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# New therapeutic avenues in multiple sclerosis: Is there a place for gut microbiota-based treatments?

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ABSTRACT

The bidirectional interaction between the gut and the central nervous system (CNS), the so-called gut microbiota-brain axis, is reported to influence brain functions, thus having a potential impact on the development or the progression of several neurodegenerative disorders. Within this context, it has been documented that multiple sclerosis (MS), an autoimmune inflammatory, demyelinating, and neurodegenerative disease of the CNS, is associated with gastrointestinal symptoms, including constipation, dysphagia, and faecal incontinence. Moreover, some evidence suggests the existence of an altered gut microbiota (GM) composition in MS patients with respect to healthy individuals, as well as the potential influence of GM dysbiosis on typical MS features, including increased intestinal permeability, disruption of blood-brain barrier integrity, chronic inflammation, and altered T cells differentiation. Starting from these assumptions, the possible involvement of GM alteration in MS pathogenesis seems likely, and its restoration could represent a supplemental beneficial strategy against this disabling disease. In this regard, the present review will explore possible preventive approaches (including several dietary interventions, the administration of probiotics, prebiotics, synbiotics, and postbiotics, and the use of faecal microbiota transplantation) to be pursued as prophylaxis or in combination with pharmacological treatments with the aim of re-establishing a proper GM, thus helping to prevent the development of this disease or to manage it by alleviating symptoms or slowing down its progression.

## 1. Introduction

Multiple sclerosis (MS), one of the most prevalent autoimmune-mediated disorders of the central nervous system (CNS), is characterized by inflammatory lesions, areas of focal demyelination in both white and grey matter of the brain and spinal cord, and neuronal loss. MS is the most common cause of non-traumatic neurological disability in young adults (aged 20-40 years) [1] and affects roughly 2.9 million individuals worldwide (www.atlasofms.org), with a higher prevalence in women [2]. Although various mechanisms have been reported to be involved in the onset of the disease, blood-brain barrier (BBB) disruption seems to be the earliest event, which is linked to the pro-inflammatory activation of brain endothelial cells and followed by the release of chemokines, pro-inflammatory cytokines, and angiogenic factors [3,4]. These events lead to cerebral blood vessels hypertrophy and to a reduction in the expression of surface tight junction molecules, resulting in BBB breakdown [4]. In this way, the BBB becomes permeable to the cells of both the innate and adaptive immune system, including macrophages, natural killer, T and B cells, which will be able to enter the CNS parenchyma, where they will release inflammatory molecules [including interferon-γ

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(IFN- γ), interleukin (IL)-12, IL-17, IL-21, IL-22, tumor necrosis factor α (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF)], resulting in neuroinflammation and neurodegeneration [5]. The clinical course is very heterogeneous; indeed, MS can be classified into four main types, which are characterized by an increased severity: (i) relapsing-remitting MS (RRMS), the most common form (85 % of patients), is identified by relapses followed by remission; (ii) secondary-progressive MS (SPMS) develops over time following a diagnosis of RRMS and it is typified by progression in disability; (iii) primary-progressive MS (PPMS), characterized by a gradual continuous neurologic deterioration, affects 8–10 % of patients; (iv) progressive-relapsing MS (PRMS) affects less than 5 % of patients and it is identified by overlapping relapses with a progressive worsening [6]. The exact aetiology remains unknown, but it is thought that environmental (including Epstein-Barr virus infection, smoking, salt intake, obesity, and vitamin D deficiency), genetic and immunological factors are involved all together in determining the susceptibility to develop the disease [7]. Given that gut microbiota (GM) is considered as an environmental risk factor in several CNS diseases, such as psychiatric, neurodegenerative, and neuroinflammatory disorders [8–11], recently, some research focused on its potential influence on both the development and progression of MS [12–14]. Physiologically, the human GM, composed of bacteria (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia, being Firmicutes and Bacteroidetes the major phyla), archaea, fungi, protozoans, and viruses, lives in a mutual beneficial relationship with the host and has a key role in several functions, including food fermentation, vitamin production, protection against pathogens and inflammation, and immune response stimulation [15]. Besides these functions, the GM may also participate in CNS development, nervous system function regulation, and neuroprotection, while the CNS, in turn, may regulate gut function (including intestinal permeability, motility, and secretion) and homeostasis [16]. In this respect, it has been documented the bidirectional interaction between the brain and the gut, known as gut-brain axis (GBA), consisting of peripheral nervous structures (mainly the vagus nerve and the enteric nervous system), microbial metabolites [i.e., short-chain fatty acids (SCFAs), tryptophan and its derivatives, as well as bile acid metabolites], which can affect directly or indirectly brain functions, and immunological factors [17]. The microbiota is a dynamic ecosystem and many factors, including environment, food habits, age, stress, host

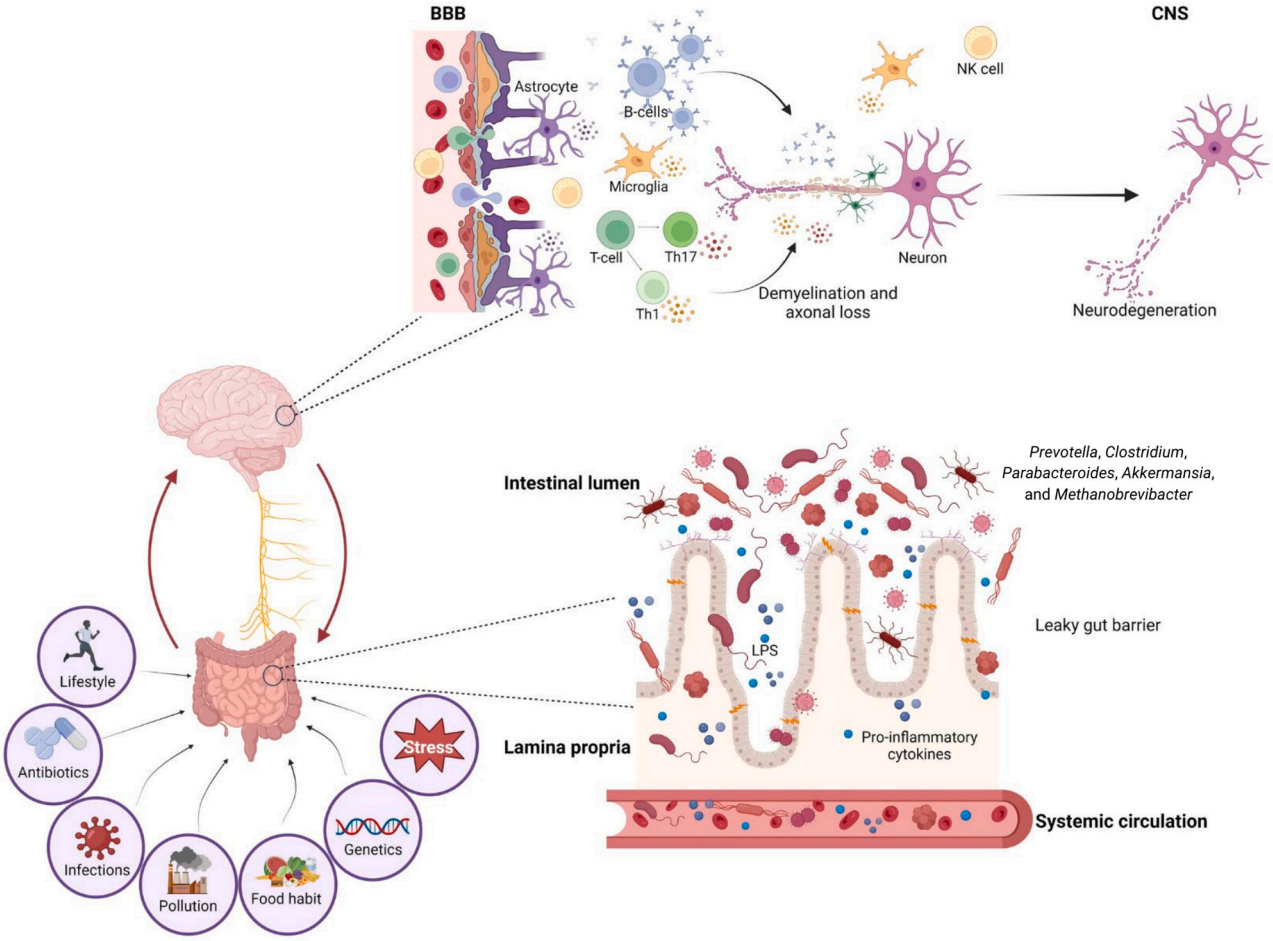


**Fig. 1.** Possible interconnection between gut and brain within the MS pathophysiology. Many factors (lifestyle, use of antibiotics, infections, pollution, food habits, genetics, and stress) may alter GM composition, leading in most cases, to a reduction in healthy bacteria (*Prevotella*, *Clostridium*, and *Parabacteroides*) and their metabolites (mainly SCFAs), and anti-inflammatory cytokines (IL-10) release, with an increase in the abundance of harmful bacteria (*Akkermansia*, and *Methanobrevibacter*), toxic metabolites (such as LPS), and pro-inflammatory cytokines (IL-6, IL-12, TNF-α, IFN-γ, IL-17A, and IL-1β). However, the exact role of *Akkermansia* is not well defined, given its pro- and anti-inflammatory roles [40,70]; as well as not all studies are concordant on the abundance of *Clostridium* [37,49].GM dysbiosis may increase the gut barrier permeability, leading to a rise in circulating microbial products and pro-inflammatory factors in the proximity of the BBB, causing its breakdown. In this way, the immune cells (B and T cells, and NK cells) can cross the BBB and enter the CNS parenchyma: B cells produce antibodies directed against the myelin, while T cells, differentiating in Th1 and Th17 cells, release pro-inflammatory cytokines. Eventually, these cells and their products, together with astrocytes and activated microglia, may contribute to neuronal demyelination, axonal damage, and neurodegeneration. Abbreviations: BBB: blood-brain barrier; CNS: central nervous system; GM: gut microbiota; IL: interleukin; IFN-γ: interferon-γ; LPS: lipopolysaccharides; MS: multiple sclerosis; NK: natural killer cells; SCFAs: short-chain fatty acids; Th: T helper cells; TNF-α: tumor necrosis factor alpha. (Created with BioRender.com).

genetics, infections, and antibiotic use, may modify its composition (the so-called intestinal dysbiosis), leading to a reduction in healthy bacteria and their metabolites, SCFAs and neurotransmitters, as well as to an overgrowth of harmful bacteria and toxic metabolites [including lipopolysaccharides (LPS)] [18,19]. Several lines of evidence revealed the influence of GM dysbiosis on CNS via the GBA, with a consequent onset or progression of several disorders, including Alzheimer's disease (AD) [9], Parkinson's disease (PD) [10], psychiatric disorders [20], depression [11], rheumatoid arthritis [21], and type 1 diabetes [22]. Concerning MS, GM dysbiosis may lead to a loss of gut integrity and functionality, increased production of toxic metabolites and pro-inflammatory cytokines, and reduced release of SCFAs and other anti-inflammatory factors. These events take part in destroying the intestinal epithelial tight and adherens junctions, thus increasing gut barrier permeability, with a rise in circulating microbial products (including LPS) and pro-inflammatory factors [19]. In turn, the presence of these gut-derived molecules in the bloodstream in the proximity of the BBB may cause its breakdown. In particular, the activation of nuclear factor kappa B (NF-kB) and mitogen-activated protein kinase (MAPK) pathways, due to the binding of LPS with the Toll-like receptors expressed on brain endothelial cells, together with the presence of pro-inflammatory cytokines (including IL-17A) may be causative factors. In this way, the immune cells may cross the BBB, with consequent neuronal demyelination, axonal damage, and neurodegeneration [5,23,24] (Fig. 1). Starting from this evidence, in this review, we will focus on the possible involvement of GM dysbiosis in MS onset and progression, and we will also discuss whether its restoration through an adequate diet, employment of probiotics, synbiotics, and postbiotics, vitamin D supplementation, as well as the use of faecal microbiota transplantation, could constitute an additional strategy to prevent or slow down the disease progression.

## 2. Gut microbiota dysbiosis in multiple sclerosis

The existence of a microbiota-gut-brain axis strengthens the relevant involvement of GM in the development and function of the CNS, especially in the control of certain neurological processes, including neurogenesis, myelination, BBB formation, and microglia maturation [25]. Nevertheless, the CNS can, in turn, influence the composition of the GM itself within a mutual interplay [26,27]. Further, a link between the commensal microbiota and the immune system has been also reported; indeed, GM is essential for immune cells maturation and, vice versa, the immune system is important for the maintenance of a healthy gut flora [28]. As mentioned above, MS is a multifactorial autoimmune demyelinating and neurodegenerative disease of the CNS characterized by inflammatory lesions, demyelinating plaques, gliosis, and irreversible axonal injury [1]. Interestingly, MS has been documented to be associated with some gut disorders: in fact, about 40 % of MS patients manifests gastrointestinal symptoms, including constipation, dysphagia, or faecal incontinence [29]. Of note, some studies described the coexistence of inflammatory bowel disease (an autoimmune inflammatory condition characterized by GM dysbiosis) and MS, thus emphasizing a possible role of GM in the disease pathogenesis [30,31]. Furthermore, alterations in different pathways commonly modulated by GM, including carbohydrate and lipid metabolism, as well as SCFAs and bile acid synthesis, have been widely reported in patients with MS [32]. In this regard, GM dysbiosis has been described in both animal models of the disease and in MS patients, suggesting the commensal microbiota as a potential additional environmental risk factor for MS pathogenesis [33–49] (Table 1).

### 2.1. Animal studies

The influence of GM on the development and clinical course of MS has been explored in animal models of MS, where the microbiota emerged to be a pivotal environmental factor contributing to the

**Table 1**
GM dysbiosis in animal models of MS and MS patients.

| Animal model/ clinical condition | Microbial dysbiosis | Reference |
|---|---|---|
| EAE C57BL/6 J mice | ↓ *Lactobacillaceae* family; ↑ *Clostridiaceae*, *Ruminococcaceae*, and *Peptostreptococcaceae* families, and *Clostridium sensu stricto 1*, *Ruminiclostridium*, *Ruminococcus*, *Romboutsia*, and *Corpococcus* genera | [33] |
| SJL/J mice with RR-EAE | ↑ *Bacteroidales* (including, *Bacteroides*, *Parabacteroides*, *Prevotella*, *Rikenellaceae*, and *Odoribacter*), and members of the Tenericutes phylum | [34] |
| C57BL/6 mice with chronic progressive EAE | ↑ *Akkermansia muciniphila* | |
| EAE mice | ↓Actinobacteria and Verrucomicrobia phyla | [35] |
| Cuprizone demyelination mice model | ↑*Akkermansia*, *Marinifilaceae*, *Lactobacillaceae*, and *Defluvitaleaceae*; ↓*Flavobacteriaceae*, *Desulfovibrionaceae*, *Haloplasmataceae*, *Veillonellaceae*, and *Thermoanaerobacteriaceae* | |
| NOD ShiLt mice model of EAE | ↑*Akkermansia*, members of the order *Clostridiales* and the genus *Turicibacter*; ↓*Lactobacillus* genus | [36] |
| 20 patients with RRMS | ↑*Bifidobacterium* and *Streptococcus* genera, *Streptococcus thermophilus/salivarius* and *Eggerthella lenta* species; ↓*Bacteroides*, *Faecalibacterium*, *Prevotella*, *Anaerostipes*, *F. prausnitzii*, and *Sutterella* genera | [37] |
| 31 patients with RRMS | ↑*Blautia*, *Dorea*, *Pseudomonas*, *Mycoplana*, and *Pedobacter*; ↓*Adlercreutzia*, *Collinsella*, *Lactobacillus*, *Erysipelotrichaceae*, *Parabacteroides*, and *Prevotella* | [38] |
| | ↑*Firmicutes/Bacteroidetes* ratio, and *Streptococcus*, ↓*Prevotella* | [39] |
| 60 patients with RRMS | ↑*Methanobrevibacter*, and *Akkermansia*; ↓*Collinsella*, *Slackia*, *Prevotella*, and *Butyricimonas* | [40] |
| 24 patients with RRMS | ↓*B. Longum*, *Clostridium Leptum*, *F. Prausnitzii*, *Bacteroides Thetaiotaomicron*, and *Prevotella* | [41] |
| 71 patients with MS | ↑*Akkermansia* and *Acinetobacter calcoaceticus*; ↓*Parabacteroides distasonis* | [42] |
| 18 RRMS children | ↑*Desulfovibrio* and *Bifidobacterium* genera, *Christensenellaceae* family, *Enterobacteriaceae* family and *Methanobrevibacter* genus ↓*Clostridiales* order (including *Lachnospiraceae* and *Ruminococcaceae*), *B. fragilis*, and *Butyricimonas* genus | [43] |
| 15 patients with PPMS | ↑*Gemmiger* and the *Ruminococcaceae* family | [44] |
| 28 patients with PPMS | ↓*Gemmiger* and *Butyricicoccus*; ↑*Methanobrevibacter*, *Sporobacter*, and *Clostridium* cluster IV | [45] |
| 44 patients with PPMS 199 patients with RRMS | ↑*Clostridium bolteae*, *Akkermansia*, *Enterobacteriaceae*, *Ruthenibacterium lactatiformans*, *Ruminococcaceae FJ366134* and *Clostridium g24 FCEY*; ↓*Blautia wexlerae*, *Dorea formicigenerans*, *Erysipelotrichaceae CCM*, and *Dorea longicatena*, *Anaerococcus vaginalis*, and *Blautia faecis* ↑*Clostridium bolteae*, *Ruthenibacterium lactatiformans*; ↓*Blautia wexlerae*, *Dorea formicigenerans*, and *Erysipelotrichaceae CCM* | [46] |
| 15 patients with SPMS | ↑*Streptococcus* and ↓*Roseburia* | [47] |
| 12 patients with SPMS | ↑*Akkermansia* and *Collinsella*; ↓*Coprococcus* and *Roseburia* | [48] |
| 45 patients with MS | ↑*Clostridium* genus | [49] |

Abbreviations: EAE: experimental autoimmune encephalomyelitis; MS: multiple sclerosis; PPMS: primary progressive-multiple sclerosis; RR-EAE: relapsing remitting-experimental autoimmune encephalomyelitis; RRMS: relapsing remitting-multiple sclerosis; SPMS: secondary progressive-multiple sclerosis; ↑: increase; ↓: decrease.

disease. In fact, germ-free (GF) or antibiotic-treated mice appear to be more resistant to develop experimental autoimmune encephalomyelitis (EAE) compared to specific pathogen-free mice. For instance, the oral administration of antibiotics (ampicillin, vancomycin, neomycin, and metronidazole) decreases the disease severity of EAE, reduces pro-inflammatory cytokines, and increases IL-10, while GF mice are protected against spontaneous EAE development or are endowed with a decreased disease severity, as well as a reduced expression of IFN-γ and IL-17 [50–53]. Of note, Lee *et al.* observed pronounced EAE symptoms when GF mice are colonized with segmented filamentous bacteria (non-pathogenic members of the commensal microbiota), which are able to induce Th17 cell differentiation in the small intestine [53]. Moreover, interestingly, other works highlighted that C57BL/6 mice colonized with *Lactobacillus reuteri* and OTU0002 strains manifest more severe symptoms compared to GF or mono-colonized mice [54]; further, mice colonized with the faecal microbiota of MS patients showed a higher EAE induction rate and severity, thus underscoring GM as a causative agent [55].

Concerning GM composition, significative differences in animal models of MS with respect to the control counterparts have been identified. For instance, Johanson *et al.* found decreased commensal *Lactobacillaceae*, and an increase in bacteria belonging to *Clostridiaceae*, *Ruminococcaceae*, and *Peptostreptococcaceae* families in EAE C57BL/6 J mice. Further, Gandy *et al.* observed a significant expansion of *Bacteroidales* (including, *Bacteroides*, *Parabacteroides*, *Prevotella*, *Rikenellaceae*, and *Odoribacter*), and members of the Tenericutes phylum in SJL/J mice with relapsing-remitting (RR)-EAE, while C57BL/6 mice with chronic progressive EAE revealed elevated *Akkermansia muciniphila*. Lastly, Moles *et al.* detected a marked reduction of Actinobacteria and Verrucomicrobia phyla during disease progression in faecal samples of EAE mice, while the cuprizone demyelination mice model showed a significant rise in Verrucomicrobia (*Akkermansia*), Bacteroidetes (*Marinifilaceae*) and in some families of Firmicutes (*Lactobacillaceae*, and *Defluvitaleaceae*), along with a reduction in Bacteroidetes (*Flavobacteriaceae*), Proteobacteria (*Desulfovibrionaceae*), Tenericutes (*Haloplasmataceae*) and in other families of Firmicutes (*Veillonellaceae*, and *Thermoanaerobacteriaceae*). A rise in *Akkermansia* was also observed by Colpitts *et al.* in the NOD ShiLt mice model of EAE, together with an increase in members of the order *Clostridiales* and the genus *Turicibacter*, as well as a reduction in the *Lactobacillus* genus [33–36].

Worthy of attention is the work of Cekanaviciute *et al.* where the authors investigated the immunomodulatory effects of GM in MS by colonizing antibiotic-treated or GF mice with *Acinetobacter calcoaceticus* (able to encode peptides that mimic the amino acid sequences of some myelin components) or *Parabacteroides distasonis*, two species resulted, respectively, more abundant or reduced in MS stool samples. In detail, in antibiotic-treated mice they found an inhibitory effect mediated by *A. calcoaceticus* on regulatory T cells (Tregs; belonging to CD4+ T cells) differentiation and a stimulatory effect due to *P. distasonis* on $CD4^{+}IL\text{-}10^{+}$ lymphocyte differentiation; instead, in GF mice they observed that the mono-colonization with *A. calcoaceticus* improved T lymphocyte differentiation into Th1 phenotype (producing pro-inflammatory cytokines), while the mono-colonization with *P. distasonis* led to a significant increase in the $CD4^{+}IL\text{-}10^{+}$T lymphocyte population (thus sustaining an anti-inflammatory response). Moreover, GF C57BL/6 mice colonized with MS donor microbiota showed a significant increase in EAE disease severity and an inhibition of Tregs differentiation [42]. The ability of specific bacteria to shift T-cells towards a pro-inflammatory profile, likely resulting in worsening or prolonged autoimmune responses, makes the GM a potential environmental factor that could contribute to the pathogenesis of MS and, hence, a valuable therapeutic target.

### 2.2. Clinical studies

Several recent studies explored the differences in faecal GM composition between MS patients and healthy individuals, detecting the presence of an intestinal dysbiosis in MS subjects. One of the first longitudinal studies of about 20 patients with RRMS and 40 healthy controls (HC) highlighted a moderate level of dysbiosis between these two groups. In detail, at the genus level, Miyake *et al.* revealed a significant increase in *Bifidobacterium* and *Streptococcus*, as well as a considerable reduction in *Bacteroides*, *Faecalibacterium*, *Prevotella*, and *Anaerostipes* in stool samples from MS patients compared to HC. Moreover, significant changes were also observed in 21 species, where S*treptococcus thermophilus/salivarius* and *Eggerthella lenta* were significantly increased in MS individuals, while the other 19 species (14 belonging to the *Clostridium* genus, including *Faecalibacterium prausnitzii*, a butyrate-producing bacterium with anti-inflammatory effects, and 5 belonging to the Bacteroidetes phylum, including *Bacteroides*, *Prevotella*, and *Sutterella* genera) were reduced [37]. Specifically, the reduction in the members of the *Clostridium* genus determines a decrease in the synthesis of SCFAs, which are able to cross the BBB and control neuroimmune homeostasis [56]. For instance, *C. tyrobutyricum* produces the SCFA butyrate, which improves the integrity and counteracts the malfunctioning of the BBB by promoting the expression of tight junction proteins [57]. Moreover, emerging evidence further supports the anti-inflammatory and immunoregulatory properties of the bacteria belonging to the *Clostridium* genus, including the ability to produce Tregs, decrease pro-inflammatory T cells and increase the production of the anti-inflammatory cytokine IL-10 [58,59]. Besides the *Clostridium* genus, also the low abundance of several *Bacteroidetes* genera has adverse effects. For instance, it leads to a consistent reduction, in the serum samples from MS patients, in the amount of lipid 654, a toll-like receptor (TLR)-2 ligand, which is hypothesized to act as an immunomodulatory factor involved in the activation and regulation of the immune responses by maintaining a specific level of the TLR-2 signaling [60]. However, in contrast, Ventura *et al.*, studying the GM from 45 treatment-naïve MS patients in early disease and 44 matched HC, found in MS patients an increase in bacteria from the *Clostridium* genus, suggesting a possible role of these bacteria in MS pathogenesis [49]. In this regard, it is possible that different species belonging to the *Clostridium* genus may make an opposite contribute to the disease course.

Another work by Chen *et al.* reported a GM alteration in stool samples from 31 RRMS patients compared to 36 HC [38]. The study revealed an increase in Firmicutes (*Blautia* and *Dorea*), Proteobacteria (*Pseudomonas* and *Mycoplana*), and Bacteroidetes (*Pedobacter*), as well as a decrease in Actinobacteria (*Adlercreutzia* and *Collinsella*), and in other species of Firmicutes (*Lactobacillus* and *Erysipelotrichaceae*) and Bacteroidetes (*Parabacteroides* and *Prevotella*) in MS patients. Interestingly, some *Dorea* species are endowed with a pro-inflammatory role, contributing to IFN-γ production and mucin degradation, thus potentially increasing gut permeability [61], although other *Dorea* species exhibit anti-inflammatory properties [62]. Moreover, *Prevotella*, *Parabacteroides*, and *Adlercreutzia* are phytoestrogen-metabolizing bacteria, while the *Erysipelotrichaceae* family is involved in bile acid metabolism [38]. Therefore, a reduction of these bacteria results in a decrease in phytoestrogens and bile acids metabolites, which have anti-inflammatory and neuroprotective effects [63,64]. Accordingly, some evidence reveals that MS patients have altered levels of bile acid metabolites, as well as elevated IL-6, TNF-α, and IL-12 protein expression [65,66]. Similarly to the studies mentioned above, also other works (performed by Cosorich *et al.*, Jangi *et al.*, and Cantoni *et al.*) underline a reduction in MS patients of the genus *Prevotella*, which is involved in the production of the anti-inflammatory propionate, a SCFA with an important immunoregulatory and neuroprotective effect [39–41,67]. Of note, the low abundance of these bacteria was seen to be associated with an increased expansion of Th17 cells at the intestinal level and with a high disease activity [39]. Interestingly, it has been observed that the administration of the human-derived *P. histicola* counteracts EAE, reducing microglia and astrocytes activation in the CNS, as well as the pro-inflammatory IFN-γ and IL-17 [68]. In detail, the study by Cosorich *et al.* documented an alteration of the microbiota isolated from the small

intestine of 19 MS patients with active disease compared to that of 17 healthy individuals [39]. The authors identified the presence of a higher Firmicutes/Bacteroidetes ratio, with a specific abundance of *Streptococcus* (phylum Firmicutes) and a decrease in *Prevotella* (phylum Bacteroidetes) strains in MS patients. Notably, different *Streptococcus* species are associated with the production of the pro-inflammatory cytokines IFN-γ and IL-1β, suggesting their harmful role [61]. Further, the study conducted by Cantoni *et al*. reported decreased levels of several bacteria, including *Bifidobacterium longum* (phylum Actinobacteria), *Clostridium leptum*, and *Faecalibacterium prausnitzii* (phylum Firmicutes), *Bacteroides thetaiotaomicron*, and *Prevotella* (phylum Bacteroidetes) in RRMS patients [41]. Lastly, Jangi *et al*. showed the abundance of *Methanobrevibacter* (phylum Euryarchaeota) and *Akkermansia* (phylum Verrucomicrobia), as well as the reduction of *Collinsella* and *Slackia* (phylum Actinobacteria), *Prevotella* and *Butyricimonas* (butyrate producers; phylum Bacteroidetes) in stool samples from 60 RRMS patients compared to 43 HC [40]. Moreover, the authors found a positive correlation of *Methanobrevibacter* and *Akkermansia* genera with the expression of innate and adaptive immune genes in T cells and monocytes, and a negative association between *Butyricimonas* and inflammatory genes. They also demonstrated that, after beta-interferon or glatiramer acetate treatment, the patients show increased *Prevotella* levels compared to untreated patients. Notably, the genus *Methanobrevibacter*, able to recruit inflammatory and dendritic cells, is involved in inflammatory processes [69], while *Akkermansia* genus has been documented to possess both pro-inflammatory and anti-inflammatory properties (i.e., as pro-inflammatory factor, it upregulates genes associated with innate and adaptive immune responses, including MAPK14, MAPK1, lymphotoxin beta receptor, and caspase 1, as well as genes involved in antigen-presentation, B- and T-cell receptor signalling, and complement activation; instead, as anti-inflammatory factor it can produce SCFAs, which can contribute to alleviate inflammation) [40, 70]. In this regard, its beneficial role was pointed out in some studies where MS patients showed increased levels of this bacterium, suggesting the possible positive role of this genus in counteracting the disease, rather than its contribution to MS course [46,55,71]. It should be also mentioned that even though several studies underscored the anti-inflammatory property of *Akkermansia* and a positive association with a lower disability, Cekanaviciute *et al*. reported its pro-inflammatory role, with the ability to promote Th1 lymphocyte differentiation [42,46]. In detail, the authors observed an abundance of *Akkermansia* and *Acinetobacter calcoaceticus* (able to reduce the number of Tregs and increase Th1 and Th2 cells), and a reduction in *Parabacteroides distasonis* (able to stimulate anti-inflammatory IL-10-expressing human $CD4^{+}CD25^{+}$ T lymphocytes) in stool samples from 71 RRMS patients compared to 71 HC [42].

Considering that the risk of developing MS is clearly associated with environmental factors (including obesity in early life, Epstein-Barr virus infection, and cigarette smoking) and that paediatric patients are generally characterized by a still lower exposure to these factors, studies on children might be, indeed, more appropriate for identifying the potential connection between GM dysbiosis and MS pathogenesis. In this regard, Tremlett *et al*. conducted some specific studies [43,72,73]. The first study documented an increase in bacteria from *Desulfovibrio* and *Bifidobacterium* genera, as well as from the highly heritable *Christensenellaceae* family, and a decrease in the members belonging to the *Clostridiales* order (including the butyrate-producing bacteria *Lachnospiraceae* and *Ruminococcaceae*) in 18 RRMS children [43]. Moreover, the authors also observed an abundance of pro-inflammatory bacteria from the *Enterobacteriaceae* family and the methane-producing *Methanobrevibacter* genus, as well as a depletion of the SCFAs-producing bacteria *Bacteroides fragilis* [43]. Of note, it has been described a protective role of this latter bacterium against EAE, via polysaccharide A production and induction of IL-10 from CD4+ Foxp3+ Tregs (FoxP3 is a transcription factor with an important role in immune regulation) in the gut [74]. In the second study, Tremlett and co-workers defined *Fusobacteria* as a relapse risk factor; indeed, they showed the existence of a positive correlation between the lack of bacteria belonging to this phylum and the risk of relapses, which was three times higher than the one observed in the patients endowed with quantifiable levels of *Fusobacteria* [73]. Lastly, the third work reports a positive association between Actinobacteria and $CD4^{+}$ T/Tr1 (type 1 regulatory cells, a subset of CD4+ T cells) cells, as well as a negative correlation between the abundance of *Bacteroidetes* and $CD4^{+}$ T/ Tregs cells by analysing stool samples and peripheral blood mononuclear cells from 15 MS children and 9 HC [72]. However, they observed an association between *Fusobacteria* or *Firmicutes* and immune markers only in paediatric HC but not in MS, thus suggesting that also the lack of communication between GM and the immune system might contribute in triggering the pathogenic state and in maintaining MS clinical course [12].

Since MS is classified into four main types, which are characterized by an increased severity, it would have been mandatory to investigate GM dysbiosis in each of these forms. Nevertheless, many studies focused on patients with RRMS, the most prevalent form, and only few works analysed the GM composition in patients with PPMS and SPMS. In this regard, recent studies conducted on PPMS subjects found significant GM differences. In particular, one study observed increased levels of bacteria belonging to the *Gemmiger* genus and the *Ruminococcaceae* family in 15 PPMS with respect to HC [44]. Another study, in contrast, reported lower levels of the *Gemmiger* genus and the butyrate-producing *Butyricicoccus*, together with a higher abundance of *Methanobrevibacter*, *Sporobacter*, and *Clostridium* cluster IV in 28 PPMS [45]. Furthermore, Cox *et al*. highlighted elevated levels of *Clostridium bolteae*, *Akkermansia*, *Enterobacteriaceae*, *Ruminococcaceae FJ366134* and *Clostridium g24 FCEY* together with a decrease in *Blautia wexlerae* (an acetate producer), *Erysipelotrichaceae CCM*, and *Dorea longicatena* [46]. Interestingly, they also found a positive association between *Clostridium g24 FCEY* and the level of disability, as well as between *Enterobacteriaceae* and fatigue; moreover, *Blautia* was described to be positively correlated with motor and cognitive functions and negatively correlated with fatigue and depression. Besides these bacteria, also *Erysipelotrichaceae CCM* was found to be associated positively with motor and cognitive function and negatively with the expanded disability status score (EDSS) and fatigue, while *Clostridium bolteae* was positively correlated with disability and fatigue. Lastly, they noticed a negative correlation between *Akkermansia* and EDSS and brain magnetic resonance imaging measures [46]. Considering SPMS patients, some works also revealed the presence of GM dysbiosis in comparison to HC. For instance, one study carried out in 15 SPMS subjects reported elevated levels of *Streptococcus* (which has been shown to correlate positively with Th17 cells and negatively with Tregs) and a decrease in butyrate-producing bacteria *Roseburia* [47]. In particular, the abundance of *Streptococcus parasanguinis* was found strongly correlated with high-severity SPMS [47]. Further, another work found higher levels of *Akkermansia* and *Collinsella*, which are associated with the production of the pro-inflammatory cytokine IL-17A and with an altered gut permeability in MS, as well as a lower abundance of SCFAs-producing bacteria belonging to *Coprococcus* and *Roseburia* genera in 12 patients with SPMS compared to 38 HC [48].

All these studies confirm the occurrence of a gut dysbiosis during the clinical course of MS, which could partially explain the inflammatory status associated with the pathogenesis. Specifically, most studies highlighted a gut microbial ecosystem of MS patients characterized by a decrease in SCFAs-producing bacteria (*Prevotella*, *Clostridium*, and *Parabacteroides*), together with an increase in *Akkermansia*, and *Methanobrevibacter*. Nevertheless, the presence of clear discrepancies among the findings might be due to the use of different methods for microbiome analysis. In this regard, more large-scale longitudinal studies are especially needed to better elucidate the potential role of GM dysbiosis in MS pathogenesis, to understand how the microbiota can influence disease onset and progression, and to clarify if these alterations could be the consequence or the cause of the disease. Moreover, it will be also important to further deepen the impact of age, gender, dietary habits,

and genetic background on GM, in order to disclose a possible association between these features and GM changes, which afterwards may lead to disease development or modify its clinical course. Lastly, although a correlation between immunity-associated genes and the presence of certain bacteria (*Akkermansia* and *Methanobrevibacter*) has been already found [40], it would be necessary to carry out studies on intestinal dysbiosis in the early stages of the disease (or even before its onset, such as in the clinically isolated syndrome and the radiologically isolated syndrome stages) [12], in order to specifically link GM alterations to the pathogenesis of MS. In this way, it will be possible to exclude the involvement of non-MS-related factors in dysbiosis, including intestinal disorders, activated systemic immune pathways, and the use of disease-modifying drugs.

## 3. Gut microbiota involvement in typical MS features

As mentioned above, the aetiology of MS is still unknown, but it is supposedly triggered by a combination of environmental and genetic factors that cause an abnormal autoimmune response, resulting in damage to myelin and axons [75]. It has been widely reported that increased intestinal permeability, disruption of BBB integrity, chronic inflammation, and altered T cells differentiation have a key role in MS onset and progression [76]. Of note, some evidence suggests the potential influence of GM dysbiosis on all these prominent pathological traits [77–79], therefore GM restoration could be an additional approach to help reduce these abnormalities, thus preventing the disease, or slowing down MS progression and alleviating symptoms.

### 3.1. Intestinal permeability

It is now clear that patients with MS have an increased gut permeability compared to HC [80]. Indeed, an interesting study conducted in 22 patients with RRMS showed a higher intestinal permeability in MS patients (73 %) following the evaluation of the capability of two sugars, lactulose and mannitol, to permeate the intestinal mucosa [81]. Moreover, another study reported increased intestinal permeability, overexpression of the tight junction protein zonulin (see also further on), and weight loss before the occurrence of MS neurological symptoms, together with morphological changes in the small intestine (including increased crypt depth and length of villi at the jejunum and ileum, as well as a more enlarged submucosal layer of jejunum and ileum) in EAE animals compared with healthy mice [82]. Interestingly, Nouri *et al.* also revealed that the transfer to healthy mice of encephalitogenic T cells, isolated from EAE animals, leads to intestinal changes similar to those observed after immunization [82]. These results underline the potential key role of circulating autoreactive T cells in altering the morphology of the intestinal wall and the barrier properties [82]. Considering that MS patients are endowed with intestinal dysbiosis, the resulting increased production of toxic metabolites and pro-inflammatory cytokines, as well as the reduction of beneficial molecules, such as SCFAs and other anti-inflammatory factors, might contribute to the destruction of the junctional protein complexes of the intestinal epithelial barrier, thus increasing the leakiness of the gut epithelium [83]. One of the biomarkers of intestinal permeability is the serum concentration of zonulin, a physiological modulator of tight junctions, which have a key role in macromolecules trafficking, in the maintenance of epithelial barrier integrity and immune response balance in the gut mucosa [84]. Of note, intestinal dysbiosis can activate the zonulin pathway and stimulate pro-inflammatory cytokines release that leads to increased permeability [77]. In this regard, the study performed by Pellizoni *et al.* described higher zonulin levels in the serum from RRMS patients compared to HC, as well as a positive correlation with the disease duration and the abundance of bacterial species belonging to the class of *Bacilli* [85].

### 3.2. Blood-brain barrier

The BBB acts as a gatekeeper, on one hand to selectively regulate the passage of specific substances, molecules, microorganisms and cells between the brain parenchyma and the circulatory system, on the other hand to limit the influx of ions, proteins and other substances into the CNS, thus maintaining homeostasis [86]. It has been reported a link between BBB disruption and the onset and progression of MS [87,88].

The impact of GM on BBB development and integrity has been already described [78]. In this regard, an animal study showed an increased BBB permeability in foetal mice from GF mothers compared to the ones from pathogen-free mice with a normal gut flora; moreover, the authors also found this alteration in GF adult mice, in which a reduced expression of the tight junction proteins occludin and claudin-5 was found [57]. Interestingly, when GF adult mice were exposed to a pathogen-free GM or were treated with *Clostridium tyrobutyricum* (a butyrate producer) or with *Bacteroides thetaiotaomicron* (acetate and propionate producer), a reduction in BBB permeability and an increase in tight junction proteins expression were seen [57]. Since MS patients have decreased levels of SCFAs-producing bacteria, this observation suggests the possible involvement of GM dysbiosis in BBB permeability alteration, as well as a plausible beneficial role of SCFAs in maintaining BBB integrity. Another positive evidence emerged treating EAE mice with *Prevotella histicola*. Indeed, these animals showed reduced BBB permeability and pro-inflammatory Th1 and Th17 cells in the CNS, thus indicating the protective role of this bacterium in preserving the BBB structure (essential for the proper trafficking of immune cells into the CNS), and in decreasing neuroinflammation and demyelination [89].

### 3.3. Inflammation

The alteration of the intestinal flora also affects the immune system, which may be overstimulated by the by-products synthesized by detrimental microorganisms [90]. In addition, gut bacteria can positively or negatively influence neuroendocrine function and brain maturation acting on the migration of $CD4^+$T cells [91]. In this respect, one study found that GF mice develop attenuated EAE compared to colonized mice, produce lower levels of pro-inflammatory cytokines, such as IFN-γ and IL-17, and exhibit increased $CD4^+CD25^+Foxp3^+$T reg cells. Moreover, segmented filamentous bacteria, which colonize the intestine, were found to produce IL-17, to induce IL-17-producing $CD4^+$T cells in the CNS and to cause the spontaneous development of EAE in GF mice, thus confirming the strong correlation between a given bacterial community and neurological inflammation [53]. Notably, the typical demyelinating inflammatory lesions within the CNS have been reported to contain $CD4^+$ and $CD8^+$ T cells, and B cells [92,93], suggesting the crucial role of the adaptive immune system in MS pathogenesis. GM has been reported to maintain the balance between pro- and anti-inflammatory immune responses, regulating the development and differentiation of T cells [79]; hence, GM dysbiosis can alter the abundance of these cells. Since MS patients have been reported to have low levels of *Prevotella histicola,* its administration might have a beneficial role in restoring the immune scenery and in modulating the systemic immune response. In this regard, HLA-DR3.DQ8 transgenic mice with EAE treated with this bacterium showed a reduced inflammation and demyelination, a higher abundance of Treg cells and a reduction of pro-inflammatory Th1 and Th17 cells, together with cytokines IL-17 and IFN-γ levels [89]. Besides these bacteria, also *Bacteroides fragilis* has been suggested as a tool to regulate the immune system. Indeed, this bacterium, which produces the capsular carbohydrate polysaccharide A, is able to induce the conversion of $CD4^+$T cells into $IL\text{-}10^+Foxp3^+$T reg cells, thus exerting an anti-inflammatory role [94].

An interesting study carried out on brain biopsies found, in the cerebral white matter, high concentrations of bacterial RNA and DNA and proteins attributable, in RRMS, to Actinobacteria strains and, in PPMS, to Proteobacteria strains [95]. Moreover, the authors revealed a positive

correlation between Proteobacteria abundance and the presence of genes belonging to the nuclear factor kappa B-signaling pathway, which is one of the key pathways implicated in neuroinflammation in MS [95].

Overall, since this evidence suggests the possible involvement of GM dysbiosis in MS pathogenesis by causing and exacerbating intestinal permeability, BBB disruption, and chronic inflammation (with an expansion of pro-inflammatory Th1 and Th17 cells, and a suppression of anti-inflammatory Treg cells), GM restoration may represent an adjunctive strategy to counteract these events and reactivate the immune system.

## 4. Dietary intervention

It has been well-documented the impact of diet on the gastrointestinal tract, by regulating GM composition and functionality, and the influence of an inadequate nutrition on the pathogenesis and progression of several disorders, including neurodegenerative diseases [96]. Diets characterized by a high intake of fat, sugar, salt, and animal proteins may lead to the development of specific pathogenic bacteria, thus promoting gut dysbiosis, inflammation, damage to the intestinal barrier, as well as an increase in BBB permeability, resulting in CNS autoimmunity [97]; conversely, a diet rich in vegetables and fibers, combined with probiotics and vitamin D administration, results in GM restoration and increase in microbiota-associated anti-inflammatory factors, such as SCFAs [98]. Considering the pivotal role of diet in shaping GM, thus preserving brain health, reducing the risk, modulating the symptoms, and improving the pathophysiological aspects related to neurodegenerative diseases [99,100], dietary intervention coupled with pharmacological treatment could represent a strategy to restore GM dysbiosis and improve MS symptoms (Fig. 2).

### 4.1. Vitamin D

Vitamin D (Vit D) is a fat-soluble vitamin whose main source is diet and its formation in the body follows exposure to sunlight and subsequent metabolic activation. Two types of Vit D can be identified: D2 (ergocalciferol), which is mainly found in fungi and algae, and D3 (cholecalciferol), which is synthesized from cholesterol in the skin following exposure to sunlight (via UVB sun rays). Vit D3 is also found in eggs and fatty fish, although the main source for humans comes from sun exposure [101].

Vit D plays a key role in calcium homeostasis, in fact, acting in synergy with parathyroid hormone, increases intestinal $Ca^{2+}$ absorption and promotes bone resorption, in order to increase calcemia. In addition to these functions, Vit D also plays an important role in immunomodulation, and its high levels are associated with a reduction in the risk of MS occurrence [102]. Conversely, a deficit of Vit D appears to be a risk factor for MS: the frequency of the disease increases with increasing latitude, in turn, it is inversely related to the duration and intensity of solar UVB rays and therefore to the formation of Vit D at the skin level. Indeed, there is a noticeable geographical distribution of MS, with higher prevalence rates in regions farther from the equator, where there is less sunlight exposure [103,104].

Concerning its activity on the immune system, Vit D is also implicated in regulating the balance between pro-inflammatory and anti-inflammatory responses. Indeed, the correlation between Vit D and MS is linked to the effect on the immune response. For instance, in macrophages and monocytes, the 1,25 dihydroxylated form acts positively increasing the expression of the Vit D receptor (VDR) and cytochrome P45027B1 (CYP27B1), an enzyme that regulates the level of the biologically active Vit D and plays an important role in calcium homeostasis. Moreover, the dihydroxylated form decreases the maturation of the dendritic cells (DC) by inhibiting the overregulation of major histocompatibility complex class II, cluster of differentiation (CD) 40, CD80, and CD86 and by reducing IL-12 production from DC while increasing IL-10 [105].

Regarding the link between neuroinflammation/ neurodegeneration and Vit D, it is necessary to take into account that both the circulating and biologically active forms of Vit D (25(OH)D3 and 1,25(OH)2D3, respectively) cross the BBB entering into the CNS, where they can act on various neuronal and glial cells [106].

In this respect, some studies performed in animal models of MS revealed a positive effect of Vit D administration. For instance, C57BL/6 mice are protected against EAE development when myelin oligodendrocyte glycoprotein (MOG) is administered together with Vit D [107]; further, Vit D3 has been seen to reduce the recruitment of inflammatory

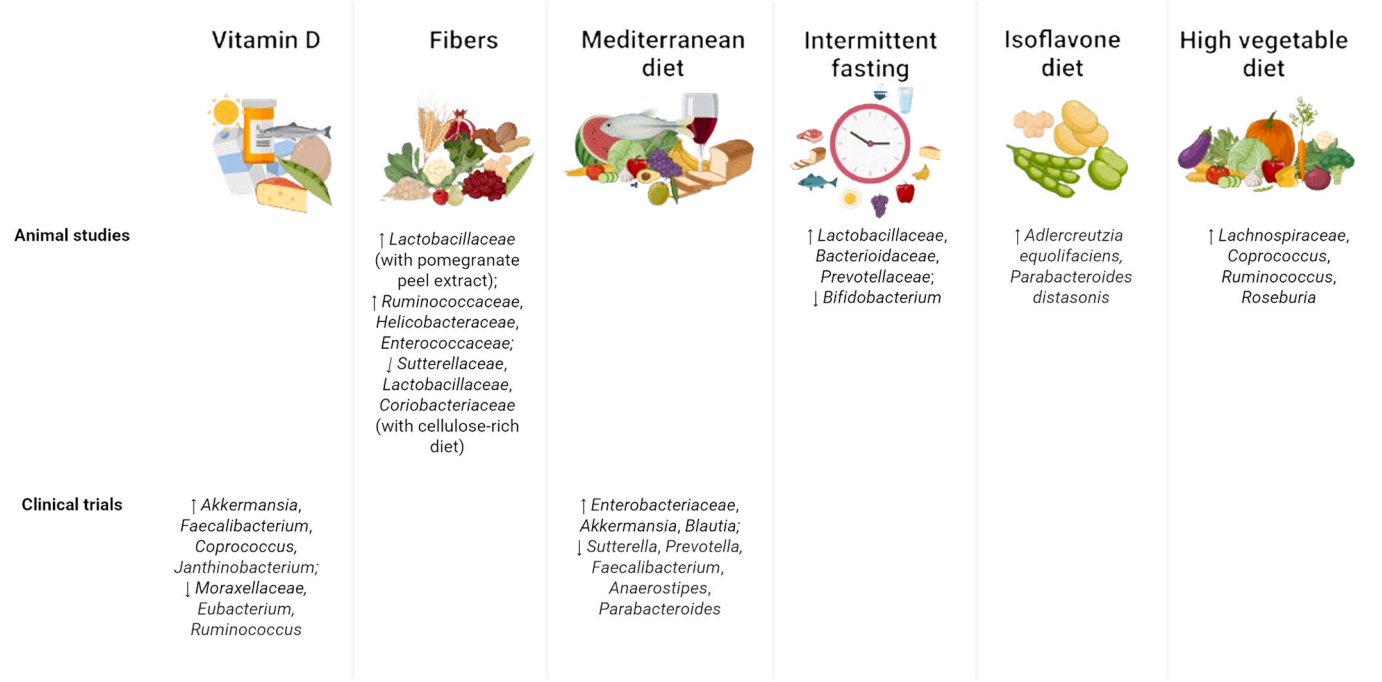


**Fig. 2.** Changes in gut microbiota composition through dietary intervention. (Created with BioRender.com).

cells, the mRNA expression of inflammatory factors [including TNF-α, NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, and the chemokine CCL17], and demyelination [108]. In addition, several clinical trials investigated the potential impact of Vit D supplementation on MS (Table 2) [109–118]. For instance, some studies reported that Vit D intake reduces the number of new brain lesions [109,110,115,117], as well as the exacerbation rate [109], improves timed tandem walk and inflammatory response [115,116], and has a positive immunomodulatory effect in MS [111,113]. However, other studies did not find beneficial results [112,114,117,118]. Specifically, no evidence that Vit D supplementation reduces MS activity, including relapses, and magnetic resonance imaging (MRI)-detected brain lesions, improves fatigue, grip strength, and disability, as well as prevents bone loss in the MS population has been documented. Moreover, a meta-analysis of a randomized, double-blind, placebo-controlled clinical trial found contradictory results, with some studies confirming a reduction in disease activity and severity, while others not reporting any favorable outcomes [119]. Overall, the findings from these trials have provided contradictory evidence regarding the effectiveness of Vit D supplementation in reducing MS activity, and further research is needed to draw definitive conclusions.

Furthermore, although consistent evidence shows an association between Vit D and GM [120–122], to date only one work documents its potential effect on GM composition in MS [123]. Specifically, this clinical trial conducted by Cantarel *et al.* reported changes in gut bacterial communities between 7 RRMS patients (Vit D deficient) [(5 patients treated with glatiramer acetate (GA) and 2 untreated)] and 8 HC. In detail, after 3 months of Vit D supplementation (5000 IU/day), they revealed an increase in *Akkermansia*, *Faecalibacterium*, and *Coprococcus* genera, as well as a decrease in *Moraxellaceae* in untreated MS patients compared to HC and GA-treated MS patients, while a rise in *Janthinobacterium*, and a reduction in *Eubacterium* and *Ruminococcus* genera were observed in GA-treated MS subjects compared to HC and untreated MS individuals [123]. Although most of these bacteria have pro-inflammatory properties when taken individually, it is worth considering the mutual interplay between commensal microbes and the host immune system at the base of the positive effects of Vit D on GM composition. Given these promising results, more clinical trials on Vit D supplementation should be encouraged.

**Table 2**
Clinical trials investigating the potential impact of vitamin D supplementation on multiple sclerosis.

| Vit D dose | Study duration | Effect | Reference |
|---|---|---|---|
| 1000 IU/d | 48 wk | Decrease in exacerbation rate; improvement in brain lesions (MRI) | [109] |
| 4000–40,000 IU/d | 28 wk | Decreased number of brain lesions (MRI) | [110] |
| 20,000 IU/d | 12 wk | Positive immunomodulatory effect in MS | [111] |
| 2800 IU/d | 96 wk | No prevention of bone loss in MS population | [112] |
| 200–10,200 IU/d | 72 wk | Positive immunomodulatory effect in MS | [113] |
| 2800 IU/d | 96 wk | No significant difference between groups in ARR, EDSS, MSFC components, grip strength, or fatigue | [114] |
| 2800 IU/d | 12 wk | Reduced new lesions and improved timed tandem walk | [115] |
| 7000 IU/d | 12 wk | Improved inflammatory response | [116] |
| 14,007 IU/d | 48 wk | No benefit for a high-dose Vit D3 as add-on to IFN-β−1a<br>Protective effects on the development of new MRI lesions in patients with RRMS | [117] |
| 5000 IU/d vs 600 IU/d | 96 wk | No evidence that high-dose Vit D supplements reduce MS activity, including relapses and MRI-detected brain lesions | [118] |

Abbreviations: ARR: annualized relapse rate; EDSS: expanded disability status scale; IFN-β-1a: interferon β-1a; MRI: magnetic resonance imaging; MS: multiple sclerosis; MSFC: multiple sclerosis functional composite; RRMS: relapsing-remitting multiple sclerosis; wk: weeks.

### 4.2. Fibers

The influence of dietary fibers as a supplementation strategy for MS has been a subject of several researches, and a detailed analysis of dietary fibers and their impact on the gut microbiome was recently published [124]. Dietary fibers, which are composed of both fermentable and non-fermentable fibers, are essential features of a vegetarian diet [125]. Earlier studies in mouse models of MS and other inflammatory diseases have mainly focused on fermentable dietary fibers. Through these studies, it has become clear that the fermentation of fibers by gut microbes increases the production of SCFAs, which have well documented beneficial properties for inflammatory and autoimmune disorders, including MS [58,59,126–128]. In this regard, some studies revealed their protective role in the EAE model, by promoting axonal remyelination and maintaining BBB integrity [57,129]. Moreover, SCFAs foster a healthy gut and determine positive systemic effects with a beneficial impact on neuroinflammation. As Hoffman and collaborators reviewed in 2022, an increasing number of studies indicate the potential of triggering SCFAs-specific responses in the context of inflammation [130]. For instance, SCFAs promote anti-inflammatory pathways that regulate the balance between Th17 and Treg responses [131]. The fermentable dietary fiber guar gum, as well, has been reported to induce increased SCFAs and significantly delay EAE [132]. A notable mention that might benefit from additional studies on GM is pomegranate peel extract, which contains dietary fibers (mainly pectins), polyphenols, vitamins, and minerals and was documented to determine increased *Lactobacillaceae* and reduced EAE severity [133].

Regarding dietary non-fermentable fibers intake, Berer *et al.* discovered that a cellulose-rich diet protected a EAE susceptible transgenic mouse strain from developing a spontaneous autoimmune CNS disease independent of SCFAs. Specifically, the authors found significant changes in the bacterial microbiota (an enrichment of *Ruminococcaceae*, *Helicobacteraceae*, and *Enterococcaceae*, and a reduction in *Sutterellaceae*, *Lactobacillaceae*, and *Coriobacteriaceae*) and in effector T cell responses, which mediate CNS autoimmunity. In particular, in the animals fed with a regular diet, the EAE disease incidence was 55 % compared to the 23 % incidence observed in animals fed with a cellulose-rich diet. Moreover, the neurological symptoms were significantly delayed of about 1 week in the cellulose-rich diet EAE group, although there was no significant difference in disease severity. Overall, despite the reduction in SCFAs-producing microbes and an increase in long-chain fatty acids, the cellulose-rich diet demonstrated beneficial effects [128].

All these studies support dietary fibers as positive modulators of EAE course, thus being potentially exploitable as therapeutic supplements for MS patients. Accordingly, a systematic review highlighted that a nutritional intervention with anti-inflammatory foods and dietary supplements, including fibers, decreases the biological synthesis of pro-inflammatory factors, which could be relevant to MS management [134]. As a whole, while the specific effects of dietary fibers as a supplementation for MS may require further investigation, the existing research suggests a potential link between dietary fiber supplementation and the modulation of autoimmune neurological diseases, including MS.

### 4.3. Mediterranean diet

It is widely reported that the mediterranean diet (MD) has protective effects against several conditions, including neurodegenerative diseases and chronic inflammatory disorders, by suppressing the expression of pro-inflammatory factors (TNF-α, IL-1, IL-6) and by reducing oxidative stress (OS) [135–137]. A high consumption of vegetables, nuts, fruits,

whole grain, and unsaturated fatty acids (mainly in the form of olive oil), a moderate intake of fish, poultry, eggs, and dairy products, and a limited use of saturated fatty acids, red wine, and red meat, are the major features of MD. Of note, most of these aliments contain antioxidant and anti-inflammatory nutrients. Indeed, olive oil contains bioactive polyphenols and tyrosol (an antioxidant derivative of phenethyl alcohol); fruits, vegetables, and whole grains are rich in fibers, folate, vitamins B, C, and E, and carotenoids; eggs contain tryptophan, vitamin B-12, folate, and choline; fish and vegetable oils are rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs) [11]. Several lines of evidence suggested the possible positive effects of MD on some MS-related symptoms; however, the studies are limited, and the results are often contradictory. For instance, although some works documented a reduced fatigue severity and EDSS score, as well as an improvement in quality of life and cognitive function in MS patients fed with MD [138–147], others did not report similar significant effects [148,149]. Concerning GM composition, very few studies have been conducted. EAE rats fed with extra-virgin oil show decreased levels of lipopolysaccharide, a Gram-negative bacterial endotoxin, suggesting a reduction of these bacteria, as well as a decrease in OS and an improvement in several clinical scores compared to non-treated EAE animals, although the authors did not examine the GM composition [150]. In this regard, the only clinical study that evaluated the potential impact of MD on GM composition was conducted in a Spanish cohort of MS patients [151]. Specifically, the authors observed increased levels of *Enterobacteriaceae*, *Akkermansia*, and *Blautia*, as well as a reduction in *Sutterella*, *Faecalibacterium*, *Prevotella*, *Anaerostipes*, and *Parabacteroides.* As some of these microbes are known to have anti-inflammatory or pro-inflammatory properties in a context dependent manner [152], further investigation is needed to better understand the mechanism through which MD positively shapes GM with a potential neuroprotective role.

### 4.4. Intermittent fasting

Several cultures have long practiced fasting, a habit that has recently gotten a lot of attention for its potential health benefits. Within this context, of particular interest is intermittent fasting (IF), which involves switching between periods of normal eating and extreme calorie restriction. When referring to IF, different protocols do exist, which are summarized as follows: time-restricted eating (eating within a range of hours each day), 5:2 (eating normally five days a week and cutting down to about 500 calories on the other two days), fasting-mimicking diet (FMD; a plant-based, low-calorie and low-protein 5-day lasting dietary intervention followed by a regular diet), alternate-day fasting (eating nothing one day and going back to the usual schedule the next one) and modified alternate-day fasting (alternating a normal calorie counting for one day and about 500 calories for the next day). In this general regard, researchers in the U.S. conducted a pilot study (*ClinicalTrials.gov*: NCT04389970) to investigate the feasibility and acceptability of a time-restricted meal intervention (8:16 protocol) in adults with RRMS. Following eight-week IF intervention, significant benefits were observed in RRMS patients who were seen to adhere to the eating plan. This study included 12 patients, with a mean age of 46 years, 83 % of whom were women. During the 8-week experiment, all participants were instructed to eat every day within an eight-hour window, and to fast the remaining 16 hours, drinking only water or black coffee. In general, the patients reported eating within the specified window for a mean of 6.8 days per week after four weeks, and 6.5 days per week at the end of the intervention. Throughout this period, the participants did not change their daily energy or nutritional intake. Considering that the exploratory results underlined a positive effect of IF on cognition, pain, and fatigue, it is worth performing future studies on large-scale in people with MS to better confirm this evidence [153].

To date, the only study performed on GM composition was conducted by Cignarella and coworkers in 2018. This work revealed an increase in *Lactobacillaceae*, *Bacterioidaceae*, and *Prevotellaceae* bacteria families and a decrease in microbes belonging to the *Bifidobacterium* genus in intermittent fasting EAE. Moreover, in a pilot clinical trial involving 17 RRMS patients, the same authors found that a modified fasting regimen induced changes reminiscent of those seen in rodent EAE models, including similar, possibly beneficial, alterations in the GM [154]. Besides these results, they also described amelioration of EAE clinical course and severity in the IF animal group, as well as less inflammatory cell infiltration, demyelination, and production of pathogenic cytokines (IL-17A, IFN-γ, and GM-CSF) [154].

Another aspect that is also important to assess is the quality of life in MS patients. In this regard, in the NCT01538355 clinical trial (*ClinicalTrials.gov*), the authors, besides suggesting that FMD is safe and feasible, observed potentially positive effects of FMD treatment in RRMS, based on changes in self-reported health-related quality of life, together with a mild improvement in EDSS [155]. Furthermore, some preclinical studies have documented that IF was able to delay the onset and to slow disease progression, as well as to increase autoreactive lymphocyte apoptosis and oligodendrocyte regeneration [155]. All this evidence suggests the potential immunomodulatory and protective effects of IF, however, since the clinical studies are limited, other works are mandatory.

### 4.5. Isoflavone diet

Isoflavones, polyphenolic compounds mainly present in legumes, including soy and beans, belong to phytoestrogens [156]. Some studies reported that a diet rich in isoflavones may reduce EAE clinical course, as well as the infiltration of pro-inflammatory immune cells into the CNS after EAE induction by boosting the abundance of specific isoflavone-metabolizing gut microbes (*Adlercreutzia equolifaciens* and *Parabacteroides distasonis*) that produce anti-inflammatory molecules [156,157]. Indeed, a research published in the *Science Advances* Journal showed, in a mouse model of MS, the protective role of an isoflavone diet against disease severity and that this protection was dependent on the presence of isoflavone-metabolizing bacteria and their metabolite S-equol [156]. The study also underlines the differences in GM composition between the mice fed with and without an isoflavone diet. In detail, EAE mice fed with an isoflavone-rich diet manifested increased levels of the anti-inflammatory isoflavone-metabolizing bacteria *A. equolifaciens* and *P. distasonis*, normally reduced in MS patients, while mice fed with an isoflavone-free diet exhibited increased abundance of *Akkermansia muciniphila* [156]. Moreover, the authors reported that the isoflavone dietary content can lead to a decrease in the severity of spinal cord pathology, a reduction in the number of inflammatory cells in the CNS, as well as a lower frequency of $CD4^+$T cells after immunization. These findings suggest that isoflavone-rich dietary-induced gut microbial changes may have a key role in alleviating MS symptoms [156].

### 4.6. High vegetable/low protein diet

It has been claimed that a diet high in vegetables and low in proteins is able to improve MS clinical symptoms. However, to date, only one study has been performed. This clinical pilot study, comparing 10 MS patients under a high vegetable/low protein (HV/LP) diet and 10 MS patients under a typical "Western diet" for one year, found promising results in favor of the HV/LP diet [158]. In detail, this study revealed that the HV/LP group showed a decrease in the pro-inflammatory $IL\text{-}17^+/CD4^+$ as well as in $CD4^+/PD\text{-}1^+$ T cells, an increase in the anti-inflammatory $CD14^+/PD\text{-}L1^+$ monocytes, and an improvement in some clinical parameters (i.e., disease severity and relapse). Regarding GM composition, the families of *Lachnospiraceae*, *Coprococcus*, *Ruminococcus*, and *Roseburia* were noticed to be more abundant in the HV/LP group compared to the western diet group. Moreover, within the HV/LP group only, *Lachnospiraceae* abundance was positively correlated with the anti-inflammatory IL-10 and TGFβ-producing $CD14^+$ monocytes, as well as with $CD4^+/CD25^+/$ $FoxP3^+$ T lymphocytes [158].

## 5. Probiotics

The Food and Agriculture Organization and the World Health Organization define probiotics as "live microbial dietary supplements that, if administered in adequate amounts, confer health benefits to the host" [159]. Probiotics have antimicrobial and anti-inflammatory properties and they reduce intestinal permeability, so they can contribute to improve the homeostasis of the intestinal microbiota and gastrointestinal symptoms [160]. The intake of probiotics is recommended to counter intestinal diseases such as inflammatory bowel disease, antibiotic-associated diarrhea, celiac disease, but also some cardiovascular diseases, obesity, diabetes, non-alcoholic fatty liver disease, and *Helicobacter pylori* infection [161–165]. Moreover, it has recently been observed the beneficial effect of probiotics intake in psychiatric and neurodegenerative disorders, such as AD, and PD [9,10,20,166].

Regarding MS a change in the GM was seen in MS patients, including an increase in *Akkermansia muciniphila* and *Streptococcus* and a decrease in butyrate-producing bacteria and various genera mainly belonging to *Firmicutes* or *Bacteroidetes* [40,167]. Some preclinical and clinical studies have shown that probiotics, such as *Escherichia coli, Lactobacillus plantarum,* and *Bifidobacterium pseudocatenulatum,* are adjuvants against the onset and progression of neurodegenerative disorders due to their anti-inflammatory and antioxidant properties [168]. Moreover, it has been reported the involvement of probiotics intake in GM restoration, preservation of the gut barrier integrity, regulation of the immune cell homeostasis, and reduction of the chronic inflammation [169]. Probiotics also affect the immune system by inducing an increase of anti-inflammatory cytokines and Tregs and a reduction of pro-inflammatory cytokines. Therefore, these features could make them a valuable aid against inflammatory and autoimmune diseases such as MS [169] (Table 3) (Fig. 3) [133,170–182].

### 5.1. Preclinical studies

The interest in probiotics in the context of MS has increased in recent years. In the most widely used animal models of MS in preclinical settings, drastic microbial changes have been observed as a function of disease progression, which, however, do not perfectly reflect changes in the microbiota of MS patients. Therefore, the valuable information provided by animal models must be supported, in parallel, by data obtained from patients. Preclinical studies have been conducted mainly in the EAE model and in the cuprizone (CPZ) model [35]. The EAE model is the most widely used animal model of MS as it well reproduces the complex inflammatory condition and immune mechanisms underlying the pathology. Experiments involving the administration of probiotics to EAE animals have yielded interesting results, especially in the control of the inflammatory cascade. Beneficial effects were produced by probiotics administered before immunization regardless of the duration of treatment. In a research conducted by Kwon *et al.* in female C57BL/6 mice, it was seen that the oral treatment with IRT5 probiotics (*L. casei, L. acidophilus, L. reuteri, B. bifidum, Streptococcus thermophilus*), for three weeks before EAE induction and continued until the end of the experiment, reduced the EAE incidence and the clinical symptoms by decreasing MOG-reactive T-cell proliferation and pro-inflammatory cytokine levels, while increasing the expression of the anti-inflammatory cytokine IL-10 and $Foxp3^+$ (a transcriptional regulator that, by binding to promoter sequences of genes involved in the development and regulation of T-cell receptors, attenuates the immune response) [170]. Moreover, Samani *et al.* investigated the efficacy of *Enterococcus durans* and Lacto-mix (*L. rhamnosus, L. casei* and *L. plantarum*) in preventing EAE. The animals were treated for two weeks before immunization and throughout the duration of the experimental model. The obtained results show a tissue-level improvement in inflammation and demyelination [171]. Another work documented that the daily treatment, started seven days before immunization, with *Escherichia coli Nissle 1917* (ECN®) reduced the severity of EAE induced by immunization with $MOG_{35–55}$ peptide. This positive effect was associated with a decrease in inflammatory cytokines and with an increased production of the anti-inflammatory cytokine IL-10 by CD4+ T cells, both in peripheral lymph nodes and in CNS [172].

Favorable results were also obtained by starting probiotics treatment on the day of immunization. Indeed, in the study conducted by Salehipour *et al.*, unlike the others, the administration of the probiotics coincided with the induction of EAE. Specifically, it was shown that an oral treatment with a mixture of *L. plantarum* and *B. animalis* for 22 days from immunization significantly delayed disease onset and decreased clinical score along with inducing CD4+, CD25+ and $Foxp3^+$ regulatory T cells. That resulted in a reduction of MOG-specific pro-inflammatory cytokines (IFN-γ, IL-17, IL-6) and in an increase in the production of MOG-specific anti-inflammatory cytokines and transcription factors (IL-4, IL-10, TGF-β and $FoxP3^+$) in the lymph nodes and spleen [173]. In a research conducted by Calvo-Barreiro *et al.* a commercial multispecies probiotic was instead administered at the onset of typical EAE symptoms. The probiotic mixture in question (Lactibiane iki®) is composed of *B. lactis* LA 304, *L. acidophilus* LA 201, and *L. salivarius* LA 302. The clinical improvement, besides being dose-dependent, is related to the inhibition of pro-inflammatory mechanisms, stimulation of immunoregulators and decreased demyelination. Furthermore, a modulation of the number and phenotype of dendritic cells was observed [174].

The model of CPZ, a copper chelator, is a purely animal model of demyelination and this makes it interesting to observe if the administration of probiotics and, therefore the modulation of the microbiota, has an impact in the processes of demyelination and remyelination. In a study published in 2020, the oral administration of *L. casei* was shown to improve motor symptoms and decrease the levels of IL-17, IFN-γ, and indoleamine 2,3-dioxygenase (IDO-1) enzyme expression. IDO-1, which is altered in the CPZ model, is induced during inflammatory processes and catalyzes the transformation of tryptophan into kynurenine, which is involved in the production of neuroactive metabolites whose altered balance may cause neurodegeneration. In this research, it was also observed that preventive administration (*L. casei* for 1 month and then cuprizone for 1 month) or therapeutic administration (cuprizone for 1 month and then *L. casei* for 1 month) show no difference. Further, the co-administration of *L. casei* and Vit D significantly increased TGF-β levels and decreased IL-17 expression in contrast to *L. casei* treatment alone, suggesting the potential role of Vit D in the anti-inflammatory process [175,183]. Similar results were obtained in the research conducted by Sadeghirashed *et al.* in which the oral administration of *B. coagulans* IBRC-M10791 in C57BL/6 mice ameliorated CPZ-induced demyelination, decreased IFN-γ and IL-17, and modulated serum IL-4 and TGF-β. As seen in the above study, also *B. coagulans* inhibits IDO-1 enzyme expression [176]. In a later study carried out by Hasaniani *et al.*, *L. casei* and *B. breve* were administered orally alone or in combination throughout the duration of the investigation. The results show an improvement in demyelination in the *corpus callosum* and in OS; it is interesting to point out that *B. breve* supplementation alone works better than *L. casei* alone or the combination of the two probiotics [177].

### 5.2. Clinical studies

Although animal studies are promising, currently, clinical studies regarding the administration of probiotics conducted in patients with MS are limited. In this regard, the administration of a probiotic mixture containing *Lactobacillus, Bifidobacterium*, and *Streptococcus* twice daily for two months in patients with RRMS induced an anti-inflammatory peripheral immune response characterized by a decrease in the amount of inflammatory monocytes [178].

In addition, Rahimlou *et al.* conducted a clinical trial in which the efficacy of a supplementation of multi-strain probiotic capsules (*L. acidophilus, L. casei, L. rhamnosus, L. bulgaricus, L. helveticus, L. plantarum, L. salivarius, L. lactis, B. breve, B. longum, B. bifidum, Streptococcus thermophilus* and *Bacillus subtilis*) for six months was analyzed. The study

**Table 3**
Preclinical and clinical studies on the effects of probiotics, prebiotics, and synbiotics treatment.

| ***PRECLINICAL STUDIES: PROBIOTICS*** | | | | | |
|---|---|---|---|---|---|
| **Probiotic** | **Experimental model** | **Animal species** | **Treatment duration** | **Treatment effects** | **Reference** |
| IRT5 (*Lactobacillus casei, L. acidophilus, L. reuteri, B. bifidum, Streptococcus thermophilus*) | $MOG_{35-55}$-induced EAE | Mouse C57BL/6 female mice (6–8 weeks old), n =10 per group | 3 weeks before immunization and until the end of the experiment (3 weeks + 1 month) | Improvement in neurological symptoms and prevention of the pathogenesis of EAE. Modulation of inflammation (↑ IL−10, ↑ FoxP3 T-reg). | [170] |
| *Enterococcus durans* and Lacto-mix (*L. rhamnosus, L. casei* and *L. plantarum*) | $MOG_{35-55}$-induced EAE | Mouse C57BL/6 female (8–10 weeks old), n =6 per group | 2 weeks before immunization and until the end of the experiment (2 weeks+1 month) | Modulation of inflammation (↓ IL−17). Effect on demyelination (↓ MMP9 in the SC and brain, ↑ MBP in the brain). | [171] |
| *Escherichia coli Nissle 1917* (ECN®) | $MOG_{35-55}$-induced EAE | Mouse C57BL/6 female (8–12 weeks old), n=40 per group | Daily treatment, started 7 days before immunizationand until the end of the experiment (7 days+ 1 month) | Development of less severe EAE. Decrease of pro-inflammatory cytokines and increase of anti-inflammatory cytokine IL−10 by autoreactive $CD4^+$ T cells, both in peripheral lymphnodes and in the CNS. Repair of impaired intestinal permeability dysfunction in EAE. | [172] |
| *L. plantarum* and *Bifidobacterium animalis* | $MOG_{35-55}$-induced EAE | Mouse C57BL/6 female mice (8–10 weeks old), n=8 per group | Daily treatment, started the same day of immunization and until the end of the experiment (22 days) | Delayed time to disease onset and clinical score and decrease of demyelination in the SC. Stimulation of $CD4^+$ T cell polarization toward regulatory T cells through induction of anti-inflammatory cytokines and transcription factors (IL−4, IL−10, TGF-β, GATA3 and FoxP3) and inhibition of pro-inflammatory cytokines (IFN-γ, IL−17, IL−6, T-bet and ROR-γt). Suppression of autoreactive T-cell proliferation and leukocyte infiltration into the CNS. | [173] |
| Lactibiane iki® (*B. lactis* LA 304, *L. acidophilus* LA 201, and *L. salivarius* LA 302) | $MOG_{35-55}$-induced EAE | Mouse C57BL/6 female mice (8 weeks old), n=18–20 per group | Daily treatment started 13–16 days postimmunization until the end of the experiment (34-day post immunization) | Improvement in clinical score and motor skills. Modulation of dendritic cell number and phenotype. Decrease in demyelination and CD3+ inflammatory infiltrating cells. Decrease in axonal damage (↓ % SMI−32). | [174] |
| *L. casei* | Cuprizone model | Mouse C57BL/6 female (8–10 weeks old) | Treatment 1 (*L. casei* for 4 weeks then cuprizone for 4 weeks); Treatment 2 (cuprizone for 4 weeks then *L. casei* for 4weeks); Treatment 3 (cuprizone for 4 weeks then *L. casei* for 4 weeks with vitamin D3 20 IU/day) | Improvement of CPZ-induced motor dysfunction in mice. Decreased IL−17 and increased TGF-β concentrations. Decreased *IDO−1* gene expression. Decreased expression of miR−155 (increased by CPZ) and increased expression of miR−25. | [175] |
| *B. Coagulans* IBRC-M10791 | Cuprizone model | C57BL/6 female (7–8 weeks old), n=7 per group | Probiotic control group (oral administration of *B. coagulans* for 4 weeks); Prevention group (oral administration of *B. coagulans* for 2 weeks and then cuprizone for 4 weeks); Improvement group I (oral administration of cuprizone for 4 weeks and then *B. coagulans* for 4 weeks); Improvement group II (oral administration of | Decreased expression of inflammatory cytokines (IL−4, IL−17, TGF-β, IFN-γ). Decreased gene expression of Cyp27b1, NLRP1, AIM2, NLRP3, IDO−1. Increased remyelination, improved spatial cognition. | [176] |

(*continued on next page*)

**Table 3** (*continued*)

| | | | | | |
|---|---|---|---|---|---|
| ***PRECLINICAL STUDIES: PROBIOTICS*** | | | | | |
| | | | cuprizone for 4 weeks then *B. coagulans* and vitamin D 20 IU/ day for 4 weeks) | | |
| *L. casei* and *B. breve* | Cuprizone model | Wistar rat male (5–6 weeks old), n=8 per group | *L. casei* group (administration of CPZ for 28 days and then treatment with *L. casei* daily for 4 weeks); *B. breve* group (CPZ administration for 28 days and then *B. breve* treatment every day for 4 weeks); *L. casei* + *B. breve* group (CPZ administration for 28 days and then treatment with combination *L. casei* and *B. breve* every day for 4 weeks) | Improvement of antioxidant activity (↑ HO−1, Nrf2) and demyelination of *corpus callosum* (↑ Olig2, MBP, PDGFRα). Increased expression of BDNF. | [177] |
| ***CLINICAL STUDIES: PROBIOTICS*** | | | | | |
| **Probiotic** | **Experimental model** | **Treatment duration** | | **Treatment effects** | **Reference** |
| *Lactobacillus, Bifidobacterium*, and *Streptococcus* | RRMS patients (n=9) and controls (n=13) | 8 weeks | | Increase of impoverished taxa in MS. Depletion of taxa associated with dysbiosis in MS. Decreased expression of MS risk allele HLA.DPB1 and HLA. DPA1. Peripheral anti-inflammatory immune response (decrease in the frequency of intermediate monocytes, decrease in MFI of the CD80 marker on monocytes). | [178] |
| Multi-strain probiotic capsules (*L. acidophilus, L. casei, L. rhamnosus, L. bulgaricus, L. helveticus, L. plantarum, L. salivarius, L. lactis, B. breve, B.longum, B. bifidum, Streptococcus thermophilus* and *Bacillus subtilis*) | RRMS patients (n=70, 35 placebo, 35 probiotic supplement) | 6 months | | A significant increase in BDNF and a significant reduction in the IL−6 levels. A significant improvement in GHQ−28, BDI-II, FSS, PRI. | [180] |
| ***PRECLINICAL STUDIES: PREBIOTICS*** | | | | | |
| **Prebiotic** | **Experimental model** | **Animal species** | **Treatment duration** | **Treatment effects** | **Reference** |
| Pomegranate peel extract (PPE) | $MOG_{35–55}$-induced EAE | Mouse C57BL/6 female (~ 8 weeks old), n=8 per group | Day 0 post-immunization (p. i.), day 10 p.i. and day 16 p.i. | Improvement of clinical disease score. Reduction of peripheral inflammation and pro-inflammatory cytokines production (IL−17, GM-CSF, IFN-γ, CD11b). Improvement in demyelination (↑ MBP in the SC). FMT from PPE mice to naïve recipients ameliorates EAE clinical course. | [133] |
| *Palmaria palmata* aqueous exctract (*Palmaria p.)* | Cuprizone model | Swiss mice (8 ± 2 weeks), n=11 males and 11 females per group | Daily for 7 weeks (5 weeks CPZ-*Palmaria p.* + 2 weeks *Palmaria p.*) | Modulation of the microbiota composition (↑ *Lactobacillus*, *Proteobacteria*, and *Bactriodia*). Improvement of damage in the *corpus callosum*. Reduction of CPZ-induced anxiety. | [181] |
| ***CLINICAL STUDIES: SYNBIOTICS*** | | | | | |
| **Synbiotic** | **Experimental model** | **Treatment duration** | | **Treatment effects** | **Reference** |
| *L. casei, L. acidophilus, L. plantarum, L. bulgaricus, B. breve, B. infantis, B. longum,* and *Streptococcus thermophilus*, with concentrated FOS | Progressive MS patients (PPMS, SPMS, and PRMS) with an EDSS score greater than 2 (n=70) | Daily consumption of synbiotics capsules anti-inflammatory-antioxidant-rich diet for 4 months | | Improvements in fatigue severity, pain intensity, bladder and bowel control, and sexual function. | [182] |

Abbreviations: AIM2: interferon-inducible protein 2 (also known as absent in melanoma 2); BDI-II: beck depression inventory-II; BDNF: brain-derived neurotrophic factor; CNS: central nervous system; CPZ: cuprizone; Cyp27b1: cytochrome P450 family 27 subfamily B member 1; EAE: experimental autoimmune encephalomyelitis; EDSS: extended disability status scale; FMT: faecal microbiota transplantation; FOS: fructo-oligosaccharide; FoxP3: forkhead box P3; FSS: fatigue severity scale; GHQ-28: general health questionnaire-28; GM-CSF: granulocyte-macrophage colony-stimulating factor; HLA.DPB1 and HLA.DPA1: human leukocyte antigen (HLA) alleles (DPA1, DPB1); HO-1: heme oxygenase 1; IDO-1: indoleamine 2,3-dioxygenase; IFN-γ: interferon gamma; IL-4: interleukin 4; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; IL-17: interleukin 17; MBP: myelin basic protein; MFI: mean fluorescence intensity; miR-25: microRNA 25; miR-155: microRNA 155; MMP9: matrix metalloproteinase-9; MOG: myelin oligodendrocyte glycoprotein; MS: multiple sclerosis; NLRP1: NLR family pyrin domain containing 1; NLRP3: NLR family pyrin

domain containing 3; Nrf2: nuclear factor erythroid 2-related factor 2; Olig-2: oligodendrocyte transcription factor 2; PBMCs: peripheral blood mononuclear cell; PDGFRα: platelet-derived growth factor receptor A; PPMS: primary-progressive multiple sclerosis; PRI: pain rating index; PRMS: progressive-relapsing multiple sclerosis; SC: spinal cord; SMI-32: antibody which recognizes an unphosphorylated epitope of NF (neurofilament)-M and NF-H subunits; SPMS: secondary-progressive multiple sclerosis; T-bet: T-box transcription factor TBX21; TGF-β: transforming growth factor beta; TNF-α: tumor necrosis factor; ↑ increase; ↓ decrease.

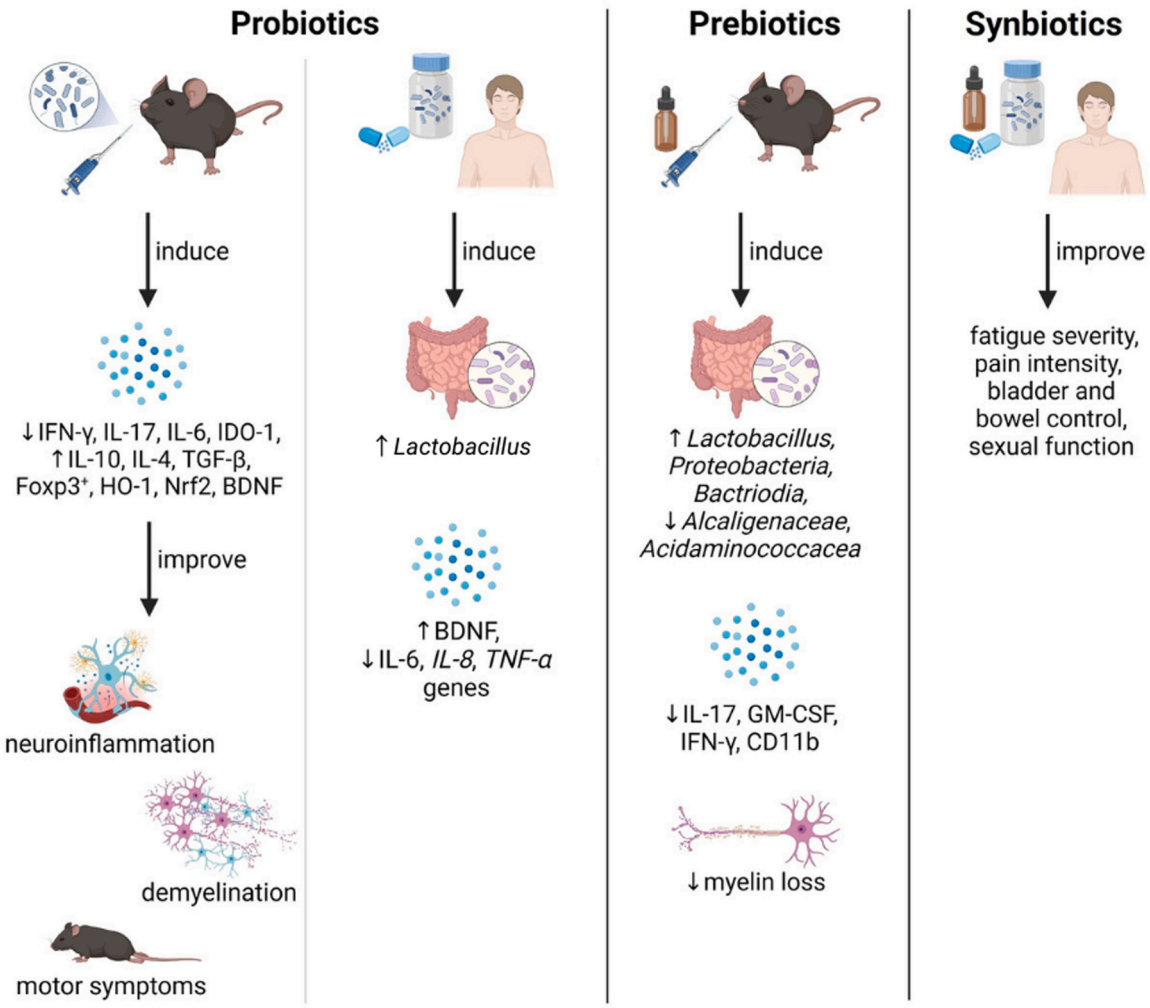


**Fig. 3.** Probiotics, prebiotics, and synbiotics administration as a potentially beneficial supplemental strategy against multiple sclerosis. **Probiotics.** Preclinical studies performed by using the EAE and CPZ models revealed that probiotics administration reduces several pro-inflammatory cytokines (including IFN-γ, IL-17, and IL-6), and the enzyme IDO-1, as well as increases numerous anti-inflammatory molecules (including IL-10, IL-4, TGF-β), FoxP3$^{+}$, the transcription factor Nrf2, BDNF, and the antioxidant enzyme HO-1, thus improving neuroinflammation, demyelination, and motor symptoms; clinical studies underscore the reshape in GM composition (increase in *Lactobacillus*, and decrease in *Akkermansia* and *Blautia*), as well as the reduction in IL-6 levels, and *IL-8* and *TNF-α* genes expression, and the increase in BDNF levels after probiotics intake. **Prebiotics.** Preclinical studies showed GM changes (increase in *Lactobacillus*, *Proteobacteria*, and *Bactriodia*, and reduction in *Alcaligenaceae* and *Acidaminococcacea*), decrease in several proteins (including IL-17, GM-CSF, IFN-γ, and CD11b), and myelin loss inhibition after prebiotics administration. **Synbiotics**. Clinical studies revealed improved fatigue severity, pain intensity, bladder and bowel control, and sexual function in MS patients treated with synbiotics. Abbreviations: BDNF: brain-derived neurotrophic factor; CPZ: cuprizone; EAE: experimental autoimmune encephalomyelitis; FoxP3: forkhead box P3; GM-CSF: granulocyte-macrophage colony-stimulating factor; HO-1: heme oxygenase 1; IDO-1: indoleamine 2,3-dioxygenase; IFN-γ: inteferon gamma; IL-4: interleukin 4; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; IL-17: interleukin 17; Nrf2: nuclear factor erythroid 2-related factor 2; TGF-β: transforming growth factor beta; TNF-α: tumor necrosis factor; ↑: increase; ↓decrease. (Created with BioRender.com).

involved 70 MS patients with an average age of around 40 years, and the findings showed a decrease in the levels of the pro-inflammatory cytokine IL-6. Interestingly, the study documented that the probiotic supplementation increased the levels of brain-derived neurotrophic factor (BDNF), thus indicating a potential neuroprotective function. However, to validate this hypothesis, it is necessary to perform controlled clinical studies [180].

## 6. Prebiotics

Prebiotics are "substrates that are selectively utilized by host microorganisms conferring a health benefit" [184]. To be defined as a prebiotic, a substance must be resistant to digestion in the upper tracts of the gut, must be fermented by the GM, and must stimulate the growth and activity of bacteria associated with a state of health and well-being [185].

Prebiotics are mainly divided into two categories: fructans, to which inulin and fructo-oligosaccharides (FOS) belong, and galacto-oligosaccharides (GOS), which are instead divided into two subcategories: GOS with excess galactose in position C3, C4 or C6 and GOS produced from lactose through enzymatic trans-glycosylation. They can be produced synthetically or also be found naturally in certain foods

such as wheat, barley, rye, honey, banana, tomato, soybean, human and cow's milk, peas, beans, algae, and microalgae [186].

Preclinical and clinical data have shown the beneficial effect of prebiotics in the CNS by improving neuroinflammation and modulating cognitive impairment, anxiety, and depression [187,188]. The studies concerning the effect of prebiotics in MS are still limited but the interesting results obtained, especially in preclinical analyses, in other neurodegenerative pathologies such as PD and AD are promising indications also for MS. In fact, in these studies an improvement of the systemic and central inflammation has been observed that, as already described above, are typical features of MS [10,189,190].

Prebiotics sustain the GM, and their degradation products are SCFAs, which are released into the bloodstream reaching not only the gastrointestinal tract but also other distant organs. SCFAs, including acetate, propionate, and butyrate, constitute 90–95 % of all microbiota metabolites produced in the colon [191]. SCFAs have been shown to reduce OS and inflammation, although studies in this regard are still few and mostly limited to butyrate [192]. Just as described above for probiotics, SCFAs also increase the expression of FoxP3$^{+}$ and the anti-inflammatory IL-10 [193].

As mentioned, MS is characterized by damage to the BBB caused by sustained systemic inflammation. The influx of immune cells and pro-inflammatory factors activates microglia in the brain, further stimulating neuroinflammation and neurodegeneration [194,195]. SCFAs have been seen to counteract the impaired BBB by restoring junctional complex proteins, affecting their transcription, intercellular localization, or proteolytic degradation [196]. In a recent study, pomegranate peel extract, a potential prebiotic dietary supplement, has been reported to inhibit the infiltration of peripheral inflammatory cells into CNS, myelin loss, and to modulate the GM, thus resulting in improvements of EAE symptoms [133]. In a later study in male and female Swiss mice, *Palmaria palmata* aqueous exctract (*Palmaria p.)* was shown to have a good potential in protecting against CPZ-induced MS. In this study, a modulation of the microbiota composition was seen with an increase in *Lactobacillus, Proteobacteria,* and *Bactriodia* communities. Furthermore, an improvement of damage in the *corpus callosum* and prevention of CPZ-induced anxiety was observed [181].

Currently, studies are ongoing in which the use of polysaccharides and the fibers contained in the *Aloe vera* and *Citrus bergamia* plants may have a remarkable effect as prebiotics [197] (Fig. 3).

## 7. Synbiotics

Synbiotics are a mixture consisting of autochthonous (probiotics) or allochthonous live microorganisms and substrates used selectively by host microorganisms to confer a health benefit to the host. Synbiotics are divided into two groups: synergistic synbiotics and complementary synbiotics. In synergistic synbiotics, the substrate is selectively utilized by the co-administered live microorganisms. The latter are chosen on the basis of their ability to provide a health benefit, and the substrate, although it may be utilized by other microorganisms within the microbiota, is chosen to promote the growth and activity of the ingested microorganisms. Complementary synbiotics, on the other hand, consist of probiotics and prebiotics and are designed to target autochthonous microorganisms [198,199]. As observed for probiotics and prebiotics, most of the studies found in the literature are focused on neurodegenerative diseases such as PD and AD. The results published in these studies show a decrease in peripheral inflammation and OS, and an improvement in cognitive deficits, suggesting an enhanced effect due to the combined components [189,200,201]. In a clinical trial by Moravejolahkami *et al.* on patients with various progressive forms of MS, a synbiotic was administered along with a diet rich in antioxidants and anti-inflammatory compounds. The synbiotic supplement consisted of *L. casei, L. acidophilus, L. plantarum, L. bulgaricus, B. breve, B. infantis, B. longum,* and *Streptococcus thermophilus*, together with a FOS. The findings after four months of treatment showed improvements in fatigue, pain, sexual function, and bowel/bladder status in patients with progressive MS. Nevertheless, this study has some limitations due to the absence of neuroimaging and analysis on serum biochemical markers [182] (Fig. 3).

## 8. Postbiotics

Postbiotics are intact non-viable microbes or cell fragments, with or without metabolites that contribute to demonstrated health benefits [202].

The official definition of Postbiotic is "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" and it was established in 2019 by the International Scientific Association for Probiotics and Prebiotics (ISAPP) following various debates [203].

Postbiotics are considered the evolution of probiotics thanks to increased safety, especially in the most vulnerable or immunodepressed patients and infants, long shelf life, and the opportunity to be easily standardized [204]. Unlike probiotics, postbiotics do not require living cells to induce health effects and so they are not subject to the food safety requirements that apply to live microorganisms [205].

Postbiotics are mainly classified as metabolites generated by the microbiota such as SCFAs, exopolysaccharides, cell wall fragments, enzymes/proteins/amino acids, and other metabolites (organic acids, vitamins, etc…) [203,206]. Until now, the most studied postbiotics in MS are SCFAs. SCFAs, including propionic acid, butyric acid, acetic acid, and pentanoic acid, are the product of the digestion of prebiotics by bacteria that, in this way, extract energy through fermentation of carbohydrates that cannot be digested by human enzymes [207] (Table 4) [59,129,208–213].

Some studies, analyzed via systematic search in PubMed in 2018, show that SCFAs are able to counteract EAE disease progression and suppress inflammation [214]. In line with these findings, the treatment of mice with pentanoate ameliorated EAE severity and reduced the number of infiltrating CD4+ and CD8+ T cells in the CNS. Pentanoate-treated mice also showed a decrease in IL-17 and an increase in IL-10 expression in effector cells [208]. On the other hand, a paper published in 2022 demonstrated a different activity of acetate depending on theadministration time/condition. Indeed, when acetate was administered together with LPS it increased the production of pro-inflammatory cytokines at the gene and protein levels (particularly TNF-α) in primary microglia cell lines and cultures; in contrast, when acetate was administered alone or as pretreatment, before stimulation with LPS, it had an anti-inflammatory effect. In addition, the *in vivo* administration of acetate during the early phase of EAE resulted in improved disease outcomes and reduced TNF-α production [209].

The studies on butyrate are extensive due to its ability to regulate inflammation not only peripherally, but also centrally given its capability to cross the BBB [215]. The administration of butyrate or methyl butyrate in EAE mice produced interesting results by reducing clinical and pathological symptoms, inflammation, and demyelination in the CNS. In particular, methyl butyrate also increased Treg and IL-10 levels [210,211]. In the *in vivo* model of CPZ-induced demyelination, the oral administration of SCFAs, especially butyrate, decreased demyelination and improved remyelination in the *corpus callosum* compared to animals treated with CPZ alone [129].

Concerning propionate, it was already tested in 2015 in the EAE model by Haghikia *et al.* that observed an increased frequency of Treg cells in the small intestine. Furthermore, the transplantation of these cells, still in EAE mice, improved the clinical score and axonal damage, demonstrating a systemic effect of gut-associated T-cell responses [59]. Later studies by Haase *et al.* further demonstrated the potential of propionate in counteracting neuroinflammation in EAE mice fed with a high-fat diet. Indeed, the treatment with propionate alleviated the clinical signs, bringing the course of the disease in line with the control group. In addition, a decrease in the expression of Th17 lymphocytes

**Table 4**
Preclinical and clinical studies on the effects of postbiotics treatment.

***PRECLINICAL STUDIES: POSTBIOTICS***

| Postbiotic | Experimental model | Animal species | Treatment duration | Treatment effects | Reference |
|---|---|---|---|---|---|
| Sodium pentanoate | $MOG_{37-50}$-induced EAE | FIR × tiger mice (8–12 weeks old), n =6 per group | Daily treatment for 4 weeks. | Improvement in EAE severity. Modulation of inflammation (↑ IL−10, ↓ IL−17). Reduction of infiltrating $CD4^+$ and $CD8^+$ T cells in the CNS. | [208] |
| Acetate | $MOG_{35-55}$-induced EAE | C57BL/6 female mice (6–8 weeks old), n =5 per group | Treatment for 8 days (−1 to day 7) before the appearance of the first symptoms | Delay in the development of EAE. TNF-α production reduction. | [209] |
| Sodium butyrate | $MOG_{35-55}$-induced EAE | C57BL/6JOlaHsd female mice (8 weeks old), n=8 per group | Once daily from 12–15 days post-immunization (dpi) to the end of the experiment (28 dpi) | Improvement of the clinical signs and reduction of CNS inflammation (↓ CD3), astrogliosis (↓ $\%GFAP^+$), and demyelination (↑ MBP in the SC). Decrease of axonal damage (↓ $\%SMI32^+$). | [210] |
| Methyl butyrate | $MOG_{35-55}$-induced EAE | C57BL/6JOlaHsd female mice (8 weeks old), n=5 per group | Daily treatment from day 0 of immunization until the end of the experiment | Improvement of the clinical signs and reduction of CNS demyelination and inflammation (↑IL−10 secretion in peripheral immune organs, ↓ IL−6). | [211] |
| Butyrate, acetate and propionate | Cuprizone model | C57BL/6 J (B6) male mice (6 weeks old) n =8 per group | Treatment for 1 week before the start of the cuprizone diet | Butyrate suppresses cuprizone-induced demyelination. Microglia are not affected by oral treatment with SCFAs. | [129] |
| Propionate | $MOG_{35-55}$-induced EAE | C57BL/6 J (B6) female mice (8–11 weeks old) n =15–29 per group | Daily treatment at either the day of immunization (DI) or at the onset of disease (OD) | Only immunized mice: improvement of clinical signs and axonal density, reduction of CNS demyelination and inflammation (↑IL−10). | [59] |
| Propionic acid and lauric acid | $MOG_{35-55}$-induced EAE | C57BL/6 J (B6) male and female mice (10–12 weeks old) n =11–12 per group | Lauric acid from 4 weeks before EAE induction and propionic acid (PA) from the day of immunization | Improvement of the clinical signs and axonal density, reduction of CNS demyelination and inflammation. Reduced infiltration of macrophages (Mac−3) and CD3-positive T cells. | [212] |

***CLINICAL STUDIES: POSTBIOTICS***

| Postbiotic | Experimental model | Treatment duration | Treatment effects | Reference |
|---|---|---|---|---|
| Sodium propionate | Initial MS cohort n=268 Healthy controls= 68 *De novo* MS cohort=36 | 500 mg sodium propionate capsules twice daily for 14 days | ↑ subcortical grey matter, ↓ annual relapse rate, ↓ disease progression, ↑ IL−10 production, ↑ Treg number and function, ↓ Th17 number. | [213] |

Abbreviations: CNS: central nervous system; EAE: experimental autoimmune encephalomyelitis; GFAP: glial fibrillary acidic protein; IL-6: interleukin 6; IL-10: interleukin 10; IL-17: interleukin 17; MBP: myelin basic protein; MOG: myelin oligodendrocyte glycoprotein; MS: multiple sclerosis; SC: spinal cord; SCFA: short-chain fatty acid; SMI-32: antibody which recognizes an unphosphorylated epitope of NF (neurofilament)-M and NF-H subunits; TNF-α: tumor necrosis factor α; ↑ increase; ↓ decrease.

and an increase of Tregs was also observed in this study [212].

The clinical studies regarding the administration of SCFAs in MS patients are still very limited although, on the other hand, there are many studies that analyzed the difference in SCFAs levels between MS patients and healthy patients [215]. In this regard, numerous studies attest to a reduction of SCFAs in the stool samples of MS patients, thus indicating their potential protective role in the disease [47,151,213,216,217]. In plasma and serum, several groups also observed a reduction of SCFAs in MS patients [48,218–220]. However, some research has produced opposite results, such as in the clinical study conducted by Perez *et al.* in patients with RRMS and the one carried out by Cuello *et al.* in patients with MS during pregnancy or *post partum*. In both works, an increase in plasma acetate levels was found. Furthermore, in the research carried out by Cuello *et al.*, it was observed that a low propionate/acetate ratio during the first trimester of pregnancy leads to an increased risk of relapses during pregnancy and *post partum* [221,222]. In a recent study by Duscha *et al.*, two-week administration of propionic acid in MS patients showed an increase in Treg and a decrease in Th1 and Th17 cells. In addition, retrospective analyses revealed a reduction in relapses, stabilization of disability, and a reduction in brain atrophy after 3 years of propionic acid intake [213].

Additional studies on SCFAs and on the effect of their supplementaion will help to further define their mechanisms of action and their possible rolein counteracting the development and progression of MS.

Within this context, the essential amino acid L-tryptophan has also been studied. Notably, it is present in many foods and is mainly metabolized by endogenous pathways, such as those of kynurenine and serotonin [223]. Some studies suggest an association of MS with an altered tryptophan metabolism. Known above all is the impact on the immune system of kynurenine metabolites generated by the IDO-1 enzyme; indeed, reduced serum concentrations of tryptophan or high kynurenine-tryptophan ratios have been associated with MS compared to healthy controls [224,225].

## 9. Faecal microbiota transplantation in multiple sclerosis

Recently, there has been a growing interest in faecal microbiota transplantation (FMT) as an alternative and effective approach aimed at restoring eubiosis in a variety of diseases [226]. Although initially approved for the treatment of *Clostridium difficile* infection [227–229], it is now under consideration for a variety of intestinal and extra-intestinal disorders [230–234], including MS [235–237].

During FMT, the faecal matter from healthy donors is transplanted into the patient's intestine through colonoscopy, oral intake, or nasogastric injection [226]. The advantage of FMT compared to probiotics, prebiotics, and synbiotics lies in the ability to produce long-lasting benefits after a single treatment, although repetitive administrations can be considered upon a case-by-case evaluation. Moreover, a therapeutic efficacy associated with limited side effects has been reported by

many studies [238,239]. Within MS context, the benefits of FMT are related to the induction of functional and compositional changes in the GM, which in turn affect immunity [232]. Specifically, the ability of GM to modulate the activity of Treg and Th cells is critical to improve MS symptoms [240].

Preclinical evidence shows the beneficial effects of FMT [241,242]. EAE C57BL/6 mice treated with faecal microbiota from healthy mice showed lower clinical EAE scores, a reduced number of infiltrating cells, and changes in GM (increase in *Prevotella*, *Adlercreutzia*, and *Sutterella*, and decrease in *Turicibacter*) compared to EAE mice [241]. Moreover, another study revealed that FMT-induced GM reshaping reduces astrocytes reactivity, lowers microglia activation, protects against axon and myelin damage, and maintains the integrity of the BBB in EAE C57BL/6 mice [242]. Conversely, the injection of faecal matter from constipated EAE mice to their microbiota-depleted counterpart is sufficient to worsen GM dysbiosis, intestinal and BBB dysfunction, together with demyelination [243]. Indeed, the constipated EAE mice showed more severe clinical and pathological scores, reduced Treg and Treg17 cells, and increased Th17 and Teff17 (Th17 effector) cells, resulting in a decrease in Treg-associated cytokines IL-21, TGF-β, and IL-10, paralleled by an increase in the Teff17-associated ones, namely IL-22, IL-17A, GM-CSF, IL-23, and IL-17F [243].

Besides transplanting a raw faecal lysate, specific faecal components can also be used as disease treatment [71]. For example, the oral intake of miR-30d is reported to improve EAE symptoms (decreased clinical EAE score and lessened demyelination and axonal loss) in mice [71]. Although miR-30d is normally found upregulated in stools of untreated MS individuals and EAE mice at the peak of the disease, the beneficial effect of miR-30d lies in its ability to increase Treg activity by promoting the intestinal growth of *A. muciniphila* [71]. This hypothesis is supported by independent evidence showing that *A. muciniphila* reduces EAE clinical symptoms [46], although the role of this bacterium is still not clear within MS context.

Although still limited, clinical evidence is accumulating [244]. Results from a case study (*ClinicalTrials.gov* identifier: NCT03975413) showed that the reduced dysbiosis following FMT increases the abundance of beneficial SCFAs and improves BDNF levels in a 48 year-old Caucasian male with RRMS [245]. Safety and tolerability of FMT have been reported in another pilot randomized controlled trial conducted in 9 MS patients [246]. Of note, this study links therapy benefits to donor-specificity, underlying that the identification of the optimal donor stool composition is of key importance to achieve clinical improvements. Moreover, the results from an ongoing phase 1b open-label prospective clinical trial (*ClinicalTrials.gov* identifier: NCT03594487; https://clinicaltrials.gov/ct2/show/NCT03594487) conducted in a total of 30 RRMS patients (split between interventional and observational arms) will be essential to evaluate FMT feasibility and tolerability.

Although an additional phase 2 interventional clinical study (*ClinicalTrials.gov* identifier: NCT03183869; https://clinicaltrials.gov/study/NCT03183869) recruited and measured the blood cytokines levels in 14 RRMS patients treated with FMT by enema, its limited sample size prevented researchers from achieving significant conclusions. Therefore, similar investigations should be undertaken in the near future.

Nevertheless, despite poor clinical evidence, the preliminary data on safety and efficacy of FMT in MS are promising, although it is very unlikely that FMT will be approved as a single MS therapy in the short term. Rather, a combination of FMT with the standard of care might be considered in the future to further stimulate immune modulation and resolution of neuroinflammation. Moreover, to be clinically approved in MS, additional investigations should solve some remaining concerns. These include a more careful assessment of short and long term risks, the establishment of standard FMT protocols indicating the optimal administration timing/frequency and the best faecal delivery method, as well as the suggested inclusion criteria [9,247–251]. Furthermore, studies on larger cohorts will be essential to clarify the disease stage/form that would benefit the most from FMT intervention. Indeed, current clinical evidence remains limited to the inclusion of relapsing-remittent MS patients. When met, these standardized criteria will increase the wide acceptance of FMT as an alternative/combined therapy for a variety of gastrointestinal and extra-intestinal disorders, including MS.

## 10. Conclusion

The bidirectional interplay between GM and CNS is emerging as a key breakthrough in better clarifying the pathophysiology and progression of various neurodegenerative diseases, including MS. Although several therapeutic strategies have been approved, so far no curative approach has been defined. In this respect, further approaches would be of great interest for the scientific community. Currently, data are evidencing an obvious gut dysbiosis in MS patients compared to healthy individuals, characterized by a decrease in SCFAs-producing bacteria *Prevotella*, *Clostridium*, and *Parabacteroides*, and an increase in *Akkermansia*, and *Methanobrevibacter* genera. These changes lead to increased intestinal permeability, disrupted BBB integrity, chronic inflammation, and altered T cells differentiation, which have all been reported in MS. Despite preliminary findings seem encouraging, the molecular mechanisms through which the GM could influence these traits are not yet fully clear, and require supportive research.

Several strategies are known to restore the GM composition. In this regard, numerous lines of evidence highlight that Vit D administration, an adequate diet, probiotics, prebiotics/synbiotics/postbiotics intake, and FMT might be effective ways to improve gut dysbiosis and positively impact brain health.

For instance, VitD, whose intake is correlated with milder MS symptoms, is an example of successful dietary support in MS patients. Although encouraging, studies are limited and the results are often contradictory, calling for the need of additional evidence.

Concerning probiotics, preclinical studies are promising. Overall, reduced inflammation, increased anti-inflammatory cytokines and antioxidants, improved neuroinflammation, reduced demyelination, and better motor symptoms have all been reported in MS patients following a probiotic regimen. Given these encouraging data, probiotics might represent an alternative and innovative therapeutic strategy for MS patients. However, further clinical research is needed. Indeed, studies are often exploratory, and small cohorts are included. Moreover, patients stratification remains a big challenge. Tailoring new treatments on symptoms and disease progression is of utmost importance, thought not yet defined.

Whilst probiotics showed encouraging data, the administration of prebiotics, synbiotics, and postbiotics remains largely unexplored, with only few and limited studies available. Similarly, although FMT resulted beneficial when tested on clinical EAE scores (i.e., lower number of infiltrating cells, reduced microglia activation, maintained BBB integrity, improved GM composition and lower axon and myelin damage), results are preliminary and currently insufficient.

Notably, although most scientific publications focused on the measurement and correlation of single genera/families or strains with the severity or resolution of the disease, it is worth considering that some bacteria are known to have pro- or anti-inflammatory properties in a context dependent manner [152]. Moreover, while single genera might harbor pro- or anti-inflammatory properties when taken individually, in the context of the GM modulation the overall pro- or anti-inflammatory response is not expected to be dependent on the contribution of single genera, but rather on a consortium of bacteria interacting with the immune system. Further studies should therefore take into account this complex interplay, thus hepling the interpretation of the results.

Overall, an adeguate diet, the use of probiotics, prebiotics/synbiotics/postbiotics intake, and FMT showed all to be effective in ameliorating MS symptoms. As most of the studies are still preclinical, further work is needed to clarify the potential effect of those strategies as

supportive interventions in MS. In this respect, it is compelling to define the optimal treatment regimen, frequency, and combination with the standard of care. Although promising, GM-based MS treatments remain far from the clinics. Despite many challenges remain, however, the scientific community should not be discouraged, and the positive data so far obtained should foster further research.

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**Angelica Varesi:** Writing – original draft. **Martina Morozzi:** Writing – original draft. **Linda Mascione:** Writing – original draft. **Giovanni Ricevuti:** Supervision. **Ciro Esposito:** Supervision. **Nicoletta Galeotti:** Supervision. **Alessia Pascale:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Lucrezia Irene Maria Campagnoli:** Writing – review & editing, Writing – original draft, Conceptualization. **Nicoletta Marchesi:** Writing – original draft.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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No data was used for the research described in the article.

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