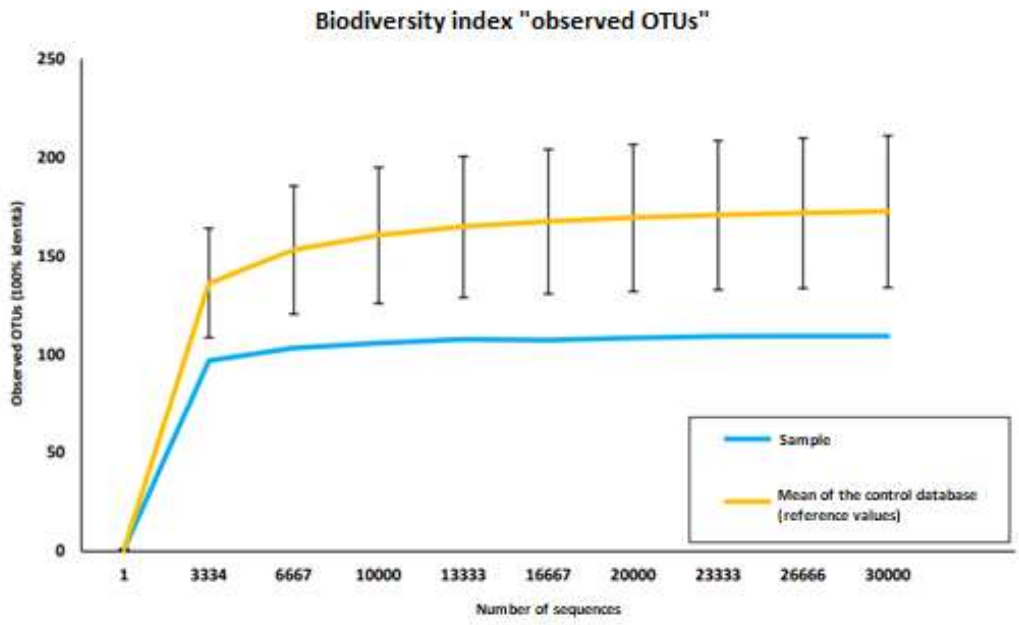
of the controls database. There was a significant reduction in *Bacteroidetes* and a significant increase in *Firmicutes* as compared to the average of the controls database, with a consequent increase in the *Firmicutes/Bacteroidetes* ratio (**Figure 12**).

Figure 12: Relative abundance ratio of Firmicutes / Bacteroidetes phyla in the analyzed sample to the control database



Finally, **Figure 13** represents the biodiversity level of GE04 gut microbiota as compared to the mean of the controls database. The higher the value reached by the curve, the greater the number of bacterial groups contained in the sample. Therefore, in the sample analyzed we found a lower number of bacterial groups as compared to the reference values.

Figure 13: Biodiversity level of the patient's gut microbiota compared to the mean of the controls database



Discussion and Conclusions

Some studies in the literature evaluated the use of probiotics or prebiotics, both in animal models and in humans, to help control epileptic seizures. Both preclinical[119,120,121,144] and clinical[127,128] studies yielded promising results. Other studies[129,131,133] evaluated the efficacy of probiotics and prebiotics in improving GI symptoms, and, as well as the other case, the literature gave positive feedback. Based on this evidence, we conducted our study. However, our results show no significant differences in either reduction of mean monthly seizures or increase in seizure-free days during the 3-months adjunctive treatment with prebiotics mixture. In addition, no significant differences were found in the sub-analysis between the different types of seizure (i.e., tonic-clonic, tonic, focal, myoclonic, absence seizures) at randomization as compared to the end of the study. However, all patients had multiple and heterogeneous seizures, so it may be difficult to analyze seizure groups. Even evaluating the subgroup of patients who reported an improvement in BST, no significant changes in the number of monthly seizures were appreciated (p=.055). Although data on seizure control are not encouraging, only 3 patients early discontinued the treatment, hence patients/caregivers observed some benefits in the treatment. Particularly, significant results were obtained when comparing scores of intestinal symptoms and fecal consistency at randomization with those at the end of treatment (p=.017). Almost all patients or their caregivers reported a "normalization" of the fecal consistency as assessed through the BST and a reduction in GI symptoms such as constipation. The analysis of the microbiota of the GE04 patient also gave positive results, showing an increase in the *Firmicutes/Bacteroidetes* ratio. Therefore, in the sample analyzed we found a lower number of bacterial groups as compared to the reference values. However, it must be considered that the treatment was carried out only for 3 months, so we hypothesize that with a longer period of

supplementation, better results can also be obtained on this aspect. In addition, some caregivers were very satisfied with the improvement in their children QoL, reporting increased attention span, greater socialization. Considering that most of our studied cohort is composed of patients with neuropsychiatric comorbidities, and borderline or severe DD even small changes in the QoL can be of great significance. For this reason, even if there were no significant improvements in seizures, many patients continued the treatment.

Some limitations may be found in the present study as only 20 patients have yet completed the 3-months treatment period. Although, the trend is quite clear with an impact of the adjunctive treatment mostly on intestinal function and participation than on seizures themselves, the results will have to be reviewed later to have a global evaluation. Furthermore, we assume that a 3-months follow-up is too short. We believe it should last at least 6 months to evaluate the effectiveness of the treatment. Another limit may be found in the low compliance to the prescribed therapy, including also the difficulty in assuming the tablet. Among the centers involved in the study, the average age of the enrolled patients is significantly different (**Figure 3**). In the centers of Naples, Pisa and Turin, only pediatric patients are visited, and this led to the selection of patients with a lower average age. This was also reflected in the smaller number of patients enrolled in these centers, as many children could not swallow the tablet and therefore could not be included in the study. Despite the inability to swallow was an exclusion criterion, two patients (GE07, TO03) had to break the tablet to allow it to be administered. In conclusion no significant difference was found in the number of seizures throughout the study. However, a significant change in fecal consistency and intestinal symptoms was recorded. This, combined with the improvements in the QoL, and with grooving evidence in literature, points towards a close relationship between gut dysbiosis and abnormal neuronal functioning.

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Supplementary materials

Supplementary Figure 1: Seizure diary

Weeks	Wake (W) or Sleep (S)	Seizures/day							Seizures/ week	Notes
		Mon	Tue	Wed	Thu	Fri	Sat	Sun		Constiaption or intercurrent events (fever)
1	W									
	S									
2	W									
	S									
3	W									
	S									
4	W									
	S									
5	W									
	S									
6	W									
	S									
7	W									
	S									
8	W									
	S									
9	W									
	S									
10	W									
	S									
11	W									
	S									
12	W									
	S									

Supplementary Figure 2: Quality of life questionnaire (QoL)

QoL questionnaire			
Do you think the number of intakes of the product is compatible with the daily routine?			
Yes			
No		Too many doses of intake during the day	
		Difficulties due to the refusal of employment by the child	
Do you perceive an improvement in your child since the beginning of the therapy?			
Yes		Reduction of seizures number	
		Reduction of seizures intensity	
No		No changes	
		Worsening compared to the previous condition	
Have you observed an increase in seizure-free days in your child since the start of therapy?			
Yes			
No			
Have you noticed an improvement in social interaction and environmental participation in your child since the beginning of therapy?			
Yes			
No			
Do you perceive an improvement in your child's intestinal well-being since the start of therapy?			
Yes, I perceive an intestinal regularization and an improvement in the consistency of the stool			
No, I don't perceive any change			
Do you perceive an improvement in your child's sleep rhythm / quality since the start of therapy?			
Yes			
No		Specify	

Supplementary Figure 3: Bristol Stool Test (BST)

		Bristol stool scale (1-7)			
		0 week	4 week	8 week	12 week
Type 1					
Type 2					
Type 3					
Type 4					
Type 5					
Type 6					
Type 7					

Type 1: separate hard lumps, like nuts, hard to pass (constipation)

Type 2: Sausage-shaped but lumpy (moderate constipation)

Type 3: Like a sausage, but with cracks on the surface

Type 4: Like a sausage or snake, smooth and soft

Type 5: Soft blobs with clear-cut edges, passed easily (diarrhea)

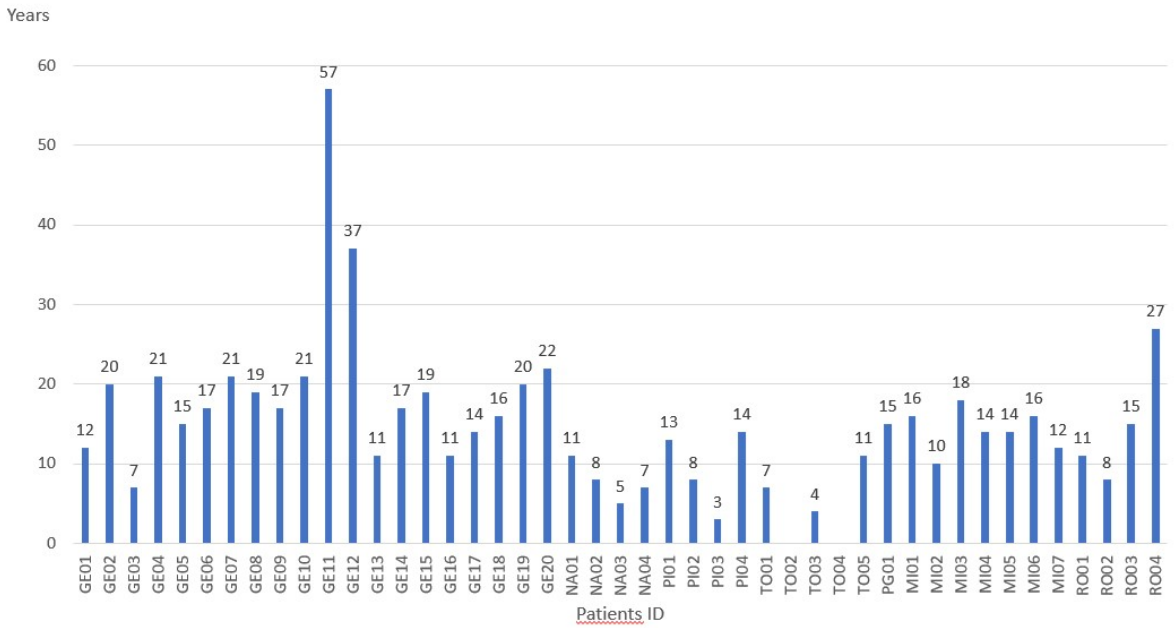
Type 6: Fluffy pieces with ragged edges, a mushy stool (severe diarrhea)

Type 7: Watery, no solid pieces, entirely liquid

Supplementary Figure 4: Food diary

Average consumption in the last 12 weeks	
Food (medium portion)	Frequency (never; 1-2/week; 3-4/week; 1/day; 2-3/day; 4-5/day; >6/day)
Red meat (roast, steak, stew)	
Chicken, rabbit, turkey	
Sausages (ham o others)	
Fish	
Bread	
Potatoes	
Wheat 00 pasta	
Ancien wheal or spelled pasta	
White rice	
Brown, basmati or venere rice	
Pizza	
Yogurt	
Cheese	
Eggs	
Cooked vegetables	
Raw vegetables	
Coffee	
Sugary drinks	
Fruits	
Average consumption in the last 12 weeks	
Condiment	Quantity per week (never; <50g/week; 50-100g/week; 100-150g/week; >150g/week)
Butter	
Oil	

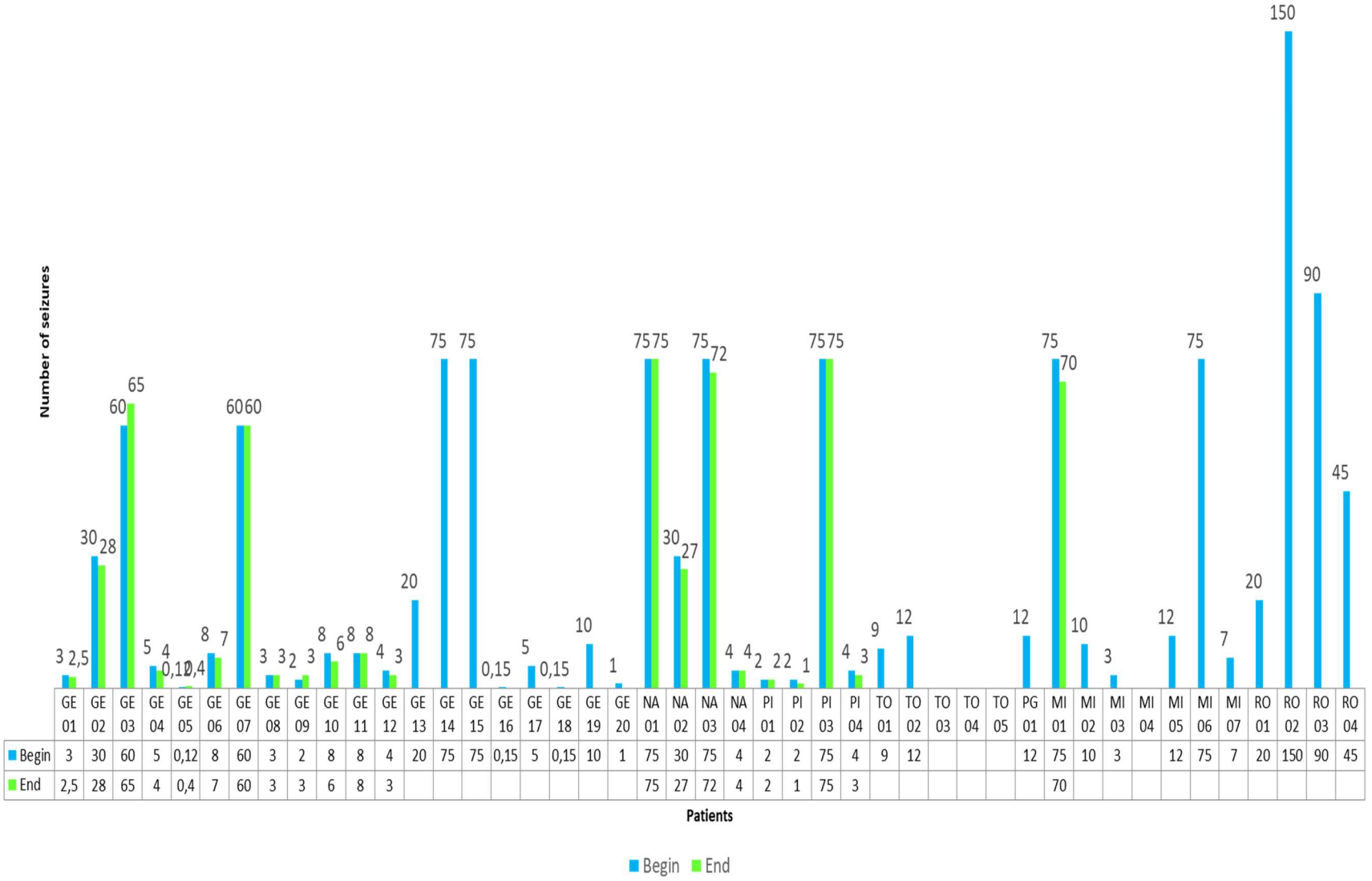
Supplementary Figure 5: Age at randomization of each patient recruited



Supplementary Figure 6: Initial and final Bristol scores

IC	Initial Bristol	Final Bristol	IC	Initial Bristol	Final Bristol	IC	Initial Bristol	Final Bristol
GE01	5	3	GE16	1		TO03	NA	
GE02	3	3	GE17	3		TO04	NA	
GE03	1	2	GE18	3		TO05	NA	
GE04	2	2	GE19	2		PG01	5	
GE05	2	3	GE20	2		MI01	3	3
GE06	2	2	NA01	3	3	MI02	NA	
GE07	1	3	NA02	2	3	MI03	2	
GE08	2	2	NA03	3	3	MI04	NA	
GE09	2	3	NA04	3	4	MI05	3	
GE10	2	4	PI01	2	3	MI06	4	
GE11	2	2	PI02	3	3	MI07	4	
GE12	2	3	PI03	1	3	RO01	4	
GE13	2		PI04	1	2	RO02	4	
GE14	2		TO01	NA		RO03	4	
GE15	2		TO02	3		RO04	3	

Supplementary Figure 7: Mean number of monthly seizures of all patients



	GE 01	GE 02	GE 03	GE 04	GE 05	GE 06	GE 07	GE 08	GE 09	GE 10	GE 11	GE 12	GE 13	GE 14	GE 15	GE 16	GE 17	GE 18	GE 19	GE 20	NA 01	NA 02	NA 03	NA 04	PI 01	PI 02	PI 03	PI 04	TO 01	TO 02	TO 03	TO 04	TO 05	PG 01	MI 01	MI 02	MI 03	MI 04	MI 05	MI 06	MI 07	RO 01	RO 02	RO 03	RO 04
Begin	3	30	60	5	0,12	8	60	3	2	8	8	4	20	75	75	0,15	5	0,15	10	1	75	30	75	4	2	2	75	4	9	12				12	75	10	3		12	75	7	20	150	90	45
End	2,5	28	65	4	0,4	7	60	3	3	6	8	3									75	27	72	4	2	1	75	3							70											

Supplementary Table 1

IC	Age at rand.	Sex (M/F)	Age at sz onset	Sz types	Diagnosis	No. sz/mo. at rand.	EEG Findings	MRI Findings	Genetic Testing
GE01	12y	F	8y	FS	FE (Idiopathic)	3	Bilateral central-parietal EA	Normal	NGS panel for epilepsy (ongoing)
GE02	20y	M	6y	TCS Atypical Abs Atonic sz	EE (Lennox-Gastaut syndrome)	30	Generalized SW	Outcomes of encephalitis	NA
GE03	7y	F	4m	TCS MS	EE (Dravet syndrome)	60	Bilateral frontal multifocal EA	NA	SCN1A
GE04	21y	F	9y	TCS FS Dyscognitive sz	FE (Symptomatic)	5	EA in central/posterior temporal R/L	hypo-ischemic damage; microcephaly	NA
GE05	15y	M	13y	Fall and unconsciousness	GGE	0,12	Generalized SW	NA	NA
GE06	17y	F	3y	Fall and unconsciousness	EE (Lennox-Gastaut syndrome)	8	Generalized SW	Hypoischemic damage Microcephaly	NA
GE07	21y	F	2y	TS MS	FE (Symptomatic-Lissencephaly 1)	60	Generalized EA	NA	LIS1
GE08	19y	F	16y	TCS FS	EE (Cornelia-De Lange syndrome)	3	Focal SW	NA	SMCA1
GE09	17y	F	8y	MS	FE (Idiopathic)	2	Generalized SW	NA	NA

GE10	21y	F	21y	MS	GGE (Janz syndrome)	8	Generalized SW	NA	NA
GE11	57y	F	6y	TCS MS	EE (Lennox-Gastaut syndrome)	8	Slow cortical activity	Cortical and cerebellar atrophy	NA
GE12	37y	F	17y	Dyscognitive sz	FE (Symptomatic)	4	EA diffuse in left hemisphere with contralateral diffusion	NA	NA
GE13	11y	F	6y	MS Abs Visual hallucinations	GGE (Janz syndrome)	20	Occipital EA	NA	To be done
GE14	17y	F	14y	TCS Typical Abs	GGE	75	Generalized SW	Normal	NA
GE15	19y	F	9y	MS	Others (PME-Unverricht Lundborg disease)	75	Occipital EA	NA	CSTB
GE16 / TO03	11y	F	9y	TCS Abs	GGE	0,15	Generalized SW	Normal	NGS panel for epilepsy (ongoing)
GE17	14y	M	3y	FS	FE (Idiopathic)	5	Temporal EA	Normal	NGS panel for epilepsy (ongoing)
GE18	16y	M	12y	TCS	GGE	0,15	Generalized SW	Normal	NA

GE19	20y	F	6 mo.	TCS FS	FE (Tuberous sclerosis)	10	EA diffuse in left hemisphere with contralateral diffusion	NA	TSC2
GE20	22y	F	2y	TCS Dyscognitive sz	FE (Idiopathic)	1	Temporal EA	Normal	NA
NA01	11y	M	9 mo.	TCS TS	FE (Symptomatic)	75	Generalized SW	Cystic supratentorial encephalomalacia, atrophic corpus callosum, reduced brainstem	CGH-Array: negative
NA02	8y	M	4 mo.	TS Infantile spasms	FE (Symptomatic)	30	EA diffuse in left hemisphere with contralateral diffusion	Cystic and gliotic encephalomalacia of the left temporo-fronto-insulo-parietal region extended to left nucleus-capsular region	NA
NA03	5y	M	3m	TC Tonic spasms	EE	75	Generalized EA	Normal	WES: CHD4 Compound heterozygosity NALCN
NA04	7y	M	At birth	FS	EE	4	Bilateral EA on the Rolandic areas	Bilateral lesions to the thalamus, basal ganglia and corticospinal tract of the midbrain and medulla	NA

PI01	13y	M	9y	FS	FE (Idiopathic)	2	Focal EA	Normal	CGH-array: negative NGE panel for epilepsy: SPTAN1 vous DEPDC5
PI02	8y	F	6y	FS	Others (Rasmussen Encephalitis)	2	Focal slow SW discharges	Rasmussen's Encephalitis	NA
PI03	3y	F	3y	MS Abs	GGE (Myoclonic absence epilepsy)	75	Generalized SW	Bilateral polymicrogyria	CGH-array: negative
PI04	14y	M	14y	TCS Atypical Abs ESES Atonic sz	GGE (ESES)	4	Generalized SW	Nodular heterotopia	Interstitial Del Cr16
TO01	7y	F	7y	Atypical Abs ESES	EE	9	Generalized SW	Normal	SHANK3
TO02	NA	M	5y	TCS MS Abs	GGE	12	Generalized and focal SW	Normal	NGS panel for epilepsy (ongoing) Normal karyotype
TO03	4y	F		NA	Others (Rett syndrome)	NA	NA	NA	NA
TO04	NA	F		NA	FE (symptomatic)	NA	NA	Cortical malformation	NA
TO05	11y	F		NA	Others	NA	NA	NA	WES (ongoing)
PG01	15y	M	14y	TCS MS	GGE	12	Generalized SW	Normal	NHLRC1

MI 01	16y	F	3 mo.	TS Infantile spasms Tonic spasms	EE (Aicardi syndrome)	75	EA diffuse in left hemisphere with contralateral diffusion	NA	NA
MI 02	10y	M	6y	TCS FS Abs	Others	10	Generalized and focal SW	Normal	NGS panel for epilepsy (ongoing)
MI 03	18y	M	1 mo.	TS	FE (Idiopathic)	3	Slow SW in fronto-temporal left	Dysmorphic signs	NA
MI 04	14y	M	2y	TS Atypical Abs Atonic sz Fall sz	EE (Lennox-Gastaut syndrome)	NA	Generalized SW	Normal	Inv-dup Cr15q11
MI 05	14y	F	9y	TCS Abs	GGE (Juvenile absence epilepsy)	12	Generalized SW	NA	NA
MI 06	16y	F	1y	TS MS Atypical Abs Atonic sz	EE (Lennox Gastaut Syndrome)	75	Diffuse slow SW discharges	Microcephaly Cerebellar hemispheres asymmetry (right and worm hypoplasia)	NGS panel for epilepsy (ongoing)
MI 07	12y	M	11 mo.	TCS FS MS Abs	EE	7	Generalized and focal SW	Normal	CHD2
RM01	11y	F	1y	TCS	EE (Dravet syndrome)	20	Generalized EA	Hydromielia C2-C7	WES: negative CGH-array: negative

RM02	8y	F	3y	Abs	GGE	150	Generalized SW	Normal	NGS panel for epilepsy (ongoing)
RM03	15y	F	1 mo.	TCS	GGE	90	Generalized SW	Normal	CDKL5
RM04	27y	F	16 mo.	TCS MS Atonic sz	EE (Lennox-Gastaut syndrome	45	Generalized SW	Normal	NA

IC: Identification code
Rand.: Randomization
No.: Number
F: Female
M: Male
Mo.: Month
Y: year
NA: Not Available
TCS: Toni-clonic seizures
TS: Tonic seizures
FS: Focal seizures
MS: Myoclonic seizures
Abs: Absences
Sz: Seizures

GGE: Genetic Generalized Epilepsy
EE: Epileptic Encephalitis
FE: Focal Epilepsy
SW: Spike-Wave
EA: Epileptic abnormalities
NGS: Next generation sequencing
WES: Whole Exome sequencing
CGH-array: Comparative Genomic Hybridization-Array
Inv-Dup: inversion-duplication
R/L: Right/Left
EEG: Electroencephalography
MRI: Magnetic Resonance Imaging

Supplementary Table 2

IC	Neurological Examination	NP Comorbidities	Other Comorbidities	ID/DD	BST	ROMA 4	Concomitant TP
GE01	Normal	Memory disturbances	Headache	No/No	5	NA	CBZ 600 mg/day
GE02	Dysarthria Hypertonia Tremors Hyperexcitable osteotendinous reflexes Adiadokokinesia	No	No	Yes/No	3	NA	FFA HCL 5mg VPA 500mg FBM 6/day CLB as needed
GE03	Tremor Gait disturbances	Autistic features	No	Yes/Yes	1	Constipation	FFA HCL 2mL x2/die STP 500 mg x2/die VPA 500 mg/day CLB 10MG
GE04	Spastic-dystonic paraparesis	Sleep disorder	No	Yes/Yes	2	Constipation	CLB 10 mg Serplus complex 2sachet/day 2 Zn tablets PER (added after)
GE05	Normal	No	No	No/No	2	NA	Serplus Complex
GE06	Spastic-dystonic paraparesis	Sleep disorder	No	Yes/Yes	2	Constipation	NA
GE07	Spastic tetraparesis	Language disorder Sleepy state	Scoliosis Weight <3°	Yes/NA	1	Constipation	RUF 150 mg CLB 10 mg as needed Sialinar oral solution 1 ml Bactopral 1 sachet

GE08	Diffuse hypotonia Dystonic and dyskinetic movements	ASD	No	Yes/Yes	2	Constipation	CBZ 900mg/day
GE09	Normal	Anxious disorder	No	NA/No		NA	None
GE10	Normal	Anxious disorder	No	No/No	2	Constipation	LTG 150 mg/day
GE11	Normal	No	No	Yes/Yes	2	Constipation	PB 100 CLB 10 mg
GE12	Normal	No	No	No/No	2	Constipation	CBZ 800 mg
GE13	Myoclonus	Sleep disorder	No	No/No	2	Constipation	VPA 500mg Lactoferrin 5 sprays x2/day CLB 10mg
GE14	Normal	Attention deficit	Dysmorphisms	No/No	2	Constipation	LEV 750mg x2/day TPM 50mg x2/day LTG 50mg x2/day
GE15	Ataxia Gait disturbances	No	No	No/No	2	Constipation	VPA 600 mg+600 mg CLB 2 mg+2 mg+1 mg PER 6 mg Atkinson's diet
GE16 / TO03	NA	ASD, ADHD, Sleep disorder	No	Yes/Yes	1	Constipation	LEV 500 mg ETX 10 ml x2/day
GE17	Normal	Sleep disorder	No	No/No	3	No	VPA 500 mg CLB 10 mg
GE18	Normal	No	No	No/No	3	No	VPA 750 mg
GE19	NA	ASD	RGE	Yes/Yes	2	Constipation	FBM 1200 mg/day CBD 20 mg/Kg/day

GE 20	Normal	No	No	No/No	2	Constipation	CBZ 600 mg/day
NA01	Spastic tetraparesis	No	RGE Recurrent respiratory infections	Yes/Yes	3	NA	NA
NA02	Right hemiparesis	Psychomotor agitation	No	Yes/Yes		NA	NA
NA03	Diffuse hypotonia	Psychomotor agitation Sleep disorder	No	Yes/Yes	3	NA	NA
NA04	Diffuse hypotonia Dystonic and dyskinetic movements	No	No	Yes/Yes	3	No	NA
PI01	Normal	Psychomotor agitation ADHD	No	Yes/No	2	No	VPA 800 mg/day OXCB 1200 mg/day
PI02	Right hemiparesis	No	No	Yes/Yes	3	No	CBZ 800 mg/day LEV 60Mg/Kg/day Mycophenolate
PI03	NA	No	Dysmorphisms	Yes/Yes	1	Constipation	VPA: 350 mg/day ETX 200 mg/day
PI04	Diffuse hypotonia	Language disorder	Dysmorphisms	Yes/Yes	1	Constipation	VPA 1100 mg/day ETX 550 mg/day. CLB 10 mg/day
TO01	Clumsiness Diffuse hypotonia Paresis in a limb	Sleep disorder	No	Yes/Yes	NA	NA	ETX Sultiame

TO02	Clumsiness	Dysgraphia	No	No/No	3	No	CZP LEV
TO03	NA	NA	NA	NA/NA	NA	NA	NA
TO04	NA	NA	NA	NA/NA	NA	NA	NA
TO05	NA	ASD	No	NANA	NA	NA	NA
PG01	Normal	No	No	Yes/No	5	No	VPA 1800mg/day ETX 1000mg/day RUF 1600 mg/day PER Metformin
MI 01	Right hemiparesis Hypertonia	Language disorder	No	NA/NA	NA	NA	NA
MI 02	Normal	Hyperactivity	No	No/No	NA	No	VPA 700 mg/day ZNS 250 mg/day CLB 20 mg/day
MI 03	Hypotonia	ASD	No	Yes/Yes	2	Constipation	VPA 1250 mg/day
MI 04	Hypotonia	Language disorder	RGE	Yes/Yes		NA	LTG 175 mg/day ZNS 150 mg/day
MI 05	Normal	Anxiety disorder	Headache	No/No	3	No	LTG 175 300 mg/day
MI 06	Diffuse hypotonia	No	Severe disability	Yes/Yes	4	Constipation	TPM 250 mg/day ETX 450 mg/day LEV 1250 mg/day CZP 20 mg/day
MI 07	Normal	ASD	No	Yes/Yes	4	No	VPA 1000mg/day TPM 100mg/day RUF 800mg/day

RM01	Stereotypies	No	Anemia	Yes/Yes	4	No	VPA 200mg/day ETX 4mlx2/day CLB 20mg/day STP 1250mg/day
RM02	Normal	Language disorder	No	Yes/Yes	4	Constipation	ETX 12ml/day CLB 3cp/day
RM03	Normal	No	No	Yes/Yes	4	Constipation	LEV 6000mg/day CLB 20mg/day VPA 2000mg/day TPM 180 mg/day
RM04	Normal	Language disorder	Dysmorphisms	Yes/Yes	3	Constipation	VPA 800mg/day RUF 600mg/day LAC 100mg/day CLB 10mg

IC: Identification code
NP: Neuropsychiatric
ID: Intellectual disability
DD: Developmental delay
BST: Bristol Stool Test
TP: Therapy
NA: Not available
Zn: Zinc
CBZ: Carbamazepine
FFA HCL: Fenfluramine hydrochloride
VPA: Valproic acid
PER: Perampanel
STP: Stiripentol
RUF: Rufinamide
LTG: Lamotrigine
PB: Phenobarbital
CLB: Clobazam
LEV: Levetiracetam
TPM: Topiramate
ETX: Ethosuximide
FBM: Felbamate
CBD: Cannabidiol
OXCB: Oxcarbazepine
CZP: Clonazepam
ZNS: Zonisamide
LAC: Lacosamide

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