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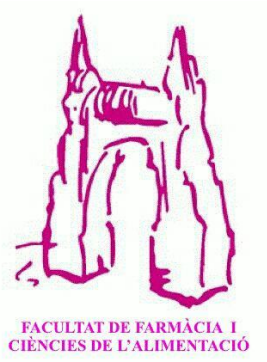

Final Degree Project

Probiotics, Prebiotics and the Nervous System

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Main Field:

FOOD SCIENCE

Secondary Fields:

PHYSIOLOGY AND PHYSIOPATHOLOGY

MICROBIOLOGY

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

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Abstract

The relationship between the gut microbiota and the central nervous system (the microbiota-gut-brain axis) is an area of increasing interest and research. Studies based on germ-free models have provided a big amount of evidence of the connection between the gut and the brain. This research has done a great step forward in recent years, due to the application of metagenomics and bioinformatics. We now know that the human gut microbiome can be classified into three enterotypes, characterized by the variation in three genera: *Bacteroides*, *Bacteroidetes,* and *Prevotella*. Type of birth, formula feeding, and antibiotic intake are among the main factors that impact on infant microbiome assembly. Moreover, the composition of the gut microbiota is strongly associated with diet. A review of the bibliographical evidence connecting the alterations in the microbiome and some central nervous system disorders (as Alzheimer disease, Parkinson disease or autism spectrum disorder) shows us that the levels of *Prevotella* and the Firmicutes/Bacteroidetes ratio are altered in these pathologies. Accumulating data reveals that the microbiota-gut-brain axis can be modulated by the administration of probiotics and prebiotics. Moreover, some traditional fermented food has been seen to have probiotic properties and high-fiber containing diets have been associated with a lower Firmicutes/Bacteroidetes ratio and higher levels of *Prevotella*.

Resum

La relació entre la microbiota intestinal i el sistema nerviós central (l'eix microbiota-intestí-cervell) és una àrea d'interès i investigació creixents. Els estudis basats en models lliures de gèrmens han proporcionat gran quantitat d'evidències de la connexió entre l'intestí i el cervell. Aquesta investigació ha fet un gran avenç en els últims anys gràcies a la metagenòmica i la bioinformàtica. Ara sabem que el microbioma intestinal humà es pot classificar en tres enterotips, caracteritzats per la variació en tres gèneres: *Bacteroides*, *Bacteroidetes* i *Prevotella*. Els tipus de naixement, el tipus d'alletament i la ingesta d'antibiòtics són els principals factors que afecten el desenvolupament del microbioma infantil. A més, la composició de la microbiota intestinal està fortament relacionada amb la dieta. Una revisió de les evidències bibliogràfiques que connecten les alteracions en el microbioma i alguns trastorns del sistema nerviós central (com la malaltia d'Alzheimer, la malaltia de Parkinson o el trastorn de l'espectre autista) ens mostra que els nivells de *Prevotella* i la relació Firmicutes/Bacteroidetes estan alterats en aquestes patologies. Cada cop hi ha més dades que revelen que l'eix microbiota-intestí-cervell pot ser modulat per l'administració de probiòtics i prebiòtics. D'altra banda, s'ha observat que alguns aliments fermentats tradicionals tenen propietats probiòtiques i que les dietes amb alt contingut de fibra s'associen a nivells inferiors de la raó Firmicutes /Bacteroidetes i superiors de *Prevotella*.

Integration of the different fields

This is a work focused on the role of probiotics and prebiotics in the treatment and prevention of neurological diseases. Even when this main objective falls into the field of Food Science, it has close connections with the proposed secondary fields. From one hand, a deep study of the potential role of probiotics in central nervous disorders needs a deep understanding of the physiology of the gut-brain axis and its relationship with brain diseases. This leads us to consider Physiology and Physiopathology as a secondary field. On the other hand, our analysis needs a detailed knowledge of the composition of the human gut microbiome, its role in the gut-brain axis and its connection with brain disorders. By this reason, we consider Microbiology as a secondary field in our work.

1. Introduction

The complex relationship between the digestive and the nervous system has been an object of attention for centuries and, to some extent, it appears in our common language, in expressions as “butterflies in the stomach”, “gut feeling”, “trust our gut instinct” or “gut check time”.

Ivan Pavlov (Nobel Prize 1904), who established the basis of the modern physiology of digestion, proved that the digestive system is influenced by the central nervous system in a complex manner, and that psychological process can influence the nature of the fluids secreted into the digestive tract (see (1)). In fact, he foreshadowed the complex and intricate net that we now call the gut-brain axis.

The gut-brain axis (GBA) consists of bidirectional communication between the gut and the brain. A major scientific breakthrough in understanding this interaction was the discovery of the enteric nervous system (ENS) in the middle of the nineteenth century (see (2)).

In the last years, there is an increasing evidence that the above-mentioned pathways are under the influence of the gut microbiota together complementing the microbiota-gut-brain-axis (MGBA) (see for example (3) or (4)). A map of the main MGBA pathways is shown in Figure 1. The brain and the ENS are connected via the autonomic nervous system and hypothalamic-pituitary-adrenal (HPA) axis. The gut microbiota interacts with the brain via the ENS and via metabolic products. The immune system communicates bidirectionally with each member of the MGBA.

Up to our knowledge (see (5)), the first evidence that microbes affect the brain chemistry was written in 1986 by Hegstrand and Hineand can be found in (6). In this ground-breaking paper, proved significant differences in hypothalamic histamine levels between germ-free and conventionally housed animals. Nevertheless, the interest in

this field did not spark until the publication in 2004 of the work by Sudo et al. (7). In this work, an exaggerated HPA stress response was reported in germ-free mice and, moreover, this response was reversed by reconstitution with *Bifidobacterium infantis*.

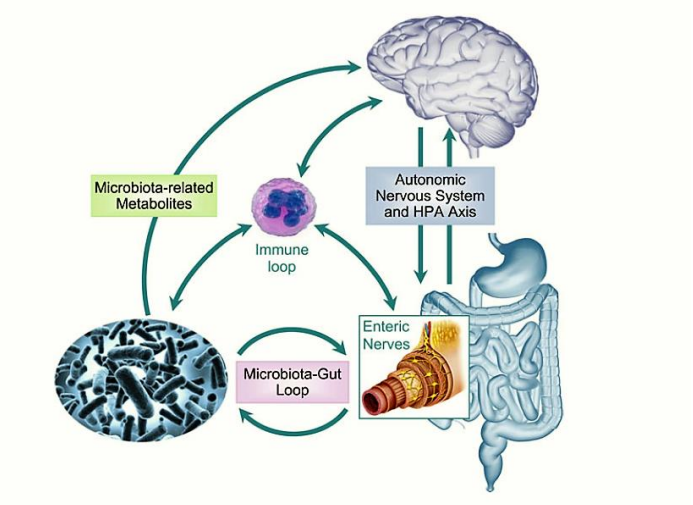

Figure 1 The microbiota-gut-brain axis (MGBA) (3)

Application of modern rapid DNA sequencing technology has transformed our knowledge of the gut microbiota. Modern metagenomic and bioinformatic techniques have allowed researchers to do a great step forward, and they have allowed us to describe the gut microbiome and to connect it with different disorders (see for example (8)). Nowadays, there is an increasing evidence that the microbiome plays a key role in the development of CNS diseases as Alzheimer's disease (AD), anxiety, autism, amyotrophic lateral sclerosis, depression, multiple sclerosis or Parkinson's disease (PD) (see for example (9)).

The interest on this field is clearly increasing. MGBA has become one of the targets of neuroscience. In the year 2013, the National Institute of Mental Health (NIMH) launched a special project to study the mechanisms of the MGBA, with a view to develop new medications or non-invasive treatments for mental diseases. On the other side, the European Union has launched a project called MyNewGut, two main objectives of which focus brain development and disorders (see (10)). The number of publications in this area is clearly increasing. For example, that a Pubmed search of the keywords 'gut brain and (microbiota OR microbiome) axis' gives a total of 323 outcomes during the year 2017, while the same quantity for the 2008 is equal to just 2 outcomes.

Despite the increasing evidences on the relationship between the microbiota and the gut-brain axis, understanding the mechanisms of these interactions require further studies. We must take into account that most of the available research in this field is still pre-clinical, and it is not clear to what extent we can generalize the results in these studies to the human physiology. Moreover, we do not know how the manipulation of the gut microbiome can be an efficient tool in the prevention and treatment of

neurological disorders. Finally, an important point is how to efficiently manipulate this gut microbiome. Even we know that its composition is affected by some environmental factors as the type of birth, stress, antibiotic intake and diet (see (11)), the design of possible treatments is not straightforward. All these points are of strong interest in research since they are a key step in the translation of all this knowledge into the prevention and treatment of diseases. A summary of the key challenges in this area can be found in (12).

In this work, our main interest is the potential applications of probiotics in the prevention and treatment of CNS diseases. This idea is not completely new. Over a century ago (see (13)), Metchnikoff theorized that senility could be delayed, by manipulating the intestinal microbiome with host-friendly bacteria found in yogurt. This theory has re-emerged from the 1990s, and today probiotics are not only the subject of medical research but also the source of a multibillion dollar global industry (see again (13)). Nevertheless, we must understand that this research, concerning the CNS, is still in its infancy. In fact, if we do a search of the keyword 'probiotic' in *Cochrane Evidence* we will find no evidences of their efficacy in the treatment of brain disorders.

2. Objectives

The main objective of this work is to carry out a bibliographical research that allows us to understand the state-of-the-art of the research in the application of probiotics in the treatment of CNS disorders. Towards this end, we want to answer the following questions:

a) What are the main tools used in the study of the microbiome-gut-brain axis? To what extent these techniques can give us enough information so that we can develop new treatments for CNS diseases?
b) What do we know about the gut-brain axis? Moreover, what do we know about the role of the microbiome in the gut-brain axis?
c) Which kind of pre-clinical and clinical results do we have about the use of probiotics in the prevention and treatment of neurological disorders?

3. Methodology

The methodology used in this work is based on bibliographical screening in PubMed, Nature Reviews, Google Scholar, and Google.

Moreover, we have consulted the web pages of several organizations and companies related to the subject of this work. Among them we can quote the websites of: The Human Microbiome Project, INFRAFRONTIER (The European Research Infrastructure

for the generation, phenotyping, archiving and distribution of model mammalian genomes), Gut Microbiota for Health (public information service of the European Society for Gastroenterology and Motility), Illumina Inc. (USA), European Food Safety Agency (EFSA), World Gastroenterology Organization (WGO), Food Agriculture Organization (FAO), International Scientific Association for Probiotics and Prebiotics (ISAPP) and the Food and Drug Administration (FDA).

4. Results

In this section, we discuss the results of our bibliographical research. In Section 4.1 we will discuss the basic tools that have been used up to now in the study of the MGBA, and we will see how, nowadays, metagenomics and bioinformatics represent a great step forward in this research. In Section 4.2 we present the basic pathways of the gut-brain axis. Section 4.3 is devoted to studying the composition of the human gut microbiome. In Section 4.4 we focus on the interactions between this gut microbiota and the gut-brain axis, while in Section 4.5 we deep on the relationship between the MGBA and CNS diseases. The modulation of the MGBA by probiotics and prebiotics is discussed in Section 4.6.

4.1 Tools in the study of the microbiota-gut-brain axis

We review the tools used in the study of the MGBA following the paper by Mayer, Tillisch, and Gupta (4), where the main techniques used up to now in the study of the MGBA are listed. These techniques are: germ-free models, microbial manipulation with antibiotics, fecal transplantation, probiotic feeding and diet (see Figure 2).

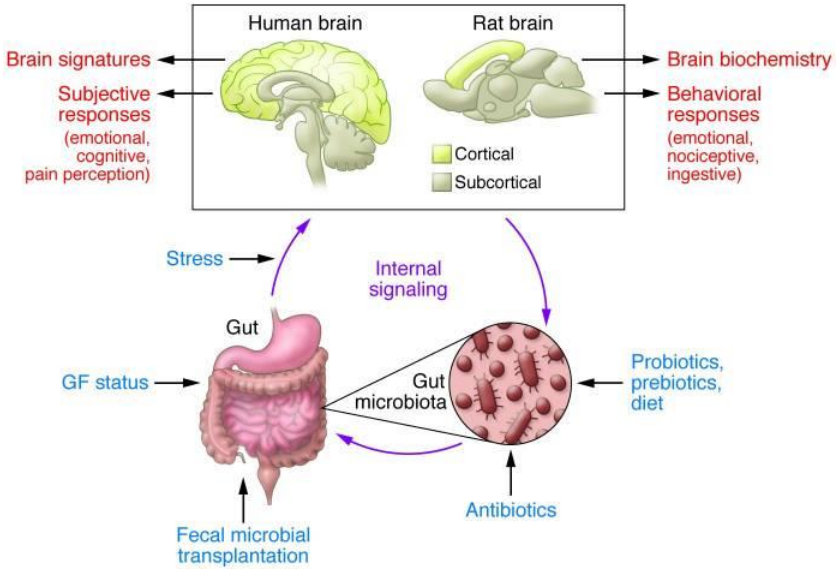

Figure 2: Classical techniques in the study of the MGBA (4).

4.1.1 Germ-free models

A common approach used to study the functions of an organ consists in abolishing its contribution. Then, it is not surprising that the first tool used in the study of the role of the microbiome in the GBA was to see how this latest is affected by the absence of microbiota. To help answer this question, germ-free (GF) animals were generated.

Germ-free (axenic) animals are animals that are free of microorganisms. As quoted in the Introduction, Hegstrand and Hine (6) demonstrated significant differences in hypothalamic histamine levels between GF and conventionally raised animals. By nearly 20 years after, Sudo et al. (7) reported an exaggerated hypothalamic-pituitary-adrenal axis response to restraint stress in GF mice. Moreover, this effect that was reversed by monocolonization with *Bifidobacterium infantis*. Many studies have been done since then by using GF models.

The main advantage of the germ-free/gnotobiotic mouse model is in proof-of-principle studies. Moreover, complete microbiota or defined sets of bacteria can be introduced at various developmental moments, which allows us to study the existence of critical windows of development that may require bacterial input. Germ-free studies are powerful in helping us to prove if the microbiome is involved in a specific aspect of brain function, as well us to study of the impact of a particular set of bacteria or dietary intervention on the microbiota-gut-brain axis in isolation.

Research using GF models has provided a big amount of evidence of the role of the microbiome in gut-brain signaling. Now we know that the microbiota is necessary for normal stress responsivity, anxiety-like behaviors, sociability, and cognition. Moreover, it keeps CNS homeostasis (by regulation of the immune system and the blood-brain barrier integrity). Furthermore, the microbiome influences neurotransmitter, synaptic and neurotrophic signaling systems and neurogenesis. Growing up in the absence of microorganisms alters behavior and brain function, as it is detailed in (14). In Figure 3 we show a summary of the results obtained up to now on GF raised mice:

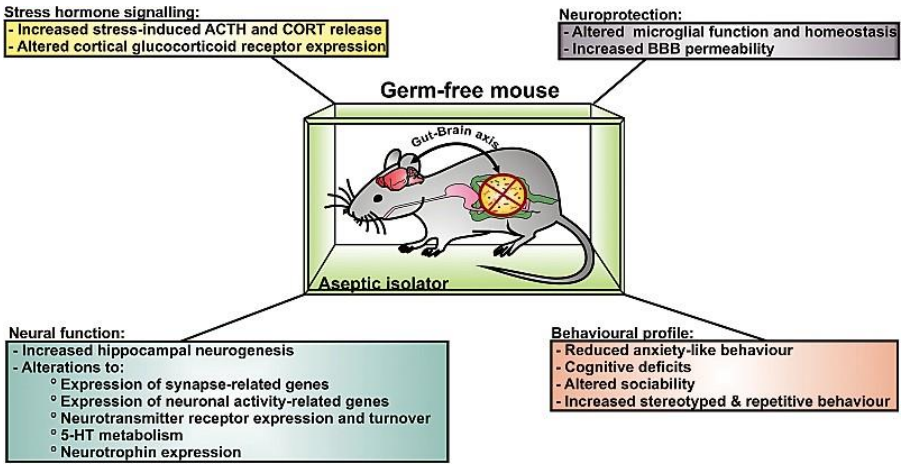

Figure 3: Germ-free (GF) mice as a tool to study the microbiota-gut-brain axis (14).

As humans are not it a GF state, GF models are often criticized and assumed to do not have clinical relevance. Another important limitation of GF animals is the fact that, as they have no bacterial exposure from conception, they are not useful for the study of questions regarding the impact of altered microbiota composition that first occurs later in life. Moreover, rearing germ-free animals implies technical difficulties and high expenses. For these reasons, alternatives to the GF model have been proposed, as we see in the following subsection.

4.1.2 Alternatives to GF models

An alternative to the use of GF mice is given by antibiotic treatment, that allows us to induce a disruption of the gut microbiota. Many of the phenotypic features associated with the GF state are also evident after this sustained disruption. Among the studies that use this technique, we can quote (15). This paper shows how microbiota depletion in the adolescence, by means of a chronic treatment with a combination of antibiotics affects the gut-brain communication in a similar way as it is reported in germ-free mice, which suggests that this technique can be an alternative to GF models. However, we notice that many antibiotics are also systematically toxic and this needs to be considered in the interpretation of the results (see for example (11)).

Another related technique consists in the introduction of bacteria in mouse models of intestinal dysbiosis. In humanization studies, human feces are transferred to GF mice and to antibiotic-treated mice (see for example (16)). The humanization technique also transfers some features of the disease to the animal (see for example (17)). Connected to this technique we can also consider Infection studies, that have been used to study the effects of pathogenic bacteria on the CNS (see for example (11)).

Another tool is the use of probiotics. Probiotics are defined *as* “living microorganisms that, when ingested in adequate quantities, confer a health benefit on the host” (18). There is an increasing evidence that certain probiotics (as *Bifidobacterium* and *Lactobacillus*) may positively impact the pathogenesis of CNS disorders (see for example (19) and (20)).

4.1.3 Metagenomics, bioinformatics and neuroimaging

Nowadays, preclinical and clinical studies can be improved significantly with the help of metagenomics, bioinformatics, and neuroimaging. Here we offer a basic introduction to these techniques in the study of the MGBA. We refer to the review (8) and to the webpage of the specialized company *Ilumina* (section ‘Human Microbiome Analysis’ (21)) for a more detailed description.

Metagenomics is the study of the microbial genetic material obtained directly from environmental (culture-free) samples. Next-generation sequencing (NGS) give us throughput and cost savings tool to study the genomes of entire communities. Among NGS methods we can quote shotgun metagenomic sequencing, 16S rRNA sequencing (a cost-saving and efficient tool in the identification of bacteria) and microbial metatranscriptomics.

These metagenomic techniques have enabled collaborative projects, as National Institutes of Health's Human Microbiome Project (HMP) and Metagenomics of the Human Intestinal Tract (MetaHIT) Project. These projects provide public databases that are available for researchers. All the information in these databases must be analyzed. In particular, the statistical analysis of this data includes:

- Microbial community composition,
- diversity analysis: alpha-diversity (biodiversity of the samples) and beta-diversity (healthy controls versus patients),
- network analysis,
- biomarker discovery and
- metabolomics (the study of the metabolites produced by the microbiome).

All these statistical analyses, jointly with the questions they can help us to answer, are summarized in Figure 4.

Figure 4: Data Analysis for metagenomic microbial data (8)

Another interesting application of metagenomics is its combination with the data from functional neuroimaging techniques that provide readouts of neural activity across the brain (see for example (3)). This is what we call *radiomicrobiomics* (see (22)): a process to extract quantitative parameters from the gut-brain axis by combining brain imaging and features of the microbiota. We notice that this process becomes possible thanks to modern mathematical and computational tools.

Vast evidence has shown that the composition of human gut microbiota has an obvious correlation with the occurrence of human diseases, such as obesity, cardiovascular disease, and tumor, but the impact on health and disease of the human brain is underway. But, even when the application of metagenomics and bioinformatics in the study of the MGBA is still in its infancy, it is a very promising approach.

4.2 The gut-brain axis

As indicated in (11), the interaction between the gastrointestinal tract and the brain has been observed since the middle of the nineteenth century through the pioneering work of Claude Bernard, Ivan Pavlov, William Beaumont, William James and Carl Lange. The impact of emotions in the secretions of the alimentary tract had been quoted by Charles Darwin in *The Expression of the Emotions in Man and Animals* (1872). In the late 1920s, Walter Cannon emphasized the role of brain processing in the modulation of gut function. A major scientific breakthrough in understanding the interaction of the nervous system with the gastrointestinal tract was the discovery of the so-called enteric nervous system (ENS) in the middle of the nineteenth century (see (2)).

It is now increasingly being recognized that the gut–brain axis provides a bidirectional pathway that uses neural, hormonal and immunological routes (see for example (23)). This relationship is important not only in normal gastrointestinal function but also plays a significant role in higher cognitive functions. Now we describe the basics of this interaction. For a more detailed exposition, we refer to (23).

The gut-brain axis is a bidirectional relationship. In response to some factors and events, some regions of the brain may be activated, which may cause different responses depending on the stimuli. The hypothalamic-pituitary-adrenal axis (HPA) may be activated to initiate the release of adrenal hormones. Projections from these brain regions to brainstem nuclei may initiate vagal output, or these may project to the spinal cord and modulate signals related to gastrointestinal spinal reflexes or pain sensitivity. Depending on which spinal cord level is activated, there may be additional parasympathetic or sympathetic outflow. These hormonal and neural outputs

influence gastrointestinal targets such as immune cells, enteric smooth muscle, enteric neurons and enteroendocrine cells.

The gut to brain interaction involves the enteric nervous system (ENS). The ENS has more than 200 million neurons and it is sometimes called the “second brain” This extensive network influences the brain via endocrine, neuronal and immune pathways. Mechanical and chemical information from the luminal environment is signaled through extrinsic (vagal and spinal) primary afferent neurons to the brain. Moreover, terminals of extrinsic primary afferent neurons are near the immune and enteroendocrine cells. These cells, in conjunction with enteric microbiota, produce several signaling molecules that can activate receptors on extrinsic primary afferent neurons. Then, endocrine, neuronal and immune signals are integrated and are sent to specific brain regions.

Due to this close relationship between the gut and the brain, it is not surprising to observe that many brain affecting disorders appear connected to gastrointestinal manifestation (24). Among them, the most explored one for this relationship is Parkinson disease (PD). Other brain disorders that have been found to be related to gastrointestinal manifestations include autism, amyotrophic lateral sclerosis, Alzheimer diseases, prion diseases, Creutzfeldt-Jakob disease, transmissible spongiform encephalopathies, depression, anxiety behavior, cognition, mood, stress, fatigue, and aging (see again (24)).

4.3 The gut microbiota

The human gastrointestinal tract is inhabited by 10^{13} to 10^{14} microorganisms (see for example (14)). This quantity is more than 10 times the number of human cells in our bodies. Moreover, this microbiome contains 150 times as many genes as our genome. It is generally accepted that the adult microbiota consists of more than 1,000 species and more than 7,000 strains.

It is well-known that gut microbiota has an important role in the development and functionality of the immune system and in regulating the gastrointestinal system. We also remark that, as quoted in Section 4.1.3, next-generation sequencing and bioinformatics have had an immense impact on our knowledge of the microbiome.

The gut microbiome is a complex ecosystem where the bacterial component is the dominant domain (see for example (25)). In fact, the term *microbiota* is usually assumed to refer to the bacteria microbiota. Bacteria represent huge quantities of microorganisms whereas fungi represent less than 0.01% to 0.1% of genes in fecal samples (see again (25)). Most studies have focused exclusively on the bacterial component, neglecting fungi and other minority kingdoms. Initially, the large-scale projects such as the quoted HMP and MetaHIT were focused exclusively on this

bacterial component. In fact, to date, the only review devoted to the role of the gut mycobiome in the MGBA is the very recent paper by Enaud et al. (25). Another recent review on the human mycobiome (not focused in the MGBA) can be found in (26). Nevertheless, even when research into the mycobiome is still in its infancy, its potential role in human disease is increasingly recognized (see (26)).

It is difficult to describe the microbial composition in health. One of the reasons is the high variation between individuals. Moreover, the composition that is usually measured is the one observed in fecal samples, that does not truly reflect the diversity of the gastrointestinal. In this section, we will describe our knowledge of the composition of the gut bacteria and the gut mycobiota.

4.3.1 The gut bacteria

Current consensus (that comes from NGS of the 16S rRNA from thousands of fecal samples) is that in health, the predominant bacterial phylum in the human microbiota of the large intestine are Bacteroidetes and Firmicutes. The next most abundant phylum is Actinobacteria, mainly comprised of the genus *Bifidobacterium* (see for example (27)).

Even when the bacterial communities vary greatly between individuals, statistical tools (Principal Component Analysis and clustering) have allowed to classify them into just 3 enterotypes (see (28)). Each of these enterotypes is characterized by the variation in the levels of one of three genera: *Bacteroides* (Bacteroidetes), *Prevotella* (Bacteroidetes) and *Ruminococcus* (Firmicutes) (see Figures 5 and 6). We remark that the classification of the microbiota into enterotypes can depend on the method for data processing and clustering. Some other works (see (29)) show two cluster, where the *Bacteroides* enterotype appears fused with the less well distinguished *Ruminococcus* enterotype.

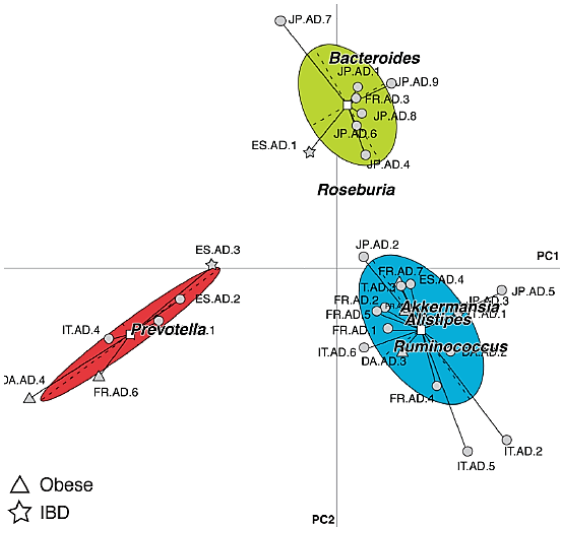

Figure 5: Results from Principal Components Analysis and clustering (28). We observe 3 enterotypes. Abbreviations: IBD inflammatory bowel disease.

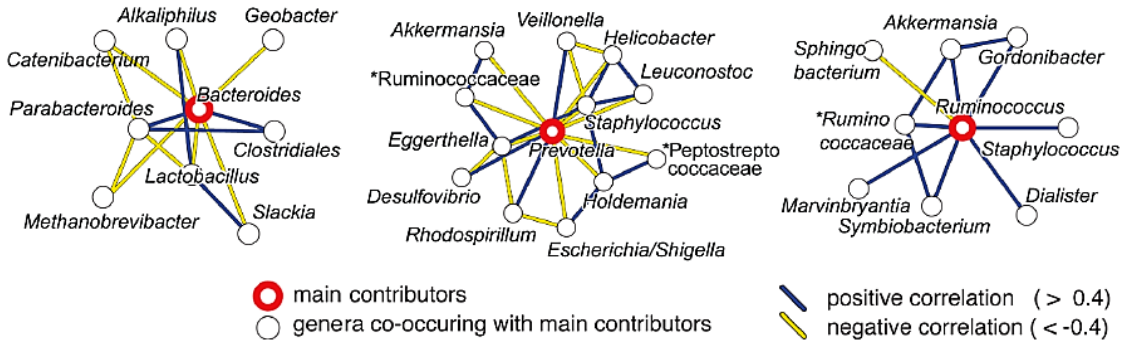

Figure 6: Network analysis of the three enterotypes (28).

Diet is one of the main factors that can alter the microbiome. Dietary effects primarily distinguish the *Prevotella* enterotype (associated with high-carbohydrate diet) from the *Bacteroides* (associated with diets that are high in fat or protein) (see again (29)). Other factors, like infection, disease, and antibiotics, may transiently alter the stability of the natural composition of the gut microbiota and, consequently, can have a deleterious effect on the well-being of the host.

The ratio Firmicutes/Bacteroidetes is an important marker since it appears increased in obesity. High-fat, high-calorie Western diets have been observed to quickly change the microbiota from a thin to an obese pattern while fat-or carbohydrate-restricted diets increase the Bacteroidetes levels and reduce the Firmicutes/Bacteroidetes ratio (see (30) and (31)).

The initial development of the neonatal microbiome is strongly determined by maternal–offspring exchanges of microbiota. It is well-known that this process is affected by several practices, as Caesarean section antibiotics, and formula feeding (see for example (32)). Babies born by Caesarian section harbor no vaginal microbes like *Lactobacillus* or *Prevotella*, but they are colonized by skin bacteria like *Staphylococcus, Corynebacterium* or *Propionibacterium*. Moreover, formula feeding has been linked with increased bacterial diversity, increased prevalence of *Clostridium. difficile*, *Bacteroides fragilis, and Escherichia coli*, and decreased prevalence of *Bifidobacterium.*

The gut microbiome of new-born has a low diversity and a relative dominance of Proteobacteria and Actinobacteria. As time passes, this microbiota becomes more diverse with the emergence and dominance of Firmicutes and Bacteroidetes. By the end of the first year of life, infants possess an individually distinct microbial profile, converging toward the characteristic microbiota of an adult. It is considered that the first 3 years of life represent the most critical period for dietary interventions to improve child growth and development (see (27)). This is the period when the intestinal microbiota is established and its alteration during this period can strongly affect host health and development. We notice that it has been proved that the

microbiota is a vital asset for neurodevelopment (see for example (33)). In the elderly, the gut microbiota is characterized by a reduced bacterial diversity, lower levels of Firmicutes, and Actinobacteria (mainly *Bifidobacterium*), and increased populations of Proteobacteria.

For the convenience of the reader, we include in Appendix 1 the taxonomic tree corresponding to the most common genera in the gut microbiota.

4.3.2 The gut mycobiome

As in the case of bacteria, fungi seem to colonize the gut shortly after birth, and the fungal composition is affected by several factors as age, diet, medication, etc. (see (25)). Despite the number of published data on the gut mycobiome is increasing, it is still difficult to describe the composition of the healthy gut mycobiome. In contrast with gut-associated bacteria, studies have found a lack of stability in the gut mycobiome over time and low abundance and diversity (see (34) and (35)). In most studies, Ascomycota is by far the most prevalent fungus phylum in the gut. We remark the recent paper (34), where the authors sequenced 317 stool samples from the American HMP project. The mycobiome in this healthy cohort was dominated by yeast and it was mainly composed of a high prevalence of *Saccharomyces, Candida*, and *Malassezia*, with *Saccharomyces cerevisiae*, *Malassezia restricta*, and *Candida albicans* being found in 96.8%, 88.3%, and in 80.8% of the samples, respectively (see Figure 7). As these fungal species persisted across a majority of samples, giving evidence that a core gut mycobiome may exist.

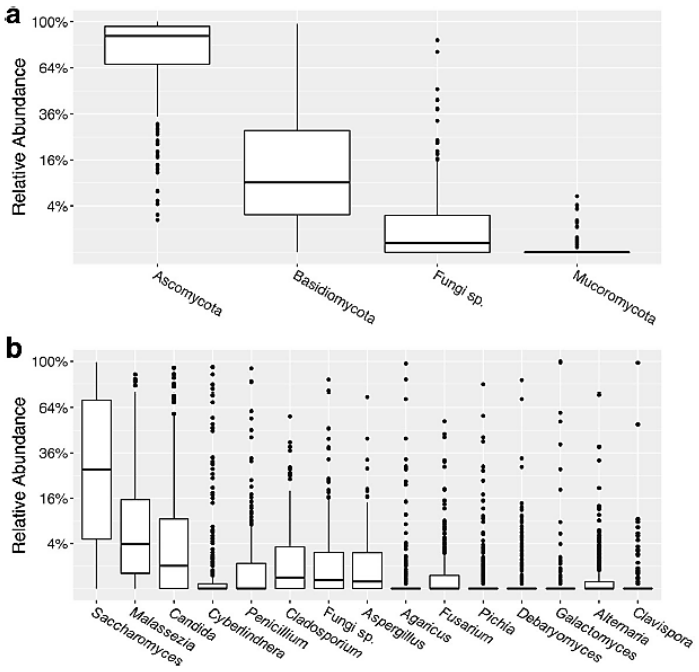

Figure 7: Composition of the human gut mycobiome (34). Relative abundance of fungi at the a) phylum level and b) genus level.

4.4. The role of the gut microbiome on the gut-brain axis

The interaction between the microbiome and the gut-brain axis relies on the following mechanisms, that are summarized in Figure 8. A more detailed description of these pathways can be found in (9) and (11).

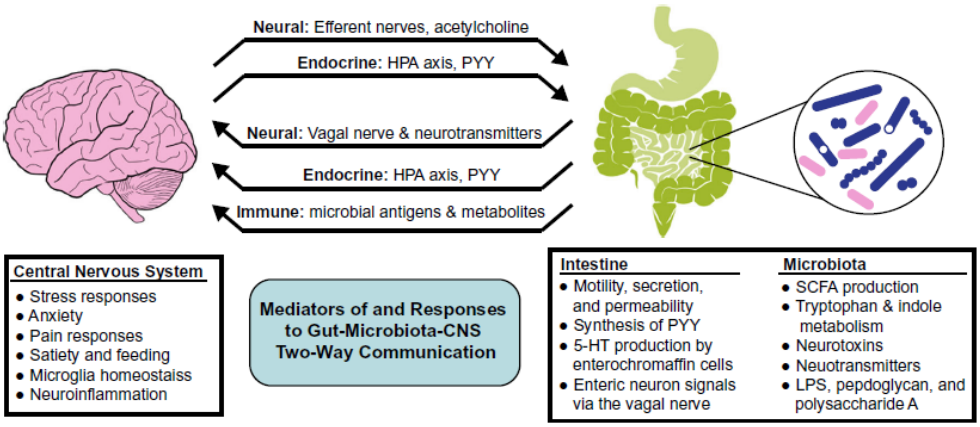

Figure 8: Pathways involved in the communication between the gut microbiota and the brain (9)

Immune activation. Microbiota and probiotic agents can have direct effects on the immune system, and the immune system also exerts a bidirectional communication with the brain. Moreover, indirect effects of the gut microbiota on the innate immune system can result in alterations in the circulating levels of cytokines that affect brain function.

Vagus nerve. Animal studies support strong evidence that gut microorganism can activate the vagus nerve and that such activation plays a critical role in mediating effects on the brain. Nevertheless, the mechanisms of this activation are still unclear.

Tryptophan metabolism. Tryptophan is an essential amino acid and is a precursor to the neurotransmitter serotonin. A growing body of evidence shows that, in many disorders affecting both the brain and the gastrointestinal tract, there is a dysregulation of the tryptophan metabolic pathway. There is some evidence to suggest that some probiotics (as *Bifidobacterium infantis*) can modulate this pathway.

Host metabolic reactions. Gut bacteria modulate various host metabolic reactions, resulting in the production of metabolites such as bile acids, choline and short-chain fatty acids (SCFA) that are essential for host health. On the other hand, complex carbohydrates can be digested and subsequently fermented in the colon by gut microorganisms into short-chain fatty acids with neuroactive properties.

Microbial neurometabolites. It has been proved that Bacteria generate neurotransmitters and neuromodulators. For example, *Lactobacillus* spp. and

Bifidobacterium spp. produce GABA; *Escherichia* spp., *Bacillus* spp. and *Saccharomyces* spp. produce noradrenalin; *Candida* spp., *Streptococcus* spp., *Escherichia* spp. and *Enterococcus* spp. produce serotonin; *Bacillus* spp. produce dopamine; and *Lactobacillus* spp. produce acetylcholine.

Bacterial cell wall sugars. Cell wall components of the microbiome are poised to induce epithelial cells to release molecules that in turn modulate neural signaling or that act directly on primary afferent axons.

Epithelial permeability. As described in (36), normal gut microbiota is essential in preventing colonization of the harmful bacteria by competing with them. If this microbiota is reduced, pathogenic organisms can colonize the epithelium. The toxins produced by these organisms, together with the local inflammation, can increase gut permeability. Moreover, several studies show that some species of probiotics (as *Lactobacillus*, *Escherichia coli*, and *Bifidobacterium*) can upregulate trans-membrane proteins and enhance mucus production, reducing then gut permeability.

Neurotoxins. Bacteria are capable of producing potent neurotoxins. One example of toxin produced in the intestine affecting the CNS is given by botulism. Even when this is a rare and extreme case, it is plausible that additional species within the microbiota can secrete highly potent neuroactive chemicals that have not yet been identified.

We finally remark that the above mechanisms can affect the brain development. In fact, the gut microbiota has been proved to be involved in mammalian brain development and subsequent adult behaviour (see for example (37) and (38)).

4.5 The gut microbiome and its connection with CNS diseases

We have seen that there is a strong evidence that the gut microbiota interacts with the brain. Then, the natural question is: to what extent does this interaction have a role in the development and/or the evolution of neurological disorders? Moreover, can we revert or prevent these diseases by modulating the gut microbiota?

As we will see in this section, some recent studies establish a correlation between the microbiota composition and different disorders (see Figure 9). This research has become possible due to the recent developments in metagenomics, as we pointed out in Section 4.1.3. Here we will present the state-of-the-art of the studies on the link between the gut microbiota and neurological diseases. For a more detailed exposition, we refer to (39) and (20).

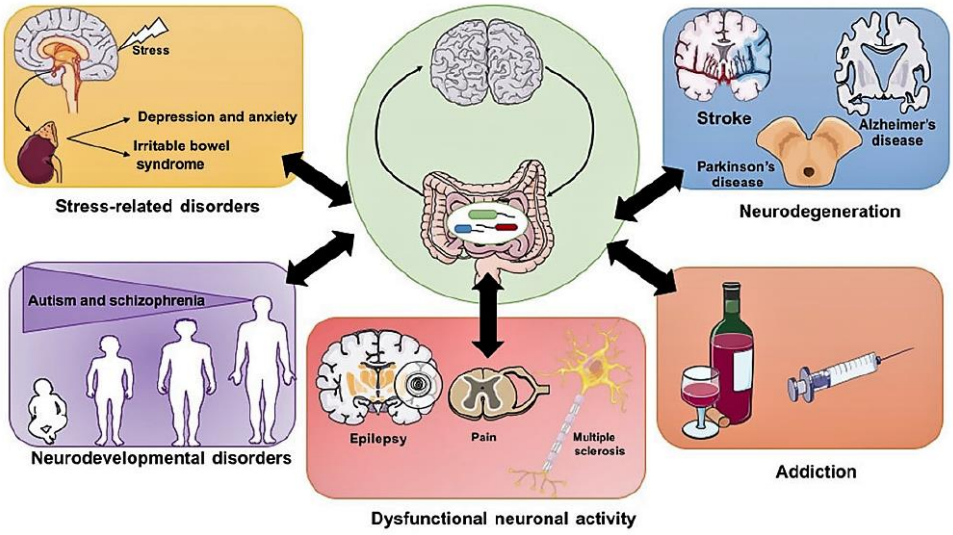

Figure 9: Overview of the effect of microbiota-gut-brain axis on neurological diseases (39)**.**

Aging Recent studies have recently characterized the microbiota composition of aged (20–21 months old) versus young (2–3 months old) mice. Aged animals have been observed to display alterations in the microbiota that have previously been related to inflammation. In aged animals, the intestinal permeability appears increased. This increased permeability can increase the risk for the translocation of bacteria or bacterial component.

Parkinson disease. Parkinson disease (PD) is the most common movement disorder. It is characterized, pathologically, by degeneration of dopaminergic neurons of the substantia nigra pars compacta and distinctive alfa-synuclein-containing cytoplasmic inclusions known as Lewy bodies. Patients exhibit motor-related impairments. Prodromal alterations in bowel function, especially constipation, are often reported before the development of the motor symptoms. Moreover, the progression of the disease is related to constipation, impaired gastric emptying. It is important to notice that, as these symptoms appear before motor manifestations, this can give us an interesting tool for prevention, early diagnosis and better treatment at the initiation stage.

Experimentally, abnormal forms of alpha-synuclein appear in enteric nerves before they appear in the brain and injection of abnormal alpha-synuclein into the wall of the intestine spreads to the vagus nerve. Ingested toxins and alterations in gut microbiota can induce alpha-synuclein aggregation and PD, however, it is not known how PD starts (see (40) and (41)).

As quoted in (39), the composition of the microbiota in feces samples has been seen to have a reduced abundance of the genera *Prevotella* (that may indicate decreased

mucin synthesis, which is associated with increased gut permeability), *Blautia* and *Roseburia* (anti-inflammatory) and increases in *Akkermansia muciniphila* (a mucin-degrading bacteria) and *Faecalibacterium* (pro-inflammatory). Altered abundances of the Bifidobacteriaceae, Christensenellaceae, Tissierellaceae, Lachnospiraceae, Lactobacillaceae, Pasteurellaceae, and Verrucomicrobiaceae families have also been found, corroborating the relationship between the altered microbiota and the evolution of Parkinson disease. Moreover, positive correlation between levels of Enterobacteriaceae and the severity of postural instability and gait difficulty was proven in PD patients. It has also been seen that the levels of SCFA are lower in parkinsonian patients and that the prevalence of *Helicobacter pillory* is higher. Even when its mechanisms have still to be understood, the close relationship between gut dysbiosis, intestinal permeability and neurological dysfunction suggests that the gut microbiota modification may provide a promising therapeutic tool in PD. Recently, an increase of the Firmicutes/Bacteroidetes ratio has been reported in an induced mice model of PD (see (42)).

Alzheimer's disease Alzheimer's disease (AD) is the most common cause of dementia. It is a neurodegenerative disease characterized by the accumulation of amyloid plaques, tau fibrils, and neuroinflammation that culminates in severe cognitive decline. Recently, it has been seen that the *Escherichia/Shigella* genera (associated with mediating inflammation) appear increased in fecal samples from Alzheimer's patients relative to healthy controls. Moreover, *Prevotella* appears decreased. In (43) it was observed a decreased Firmicutes/Bacteroidetes ratio and decreased *Bifidobacterium* in AD patients. Moreover, it was observed a correlation between levels of differentially abundant genera and cerebrospinal fluid (CSF) biomarkers of AD.

It has been proposed that, as intestinal permeability increases with age, some bacteria or bacterial components as LPS (lipopolysaccharide), found in amyloid plaques, may transport from the gut into the systemic circulation and mediate neuroinflammation. We also notice that risk factors for AD such as metabolic syndrome, type 2 diabetes and obesity are associated with gut microbiota alterations.

A very interesting recent study by Pisa et al. (44) found that 100% of the AD patients analyzed presented fungal cells and fungal material in brain sections. Moreover, fungal macromolecules were found in blood serum from AD patients. We remark that Aβ peptide has a potent antimicrobial activity, in particular against *C. albicans*. Then, it is possible that the presence of a chronic fungal infection in the CNS promotes the synthesis of Aβ peptide, which in turn leads to amyloid deposits. The results in this paper support the hypotheses that AD may be caused by fungi, even when more research should be necessary to prove casualty. It is interesting to point out that the gastrointestinal tract is the main reservoir of *C. albicans,* from where systemic infections

originate as a consequence of the disruption of the intestinal mucosal barrier. Moreover, the antagonistic interkingdom interactions between *C. albicans* and common intestinal commensal bacteria have been recently showed (see (45)).

Multiple sclerosis Multiple sclerosis (MS) is an autoimmune neurodegenerative disorder characterized by the progressive loss of myelin surrounding the axons of neurons. It has been seen that patients with MS present significant reductions in *Faecalibacterium*, *Prevotella* and *Araerostiples*. Nevertheless, it is not clear if these alterations in the gut microbiota are a cause or a consequence of the disease. We also notice that pre-clinical studies with GF mice have shown that the gut microbiota influences myelination within the CNS.

Depression The role of the gut microbiota in depression and other stress-related disorders has predominantly studied in animal models. Recent research has proved that stress in rats is related to a decrease in the Firmicutes/Bacteroidetes ratio. More precisely, decreases in the relative abundances of *Lactobacillus* and increases in *Oscillibacter.* Even when pre-clinical research gives us strong evidences of this relationship, only a few clinical studies to have performed microbiota analysis in depressed patients to assess for any potential dysregulation. In these works, depressed patients were found to have a dysregulated microbiota, observed as a reduction in species richness and microbial diversity. Moreover, a negative correlation between *Faecalibacterium* and severity of depressive symptoms has been reported. We notice when of fecal microbiota of depressed patients were transplanted to microbiota-depleted rats, the depression phenotype was also transferred to the animals. These animals presented an increased kynurenine/tryptophan ration. This means that the altered microbiota affects the tryptophan pathway, which is implicated in depression.

Autism spectrum disorder (ASD) The BTBR animal model (that presents a spontaneous deletion of the DISC1 gene) is a model of autism. It has been demonstrated that BTBR mice present a decrease in the Firmicutes/Bacteroidetes ratio, together with increases in the abundance of species such as *Akkermansia mucinphilia* and reductions in *Bifidobacterium*, suggestive of microbiota dysregulation. On the contrary, In clinical studies, it has been seen a significant increase in the Firmicutes/Bacteroidetes ratio due to a reduction of the Bacteroidetes relative abundance ((46)). Moreover, *Prevotella* and other fermenters has been found to decrease (see (47)).

Addictions Little is known with regard to the role of the gut microbiota in substance abuse disorders. Nevertheless, there is growing evidence on the capability of the gut microbiota to modulate behaviors relevant to substance abuse.

Even when research on the link between the gut microbiome and brain diseases is still dispersed, we observe we can extract the following information from the above review. From one hand, a reduction in *Prevotella* seems to be decreased in many of these disorders, as PD, AD MS and ASD. On the other hand, the Firmicutes/Bacteroidetes ratio appears to be altered. Some diagnostics companies, (see for example (48)) offer the analysis of this ratio and indicate optimal ranges.

4.6 Probiotics, prebiotics and synbiotics

4.6.1 Basic definitions

Probiotics According to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (18). We notice that this definition points out the need for providing an adequate dose of probiotic bacteria in order to obtain the health benefits. It is generally accepted that probiotic products should have at least a concentration of 10^6 CFU/mL and that a total of some 10^8 to 10^9 probiotic bacteria should be ingested daily for the probiotic effect to be conferred to the consumer (see (49)).

From the legal point of view, it is difficult to precise when a food product can be classified as probiotic. To date, there is not, in the European Union, a legal framework defining probiotic bacteria or the food category "probiotics". Nor is there a harmonized EU legal framework establishing the conditions for a strain to be considered as probiotic. Moreover, the 2007 European Commission Guidance on the implementation of the Nutritional Health Claim Regulation (NHCR) (50) considered the phrase "contains probiotics" to be a health claim instead of a nutrition claim. Many applications for health claims on probiotics have been submitted for evaluation to the European Food Safety Authority (EFSA) but to date, no application has received a positive opinion (see the EU register of nutritional and health claims (51)). We can see from this register that the main reason for these rejections is the lack of sufficient scientific evidence.

Nevertheless, we point out that the regulation of probiotics is not the same worldwide. Some countries (as for example Canada) include a list of species considered as probiotics in their regulatory guidelines (see for example (52))

In order to fix an appropriate use and scope of the term 'probiotic', an expert panel was convened in October 2013 by the International Scientific Association for Probiotics and Prebiotics (ISAPP). The conclusions of this consensus meeting can be found in (53).

In this consensus, the categories of living microorganisms for human use are classified as in Table 1:

Non-probiotics	This category includes traditionally associated with fermented foods and for which there is no evidence of a health benefit
Probiotics	
Probiotic in food or supplement without health claim	A member of a safe specie, with sufficient evidence for a general benefit in humans
Probiotic in food or supplement with a specific health claim	A specific strain, in an efficacious dose, and with sufficient evidence in the specified health condition
Probiotic drug	What constitutes a drug claim varies among countries

Table 1: Categories of living microorganisms for human use (adapted from (46)**)**

Prebiotics In Europe, EFSA follows the FAO prebiotic definition, that states that "A prebiotic is a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota" (see for example (54)). Prebiotic is considered a health claim, and no application for a health claim on prebiotics has been approved (see again the EU register for nutritional and health claims (51)). The current ISAPP consensus panel now proposes the following definition of a prebiotic: *a substrate that is selectively utilized by host microorganisms conferring a health benefit* (see (55)).

Synbiotics are Products that contain both probiotics and prebiotics, with conferred health benefits (see the WGO guidelines (56)).

Psychobiotics are probiotics that can affect cognitive functions (see for example (57)).

4.6.2 Genera, species and strains used as probiotics

The most common species used as probiotics are species of the genera *Lactobacillus* and *Bifidobacterium*. The yeast *Saccharomyces boulardii* and some species from other genera as *Escherichia* and *Bacillus* have also been used. Newcomers include also *Clostridium butyricum*, recently approved as a novel food in European Union.

A probiotic strain is identified by the genus, species, subspecies (if applicable) and an alphanumeric designation that identifies a specific strain. Marketing and trade names are not controlled by the scientific community. According to FAO/WHO guidelines (18),

probiotic manufacturers should register their strains with an international depository. Depositories will give an additional designation to strains. An example of this identification system can be seen in the following table:

Genus	Species	Subspecies	Strain designation	International strain depository designation	Strain nickname
Lactobacillus	*rhamnosus*	None	GG	ATTC 52103	LGG
Bifidobacterium	*Animalis*	*lactis*	DN-173 010	CNCM I-2494	Bifidus Regularis
Bifidobacterium	*longum*	*longum*	35624	NCIMB 41003	Bifantis

Table 2: Nomenclature used for probiotic microorganisms (adapted from (56))

As we will see in the following subsections, some properties appear, in the literature, linked to a specific strain, while some mechanisms of probiotic activity are shared among different strains, species, or even genera.

The probiotic organisms that we can find in commercial products have been mainly sourced from the gut or from traditional fermented foods. Nevertheless, modern biotechnology allows us to consider and develop probiotics that address specific needs and issues. In particular, the sequencing of the human gut microbiome has dramatically extended the range of organisms with potential health benefits. These organisms are called next-generation probiotics (NGPs), but may also be termed live biotherapeutic products (LBPs) (see (58)).

4.6.2 Probiotics: general mechanisms of action

The mechanisms of action of probiotics are not completely clarified (see for example (59) or (60)). Moreover, most of the available research is still pre-clinical. Nevertheless, some mechanisms have been postulated (see Figure 10). Here we summarize these mechanisms. For a more complete exposition we refer to (59) and (60)).

One of these mechanisms is a competition for adhesion sites. Some strains of *Bifidobacterium* and *Lactobacillus* can adhere to the epithelium, preventing pathogens from adhering to the mucosa. For example, *Lactobacillus rhamnosus* strain GG and *L. plantarum* have been proven to in vitro inhibit attachment of *E. coli* to human colon cells.

Another possible mechanism of action is the synthesis of antimicrobial compounds that modify the microbiota, as many species of the genera *Lactobacillus* and *Bifidobacterium.* Moreover, acid lactic bacteria produce some biological active compounds, as hydrogen peroxide, diacetyl, and short-chain fatty acids.

It has been seen that probiotics can stimulate the immune response. This immune response may decrease numbers of pathogenic organisms in the gut, thus improving the microbiome composition. Because of this immunomodulation effect, it is reasonable that probiotics can be useful in the prevention or treatment of other diseases.

Probiotics may also compete for nutrients that would otherwise be utilized by pathogens (as for example with *Clostridium difficile*).

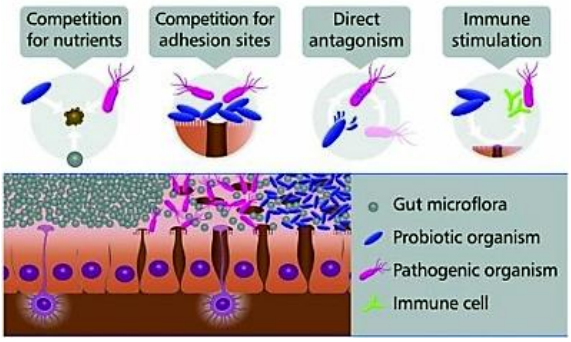

Figure 10: Probiotic mechanisms of action (61)

4.6.3 Probiotics and the gut-brain axis: pre-clinical research

As quoted in the previous sections, several CNS disorders have been associated with changes in the gut microbiome. This fact has led to an increasing interest in the regulation of the gut microbiota through probiotics. There is an increasing evidence that certain probiotics can impact the pathogenesis of CNS disorders. Clinical data is less compelling than the animal model data but is rapidly emerging.

Several probiotics have been investigated in animal models of neurological disorders. *Bifidobacterium* and *Lactobacillus* are the main genera that have shown beneficial effects on neurological diseases. In (62) we can find a systematic review of this research (to 2016). In particular,

Anxiety Reduction of the anxiety-like behavior in animals (mice or rats) was observed using single strains of *Bifidobacterim. longum, B. breve, Lactobacillus helveticus, L. plantarum,* and *L. fermentum*, as well as with multi-strain probiotic combinations of *L. rhamnosus* + *L. helveticus* and *B. longum* + *L. helveticus.*

Depression Antidepressant effects observed using single strains of *B. longum*, *B. breve*, *L. rhamnosus,* and *L. helveticus,* as well as using multi-strain combination of *B.longum* + *L. helveticus.*

Cognitive function Single strains of *B. longum, B. breve*, and *L. helveticus* were effective on both spatial and non-spatial memories. Single strains of *L. fermentum* and

Clostridium butyricum improved spatial memory ability. Multi-strain combinations of *L. rhamnosus + L. helveticus*2 and *B. longum + L. helveticus* were assessed to be effective with regard to non-spatial memory, and combinations of *Lactobacillus acidophilus + B. lactis + L. fermentum* and *L. plantarum + L. curvatus* were reported to be effective with respect to spatial memory.

Autism spectrum disorder and obsessive-compulsive disorder *B. fragilis* improved behaviors related to the ASD in maternal immune activation mice, but not social interaction behavior. On the other hand, *L. rhamnosus* was decreased some obsessive-compulsive disorder-like behaviors in mice, but with no effect in communicative or social interaction behaviors.

Stress response A probiotic combination of *L. rhamnosus + L. helveticus* prevented non-spatial memory dysfunction induced by acute stress. L. *casei* Shirota was related to a significant decrease of plasma corticosterone levels in response to acute. Moreover, *B. longum* biotype *infantis* was found to normalize depression- like behavior induced by maternal separation.

Mechanisms of action Apart from behavioral changes, pre-clinical research also gives us information about the mechanisms of the relationship between probiotics and the gut-brain axis. Pre-clinical studies give us the following evidence:

- Serum corticosteroid levels are found to be decreased by *L. plantarum, L. helveticus, L. fermentum, L. rhamnosus,* and *L. casei Shirota*.
- Adrenocorticotropic hormone (ACTH) could also be decreased by *L. helveticus* and *L. fermentum*.
- Inflammatory cytokines such as were decreased, and anti-inflammatory cytokines were increased with *L. plantarum, L. helveticus, L. fermentum, L. acidophilus, B. longum,* and *L. rhamnosus*.
- Brain monoamines (as for example 5-HT and DA) could be increased by *L. plantarum, L. helveticus,* and *B. infantis*, while their metabolites reduce.
- GABA receptor expression could be affected by *L. rhamnosus*, depending on the brain area.
- Brain BDNF and c-Fos mRNA expression was observed to increase with *L. helveticus, L. plantarum, L. rhamnosus, B. longum*, and *Clostridium butyricum*
- c-Fos in the hypothalamus paraventricular has observed to decrease with *L. casei* Shirota.
- Some effects of *L. rhamnosus* and *B. longum* could be mediated via the vagus nerve. It has been seen that *L. casei Shirota* improve gastric vagal afferent activity. *B. longum* was found to inhibit enteric neuron excitability. It has been

observed that a combination of *B. longum + L. helveticus* reduced intestinal barrier permeability.

- Serum tryptophan levels were increased by *L. helveticus, B. infantis,* and *B. fragilis* at the same time its metabolites decreased.
- The fecal microbiota has been observed to be altered by probiotics. For example, *Bacteroides* and *Lactobacillus* were increased and Firmicutes decreased by *L. fermentum*.

4.6.4 Probiotics and the gut-brain axis: human studies

In the 2016 review (62) a total of 15 human studies were included. All the selected studies had strong ratings in the quality assessment tool for quantitative studies. Eight of these 15 studies found significant effects of the probiotic treatments. The main results of these works are the following. For a more detailed exposition we refer to (62).

Psychiatric conditions 15 studies tested participants with respect to anxiety, depression, distress levels, mood state, and behavior disorders. The studies used different probiotic formulations containing different strains of *Lactobacillus* spp. and *Bifidobacterium* spp. The changes were measured using different scales as the Leiden Index of Depression Sensitivity-Revised (LEIDS-r), the Hospital Anxiety and Depression Scale (HADS), the State-Trait Anxiety Inventory (STAI), the Positive and Negative Syndrome Scale (PANSS) (used in schizophrenia) and the Developmental Behavior Checklist (DBC) (used in ASD). Some other indicators as the salivary cortisol levels were also considered. Among these studies, 7 of them reported significative changes, while other studies found no significant effects in the treatment with probiotics.

Memory and other cognitive abilities Different memory and cognitive abilities were evaluated in healthy participants. *L. casei* Shirota was found to slightly decrease memory abilities in all participants, with no effect on fluency or eating-associated behavior (see also (63)).

Neuroimaging study There was only one neuroimaging study, that used functional magnetic resonance imaging (fMRI). In this study, a fermented milk product with probiotic (FMPP) containing *Bifidobacterium lactis* with yogurt starters (*Streptococcus thermophilus, Lactobacillus bulgaricus*, and *Lactococcus lactis* subsp. *lactis*) was found to reduce task-related response of a distributed functional network.

Mechanisms of action Some evidences on the mechanism of action that we can deduct from the previous works in human studies can be summarized as follows:

- The cortisol levels in saliva and urine after probiotic interventions were found to decrease with *Lactobacillus casei* Shirota and multi-strain *L. helveticus* + *B. longum*, respectively.
- *L. casei* reduced pro-inflammatory cytokines, increased regulatory cytokines, and increased natural killer cell activity in smokers.
- Only one study in humans investigated the tryptophan pathway, but no significant changes were found.
- Changes in the fecal microbiota were observed: *Lactobacillus* increased, and C*lostridium* was decreased by *L. plantarum*, and *Bifidobacterium* and *Lactobacillus* increased after a treatment by *L. casei* Shirota.

4.6.5 Prebiotics and the gut-brain axis

Prebiotics comprise certain non-digestible oligosaccharides (NDOs), soluble fermentable fibers (as inulin and fructooligosaccharides), and human milk oligosaccharides (HMOs). The use of NDOs as prebiotics has rapidly increased, due to the fact that the enrichment of the diet with NDOs provides the opportunity to improve the gut microbial ecosystem (see for example (64)).

It has been proved that polysaccharides can improve brain function (see for example (19)). Moreover, plant polysaccharides have major influences on gut microbiota. For example, arabinoxylan was reported to increase the growth of butyrate producers (*Roseburia intestinalis*, *Eubacterium rectale*, *Anaerostipes caccae*); fucoidan was found to reduce Enterobacteriaceae population in the newly weaned pig and glucan increased the growth of *Lactobacillus* strains in the human intestine. On the other hand, prebiotics directly influence signaling molecules in the brain (see again (19)).

We also remark that one of the advantages of prebiotics is given by the presence of survival problems in the GI tract for probiotics (for example, for some genotypes). On the other hand, probiotics are usually supplemented by some few species at one time, whereas prebiotics could stimulate several beneficial species simultaneously.

4.6.6 Probiotics and prebiotics in food

Here we summarize the recent review on the most common probiotic and prebiotic foods we can find in the traditional gastronomy in our cultural environment.

4.6.6.1 Probiotics in food

The most classical source of probiotics in the diet is fermented food. Fermented products have a long history and they have been consumed by nearly every culture worldwide. Recently, some groups recommend their inclusion in national dietary guidelines (see (52)). Here we review some of the latest research on specific probiotic foods (in the European context). We remark that there is a need for more clinical studies to elucidate the effects of different fermented foods on human health.

Kefir An interesting pre-clinical study on the modulation of the intestine microbiota in mice by kefir administration can be found in (65). Here we summarize the main results presented in this paper. Kefir, a traditional food originated in the Caucasus Mountains, is a multi-species complex probiotic containing lactic and acetic acid bacteria and yeast, in a symbiotic mixture of more than 50 species of microorganisms. In the study, the number of total bacteria was found not to be significantly different during the experiment between the control and kefir groups. Nevertheless, interesting changes were observed in the composition of the gut microbiota. Moreover, these changes were observed to increase gradually during the administration period. In comparison with the control group, numbers of Firmicutes, Proteobacteria, Enterobacteriaceae in the kefir group significantly decreased while the numbers of Bacteroidetes, *Lactobacillus, Lactococcus* and yeast increased. In particular, the Enterobacteriaceae/*Lactobacillus* ratio increased and Firmicutes/Bacteroidetes ratio decreased (see Figures 11 and 12).

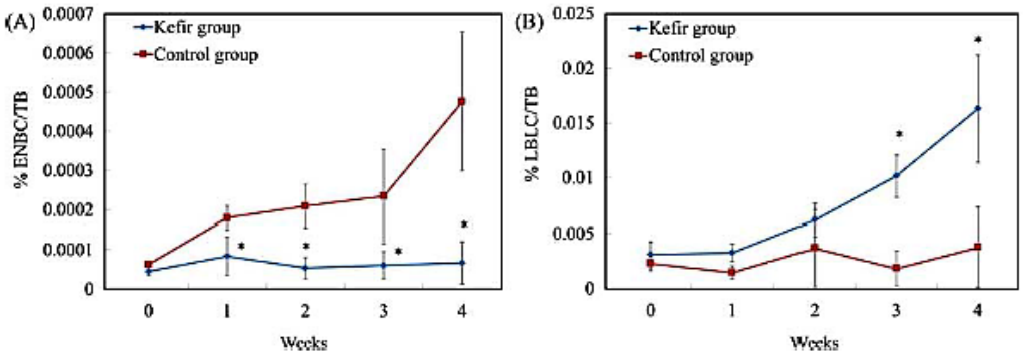

Figure 11 Composition of (A) Enterobacteriaceae and (B) Lactobacillus and Lactococcus in total bacteria during the experimental period (65). * indicates a significant difference compared to the control group Abbreviations: TB total bacteria, LBLC *Lactobacillus* and *Lactococcus*, ENBC Enterobacteriaceae

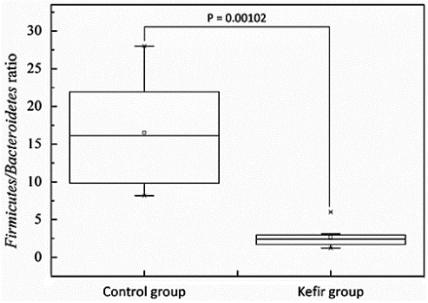

Figure 12 Box-and-whisker plot of Firmicutes/Bacteroidetes ratios (65).

Yogurt According to the USA Food and Drug Administration (FDA) regulations (see (66)),
yogurt is produced by culturing dairy ingredients with a characterizing bacterial culture that contains *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Recent research coincides in pointing out that the consumption of dietary yogurt does not produce significant changes in the composition of the gut microbiota (see for example (67) and (68)). Nevertheless, we remark that this does not mean that yogurt has no effect on the microbiota. For example, in (68), 7 healthy adult female twins consumed a probiotic yogurt (containing *Bifidobacterium animalis* subsp. *lactis* and 4 strains of lactic starter bacteria) for 7 weeks. Even when there were no significant changes in the microbiota composition, there were changes in transcriptional responses (primarily in carbohydrate metabolism pathways) and in urinary metabolites. These results highlight that one of the mechanisms of probiotics may be not to alter the microbiota composition, but to affect its metabolic pathways.

Cheese Some recent papers indicate that some cheeses can be considered as probiotics. For example, it has been seen that starter acid lactic bacteria survive in Cheddar cheese (see (69)). Notice that this fact opens the door to enrich cheese with probiotics. In particular, it has been suggested to enhance the quality of Mozzarella (70) and Feta (71) cheeses by using different strains of *Lactobacillus.* On the other hand, some peptides derived from simulated gastrointestinal digestion of Parmesano Reggiano have been proved to stimulate the growth of most *Lactobacillus* and *Bifidobacterium*.

Non-dairy probiotic food Some probiotic fermented food include olives (the main microbial genus in more olive fermentations are *L. plantarum* and *L. pentosus*, as quoted in (72)), and fermented cabbage as sauerkraut or choucroute (see for example(73)).

4.6.6.2 Prebiotics in food

The main source of prebiotics in food is dietary fiber. A diet rich in the fructan-type resistant starches, especially oligofructose and inulin, is known to promote “good” species of colon bacteria (see for example (74)). Such prebiotics are found in the diet mainly associated with wheat, barley and onions.

An interesting study on the effect of the fiber content on the gut microbiota can be found in (75). In this work, the fecal microbiota of a group of European children (EU), consuming a ‘Western’ diet rich in animal fat and low in legume and fruit dietary fiber, was compared to a group of children from a rural African village of Burkina Faso (BF), consuming a plant-based diet, rich in fruit and legume fiber. BF children showed a lower Firmicutes/Bacteroidetes ratio, with abundance of *Prevotella* and *Xylanibacter*, known to contain a set of bacterial genes for cellulose and *xylan* hydrolysis, genus that is lacking in the EU children. Moreover, it was found significantly more SCFA in BF than

in EU children. On the other hand, Enterobacteriaceae (*Shigella* and *Escherichia*) were significantly underrepresented in BF than in EU children, as we can see in Figure 13:

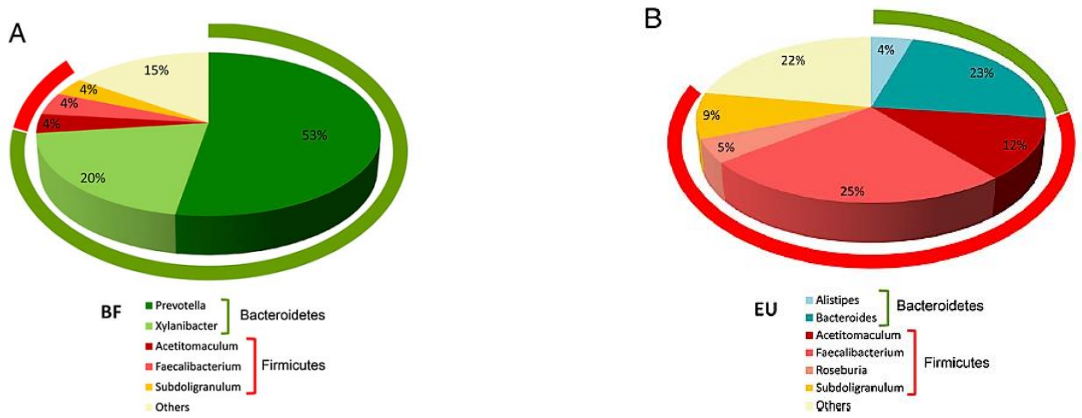

Figure 13: Diet and the microbiome (in green: Bacteroidetes, in red: Firmicutes) Adapted from (75).

We finally mention that, apart from fiber, some other components of the diet have been proved to be prebiotics, as for example polyphenols in wine (see (76)).

5 Conclusions

The conclusions of this work can be summarized as follows

1. Germ-free models have provided a big amount of evidence of the connection between the gut and the brain. In recent years, metagenomic techniques, jointly with computational and statistical tools, have allowed researchers to do a great step forward the study of the gut microbiome.

2. The predominant bacterial phyla in the human microbiota of the large intestine are Bacteroidetes and Firmicutes. We can classify the human gut microbiome into three enterotypes, characterized by the variation in three genera: *Bacteroides*, *Bacteroidetes,* and *Prevotella.* The *Prevotella* enterotype is connected with high-fiber diets, while the *Bacteroides* enterotype is linked to high-fat, low-fiber diets. Moreover, the Firmicutes/Bacteroidetes ratio has been observed to increase in obesity.

3. Several neurological diseases are correlated to changes in the gut microbiome. Among the reviewed results, we remark that *Prevotella* has been found to decrease in Alzheimer's disease, Parkinson disease, and autism spectrum disorder. On the other hand, the ratio Firmicutes/Bacteroidetes is altered in several brain disorders. Moreover, recent studies link Alzheimer's disease with a fungal infection. An

interesting question to elucidate is if this fungal infection is the cause of Alzheimer's disease and if the alterations in the gut permeability due to changes in the microbiota can be the cause of this fungal infection.

4. One of the tools to modify the composition of the gut microbiota is the use of probiotics and prebiotics. The most common species used as probiotics are species of *Lactobacillus* and *Bifidobacterium*, even when modern biotechnology has extended the range of organisms with potential health benefits. The study of the effect of probiotics in health is still in its infancy, and in fact, to date, no probiotic claims have been approved in the European Union.

5. The main source of probiotics in the diet are some fermented products. It has been seen in pre-clinical studies that kefir is able to change the microbiome composition. This effect has not been observed in the dietary consumption of yogurt, even when studies have reported changes in transcriptional responses. These results point out that one of the mechanisms of probiotics may be not to alter the microbiota composition but to affect its metabolic pathways. The main source of prebiotics in the diet are some types of fiber. As quoted before, high-fiber diets are associated with an increase in *Prevotella*.

6. From the evidence we can state that the microbiome plays a relevant role in human health. More research is needed to understand the mechanisms of the microbiota-gut-brain axis and its interaction with brain disorders, as well as to develop new probiotic products that target specific diseases. Given the importance of the microbiome, we consider convenient to pay attention to those factors that can affect its development, like Cesarean delivery, perinatal antibiotics, and formula feeding. Moreover, we consider important, as some groups propose, including probiotics in national dietary guidelines, as well as to promote the consumption of dietary fiber.

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Appendix 1: Taxonomic tree of the main genera in the gut microbiota

(Generated from (77))