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REVIEW ARTICLE

Microbiota modulation as preventative and therapeutic approach in Alzheimer's disease

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The gut microbiota coevolves with its host, and numerous factors like diet, lifestyle, drug intake and geographical location continuously modify its composition, deeply influencing host health. Recent studies demonstrated that gut dysbiosis can alter normal brain function through the so-called gut–brain axis, a bidirectional communication network between the central nervous system and the gastrointestinal tract, thus playing a key role in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease (AD). In this perspective, in the constant search for novel treatments in AD, the rational modulation of gut microbiota composition could represent a promising approach to prevent or delay AD onset or to counteract its progression. Preclinical and human studies on microbiota modulation through oral bacteriotherapy and faecal transplantation showed anti-inflammatory and antioxidant effects, upregulation of plasma concentration of neuroprotective hormones, restoration of impaired proteolytic pathways, amelioration of energy homeostasis with consequent decrease of AD molecular hallmarks and improvement of behavioural and cognitive performances. In this review, we dissect the role of gut microbiota in AD and highlight recent advances in the development of new multitarget strategies for microbiota modulation to be used as possible preventative and therapeutic approaches in AD.

Introduction

Microbiota is a community of symbiotic microorganisms that can be neutral, beneficial or detrimental to the host, with important regulatory functions in health and disease. The human body hosts trillions of microorganisms (bacteria, archaea, fungi and viruses) that colonize the skin surface, the respiratory tract, genitourinary organs and, most importantly, the gastrointestinal tract. Approximately 95% of the symbiotic organisms of the human microbiome can be found in the gut (gut microbiota) [1]. Gut microbial ecosystem consists mainly of bacteria, mostly obligate anaerobes, fungi and viruses [2]. These diverse groups of microorganisms play multiple roles in humans, such as the fermentation of undigested carbohydrates, the production of short-chain fatty acids (SCFAs) and other metabolites, the synthesis of vitamins B and K, the metabolism of important substances (bile acids, sterols and drugs), and the protection against exogenous pathogens [3]. *Bacteroidetes* (~ 48%), *Firmicutes* (~ 51%), *Proteobacteria* and *Actinobacteria* (1%) are

Abbreviations
AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid beta; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; FMT, faecal microbiota transplantation; GABA, γ-aminobutyric acid; GIP, glucose-dependent insulinotropic polypeptide; GLP1, glucagon-like peptide-1; LPS, lipopolysaccharide; MCI, mild cognitive impairment; NFT, neurofibrillary tangles; SCFAs, short-chain fatty acids; TLRs, Toll-like receptors; UPS, ubiquitin–proteasome system.

the main four bacterial phyla present in adults [4]. The gut microbiota coevolves with humans and regulates host pathophysiology and health status via symbiotic interactions [5–8]. An example of this tight relationship is the crosstalk between microbiota and mitochondria [9–11], and these subcellular organelles (which evolved from ancestral bacteria) mediate the transduction of stress and metabolic signals, and are particularly sensitive to metabolites produced by other microbes associated with the gut and other mucosa [12].

The gut microbiota communicates with the central nervous system (CNS) through the bidirectional gut–brain axis involving different pathways: the neuroimmune system, sympathetic and parasympathetic branches of the autonomic nervous system, and neuroendocrine system [13] (Fig. 1). The gut–brain axis is responsible not only for the proper function of the digestive tract, but it also represents a biochemical signalling system linked to the functionality of sympathetic nervous system, endocrine glands and specific brain regions, such as the hypothalamus and the frontal cortex. Moreover, the gut–brain axis influences CNS development and behavioural performances in both normal and pathological conditions.

Although most bacterial metabolites are required for anabolic and catabolic functions, many of these compounds have other physiological roles. The gut microbiota synthetizes neurotransmitters such as γ-aminobutyric acid (GABA), noradrenaline and dopamine, modulates systemic immune cells, produces metabolites like SCFAs, metabolizes essential amino acids like tryptophan and activates the secretion of the nerve growth factor (NGF), the glial-derived neurotrophic factor (GDNF) and the brain-derived neurotrophic factor (BDNF) with consequent implications in neurodegenerative disorders [14–16]. In particular, BNDF plays a crucial role in the normal function and survival of neurons in mature peripheral and central nervous system [17]. Interestingly, germ-free mice showed a decreased expression of BDNF in the hippocampus, at both protein and mRNA levels, associated with impaired cognition [18,19].

Bacterial fermentation of indigestible carbohydrates in the colon produces SCFAs, metabolites implicated

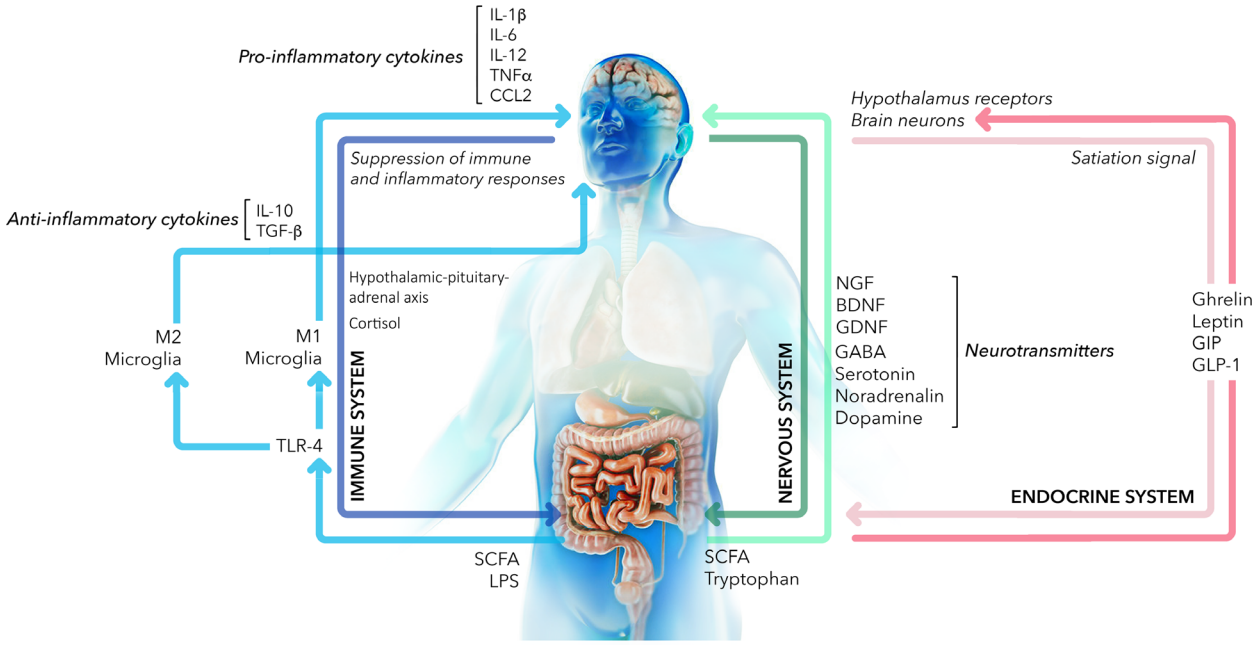

Fig. 1. Gut–brain axis. Schematic representation of the bidirectional communication network between the gut microbiota and the brain. Principal molecular mediators in the nervous system (green arrows), immune system (blue arrows) and endocrine system (pink arrows) are reported. In detail, gut bacteria produce metabolites like SCFAs and process essential amino acids like tryptophan triggering the secretion of the nerve growth factor (NGF), the brain-derived neurotrophic factor (BDNF) and the glial-derived neurotrophic factor (GDNF) and the synthesis of neurotransmitters such as γ-aminobutyric acid (GABA), noradrenaline and dopamine. Bacterial LPS stimulates TLR4 thus modulating systemic immune cells. Microglia cells are activated and polarized to the pro-inflammatory (M1) phenotype, resulting in the production of cytokines and chemokines like IL-1β, IL-6, IL-12, TNF-α and CCL2. Differently, SCFAs producing bacteria favour the anti-inflammatory M2 phenotype, with the secretion of IL-10 and TGF-β. Moreover, SCFAs stimulate endocrine cells of the gastrointestinal tract to synthetize neuroactive compounds like ghrelin, leptin GIP and GLP-1 that exert neuroprotective effects and regulate important metabolic functions.

also in neurotransmission, since they modulate the synthesis of several neurotransmitters regulating behaviour and cognition [20]. Acetate, propionate and butyrate are the most abundant gut bacterial metabolites that can act either as substrates for host metabolism or as signalling molecules. In particular, butyrate and its protonated form, butyric acid (pKa = 4.82), exist in different parts of the gastrointestinal tract (stomach: $1.5 < pH < 3.5$; intestine: $5.5 < pH < 7.4$), where both forms exert beneficial health effects, improving food digestion and nutrient absorption, downregulating the proliferation of pathogenic microflora and favouring the colonization of anti-inflammatory bacteria [21].

Specifically, systemic administration of butyrate determines an antidepressant-like behavioural response [22] and butyric acid, as well as valeric acid and propionic acid, was shown to successfully counteract the conversion of Aβ peptides to neurotoxic aggregates *in vitro* [23].

The gut microbiota exert a fundamental role in the digestion and absorption of amino acids that are important not only in protein synthesis, but also in the production of bioactive molecules that regulate key signalling pathways and metabolic pathways of the host [24]. Dysregulation of amino acid homeostasis can contribute to AD pathogenesis. In fact, changes in glutamate metabolism in AD brain consequently alter GABA concentrations thus affecting neural functioning. GABA is produced from glutamate metabolism by different bacterial species, mainly lactic acid bacteria [25,26]. It is the major inhibitory neurotransmitter in the brain that regulates adult brain function, synaptic plasticity, and cortical adaptation and reorganization thus representing an important mediator through which bacteria can modulate brain chemistry [27]. Altered concentrations of this neurotransmitter were detected in several conditions, including epilepsy [28] and schizophrenia [29], and dysfunctions of the GABA system were implicated in the pathophysiology of several chronic neurological diseases [30].

Changes in methionine, tryptophan, tyrosine and purine metabolism pathways were observed in both mild cognitive impairment (MCI) and AD subjects [31]. Interestingly, tryptophan is an essential amino acid largely found in meats, dairy products, fruits and seeds. It is not only absorbed through the intestinal epithelium to enter the blood circulation, but also directly and indirectly metabolized by the gut microbiota into several compounds with an active role in gut–brain axis [32]. It is the precursor of several metabolites, most notably kynurenine and serotonin [33]. Kynurenine can cross the blood–brain barrier (BBB) [34] and, once in the brain, it is the precursor of neuroactive glutamatergic compounds, including the neuroprotective kynurenic acid. Serotonin, a neurotransmitter active both in the central nervous system and in the gut, plays an important role in maintaining mood and cognition [35]. Alterations in the levels of serotonin can be associated with the onset of gastrointestinal and mood disorders, and tryptophan dysregulation is linked to detrimental conditions both in the brain and in the gastrointestinal tract [36].

Microbiota and related metabolites regulate BBB permeability. Among them, SCFAs can access the BBB via the bloodstream and can regulate its integrity through the upregulation of tight junction proteins [37]. Interestingly, the lack of gut microbiota is associated with increased BBB permeability and altered expression of tight junction proteins. Braniste *et al.* demonstrated the tight communication between gut microbiota and BBB that initiates during gestation and propagates throughout life. They showed that faecal transfer from mice with pathogen-free gut flora into germ-free mice or treatment of germ-free mice with bacteria that produce SCFAs decreased the permeability of the BBB and upregulated the expression of tight junction proteins [38].

The composition of the microbiota loses diversity and the abundance of beneficial bacteria decreases with ageing. This alteration directly and indirectly influences mitochondria functionality and energy production in intestinal cells, reducing the integrity of the intercellular junctional apparatus and increasing the translocation of bacterial products (principally lipopolysaccharide, LPS). This condition favours the so-called '*inflammaging*', which is strongly associated with numerous age-related diseases, whereas a healthy microbiota represents a key condition for longevity [25,39].

Moreover, dietary changes, antibiotic exposure and infections impair intestinal homeostasis promoting a condition known as dysbiosis [15], during which altered gut microbiota composition can damage the normal function of the intestinal barrier and increase intestinal permeability. Consequently, neuroactive compounds and gut microbial metabolites can reach areas of the central nervous system that regulate cognition [25]. Dysbiosis is observed in neurodevelopmental diseases such as autism and in neurodegenerations such as Huntington's disease, Parkinson's disease and AD [40]. Current research is trying to gather new knowledge on the underlying mechanisms.

In the following sections, the impact of dysbiosis on gut–brain axis in AD is described and recent advances in the identification of multitarget strategies for microbiota modulation to counteract the onset and progression of AD are summarized.

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder in the elderly and is a major challenge for the healthcare system given the impact it has on both economy and society. It is the main cause of dementia, and it is characterized by loss of neurons in the hippocampus and cerebral cortex, shrinkage of the cortex, enlargement of ventricles, resulting in the progressive decline in cognitive function. Memory impairment accompanied by visual space dysfunction and sleep deprivation was also linked to AD progression [22]. Key molecular hallmarks of the disease are the extracellular amyloid beta (Aβ) plaques and the intraneuronal neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein (Fig. 2).

Plaques are extracellular deposits of amyloid peptides deriving from the amyloid precursor protein (APP), a type I membrane protein. APP was considered a good target for therapeutic interventions in the early treatment of AD. APP can undergo nonamyloidogenic cleavage process mediated by α-secretase and γ-secretase that generates a soluble APP fragment (sAPPα) and a membrane-bound C-terminal fragment of APP (αCTF). Alternatively, APP can be cleaved by β-secretase and γ-secretase releasing two major fragments, sAPPβ and a C-terminal fragment located in the membrane (βCTF). Further cleavage of βCTF results in the production of Aβ peptides ranging from 37 to 43 amino acid in length, with Aβ(1-40) and Aβ (1-42) being the most dominant and neurotoxic [41,42]. Aβ monomers form oligomeric structures that can further aggregate into regular fibrils. According to the Aβ oligomer hypothesis, small soluble Aβ oligomers are considered more neurotoxic than insoluble fibres or amyloid plaques [43].

Defective proteolysis is another important contributor to AD pathogenesis, because it favours the accumulation of detrimental aggregates. In addition, it may also be secondary to accumulation of aggregates that can act as inhibitors for cellular proteolytic machineries, mainly the ubiquitin–proteasome system (UPS) and autophagy. The UPS is the major intracellular degradation system, responsible for the removal of short-lived, misfolded and defective proteins [44], and proteasome inhibition impairs both APP processing and Aβ production [45]. Autophagy includes degradation pathways that finally transport their targets to lysosomes, acidic membrane-surrounded compartments containing hydrolytic enzymes involved in the intracellular breakdown of long-lived proteins, organelles and substrates with limited access to catalytic chamber of

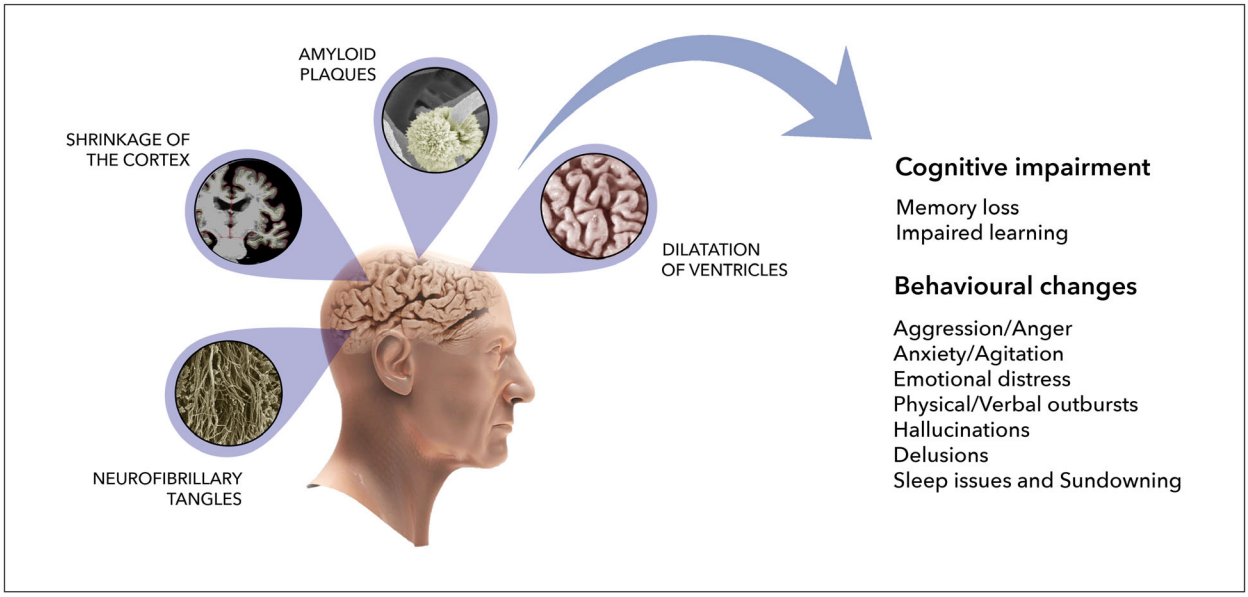

Fig. 2. Schematic representation of cerebral modifications in Alzheimer's disease. AD is characterized by loss of neurons in the hippocampus and cerebral cortex, shrinkage of the cortex, enlargement of ventricles, resulting in the progressive decline in cognitive function. The principal AD molecular hallmarks are the extracellular amyloid beta plaques and the intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau protein. SEM micrographs for neuronal deposition of amyloid plaques and neurofibrillary tangles are from Meyer *et al.* [194] and Itoh *et al.* [195], respectively. M.R.I. of the brain is from Ledig *et al.* [196].

the proteasome, such as larger aggregates [46]. Lysosomal enzymes, particularly cathepsin B and cathepsin L, can interfere with APP processing, thus altering Aβ formation [47]. Aβ deposition and removal are finely regulated by the UPS and autophagy [48–51]. Impairment of proteolysis, which is typical of AD neurons, favours the accumulation of harmful Aβ structures, which in turn alter both proteasome and autophagy functionality [52]. Failure of autophagy–lysosome-mediated proteolysis in AD brain leads to a massive accumulation of autophagic vacuoles and lysosomes in dystrophic neurites. Vacuoles contain incompletely digested proteins, including toxic autophagic substrates, ubiquitinated proteins and Aβ, indicating the importance of targeting autophagy to ameliorate neuropathology and cognitive deficits in AD [53]. Regarding UPS, some authors considered the proteasome a potential target in AD therapy because this multicatalytic protease complex regulates the intracellular concentration of both presenilins 1 and 2, and/or their presenilinase-derived C-terminal maturation fragments, thereby modulating both α- and β/γ-secretase-derived products APPα and Aβ(1-40) and Aβ(1-42). Based on the amyloidogenic hypothesis, proteasome activators would enhance presenilin degradation and lower Aβ peptide secretion [54]. Later, deficiencies of the amyloidogenic hypothesis were identified and a major role of tau in the development and progression of AD emerged [55]. Failure of proteasomal- and autophagy-mediated clearance of tau and its aggregates leads to a progressive neurofibrillary degeneration in AD [56,57]. Nontoxic approaches to restore both UPS and autophagy represent an important challenge to achieve Aβ and tau proteins correct balance.

The proteasome system is also responsible for the clearance of oxidized proteins [58,59]. Oxidative stress is one of the mechanisms through which Aβ neurotoxic peptides and tau protein cause impaired synaptic plasticity, neuroinflammation, neuronal and synaptic loss, and neurotransmitter imbalance in AD [60], contributing to the observed behavioural disturbances [61]. The key role of oxidative stress in the onset and progression of AD is largely documented: inadequate antioxidant defence systems, high O_2 consumption, the presence of excitotoxic amino acids and high iron content promote the production of unstable reactive oxygen and nitrogen species (ROS and RNS) in the brain [62,63]. ROS and RNS easily react with proteins, lipids, carbohydrates and nucleic acids, causing oxidative modifications that finally result in dysfunctions of cellular processes [64,65], among them impaired proteasome-mediated proteolysis [66]. Numerous evidences have described a crosstalk between proteasome and autophagy, with the overexpression of APP correlating with a reorganization of the cellular proteolytic machineries and with an increased oxidative status [48]. Also tau and tau aggregates are degraded by both the proteasome [67] and lysosomes [68].

Alzheimer's disease subjects are characterized by a compromised blood–brain barrier (BBB) that is permeable to neurotoxic components as well as pathogens and favours neuroinflammatory and neurodegenerative processes [69]. Both genetic and environmental factors can be involved. Apolipoprotein E variant 4 (APOE4) was found to be responsible for the increased risk of AD due to a leaky BBB. Individuals carrying APOE4 have a breakdown of the BBB in the hippocampus and parahippocampal gyrus, the regions of the brain responsible for memory and cognition. This condition is already detectable in individuals with MCI [70]. The increased permeability of BBB is also favoured by a dysregulated microbiota and causes the penetration of microbiota-derived products from the blood into the brain. In particular, gut microbes can excrete complex and immunogenic factors like LPSs and amyloids which may leak from the gastrointestinal tract and accumulate at the systemic and brain level contributing to the production and release of pro-inflammatory cytokines and reactive oxygen species, both responsible for AD neuroinflammation [71].

Neuroinflammation plays a major role in the pathogenesis of AD. A cascade of molecular events involves the activation of microglia, primary immune cells of CNS. Dysbiosis and intestinal infection trigger systemic immune response and cerebral inflammatory processes in AD [72]. Toll-like receptors (TLRs), which are present throughout the intestinal mucosa and in CNS, are fundamental in transducing molecular pathogenic patterns from the gut to the brain by modulating microglia response. TLRs are involved in commensal colonization, homeostasis maintenance and intestinal barrier integrity [73]. Aβ binding to TLRs was demonstrated to trigger an inflammatory process in the gut and the brain, contributing to the development of AD and other neurodegenerative diseases. Although all TLRs are highly expressed in intestinal epithelial cells, they have been also detected in CNS: in particular, TLR1-9 encoding mRNA is present in microglia; TLR2 and TLR3 are expressed in astrocytes and oligodendrocytes, in association with TLR1 and TLR4 [74]. Besides, certain TLRs have been also found in neurons [75].

Under physiological conditions, the CNS-resident macrophages constituting microglia ensure neuronal health by secreting trophic factors such as BDNF [76], by playing a role in synaptic pruning [77,78] and by

exerting phagocytic activity towards senescent cells [79] and toxic forms of Aβ [80]. Aberrant microglia activity or age-related decline of phagocytosis can cause inflammatory processes in CNS leading to cognitive impairment [81]. It was demonstrated that microglia activation by Aβ requires CD14 (lipopolysaccharide receptor), TLR2 and TLR4 [82]. These receptors are overexpressed in 3xTgAD mice [83] as well as in the brain of AD patients [84]. Contrasting studies indicate that microglia can exert a protective role in AD [85,86], or can induce inflammation [87] and accelerate the disease [88]. Since microglia and blood-derived macrophages share many membrane determinants and possess phagocytic activity [89], peripheral monocytes can enter the BBB and, upon differentiation, can exert an essential role for reparative and regenerative functions in AD [90].

TLR4, localized on the surface of microglia, contributes to induce amyloid phagocytosis [91]. Aβ is highly hydrophobic and can induce innate immune responses like those triggered by LPS. Furthermore, it has been shown that acute or chronic administration of LPS into the brain ventricles of rats and mice results in gliosis, cytokine production, increased Aβ concentrations and occasional cognitive deficits [92]. Glial activation due to LPS administration was thought to mimic some features of AD [93]. In this perspective, several works were conducted to directly stimulate TLR4 or to administer TLR4 agonists to enhance the activation of monocyte-derived macrophages and microglia, and to increase the natural clearance of amyloid deposits. Active phagocytosis of Aβ(1–42) deposits in the brain of AD patients can be obtained through the natural stimulation of the innate immune response by stimulating TLR4-mediated phagocytosis, or the active immunization through the administration of adjuvanted Aβ(1-42).

Alteration of gut microbiota in Alzheimer's disease

Gut microbiota can influence brain activity [19], and changes in relative abundance of taxa are documented in cognitively impaired subjects. In detail, a comparative analysis of the microbiome between healthy subjects and AD patients showed a lower prevalence of bacteria synthesizing the anti-inflammatory and neuroprotective SCFA butyrate and higher levels of pro-inflammatory taxa [94]. Older adults showed increased abundance of the pro-inflammatory bacteria *Escherichia/Shigella*, whereas individuals with evidence of amyloid deposition on PET imaging exhibited decreased abundance of the anti-inflammatory bacteria *Eubacterium rectale* [95]. Bacterial taxa correlate also with cerebrospinal fluid (CSF) biomarkers of AD pathology. Brandscheid *et al.* [96] observed an increased abundance of *Firmicutes* with a significant decrease in *Bacteroidetes* in 5xFAD mice, a model with severe amyloid pathology. Additionally, an increase in *Clostridium leptum* group was observed in AD mice [96]. Consistently, a study from Vogt *et al.* revealed reduced richness and abundance in alpha diversity and beta diversity in AD patients compared to healthy subjects. They evidenced differences in bacterial abundance such as decreased *Firmicutes*, increased *Bacteroidetes* and decreased *Bifidobacteria* in the microbiome of AD participants [97]. Colonization of *Bacteroides fragilis* was linked to exacerbation of AD along with increasing Aβ plaques in AD mice [98]. Interestingly, *Helicobacter pylori* was largely studied not only for its pro-inflammatory properties, but also for its ability to stimulate Aβ deposition [99] as well as tau hyperphosphorylation [100]. A recent comparative microbiome study confirmed a decreased microbial diversity in human AD patients with respect to mild cognitive impairment (MCI) patients and healthy controls. The study highlighted a progressive growth in the abundance of *Gammaproteobacteria, Enterobacteriales* and *Enterobacteriaceae* from healthy controls to AD subjects, increased glycan biosynthesis and metabolism in AD and MCI patients and decreased immune system-related pathways in AD patients. Among *Firmicutes, Lachnospiraceae, Clostridiaceae* and *Ruminococcaceae* were major SCFAs producers found to be lacking in AD patients [101]. Zhang *et al.* observed in APPSwe/PS1E9 transgenic mice a significant alteration of gut microbiome on the level of phylum, genus and species with age. Changes in the diversity and the composition of the faecal microbiota and SCFAs in these mice correlated with alterations of important metabolic pathways, which are associated with amyloid deposition and ultrastructural abnormalities in AD mouse intestine [102].

Microbiota modulation influences multiple molecular mechanisms through the gut–brain axis

The modulation of intestinal homeostasis triggers multiple mechanisms, among them the increase of anti-inflammatory microbial metabolism. As abovementioned, the gut microbiota is an important source of LPS and amyloid peptides. LPS stimulates TLR4 via CD14. Bacterial amyloid proteins help bacterial cells to aggregate in biofilms and to resist destruction by physical or immune factors [103,104] and induce CNS

inflammation in different ways. Despite the differences in the sequence, bacterial amyloid peptides share similarities in their tertiary structure with CNS amyloids [105,106]. The exposure to bacterial amyloid proteins in the gut can cause priming of the monocytes/macrophages system, leading to endogenous production of neuronal amyloid in the brain [104]. Microglia can recognize and phagocyte Aβ by receptor-mediated endocytosis that activates signalling pathways and cytokine production in a ligand-dependent manner [107]. In AD, microglia are activated and polarized to the proinflammatory (M1) phenotype. M1 microglia produce cytokines and chemokines (IL-1β, IL-6, IL-12, TNF-α, CCL2) and express NADPH oxidase generating reactive oxygen and nitrogen species. M1 microglia also express MHC-II, integrins (CD11b, CD11c), costimulatory molecules (CD36, CD45, CD47) and Fc receptors. Differently, M2 microglia produce anti-inflammatory cytokines (IL-10, TGF-β), growth factors (IGF-1, FGF, CSF1) and neurotrophic factors (nerve-derived growth factor (NGF), BDNF, neurotrophins, glial cell-derived neurotrophic factor (GDNF)) [108].

Scientists totally agree on the fundamental neuromodulatory role of SCFAs. These well-recognized anti-inflammatory bioactive bacterial metabolites provide cells (including nervous cells) with energy; they are involved in cell signalling systems by influencing the intracellular levels of potassium, and directly affect brain neurochemistry by regulating the expression of the gene coding for tryptophan hydroxylase, the key enzyme in the serotonin biosynthesis pathway [109]. SCFAs interfere with the activity of DNA repair enzymes by decreasing the activity of chromosome histone deacetylases (HDACs); for example, the HDAC inhibitor 4-phenylbutyrate was investigated for its ability to restore dendritic spine density in the hippocampus of Tg2576 mice, coupled with decreased Aβ load and tau phosphorylation, producing positive effects on cellular protein homeostasis [110], with the main disadvantage of a high therapeutic dosage required (up to 15 g/day) [111]. Undoubtedly, microbiota modulation strategies that favour SCFAs producing bacteria represent a feasible and sustainable approach to ameliorate intestinal ad neuronal homeostasis.

Interestingly, SCFAs are able to stimulate endocrine cells of the gastrointestinal tract to synthetize neuroactive compounds like histamine, serotonin, γ-aminobutyric acid, β-alanine, peptide YY, leptin and glucagon-like peptide-1 (GLP-1) [112-114]. Gut peptide hormones have a documented role in AD as they regulate energy homeostasis and food intake and modulate nervous functions like learning and memory [115-117]. For example, ghrelin and leptin are neurotrophic hormones [115,118]. Specifically, ghrelin affects both glucose and lipid metabolism, and it influences mitochondrial respiration and exerts neuroprotective effects and therefore is involved in the aetiopathogenesis of neurodegenerative disorders, representing a link between metabolism and neurodegeneration [119]. In AD, memory and learning impairment is closely associated with the age-related decline of plasma ghrelin concentration [120]. Conversely, plasma leptin concentration is negatively correlated with Aβ levels due to its direct regulatory effect on γ-secretase [121]. Interestingly, treatment with leptin reduced both Aβ and p-tau levels in AD animal models [122,123]. Also, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) act as neuroprotective hormones. Several studies described GLP-1 ability to protect cultured neurons from oxidative damage and Aβ plaque formation, and the capability to improve synaptic plasticity in mice [124,125]. Additionally, increased plasma levels of gut hormones such as GLP-1, leptin, ghrelin and GIP partially restore hippocampus functions in AD subjects [119,126]. In this framework, the use of GIP analogues has emerged as promising strategy in AD treatment [127,128].

Since in many cases of early-onset AD, Aβ peptides originate from mutations in genes encoding APP and presenilins (1 and 2), past and present therapies focused on the improvement of α-secretase activity to promote the nonamyloidogenic pathway, and on the modulation of proteolytic systems in charge of the clearance of amyloid deposits. The UPS and autophagy are the main cellular proteolytic systems [48–51], and there is a mutual effect of harmful Aβ structures on both proteasome and autophagy functionality [52]. Both autophagy [53] and UPS have been identified as attractive therapeutic targets in AD, not only to degrade presenilins and lower Aβ peptides production [54], but also for the clearance of tau and its aggregates [56,57]. Drug-based pharmacological modulators of proteolytic machineries are detrimental because of their toxicity, whereas natural product-derived approaches represent nontoxic options to restore both UPS and autophagy in several pathologic conditions including AD. A partial but significant recovery of both proteolytic pathways was obtained through microbiota modulation in AD transgenic mice, evidencing another important molecular target to achieve Aβ and tau proteins correct balance. Moreover, autophagy induction can mediate the inhibition of Wingless-related integration site (Wnt) signalling, which affects differentiation of the CNS [129] and causes the

reduced expression of fibroblast growth factor 9 (FGF9) in the CNS, thus explaining the improvement of behavioural performances in probiotic treated animals [130]. The crucial role of the proteasome in the clearance of oxidized proteins has been described [58,59]. A proper modulation of gut microbiota can directly and/or indirectly influence the oxidative balance in the CNS, increasing the degradation of oxidized proteins and positively interfering with antioxidant systems [131]. Interestingly, probiotics oral administration ameliorated oxidative status in transgenic AD mice by improving the functionality of antioxidant enzymes and decreasing oxidized proteins, lipids and DNA also through the activation of sirtuin-1 (SIRT-1) [132], a deacetylase enzyme with established neuroprotective action, able to lower ROS levels and promote cell survival [133].

Gut microbiota modulation inevitably impacts key metabolic functions such as glucose homeostasis which is impaired in AD. Defective insulin signalling strongly contributes to brain metabolic impairments in AD. For example, deficient cerebral amount of insulin growth factor I (IGF-I) is associated with compromised Aβ clearance [134] and reduced proteolysis of oxidized proteins by the proteasome [135]. The levels of both IGF-I and its receptor significantly decrease in the hippocampus and somatosensory cortex of aged mice, causing age-related changes in the brain [136]. Moreover, tau hyperphosphorylation occurs in mice having abnormal brain insulin levels [137]. Specific gut microbial alterations can indicate a signature of the pathology. In this perspective, personalized intervention, using, for example, probiotics and prebiotics, represents a successful revolutionary approach to restore the optimal concentrations of healthy promoting bacteria that contribute to normalize host glycaemic response and insulin sensitivity.

Figure 3 summarizes the multiple molecular and metabolic mechanisms that are influenced by gut–brain axis modulation. Studies on animal models and human subjects show that modifications of gut microbiota reflect changes in genes involved in inflammatory and neuronal plasticity processes, with a positive impact on neuronal function [138,139].

Novel therapeutic strategies: diet-based approaches and faecal transplantation

As previously described, ageing, infections, unhealthy dietary habits and lifestyle behaviours can alter gut microbiota composition and diversity favouring the onset and progression of neurodegenerative disorders including AD. Since dysbiosis is strictly correlated with alterations of intestinal permeability, dysfunctions of BBB and neuroinflammatory processes [140,141], strongly participating in the development of AD, gut microbiota can also represent a key to tackle AD. Specifically, in the absence of a definitive treatment for AD, with most therapies simply delaying the loss of cognition and memory, recent studies have focussed on the role of the human microbiome in regulating multiple neurochemical pathways through the gut–brain axis and on looking for new therapeutic approaches for microbiota modulation [142–144]. A diet rich in saturated fat, carbohydrates and highly processed foods may have detrimental effects on health contributing to the reduction of microbiota diversity, neuroinflammation and cognitive impairment. On the other hand, healthy dietary patterns show

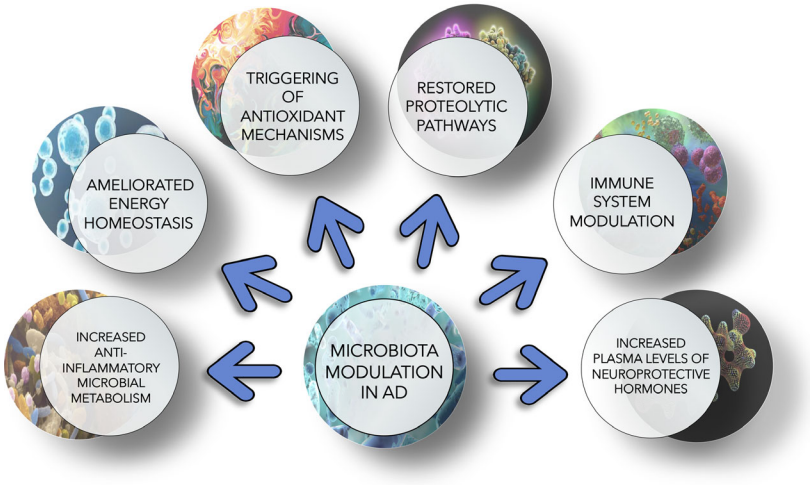

Fig. 3. Schematic representation of metabolisms and pathways affected by gut microbiota modulation. Microbiota modulation targets multiple molecular mechanisms to ameliorate AD condition, among them inflammatory and oxidative processes, proteolytic pathways, gut–brain axis, immune system components and energy metabolism.

neuroprotective properties and can be beneficial to host cognitive health. On this regard, the Mediterranean diet is rich in many components considered helpful for AD subjects, among them vegetables, legumes, fruits, cereals and a high intake of unsaturated fatty acids and polyphenols [145]. In addition, oral bacteriotherapy has been recently identified as an accepted practice for the prevention and treatment of gastrointestinal infections [146] and inflammatory conditions [147,148]. Beneficial effects of lactic acid bacteria and bifidobacteria in CNS-related diseases have been reported [149–154]. Diet-based therapeutic interventions include probiotics administration through specific supplements or probiotics enriched foods, foods rich in prebiotics, supplementation with polyphenols, calorie restriction and consumption of digestion-resistant fibres.

Besides dietary interventions, faecal microbiota transplantation (FMT) is another promising therapeutic option against AD that targets intestinal microbes, which involves the transfer of stool from a healthy donor into the gastrointestinal tract of a patient in order to restore diversity and function of the microbial population. This approach is currently considered a valid treatment for recurrent *Clostridioides difficile* infections, and it was successfully tested in intestinal conditions including inflammatory bowel disease (IBD), diarrhoea, irritable bowel syndrome (IBS) and constipation, and is now being investigated for its possible application in extraintestinal conditions such as metabolic and neuropathological conditions (Fig. 4) [155]. FTM is considered a safe therapeutic procedure with minor and transient side effects due to the introduction of live microorganisms and associated metabolites. However, it is of extreme importance to properly screen the donor and the faecal material in order to avoid contamination of the patient with pathogenic microorganisms that could lead to serious infections.

In the following sections, we report recent findings on the use of these new microbiota-modulating strategies.

Preclinical studies in animal models

Acting on the microbiota through specific strategies such as intervention with beneficial microbes or diet modifications could be considered a promising preventative and therapeutic approach in AD. Particularly, single- or multistrain probiotic preparations turned out to be successful examples of oral treatments. These formulations are usually made of *Lactobacillus* and *Bifidobacterium* species since members of both groups have been used extensively in promoting human health and are classified as GRAS (generally regarded as safe) for human consumption [156]. We recently reported on the beneficial properties of SLAB51, a formulation of lactic acid bacteria and bifidobacteria able to modulate microbiota in 3xTg-AD mice increasing the

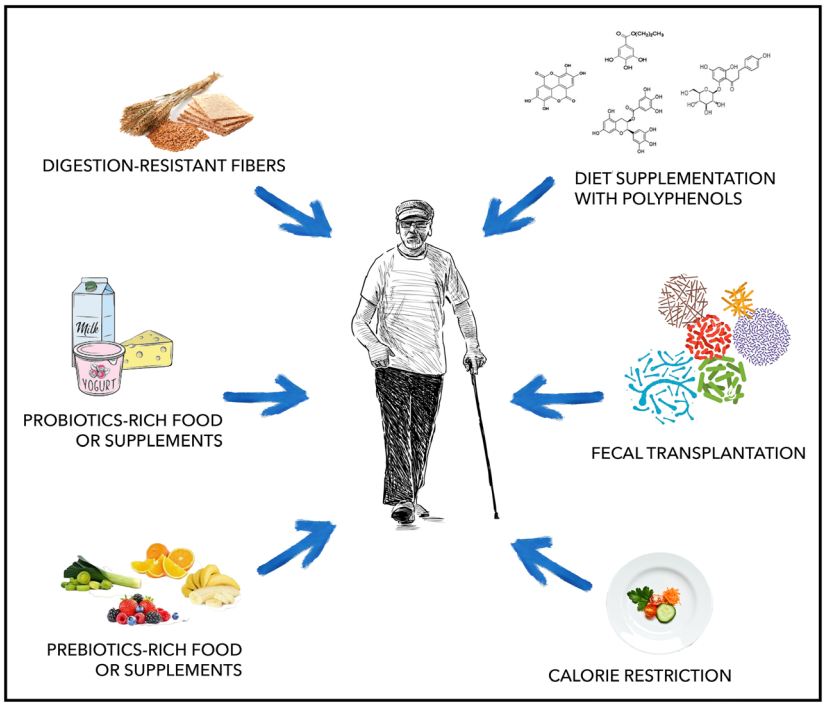

Fig. 4. Strategies used to modulate gut microbiota composition. Diet-based strategies and faecal transplantation are considered promising approaches to regulate function and composition of gut microbial population, favouring the abundance of beneficial bacterial groups.

relative abundance of *Bifidobacterium* spp. and decreasing *Campylobacterales*, bacterial groups differently involved in the regulation of inflammatory pathways. These changes in microflora composition together with enriched gut concentration of SCFAs and increased plasma levels of neuroprotective gut peptide hormones contributed to counteract cognitive decline through a reduction of Aβ aggregates and brain damages, and a partial restoration of impaired neuronal proteolytic pathways [130]. SLAB51-mediated microbiota modulation also mitigated oxidative stress by activating SIRT1-dependent mechanisms and restored glucose homeostasis in 3xTg-AD mouse brain [132,157]. ProBiotic-4, another formulation containing *B. lactis*, *L. casei*, *B. bifidum* and *L. acidophilus*, significantly improved cognitive functions and attenuated intestinal and BBB injury in aged SAMP8 mice through inhibition of both TLR4- and RIG-I-mediated NF-κB signalling pathways and inflammatory responses [158]. The mixture exerted a modulation of SAMP8 mouse microbiota with a marked decrease of *Proteobacteria* (phylum), *Pseudomonas* (genus) and *Lachnospiraceae_NK4A136_group* (genus) and a significant lower Firmicutes/Bacteroidetes ratio than vehicle-treated SAMP8 mice [158]. A probiotic mixture containing *Lactobacillus acidophilus*, *L. fermentum*, *Bifidobacterium lactis* and *B. longum* administered for 8 weeks to Aβ(1-42)-injected rats improved spatial memory and learning deficits and decreased oxidative stress by modifying microbiota [159]. Manipulation of the gut microbiota with a combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 significantly decreased serum and hippocampus levels of pro-inflammatory cytokines, alleviated hippocampal apoptosis and attenuated the detrimental effect of LPS on memory through BDNF protein expression in LPS-induced rats [160,161]. Short-term administration of *Bifidobacterium breve* strain A1 prevented cognitive decline in AD mice, with a reduction in the immune response and neuronal inflammation. However, the authors did not detect a marked effect on intestinal microbiota composition, indicating the involvement of other mechanisms in the probiotic final effect, such as the gut–brain communication via stimulation of the vagus nerve [162]. The effects of bacteria on cognition can also depend upon the type of strain that is administered. On this regard, Savignac H.M. *et al.* demonstrated that treatment with *B. longum 1714* improved the ability of learning and memory in an anxious mouse model, whereas *B. breve 1205* had little or no positive impact on memory [163,164]. A recent article introduced the possibility for probiotics to be used also in combination with traditional AD drugs to potentiate their beneficial effects. In details, *L. plantarum* augmented the therapeutic efficacy of memantine in APP/PS1 mice by remodelling the intestinal microbiota, inhibiting the synthesis of trimethylamine-N-oxide (TMAO), a gut microbial metabolite able to promote AD progression, and reducing clusterin levels. Moreover, a 12-week treatment with memantine in combination with *L. plantarum* ameliorated cognitive deterioration, decreased hippocampus Aβ levels, and protected neuronal integrity and plasticity [165].

Several studies on microbiota and probiotic interactions involve *Drosophila melanogaster*, which is considered an excellent model for microbiota research in view of its easily manipulated microbiome, high-throughput screening capabilities, low costs, and fast reproduction and is currently used to investigate the mechanisms at the basis of AD [166,167]. The administration of *Lactobacillus plantarum* DR7 to an AD-induced *Drosophila melanogaster* model rescued the rough eye phenotype and restored the gut microbiota diversity with a significant reduction in *Wolbachia*'s relative abundance, positively correlated with neurodegenerative disorders, accompanied by an increase of *Stenotrophomonas* and *Acetobacter* [168]. A symbiotic preparation containing three metabolically active probiotics and a polyphenol-rich prebiotic increased survivability and motility, rescuing Aβ deposition and acetylcholinesterase activity in a transgenic humanized *Drosophila melanogaster* model of AD through the effect on gut–brain axis components and on PPARγ activity [169].

Numerous studies also highlighted that dietary interventions with specific nutrients or combination of nutrients may act on gut microbes and their metabolites to ameliorate AD neuropathology. Diet supplementation with prebiotics was demonstrated to be a possible strategy to attenuate AD symptoms by modulating the microbiota. Prebiotics are dietary supplements used as food source by the microflora that offer a health benefit to the host regulating gut microbiota composition. Inulin, a well-studied prebiotic compound, enhanced systemic metabolism and decreased hippocampus inflammatory gene expression modulating gut microbiome composition in E4FAD mice even before the development of Aβ [170]. Treatment of APP/PS1 transgenic mice with fructooligosaccharides (FOS), commonly found in fruits and vegetables, changed microbiota composition and activated the GLP-1 pathway with consequent amelioration of cognitive deficits and pathological changes. In details, FOS reduced the groups of *Proteobacteria*, associated with dementia and immunological reactions and inflammation, of *Helicobacteraceae* and *Desulfovibrionaceae* and

reversed the decrease of *Lactobacilli* observed in untreated transgenic animals [171]. Oligosaccharides from *Morinda officinalis* administered to APP/PS1 transgenic mice significantly improved memory, brain tissue swelling and neuronal apoptosis and downregulated the expression of Aβ(1-42). These molecules were able to regulate the composition and metabolism of the gut microbiota in treated transgenic mice [172]. Syeda *et al*. demonstrated that the ingestion of bioactive food composed of nopal, soy protein, chia seed and turmeric reduced the amount of pro-inflammatory bacteria simultaneously increasing the anti-inflammatory ones, including *A. muciniphila* and *F. prausnitzii,* in 3xTg-AD mice [173]. This variation in microflora exerted numerous beneficial effects on the main pathological markers of AD such as a better cognitive outcome, decreased amyloid aggregates and hyperphosphorylation of tau, diminished oxidative damage, neuroinflammation, synaptic and metabolic alterations [173]. Sesamol, an antioxidant lignan from sesame oil, was able to reshape gut microbiome and improve the generation of SCFAs in high-fat diet rats. The effect on the gut was then accompanied by an ApoE-dependent improvement of cognitive deficits, anxiety, and synapse ultrastructure and inhibition of Aβ accumulation [174]. Treatment with jatrorrhizine, a main component of Coptidis rhizome, a traditional Chinese herbal, alleviated learning and memory deficits, reduced the levels of Aβ plaques in the cortex and hippocampus of APP/PS1 mice. Jatrorrhizine administration clearly affected mouse microbiota modulating the relative abundance of the most predominant phylum Firmicutes and Bacteroidetes in transgenic mice [175]. Also calorie restriction (CR) was effective in promoting neuroprotective effects and health benefits in different animal models, being also able to counteract AD symptoms [176-178]. Cox *et al*. recently reported on the effects of CR on the microbiome of Tg2576 mice during ageing, showing that CR can rescue age- and APP-related microbiome alterations and prevent the enrichment of microbes associated with AD age-related cognitive decline [98].

Besides oral administration of bacterial strains or nutrients, faecal microbiota transplantation (FMT) from a healthy donor to a patient or diseased animal is an alternative microbiota-targeted intervention, which represents a potentially attractive therapeutic approach against AD. A recent article by Kim *et al*. focused on this strategy, further contributing to elucidate the intricate relationship among the gut, blood and brain axis and AD. They demonstrated that fresh faecal matters oral transfer of healthy wild-type mice in ADLP^{APT} mice for 16 weeks and faecal microbiota transplantation in antibiotics-pretreated ADLP^{APT} mice for 4 weeks alleviated Aβ deposition, tau pathology, reactive gliosis and memory impairment in these transgenic AD mice. Interestingly, FMT successfully reversed abnormalities in intestinal macrophage activity and circulating blood inflammatory monocytes in the ADLP^{APT} recipient mice [179]. 16S ribosomal RNA sequencing analyses revealed that FMT reversed the alterations observed in microbial composition of APP/PS1 transgenic mice such as the abnormal enrichment in *Proteobacteria*, *Verrucomicrobia, Akkermansia* and *Desulfovibrio*, and the downregulation of Bacteroidetes and *Alloprevotella*. Modulating transgenic mouse microbiota and the associated SCFAs production, FMT improved cognitive deficits, decreased phosphorylation of tau protein and the levels of amyloid peptides, and ameliorated synaptic plasticity [180]. Cognitive dysfunctions and α- and β-diversity indices of pseudo germ-free mice (in detail, C57BL/6 mice receiving broad-spectrum antibiotics dissolved in drinking water for 14 consecutive days) were deeply ameliorated upon FMT from senescence-accelerated mouse resistant 1 (SAMR1) mice, but not from SAMP8 mice, further confirming that improving unhealthy gut microbiota may provide valid treatment for AD [181]. Interestingly, FMT associated with calorie restriction improved glucose tolerance, insulin sensitivity and lipid metabolism and regulated immune system in mice, indicating positive implications in metabolic disorders [182] and suggesting larger therapeutic potential, also in AD, considering that glucose intolerance and impairment of insulin metabolism are strictly connected with a higher risk of developing AD [183].

Human clinical studies on microbiota modulation

An increasing number of nutritional interventions to modify gut microbiota are documented in humans, principally involving old adults with memory complaints and healthy volunteers, also considering that AD, insulin resistance, diabetes, obesity and cardiovascular disease are strongly interconnected [152] and that there is an urgent need to establish preventative strategies. Specifically, it was reported that chronic supplementation with *Bifidobacterium breve A1* restored cognitive functions in old people with impaired memory [184]. Conversely, in some cases, experimental groups receiving placebo, instead of probiotics, obtained significantly better memory scores [185]. Nagpal *et al*. identified significant differences between the gut microbiome of MCI patients and cognitively

normal subjects, highlighting specific gut microbiome signatures that are associated with MCI and that correlate with cerebrospinal fluid levels of Aβ, total tau and phosphorylated tau [186]. In the same study, a modified Mediterranean ketogenic diet influenced the abundance of specific bacterial taxa (increasing the abundance of *Enterobacteriaceae*, *Akkermansia*, *Slackia*, *Christensenellaceae* and *Erysipelotriaceae* and reducing *Bifidobacterium* and *Lachnobacterium*) and SCFAs (increasing propionate and butyrate) consequently improving AD biomarkers in the cerebrospinal fluid of treated MCI patients [186].

Interestingly, a randomized, double-blind and controlled trial conducted by Akbari and collaborators evidenced that chronic supplementation with milk enriched with *L. acidophilus*, *L. casei*, *B. bifidum* and *L. fermentum* improved learning and memory in AD patients. Probiotics positively influenced the levels of malondialdehyde and high-sensitivity C-reactive protein, improved insulin resistance, pancreatic beta cell secretion, and metabolic status with respect to controls [187].

Another study reported that AD patients supplemented with a multispecies probiotic formulation influenced gut bacteria composition decreasing faecal zonulin concentrations and increasing *Faecalibacterium prausnitzii*. In these patients, enhanced kynurenine serum concentrations influenced tryptophan metabolism and stimulated the immune system [188].

No data on FMT in human AD patients were found but results from ongoing clinical trials are expected in the near future. Although animal models represent great opportunities to reveal new insights into microbiota–host interactions, they cannot fully nor accurately reproduce the human phenotype and there is an urgent need for additional human clinical studies with well-defined targets, and standardized protocols and outcome measures.

Conclusions

Alzheimer's disease is the most diffuse incurable dementia, and the identification of a definitive therapeutic intervention is a major challenge of our time. Dysbiosis was demonstrated to be a relevant risk factor for AD [101], with lifestyle, geographical location, drug assumption and dietary habits continuously being capable of modifying the gut microbiota composition. Diet rich in saturated fat and simple carbohydrates increases the risk of dementia and a suboptimal diet is associated with a more severe impaired cognition in AD [189]. Differently, a high quality diet like the Mediterranean diet correlates with better cognitive status in healthy people with reduced risk of developing MCI and AD [190]. In this context, the possibility to modulate the composition of gut microbiota using probiotics, prebiotics and other dietary intervention represents a promising and sustainable approach. Dietary interventions are generally safe and more advantageous than drug-based therapies since probiotics, prebiotics and synbiotics are cheap and easy to handle, thus reducing the burden also for AD patient caregivers. Similarly, FMT was described as a promising procedure, although some adverse effects were documented in infections from *Clostridium difficile* [191] or ulcerative colitis [192], indicating that intersubject variability must be considered and that a long-term follow-up is necessary to assess the risks and benefits. Moreover, standardization of methods used for microbiota analysis (sampling, preservation and storage of samples, and analytic procedures) will facilitate comparison between studies, enhancing the reproducibility [193]. The rapid advances of metabolomics and informatics will help in managing the vast databases deriving from ongoing and short-coming microbiota studies.

Successful results depend upon the optimization of different factors including proper combinations of bacterial strains and nutrients, time of treatment, disease stage therefore the presence of specific procedures and guidelines are necessary to enhance effectiveness of gut microbiota modulation.

The identification of AD-specific signatures in gut microbiota together with a better knowledge of the molecular mechanisms triggered upon microbiota modification will contribute to identify multiple personalized interventions for decreasing AD risk, delaying the onset of the pathology, and counteracting the appearance or improving the clearance of AD hallmarks.

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Author contributions

LB organized the contents, and drafted and wrote the manuscript. VC wrote, edited and revised the article. OG and CG drafted and wrote the manuscript. MC designed the figures and revised the article. GR and MA wrote and critically revised the article. AME conceived and designed the work, critically revised the manuscript, and edited and approved the final version to be published.

Conflict of interest

The authors declare no conflict of interest.

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