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The Second Brain: Is the Gut Microbiota a Link Between Obesity and Central Nervous System Disorders?

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Abstract The gut-brain axis is a bi-directional integrated system composed by immune, endocrine, and neuronal components by which the gap between the gut microbiota and the brain is significantly impacted. An increasing number of different gut microbial species are now postulated to regulate brain function in health and disease. The westernized diet is hypothesized to be the cause of the current obesity levels in many countries, a major socio-economical health problem. Experimental and epidemiological evidence suggest that the gut microbiota is responsible for significant immunologic, neuronal, and endocrine changes that lead to obesity. We hypothesize that the gut microbiota, and changes associated with diet, affect the gut-brain axis and may possibly contribute to the development of mental illness. In this review, we discuss the links between diet, gut dysbiosis, obesity, and immunologic and neurologic diseases that impact brain function and behavior.

Keywords Gut microbiota · Dysbiosis · Diet · Obesity · Gut-brain axis · CNS diseases

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Introduction

In the evolutionary process, microbes colonized both plants and animals, establishing a complex host-microbial relationship, and evolved together. This interaction shaped their genotype and phenotype and generated an extremely wide range of co-dependent physiologic activities that have led to the health and well being of the host whether plant or animal.

Microbes colonize many external and internal areas of vertebrates. Collectively, the microbial community residing in our bodies is termed microbiota. Microbes residing in the gastrointestinal (GI) tract outnumber us by tenfold, and the microbial genome is hypothesized to be 100 times larger than our own [1]. Joshua Lederberg defined the "the ecological community of commensal, symbiotic, and pathogenic microorganisms that share our body space" as microbiome [2]. The microbial colonization begins at birth, although one study suggests that maternal microbiota can be directly transferred to the neonate [3]. Recent studies suggest that the delivery method determines early colonization. For example, C-section promoted a significantly different microbiome in offspring when compared to those born to mothers and colonized by natural vaginal microbiota [4]. This same study revealed that breast-fed versus bottle-fed nutrition affected the overall microbial composition of the infant's gut. Further, family and shared environment appear to favor a common microbial profile, as reviewed recently by Marietta and colleagues [5]. The GI tract has near 200 m^2 of mucosal tissue that can be inhabited by microbes although colonization is primarily within the distal ileum and colon. Within the GI tract, *Bacteroidetes* and *Firmicutes* are the predominant phyla. However, the relative abundances depend on the anatomical location, and distinct fractions of the small and large intestine harbor significantly different populations [6]. The ecosystem is more complex due the different microbial vertical

distribution, from lumen and mucus to the intestinal epithelium [7]. Furthermore, these microbial populations are not homogeneous in time as the gut microbiome changes with age, disease, and many external factors. There are a number of environmental and genetic factors that are known to affect significantly the microbiome [8].

In this review, we discuss the role of the gut microbiome in the regulation of metabolism and obesity and hypothesize that the gut-brain axis significantly influences CNS health and disease.

Gut Microbiome and Diet

Recent studies on the microbiome show that its composition is highly heterogeneous and that rapid changes in the relative abundances in the main microbial populations occur in individuals. External factors that have been shown to shape the gut microbiome have been postulated as risk factors in different autoimmune diseases, such as stress, smoking, or vitamin D deficiency [8], and diet, perhaps the most relevant influencing element when considering the composition of the microbiota [9]. Among the key beneficial effects of harboring a permanent gut commensal community comes from their ability to digest complex dietary macromolecules into metabolites that vertebrates can then utilize nutritionally. The catabolism of fibers, large plant polysaccharides, requires enzymes that are expressed by the microbes and not the host. Thus, by using food fibers for their own metabolic benefit, microbes provide the host with end products such as simpler polysaccharides. The different metabolic pathways and requisites of distinct microbes provided in the host diet might be a key factor shaping the microbiome.

The study of the microbiome has in recent years undergone a period of expansion that translated into a significant increase in our understanding of its role in health and disease. Many studies have focused on the effects of diet and other external stimuli in the composition of the gut microbiota that may be reflected on changes based on the geographical locations of the samples [10]. As an example, when the microbiota of children growing up in rural areas of Africa, breast-fed for 2 years and fed with a diet high in fibers and low in animal fat and dairy, was compared with the microbiota of samples isolated from children from urban areas of western Europe, important differences were observed in the overall composition of the microbiome [11]. In this study, European children, breast-fed for 1 year and then fed with a diet high in animal proteins and fat, processed sugars, and starch, the rural African gut microbiota showed an abundance of species belonging to the Phylum *Bacteroidetes* and a reduction in *Firmicutes*. Furthermore, children from rural areas of Africa had enhanced levels of short-chain fatty acids (SCFA), a metabolite derived from the catabolism of fibers that has been shown to enhance immune regulation.

Diet of vegetarians, vegan, strictly composed of vegetables or derived products and excluding meat and any animal products, or omnivores modulate the composition of the gut microbiome [12]. More recently published are the results obtained when the gut microbiome isolated from two different tribes from Papua New Guinea were compared among them, and with samples isolated from the USA [13]. The analysis described a microbiome with a higher biodiversity (α-diversity), with higher average number of bacterial operational taxonomic units (OTU) in the two tribes from Papua New Guinea when compared with the samples isolated from matched individuals in the USA. No differences in the α-diversity of both tribes were found. By contrast, the β-diversity, that evaluates the similarities in the OTUs of different samples, was lower in the two PNG tribes when compared with USA samples, indicating a less individualized, more homogeneous composition. Authors hypothesized that lifestyle explains the patterns observed and that westernization may decrease bacterial dispersal rates, altering microbiota structure. Interestingly, Papua New Guinea is considered, based on global socioeconomic parameters, one of the least urbanized areas in the world, where the majority of the population has an agriculture-based lifestyle. The country also has high infant and maternal mortality rates and general low life expectancy, with high prevalence of infection disease deaths. Similarly, it is characterized by relatively low rates for autoimmune diseases. These observations could be associated with the *hygiene hypothesis* that postulates that the increased use of antibiotics, enhanced hygienic standard procedures, and the tendency of the industrialized world to prevent infections during early stages of life will promote a shift in the disease patterns, with a reduction in the infectious diseases rates and a concomitant enhanced incidence in autoimmune disorders [14, 15]. In a different study by Clemente and collaborators, the microbiome of the skin, oral, and the GI tract of the Ameridians, a small community that lives in the Yanonami territories of the Amazon was compared to the microbiome of US residents [16]. Remarkably, although it is believed that their ancestors settled in the area over 11,000 years ago, the Ameridians were not contacted until late in the twentieth century, in the mid sixties. Furthermore, despite their first contact, the Ameridians remain semi-isolated and follow their traditional lifestyle, as hunter-gatherers and no use of antibiotics. The study showed that the Ameridians harbor a highly diverse microbiome when compared with western US and Italian, agrarian Malawi and Guahibo populations, and hunter-gatherer populations of Tanzania, with the highest gut diversity found thus far.

Specific dietary components that promote the changes in the gut microbiota have now been examined. As reviewed by Tilg and Moschen [17], an increasing number of factors present in our daily diets are associated with inflammatory

pathways. Inflammatory signal pathways triggered by dietary elements have been associated with central nervous system (CNS) inflammatory diseases, such as multiple sclerosis (MS), and beneficial diets hypothesized as potential methods to improve disease outcome [18]. Those inducing inflammatory signals are preferentially found in red meats, eggs, saturated, and poly-unsaturated fatty acids (PUFA) such as arachidonic acids, omega-6 long-chain PUFA from animal origin, trans-unsaturated fatty acids derived from vegetable fats, fructose, milk-derived fats, or salt among others [17]. High-fat diets administered experimentally to lean mice provoke significant enhancement of free fatty acids that have been associated with inflammatory syndrome. Diets rich in fatty acids promoted intestinal inflammation characterized by enhanced infiltration of neutrophils and macrophages [19] High levels of plasma-free fatty acids measured in mice fed with a high-fat diet were linked with exacerbated inflammation, increased oxidative stress by the release of reactive oxygen species that lead to insulin resistance, and diabetes [20]. Remarkably, plasma-free fatty acid levels are significantly reduced after the treatment with antibiotics targeting the gut microbiome [21]. The molecular mechanisms associated with free fatty acids correspond to enhanced activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Palmitic acid enhances NF-κB and NACHT-, LRR- and PYD-containing protein 3 (NALP3) inflammasome activity that result in enhanced production of interleukin (IL)-1β and IL-18. Furthermore, high-fat diets increase intestinal inflammation and the toll-like receptor (TLR) signaling. Salts have been associated with increased activity of T helper (Th)-17 (Th17) cells through p38/mitogen-activated protein kinase (MAPK) [17, 22], and the induction of Th17 cells by sodium chloride was directly associated with an exacerbated severity of CNS inflammatory demyelination [22]. Red meats, fats, milk, and milk-based products and salts are the main components of westernized diets, proposed as principal environmental factors associated with obesity, and with the geographical differences observed in the gut microbiota composition.

Specific dietary factors such as omega-3 long-chain PUFA acquired from fish, nuts, and seeds have been shown to promote brain development during early stages of life, with effects on neuronal differentiation and growth, and synaptic function. Perinatal supplementation of diet with omega-3 in rats improved balance and motor coordination [23]. Further, direct effects of omega-3 in primary cultures of rat microglia, population with a significant role in the immunopathogenesis of multiple sclerosis, have been described [24]. Other dietary factors influence on anti-inflammatory pathways through their recognition by specific receptors such as the aryl hydrocarbon receptor (AhR). AhR are specific for different ligands found in vegetables but also for microbial metabolites. The interaction between the ligand and AhR, a transcription factor, induces its nuclear translocation and affects the production of cytokines with anti-inflammatory properties, such as interleukin-22 (IL-22) [25]. Broccoli and cabbage are two examples of vegetables that could promote such effects [26]. Reductions in AhR ligands have been shown to exacerbate experimental colitis in mice [26], and reduced AhR expression has been shown in intestines of inflammatory bowel disease patients [27]. Tryptophan, found in fish and vegetables, is converted only by bacteria to indole-3-aldehyde, a ligand of AhR, and it also regulates anti-inflammatory pathways through mTOR activation [17].

As opposed to omega-6 long-chain PUFA, omega-3 PUFA promotes anti-inflammatory responses through their binding to G-coupled receptor-120 (GPR120) in immune cells [28]. GPCRs are receptors also identified in the binding of short-chain fatty acids with anti-inflammatory effects, such as butyrate, acetate, and propionate, metabolites produced by gut microbes in response to dietary fibers. Binding of short-chain fatty acids to GPR43 in T cells promotes the acquisition of a regulatory phenotype and enhances blood-brain barrier integrity [29••]. Remarkably, GPR43-deficient mice are obese when fed with a normal diet, and transgenic mice that overexpress the receptor remain lean when administered with a high-fat diet (HFD). These observations were not found in germ-free mice and also after the treatment of conventionally housed mice with antibiotics [30].

One of the key beneficial implications of harboring microbes in the gut results from their ability to process dietary fibers, using specific enzymes that we lack. Besides the nutritional mutualistic benefit, when catabolizing fibers, microbes produce metabolites that have been shown to affect the immune system. Metabolites derived from gut microbes might have a profound impact on other immunologic and metabolic pathways and modulate health and disease. It has been described that the metabolite pool (metabolome) of mice that suffer from experimental encephalomyelitis, animal model that is widely used to study multiple sclerosis, and rats differ from healthy animals. In the CSF of experimental autoimmune encephalomyelitis rats, significant changes in metabolite patterns are found at the onset and peak of the disease [31], and in blood of SJL experimental autoimmune encephalomyelitis mice, approximately 15 % of the metabolites detected (283) differed in their concentrations during disease [32]. Changes in the gut metabolome are observed during dietary interventions that affect the gut microbiome [33]. These changes might have significant effects in the immune system and neuroinflammation. Because of the influence of diet and disease, the metabolome constitutes a prominent area of research in the context of metabolic and autoimmune diseases.

The pro- and anti-inflammatory effects of dietary factors suggest their relevance for the control of autoimmune disorders of the CNS, such as multiple sclerosis [34]. Riccio and colleagues suggest avoiding the excess in the intake of animal fats and milk fat globule membrane proteins, and promoting

the use of diets rich in polyphenols and carotenoids, with described anti-oxidant activity, poly-unsaturated fatty acids (PUFA) derived from vegetables and fish, such as omega-9 monounsaturated oleic acid and alpha-linolenic acid (omega-3). Addition to diet of supplements, such as vitamin D and vitamin B12 could also have beneficial effects [34]. The direct effect of diet in the improvement of MS patients remains yet to be elucidated.

The use of probiotics, microbial populations that promote direct immunological benefits (such as the induction of anti-inflammatory cells and cytokines) and indirect effects by their production of metabolites that promote anti-inflammatory functions in immune cells, is currently being extensively investigated. Some of their effects in autoimmune CNS diseases are summarized in Table 1 and further discussed later. Also, prebiotics, compounds that promote the abundance of microbes with effects that are believed to be beneficial are under current scrutiny. However, more and larger studies in human individuals are necessary in order to evaluate their value as preventive and therapeutic strategies.

Gut Microbiome and Obesity

Obesity is a major health and socio-economical challenge. Both genetic and environmental factors are believed to play a significant role in obesity and diet is perhaps the most modifiable of all. Dietary patterns are determined by a number of different determinants that include geographical location, familiar and educational habits, mood, and physiological conditions. The diet-induced changes in the gut microbiome occur rapidly [52, 53••]. In a study by Walker and colleagues [52], the microbiota of human volunteers fed for 3 weeks with four different diets based on their protein/carbohydrate/fat percentages and addition of non-digestible carbohydrates, showed significant alterations in microbial abundances and phylum distributions [52]. A more recent study shows that changes occur faster than a few weeks [53••]. The administration of a plant-based diet, enriched in grains, vegetables, beans and fruits, for 5 days induced significant changes in the gut microbiota when compared with the administration of an animal-based diet, composed of meat, eggs, and cheese [53••]. Both

Table 1 Gut microbiota species that have been shown to affect the outcome of experimental models of CNS diseases

CNS experimental disease	Microbe or microbial product	Animal model	Major effects observed	Refs
EAE	PSA produced by *Bacteroides fragilis*	Mouse	Administration of purified form of PSA to conventional mice, disease protection by interleukin-10 (IL-10)-producing CD39+ and FoxP3 + Treg induction	[35–37]
	PSA-producing *B. fragilis*	Mouse	In mice colonized with living *B. fragilis* that produces PSA, but not *B. fragilis* deficient in production of PSA, render protected after treatment with antibiotics	[38]
	Segmented filamentous bacteria	Mouse	Mono-colonization of GF mice restores EAE susceptibility	[39]
	Lactococcus spp.	Mouse	EAE protection by induction of IL-10-producing Tregs	[40]
	Bifidobacterium animalis	Rat	Reduces EAE severity	[41]
	Pediococcus acidilactici R037	Mouse	Reduces the severity of EAE by induction of IL-10-producing Tr1 cells	[42]
	Lactococcus lactis Hsp65	Mouse	Reduces the severity of EAE by induction of Tregs cells and LAP^+ $CD4^+$ Tregs	[43]
Stress-induced anxiety	*Bifidobacterium infantis*	Mouse	Mono-colonization reverses stress exacerbation in germ-free mice	[44]
	Bifidobacterium infantis	Rat	Reduces depression induced by maternal separation in adult rats	[45]
	Lactobacillus helveticus ROO52	Mouse	Reduce anxiety behavior and memory dysfunction	[46]
	Lactobacillus rhamnosus JB-1	Mouse	Improves behavior in anxiety and depression	[47]
	Mycobacterium vaccae	Mouse	Improves behavior in anxiety	[48]
	VSL-3 probiotic mixture	Rat	Reverse age-induced deficits in memory	[49]
	L. rhamnosus + *L. helveticus*	Mouse	Reversed memory dysfunction in infection-induced stress	[50]
Autism spectrum disorder	*B. fragilis*	Mouse	Reduced behavioral and physiological dysfunctions	[51••]

diets modified the intake of fats, fibers, and proteins, with significant increases in the fibers levels, and reduced fat and protein intake levels found in individuals fed with the plant-based diet. The animal-based diet, in turn, did not affect the fiber levels whereas an increase of dietary fat and protein intake levels were found. The study showed no differences in the α-diversity (diversity of the microbial population within individuals at a certain time); however, the animal-based diet promoted a significant change in the β-diversity (difference in individuals between base-line and after-diet microbiota) only 1 day after diet reached the distal gut microbiota, that was measured with a food tracking dye. The same study showed that the levels of short-chain fatty acids were reduced in fecal samples obtained from individuals fed with the animal-based diet when compared with the plant-based diet samples [53••].

Host genetics play a critical role in the interaction of the environment and microbes. Experimentally, drastic alterations of the host's genome that drives microbial interaction pathways affect significantly the host metabolism. Knockout mice in toll-like receptor 5 (TLR5), a flagellin ligand, results in hyperlipidemia, increased triglycerides and cholesterol, hypertension, adiposity, and a 20 % increase in the average body weight of mice [54]. Bacterial lipopolysaccharide (LPS) interaction with its immune ligand TLR4 is also involved systemic inflammation in response to the experimental administration of a high-fat diet (HFD). HFD administration to conventional mice induced obesity, glucose intolerance, and the development of type 2 diabetes (T2D) [55]. HFD induced significant changes in the gut microbiota, and increased in the intestinal permeability, inflammation and oxidative stress. The treatment of HFD-fed mice with antibiotics reduced diet-induced metabolic endotoxemia, inflammatory cytokine and markers, macrophage infiltration, and oxidative stress. Further, antibiotics reduced the obesity and restored the glucose tolerance and improved the metabolic parameters associated with the development of T2D. Mice deficient in the cluster of differentiation-14 (CD14), required for the signaling of LPS in combination with TLR4, had similar effects in HFD-fed mice than those observed after the treatment with antibiotics [55].

The microbiome composition of lean humans is significantly different to obese mice and individuals [56]. The analysis of the gut microbiota of fecal samples obtained from obese and lean monozygotic and dizygotic twin pairs and their mothers was compared by PCR and sequencing of 16S rRNA. The structure and diversity of the microbiota of members from the same family had more similarities than individuals from different families. However, the results showed a significant reduced bacterial diversity in obese when compared with lean individuals, despite the similarities found among family members. Obese individuals had a reduced proportion of *Bacteroidetes* species and increased proportions of *Actinobacteria* species when compared with samples obtained from lean individuals [56]. The administration of a westernized diet to mice significantly altered the microbiome and promoted a relative increase in *Firmicutes* and concomitant reduction in members of the *Bacteroidetes* phylum, and was associated with the acquisition of an obese phenotype [57] that could be reversed with diet. When a HFD was administered to gnotobiotic, also termed germ-free mice, that are born and raised in sterile incubators, these showed a marked reduction in weight gain and fat mass when compared to animals raised with "normal" microbiota. Furthermore, germ-free mice were more resistant to glucose intolerance than conventional mice [58]. Germ-free mice are artificially generated to lack microbes and microbial antigens. Although very relevant experimental tools, these mice show marked anatomical and immunological alterations, such as reductions of specialized lymphoid tissues in the small intestine, Peyer's patches, thinner lamina propria, and reduced and smaller isolated lymphoid follicles, as well as a reduced number of T cells in the gut. Furthermore, a biased immune system, with lower frequencies in pro-inflammatory cell subtypes in the small intestine, such as Th17 cells among others is observed [59]. The significant alterations in germ-free mice indicate an improper development of the immune system, and as we will discuss later, also altered brain function and suggest a very significant influence of gut microbes in the development of a fully competent immune system. However, because of their intrinsic deficiencies the effects observed in germ-free animals should not be compared with the scenarios that the hygiene hypothesis proposes for individuals born and raised in natural non-germ-free conditions.

The gut microbiome can be modified by diet rapidly, as earlier discussed. The changes that diet induces in the abundances and diversity of the gut microbial populations could affect the function of the immune, endocrine, and neuronal systems and cause disease. The hypothesis that the alteration of the gut microbiome can lead to disease is termed "dysbiosis". Dietary factors are direct inducers of gut dysbiosis [19]. It has been hypothesized that dysbiosis plays a role in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). Microbiome analysis show remarkable differences in the gut populations of Crohn's disease and ulcerative colitis patients when compared to matched healthy controls [6]. Significant differences were also observed between the gut microbiome of IBS patients and healthy donors [60]. Intestinal syndromes and perhaps obesity share an inflammatory component that could be modulated by the gut microbiome.

Obesity has been traditionally considered a metabolic disease, but inflammation emerged as a key effect and/or mediator. In obesity, hyperplasia of the white adipose tissue is a hallmark. During this process, adipocytes produce mediators with modulatory functions in both the endocrine and immune systems, such as pro-inflammatory cytokines and satiation

hormones, such as leptin [61]. How much fat the body has will regulate the production of leptin, which then controls food intake. Leptin is an anorexigenic factor by interacting with the hypothalamic nucleus. Knockout mice unable to produce leptin are obese, and fasting reduces the serum levels of leptin and regulates experimental autoimmune encephalomyelitis severity [62]. Interestingly, leptin-deficient mice are resistant to EAE [63]. Brain lesions and cerebrospinal fluid of multiple sclerosis patients also show enhanced leptin levels when compared with controls [64]. The therapeutic implication of these observations augments the previous data reported in EAE mice, when the in vivo neutralization of the hormone impacted the severity of the disease [65]. A link between leptin levels and Alzheimer's disease (AD) has been also postulated [66, 67]. Leptin effects are examples of the tight interactions between the endocrine, immune, and neuronal systems affected by all dietary factors and that can in turn regulate dietary habits.

Systemic inflammation has been postulated in obese patients, and increased levels of pro-inflammatory cytokines are observed in their serum, supporting the hypothesis [68]. Among the factors that have been shown to regulate inflammatory and anti-inflammatory pathways and diseases, the effects of the gut microbiota are crucial. Here, we hypothesize that gut microbes have the ability to modulate the inflammatory balance in the gut, and also in the brain, by benefiting from a bi-directional communication system that regulates obesity, and neuroinflammation that can lead to mental illness.

The Gut-Brain Axis and Experimental CNS Inflammatory Demyelination

As earlier discussed, germ-free mice are important experimental tools for the study the effects of the absence of microbes or microbial colonization of the gut in CNS inflammatory demyelination diseases. Germ-free mice and the use of antibiotics set the ground for more complex studies in animal disease models, including experimental autoimmune encephalomyelitis, an inflammatory model of demyelination of the axons of neurons of the central nervous system (CNS) that shares many features with multiple sclerosis. Germ-free mice show reduced severity of spontaneous [69] and actively induced CNS demyelinating inflammation [39]. Mono-colonization with *Segmented filamentous bacteria* (SFB), known murine pro-inflammatory Th17 cell inducer restores experimental autoimmune encephalomyelitis severity [39]. The use of antibiotics that modify the gut microbiome quantitative and qualitatively also modulates CNS disease severity [70, 71]. These observations suggest that the significant alterations in the immune system of germ-free conditions and the effects that antibiotics promote in the gut and peripheral immune responses ultimately influence CNS disease severity.

Microbes, such as *segmented filamentous bacteria*, enhance pro-inflammatory responses and could exacerbate CNS inflammation in conventionally housed mice. *Porphyromonas gingivalis*, a commensal microbe of the oral cavity potentiates glial activation and enhances pro-inflammatory responses and experimental autoimmune encephalomyelitis severity [72, 73]. However, other microbes have been shown to regulate disease, such *Bifidobacterium animalis* in Lewis rats [41] and the administration of a mixture of *Lactobacillus* spp. in mice [40]. A list of gut commensal bacteria that regulate experimentally the severity of experimental autoimmune encephalomyelitis is shown in Table 1. The work of our laboratory focused on the polysaccharide A (PSA), produced by the human gut commensal *Bacteroides fragilis*. PSA is a capsular polysaccharide that induces regulatory T cells (Tregs) that are immunosuppressive by the production of anti-inflammatory IL-10 [35, 74]. PSA also promotes the induction of a subclass of regulatory T cells that express an ectoenzyme, the ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), also known as CD39, responsible for the catabolism of ATP into AMP ($CD39^+$Tregs) [36], and also IL-10-producing T cells defined as Tr-1, are immunosuppressive but do not express the transcription factor foxhead box P3 (FoxP3) that defines Tregs [75–77]. PSA is protective against experimental CNS inflammatory demyelination [35–37]. The induction of regulatory T cells by PSA is dependent on the recognition of the polysaccharide by dendritic cells, both conventional [36, 75] and plasmacytoid dendritic cells, in a TLR2-dependent mechanism [78]. The prophylactic and therapeutic oral treatment with purified PSA induces/promotes an accumulation of regulatory T cells in CLNs of EAE mice. Remarkably, no accumulation is observed in CLNs of non-EAE mice treated with PSA. We hypothesize that an unknown CNS-derived inflammatory stimulus is promoting a migratory effect in gut-induced immunomodulatory cells. The regulatory T cells that PSA induce in EAE mice express high levels of CD39, in a process that is TLR2-dependent [36]. These $CD39^+$T cells show an augmented regulatory phenotype and produce high levels of anti-inflammatory IL-10. CD39 deficient mice develop EAE when treated with PSA. Further, the acquisition of a $CD39^+$ phenotype potentiates their migratory phenotype increasing the levels of expression of chemokines CCR5, CCR6, and CXCR3, and adhesion molecules such as CD49b and CD29 [37]. In vitro, $CD39^+$ T cells isolated from PSA-treated EAE mice migrate more efficiently toward inflamed EAE CNS tissue. In vivo, we observed that $CD39^+$ T cells, independently of FoxP3 expression that as discussed earlier defines the Treg cell population, accumulate in the CNS of EAE mice treated with PSA when compared to untreated EAE mice [37]. The importance of such finding relies on the impaired anti-suppressive function of $CD39^+$ Tregs isolated from MS patients, that are unable to control the proliferation of Th17 cells

in vitro and their production of IL-17 [79]. Figure 1 represents the major findings for PSA in their ability to protect mice against CNS inflammatory demyelination, based on animal models. We have recently shown that PSA modulates the phenotype of peripheral blood mononuclear cells isolated from circulating blood of healthy donors. In vitro culture of naïve $CD4^+$ T cells with dendritic cells, but not macrophages or B cells, acquire a $CD39^+FoxP3^+$ phenotype in the presence of purified PSA [80]. When circulating $FoxP3^+CD4^+$ T cells are exposed to dendritic cells and PSA, the CD39 relative expression increases. Furthermore, IL-10 production by the PSA-sensitized $CD39^+FoxP3^+$ T cells increases, as well as their in vitro ability to reduce TNF-α production by monocytes stimulated with LPS [80]. To date, PSA is the only known symbiotic factor isolated from a human commensal microbe that promotes regulatory T cells, suppressive in vitro and in vivo, that are also able to promote a tolerogenic T cell phenotype in human samples. The experimental evidence that suggest a primary effect of the gut microbiota regulating EAE lead to hypothesize that similar modulatory pathways will be revealed in MS patients. Dysbiosis, a shift in the microbial populations of the gut, would then be considered a novel risk factor for MS and perhaps other CNS diseases [8, 81, 82].

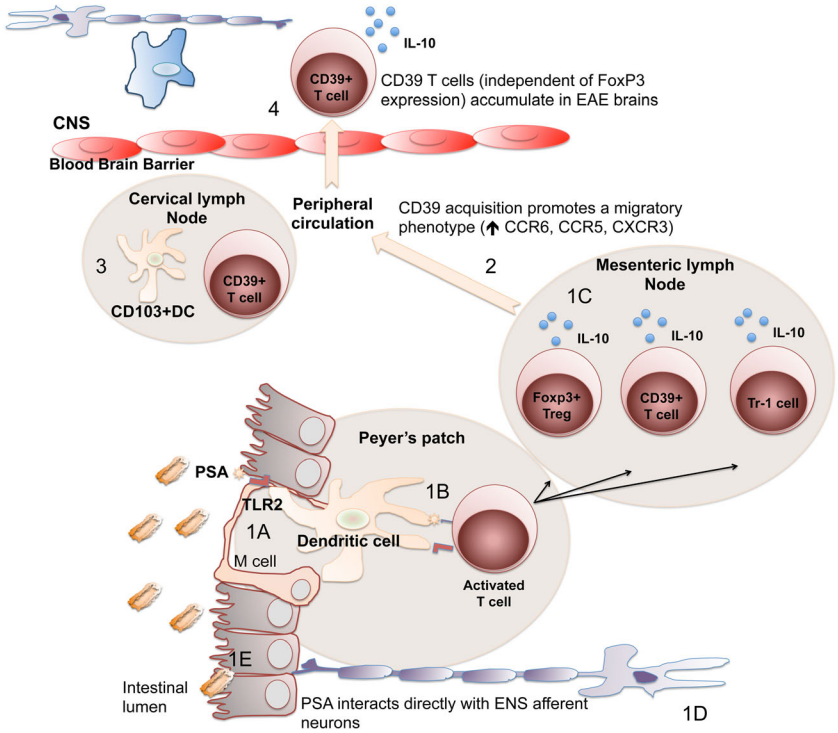

Fig. 1 The gut-brain axis concept applied for the symbiont factor polysaccharide A (PSA) of *B. fragilis*. The gut-associated lymphoid tissue (GALT) is the largest reservoir for immune cells. Because its relevance sampling the environment, the GALT is composed of specialized areas where microbe-host interactions occur; the intestinal epithelium is a physical and biochemical barrier that separates the lumen, where microbes inhabit, from the lamina propria, enriched in immune cells. The intestine also contains the Peyer's patches, lymphoid tissues with cells that facilitate antigen sampling, such as the M cells, and antigen presenting cells (dendritic cells, monocyte/macrophages, and B cells), and effector cells such as T cells. (*1A*) Conventional and plasmacytoid dendritic cells have been shown to recognize polysaccharide A (PSA) produced by the human commensal *B. fragilis* in the context of toll-like receptor-2 (TLR2). PSA is internalized by dendritic cells and presented to naïve T cells by the major histocompatibility class – II. (*1B*) With required costimulation, naïve T cells are activated and differentiated into three distinct types of regulatory T cells: regulatory T cells (Tregs) that express the transcription factor foxhead box P3 (FoxP3); T cells that express the ectoenzyme CD39, responsible for the catabolism of ATP into AMP ($CD39^+$Tregs); and T cells that are regulatory but do not express FoxP3, called Tr1 cells. All three subtypes induced in vivo and in vitro by PSA are characterized by their production of anti-inflammatory IL-10 cytokine, and by their immunosuppressive function. (*1C*) The dendritic cell—T cell interaction can occur in the Peyer's patches but also in the mesenteric lymph nodes where dendritic cells may migrate once exposed to antigens, such as PSA. (*1D*) PSA has also been shown to interact directly with afferent neurons of the enteric nervous system (ENS). (*1E*) *B. fragilis* has been shown to stabilize a leaky gut. (*2*) The acquisition of a CD39 phenotype by T cells once activated by dendritic cells exposed to PSA, independently of FoxP3 expression, enhances the migratory function of T cells. (*3*) CD39 + T cells, both $FoxP3^+$ and $FoxP3^-$, accumulate in the cervical lymph nodes of EAE mice. Also, a specialized subset of generally gut-derived dendritic cells that express CD103 that accumulate upon oral treatment of EAE mice with PSA. CD103 + DCs accumulated in response to PSA are tolerogenic, and induce Treg differentiation. (*4*) Enhanced frequencies of $CD39^+$T cells, both $FoxP3^+$ and $FoxP3^-$, are found in the brains of EAE mice. Accumulation of these regulatory cells occurs in cervical lymph nodes and brains in mice treated orally with PSA only when EAE is induced, and not in healthy animals immunized with the polysaccharide

The Gut-Brain Axis is a Bi-directional System

The experimental evidence suggests that the mucosal immune responses elicited in the gut modulate animal models of CNS inflammation. Recent findings suggest a more complex scenario, where CNS disease regulates intestinal inflammation. Thus, the gut-brain axis might involve bi-directional pathways of interaction. A recent work by Lavasani and colleagues describes that the induction of EAE promotes significant increases in the small intestine permeability, an enhanced infiltration of IFN-$\gamma^{+}CD4^{+}$ (Th1) and IL-$17^{+}IL\text{-}22^{+}CD4^{+}$ (Th17) cells, as well as enhanced frequencies of increased expression of IL-17^{+} γδ T cells in the small intestine gut mucosa and Peyer's patches. Antigen presenting cells isolated from the small intestine of EAE mice produced enhanced levels of pro-inflammatory IL-6 and TNF-α when compared to healthy animals [83•]. Changes were observed as soon as 7 days post-EAE induction. EAE mice showed altered intestinal morphology and function when compared with control mice, immunized only with the adjuvants, and involved an increased expression of the tight junction regulator zonulin in duodenum, jenunum, and ileum. Although not covered in the study, it would be relevant to determine whether the "leaky gut" syndrome described by authors for EAE mice are associated with significant changes in the gut microbiota. Further, the study of Lavasani and collaborators acquire further significance when compared with the also recent study published by Braniste and colleagues [29••]. In this study, investigators used the germ-free murine model to evaluate the importance of the gut microbiota, or its absence, in the permeability of the blood-brain barrier. In their study, authors showed that mouse embryos from mother devoid of gut microbiota presented increased blood-brain barrier permeability. Remarkably, its integrity is compromised in different inflammatory conditions of the CNS [84]. The blood-brain barrier integrity is critical for the normal and healthy development of the brain because of its control of the passage of nutrients and other molecules that influence neuronal growth. The increased blood-brain barrier permeability was linked molecularly to a reduced expression of tight junction modulator occludin in germ-free embryos when compared with conventional embryos. Furthermore, the enhanced blood-brain barrier permeability was also observed in mature GF mice that showed an altered expression of tight junction regulators. The expression of occludin and claudin-5 was significantly reduced in frontal cortex, striatum, and hippocampus of brains isolated from germ-free adult mice when compared to SPF brains. When GF mice where colonized with conventional murine microbiota, the BBB integrity was restored. Further, authors evaluated the role of short-chain fatty acids that as discussed earlier are metabolites produced by gut microbes as a result of the catabolism of dietary fibers. For that, GF mice mono-colonized with SCFA-producing gut microbes restored the blood-brain barrier integrity [29••]. In light of these results, and since blood-brain barrier development is initiated previous to birth and continues during early stages of life, it is plausible to hypothesize the essential role of the gut microbiota on its appropriate integrity. Thus, gut commensal microbes and metabolites are essential in regulating intestinal integrity and function, immune homeostasis in the gut and the periphery, and the recent experimental data discussed above suggest their relevance controlling BBB development and integrity. Also, CNS inflammation leads to changes in the intestinal integrity, as demonstrated by Lavasani and colleagues [83•].

Although most of existing literature describes the effects of the gut microbiota in the immune compartment of the gut-brain axis, recent evidence suggests that the hormonal and neuronal systems are key regulatory components of the axis. The endocrine system is an essential regulator of nutritional patterns. Experimental models of inflammatory demyelination show that simultaneously, hormones regulate CNS disease. The autonomic nervous system and the hypothalamus-pituitary adrenal axis effects on food intake could influence the gut microbiota composition. The gut microbiota regulates neuronal functions of the enteric nervous system (ENS). PSA-producing *B. fragilis* and *Lactobacillus rhamnosus* (JB-1) reach ENS afferent neurons of mice and influence their function [85•]. Gut microbes directly affect hormone synthesis, such as serotonin, 5-hydroxytryptamine (5-HT). Serotonin is a neurotransmitter that exerts essential brain functions and has essential roles regulating GI function. Serotonin is produced by enterochromaffin cells, endocrine cells of the gut, in response to spore-forming commensal bacteria [86]. The colon, but not the small intestine of GF mice, showed significant lower levels of 5-HT when compared to SPF mice. When GF mice where mono-colonized with non-spore forming bacteria no differences in the 5-HT levels where observed when compared to non-colonized GF mice. However, when GF mice where mono-colonized with spore-forming bacteria the levels of 5-HT in serum and colon were restored. The control of serotonin by some components of the gut microbiota, or their metabolic products [86] resulted in regulation of GI motility, suggesting a direct role of the gut microbiota regulating the effects of the endocrine system in the autonomous nervous system.

Also, hormones that control dietary habits are strong immunomodulatory factors that regulate CNS inflammation while serving as a link with the autonomic nervous system and the hypothalamus-pituitary adrenal axis, such as the

already discussed leptin [62–65]. The vasoactive intestinal peptide (VIP) also regulates the immune system and modulates CNS inflammation [87]. The interaction between the gut microbiota, the endocrine, and neuronal system is also supported by the effect of gut microbes in the fat-gene regulation in the CNS [88]. In this work, investigators compared the mRNA expression levels of neuropeptides that regulate food intake in the CNS of germ-free and conventional mice. When compared to germ-free mice, conventional mice had reduced expression levels of glucagon-like peptide-1 (GLP-1) precursor proglucagon (Gcg). GLP-1 plays a key role regulating food intake and body, and its levels are reduced during food restriction. The CNS of conventional mice also showed reduced levels of brain-derived neurotrophic factor (BDNF) that inhibits appetite limiting obesity levels. Based on their results, authors propose that the gut microbiota reduces the expression of genes that regulate food intake and fat mass. Thus, the effects observed in the presence or absence of gut microbiota suggests the key role regulating the expression of genes that regulate nutritional habits. Reciprocally, dietary habits modulate the shape of the gut microbiota, supporting the bi-directional mechanism of regulation that characterizes the gut-brain axis.

Does the Gut Microbiome Regulate Brain Function and Illnesses?

The brain has been considered historically sealed from the microbial world unless infection occurs. However, the recent studies covering the gut-brain axis and the evidence that the gut microbiota interplays with different systems ultimately impact the brain [89]. Does the microbiota control brain development and function? Moreover, can gut microbes trigger or modulate brain diseases associated with the mood and behavior? Figure 2 represents the potential interaction between diet and changes in the microbiota of the gut with effects in the gut-brain axis that can lead to obesity and mental diseases.

Among mental illnesses, autism has been related to the gut microbiome [90, 91]. Of interest, autism spectrum disorder (ASD) patients suffer from gastrointestinal dysfunction, including deficiencies in GI motility and enhanced intestinal permeability. There is an increased prevalence in IBD among ASD young individuals when compared to controls [92]. When comparing the gut microbiota of ASD individuals with control donors, significant shifts in the microbial populations have been documented [93–96]. ASD is associated with immunological abnormal parameters, such as enhanced pro-inflammatory cytokines in periphery as well as evidence of astroglia and microglia activation within the CNS [97, 98]. In an experimental work performed using a murine model of ASD, Hsiao and colleagues evaluated the contribution of the gut microbiota in regulating behavioral and physiological dysfunctions associated with ASD [51••]. The mice used for the study were the offspring of immune-activated mouse mothers (maternal immune activation model, or MIA model). In this model, mothers are administered with the viral mimic poly (I:C), which results in offspring with ASD social and behavioral impairments. ASD mice exhibited GI dysfunction and gut dysbiosis. Remarkably, the treatment of ASD mice at 8 weeks of age with *Bacteroides fragilis* contributed to the recovery of the GI integrity and function. Furthermore, the treatment with *B. fragilis* restored the microbiota dysbiosis in ASD mice and regulated behavioral parameters, such as motility and anxiety-like motion determined by an open field exploration, sensomonitor gating, that determines the response of an animal to inhibit movement in response to an acoustic tone when it is preceded by a lower-intensity stimulus, and deficits in communication, determined by the duration of ultrasonic vocalizations in response to a social encounter with a novel mouse and to an object, impaired in ASD mice [51••]. Treatment with *B. fragilis* did not improve social interaction in ASD mice. The behavioral effects were neither *B. fragilis*-specific nor dependent on the immunomodulatory role of PSA as previously described for EAE, IBD, and asthma. Further, no significant effects were observed in the peripheral immune parameters compared. By contrast, the beneficial effects of *B. fragilis* were associated with metabolites that were significantly enhanced in serum samples of ASD mice treated with *B. fragilis*, such 4-ethylphenylsulfate (4EPS), a uremic toxin chemically related to others that, as noted by Hsiao and colleagues, are proposed as urine biomarkers for autism. 4EPS has been associated with the communication skills in mice [99]. Injection of 4EPS to naïve mice induced an autism-like phenotype [51••]. 4EPS was drastically (46-fold) enhanced in MIA mice used as an ASD model. The treatment of ASD mice with *B. fragilis* restored those levels.

The beneficial effect of *B. fragilis* in a mouse model of autism suggests that gut microbes, contrary to what it was historically hypothesized, affects brain function and modulates mental disorders. Evidence that links the gut microbiota with brain function is the observation that germ-free mice show an enhanced response to stress modulated by the hypothalamic-pituitary-adrenal axis [44], an axis that is sensitive to stress events that occur early in life [100]. Furthermore, the mono-colonization with *Bifidobacterium infantis* reversed the stress induced in these mice [44, 45], suggesting again that the neuroendocrine system is tightly regulated by the gut microbiota. Related behavioral effects induced by stress in germ-free mice have been more recently described in rats [101]. Other studies have evaluated the beneficial effects of gut microbes on brain function and illness that are used as probiotics [102] and summarized in Table 1.

The mechanisms by which gut microbes are regulating brain function remain to be elucidated, although recent observations suggest a potential overlap between the immune, endocrine, and neuronal system. For example, *B. fragilis* not only directly interacts with antigen-presenting cells of the

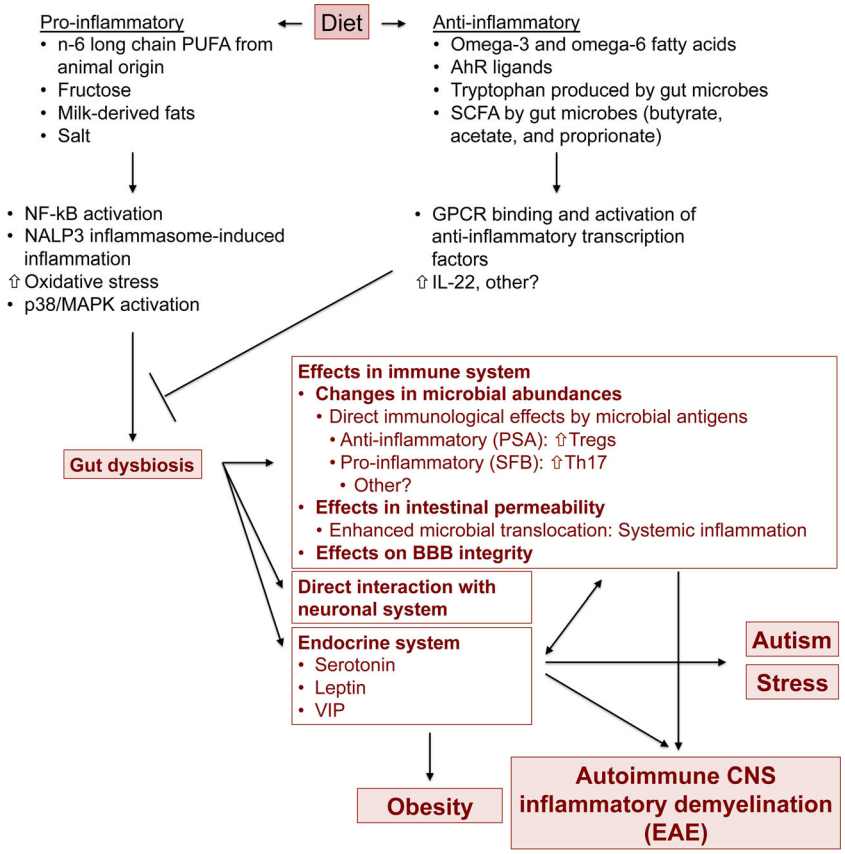

Fig. 2 Diagram of the hypothetical effects of diet in gut dysbiosis, obesity and CNS diseases, based on experimental models. Different dietary components that are believed to promote diverse pro-inflammatory pathways have been associated with gut dysbiosis, postulated as major factor for gut inflammatory diseases, but also hypothesized to be responsible for obesity, autoimmune CNS inflammatory diseases, such as the demyelinating MS, and for affective neurological disorders. Several pro-inflammatory triggering dietary factors have been associated with the significant changes that westernized diet in the gut microbiota. Other dietary components promote anti-inflammatory responses that could help in maintaining a balanced gut microbiota. Both pro- and anti-inflammatory-triggering dietary factors can have direct effects on the immune, endocrine, and neuronal system, but also indirectly affect them by their catabolism by gut microbes. For instance, some short-chain fatty acids (SCFA) and tryptophan are produced by gut microbes in response to diet and constitute essential ligands for immune GPCR receptors that mediate in anti-inflammatory responses. Furthermore, bacterial antigens, such as PSA produced by *B. fragilis* have major immunomodulatory roles. The mechanisms by which dietary factors regulate CNS diseases could involve changes in the microbial populations that express immunomodulatory factors that directly interact with immune cells, but also indirectly by their production of metabolites (such as short-chain fatty acids) as products of food catabolism with effects in the immune, neuronal, and endocrine systems. Furthermore, the different compartments of the gut-brain axis interact significantly. Although very substantial research on this field is currently ongoing, the bi-directional gut-brain axis is a highly complex system, and many aspects of the multifactorial interactions need to be elucidated using experimental models, but more importantly in human studies

immune system to promote tolerogenic Treg responses [35, 103, 104], but also affects brain function by promoting specific metabolites [51••], and also directly activates intestinal neurons [85•]. Another gut microbe, *Bifidobacterium longum*, directly interacts with the vagal nerve [105]. Remarkably, a significant reduction in the levels of brain-derived neurotrophic factor (BDNF) was documented in the hippocampus of germ-free mice when compared to conventional mice. BDNF plays a pivotal role in neuronal development and function [106], as well as constituting an essential regulator of food intake, controlled by the presence of gut microbiota [88]. The effects of the gut microbiota in neuroinflammatory processes associated with diseases have emerged as a central field of study [91, 107, 108], although direct mechanisms of interaction have yet to be described. Among those factors that are associated with neuroinflammation during mental illnesses, obesity is hypothesized to play a major role [68]. Table 1 summarizes the experimental evidence that suggests a role of gut microbes regulating CNS disorders.

Does Microbiota Associated with Obesity Induce Mental Illnesses?

Epidemiological analysis of patient populations propose obesity as a risk factor for brain pathologies and mental diseases that include anxiety, dementia, and depression [109–112]. Obese individuals suffer from increased symptoms associated

with depression when compared to control individuals [113]. However, not all studies show a correlation [114]. Despite the contradictory literature, the effects of diet in obesity and impact on the gut microbiota appear to be more established, as previously described. In light of the experimental evidence that suggests that probiotic microbes reduce severity of the behavioral murine models of anxiety and stress, it would be of importance to establish whether changes in the gut microbiome associated with obesity or obesity-induced diets are triggering factors for affective mental disorders. In a study performed using non-obese conventional mice, Bruce-Keller and collaborators evaluated the effects of gut microbiota replenishment of antibiotic-depleted mice in the behavior, comparing an array of behavioral tests and biochemical analysis in gut, periphery, and brain [115]. The recipient mice were initially treated with antibiotics and subsequently were transferred with the fecal content of mice either treated with conventional diet or with a high-fat diet (HFD). The transfer of the gut microbiota of mice fed with HFD resulted in an enhanced intestinal permeability in recipient mice, when compared with animals that received gut microbiota from mice fed with conventional diet, with no significant changes in the body weights of recipient mice observed. Furthermore, HFD-induced gut microbiota promoted systemic and brain inflammation, enhanced microgliosis, and increased the expression of TLRs in the brain, when compared with mice that received normal microbiota. The HFD microbiota enhanced the anxiety levels quantified by elevated plus and open field methods and stereotypical behaviors measured by quantifying marble burying. HFD microbiome reduced memory in recipient mice. To evaluate the effects of the transplantation in the brain, investigators compared the expression of the synaptic marker proteins, such as synapse-associated protein 97 and synapsin 1, that were found to be similar. However, levels of phosphorylated synapsin 1 were reduced in mice transferred with HFD microbiota. Although the immunological analysis performed did not phenotype the inflammatory cells nor evaluate the cytokine profile of these mice, the study provides relevant information with regard to the effects of a microbiota induced by HFD, that alters the gut microbiota as shown in this work, and associated with obesity, as previously covered in this review.

Conclusions

Both experiments using animal models and some initial large studies in humans suggest that the gut microbiota plays very significant regulatory roles in a wide array of diseases, including not only those of the gut, but also cardiovascular and autoimmune diseases that affect distal organs, such as the pancreas, joints, or the CNS. Although initial human studies suggest a correlation with obesity and mental diseases, further and larger studies are necessary. Mechanistically, the animal models used this far have relevant limitations that need to be considered. As it was discussed earlier, germ-free mice, widely used to test the hypothesis that gut microbes affect immune, endocrine, and neurological diseases, suffer from important alterations in these systems. Although these limitations can be used as proof of the importance of the absence of microbes in the development of diseases, they also add additional pitfalls when evaluating the effects since significant cellular mechanisms are affected. Also, mono-colonization with one or multiple microbial species is also an experimental scenario that is far from what occurs environmentally.

The potential role of the gut microbiota in behavioral affective disorders has been demonstrated in multiple studies in which the microbiota has undergone its experimental manipulation. Recent observations that implicate an association between dietary components present in westernized diet, strongly correlated with changes in the gut microbiota and obesity, establishes new a paradigm for mental disorders. In this scenario, a diet that potentiates a significant change in the microbiota and metabolites can significantly modulate the immunological, endocrine, and neurological interrelated branches of the gut-brain axis, and affect unknown neurological and immunological shifts that could trigger brain dysfunction. According to this hypothesis, commensal microbes or their metabolic products could be considered potential therapeutic tools.

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Compliance with Ethical Standards

Conflict of Interest Javier Ochoa-Repáraz and Lloyd H. Kasper declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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