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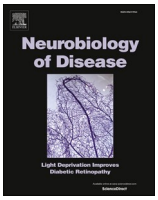

Gut microbiota manipulation as an epilepsy treatment

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ABSTRACT

Many studies have documented the important role of the gut microbiota (GM) in the regulation of several central nervous system (CNS) processes through the microbiota-gut-brain (MGB) axis. This latter represents the connection between the CNS, the enteric nervous system, the gut and its microbiota through several ascending and descending pathways. The variation of the GM composition is associated with the pathogenesis and/or progression as well as severity of various neuropsychiatric/neurological diseases such as depression, autism spectrum disorder, multiple sclerosis, Parkinson's, and Alzheimer's diseases. Recently, changes in the bacterial composition of the GM have also been linked to epilepsy and seizures, with some studies exploring the potential role of GM in the regulation of neuronal hyperexcitability, seizure occurrence and epileptogenesis. Accordingly, there are potential novel treatments which are currently being investigated such as probiotics, prebiotics and symbiotic that may represent innovative therapeutic approaches. The aim of this review is to explore the effect of gut microbiota manipulation as a therapeutic strategy in epilepsy and the methodological challenges to design (translational) clinical trial investigating the gut microbiota.

1. Introduction

A large amount of preclinical and clinical studies documented the crucial role of the gut microbiota (GM), the complex of microorganisms colonizing the intestinal tract, in regulating the central nervous system (CNS) homeostasis, cognitive development, and behaviour through the microbiota-gut-brain (MGB) axis, beginning a new frontier for neurological disorders. The role of GM composition in the genesis or progression of a variety of neurological disorders (Chatzikonstantinou et al., 2021) such as autism spectrum disorder (Coretti et al., 2017), multiple sclerosis (Jangi et al., 2016), Parkinson's and Alzheimer's (Jiang et al., 2017) diseases has been recently highlighted. Changes in the gut bacterial composition have also been linked to epilepsy (Citraro et al., 2021). Epilepsy is one of the most common neurological disorders affecting about 50 million people worldwide (Devinsky et al., 2018). About 35% of epilepsy cases are directly linked to a genetic background, whereas in the remaining cases genetic risk in addition to acquired and environmental factors contribute to epileptogenesis. Among environmental causes there are trauma, tumours, strokes, traumatic brain injury, or infections. Epilepsy treatment involves the use of antiseizure medications (ASMs), surgery, vagus nerve stimulator (VNS), and ketogenic diet (KD). Notwithstanding the availability of several ASMs, about 30% of patients will not respond to an appropriate pharmacological treatment, being then classified as drug or treatment resistant (Chen et al., 2018).

Novel scientific approaches, such as metabolomics, metagenomics and even faecal microbiota transplantation (FMT) have deeply contributed to a better understanding of the potential impact underpinned by the gut microbiota on neuroinflammation, metabolic and neuroendocrine signalling pathways among others (Cryan et al., 2020). It is assumed and widely scientifically supported that, the intestinal microbiota might exert changes on the CNS *via* the MGB axis (Wang et al., 2018). During the last decade, numerous researchers and clinicians keenly investigated the potential role of the microbiota to regulate hyperexcitability, seizures and its impact on epileptogenesis. Furthermore, the possibility to control seizures modifying directly and/or indirectly MGB axis (Sandhu et al., 2017) using supplements (*e.g.*, prebiotics, probiotics and symbiotic), diets (*e.g.,* ketogenic diet) and FMT has been explored. The administration of probiotics, prebiotics and their combination called symbiotic, is increasingly used in clinical practice for several conditions such as inflammatory bowel disorders (Naidoo et al., 2011; Rolfe et al., 2006), systemic diseases (*e.g.*, diabetes (Zhang et al., 2016), hypertension (Khalesi et al., 2014)) but also for neuropsychiatric conditions (*e.g.,* depression (Huang et al., 2016) and autism spectrum

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disorders (Shaaban et al., 2018)); however, in comparison, only few studies in the field of epilepsy are available. Moreover, it is well known that also antibiotics modify GM community clearly representing the most direct and effective way of targeting intestinal microbes (Smaga et al., 2020). This review aims to explore the effect of GM manipulation as a novel therapeutic strategy in epilepsy focusing on human findings and the methodological challenges to design (translational) clinical trial investigating this approach.

2. Overview of MGB axis in epilepsy

The MGB axis, among others, is formed by the CNS, the enteric nervous system, and the GM communicating by ascending and descending routes (Ambrosini et al., 2019). More specifically, the ascending pathway of the axis is involved in neural changes including microglia's functions (Abdel-Haq et al., 2019); this reflects its importance in neurogenesis, synapse modelling, and prevention of excitotoxicity (Kettenmann et al., 2013). Accordingly, several studies on GM and MGB axis evidenced their role in neural development (Warner, 2019), neuroinflammation (Sundman et al., 2017), stress response and neurotransmission (Foster et al., 2017). On the other hand, the brain regulates gut peristalsis, sensory and secretion function, mainly acting on the enteric nervous system through the vagus nerve (Bonaz et al., 2018); all of them impacting on GM composition.

Overall, many clinical and experimental studies investigated the role played by GM in several pathologies such as cancer (Ianiro et al., 2020), obesity (Lu et al., 2020) diabetes (Lecronier et al., 2020), and neurological diseases such as Alzheimer's (Sun et al., 2019), Parkinson's (Xue et al., 2020) and epilepsy (De Caro et al., 2019a; Iannone et al., 2019). Regarding the brain, It was shown that GM's alterations can interfere in the development and maintenance of some neurological/neuropsychiatric disorders (Cryan et al., 2019; Cryan and Dinan, 2012). More specifically for epilepsy, seizures and epileptogenesis could be influenced by GM through: 1) the intestinal production of neurotransmitters such as γ-aminobutyric acid (GABA), glutamate and serotonin (5-HT) (Mittal et al., 2017); 2) by pro-inflammatory effects mediated by the immune system with the release of cytokines and chemokines, and an increase in lipopolysaccharide (LPS) levels contributing to gut- and blood-brain barrier increased permeability and increased neuroinflammation (Blander et al., 2017; Riazi et al., 2008, 2010); 3) by modifying the amount of gut-derived metabolites (Olson et al., 2018) such as short chain fatty acids (SCFAs) mainly known for CNS protective effects (De Caro et al., 2019b; Stilling et al., 2016). Furthermore, neural and neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis (Sudo et al., 2004), as well as the endocannabinoid system and the levels of brain-derived neurotrophic factor (BDNF) (Maqsood and Stone, 2016) can be influenced by GM interfering with seizures mechanisms (Fig. 1).

Some studies have already demonstrated that the GM and even gastrointestinal functions are altered in epilepsy (Avorio et al., 2021; Russo, 2022). As an example, Xie and colleagues published the first paper sequencing and comparing faecal samples of 14 drug-resistant epilepsy (DRE) children and 30 healthy controls (Xie et al., 2017) highlighting a reduction of the *Bacteroidetes* phylum in DRE patients. This was later confirmed by Peng and colleagues comparing 42 DRE patients with 49 patients with drug-sensitive epilepsy and 65 healthy controls (Peng et al., 2018). In this study, it was evidenced that DRE patients had higher levels of rare microbial genera, including *Clostridium XVIII, Atopobium, Holdemania, Dorea, Saccharibacteria, Delftia,*

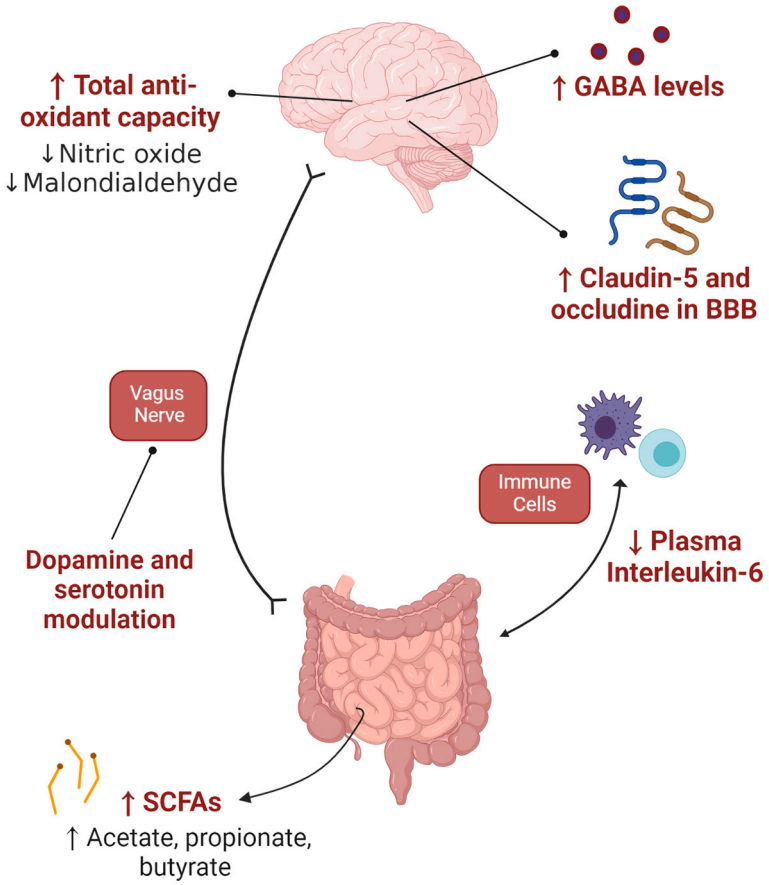

Fig. 1. An overview of the microbiota gut brain axis in epilepsy.
5-HT, serotonin; BBB, blood brain barrier; BDNF, brain-derived neurotrophic factor; FMT, faecal microbiota transplantation; GABA, γ-aminobutyric acid; HPA, hypothalamic-pituitary-adrenal; IL, interleukin; LPS, lipopolysaccharide; SCFAs, short chain fatty acids; TNF, tumour necrosis factor. Created with Biorender.com.

Coprobacillus, Paraprevotella, Ruminococcus, Gemmiger, Akkermansia, Neisseria, Coprococcus, Fusobacterium, Methanobrevibacter, Phascolarctobacterium, and *Roseburia*. Some other subsequent studies identified differences between patients with epilepsy and healthy controls, reviewed in Russo (2022). Similar results have been achieved in animal models although even less studies are yet available. For example, the first study in a genetic model was performed by Citraro et al. demonstrating that WAG/Rij rats, a genetic animal model of absence epilepsy, presented alterations in the GM composition when compared with control non epileptic Wistar rats. No changes in α-diversity were observed although β-diversity was influenced differently at different ages with decreased abundance of *Bacteroidetes* phylum and increased abundance of *Firmicutes* and *Proteobacteria* phyla, and a reduction of intestinal level of SCFAs (Citraro et al., 2021). In agreement, a subsequent study found a difference in GM composition also in a genetic animal model of Dravet's Syndrome (Miljanovic and Potschka, 2021).

Very few studies investigated the impact of ASMs on the gut microbiota. In the large study by Maier et al. (2018) have shown that several non-antibiotic drugs (27% on 1197) inhibited at least one bacterial strain, although none ASMs have shown antimicrobial effects. However, other *in vitro* studies demonstrated that lamotrigine inhibit *E. coli* ribosomal biogenesis (Stokes et al., 2014) and can inhibit aerobic or facultative aerobic strains as well as an antibacterial activity against some Gram-positive species (Qian et al., 2009). Valproate demonstrated to affects microbiota composition in an animal model and zonisamide is metabolized to 2-sulfamoylacetylphenol by gut microbiota (Kitamura et al., 1997). Recently, Ilhan et al. (2022) showed that carbamazepine, lamotrigine, and topiramate reduced the growth of more than ten strains (including *E.coli* and *Lactobacillus reuteri*). Altogether, we do not completely understand the mechanisms involved in the complex mechanisms on ASMs but indeed MGB axis represents a suitable target for the development of innovative therapeutic interventions for patients with epilepsy.

3. Modulation of the gut microbiota through functional foods

It has to be specified that the GM may have both a beneficial or detrimental effect on human health status contributing to different diseases; this depends on the ratio between protective and harmful species in the gut and its continuously maintained homeostasis (Arulsamy et al., 2020). Some Authors have hypothesized the possibility of preventing or improving the progression of certain diseases by re-establishing an altered microbiota towards beneficial flora or adding and increasing beneficial microbial species in the gut. Different methods have been used with the aim of altering gut microbiota improving dysbiosis and GM health such as ketogenic diet, functional foods (probiotics, prebiotics, symbiotic, postbiotics), antibiotics and faecal microbial transplantation.

3.1. Prebiotics and probiotics

Functional foods can be defined as "*any food that has a positive impact on an individual's health, physical performance, or state of mind, in addition to its nutritious value*" (Nataraj et al., 2020). Substantially, they should serve to regulate particular body process(es), such as enhancement of biological defense mechanisms, prevention of specific diseases, control of physical and mental disorders, and slowing of aging. Prebiotics and probiotics are included among functional food products. The World Health Organization (WHO) defines probiotics as "*vital microorganisms that provide health advantages to their consumers when digested in sufficient quantity*" (Morelli and Capurso, 2012). Prebiotic concept was first defined in 1995 as a "*non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon*" (Gibson and Roberfroid, 1995).

Trials about probiotics and epilepsy are very limited although some are currently being performed (Tables 1 and 2) and the potential mechanisms of action proposed come mainly from animal studies (Fig. 2 or graphical abstact and Table 3) (Tahmasebi et al., 2020). For example, one of the potential mechanisms is linked to increased brain GABA levels or improved antioxidant/oxidant ratio (total antioxidant capacity with decreasing nitric oxide and malondialdehyde) as observed in kindling model (Bagheri et al., 2019).

To date, only two human studies have been concluded. The first one was ruled out in neonates (Yeom et al., 2019). The authors designed a prospective study enrolling 228 neonates who were admitted in intensive care units. Rotavirus infection was found as an independent risk factor for neonatal seizures. Immediate administration of probiotics (*i.e.*, *Saccharomyces boulardii* and only one *Lactobacillus casei*) after birth (for a non-specified reason reported) reduced rotavirus-associated neonatal seizures by 10-fold (OR 0.09; $p < 0.001$). They proposed that *S. boulardii* reduces seizures through inhibition of rotavirus structural protein 4 (NSP4) or by anti-inflammatory effects, considering that NSP4 is a viral enterotoxin associated with neurological injury produced by rotavirus.

was Another study conducted in adults with DRE (Gómez-Eguílaz et al., 2018). Probiotics was administered as a supplementation of their stable ASMs treatment for 4 months. After the probiotic was interrupted, patients were followed-up for a further 4 months. A higher than 50% reduction in seizures' number was observed in 28.9% of the patients (ITT population). Quality of life significantly (QoL) improved in those in which probiotics were effective to reduce seizures (mean ± SD: 19.23 ± 6.04 *vs* 26.45 ± 9.7; $p = 0.013$). This latter improvement was observed both during probiotic supplementation and in the 4 months after suspension, no data on seizures after discontinuation has been reported. The plasma levels of IL-6 (*i.e.*, an inflammatory cytokine) decrease and GABA levels increase during treatment, without achieving statistical difference. It was proposed that both could be involved in seizure control and improved QoL, although the sample size was too small and further studies are needed to confirm this hypothesis. Ongoing clinical trials investigating supplementations/modulations in epilepsy are listed in Table 4.

Prebiotics and probiotics are safely used, and safety outcomes are frequently reported in clinical trials, supporting the assumption that they are generally secure. However, probiotics could be not universally safe, above all in vulnerable populations (*e.g.*, premature infants, severe clinical conditions and during immunosuppressive treatments), and potential risks have been described in experimental models, trials, and case series (Kothari et al., 2019).

These risks include sepsis, localized infections, metabolic disturbance, excessive immune activations as well as gastrointestinal adverse effects (Doron and Snydman, 2015). Therefore, the production and distribution of probiotics need to be regulated and more research focused on safety need to be performed, with active surveillance for potential related-infections and other adverse events.

3.2. Postbiotics

Probiotics use has some limitations: 1) difficulty to achieve and use effective concentrations (Reid et al., 2011; Shenderov, 2013); 2) alterations of the gastrointestinal tract (GIT) activating several bacterial genes for degradation and production of different nutrients by various metabolic pathways (Baugher and Klaenhammer, 2011; Bron et al., 2004). To overcome these problems, postbiotic have been developed, being produced by probiotics and representing a promising alternative supplement for human health (Nataraj et al., 2020). Postbiotics may be defined as "*non-viable bacterial products or metabolic products from microorganisms that have biological activity in the host*" (Martín and Langella, 2019). They are the complex mixture of healthy metabolic products or secreted components of probiotics in cell-free supernatants such as enzymes, secreted proteins, short chain fatty acids, vitamins, amino acids, peptides, organic acids, *etc.* (Nataraj et al., 2020). These have several advantages over the traditional probiotics such as a known molecular

Table 1
Preclinical and clinical studies on epilepsy and probiotics.

Title	Year	Population	Outcomes	Strength	Limitations	References
Probiotics and *Nigella sativa* extract supplementation improved behavioral and electrophysiological effects of PTZ-induced chemical kindling in rats	2020	Epileptic models rats	Probiotics and *Nigella sativa* extract supplementation had inhibitory effects on kindling	High sample size and model of kindling		(Tahmasebi et al., 2020)
Effect of probiotic supplementation on seizure activity and cognitive performance in PTZ-induced chemical kindling	2019	Epileptic models rats	Probiotics diminish epileptic activity	Model of kindling	Limited sample size	(Bagheri et al., 2019)
Neonatal seizures and white matter injury: Role of rotavirus infection and probiotics	2018	Neonates	Immediate administration of probiotics after birth may reduce rotavirus-associated neonatal seizures	High sample size		(Yeom et al., 2019)
The beneficial effect of probiotics as a supplementary treatment in drug-resistant epilepsy: a pilot study.	2018	Drug-resistant epilepsy patients	Improve frequency of seizures and quality of life	Paired samples	Not placebo arm	(Gómez-Eguílaz et al., 2018)

PTZ, Pentylenetetrazol.

Table 2
Different probiotics used in epilepsy and related studies.

Article	Tipe of product	Composition	Commercialized	References
Probiotics and *Nigella sativa* extract supplementation improved behavioral and electrophysiological effects of PTZ-induced chemical kindling in rats	Probiotics and *Nigella sativa*	*Lactobacillus (L. casei, L. acidophilus)* and *Bifidobacterium (B. bifidum)*	Provita©	(Tahmasebi et al., 2020)
Effect of probiotic supplementation on seizure activity and cognitive performance in PTZ-induced chemical kindling	Probiotics	*Lactobacillus rhamnosus, Lactobacillus reuteri* and *Bifidobacterium infantis*	Pedilact©	(Bagheri et al., 2019)
Neonatal seizures and white matter injury: Role of rotavirus infection and probiotics	Probiotics	*Saccharomyces boulardii* and only one took *Lactobacillus casei*	Not Commercialized	(Yeom et al., 2019)
The beneficial effect of probiotics as a supplementary treatment in drug-resistant epilepsy: a pilot study	Probiotics	*Lactobacillus acidophilus DSM32241, Lactobacillus plantarum DSM32244, Lactobacillus casei DSM32243, Lactobacillus helveticus DSM32242, Lactobacillus brevis DSM11988, Bifidobacterium lactis DSM32246, B. lactis DSM32247* and *Streptococcus salivarius* subsp. *thermophilus DSM32245.*	Sivoy©	(Gómez-Eguílaz et al., 2018)

PTZ, Pentylenetetrazol.

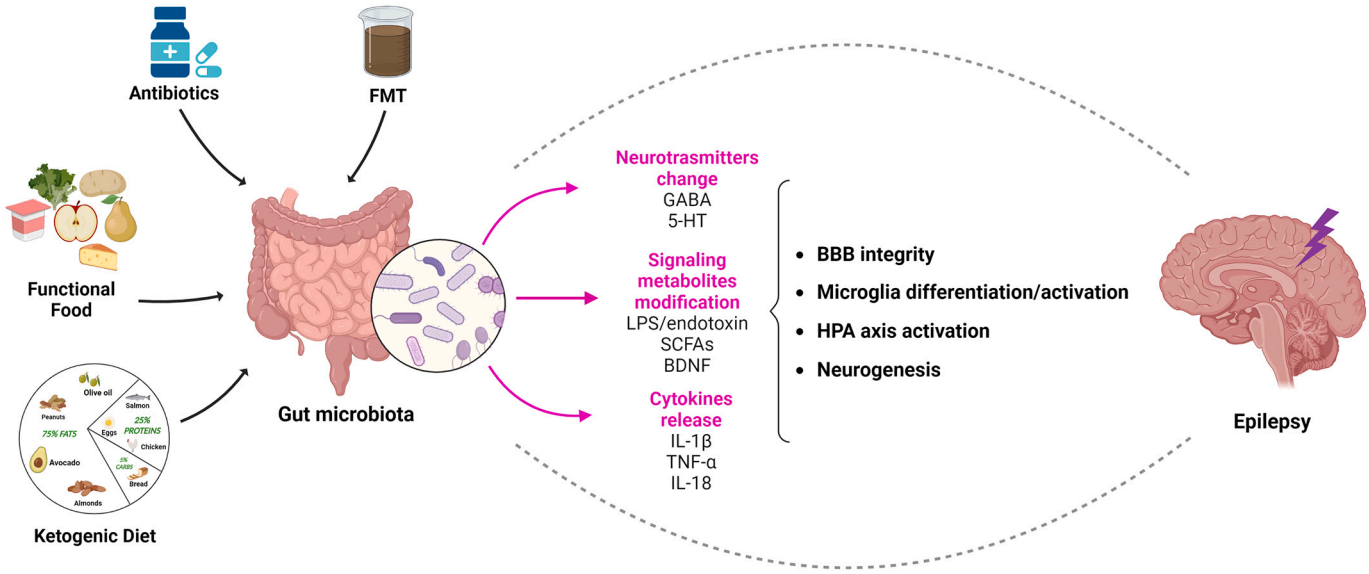

Fig. 2. Supposed mechanisms and effects of prebiotics and probiotics in epilepsy.
BBB, blood brain barrier; GABA, γ-aminobutyric acid; SCFAs, short chain fatty acids. Created with Biorender.com.

structure, can be used in purified forms and possess a defined mechanism of action. Greater and easier production along with easier storage are other advantages. The postbiotics benefits include, among others, anti-inflammatory, immunomodulatory, antihypertensive, hypocholesterolemic, antiproliferative, and antioxidant effects. (Nataraj et al., 2020) but seem also to effect seizure occurrence in animal models of absence epilepsy (Citraro et al., 2020; De Caro et al., 2019a, 2019b; Leo et al., 2021). These attributes suggest the potentiality of postbiotic molecules to improve the host health by modulating the host physiology. But human/clinical trials are needed to confirm these results. At present, there are no published clinical studies on postbiotics in epilepsy.

Table 3
Potential mechanisms/targets implicated in epilepsy of probiotics and prebiotics through gut brain-axis.

Blood brain barrier	Modification in BBB permeability (increasing occludin and claudin-5 expression)
Neuromodulators	GABA modulations directly in the brain 5-HT and dopamine (influence brain function indirectly acting *via* the ENS and vagus nerve)
SCFAs	Acetate, propionate, and butyrate can influence microglia maturation, stimulate autonomic nervous system by enteric neurones or act as epigenetic modulators by histone deacetylases
Endocannabinoid system	Influencing endocannabinoids in the gut, reducing peripheral inflammation and seizure susceptibility

5-HT, 5-hydroxytryptamine; BBB, blood brain barrier, GABA, gamma-aminobutyric acid; ENS, enteric nervous system; SCFAs, short chain fatty acids.

4. Antibiotics and seizures/epilepsy

Findings from preclinical models have studied potential pathways by which antibiotics regulate seizure susceptibility (Lum et al., 2020) that could be directly or indirectly related to GM modulation. However, no specific studies have been performed so far to investigate if antibiotics pro/anti-seizures effects are related to GM. Overall, several mechanisms have been related to antibiotics neurotoxicity and seizure induction.

β-lactams are the class of antibiotics most widely used (Esposito et al., 2017), including penicillins, cephalosporins, carbapenems and monobactams (Raposo et al., 2016). The neurotoxicity of penicillin was first reported in 1945 by Johnson and Walker, who observed myoclonic twitching after intravenous administration (Walker et al., 1945). The theory of epileptogenesis related with the β-lactams is due to the interference in the inhibitory effect on GABA binding to GABA-A receptors. The ring structure of β-lactams is similar to the architecture of GABA neurotransmitters. The inhibition of GABA led to hyperexcitability of neurons and depolarization of the postsynaptic membrane, lowering the seizure threshold. The molecular structure of the β-lactams is not the same, that could explain the different risk of epileptogenesis among them.

One study reports the effect of ceftriaxone and cefepime (β-lactam antibiotics) on the seizures (Amakhin et al., 2018). The authors demonstrate in rats, that cefepime and ceftriaxone have different mechanisms of action on GABA-A receptors. Cefepime blocks GABA-A receptors in a competitive manner, while ceftriaxone has a non-competitive mechanism of GABA receptor inhibition. Both antibiotics decrease the amplitude of evoked inhibitory postsynaptic currents (IPSCs), but with high concentrations that do not use in normal prescription. The direct inhibitory synaptic transmission blockade may not be the primary mechanism underlying cephalosporin-induced seizures. Nevertheless, they analyzed the side-effects of cephalosporins on epileptogenesis and hypothesize that they could be related with renal insufficiency, increasing circulating cephalosporin concentrations and results in an electrolyte disturbance. Hikida and colleagues showed that the interaction with carbapenems and GABAA receptors depends primarily on the side chain on the second carbon atom in the carbapenem nucleus (Hikida et al., 1993). Imipenem has a basic C-2 side chains, instead of a less basic side chain of meropenem, that led to increased risk of seizures (Sunagawa et al., 1995). Furthermore, carbapenems due to their greater ability to cross the blood brain barrier (BBB) and reduction of the action of ASMs, have an elevated potential to promote seizures (Lum et al., 2020).

The neurotoxic effects of the different β-lactams could be explained by the molecular characteristics, though other factors may further influence their occurrence and severity as age (infants and elderly patients) or renal insufficiency that could led an increased levels of antibiotics. Fluroquinolones could produce seizures, but usually in subjects who already suffer from a neurological disease. The mechanism of action in similar to β-lactams, the inhibition of GABA-A receptors. Moreover, it has been postulated that these antibiotics activate *N*-methyl-D-aspartate (NMDA) receptors, causing excitotoxicity and damage to neuronal cells (Takayama et al., 1995). There is no evidence about the implications of aminoglycosides, tetracycline polymyxins, or macrolides in epileptogenesis (Esposito et al., 2017).

On the other hand, Braakman et al., in a small case series, reported that six patients with DRE become seizures free during the administration of antibiotics. The Authors hypothesize that the effect of the antibiotics on the gut microbiota could be the explanation of the seizure freedom achievement in these patients. The mechanism by which antibiotics could decrease seizure frequency is not known (Braakman and van Ingen, 2018).

Previously, Wang et al., published that minocycline and tetracycline-class antibiotics are protective against partial (focal) seizures in animal models (Wang et al., 2012) but they not relate this effect to GM modulation. The authors postulate that the anti-inflammatory effect of minocycline, doxycycline, and tetracycline was the putative antiseizures mechanism.

5. Faecal microbiota transplantation and ketogenic diet

No clinical trials have been performed with FMT in epilepsy so far. There is only one ongoing trial (NCT02889627) with recruitment in progress. Overall, FMT has only indication for the treatment of *C.Difficile*-intractable colitis.

Only one case report was published (He et al., 2017) with FMT performed on a 20-year-old patient with Crohn's disease and a 17-year history of epilepsy that led to a 20 months seizure-free period without concomitant ASMs. However, the report lacks several clinical details, and no gut microbiome analysis was realized as well as no confirmed focal pathology. On the other hand, ketogenic diet and its effect mediated by the gut microbiota was more investigated, as recently summarized in (Fan et al., 2019; Spinelli and Blackford, 2018; Tang et al., 2021). Further studies are needed to define the potential of KD and FMT in epilepsy and to define a consensus on their use.

Table 4
Ongoing clinical trials investigating gut-microbiota modulation in epilepsy.

Treatments and comparators	Status	Phase	Diagnosis	Age range (years)	Primary endpoint	Planned treatment duration	Trial number
Prebiotic or placebo (Oligofructose-enriched Inulin)	Recruiting	IV	Paediatric refractory epilepsy	2 to 18	-Change in alpha and beta bacterial diversity measures in stool -Change in Short Chain Fatty Acid (SCFA) levels in stool	12 weeks	NCT04705298
Faecal microbiota suspension	Recruiting	III	Epilepsy	3 to 70	Seizures frequency	3 months	NCT02889627
Ketogenic diet	Withdrawn *(sponsor decision)*	NA	Paediatric refractory epilepsy	1 to 18	Microbiome features associated with Ketogenic Diet response	24 months	NCT04311242

NA, not applicable.

6. Challenges in translational clinical trials

Nowadays, few human studies investigating the role of GM in epilepsy have been performed, and the increasing number of preclinical evidence linking epilepsy with different GM compositions, changes during ASMs' treatment, antibiotics or supplements need to be confirmed in translational clinical studies. Although pre- and probiotics are used in clinical routine for numerous GI and systemic disorders, no one can be considered "*evidence based*" in neurologic diseases (Rondanelli et al., 2017).

Designing appropriate clinical trials to address GM changes before/after an intervention or highlighting differences among different populations (*e.g.,* patients with a disease and healthy controls) can be difficult. Indeed, the microbiome is a complex and dynamic system affected by multiple endogenous and exogenous influences, showing a significative intra- and inter-subjects variability (Cryan et al., 2019). Variations in lifestyle, diet, and medication use, as well as age and geographic background can lead to issues in data reproducibility and consistence, performing statistically underpowered studies, where treatment groups are significantly different in demographics, clinical and microbiological features. Furthermore, several techniques for microbiome measurement and analysis, as well as sampling timing and storage, are available, each with different limitations and advantages while some others may appear (Qian et al., 2020; Shankar, 2017).

Given the complexity of diseases and their relationship with GM, it is predictable that populations of patients are heterogeneous in their clinical phenotypes, tempering the effect size of the microbiome, with an even more acute attenuation for diseases with several complex phenotypes (Qian et al., 2020; Shankar, 2017; Swann et al., 2020). Epilepsy *per se* provides several variables and risk of biases in design and evaluating the results of clinical studies. Indeed, epilepsy cannot be considered as a homogenous disorder due to differences in pathogenesis, age of onset, clinical manifestations, and different neuropsychiatric/systemic comorbidities (Devinsky et al., 2018). Therefore, if a substantial heterogeneity is known *a priori*, could be useful to recruit a relatively homogeneous study population, using well-defined inclusion and exclusion criteria, increasing the power of the study.

The choice of the control group is also essential for an appropriate study design. In studies that investigate microbiome-host interactions to identify differences in pathways and small-molecules, a control group could be one with healthy, age-related controls. Multiple control groups based on different criteria and methods could offer a better understanding of the heterogeneous effects of the microbiota compared to studies with only a single control group (Shankar, 2017; Swann et al., 2020).

Regarding methods of profiling, the 16 s RNA and metagenomic sequencing are the most common measurements for GM (Durazzi et al., 2021; Lloyd-Price et al., 2017). The choice between 16S and metagenomic sequencing is determined by the aims of the study and more often the resources available. If the purpose is an in-depth characterization of the most abundant bacterial species or strains along with their functional and metabolic profiles, metagenome sequencing could be more useful. However, if the objective is to monitor changes in the entire microbiome community, 16S sequencing could be a better alternative (Shankar, 2017). Moreover, translational studies can also combine measurements such as metabolomics and proteomics to enhance findings. Indeed, every bacteria strain seems to have a role in the microbiota-host association and in the production of specific metabolites. However, these characteristics are not unique and different bacteria can produce redundant metabolites and exert the same role in pathophysiology.

It is worth noticing that all the studies on epilepsy and GM have been focused on bacteria populations, but the GM also includes fungi, yeasts, viruses, archaea, and protozoa; all microorganisms with a potential role in MGB axis that need further investigations (Cryan et al., 2020). Understanding the microbiome might be the central focus of the study, but concomitant clinical, laboratory and "*-omics*" measurements can increase the scope of microbiome-related findings.

Rigorous testing and RCTs can determine whether differences of gut microbial compositions are a cause or consequence of a disease. The cause-effect relationship has important implications from an efficacy and a safety perspective but understanding what correlates and what is causative has shown to be challenging. Indeed, correlation does not imply necessarily causation and if epilepsy outlines the microbiota, or if differences in microbiota-composition can have a role in seizures onset and maintenance is not known yet.

7. Conclusions

Rising evidence are linking GM with epilepsy and other neurological diseases. In the next years, clinical studies assessing the differences in GM in patients with epilepsy, the mechanisms involved in the MGB axis, the role of ASMs and antibiotics and the effects of pre- and probiotics, will be performed. New therapeutic options and a better understanding of the pathophysiology of epilepsy is needed, and the gut microbiota could be an important component. Randomized clinical trials are mandatory to establish if microbiota-based treatments can be effectively and safely used for clinical improvement of seizure frequencies, severity and epilepsy-related disorders.

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Data availability

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