Probiotics treatment can improve cognition in patients with Mild Cognitive Impairment.

A Systematic Review

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Runing title: Probiotics an effective tool to prevent cognitive decline.

Abstract

Background. In recent years, the existence of the gut-brain axis and the impact of intestinal microbiota on brain function has received much attention. Acumulated evidence has prompted to the postulation of the *infectious hypothesis* underlying or facilitating neurodegenerative diseases, such as Alzheimer's disease. Under this hypothesis, the intervention with probiotics could be useful at a preventive and therapeutic level. *Objective***.** The objective of this systematic review is to reveal the benefit improving cognitive function following the use of probiotics in individuals with mild cognitive impairment. *Methods***.** We searched bibliographic databases and analyzed in detail the evidence and methodological quality of five recent randomized, double-blind, placebo-controlled clinical trials using the Cochrane Tool and the SIGN checklist. *Results***.** Overall, and with satisfactory methodological quality, the studies evaluated support the use of probiotics as a weapon to slow the progression of cognitive decline in subjects with mild cognitive impairment. The literature review also indicates that maximum benefit of probiotics is found in subjects with incipient cognitive dysfunction and has no effect in those with advanced disease or absence of disease. *Conclusion.* These results support the intervention with probiotics, especially as a preventive approach. However, caution is required in the interpretation of the results as microbiota has not been evaluated in all studies, and further large-scale research with a prolonged study period is necessary to ensure the translatability of the results into real practice.

Keywords:

Brain-Gut Axis, Cognitive Dysfunction, Alzheimer Disease, Gastrointestinal Microbiome, Probiotics.

Introduction

Gut microbiota

The microbiota is a community of symbiotic micro-organisms that can be neutral, beneficial, or detrimental to the host, with important regulatory functions in health and disease. At the genetic level, more than 99% of the genes in our body are microbial, amounting to more than 10 million [1, 2]. There is a distinct microbiome in almost every niche of the human body. Bacterial are found mainly in the skin, eyes, respiratory, urogenital, and gastrointestinal tract. In order of abundance, while the oral [3] and pulmonary [4] microbiota are important, the majority (approximately 95%), reside in the gut, referred to as the "gut microbiota" [5].

Within the tract, vast majority resides in the distal part of the tract, since the hydrochloric acid in the stomach and bile and pancreatic secretions in the proximal small intestine prevent colonisation, allowing concentration ranges between 10^1 and 10^3 CFU/ml. This bacterial density increases progressively in the small intestine with 10^4 to 10^7 CFU/ml from jejunum to ileum and reaches an estimated 10^{11} to 10^{12} CFU/ml bacteria per gram of intestine in the colon [6]. The advance of next-generation sequencing technologies over the last decade together with the development of bioinformatics is making the analysis of the composition of the microbiota more sophisticated and affordable, leading to an exponential advance in the knowledge of microorganisms that colonise human gastrointestinal tract [7]. Thus, in the gut microbiota Bacteroidetes and Firmicutes (stand out as the two dominant phyla (70-75% of the total). In a healthy microbiota, the genus within these phyla must be balanced. For example, within the Firmicutes phyla Lactobacillus presence is healthier that the presence of Clostridium or Enterococcus. Other phyla, such as Proteobacteria and Actinobacteria are less represented. Fungi, viruses, yeasts, archaea and protozoa are also present in approximately 1% [8].

While the inherited genome is essentially stable throughout the life of the host, the microbiome is immensely diverse and dynamic [9]. The composition of these microbes can be influenced by different factors early in life (with relevance to the birth (vaginal or caesarean) [10, 11]). With development and aging, intrinsic and environmental factors including diet, commonly used drugs and antibiotics, smoking, lifestyle, host genetics and disease will greatly influence gut microbiota [12].

Although significant variations may exist between the microbiota of different individuals, the gut microbiota present in the same anatomical region between two people is much more similar than the microbiota of two regions (e.g. gut and vaginal microbiota) in the same person. Despite the inter-individual variability, the functions that the microbiota exert on the organism is stable and constant in each location, allowing the functions of each microbiota to be generalised.

Under healthy conditions, the gut microbiota plays a vital role in gut homeostasis and host energy metabolism, performs immunomodulatory, metabolic, anti-inflammatory and, more recently recognised, neuromodulatory functions via the gut-brain axis [12] (Figure 1).

Some of the important functions performed by the microbiota include: **1. Vitamin synthesis**. Strains of the genus Escherichia produce vitamin K, B6 and B12; other beneficial bacteria produce pantothenic acid, folic acid, thiamine (vitamin B1), riboflavin (vitamin B2) and promote the absorption of calcium and iron in the colon. They are also capable of neutralising nitrates, xenobiotics and other toxic substances [13]. **2. Fermentation of undigested carbohydrates.** Some plant-derived carbohydrates such as cellulose and pectins reach the large intestine virtually intact. Primarily in the caecum and descending colon, the microbiota can ferment undigested carbohydrates, generating H_2, CO_2 and short-chain fatty acids (SCFA). Acetate, propionate and butyrate account for 95% of SCFA [14]. They modulate intestinal function by increasing luminal osmotic pressure, inducing water secretion and, together with the gases generated, increasing stool bulk, all of which stimulate intestinal peristalsis [15]. SCFA can also be absorbed and influence host energy homeostasis, including appetite regulation. Butyric acid is used by enterocytes as an energy source, while acetate and propionate go to the liver and enter the sugar and lipid metabolism pathways. This results in energy recovery from the diet and promotes ions absorption in the caecum. Anaerobic metabolism of peptides and proteins (putrefaction) occurs in more distal segments of the colon and is also a source of SCFA, but, at the same time, it can generate potentially toxic substances including ammonia, amines, phenols, thiols, and indoles [16, 17]. **3. Colonisation resistance**. Microbiotas prevent mucosal colonisation by pathogens by three main mechanisms: physical interference, production of antimicrobial compounds and co-aggregation with pathogens. Bacteria develop adhesins on their surface that bind to the mucosal glycocalyx, forming biofilms that prevent the establishment of pathogens. On the other hand, they produce antimicrobial

compounds (from toxic metabolites to bactericidal substances) and induce pH changes. Finally, co-aggregation with pathogens prevents their binding to the mucosa and facilitates their elimination [18]. **4. Differentiation of the immune system**. The gastrointestinal microbiota stimulates maturation of the immune system both locally and systemically. Bacteria increase the proportion of mucus-secreting goblet cells and promote deeper crypts, where Paneth cells settle, improving intestinal barrier permeability. Microbes provide a potent stimulus for the expansion of mucosa-associated intraepithelial lymphoid tissue (MALT), including Peyer's patches and mesenteric lymph nodes, and lead to the differentiation of T-helper lymphocytes and cytokines, conditioning the functions of dendritic cells, B-lymphocytes, and epithelial cells themselves [19]. **5. Interaction with the nervous system.** Microbial metabolites are precursors of several amino acids (e.g. tryptophan, y-aminobutyric acid GABA) and monoamines such as serotonin and dopamine, assuming an important role in neurotransmission and cognition [20]. Many other mechanisms describe bidirectional communication along the gut-brain axis (*see section 1.2*) and is a fundamental aspect of the synergy between microbiota and host, capable of modulating host behaviour.

Gut microbiota and ageing

The microbiota of an individual varies throughout life (Figure 2). Thus, in the early years, the microbiota is strongly conditioned by the type of birth (natural or caesarean) and feeding (maternal breastfeeding or formula feeding). Bacteria of the phylum Actinobacteria (genus Bifidobacteria) predominate at early ages [21]. This evolves, and in adulthood, Bacteroidetes and Firmicutes phylum predominate, but the intestinal ecosystem achieves the greatest variety. Many external factors such as diet and medication cause changes in the gut microbiota composition. In the elderly, the microbiota loses biodiversity, and pathogenic bacteria such as Clostridium become more important [22]. The fact that this trend is much more pronounced in developed countries [23] highlights the negative influence of certain environmental factors specific to these societies on the microbiota (e.g. contaminants, sedentary lifestyles, obesity, stress, high-calorie diets or diets with excess sugar and/or fat).

While the composition of the adult gut microbiota is generally stable, ageing, and age-related inflammation have been linked to deterioration of this stability [24,26]. Moreover, in aged humans, a decrease in Firmicutes phyla and an increase in Bacteriodetes phyla, resulting in a reduction in the

Firmicutes/Bacteriodetes (F/B) ratio was found in an Ukranina population [27], promoting progression, together with the increased presence of pathogenic species. Age-related changes in gut microbiota composition are linked to various parametres of functional health, including frailty, cognition, depression, and inflammatory markers [28, 29].

Gut-Brain Axis

In the 1880s, William James and Carl Lange first introduced the concept that bidirectional communication between the CNS and gut organs plays a role in emotional regulation. Forty years later, the idea that the brain plays an important role in regulating gastrointestinal function was developed by physiologist Walter Bradford Cannon (1871-1945) (for review see [29]. However, although communication between the gut and the brain has long been known, it is only in the last decade that research have started to use a holistic view of the human body. This idea began in the 1990s when pharmacological gastric management (ignoring brain relationship) was questioned [30, 31]. This holistic approach represents a paradigm shift in medicine, where a better understanding of the microbiome will impact clinical practice [32].

Beyond the proper functioning of the digestive tract, the gut-brain axis is linked to the functionality of the autonomous nervous system, endocrine glands, and even specific brain regions, such as the hypothalamus and frontal cortex. Furthermore, gut-brain communication influences CNS development and behaviour under both normal and pathological conditions. In this section, the different pathways involved in this axis, the nervous, immune, and endocrine systems, and their relationship with the gastrointestinal microbiota are discussed.

Three major pathways constitute the gut-brain axis: the autonomic nervous, endocrine, and immune system. i) The autonomic nervous system controls gastrointestinal functions (e.g. intestinal motility and permeability, luminal osmolarity, bile secretion, and mucus production) [33]. Moreover, the vagus nerve exerts anti-inflammatory effects [34] and its stimulation is used therapeutically for Crohn's disease [35], refractory depression [36], chronic pain [37] and epilepsy [38]. Interestingly, vagotomy performed for peptic ulcer disease treatment was found to increase the incidence of psychiatric disorders [39]. Furthermore, vagal signalling can mediate the dialogue between the microbiota and the CNS since vagotomy overrides responses to psychobiotic administration [40-42]. ii) Endocrine System. The

enteroendocrine cells in the gut epithelium respond to luminal nutrients and microbiota metabolites by secreting peptides that, (in addition to a digestive function) influence certain behaviours [43, 44]. For example, L-cells produce GLP-1 and PYY, stimulate insulin secretion and suppress appetite [44]. Moreover, over 95% of serotonin (5-HT) synthesis takes place in enteroendocrine cells of the GI tract [45], indirectly regulated by microbial production of serotonergic precursors, such as tryptophan, the 5-HT transporter, and the trytophan hidrolase (TPH) [46,47]. Moreover, the gut microbiota synthesises GABA, melatonin, histamine, acetylcholine, norepinephrine and dopamine, all important neuromodulators [48,49]. iii) Immune System, the microbe-associated molecular patterns (MAMPs), activate Toll receptors modulating innate and adptative immune responses [50]. In addition, bacterial metabolites are immunomodulators; one of the most studied are the SCFAs [51]. Furthermore, loss of gut microbiota diversity has been associated with defects in microglial morphology and differentiation [52,53].

Dysbiosis And Alzheimer's Disease

Dysbiosis is the loss of microbial homeostasis and involves local inflammation with increased intestinal permeability, ultimately leading to systemic inflammation, which is associated with several diseases, beyond intestinal nature (Table 1). Studies are warranted to provide a deeper understanding of the role of the microbiota in the pathogenesis of these diseases, to generate promising therapies.

Neuropathological findings in the brain of patients with Alzheimer's disease (AD) are amyloid plaques composed of amyloid β-peptide, neurofibrillary tangles, with hyperphosphorylated tau accompanied by astrogliosis and activation of microglia, mainly in the region of the hippocampus and cortex. These changes will lead to loss of neurons, neuropil, and synaptic elements [54-56].

The aetiology of AD encompasses multiple genetic and environmental factors, and the most important known risk factor is age [56]. In recent years, accumulated evidence highlights the role of gut dysbiosis in AD (Figure 3). Dysbiosis can occur by loss of key taxa, loss of biodiversity, changes in metabolic capacity or proliferation of pathogens [52]. Ageing is a major factor in loss of biodiversity. AD patients exhibit further reduction in gut microbial biodiversity compared to aged- matched controls, consistent to an accerelated aging. A clear decrease in anti-inflammatory bacteria (e.g. Bifidobacterium, del phylum actinobacteria); and an increase in pro-inflammatory bacteria such as Shigella (Proteobacteria)

[57] is found, correlating with pathological Aβ levels and phosphorylated tau in the cerebrospinal fluid (CSF) of patients. Furthermore, these imbalances in a non-elderly population correlate specifically with cognitive impairment [58].

In aging there is an increased intestinal permeability that can be caused, amongst others, by alteration of the microbiota profile. Pathogenic bacteria (e.g. *Salmonella, Shigella, Helicobacter pylori, Vibrio, Clostridium, Bacteroides fragilis)* produce exotoxins that disrupt the integrity of the tight junctions that bind enterocytes through E-cadherin adhesion, thereby increasing intestinal permeability [59-61]. Tight junctions are also affected by the reduced production of SCFA. Increased permeability favours chronic inflammation [62]. Studies in AD patients have revealed an increased level of calprotectin in faeces, CSF and brain [63]. In addition, pathogenic bacteria **(**some strains of *E.coli*, *Salmonella enterica*, *Bacillus subtilis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*) can produce extracellular amyloid fibres [64]. These amyloid proteins help bacterial cells to form biofilms that tightly hold them together [64]. Bacterial amyloids are similar from CNS amyloids in their tertiary structure [65]. Bacterial amyloid can act as a prion 'protein' causing a cross-seeding phenomenon, i.e., capable of inducing other host proteins to form pathogenic β-sheet structure [66,67]. Thus, dysbiosis may contribute to the onset of β-amyloid peptide aggregation in AD, via TRL2 activation [68]. Lipopolysaccharide (LPS) is the main component of the outer membrane of gram-negative bacteria act as endotoxins, and it is recognized by microglia TLR4 inducing a proinflammatory response [69,70]. LPS levels are higher in AD patients compared to healthy adults [71]. Strong evidence supports that neuroinflammation is key in AD progression [72,73]. Activation of microglia can arise from oligomers Aβ 40/42 accumulation [74], which in turn secrete pro-inflammatory molecules (reactive oxygen species, nitric oxide, and cytokines) that, chronically, cause toxic effects [73]). Thus, in pathological conditions like in AD, Aβ and microglia enter a vicious cycle leading eventually to neuronal degeneration [75]. Astrocytes, the most abundant glial cells in the CNS are also involved in neuroinflammation. Astrogliosis has been observed in AD brains [76]. Like microglia, over-activation of astrocytes leads to chronic inflammation and oxidative stress that ultimately induces neuronal death [77,78].

Justification of the study

Alzheimer's disease (AD) is a progressive multifactorial neurodegenerative disorder that accounts for ~80% of dementias worldwide, specifically in adults over 60 years of age. Clinically, AD is characterised by severe deficits in memory, cognitive and motor functions, leading to a decline in mental, behavioural, and functional activities that affect the quality of daily life [79]. The epidemiological survey conducted by the Global Burden of Diseases, Injuries, and Risk Factors 2016 study revealed that, worldwide, about 43.8 million people had AD in 2016 [80]. According to the World Alzheimer Report 2015 projections, the total number of people with AD will reach 74.7 million by 2030 and 131.5 million by 2050, making it a global health challenge for which there is currently no satisfactory treatment [80].

As a result of a better understanding of the gut-brain axis, probiotics have been postulated to have beneficial effects improving cognition and memory. This is an emerging area of research that may generate new insights into individual variations and perhaps enable the development of new treatments for AD and other neurodegenerative disorders.

Objectives and research questions

This systematic review aims to evaluate the evidence from the latest published clinical trial in patients with mild cognitive impairment (MCI), to elucidate the potential application of probiotics in AD prevention. The main objective of this systematic review is to determine whether probiotic intervention provide significant cognitive improvements, preventing the progression of sporadic forms of AD.

Thus, this systematic review will answer research questions:

i. Does probiotic treatment have preventive and/or therapeutic potential in patients with MCI?

ii. What are the utilities and limitations of probiotics?

iii. Is intestinal dysbiosis a key trigger, enhancer or secondary factor in AD?

Materials and Methods

Search strategy and selection criteria

This systematic review is reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement [81]. PubMed, Web of Science, Cochrane Library, Scopus and ClinicalTrials.gov were searched for relevant studies published up to 3 November 2021. The following MeSH terms and combined text were used to search the databases: cognitive dysfunction, cognitive impairment, mild cognitive impairment, neurocognitive disorder, probiotics, randomised controlled trial, clinical trial, placebo. Search strategies used in specific databases are provided in Supplementary data Fig S1. Reference lists of retrieved studies were also handsearched for relevant articles.

Study eligibility criteria

Only randomised controlled trials (RCTs) were included in this systematic review. RCTs were eligible if they met the PICO (Patient, Intervention, Comparison, Outcomes) criteria (Table 2). Studies were included if they (1) were randomised clinical trials conducted in participants with mild cognitive impairment; (2) included a probiotic intervention; (3) compared the efficacy of the intervention with a control or placebo; and (4) reported the main outcomes of cognitive function assessed using a validated rating scale. Additional outcomes reported could be changes in metabolic variables and biomarkers of oxidative and inflammatory stress.

Studies were excluded if they (1) did not have a cohort of people with MCI, (2) included subjects with advanced AD or other dementias, (3) were observational or retrospective, (4) were based on a prebiotic, synbiotic or mixed intervention, or (4) did not assess intervention outcomes with validated tests.

Data extraction

The following data were extracted from the included studies:

- General information: title, authors, year of publication, trial registration number and country.
- Participant information: sample size, age, demographics, and baseline characteristics.
- Methodological information: study design, intervention, comparison, treatment allocation, description of the intervention and duration of the intervention period.
- Outcome-related information: recruitment, primary outcome data, secondary outcome data, adverse events recorded, and study completion rates.

Assessment of risk of bias according to the cochrane tool

The risk of bias of each RCT was assessed using the specific questions listed in the Cochrane risk of bias tool [82] and internal validity using The Scottish Intercollegiate Guidelines Network (SIGN) clinical trials checklist [83].

The Cochrane Handbook proposes a tool that assesses the risk of bias through 7 items for each included study: random sequence generation, allocation concealment, blinding of participants and staff, blinding of outcome assessors, incomplete outcome data, selective reporting of results, and other biases not addressed in the previous sections. For each of these, criteria are explained to classify the assessment of risk of bias as low / high / unclear.

The SIGN checklist assesses internal validity for each included study using 10 items: appropriate and focused research question, random allocation, appropriate method of concealment, blinding of participants and investigators, similarity of treatment and control groups at baseline, the only difference being the intervention performed, standard, valid and reliable measurement of outcomes, participant dropout rate, intention-to-treat analysis of results, and comparability between different centres if any. After examining these items SIGN proposes an overall assessment of the study reflecting on the risk of bias, the causal relationship between the intervention and the observed effect and the external validity of the study.

Results

Literature Search and Study Selection

A total of 41 results were obtained after the initial search of electronic databases, and 1 study was identified by hand searching the reference lists of relevant published reviews. Of these 42 studies, 19 were duplicates and removed; 11 publications were excluded after reviewing the title and abstract (with reasons: they were not randomised clinical trials (n=2), they referred to other pathological processes rather than cognitive impairment (n=8) or the study population was different (n=1)). The remaining 11 articles were analysed by reviewing the full text, after which 6 were excluded for different reasons (intervention was combined (n=1), participants did not meet the pre-determined inclusion criteria (n=3) or results had not yet been published (n=2)). Ultimately, 5 studies were considered eligible for this systematic review (Figure 4).

Characteristics of the included studies

The characteristics of the included studies are summarised in Table 3, and in more detail in Supplementary data Fig S2. The five included studies were published in the last three years (2019-2021), all randomised, double-blind, placebo-controlled clinical trials (RCTs). Two of them were conducted in Japan, two in Korea and one in the United States. The aggregate sample of all of them is 509 subjects (an average of 102 per study), and the minimum age of inclusion of participants was 50 years. In all of them, the intervention consisted of administering 2 capsules of a probiotic strain per day for 12 weeks, except in one study where the duration was 16 weeks. Importantly, the genera used were Lactobacillus and Bifidobacterium, the two best known and most used. The outcome of the intervention was measured as the difference in cognition compared to placebo, with different tests in each study (RBANS, NIHTH, CNT, MMSE, CERAD-K), all validated for this purpose. The main findings included a significant improvement in, at least, one aspect of cognition (immediate, visuospatial, constructive, immediate memory, and/or attention and mental flexibility), in four studies. In one study, significant differences were observed only if patiEnts were stratified, and disregarding more advanced stages of disease (RBANS>41) from the analysis.

Risk of Bias Assessment

Cochrane Tool. The results of the risk of bias assessment for the included studies are shown in Figure 5, and for each study individually in Supplementary data Fig S3. As all five studies were double-blind RCTs, all were classified as having a low risk of selection and conduct bias. However, two studies, Kobayashi *et al* (2019) and Xiao *et al* (2020) were unclear in describing the form of allocation concealment [84, 85]. Also, Kobayashi *et al* (2019) does not describe the blinding of outcome assessors [84], which could introduce selection and detection biases respectively. Unclear risk of attrition bias due to incomplete outcome data was reported in Sanborn *et al* (2020) study [86]. None of the included studies stopped their trials early and therefore all were assigned a low risk of reporting bias. None of the included studies were considered to have high risk of bias, and generally all implemented strict procedures in their methodology.

SIGN methodology checklist for clinical trials Overall, the parameters assessing internal validity according to the SIGN scale are positive for the studies included in this review (Table 4). The breakdown for each study can be found in Supplementary data Fig S4. In all studies the research question was

adequate, randomisation and double blinding were performed, there were no differences in the intervention in each group and no excessive loss to follow-up, and the relevant outcomes were measured in a standard, valid and reliable way. All studies indicated that the analysis was both per protocol and intention-to-treat. Loss to follow-up and missing or atypical data are adequately accounted for in each study, and the investigators note that participants excluded from the analyses were not significantly different from included participants, so it is unlikely that the balance achieved by randomisation was broken for these reasons. Again, it should be noted that in two cases the method of concealment was not specified [84,85]; and in Kombayashi *et al*, the treatment and control groups showed differences in cognition score at baseline, which led the authors to stratify the results for analysis [84].

As an overall assessment, the RCTs included in this systematic review are of high methodological quality, with low risk of confounding, bias, or chance, and with a high probability that the relationship is causal. The results of the studies are directly applicable to the patient group targeted by the guidelines, with internal validity being an outstanding quality. A possible lack of external validity of the RCTs should be acknowledged, as the exclusion criteria of the studies ruled out subjects with common characteristics such as having a relevant disease or being a user of drugs that could interact with probiotics, among others.

Discussion

Role of probiotics and cognition: current evidence from preclinical and clinical studies

As discussed above, strong evidence suggests that the gut microbiota is pathophysiologically involved in the progression of AD. Of note, two recent systematic reviews show evidence that in preclinical studies, probiotics are very effective in promoting cognition in rodents with AD or cognitive impairment [89, 90]. Furthermore, the cognitive improvement correlate with biochemical and histological measures, ie. reduction in brain oxidative stress biomarkers, amyloid plaques, and proinflammatory microglia in rat model of diseases [91]. Generally, the risk of bias of the studies included in these systematic reviews was low, although the heterogeneity among them was moderate. In addition, it should be stressed that animal models may have limitations mimicking human disorders.

In humans, increasing evidence is available. To date, four systematic reviews have been published on the subject [92-95], all within the last 4 years, suggesting an exponential trend in scientific production.

The results of the systematic reviews are generally favourable for the consideration of probiotics as a weapon to slow the progression of cognitive decline. However, all of them acknowledge important limitations in relation to the small number of included studies and the high risk of bias attributed to most of them. The high heterogeneity among the included studies is obvious, and could be attributed to (1) differences in the intervention performed in terms of duration of the intervention and probiotic strains used (dose, number and type of species); (2) very loose inclusion and exclusion criteria of the studies, so that the results obtained with subjects at very different stages of the disease, from healthy subjects to those with severe AD, are evaluated together; (3) the measurement of cognitive function as an outcome variable is not always assessed by objective methods. Moreover, many studies did not record changes in microbiota composition after the intervention, so the causal relationship between supplementation and cognitive improvement is weak and may be due to yet unexplained intermediate links. Krüger *et al* (2021) systematic review found no beneficial effect of probiotic supplementation on cognitive function [93]. Therefore, interpretation of the results should be very cautious. The present systematic review attempts to focus on studies with more homogeneous characteristics, to elucidate whether, in this group of subjects there is a significant effect of probiotics.

Importance of severity of cognitive impairment on the usefulness of probiotics

One of the most important aspects to question about the usefulness of probiotics is their effectiveness at different stages of cognitive decline. Several studies in both animals and humans indicate that in subjects with intact cognitive abilities, probiotic intervention has a limited impact [93], likely due to the ceiling effect. Thus, high baseline cognitive function may limit the scope for improvement. On the other end, probiotic supplementation has not led to significant changes in cognition in individuals with advanced AD [96,97], which can be due to several reasons, i) the margin for improvement in advanced disease is very small, ii) the follow-up in studies is insufficient to reveal significant changes, iii) the histopathological changes in severe disease stages are already irreversible. Yet, even in these cases, metabolic improvements have been reported, such as in plasma triglyceride levels, very low-density lipoproteins, insulin resistance and plasma malondialdehyde.

Current knowledge supports positively the usefulness of probiotics in improving cognitive ability in the early stages of AD or mild cognitive impairment (MCI) [98] and reflected in the present systematic

review. MCI refers to a state of cognitive impairment preceding the clinical diagnosis of Alzheimer's disease, which does not yet compromise daily functioning.

This systematic review analyses five RCTs: **Hwang *et al*** (2019) evaluated the efficacy and safety of the probiotic DW2009 as a nutritional supplement for cognitive enhancement in 100 people with MCI aged 55-85 years. After 12 weeks, improvements were found in cognitive performance, as measured by the CNT scale, especially in attention [87]. **Kobayashi *et al*** (2019) conducted a similar intervention on 121 subjects with memory problems with *Bifidobacterium breve A1* capsules. After 12 weeks of intervention, significant differences in RBANS and MMSE scores were only observed in the subgroup of individuals with incipient dementia, but not in those with near normal memory function [84]. These results support probiotics rescue cognitive function in subjects with MCI. It invites future studies to clarify the benefit of probiotics in healthy individuals. The study by **Xiao J *et al*** (2020) analysed the effect at 16 weeks of *Bifidobacterium breve A1* administration in 80 subjects aged 50-79 years with MCI (MMSE score >22). The RBANS total score improved significantly in the probiotic group compared to placebo, markedly in the immediate, visuospatial, constructive, and delayed memory domains. The JMCIS score also improved [85]. Similarly, **Sanborn *et al*** (2020) investigated whether *Lactobacillus rhamnosus* 12 weeks of GG supplementation improved cognitive function in 200 middle-aged and older adults. The results, measured with the NIH ToolBox scale, were favourable, showing an improvement in total cognition in those with previous cognitive impairment. No effect was observed those with intact cognitive function or in placebo group [86]. Finally, **Kim CS *et al*** (2021) evaluated the effect of *Bifidobacterium bifidum* and *Bifidobacterium longum* probiotics in 63 subjects over 65 years of age. After 12 weeks significant improvements in scores on the cerad-k mental flexibility test were found in the probiotic group compared to the placebo group [88].

This systematic review strongly supports a positive effect of probiotic supplementation on cognitive function in people with MCI. Importantly, only this study [88] performed an analysis of the gut microbiome profile in their participants, before and after the probiotic intervention. A decrease in the relative abundance of pro-inflammatory bacteria (*Eubacterium, Allisonella, Clostridiales and Prevotellaceae*) at week 12 in the probiotic group was reported. The study by **Hwang *et al*** (2019) [87] correlated the serum levels of BDNF with cognitive performance for each treatment group: serum BDNF

levels increased after *Lactobacillus Plantarum C29* probiotic administration, which may suggest that this metabolite may have mediated the cognitive enhancement.

The improvement in cognitive function may be due to a direct effect of the gut microbiota via the gut-brain axis; or indirectly, improving metabolic parameters (insulin sensitivity), and reducing inflammatory conditions.

In terms of safety, probiotics were well tolerated as no adverse events were reported in any of the RCTs evaluated. There were also no differences in vital signs (blood pressure, pulse rate), body mass index and laboratory results between treatment groups. Probiotics are classified as safe by the US Food and Drug Administration (FDA). However, they should not be administered in certain patients, particularly those receiving immunosuppressive treatments such as chemotherapy, as some cases of sepsis, fungemia and bacteraemia have been reported in people receiving *S. boulardii*. Although no adverse events were found in these studies, exceptionally, probiotic bacteria may contain antibiotic resistance genes that can be transmitted to other bacteria, including harmful genera [99].

Gut health beyond probiotics

In addition to probiotics, other measures must be taken to preserve the gut health in the elderly, such as monitoring the excess of medication. The most used non-antibiotic drugs affecting the gut microbiota profile are proton pump inhibitors [100], antipsychotics and antidepressants [101}. Also, non-steroidal anti-inflammatory drugs [102], laxatives, statins, anti-diabetic drugs (e.g., metformin) [103] and anti-rheumatic drugs such as methotrexate can affect microbiota [104,105]. Polypharmacy (defined as 5 or more drugs) can cause changes in luminal pH, local mucosal inflammation, leading to dysbiosis, depending on the type of medication used [106]. Polypharmacy is very common in developed countries. According to the 2017 Spanish National Health Survey, the 37.5% of non-institutionalised older adults consume 5 or more medicaments [106]. However, other national [107], French [108], and Swedish [109] studies report even higher prevalences, close to 50% of polypharmacy and up to 20% of hyperpolypharmacy (defined as 10 or more medicines). In this context, it is worth noting a study on hospitalised elderly patients in which polypharmacy was significantly associated with gut microbiota dysbiosis and mortality. Differences in more than 15 taxa were found associated to polypharmacy (e.g. positive association was observed in *Bradyrhizobium*, *Coprobacter, Helicobacter* and *Prevotella),* in

comparison with healthy, active elderly subjects without polypharmacy [110]. Therefore, although the efficacy and safety of medication remain highly favourable, increasing evidence reveals the importance of identifying inappropriate polymedication by assessing the long-term effects in the gut ecosystem.

Strengths and limitations of this review

This systematic review has some notable strengths. First, the systematic review strictly follows the recommendations of the Cochrane handbook. Secondly, we applied strict inclusion and exclusion criteria to have as homogeneous studies as possible, which, together with the high methodological quality of the studies. Also, the primary outcomes were measured with scales validated for this purpose. Despite these strengths, this systematic review is not without some limitations. First, despite thorough literature searches, it is possible that some eligible studies may have been missed, especially those published between the search deadline and the publication of this systematic review. Furthermore, the research question of whether gut dysbiosis is a key trigger, enhancer or secondary factor in AD has not been satisfactorily elucidate since most studies did not evaluate the bacterial profile before and after the intervention. Further studies are warranted to clarify the directionality of this causal association.

Although the current scientific evidence invites optimism about the role of probiotics in not only gastrointestinal but also systemic and neurodegenerative diseases, such as AD, there are many unresolved questions that need to be clarified before the use of probiotics in clinical practice can be firmly considered. Some of these questions include: Are there differences in therapeutic intervention with prebiotics and probiotics? Does the brain adapt to probiotic intake over the long term? Is there a "ceiling effect" on probiotic benefits? Do factors such as diet, genotype, gender, and age moderate the effects of probiotics? How do probiotics interact with other drugs?

Furthermore, given the taxonomic disparity at the individual level, personalised medicine should be considered in the use of probiotics based on the pre-existing microbiota and patient symptomatology. Future studies with longer treatment are needed to address these questions.

Conclusions

Taken together, the results of this systematic review indicate that *Bifidobacterium and Lactobacillus* probiotics, when supplemented for minimum of 12 weeks, can improve cognitive function in people with MCI and/or incipient AD, with a very favourable safety profile. Due to the gradual nature of AD

progression, and considering its societal importance, it is imperative to explore and develop intervention strategies for early AD.

Preserving gut health, counteracting the dysbiosis associated with ageing would have a great health, social and economic impact, by preventing or delaying progression of diseases associated to aging.

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Conflict of Interest/Disclosure Statement

Authors declare no conflict of interest.

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Figure Legends

Figure 1. Basic functions of the gut microbiota.

Figure 2. Composition and diversity of the gut microbiota throughout life and the main external conditioning factors.

Figure 3. Schematic representation of the pathophysiology of the sporadic form of AD. Both intrinsic and environmental factors can lead to gastrointestinal dysbiosis in the elderly, resulting in local inflammation that spreads systemically through the bloodstream and vagus nerve to the CNS, disrupting the blood-brain barrier and promoting neuroinflammation, a key factor underlying AD.

Figure 4. Flow chart for literature search.

Figure 5. Summary Graph of Cochrane risk of bias.

Tables

Table 1. Examples of publications were microbiota dysbiosis is associated with human pathologies

Table 2. PICO criteria; Patient, Intervention, Comparison, Outcomes

Table 3. Extracted data from revised trails. For the extended version see Fig S2.

Table 4. Cochrane revision. For details see Fig S3

Suplementary data

Figure Supplementary 1. Search strategies used in specific databases

Figure Supplementary 2. Detailed Data extracted from the revised trails.

Figure Supplementary 3. Detailed Cochrane risk of bias tool.

Figure Supplementary 4. SIGN checklist for clinical trials.