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## CORSO DI LAUREA IN MEDICINA E CHIRURGIA

**Department of neuroscience, rehabilitation, ophthalmology, genetics and maternal and child sciences - DINOGMI**


# Epilepsy and nutritional habits in a pediatric cohort: how do they influence the gut microbiota profiling?


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# Primo Approdo ad Itaca.

A mio papà, a mia mamma, a mia sorella
ed a chi mi guarda da lassù.

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# Abstract

**Background:** The microbiota-gut-brain axis is a bi-directional communication pathway modulated by environmental factors and increasingly involved in epilepsy.

We aimed to investigate the gut-microbiota in pediatric epileptic patients, evaluating the influences of the response to anti-seizures medication (ASMs) and of nutritional habits.

**Methods**: Child with epilepsy and age-matched neurotypical children were recruited. Clinical and treatment data were collected. Stool samples were processed through 16S rRNA. The Rome IV questionnaire and the Bristol Stool Scale were used to record gastro-intestinal symptoms and stool consistency. A food diary was used to evaluate nutritional habits. Patients were sub-grouped into drug-sensitive (DS) and drug-resistant (DR). Alpha-diversity (AD) and beta-diversity (BD) were calculated using linear models (statistical significance $p<0.05$ or $p<0.1$).

**Results**: Forty-six patients with a median age of 6.5 years (IQR:8; range:0-17 years) and 33 healthy (H) children were recruited. Thirty-one (67%) patients had a developmental and epileptic encephalopathy, 7(15%) had genetic generalized epilepsy, 7 (15%) had focal epilepsy, and 1 (2%) had progressive myoclonic epilepsy. 25 (54%) patients were classified as DS and 21 (45%) as DR. AD and BD showed to be significantly associated with age in all groups. Both the DR and the DS subgroups showed a significantly different BD as compared to the controls (DS vs H: $p=0.001$; DR vs H: $p=0.001$). Carbohydrates showed to be significantly associated with a difference in BD (DS vs DR: $p=0.033$; DR vs H: $p=0.040$; DS vs healthy: $p=0.042$). The main significantly changing phyla were *Actinobacteriota, Desulfobacterota, Firmicutes,* and *Proteobacteria*.

**Conclusions**: Our study gives evidence that in epileptic patients the GM is influenced by age, diet, and response to ASMs. Differences in the inter-sample's composition give relevance to some bacterial phyla, whose role needs to be further investigated in future studies.

# 1. Introduction

**"All disease begins in the gut".**

*Hippocrates of Kos (Hippokrátes ho Kőos: c. 460 – c. 370 BCE)*

It was the 5th century B.C. when Hippocrates, the father of modern medicine, with this sentence resumed the essential link between brain and body, and particularly brain and gut.

An innovative concept, which breaks down the idea that each organ is independent of another, and instead proposes an innovative idea of communication between organs, even distant, placing the focus on an organ that will be revalued over the centuries: the intestine.

The human gastrointestinal tract is the largest micro-ecosystem in the human body, harbors a complex and dynamic population of microorganisms and counting approximately $10^{14}$ bacteria of more than 2000 different species living inside it.[1] The collection of bacteria, archaea, and eukarya, and to a lesser extent yeasts, parasites, and viruses, colonizing the GI tract is called the "**gut microbiota"**. This complex system, made of various forms of life, has co-evolved with the host over thousands of years to form an intricate and mutually beneficial relationship.[1] One should not confuse the microbiota with the **microbiome**, which represents the totality of the genetic information of the microbiota, that is gut microbial population. Inspired by the revolution of the master of medicine Hippocrates, according to which the gut is important to such an extent that the main factor of every disease lies in the intestine, several scholars have questioned the importance of this rich intestinal population. After several years of research, it was discovered that intestinal bacteria play a crucial role in maintaining immune and metabolic homeostasis and protecting against pathogens. Of the 2172 species isolated from human beings, classified into 12 different phyla, 93,5% belongs to four phyla, which can be considered the main gastrointestinal bacteria phyla: *Proteobacteria, Actinobacteria, Firmicutes, and Bacteroides*. When the bacterial community lives in equilibrium, there is a condition defined as **eubiosis**, which is essential to allow

the various components of the intestinal microbiota to be functionally effective for the host, performing metabolic functions (such as the synthesis of certain neuroactive metabolites, neurotransmitters or their precursors), enzymatic functions, stimulating the immune system and eliminating toxins. An alteration of the gut bacterial composition is called **dysbiosis.** Dysbiosis is called **acute** when caused by infections that come from outside, or when caused by antibiotics causing symptoms such as diarrhea, abdominal pain, and meteorism. On the contrary, it is called **chronic dysbiosis** when caused by more subtle and slower-acting factors such as high-protein diets or diets with too many carbohydrates, unhealthy lifestyles (limited physical activity, smoking, alcohol abuse, etc.), or also drugs taken chronically, such as proton pump inhibitors (PPIs), corticosteroids, and oral contraceptives. Regardless of the cause, when dysbiosis occurs, it has been associated with the pathogenesis of many diseases, affecting not only the gastrointestinal tract, such as diabetes, obesity, dermatitis, cardiovascular and neurological diseases.[2,3] Indeed, it was seen that not only an alteration of the intestinal tight junctions can promote the passage of toxic substances, allergens, and microbes from the intestinal lumen to the circulatory stream, leading to cardiovascular and inflammatory diseases, but also certain changes modulated by the microbiota, for instance those in neurotransmitter/precursor synthesis, may worsen alterations in brain function and influence outcome in neurological and psychiatric diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Autism Spectrum Disorder (ASD), schizophrenia and mood disorders.[4,5,6,7]

A key prerequisite for supporting the causality between gut microbiota composition and metabolic disorder states is knowledge of the composition and function of the gut microbiome of persons defined as metabolically healthy. On a taxonomic level, healthy human gut microbiota has not yet been well defined due to the individual uniqueness of the relative distribution of bacteria, fungi, viruses, and archaea and the high variability of strains, microbial growth, and structural variants within microbial genes. Exposure to environmental factors and host genetics plays a major role in determining microbial diversity

between individuals. In general, it is observed that gut microbial communities in healthy individuals are characterized by: high diversity of microbial taxa; high microbial gene richness; and stable functional cores of the microbiome.

Although the exact mechanisms underlying the communication pathways between intestinal microbial systems and the host and the ones by which the gut can determine the onset of several ailments remain rather elusive, its connections with the brain and other organs are certainly deeper than one might think, and the seed planted by Hippocrates in the minds of scholars of a human body as a complex system of organs in communication with each other, with the gut probably at the core, the matrix of every ailment, has triggered numerous studies in what might be called the 'Hippocratic revolution'.

## 1.1 Origin of the gut microbiota and its place in early life

The gut microbiota originates before the baby is even born. Various factors can influence the cognitive and microbial development of the child, such as mental health, the mother's lifestyle, antibiotic exposure, environmental factors, infections, etc.[8] Particularly, dietary habits during the period of gestation can shape the maternal intestinal microbiota with the subsequent release of SCFAs, microbial by-products which are able to pass from the gut lumen to the circulation and travel to the fetus via the placenta and that can also imprint in-utero development with possible health outcomes on the offspring across the lifespan.[8,9] Indeed, it is well-known that unhealthy eating habits, leading to maternal overweight and obesity, have an impact on the infant microbiota and can influence the risk of obesity later in life.[9,10,11]

The microbiota in late pregnancy has reduced alpha-diversity but higher beta-diversity compared to non-pregnant women or early pregnancy. In the third trimester, there is a greater representation of lactic acid bacteria (*Lactobacillus, Streptococcus, and Enterococcus*), which are highly prevalent in the infant's gut, whereas butyrate-producing bacteria (such as *Faecalibacterium, Blautia, and Ruminococcus*), which dominate the gut in adulthood, are enriched in early pregnancy. The greater representation of

*lactic acid bacteria* may be seen as an adaptation to prepare the mother for the transfer of these organisms in the birth and perinatal period to her offspring to take maximum advantage of the main energy source for the child, lactose in its mother's milk.[10]

When the baby is born, it is immediately confronted with an interval of time that will be crucial for its life. The first thousand days of life represent the period from conception to 2 years of age, a critical window of perinatal development susceptible to insults that can have long-lasting effects on the microbiota-intestine-brain axis, and a delicate phase in which the balance between the various organs and apparatuses is essential for the child's future health.[8,12]

At the time of delivery, when the newborn's skin and mucosal surfaces are seeded with maternal vaginal and fecal microorganisms, a new microbiota origin. This initial microbial exposure constitutes the first moment leading to extensive microbiota colonization of the neonate and establishes an early-life microbiota that engages in a mutualistic relationship with the host and leaves a lasting impression on childhood development that can control the balance between health and disease.[8,13] The intestinal microbial population may vary from one infant to another depending on the type of delivery[14]. An infant delivered vaginally (VB) presents a representative colonization of the vaginal tract of the mother, with quick colonization by anaerobes, such as *Escherichia coli and Streptococcus, followed by Bifidobacteria, Lactobacillus, Bacteroides, and Gram-positive bacteria* in the first month. From one year of age, the microbiota increasingly resembles that of the adult, and the bacterial groups isolated are *Staphylococcus aureus (4%), S. epidermidis (20%), Streptococcus fecalis (30%), Streptococcus faecium [10], nonhemolytic streptococci [10], and Enterobacteriaceae (E. coli [20%], Klebsiella aerogenes [20%], Proteus mirabilis [2%], Enterobacter cloacae [1%], Serratia sp. [1%], and Pseudomonas aeuroginosa [0.5%]).*[15]

Babies born by cesarean section (CS) instead, lose contact with the microbes in the mother's vaginal canal, showing a significantly lower abundance of *Bacteroides and Bifidobacterium spp*., while species associated with the hospital environment (often possessing virulence and antibiotic resistance

genes) and bacterial communities similar to those found on the skin surface, dominated by *Staphylococcus, Corynebacterium,* and *Propionibacterium spp.,* are overrepresented.[16,17,18] Moreover, compared to VB infants, the gut microbiome of CS infants also exhibits a higher degree of strain turnover in early life, with fewer maternally-derived strains, leading to functional differences in the immunostimulatory potential of the gut community.[19,20] There is growing concern that disruption of microbial transmission from mother to neonate is linked to conditions more frequently observed in CD-born individuals, including allergies, chronic immune disorders and metabolic disorders[20,21,22] (Figure 1).

Another important factor influencing the newborn microbiota is the milk, and changes are observable in breastfeeding infants versus infants alimented with formula.

Indeed, breastfeeding affords protection against a wide variety of medical conditions that may emerge at different time points over the lifespan, including hospital admissions for respiratory infections and neonatal fever, childhood obesity and cancer, sudden infant death syndrome (SIDS), as well as an array of other medical conditions, such as cardiovascular disease, obesity, hyperlipidemia, hypertension, types 1 and 2 diabetes, depression and Alzheimer's disease.[23,24] On the contrary, Infant formula is an industrially produced substitute for infant consumption that attempts to mimic the nutritional composition of breast milk as closely as possible. The infant formula has different limits, that compared to breast-feeding milk, make it a non-preferable choice for the child's nutrition: the high casein content makes it less digestible, and of the K vitamin contained in it, only limited amounts are transferred from the placenta to fetus, making the child deficient in vitamin k, with a higher risk of developing hemorrhagic disease.[25]

It has also been found that non-breastfed infants had a higher abundance of *Bacteroidaceae, Bacteroidetes, Clostridia*, and *Bifidobacteria,* with an overall increase in facultative anaerobic bacteria such as *Staphylococci, Enterobacteriaceae*, and *Streptococci*.[26,27,28]

Moreover, breastfed infants have fewer bacterial species associated with pathogenesis such as *Escherichia coli, Bacteroides fragilis*, and *Clostridium difficile* compared to formula-fed infants.[28]

Thus, it is possible to conclude that the microbiota is a complex, evolving system, perennially stimulated by factors of different kinds from the very beginning, during the critical window of the first 1000 days of life, and on which the health of the individual, or the onset of numerous diseases, will depend.

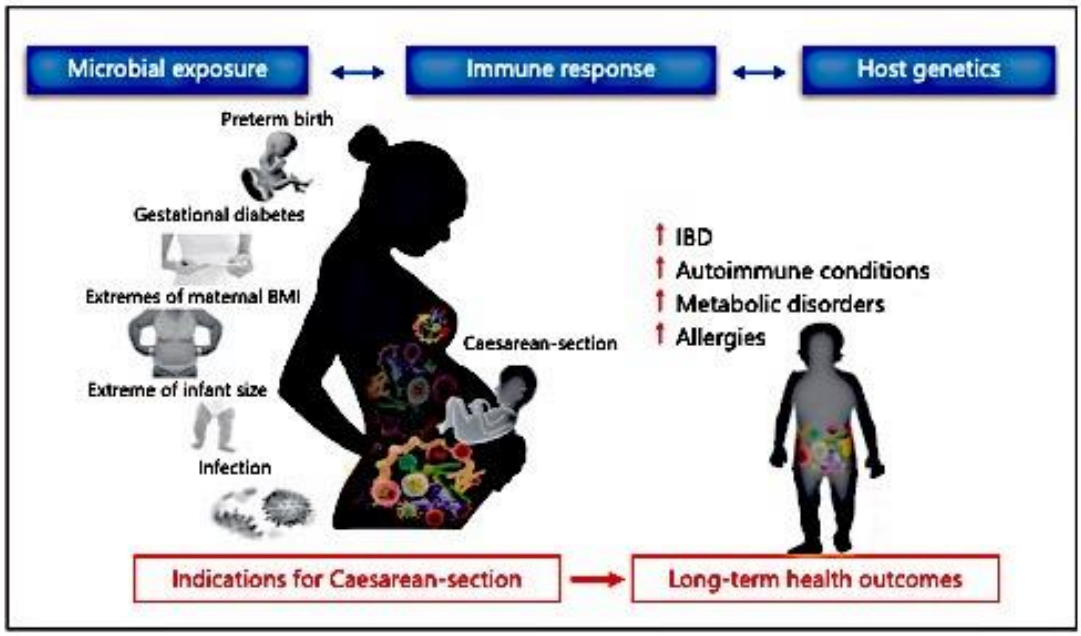



**Figure 1** *A summary of our current understanding of factors that indicate conditions linked with a Caesarean-section delivery. The developing gut microbiota, microbial exposures from the environment, and host genetics interact to mediate infant immune responses. Several indicators of Caesarean-section, including preterm birth, extremes of maternal body mass index (BMI), infection, extremes of infant size, and gestational diabetes, may independently cause microbial dysbiosis and confound our understanding of the effects of Caesarean-section. Microbial dysbiosis caused by Caesarean-section delivery is linked with an increased risk for inflammatory bowel disease (IBD) and a wide range of autoimmune, allergic, and metabolic conditions.* | [Taken from reference 18: Salas Garcia MC, Yee AL et al.; Dysbiosis in Children Born by Caesarean Section. Ann Nutr Metab]

## 1.2. The microbiota-gut-brain axis: pathways of communication

Over the course of time, the belief that the organs of the human body are inseparably connected has developed and strengthened, and two organs, in particular, have become the true paradigmatic examples of this connection: the brain and the intestine, so much so that they are now considered almost like brothers. Precisely in recent years, the intestine has been given the appellation “second brain”, and we are now beginning to refer to the complex of microbes (which, although invisible to the naked eye, reach an overall weight of over 2 kg) as the “third brain”. This connection finds its origin in the embryological development of these organs, both originated from the same issue –the neural crest- tightly working in tandem, each influencing the other. The brain and gut communicate thanks to a bidirectional communication network that comprises the central nervous system (CNS) –both the brain and spinal cord-, the autonomic nervous system (ANS), the enteric nervous system (ENS), and the hypothalamic-pituitary-adrenal axis (HPA), forming a complex system known as “gut-brain axis”.[29,30] (Figure 2)

Gut microbiota can release molecules that may activate the neuro enteric plexus and stimulate brain production of neuropeptides, as well as increase gut–blood barrier and blood–brain barrier (BBB) permeability. Indeed, the diet- or chronic stress-induced alterations of the intestinal barrier, triggers the activation of dendritic as well as of other immune cells and leads to the loosening of the tight junctions between epithelial cells, allowing microorganisms to translocate across the intestinal barrier, which in turn enables further local immune cell activation and dissemination throughout the systemic circulation.[31,32]

At the same time, the brain releases molecules that stimulate the neuroenteric plexus and gut function, to such an extent that the ENS can be viewed as a peripheral extension of the limbic system into the gut or, alternatively, parts of the CNS (in particular, pontine, autonomic and limbic circuits) can be viewed as an encephalized portion of the ENS.[33] The impact of the microbiota on the

axis has been further supported by studies aimed at manipulating the gut microbiota using probiotics or antibiotics, which also confirm how the microbiota influences anxiety and the HPA system by acting on the neurochemistry of the brain.[34] Finally, the vagus nerve sends orthodromic information, regulating the contraction of smooth muscles and glandular secretion in the intestines, and antidromic information to several regions of the CNS, such as the locus coeruleus (LC), the rostral ventrolateral medulla, the amygdala, and the thalamus.[35] Various studies showed that vagotomised mice failed to show any improvement in anxiety or depressive-like behaviors following treatment with a potential probiotic *Lactobacillus rhamnose,* indicating that behavioral properties of this bacterial strain are dependent upon gut-brain signaling via the vagus nerve.[28] Similarly, a potential probiotic *Bifidobacterium longum* failed to produce an anxiolytic effect in a vagotomised colitis mouse model.[28]

Both neural (vagus) and hormonal (HPA axis) lines of communication combine to allow the brain to influence the activities of intestinal functional effector cells, such as immune cells, epithelial cells, enteric neurons, smooth muscle cells, interstitial cells of Cajal, and enterochromaffin cells.[35,36]

When a rupture of the microbiota-gut-brain axis (MGB-axis) occurs, multiple pathologies of different types can arise: irritable bowel syndrome (IBS), depression, PTSD, autism spectrum disorders, obesity, diabetes, etc.[35,36]

Although it is not yet completely clear the exact mechanism by which the MGB-axis can lead to different pathologies, the bidirectional relationship between the gut microbiota and the brain, termed the MGB-axis, plays a key role in maintaining the homeostasis of the individual and assuring his health.

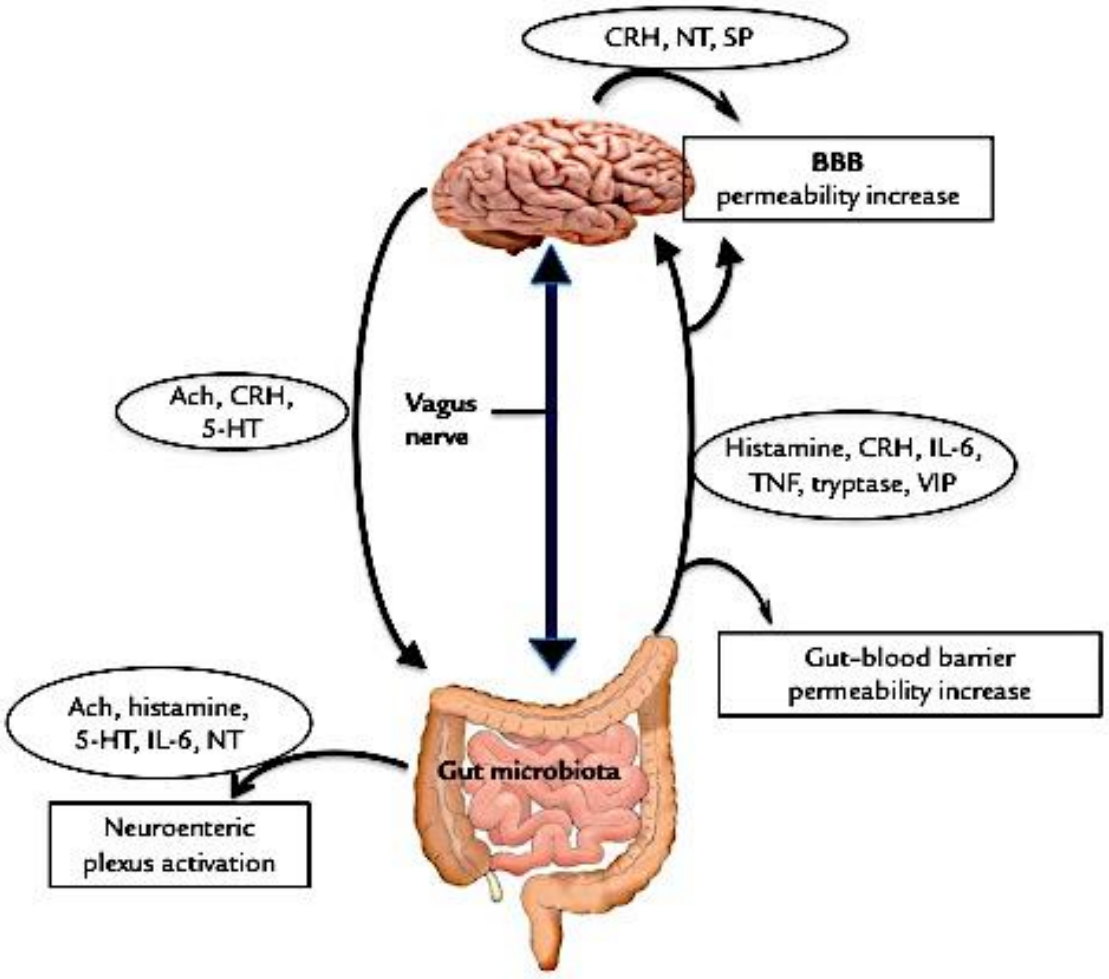



**Figure 2 |** *Diagrammatic representation of the microbiota–gut–brain (MGB) axis highlighting the proposed bidirectional communications. CRH = corticotropin-releasing hormone; NT= neurotensin; SP=substance P; Ach = acetylcholine; IL-6 = interleukin 6; TNF= tumor necrosis factor; VIP = vasoactive intestinal peptide; 5-HT= 5-hydroxytryptamine.* [Taken from reference 30: Petra AI, Panagiotidou S, et al.; Gut-Microbiota-Brain Axis and Its Effect on Neuropsychiatric Disorders With Suspected Immune Dysregulation. Clin Ther. 2015 May 1;37(5):984-95]

## 1.3 MGB axis and epilepsy

The possible relationship between the gut microbiota and epilepsy was supposed already at the beginning of the 20th century with the hypothesis of a Bacillus Epilepticus related to the onset and maintenance of epilepsy and constipation.[37]

Nowadays, researchers and clinicians in the epilepsy field are becoming focused on the potential of the microbiota to regulate seizures and its possible impact on epileptogenesis, in addition to the possible use of gut supplements (i.e. prebiotics, probiotics, and symbiotics) as well as diets (e.g. ketogenic diet) in add-on with antiepileptic drugs (AEDs), and fecal microbiota transplantation (FMT). Recent studies seem to demonstrate that acting locally, through different interventions on gut microbiota, could perturb directly and indirectly the MGB axis. Many highly modifiable pathways of the MGB axis could be correlated with epilepsy and are currently extensively studied with multiple lines of approach involving especially animal models and assessing the role of external interventions (e.g. diet, gut supplements, drugs) or other factors on gut microbiota composition that consequentially shape small molecule messaging systems, in the gut and the brain.[37]

Epilepsy is a complex chronic disease with multiple risk factors affecting more than 70 million people worldwide: in advanced countries, the incidence is around 40-70 per 100,000 people every year, while in less advanced countries it is higher, around 100-190 per 100,000/year.

The word epilepsy comes from the Greek ***epilambanein***, which means “to seize” or “to attack” since this pathology is characterized by recurrences and spontaneous unprovoked seizures. The International League against Epilepsy (ILAE) recently defined epilepsy based on at least one of the following conditions: (1) at least two unprovoked (or reflex) seizures occurring more than 24 hours apart; (2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two

unprovoked seizures, occurring over the next ten years; (3) diagnosis of an epilepsy syndrome.[38]

An epileptic seizure is the behavioral manifestation of abnormal neuronal activity characterized by excessive hypersynchronous neuronal discharges. Indeed for an epileptic seizure to occur, two basic phenomena must coexist: there must be excessive neuronal excitability and abnormal neuronal synchronization. This electrophysiologic disturbance is called the paroxysmal depolarizing shift: a prolonged high-voltage depolarization of the neuron caused by a massive influx of sodium and calcium.

It is known that a variety of genetic, environmental, and even normal physiologic factors are important contributing factors to the appearance of seizures. The importance of family history for patients with epilepsy has been well known since the publication of "*The Sacred Disease*" more than 2000 years ago, and further studies have highlighted that some epilepsies, mainly generalized epilepsies, have a primarily genetic component.[40]

But the genetic predisposition is not everything: a healthy person may experience seizures if given a potent enough precipitant. Precipitants increase neuronal hyperexcitability by disrupting the ionic balance in the extracellular space or interfering with cellular ionic pumps, and they include stress, sleep deprivation, known convulsive drugs, alcohol or sedative withdrawal, fever or infection, and toxic and metabolic processes such as hypoxia, ischemia, hypoglycemia, DM, hepatic encephalopathy, and electrolyte disturbance.[39]

In addition to seizures, epileptic patients often have gastrointestinal symptoms, while patients with inflammatory bowel disease (IBD) have an increased susceptibility to epilepsy.[40] Based on these clinical facts, the role of the microbiota and the gut-brain axis in epilepsy cannot be ignored.

It is also now well-known that gut microbiota (GM) is involved in epilepsy in terms of seizure control and psychiatric comorbidities. Particularly, SCFAs play an important role in maintaining brain balance, since they have neuroprotective effects on the epileptic brain.[41] Indeed, they might directly influence the brain by reinforcing the integrity of the blood–brain barrier (BBB), modulating neurotransmission, influencing neurotrophic factor levels, and enhancing

memory consolidation, as well as controlling the maturation and function of microglia.[41] Multiple studies have evidence that butyrate is implicated in reinstating BBB integrity in germ-free mice by increasing the expression of tight junction proteins and attenuating neuronal deficits.[42,43]

Moreover, SCFAs protect the BBB from inflammation and oxidative stress (OS), which is known to be interconnected with inflammation, and OS and neuroinflammation often co-exist in the brains of epileptic patients.[41] Propionate itself has also shown significant antiseizure effects. Diet can also contribute to the onset of comorbidities related to epilepsy, such as two of the most common: depression and anxiety.[41,42] More than 2000 years ago, Hippocrates described a bidirectional relationship between depression and epilepsy.[44] He wrote, “Melancholics ordinarily become epileptics, and epileptics melancholics: what determines the preference is the direction the malady takes’’. Many physicians have since noticed a connection between epilepsy and depression and anxiety. Differences in bacterial taxa indicated that depression and anxiety could be characterized by an increase in proinflammatory species and a decrease in SCFA-producing bacteria.[44]

It was evidenced that diet, and thus the abundance of specific gut microbes, such as the families of *Bacteroidaceae, Clostridiales, Clostridiaceae,* and *Ruminococcaceae*, can mediate genetic effects on anxiety through modulating, suggesting links between host genetics and anxiety via intestinal health[45] (Figure 3). Studies suggest that Fecal microbiota transplantation (FMT) could be a therapy strategy for depression. Health microbiota contains a higher number of *Lachnospiraceae* compared to patients with a story of mental depression. *Lachnospiraceae* break down carbohydrates into short-chain fatty acids (SCFAs) and a reduction of this species would thereby result in the loss of SCFAs, whose decrease can consequently reduce the intestinal production of 5-HT, an important neurotransmitter in the fight against depression. This supports the hypothesis that FMT can optimize the intestinal microflora of patients with depression and relieve depression-related symptoms by restoring or reconstructing the constitution of the intestinal microflora.[46]

Although mechanisms through which GM can lead to a worsening of epileptic symptomatology, recent studies have revealed that modifying the patient's diet could be a valid therapeutic option, as explained below.

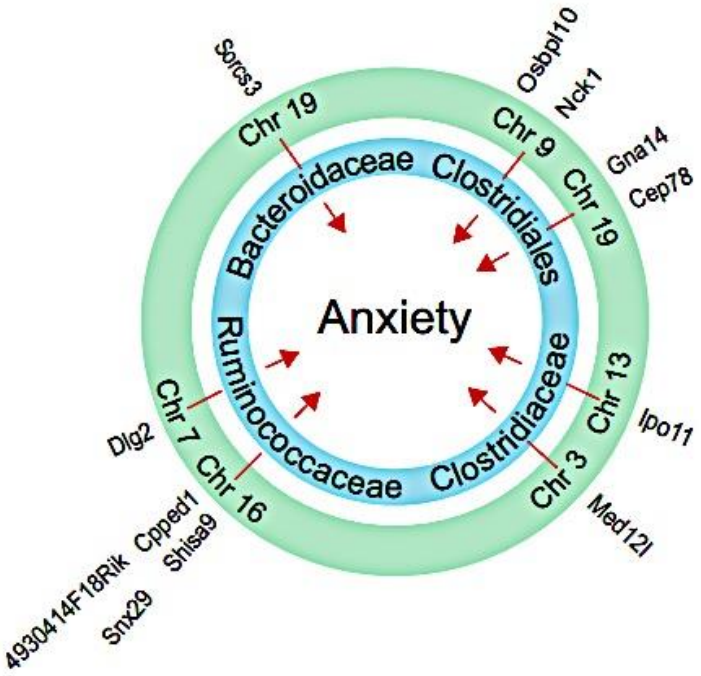



**Figure 3 |** *Microbial families mediate the effect of host genetics on anxiety. Four microbial families were identified as mediators between genetic variants and anxiety. The green ring indicates chromosomal locations associated with anxiety and abundance levels of microbial families indicated in the blue ring. Candidate genes within each genetic locus are listed on the outside of the green ring.;* [Taken from reference 45: Jin X, Zhang Y, Celniker SE et al.; Gut microbiome partially mediates and coordinates the effects of genetics on anxiety-like behavior in Collaborative Cross mice. Sci Rep. 2021.]:

## 1.4 Microbiome sequencing

Culture-independent laboratory techniques have enabled the characterization of gut microbial communities, improving the ability to identify individual species of micro-organisms and their quantities in feces samples.

Advances in technology like next-generation sequencing (NGS) have led to an explosion in the discovery and characterization of microbes because NGS

methods do not rely on traditional culture techniques and can thus detect unculturable microbes. Complementing traditional culture methods with NGS has already been implemented in many clinical microbiology laboratories because of its potential to address severe, insidious infections.[47] NGS methods sequence microbial DNA or RNA in fecal, blood, and/or tissue samples. The two primary NGS methodologies now in use are amplicon sequencing and shotgun metagenomic sequencing. Amplicon sequencing involves first amplifying a region of the DNA via PCR, and then sequencing the resultant product. The target for PCR amplification is, most commonly, the bacterial 16S ribosomal RNA (rRNA) gene (Figure 4). The 16S rRNA gene is an ideal target because it is highly conserved and ubiquitous among bacteria (without it, bacteria would be unable to translate mRNA into proteins and thus be nonfunctional) and it also contains nine hypervariable regions (V1–V9) that differ between bacterial species and genera.[47] (Figure 4).

After PCR amplification of the selected hypervariable regions, the resulting amplicons are sequenced, followed by data “cleaning”, a process involving multiple steps, such as adapter and primer sequence trimming, removal of low-quality bases and sequences from reads, and removal of sequences matching a control library such as the PhiX Control (Illumina), chimeric sequences, and human contaminant reads, as well as chloroplast and mitochondrial contaminants.[47] The amplicon sequencing ends with taxonomic identification.

In contrast to amplicon sequencing, metagenomics explores the global composition of microbial communities, combining molecular biology and genetic techniques to identify and characterize genetic material from complex microbial environments. Shotgun metagenomic sequencing and RNA sequencing analyze all the DNA or RNA in a given sample, respectively. For shotgun metagenomic sequencing, after extraction, the DNA is randomly fragmented, and barcodes and adapters are ligated to the ends of each segment to facilitate sample identification and DNA sequencing. The resultant reads are cleaned and subsequently aligned to a reference database, such as Reference Sequence and GenBank to identify taxa and functional potential.[47]

The RNA sequencing workflow is similar to that for shotgun metagenomic sequencing; however, after fragmentation, the RNA segments are reverse transcribed, using PCR, into complementary DNA (cDNA), which is then processed using the DNA sequencing pipeline.[47]

Importantly, a distinct difference between shotgun metagenomics and RNA sequencing is that shotgun metagenomics provides a random selection of all genes encoded by the microbes (predictive functional potential) whereas RNA sequencing identifies which genes are actively being transcribed (active functional profile).[47]

16S rRNA, shotgun metagenomic, and RNA sequencing can all be used to determine what bacteria are present in a microbiome; however, the latter two also detect members of other domains such as fungi and parasites, as well as viruses. Only RNA sequencing examines RNA viruses.[47]

Once the bacterial genome present at the fecal level has been extracted and the genomic data obtained digitalized, an analysis is made of the different species present, considering relative abundance and diversity. Precisely, three classes of diversity can be identified: **α, β, ανδ γ** diversity. **Alpha diversity** describes the diverseness within a sample, while **Beta diversity** describes the diversity, or dissimilarity, between samples.[48,49] **Gamma diversity** is rarely used and describes the total species diversity over all samples, comparable to alfa diversity in a single sample.[48]

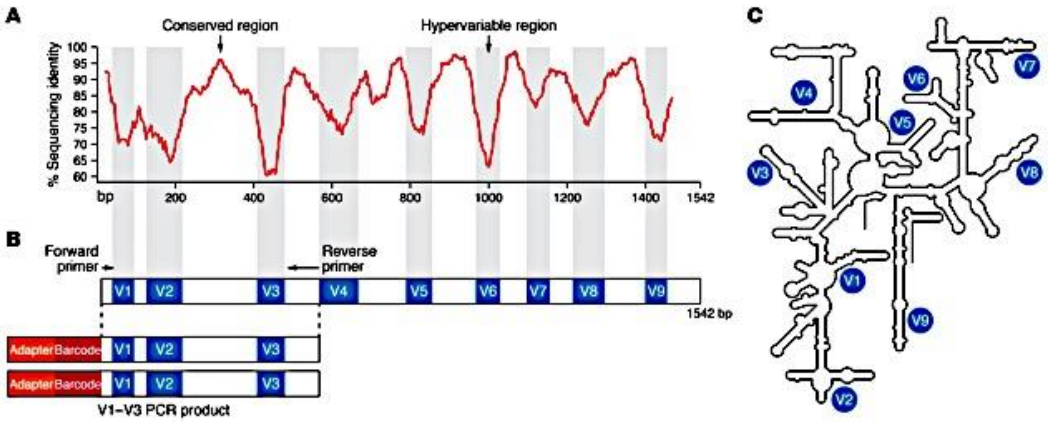



**Figure 4 |** *Bacterial 16S rRNA gene. (A) Percentage sequence identity of conserved and hypervariable regions of the bacterial 16S rRNA gene. (B) Illustration of conserved and hypervariable regions corresponding to A and PCR amplification of the V1–V3 region of the bacterial 16S rRNA gene. (C) Schematic of 16S rRNA gene*

*structure with hypervariable regions (V1–V9) labeled.* [Taken from reference 47: Wensel CR, Pluznick JL et al; Next-generation sequencing: insights to advance clinical investigations of the microbiome. J Clin Invest.]

# 2. Diet

## 2.1 Diet Correlation with Gut Microbiota

Studies have proven that diet is the main responsible for the alteration in gut microbiome diversity. The main species of bacteria include *Prevotella*, *Ruminococcus*, *Bacteroidetes*, and *Firmicutes*.[2] Moreover, our gut microbiota includes viruses, especially phages, *Eukarya*, as *Fungi*, *Blastocystis*, *Amoebozoa*, and *Archaea*.[50] Bacteria within the gut microbiome are involved in harvesting energy from food, balancing the beneficial and opportunistic bacterial composition, and manufacturing neurotransmitters, such as serotonin, enzymes, and vitamins. As a result, when there is an imbalance in bacterial species, disease could result.[2]

It has been reported that *bifidobacteria* and *lactobacilli*, which are members of the intestinal microbiota, are beneficial bacteria.[51] Evidence is accumulating that a higher diversity is related to health, whereas a lower diversity is observed in relation to various diseases.[52] In diseases including IBD, diabetes and obesity, diet is implicated as a contributing factor by having direct effects on host metabolism and/or immune responses.[53]

Western diet, animal-based and vegetarian diet are suitable examples of how the diet can alter the microbiome diversity and eventually lead to different pathologies, such as diabetes, inflammatory bowel disease, colorectal cancer, obesity, depression, epilepsy, and many other conditions. Vice versa, multiple studies show how a low-sugar, low-fat diet, but also the Ketogenic diet, high in fat and low in carbohydrates, can be useful for the mitigation of symptoms and become the appropriate therapy to follow.

## 2.2 Food essentials

### 2.2.1 Proteins: Animal-based diet and plant-based diet

The effects of dietary protein on the gut microbiota were first described in 1977.

Thanks to the advances of 16S rRNA, different studies investigated the role of an animal-based diet on gut microbiome giving participants different forms of protein, such as heavy animal-based protein from meats, eggs, and cheeses; whey protein; or purely vegetarian sources such as pea protein. Most of the studies noted that protein consumption positively correlates with overall microbial diversity. It was seen that a diet high in animal and saturated fats has the potential to alter the gut microbiota, by increasing lipopolysaccharides (LPS), increasing trimethylamine-N-oxide (TMAO), which is a proatherogenic compound that increases the risk of cardiovascular disease, and decreasing short-chain fatty acids (SCFA).[2,3] In agreement with these findings, a study on mice showed that the proportion of proteins derived from animal sources accounted for a significant proportion of the association between overall protein intake and all-cause and cancer mortality, whereas, the progression of both melanoma and breast cancer was strongly inhibited by the low protein diet, indicating that low protein diets may have applications in both cancer prevention and treatment.[54] Moreover, children with an animal-based dietary regime showed an increase in bile-tolerant anaerobes such as *Bacteroides, Alistipes,* and *Bilophila*, and a decrease in the beneficial bacteria *Lactobacillus spp., Roseburia spp.,* and *E. rectale*, (Figure 5). As an interesting clinical correlate, several studies have demonstrated that IBD patients possess lower fecal counts of *Roseburia* and other butyrate-producing bacteria than healthy subjects. Healthy subjects, on the other hand, have 10-fold more abundant *E. rectale* in their intestines.[2,3]

On the contrary, the consumption of plant-derived proteins, such as whey and pea protein extract increases gut-commensal *Bifidobacterium* and *Lactobacillus*, while whey additionally decreases the pathogenic *Bacteroides fragilis* and *Clostridium perfringens*. Pea protein has also been observed to increase intestinal short-chain fatty acid (SCFA) levels, which are products of

microbial fermentative activity in the gut and has been considered anti-inflammatory and important for the maintenance of the mucosal barrier. The concentration of SCFAs varies along the length of the gut, with the highest levels in the cecum and proximal colon, while it declines toward the distal colon.[55] Plant-based diets were associated with a lower risk of incident CVD and coronary heart disease (CHD). It was also found that the vegetarian subjects had microbiomes enriched with *Prevotella* when compared to non-vegetarians.[2,3,56,57]

The study of Hayashi, Sakamoto et al., using the 16S rDNA library, T-RFLP, and cultivation, also detected many phylotypes that had not been discovered previously, yet unknown in the intestinal tract, probably related to vegetarian intestinal tract ecology.[58] It was also discovered that plants use exosome-like nanoparticles (ELNs) to communicate with microbes and fungi through the transport of various lipids, proteins, and RNAs. Recently, was proposed a model that each plant species has unique ELNs which differentially modulate bacteria within the gut.[59] Additional studies investigated the role of bioactive plant-derived miRNA and its positive impact on host health. Further studies with more sensitive technologies will be required to demonstrate the direct impact of dietary plant miRNAs on the microbiome.[60]

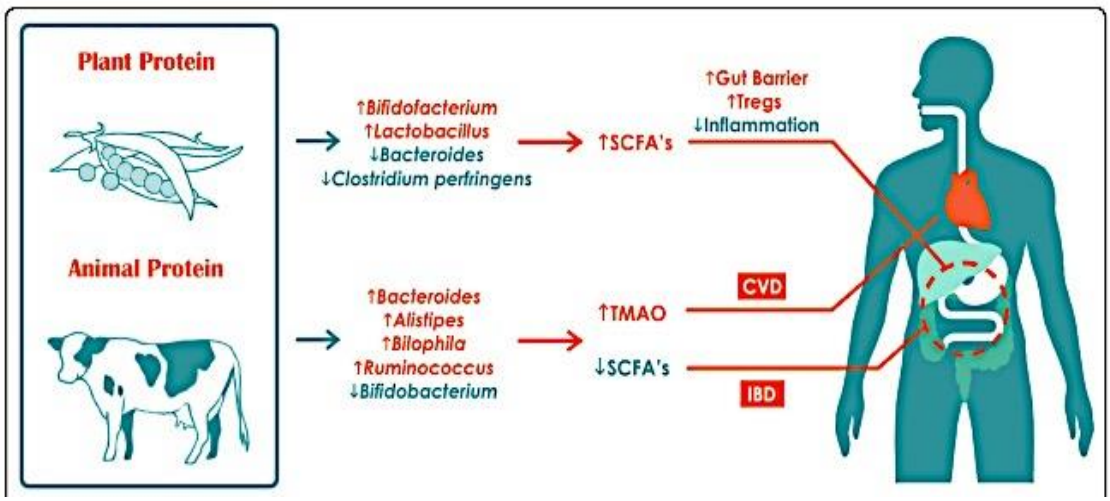



**Figure 5 |** *impact of dietary protein on intestinal microbiota and health outcomes. SCFA's short chain fatty acids, TMAO trimethylamine N-oxide, Tregs T regulatory cells, CVD cardiovascular disease; IBD inflammatory bowel disease.* [Taken from

reference 3: Singh RK, Chang HW, et al; Influence of diet on the gut microbiome and implications for human health. J Transl Med. 2017]:

### 2.2.2 Fats

Several human studies have suggested that a high-fat diet increases total anaerobic microflora, counts of *Bacteroides*[3,61], and, for those people following a high saturated-fat diet, an increase of *Faecalibacterium Prausnitzi* was noted. A high-fat diet also decreases Lactobacillus intestinalis and results in more propionate and acetate-producing (SCFAs) species, including *Clostridiales, Bacteroides, and Enterobacteriales*.[3] Moreover, germ-free mice colonized with the microbiota from obese mice display higher concentrations of fecal SCFAs.[62]

On the contrary, a low-fat diet increases the fecal abundance of *Bifidobacterium* with concomitant reductions in fasting glucose and total cholesterol.[3]

It is interesting to report that GIM variations also depend on the type of fat: a comparison of lard-derived and fish oil-derived lipids revealed that *Bacteroides* and *Bilophila* were increased in lard-fed mice, while *Actinobacteria* (*Bifidobacterium and Adlercreutzia*), lactic acid bacteria (*Lactobacillus and Streptococcus*), and *Verrucomicrobia (Akkermansia muciniphila)* were increased in fish-oil-fed mice.[3]

### 2.2.3 Carbohydrates

Carbohydrates can be divided into two varieties: digestible carbohydrates, which are enzymatically degraded in the small intestine and include starches and sugars, such as glucose, fructose, sucrose, and lactose[3]; and non-digestible carbohydrates, such as fibers, which are not enzymatically degraded in the small intestine, but they travel to the large intestine where they undergo fermentation by resident microorganisms and can modify the intestinal environment, an ability that warrants their additional designation of prebiotics.[3]

## Digestible Carbohydrates

Different studies show how carbohydrates affect the gut microbiota. A study unexpectedly shows that lactose is able to modify the gut microbiota of allergic infants by significantly increasing the total fecal count of *Bifidobacteria* and *Lactobacilli* and decreasing the numbers of *Bacteroides, Prevotella*, and *Clostridia*. In turn, the modification of the GI microbiota is associated with an increase in the median concentrations of total SCFA and a decrease in ketones and alcohol. Essentially, this study demonstrates that lactose in the diet could promote SCFA-producing bacteria, whose beneficial effects are well known: they protect the intestinal epithelial cells from mechanical, chemical, and microbial damage by enhancing the production of MUC-2 (mucin is a glycoprotein made by the host that is believed to maintain the integrity of the gut epithelium) by intestinal epithelial cell and indirectly reduce the risk of several acute and chronic diseases by acting as stimulating substrates for potentially health-promoting bacteria, such as *lactobacilli* and *bifidobacteria*, that are able to inhibit the proliferation of pathogenic/harmful bacteria through colon acidification, competition for the occupation of colonization sites and nutrients, synthesis of antimicrobial compounds and stimulation of the immune system.[63,64] These findings are quite unexpected given that lactose is commonly thought of as a potential gastrointestinal irritant (e.g. lactose intolerance).[3]

The artificial sweeteners saccharin, sucralose, and aspartame represent another dietary controversy: they were marketed as a no-calories food option to replace natural sugar, but contrary to popular belief, recent shreds of evidence suggest that they can alter the microbiota, leading to dysbiosis, and induce glucose intolerance.[3,65]

## Non-digestible carbohydrates: Fibers

In 2001, the American Association of Cereal Chemists adopted the following definition: "Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary

fiber includes polysaccharides oligosaccharides (OS), lignin, and associated substances. Dietary fibers promote beneficial physiologic effects including laxation or blood cholesterol attenuation or blood glucose attenuation".[66]

Indeed, in contrast to digestible carbohydrates, non-digestible carbohydrates such as fiber and resistant starch are not enzymatically degraded in the small intestine and can be called prebiotics. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of bacterial species already resident in the colon, and thus attempt to improve host health.[67] Intake of prebiotics can significantly modulate the colony microbiota by increasing the number of specific bacteria and thus changing the composition of the microbiota.[67] Sources of prebiotics include soybeans, inulins, raw oats, and non-digestible oligosaccharides such as fructans, fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), and arabinose-oligosaccharides (AOS). A diet that is low in these substances has been shown to reduce total bacterial abundance, whereas a diet rich in non-digestible carbohydrates most consistently increases intestinal *bifidobacteria and lactic acid bacteria*.[3] Many well-fermented fibers form viscous solutions in the gut (guar gum), and form gels (pectins), whereas others have a high water-holding capacity (cellulose).[66] Cellulose derivatives are very poorly fermented, whereas instead soluble and viscous fibers are more fermentable and therefore a better source of SCFA.[66]

It has been described that apple pectin, which is dietary fiber contained in apples, might strongly influence the intestinal microbiota because it has a strong bacteriostatic action on *Staphylococcus aureus, Streptococcus fecalis, Pseudomonas aeruginosa, and Escherichia coli,* suggesting that the regular consumption of this fruit, one of the most popular in the world, improves the intestinal environment and has beneficial effects.[51] Supporting this study, another study investigated the effects of six-weeks consumption of a wild blueberry (*Vaccinium angustifolium*) drink, versus a placebo drink, in modulating the intestinal microbiota. Relative to total eubacteria, *Bifidobacterium spp.* significantly increased following blueberry treatment, while *Lactobacillus acidophilus* increased after both treatments.[6]

Inulin-type fructans (a mixture of oligofructose and inulin) and galacto-oligosaccharides are the most studied prebiotic fibers. A study conducted on patients with type 2 diabetes with a daily supplement of inulin-type fructans induced a moderate, but significant increase in fecal levels of *bifidobacteria*, total SCFA, acetic acid, and propionic acid, suggesting that these prebiotic fibers could improve the intestinal environment in patients affected by type 2 diabetes.[69] Another study on human volunteers demonstrated how biscuits containing partially hydrolyzed guar gum (PHGG) and fructo-oligosaccharides (FOS) have prebiotic effects. Fructo-oligosaccharides are widely distributed in plants such as onions, asparagus, wheat, etc. are not hydrolyzed by human digestive enzymes, but are utilized by intestinal bacteria such as *bifidobacteria*.[70] Indeed, the fluorescent in situ hybridization using oligonucleotide probes (FISH) showed an increased number of fecal *bifidobacteria* in volunteers ingesting the experimental biscuits compared with pre-treatment and placebo population levels[71], supporting previous studies.[70,71,72,73,21,74]

### 2.2.4 Probiotics and Prebiotics: modulation of the gut microbiota

In recent years, the growing interest in the use of pre- and probiotics to optimize the gut microbiota has led to their further study also in the treatment of neurological/neuropsychiatric disorders. One of the tools at our disposal is prebiotics which, alone or in combination with probiotics, can be used to modulate the gut-brain axis.

A **prebiotic**, as proposed by the International Scientific Association for Probiotics and Prebiotics (ISAPP), is "a substrate that is selectively utilized by host microorganisms and confers a health benefit". These compounds include highly fermentable soluble fibers, non-digestible oligo-saccharides (NDO), and human milk oligosaccharides (HMO). Although prebiotic therapies may be potentially useful by enhancing *Lactobacilli* and *Bifidobacteria*, few studies have been published on the beneficial effects of prebiotics on the gut-brain

axis. One such study was performed on mice and showed that fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), or a combination thereof for 3 weeks could have antidepressant and anxiolytic effects and produce reduced stress-induced release of corticosterone.

According to the World Health Organization (WHO) definition, **probiotics** are "live microorganisms that, when administered/injected in adequate amounts, confer a health benefit on the host". Probiotics are mainly composed of two genera of lactic acid-producing bacteria (*Lactobacillus and Bifidobacterium*). Studies on mice treated with *Lactobacillus rhamnosus, helventicus,* and *fermentum* after treatment with ampicillin have shown that they have a positive effect on spatial memory impairment due to stress and alterations in the microbiota. Combining a balanced diet with treatment with pre- and/or probiotics could therefore be the key to improving the quality of the intestinal microbiota and optimizing the general condition of patients, thanks to their synergic action.[75,76,77]

### 2.2.5 Food additives

Food additives are substances commonly used during food processing to increase shelf life and improve the quality and taste of pre-packaged foods. Food additives can also be used as stabilizers, coating, or filler agents, and their presence in food products is often indicated with a unique "E-number".[80] With the development of the ultra-processed foods that characterize mainly Western diets, the number of food additives, such as preservatives (sodium chloride, propionates, sorbates), non-nutritive sweeteners, and emulsifiers, has increased dramatically in recent decades. The intake of preservatives, non-nutritive sweeteners, and emulsifiers is often associated with a loss of intestinal microbial richness in favor of a few microbial groups that are often involved in intolerance, loss of intestinal permeability, chronic intestinal inflammation, promoting colitis, and metabolic syndrome.

For instance, non-caloric artificial sweeteners (NAS) are common food additives that provide a sweet taste and a low-caloric content. NAS, such as

neotame, are largely present in soft drinks, snack foods, and dairy products. Suez et al. showed that long-term oral intake of saccharin, a commonly used NAS, induced glucose intolerance and dysbiosis in mice, whereas treatment with antibiotics protected animals from the disease.[78,26]

Moreover, in later years, titanium dioxide (TiO2) has attracted the attention of the scientific community, as it is one of the most widely used food colorants. Studies showed that high doses of microparticles synergized with bacterial antigens to boost IL-8, tumor necrosis factor-α (TNF-α), and IL-10 production by macrophages, as well as to impair their TGF-β secretion/phagocyte activity, resulting in an exacerbation of intestinal inflammation in mice fed a TiO2-enriched diet.[78,79] In particular, it was shown a marked change in the microbiota composition, together with enhanced release of ROS, NLRP3 inflammasome, IL-1β, IL-18 cytokines, alteration of epithelial barrier permeability, and consequent bacterial translocation.[78]

### 2.2.6 Fermented Foods

Fermented foods are defined as "foods or beverages produced through controlled microbial growth, and the conversion of food components through enzymatic action".[80]

Fermented foods such as yogurt, cheese, sauerkraut, pickles, cocoa, coffee, kimchi, and kefir, hold a firm place in cuisine from almost every culture in the world, and thus it is important to understand the beneficial effects that they may exert effects in health and disease on human health.

They contain potentially probiotic microorganisms, such as lactic acid bacteria, and reach the gastrointestinal tract, where they can potentially exert a physiological benefit in the gut, through competition with pathogenic bacteria and the production of immune-regulatory and neurogenic fermentation by-products. For instance, a wide range of microbial species have been identified in kefir grains, commonly including *Lactobacillus brevis, L. paracasei, L. helveticus, L. kefiranofaciens, L. plantarum, L. kefiri, Lactococcus lactis, Streprotcoccus thermophiles, Acetobacter lovaniensis, Acetobacter orientalis, Saccharomyces cerevisiae, S. unisporus, Candida Kefyr, Kluyveromyces*

*marxianus, and Leuconostoc mesenteroides* and in vitro studies have shown that kefir exhibits antimicrobial activity against *Candida albicans, Salmonella typhi, Salmonella enterica, Shigella sonnei, Escherichia coli, Bacillus subtilis, Enterococcus faecalis and Staphylococcus aureus*.[80,81]

Furthermore, fermentation-derived metabolites, such as lactic acid bacteria, generating bioactive peptides and polyamines may exert benefits on cardiovascular, immune, and metabolic health. Lastly, fermentation can reduce toxins and anti-nutrients: for instance, sourdough fermentation can reduce the content of fermentable carbohydrates (e.g., fermentable oligosaccharides, disaccharides, monosaccharides and polyols, FODMAPs), which may increase the tolerance of these products in patients with functional bowel disorders such as irritable bowel syndrome.[80]

## 2.3 Diet around the world: Western, Mediterranean, and Nordic diet

Several popular diets, including Western, gluten-free, omnivore, vegetarian, vegan, and Mediterranean, have been studied for their ability to modulate the intestinal microbiota.[3]

The Western diet (WD) - an unhealthy diet with high consumption of fats - can be broadly characterized by overeating, frequent snacking, and a prolonged postprandial state.[82] WD is generally characterized by high intakes of refined sugars (candies and sweets, and high-sugar soft drinks), animal fats (high intake of saturated and omega-6 fatty acids, reduced omega-3 fat intake), processed meats (especially red meat), refined grains, high-fat dairy products, conventionally-raised animal products, salt, eggs, potatoes, corn, mainly processed, refined, fried, and pre-packaged, with low intakes of unprocessed fruits, vegetables, whole grains, grass-fed animal products, fish, nuts, and seeds, hence, is low in fiber, vitamins, minerals, and other plant-derived molecules such as antioxidants.[82]

Besides the effects of a low-fiber dietary (as previously specified in the dedicated paragraph), WD's harmful metabolic properties can be related to the gut-barrier function impairment caused by food additives like emulsifiers or sweeteners. In several studies, WD led to a marked decrease in the numbers of total bacteria and beneficial *Bifidobacterium* and *Eubacterium* species.[3] A study comparing the Ugandan, Japanese, and Indian diets to the WD, noticed that the latter presented a higher number of *Bacteroides* and *Enterococci* in their feces.[83] The alteration of gut microflora, resulting in dysbiosis, gut barrier dysfunction, increased intestinal permeability, and leakage of toxic bacterial metabolites into the circulation, contribute to the development of low-grade systemic inflammation, with the increasing of reactive oxygen species (ROS) and the consequent enhancement of oxidative stress.[82] (Figure 6)

These pathophysiologies have been linked to diseases like obesity, type 2 diabetes mellitus, dyslipidemia, inflammatory bowel disease, neoplasms, and cardiovascular diseases (including atherosclerosis, cardiomyopathy, hypertension, and heart failure).[82] A study also evidenced that increments in the level of circulating insulin are associated with progressive elevations in plasma norepinephrine (NE) concentrations and with increasing evidence of cardiovascular stimulation.[84] Consumption of a Western diet has also been associated with the production of cancer-promoting nitrosamines[3] and overstimulation of the sympathetic nervous system.[82]

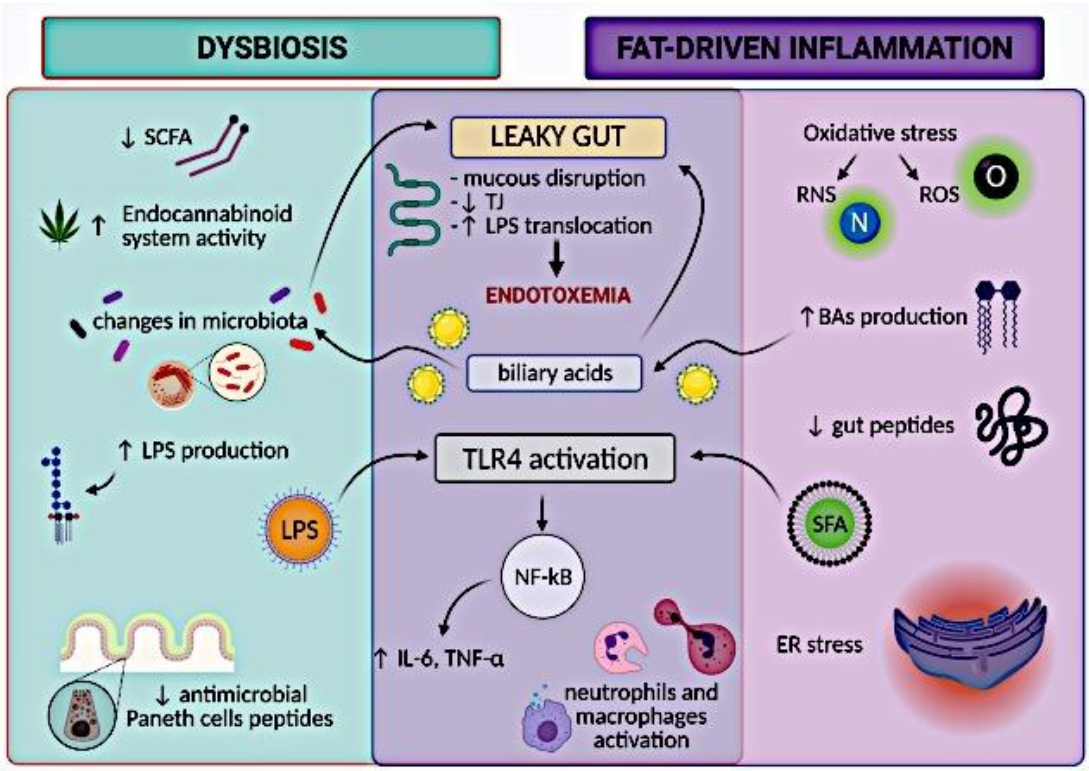



**Figure 6 |** *Western diet-associated pathologies. SCFA, short-chain fatty acids; LPS, lipopolysaccharide; TJ, tight junctions; TLR4, toll-like receptor 4, NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL-6, interleukin 6; TNF-α, tumor necrosis factor-alpha; RNS, reactive nitrogen species; ROS, reactive oxygen species; ER, endoplasmic reticulum; SFA, saturated fatty acids.* [Taken from reference 82: Malesza IJ, Malesza M. et al; High-Fat, Western-Style Diet, Systemic Inflammation, and Gut Microbiota: A Narrative Review. Cells. 2021 Nov 14;10(11):3164.]

As opposed to WD, the Mediterranean diet (MD) is highly regarded as a healthy balanced diet, with demonstrated benefits for obesity treatment and improvement in associated cardiovascular risk markers.[3,85]

MD is distinguished by a beneficial fatty acid profile that is rich in both monounsaturated and polyunsaturated fatty acids, high levels of polyphenols and other antioxidants, high intake of fiber and other low glycemic carbohydrates, and relatively greater vegetable than animal protein intake.[3,85] It includes the consumption of high intakes of olive oil, fruits, nuts, vegetables, and cereals; a moderate intake of fish and poultry; a low intake of dairy

products, red meat, processed meat, and sweets; and a moderate consumption of red wine with meals.[3,85]

Studies show that MD, a fat-restricted diet, increases both *Bacteroides* and *Bifidobacterium*, *Prevotella* bacteria, and other *Firmicutes* and also levels of SCFAs. At the same time, low adherence to the Mediterranean diet was associated with elevated urinary trimethylamine oxide, which is associated with increased cardiovascular risk.[3,85]

A diet that has shown similar beneficial effects to the Mediterranean diet is the Nordic diet (ND), which has been proposed as an alternative to the Mediterranean diet in Nordic countries.[86] The key dietary items of the ND are whole-grain cereal products; local berries, fruits, and vegetables; fish; low-fat or fat-free milk products; rapeseed oil; and vegetable oil–based margarine. All these foods have been shown to have beneficial effects on health and may affect adipose tissue metabolism, preventing obesity and cardiovascular disease.[86]

## 2.4 SCFA: the fecal proof of a link between diet and pathologies

SCFAs are bacterial waste products that are produced by the bacteria to balance the redox state in the gut.[55,87] The major SCFAs are acetate, propionate, and butyrate.[55] SCFAs constitute a substantial source of energy, serve as fuel for colonocytes, have a trophic effect on the mucosa, and foster the reabsorption of sodium and water.[66]

Recent studies demonstrate how an alteration of the GI microbiota is linked to an increased quantity of SCFAs in fecal samples, and how SCFAs can play a relevant role in the occurrence of some pathologies using different mechanisms (Figure 7).

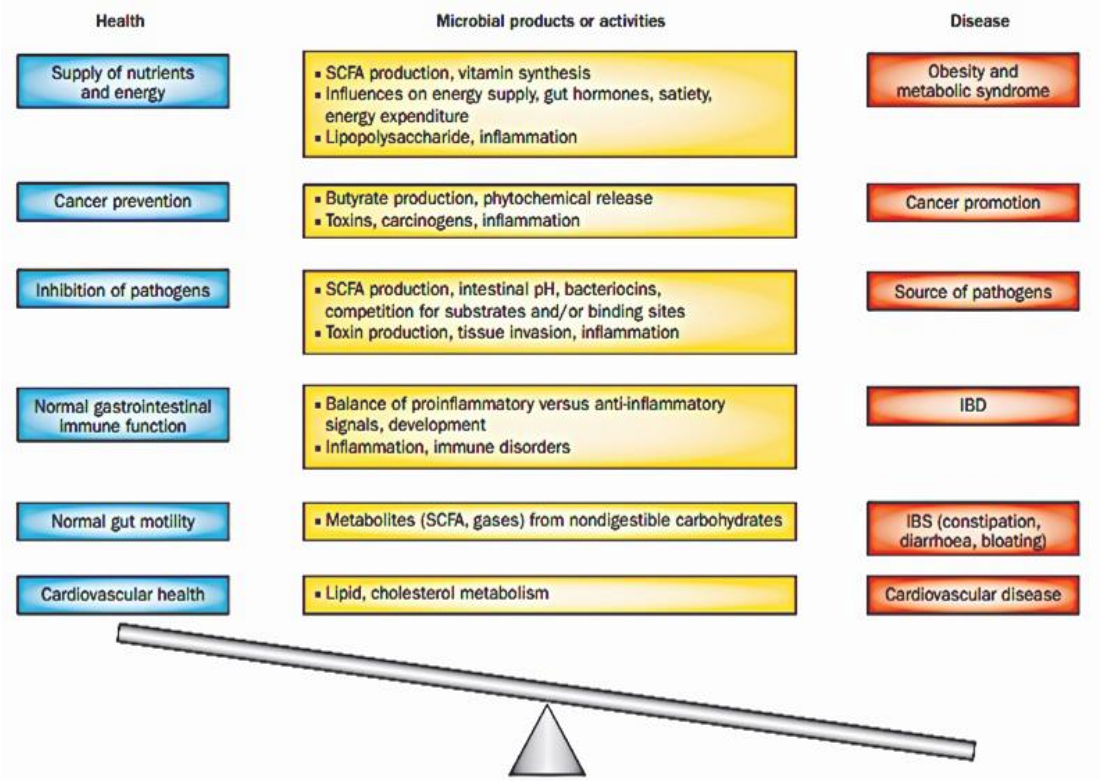



**Figure 7** | *Influence of gut microbial communities on health. Most of the microbial activities indicated in the center column are functions of the whole community of gut microbiota rather than being attributable to a single species. The balance of the community and its output determines the net contribution to health or disease. Abbreviation: SCFA, short-chain fatty acid.* [Taken from reference 95: Flint HJ, Scott KP et al.; The role of the gut microbiota in nutrition and health. Nat Rev Gastroenterol Hepatol. 2012 Sep 4; ]

## 2.4.1 SCFA in Newborns and Preterms

At birth, the infant's colon is sterile. As previously mentioned, colonization starts within the next few days and depends on the bacterial load of the environment, the mode of delivery, and more importantly, on nutrition.[66] Irrespective of the type of delivery, the development of intestinal flora is mainly influenced by the kind of feeding. Breast-fed infants show a microbic flora characterized by a definite predominance (about 90%) of *bifidobacteria* and *lactobacilli* (bifidogenic flora), which has been shown to be beneficial.[66,88] On the contrary, the bottle-fed infant develops mixed flora with a lower number of *bifidobacteria* (40–60%) and the presence of other germs that, in some

situations, may also have pathogenic effects (*Clostridium, Staphylococcus, Bacteroides*).[88,89] Oligosaccharides (OS) are carbohydrates made up of 3-9 monosaccharide units and are quantitatively the third largest component of human milk after lactose and lipids, reaching the highest concentration in colostrum and then decreasing after about 2 weeks.[66,88] When they reach the colon, they favor the growth of selective bacteria, more specifically, of the bifidogenic flora, and undergo bacterial fermentation, leading to the production of SCFA, which are largely absorbed (Figure 8). A recent study examined the relationship between OS in human milk and the composition of the infants' fecal flora, suggesting that the number of *bifidobacterium* species is correlated with the content of OS, thereby proving that human milk OS are effective prebiotics. It is of interest to note that in premature infants a larger percentage range (24%–74%) of dietary lactose may end up in the colon, which then becomes truly part of the absorptive apparatus.[66] Moreover, since the counts of *enterobacteria* and *enterococci* are higher than in full-term infants and remain predominant, shreds of evidence suggest that access to human milk is pivotal and may play an important role in decreasing the prevalence of the GI disorder Necrotizing Enterocolitis (NEC), affecting mostly premature and low birth weight infants.[66] Furthermore, *Bifidobacteria* in preterm infants do not appear during the first few days of life and are observed later than in full-term formula-fed infants.[66,90]

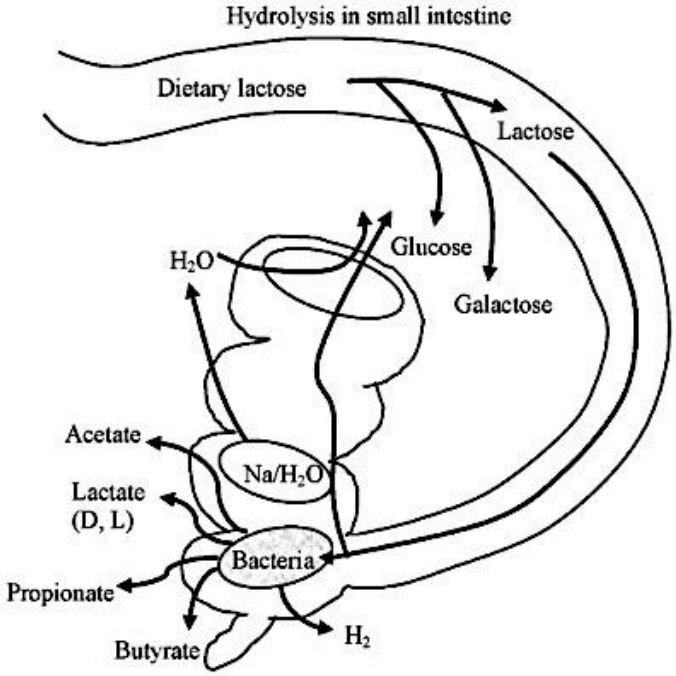



**Figure 8** | *In newborns and infants, the bulk of lactose is digested by lactase and absorbed as glucose and galactose. However, 20% escapes digestion and/or absorption and is fermented by the flora into the SCFA (acetate, propionate, and butyrate) as well as into both the L- and the D-form of lactate. If fermented, lactose may foster the absorption of sodium and water, whereas unfermented it may lead to an osmotic diarrhea.* [Taken from reference 66: Roy CC, Kien CL, Bouthillier L, Levy E. Short-chain fatty acids: ready for prime time? Nutr Clin Pract. 2006 Aug;21(4):351-66.]:

### 2.4.2 Intestinal Diseases: colorectal cancer (CRC), Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS)

Several studies have evidence that SCFAs have an effect on colonic blood flow and muscular activity of the colon[91]: Human volunteers who underwent a rectal infusion of SCFA, showed a decrease in gastric tone at manometric records[91,92], and patients with gastroesophageal reflux syndrome (GERD) had a worsening of symptom score.[91,93]

Histone acetylation emerges as a central switch that allows interconversion between permissive (via acetylation) and repressive chromatin structures (via

deacetylation).[94] During acetylation, acetyl groups are added to histone tails by histone acetyltransferases (HATs) and are removed by histone deacetylases (HDACs) during deacetylation. Among the SCFAs, butyrate has been investigated extensively and was seen to act as an HAT activator in normal cells and as an HDAC inhibitor in cancerous cells, working as an anti-tumor agent. SCFA-mediated HDAC inhibition is also a potent anti-inflammatory agent.[94,95]

When fermentable fibers are in short supply, microbes switch to energetically less favorable sources for growth such as amino acids from dietary or endogenous proteins, or dietary fats resulting in reduced fermentative activity of the microbiota and SCFAs as minor end products.[94] As a result, a prolonged or chronic diet rich in fiber is implicated in higher levels of inflammation, which has been observed how chronically as a well-established risk factor for colorectal cancer (CRC).[94,96] Supporting the hypothesis of a link between GI-microbiota and CRC, there is also the fact that *Streptococcus Bovis* infections have long been recognized as signs of occult carcinomas of the colon.[96] Moreover, in vitro studies prove that SCFA butyrate has the potential to inhibit the growth of premalignant and malignant cells.[66]

Several species have been found higher in patients, and thus associated to CRC: *Fusobacterium, Porphyromonas, Parvimonas, Peptostreptococcus, Gemella, Prevotella, and Solobacterium*.

These species were often undetectable in metagenomes from non-neoplastic controls.[97]

Another class of diseases related to high inflammatory levels due to dysbiosis is the one of Inflammatory Bowel Disease (IBD). Indeed, it was demonstrated that nonpathogenic Escherichia coli strains present in patients affected by IBD strongly stimulate the release of proinflammatory cytokines (tumor necrosis factor, IFN-γ, IL-6, IL-23p19, IL-12p35, and IL-17F) and chemokines (IL-8, CXCL1, and CXCL2), thus stimulating the inflammatory cascade.[98,99]

The inflammatory process activates matrix metalloproteinases that provoke matrix degradation, epithelial cell detachment, and ulceration;[98] Moreover, an

interesting study shows that mucosal antibodies in IBD are directed against intestinal bacteria. Particularly, the intestinal IgG is specifically directed against cytoplasmic proteins from commensal bacteria in active CD and UC, implying that these may be of major importance in the relapse of the two diseases.[100] Studies that used gene-sequencing technologies and powerful bioinformatic tools indicate that dysbiosis and decreased complexity of the gut microbial ecosystem are common features in patients with Crohn's disease (CD) or ulcerative colitis (UC).[98,101] Patients with ulcerative colitis (UC) show an increased load of Gram-negative, anaerobic and sulfate-reducing bacteria called *Desulfovibrio* subspecies, which are able to generate sulfides, involved in the pathogenesis of UC.[96] Similarly, *E. coli* has been detected at increased levels by molecular techniques in fecal samples, whereas *F. Prausnitzii,* a major representative of the *Clostridium leptum* group with known anti-inflammatory properties, is under-represented in patients with UC who have active disease and during remission[98,102,103,104] (Figure 9). Regarding patients with CD, not only was seen a difference in terms of microbial communities compared to healthy individuals, but patients with CD had differences from each other depending on whether the disease predominantly involved the ileum or the colon. Changes specific to patients with ileal Crohn's disease included the disappearance of core genera, such as *Faecalibacterium* and *Roseburia*, and increased amounts of *Enterobacteriaceae* and *Ruminococcus gnavus*.[98,105]

Finally, Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder whose pathogenesis is unknown, but basing on the epidemiological studies of IBS patients, altered gut microbiota was proposed as one of the possible causes of IBS. Acute bacterial gastroenteritis can cause chronic, asymptomatic, low-grade intestinal wall inflammation sufficient to alter neuromuscular and epithelial cell function.[106] In 36% of patients infected with *Campylobacter jejuni and Escherichia coli O157:H7*, IBS presented itself with heightened intestinal permeability, which was observed even after 2 years post-infection. Thus, it has been suggested that changes to the gut microbial community may trigger IBS symptoms. The importance of gut microbiota in the

etiology of IBS becomes more apparent after special diets and antibiotics have improved IBS symptoms.[106]

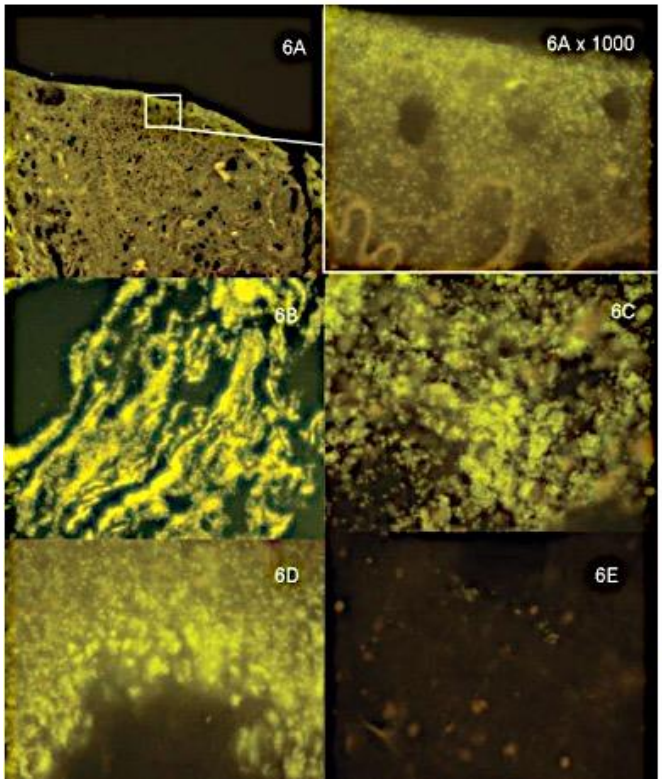



**Figure 9 |** *Examples of alteration within the web structure of habitual bacterial groups. (A) Hybridization silence (Cy3, 100 left, 1000 right micrograph) in an IBS patient. The fluorescence signals of Bacteroides gradually fade from the surface to the center of the fecal cylinder. Bacterial counts remain unchanged in zones of high and intermediate fluorescence. (B) Mucus striae interrupt the web of the habitual bacteria in a patient with diarrhea (Bac, Cy3, 400). (C, D) Spheroid precipitation of Bacteroides in a patient with active UC. (E) Subtotal depletion of Faecalibacterium prausnitzii in CD. B–E at magnification of 1000, Cy3*

[Taken from reference 104**:** Swidsinski A et al.; Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora. Inflamm Bowel Dis.]

## Non-intestinal diseases

## Asthma

The active role of diet in disease onset is not limited only to purely intestinal disease, but is also associated with systemic diseases, showing a connection between different systems. Indeed, different studies show how dysbiosis is related to asthma[107]: a high-fiber diet (producing high amounts of acetate) suppresses allergic airway disease by enhancing regulatory T cells (Treg)

through HDAC9 inhibition[94,108] and inducing hematopoiesis of dendritic cells that seed the lungs and reduce Th2 effector function in a GPR41-dependent fashion.[94,109,110] Similarly, intestinal helminth infection causes changes in commensal communities, resulting in an increase in SCFAs and a reduction of allergic asthma in a GPR41-dependent manner.[94]

## Obesity

The food that is consumed affects the bacterial composition within the gut microbiome, and the gut microbiome plays a vital role in food absorption, nutrient, and energy extraction, and low-grade inflammation, all of which have the potential to lead to obesity and type II diabetes.[2,87] Due to the exponential increase in obesity rates and its associated complications such as diabetes (currently, more than 34 million Americans have diabetes and 90–95% of those have type II diabetes) in the past few decades, tremendous attention has been given to understanding underlying mechanisms.[2,87] SCFAs such as acetate and propionate _mostly produced by the *Bacteroidetes phylum*_ and butyrate _produced by the *Firmicutes phylum*_ have been shown to exert beneficial effects on body weight, glucose homeostasis, and insulin sensitivity.[87] Particularly, butyrate dietary supplementation reduces diet-induced insulin resistance in mice, whereas butyrate and propionate are protective against diet-induced obesity.[87,111] Another example of the role of microbiota in obesity has been seen with patients undergoing Roux-en-Y gastric bypass: after the surgery, changes in gut microbiota have been shown to play a role in this improvement as a shift in bacterial population and a consequent metabolic improvement.[87,112]

In a study of Parks, Nam et al. it was observed a modest but statistically significant negative correlation between the abundance of the genera *Akkermansia* (phylum *Verrucomicrobia*) and body fat percentage growth after 8 weeks of high-fat/high-sucrose (HF/HS) feeding. Furthermore, body fat percentage growth after HF/HS feeding was positively correlated with the relative abundances of *Lactococcus* from phylum *Firmicutes* and with the genera *Allobaculum* (phylum *Bacteroidetes*). Additional studies are warranted

to validate the connections between these specific gut microbiota and dietary interactions.[113] Finally, it was observed that fruits and vegetables (FV) and whole grains (WG) have a positive impact on metabolic health in individuals affected by overweight or obesity with normally low intake of WG and FV since their intervention significantly and uniquely reduced biomarkers of inflammation: FV decreased circulating IL-6 and lipopolysaccharide-binding protein (LBP), while the WG treatment decreased TNF-α and LBP. Both treatments had individualized effects on the gut microbiota, with a significant increase in α-diversity in the FV treatment. These data support the positive impact that WG and FV intake can have on metabolic health in individuals affected by overweight or obesity with normally low intake of WG and FV.[114]

## Diabetes

It's becoming increasingly evident that gut microbiota is contributing to many human diseases including diabetes both type 1 and type 2.[87]

## Type1-Diabete and GIM

Type 1 Diabetes (T1D) is a multifactorial, immune-mediated disease, which is characterized by the progressive destruction of autologous insulin-producing beta cells in the pancreas.[115] Even though T1D is mainly caused by genetic defects, epigenetic and environmental factors have been shown to play an important role in this disease such as diet, hygiene, and antibiotic usage that can directly affect microbiota.[52] Supporting the hygiene hypothesis, which states that increased exposure to microbes (such as the gut flora and helminth parasites) in early childhood may prevent diseases, it has been shown that diabetes incidence in the germ-free non-obese diabetic subjects or patients (NOD) was significantly increased. This evidence is in line with the observation that the rates of T1D are higher in countries with stringent hygiene practices.[87] Moreover, the intestinal epithelial barrier is an important player in preventing food antigens, pathogenic as well as commensal bacteria from leaving the gut lumen and inducing a systemic immune response, and a disruption of this

crucial intestinal barrier is associated with intestinal autoimmune disorders including inflammatory bowel disease, celiac disease, and irritable bowel syndrome. It was seen that GIM is a key determinant in the fate of lactate and consequently in gut health. Indeed, when *Lactobacilli* and *Bifidobacteria* produce lactate, this can take different paths, depending on the GI-flora: lactate is converted into butyrate by butyrate-producers' bacteria, such as *Faecalibacterium* and *Subdoligranulum*, and this results in more mucin synthesis and tighter junctions, with the consequence of a healthy gut. On the contrary, the presence of bacteria such as *Alistipes*, *Bacteroides*, and *Veillonella*, leads to the conversion of lactate to other SCFAs: propionate, succinate, and acetate, which reduce mucin synthesis and tight junctions, with a consequent permeable gut and the occurrence of autoimmune disorders.[116] (Figure 10) Supporting these studies, it was seen that cases have a much larger population of bacteria such as *Bacteroides, Veillonella*, and *Alistipes* compared to controls[116]. In addition, patients with T1D exhibit changes in the ratio of *Firmicutes* to *Bacteroidetes*[115,116], and prediabetic children harbor more *Bacteriodetes* compared to controls.[115] A decreased abundance of *Faecalibacterium prausnitzii* (butyrate-producing bacterium) in children who had more than two diabetes-related autoantibodies has also been observed.[115]

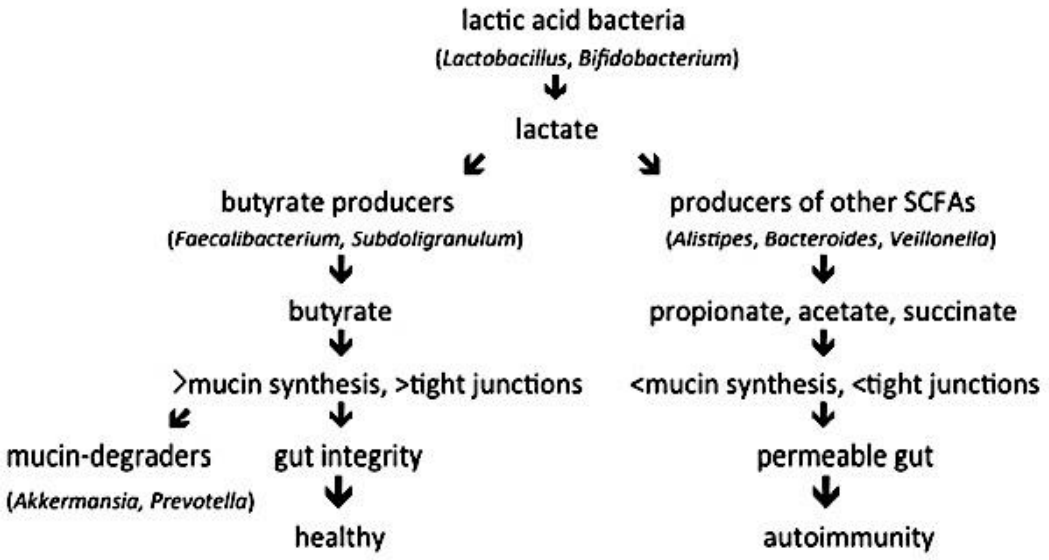


**Figure10** | *Model for a bacterial role in gut integrity leading to either a healthy state or autoimmunity for type 1 diabetes. In this model, the fate of lactate is crucial in determining gut health. Conversion to butyrate results in more mucin synthesis and tighter junctions. Conversion to other short-chain fatty acids (SCHAs) reduces mucin synthesis and tight junctions. The bacterial genera listed are examples of a given phenotype. Other bacteria may also be involved in these characteristics.* [Taken from reference 116: Brown CT, Davis-Richardson AG, et al.; Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes.]

## Type2-Diabete and GIM

The SCFA, especially butyric acid, may also act as signaling molecules by binding to receptors on the enteroendocrine cells, with the potential to increase the postprandial secretion of gut hormones and improve the regulation of blood glucose.[69] Observational studies have shown that gut microbiota in type 2 diabetes differs from healthy individuals with lower diversity of the microbial community, less butyrate-producing bacteria, lower fecal concentrations of SCFA, and elevated levels of pathogenic bacteria.[69] Alterations in gut homeostasis such as these are suspected to contribute to the pathophysiology of type 2 diabetes.[69]

Studies also showed that type 2 diabetes has often been associated with a lower abundance of *Firmicutes*, while *Bacteroidetes* and *Proteobacteria* tended to be more abundant.[117] Other data showed a lower abundance of *Bacteroidetes vulgatus* and *Bifidobacterium* spp. and a higher one of *Clostridium leptum* cluster in diabetics compared to healthy volunteers.[117] Moreover, the gut microbiota has been shown to affect the production of key insulin signaling molecules such as GLP-1 and PYY through SCFAs and various physiological pathways, including components of the central nervous system.[69,87] Thus, SCFAs can modify the levels of several gut hormones involved in glucose and energy homeostasis.[118]

## 2.5 Diet as Therapy for Epilepsy: Ketogenic Diet and its Variants

The Ketogenic diet (KD) is an effective, relatively safe, and tolerable dietary treatment for adults and children with refractory epilepsy which has been used to treat refractory epilepsy since the 1920s. In the last decades, less restrictive and more liberal KD variants, such as the Atkins diet (MAD), low-glycemic index treatment (LGIT), and medium-chain triglyceride diet (MCTD), have been developed to make the diet more feasible and palatable while reducing side effects and making it available for a larger group of patients.

## Classic Ketogenic Diet

The classic KD was first introduced in 1921 by Wilder for the treatment of epilepsy to avoid malnutrition that occurs with prolonged fasting.[119] The KD is a strict diet high in fat and low in protein and carbohydrates, with the aim of increasing the ketone body concentrations that could lead to an enhancement of inhibitory neurotransmission and thereby possibly reducing the seizure frequency. The exact mechanism of the KD is under investigation, and ketone bodies could exert anti-oxidative, anti-inflammatory, cellular, epigenetic, and gut-microbiome alterations.[119,120] In general, studies have failed to demonstrate a direct action of ketone bodies on GABA or glutamate receptors at physiologically relevant concentrations[121], and may therefore induce homeostatic effects on these neurotransmitter systems. The metabolism of ketones may cause the reduction of aspartate, an inhibitor of an enzyme involved in the generation of GABA from glutamate called glutamate decarboxylase, and therefore promote the synthesis of GABA, an inhibitory neurotransmitter in the brain.[122] This could explain why the classic KD and other more palatable versions have a positive effect on infantile spasms, severe myoclonic epilepsy, tuberous sclerosis complex, and children with refractory status epilepticus.[123]

Moreover, although the exact mechanisms through which KD promotes benefits in epileptic patients are still unknown, studies show gut microbiome composition was significantly changed after KD, and some specific bacteria may be associated with different efficacy.[124] It has been observed that KD reduces the diversity and richness of intestinal microbiota in children with

epilepsy, which could explain the occurrence of hyperlipidemia after KD may. On the other hand, it is reported that altered diversity of gut microbiota may influence cognition and depressive symptoms so the improvement of cognitive function in children with KD may be related to the bacteria, and particularly it resembles that there is an abundance ratio of *Bacteroidetes* after KD treatment, while the abundance of *Firmicutes*, and *Actinobacteria* decreased obviously. Moreover, it was also noted that the non-responders after KD had significantly increased the abundance of *Clostridiales, Clostridia, Ruminococcaceae*, *Lachnospiraceae, Alistipes,* and *Rikenellaceae*. This is interesting to note since it has been reported that Clostridial neurotoxins can affect central nervous system function through the axon transport pathway and therefore, inhibiting the overproliferation of these colonies helps to protect the host's neurological function and may also help to control seizures.[124]

The KD has a major benefit compared with standard anticonvulsant treatment with anti-epileptic drugs AEDs, which is associated with long-term side effects.[119] Nevertheless, the KD has been described as unpalatable and difficult to tolerate, thus leading to poor compliance. The main adverse effects were gastrointestinal symptoms, including vomiting, constipation, and diarrhea. For these reasons, KD may be used as an adjunct treatment to AEDs in children and adults with refractory epilepsy. However, in some metabolic diseases, such as type 1 glucose transporter and pyruvate dehydrogenase deficiencies, and mitochondrial complex I defect, KD may be considered a first-line of treatment.[123]

## Variants of the Ketogenic Diet

Alternatively to KD, more feasible and palatable diets were developed.

### Modified Atkins diet (MAD)

The modified Atkins diet (MAD) has been used since 2003 to treat children and adults with refractory epilepsy. It is a variation of the KD: more liberal, less restrictive, and more palatable, which yields high compliance and similar effectiveness as a classical, more restrictive KD. It induces ketosis, but without

fluid, caloric, or protein restriction, nor the requirement for fasting, food weighing, or hospitalization.[125] It consists of a nearly balanced diet (60% fat, 30% protein, and 10% carbohydrates by weight), without the restriction of recommended daily calories according to patient age.[126] (Figure 11)

Studies conclude that MAD is similarly effective as the classical KD in reducing seizure frequency in children with medically resistant epilepsy.[127]

**Figure 11 |** *A typical daily portion of a modified Atkins diet, allowing large amounts of protein (30% by weight) with the restriction of carbohydrates to 10 g/day. From the upper and right corner to the lower and left corner, boiled chicken, roasted fish, fresh tomato, kimchi (Korean pickled vegetables), milk, bean sprouts, anchovy soup, and olive oil.*

[Taken from reference 126: Kang HC, Lee HS, You SJ, Kang du C, Ko TS, Kim HD. Use of a modified Atkins diet in intractable childhood epilepsy. Epilepsia. 2007]

## Low-Glycemic Index Treatment (LGIT)

Low-Glycemic Index Treatment (LGIT) was first used for refractory epilepsy treatment in 2005 as an alternative to KD. It is a less restrictive diet that emphasizes complex carbohydrates (with a typical goal of 40-60 grams per day) over simple sugars and is not intended to promote ketosis. Contrary to the KD, food quantities are not weighed out to the gram but are based on portion sizes, so that patients can live a more flexible lifestyle that includes

eating at restaurants. Similarly to the ketogenic diet, the mechanism of action of the LGIT remains unknown, and it is thought that metabolic changes that occur with the diet (such as the decrease in blood glucose levels and production of ketones) may have a therapeutic effect on the brain.

Studies show that the LGIT diet is an efficacious therapy in terms of reduction of seizures for patients with drug-refractory epilepsy, especially if it is added to an ongoing antiepileptic drug (AED) therapy.[128] However, results are inconclusive concerning the noninferiority of the MAD and LGIT diet over KD, and all 3 dietary regimens— KD, MAD, and LGIT diet— may therefore be used as equivalent therapies, significantly reducing the seizure burden in children with drug-resistant epilepsy. Nevertheless, the risk profiling illustrates that the LGIT diet is associated with the least number of and least severe adverse events, while the other 2 diets are more likely to be associated with serious and life-threatening events.[129]

## Medium-Chain Triglyceride Diet (MCTD)

Medium-Chain Triglyceride Diet (MCTD) is a very flexible diet, with a high-fat content (30–60%), low protein (10%), and carbohydrates (15–19%), through which patients consume more food. Medium-chain triglyceride (MCT) fat produces more ketones per gram than long-chain triglyceride (LCT) fat, used in the classic KD.

This high ketogenic potential allows to reduce the intake of fatty acids in favor of greater consumption of proteins and carbohydrates, but also to consume larger portion sizes and more fruits and vegetables, making the diet more palatable and usable for children compared to KD.[130] Moreover, children on MCTD tend to grow better, require fewer micronutrients, and significantly have lower total cholesterol/high-density lipoprotein ratios compared to the classic KD.[119] Finally, the MCTD should remain a viable dietary option for children with refractory epilepsy who have large appetites, can tolerate more calories, or cannot accept the restrictions of the classic KD.[130]

Studies show that MCTD is successful in suppressing a similar spectrum of seizures as the classic KD (e.g., minor motor, akinetic, and myoclonic

seizures). Moreover, it has shown fewer incidents of kidney stones, hypoglycemia, ketoacidosis, constipation, low bone density, growth retardation, and acidosis as in classic KD.[130] Unfortunately, MCTD was frequently associated with gastrointestinal (GI) side effects including diarrhea, vomiting, bloating, and cramps, and for this reason, has been underutilized for children with intractable epilepsy.[130]

# 3. Aims of the study

In this study, we aimed at investigating the composition of the gut microbiota (at *phylum* and *genera* level) in a population of pediatric epileptic patients. Moreover, we looked for a possible correlation of such bacterial ecosystem with disease features (i.e., drug-sensitiveness and drug-resistance) and nutritional habits.

# 4. Methods

## 4.1 Patients

Pediatric patients (0-18 years) with epilepsy of different etiologies were recruited at the Department of Pediatric Neurology and Muscular Diseases Unit, IRCCS Giannina Gaslini, from April 2020 to November 2022.

Parents/caregivers gave written informed consent to the study, which was conducted following the Helsinki protocol.

The clinical and instrumental data, including the results of the cerebral magnetic resonance imaging (MRI) and electroencephalography (EEG), as well as drug treatment data and genetic results, were collected through the local database.

A stool sample was collected from each patient at the time of the enrollment. The presence or absence of gastrointestinal (GI) disorders was assessed by referring to the validated Rome IV Diagnostic questionnaire (the Rome Foundation, Inc.; https://theromefoundation.org), which investigates the frequency and intensity of the following gastrointestinal symptoms: abdominal pain, constipation, diarrhea, reflux, bloating, dyspepsia, nausea, and vomiting (see appendix 1).

In addition, the type and frequency of bowel movements were investigated using the Bristol Stool Scale (BSS) (see Appendix 2), which illustrates the shape of stools together with precise descriptions regarding their similarity to an ordered scale of types ranging from the hardest (type 1) to the softest (type

7): types 1 and 2 are considered abnormally hard stools while types 5, 6 and 7 are considered abnormally liquid stools. Types 3 and 4 are generally considered normal stool forms.

The subjects recruited in the study were also given a dietary semi-quantitative questionnaire, which has been either administered by the physician or self-administered, developed ad hoc in order to track nutritional habits (see Appendix 3). The daily frequency was converted into weekly frequency, based on average daily intake levels. Foods were distributed into the main constituent macronutrient categories: proteins, fats, and carbohydrates. For the fat sources (oil and butter), which in the questionnaire are expressed in weight ranges, it was considered as a reference value 10g of oil (about 1 tablespoon) and 10g of butter respectively, always based on average daily intake levels and then converted into weekly frequency.

Patients were classified into two different subgroups based on their response to ASMs according to the International League Against Epilepsy (ILAE) recommendations.[131]

- Patients with drug-sensitive epilepsy (DS): taking <2 ASMs
- Patients with drug-resistant epilepsy (DR): taking ≥ 2 ASMs

## 4.2 Metatassonomy

Fecal samples were processed for 16S rRNA sequencing. Total DNA was extracted from the fecal material using the Zymo BiomicsDNA/Rna “Miniprep Kit” extraction protocol in accordance with the guidelines of the company (Zymo Research). Then, the samples were stored at -80°C for their preservation and a later analysis.

The final genomic library was first prepared by amplifying the V3/V4 hypervariable regions of the bacterial 16S rRNA gene using the protocol "Zymo Quick 16 S Library preparation Kit" and subsequently assembled; finally, paired-end sequencing (2x250 bp) was performed with the MiSeq platform (Illumina). The results were processed with the bioinformatics software

Quiime. The resulting files were mapped with the SILVA23 database using MALT24 and taxonomic positioning was performed using the Lowest Common Ancestor algorithm implemented in MEGAN6 Ultimate Edition.25. Taxa with relative sequence abundance > 0.001% were considered.

## 4.3 Statistical Analysis

Descriptive statistical analyses were performed to evaluate demographic and clinical characteristics, with continuous data presented as mean – standard deviation (SD) or median (interquartile range [IQR]) as appropriate, and ordinal data expressed as number (percentage).

The metadata was organized in a table including biographical data; syndromic diagnosis; type and frequency of seizures; drug treatment and possible drug resistance; neurological examination; the presence of intellectual disability; treatment with supplements; results of the Rome IV questionnaire; type and mode of delivery; age at weaning; and the results of the nutrition survey.

For the statistical analysis of the data, we used MaAsLin2, a comprehensive R package for efficiently determining multivariable associations between phenotypes, environments, exposures, covariates, and microbial meta'omic features. MaAsLin2 relies on general linear models to accommodate most modern epidemiological study designs, including cross-sectional and longitudinal, and offers a variety of data exploration, normalization, and transformation methods.

The study population was divided into three groups:

1. healthy (control group)
2. drug-sensitive patients with epilepsy (DS)
3. drug-resistant patients with epilepsy (DR)

These three groups (healthy, DS, and DR), were submitted to MaAsLin2 and adjusted for group, age, carbohydrate, and protein, which were seen to have a significant association.

Alpha and Beta diversity were calculated in the statistical analysis, using the R software. *Alpha diversity* identifies the bacterial diversity in a specific sample and was calculated with the Chao 1 index (a non-parametric method to estimate the number of species in a bacterial community), Shannon, Fisher, Simpson, ACE, and se.ACE index. *Beta diversity* identifies bacterial dissimilarity between different samples and in the present study was calculated using the Bray-Curtis index and the Jaccard index. The association was considered statistically significant for $p<0.05$ and it was also considered an approaching significance for $0.05<p<0.1$.

# 5. Results

## 5.1 Clinical features of our population

Forty-six pediatric patients (24 female) with a median age of 6.5 years (IQR:8; range:0-17 years) and 33 age-matched neurotypical subjects were recruited. (Figures 12,13)

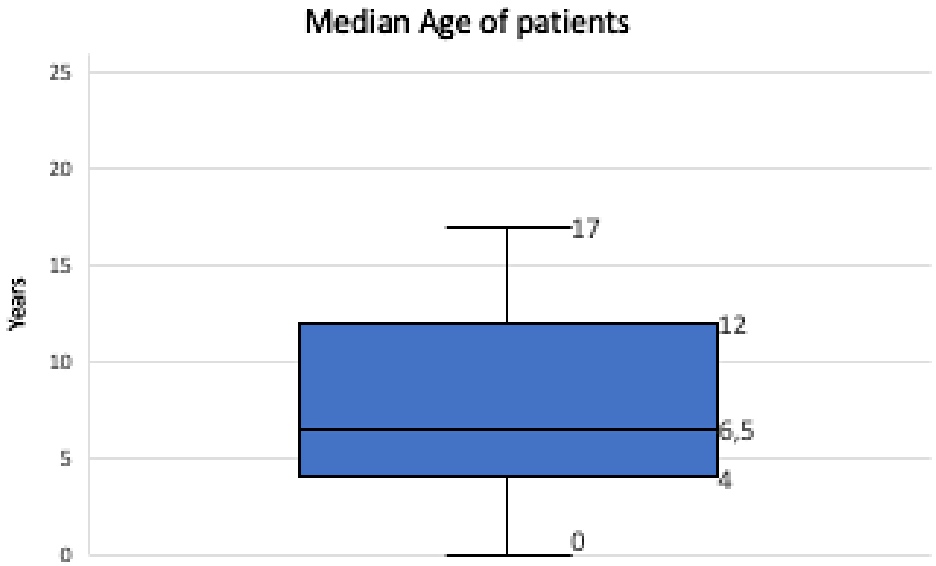



**Figure 12**: *Median age of the epileptic patients in our cohort.*

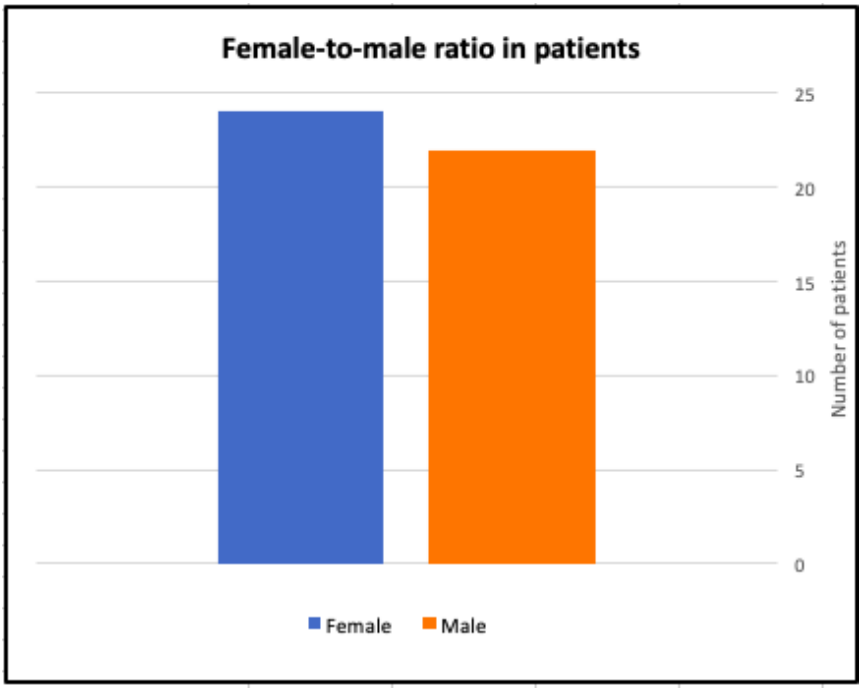



**Figure 13:** *Female-to-male ratio in patients.*

At the Rome IV Diagnostic questionnaire, the results for epileptic patients showed: 35 (76%) had no GI symptoms (“negative”), 5 presented “functional constipation”, 3 had “non-retentive fecal incontinence”, 1 had “aerophagia”, 1 had irritable bowel syndrome with gastrostomy (IBS gastrostomy), 1 had “functional constipation and aerophagia” and 1 had “functional vomiting” (Figure 14). No relevant GI disorders were detected in the healthy subjects group.

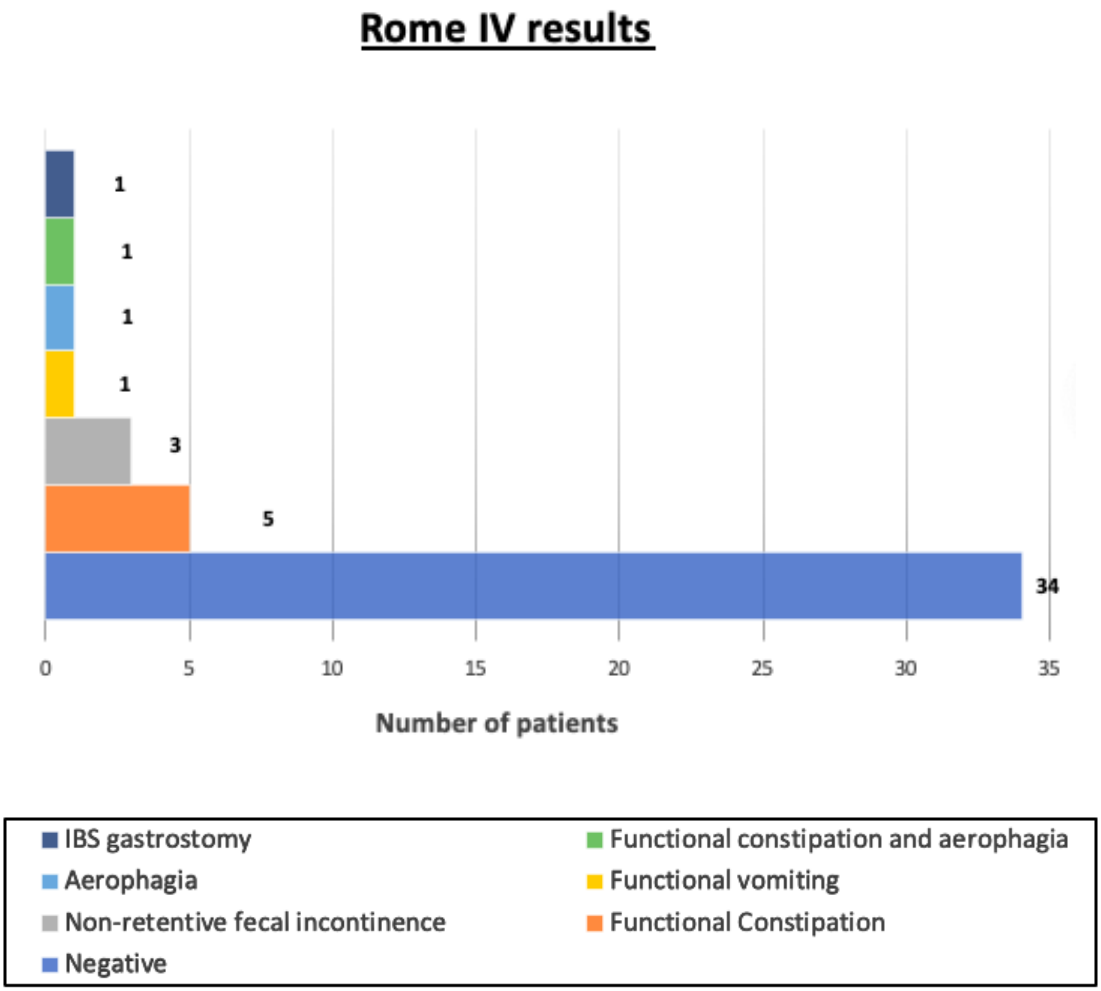



**Figure 14:** *Rome IV results in epileptic patients*.

At the Bristol Stool Scale, 34 (74%) patients had normal stool forms (types 3 and 4), while 12 (26%) patients reported abnormal stools consistency.

The patients recruited were suffering from epilepsy of various etiologies. The main categories of diagnosis were: DEE (developmental and epileptic encephalopathy); FE (focal epilepsy); GGE (genetic generalized epilepsy); progressive myoclonic epilepsy. Some patients also had comorbidities: 2 patients (4,35%) were suffering from autism. Of all patients, 31 (67%) had DEE, 7 (15%) GGE, 7 (15%) FE, and 1 (2%) progressive myoclonic epilepsy (Figure 15).

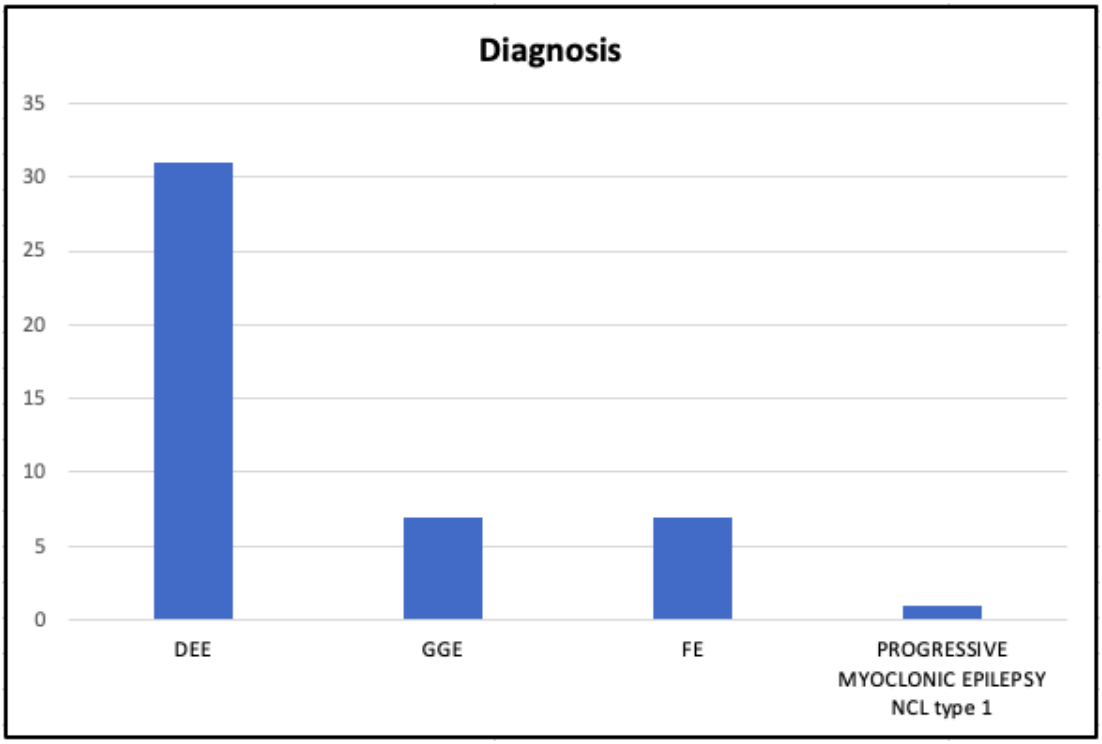



**Figure 15:** *epilepsy of various etiologies and number of patients encountered.*

Of the patients analyzed, 5 had genetic mutations, corresponding to 10% of the total number of patients (46): 1 (2.17%) patient had *CDKL5* mutation; 1 (2.17%) patient had *BRAF* mutation; 1 (2.17%) patient had *CLN1* mutation.2 (4.34%) patients had *BRAT1* mutations;

Based on the International League Against Epilepsy (ILAE) guidelines, 21 (45%) patients were DR, while 25 (55%) patients were DS (Figure 16).

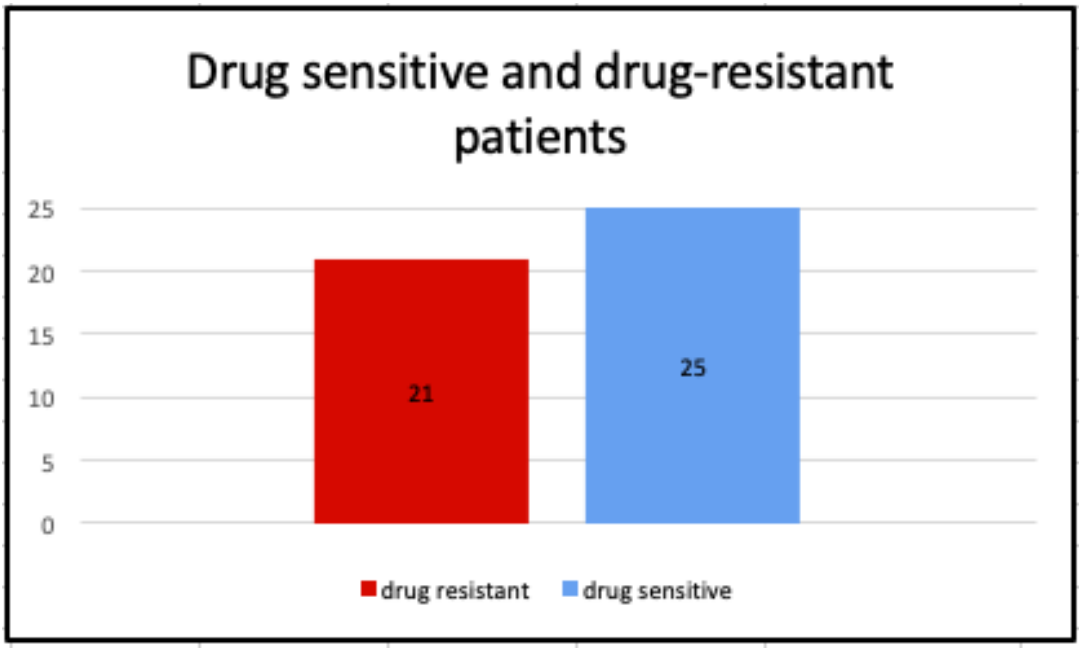



**Figure 16**: *Drug-sensitive and drug-resistant patients*

## 5.2 Alpha diversity

Alpha diversity identifies the bacterial diversity in a specific sample and was calculated with the Chao 1 index, Shannon, Fisher, Simpson, ACE, and se.ACE index. All these methods showed a significant logarithmic association with age (Shannon:p=0.0007; InySimpson:p=0.0009; Observed:p=0.001; Fisher:p=0.001; Simpson:p=0.002; Chao1:p=0.0025; ACE:p=0.0025; Se.ACE:p=0.0025), whereas Shannon and InySimpson were the only methods to show a significant logarithmic association with carbohydrates (Shannon (p=0.0176); InySimpson (p=0.0245)). (Figures 17-26)

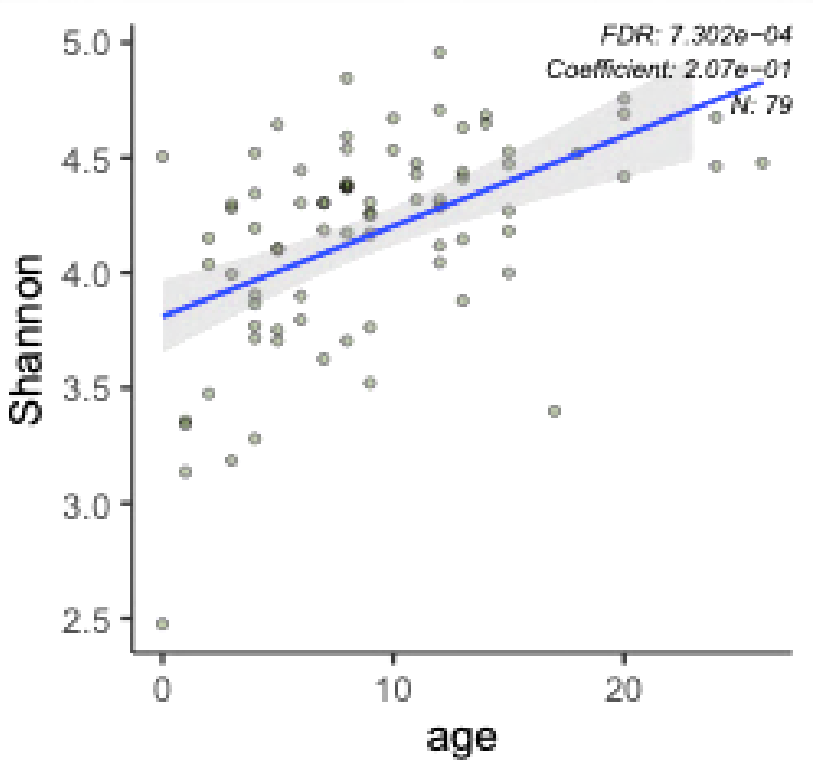



**Figure 17**: *linear relationship between alpha-diversity and age using the Shannon model*

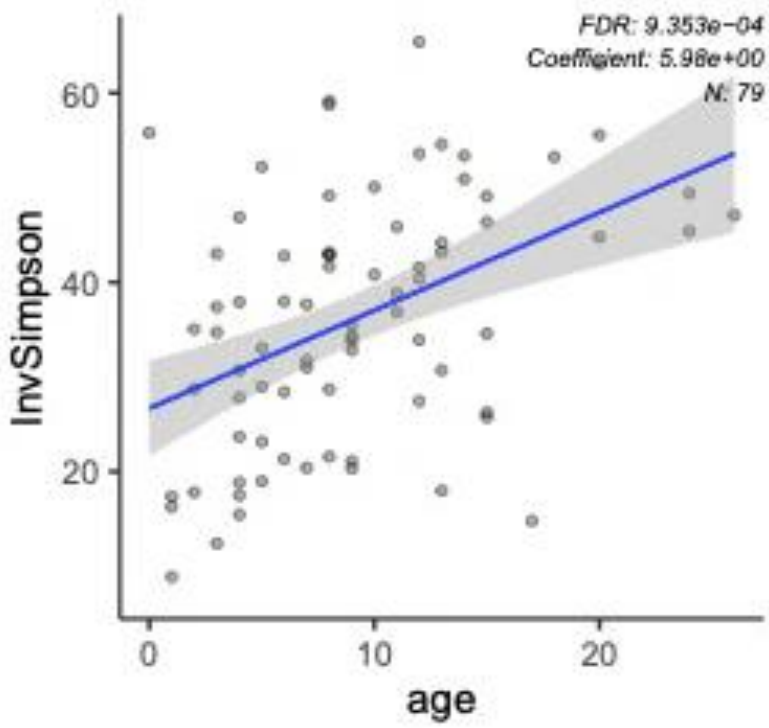



**Figure 18:** *linear relationship between alpha-diversity and age using the InvSimpson model*

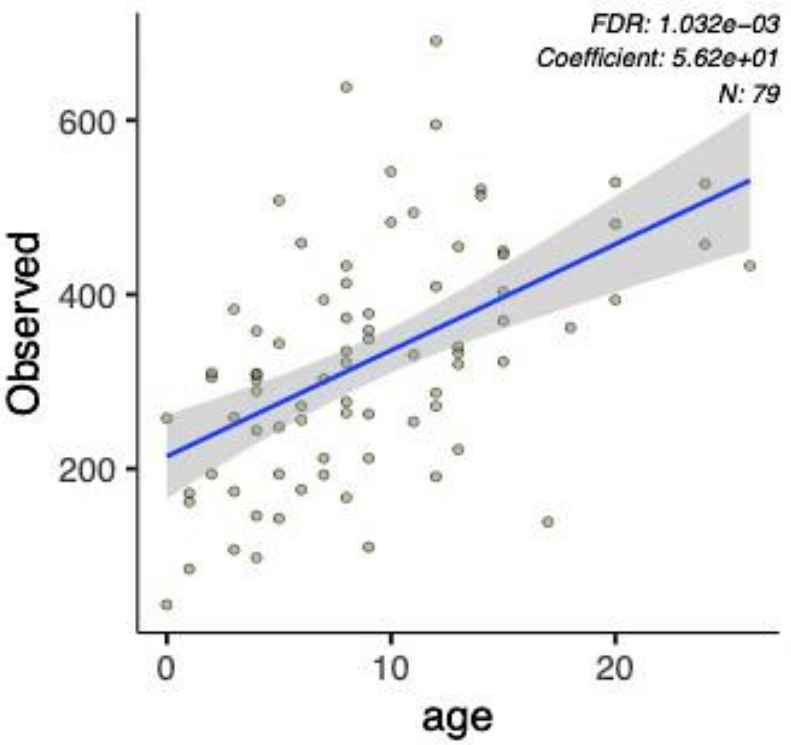



**Figure 19:** *linear relationship between alpha-diversity and age using the Observed model*

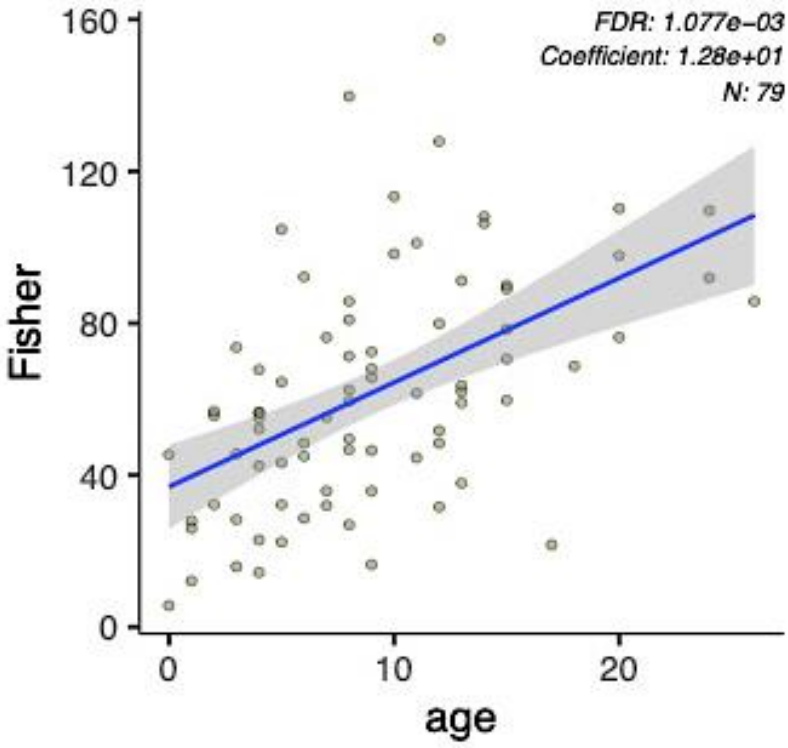



**Figure 20:** *linear relationship between alpha-diversity and age using the Fisher model*

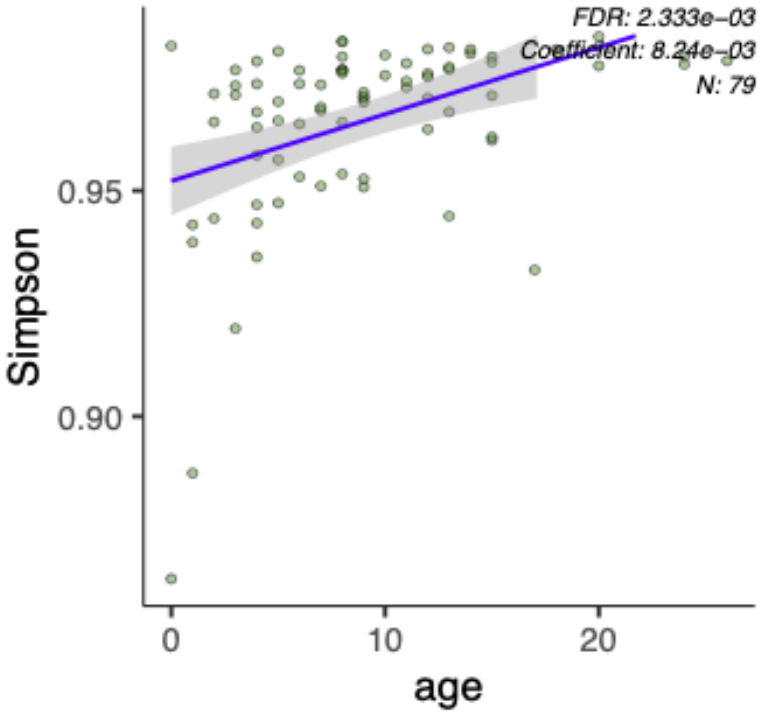



**Figure 21:** *linear relationship between alpha-diversity and age using the Simpson model*

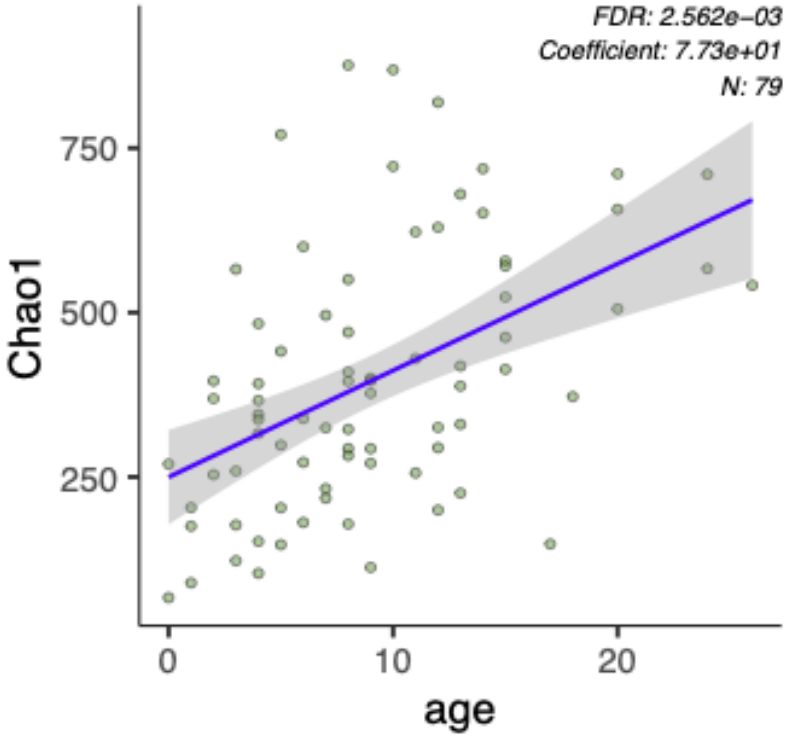



**Figure 22:** *linear relationship between alpha-diversity and age using the Chao1 model*

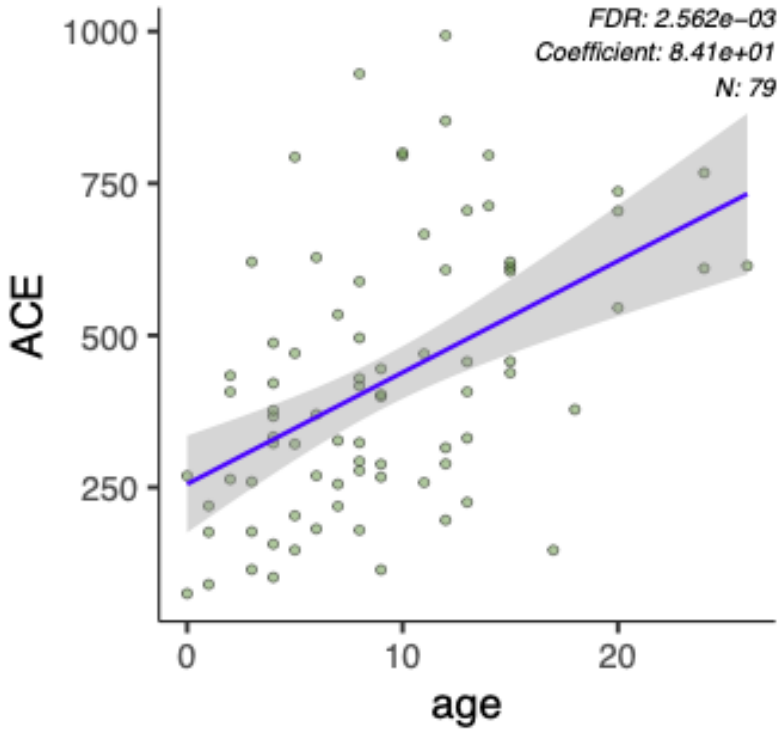



**Figure 23:** *linear relationship between alpha-diversity and age using the ACE model*

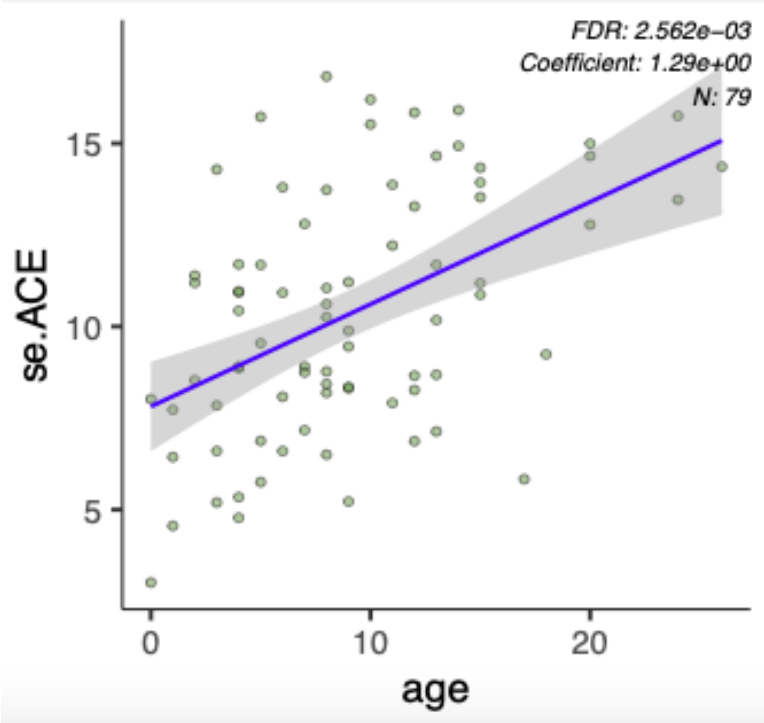



**Figure 24:** *linear relationship between alpha-diversity and age using the se.ACE model*

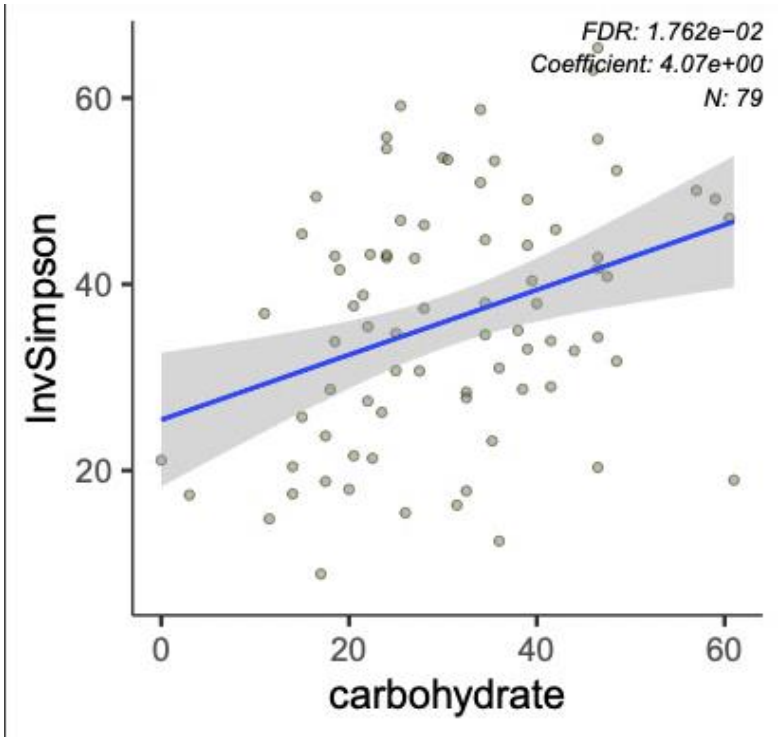



**Figure 25:** *linear relationship between alpha diversity and carbohydrates using the InvSimpson model*

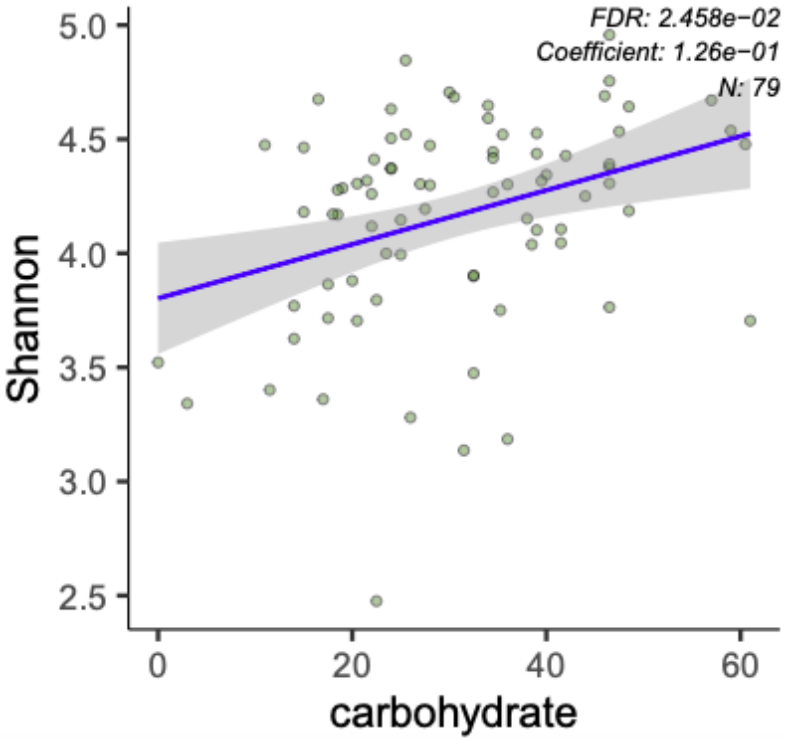



**Figure 26:** *linear relationship between alpha diversity and carbohydrates using the Shannon model*

## 5.3 Beta diversity

As already mentioned, Beta diversity identifies bacterial dissimilarity between different samples and in the present study was evaluated using both the Bray-Curtis (BCM) and Jaccard model (JM) (Figures 27,28). Bacterial diversity was found comparing three groups: healthy, DS, and DR. Specifically:

- In both models, the comparison between DS and DR, showed a statistically significant bacterial diversity related to age (BCM:p=0.022; JM:p=0.029) and carbohydrates (BCM: p=0.048; JM: p=0.033).
- In both methods, comparing DR patients and the healthy group, bacterial diversity was statistically significant related to group (BCM,JM:p=0.001) and age (BCM:p=0.031; JM:p=0.013). Moreover, in the Jaccard model, also carbohydrates showed a significant association (p=0.040) in between these two groups.
- Using both methods, the comparison of DS patients versus the healthy group, showed a statistically significant association with group (BCM,JM:p=0.001), age (BCM,JM:p=0.001) and protein (BCM:p=0.044; JM:p=0.008). At the Jaccard model also carbohydrates showed a significant association (p=0.042).

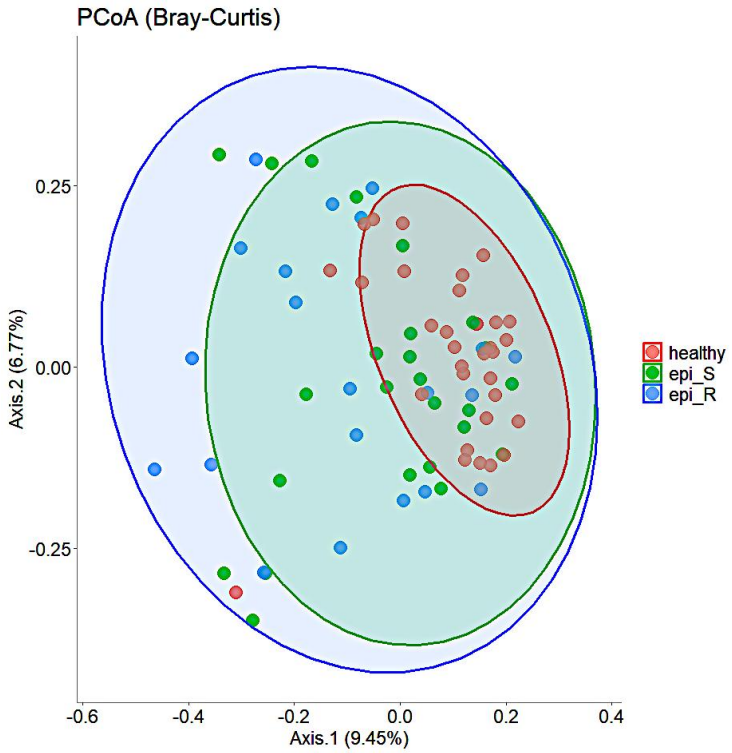



**Figure 27**: *Beta diversity and Bray Curtis index*

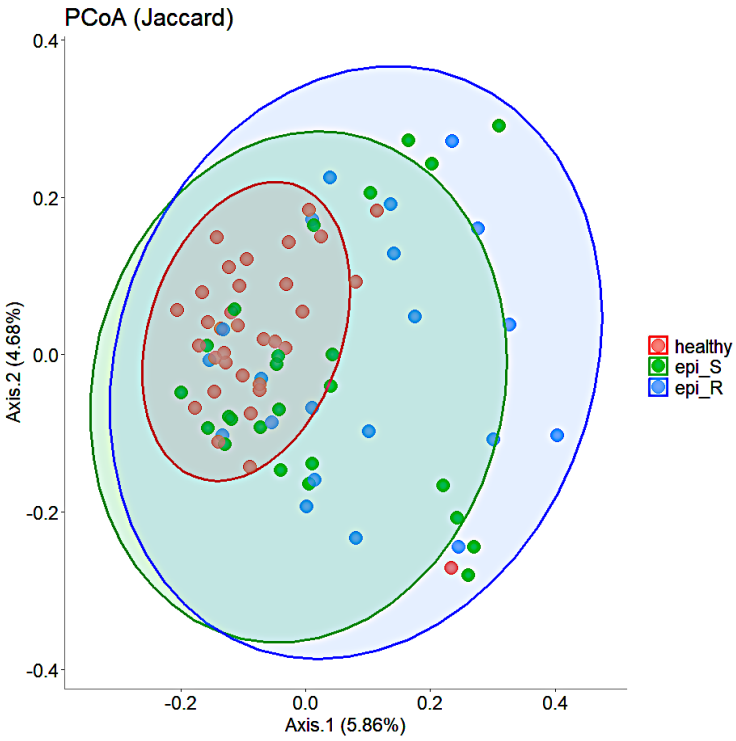



**Figure 28**: *Beta diversity and Jaccard index*

## 5.4 Genus

At the genus level, two analyses were conducted. The first analysis was conducted comparing two groups: healthy (set as controls) and epileptic patients (DR and DS); the second analysis was conducted only within the patients with epilepsy, between drug-sensitive (DS) and drug-resistant (DR) groups. For completeness, both sub-analyses are reported below.

**Analysis n.1 comparing two groups (healthy vs patients with epilepsy):**

**1. evaluating the association between genus and groups (DS, DR), age, carbohydrate, protein, fat,** and **fiber**, a significance was detected as follows:

- **DS group** presented a significant reduction of *X. Eubacterium* (oxidoreducens group);
- **DR group** presented a significant increase of *Sellimonas* and *Hungatella* genus, whereas *X. Eubacterium* (oxidoreducens group) was significantly decreased;
- **Age** positively correlated with the abundance of *X.Ruminococcus* (gauvreauii group), and *Negativibacillus* genus*;*
- A diet rich in **protein** negatively correlated with *Negativibacillus* genus.

**Figures n. 29-31**

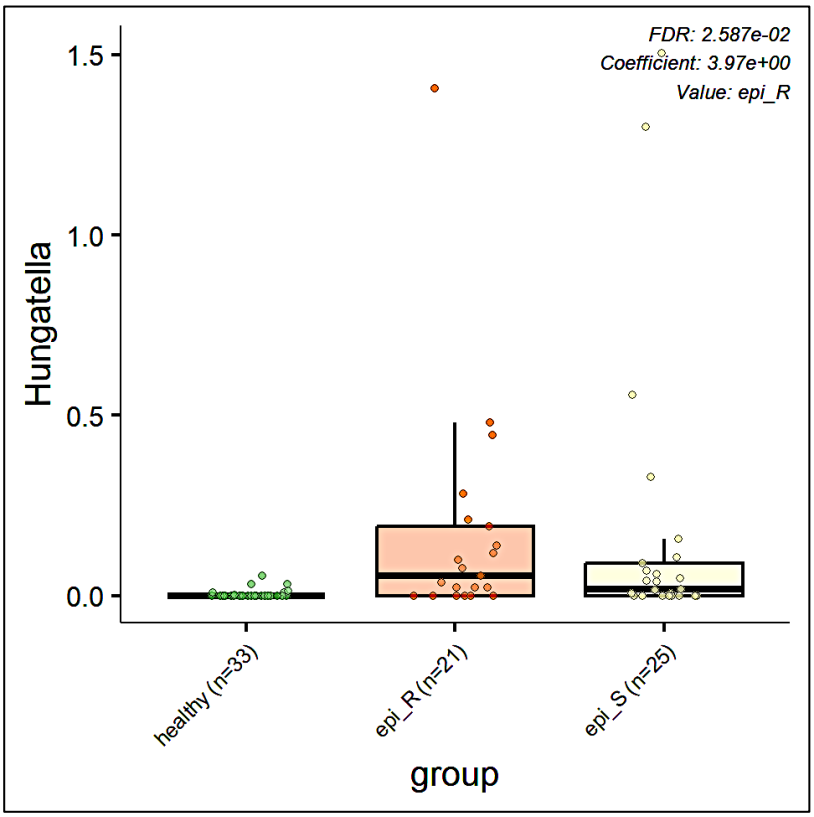



**Figure 29:** *Hungatella* genus in the three different subgroups

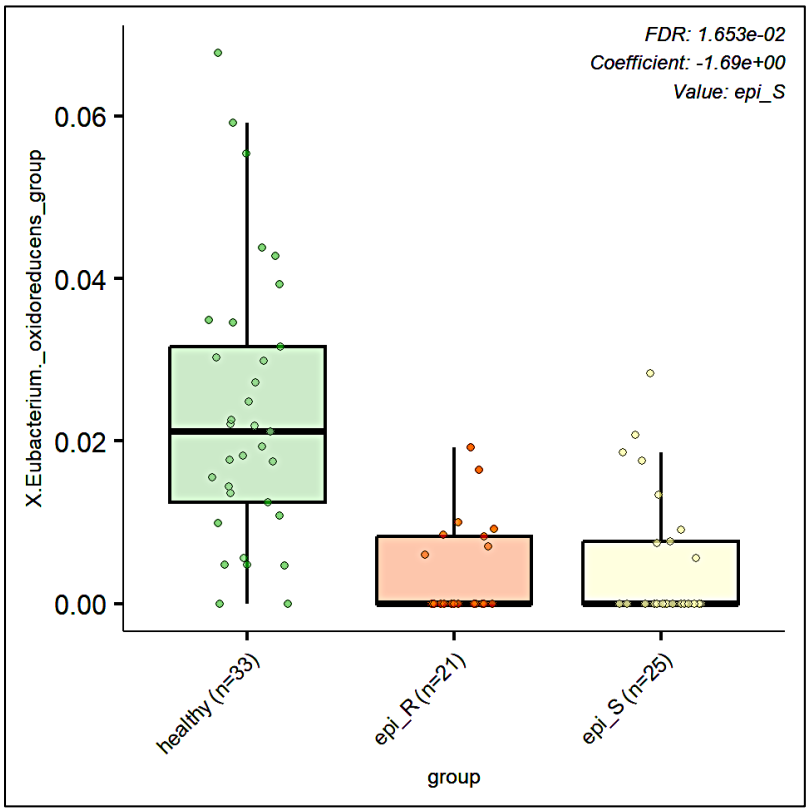



**Figure 30**: *X. Eubacterium* (oxidoreducens group) in the three different subgroups

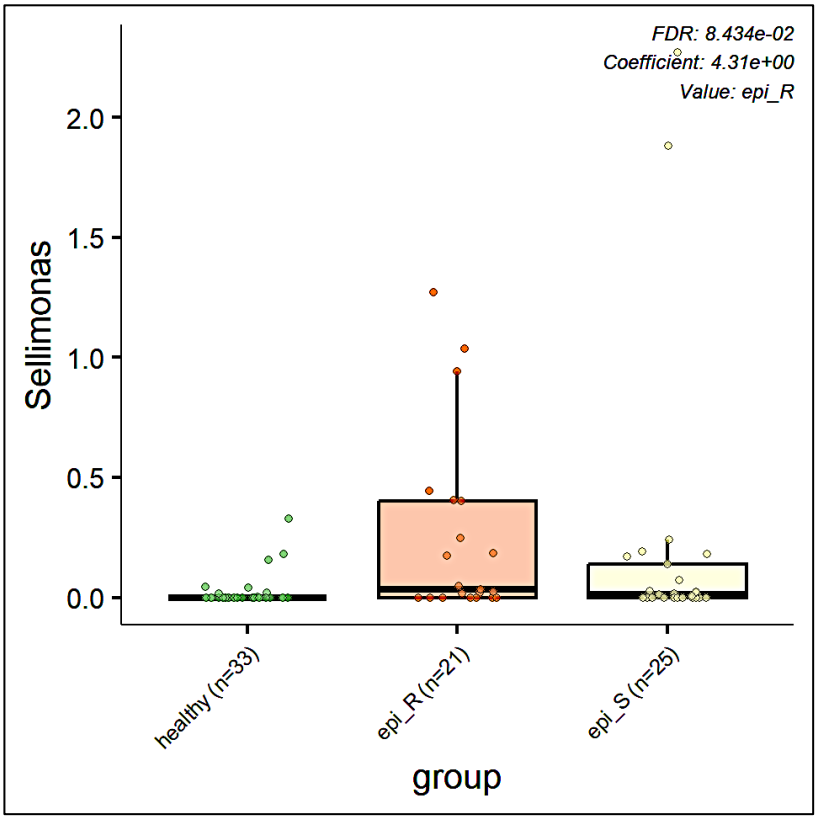



**Figure 31**: *Sellimonas* genus in the three different subgroups

2. **evaluating the association between genus and groups (DS, DR), age, carbohydrate,** and **protein,** a significance was detected as follows:

- **DS group** presented a significant positive correlation with *Hungatella* genus, and a negative correlation with *Lachnospiraceae (ND3007 group), Lachnospiraceae* (*UCG.004),* and *X. Eubacterium* (oxidoreducens group) genus.
- **DR group** presented a significant positive correlation with *Hungatella* and *Sellimonas* genus, and an inverse relationship with *Lachnospiraceae (NC2004 group), Erysipelotrichaceae (UCG003), UCG003, Coprobacter, Lachnospiraceae (UCG 004), X.Eubacterium (ventriosum group),* and *X. Eubacterium* (*oxidoreducens group)* genus.
- **Age** positively correlated with the abundance of *X.Eubacterium (hallii group), Christensenellaceae (R7 group), Alistipes, Odoribacter,*

*Merdibacter, X.Ruminococcus* (gauvreauii group), *Family XIII (AD3011 group),* and *Negativibacillus* genus*.*

- A diet rich in **proteins** negatively correlated with *Negativibacillus* genus.
- A diet rich in **carbohydrates** showed a significant positive correlation with *Subdoligranulum, Roseburia, Terrisporobacter, Butyricicoccus,* and *Romboutsia.*

**Figures n.32-41**

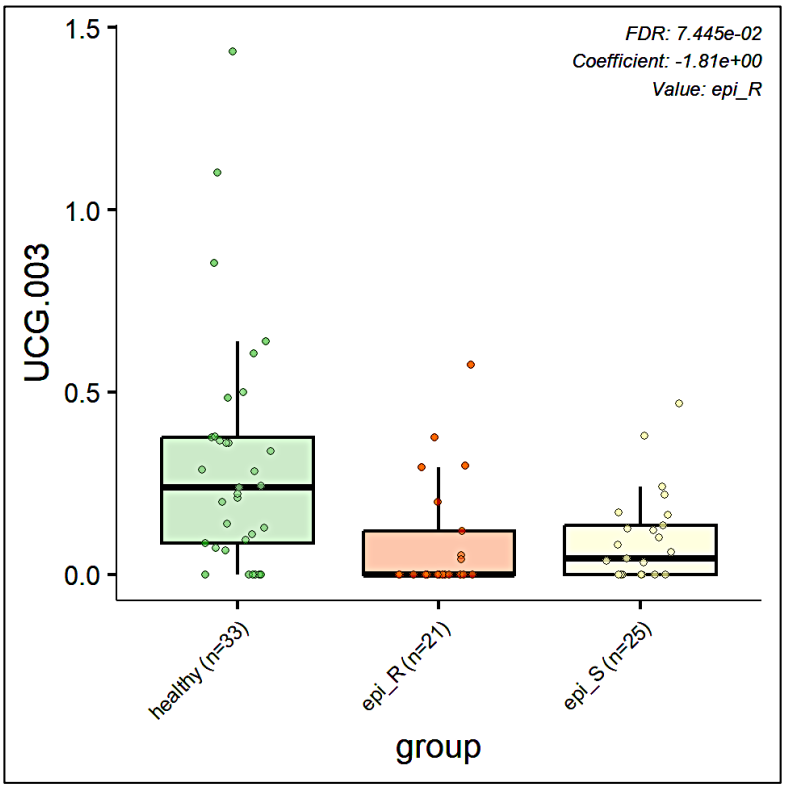



**Figure 32:** *UCG003* in the three subgroups

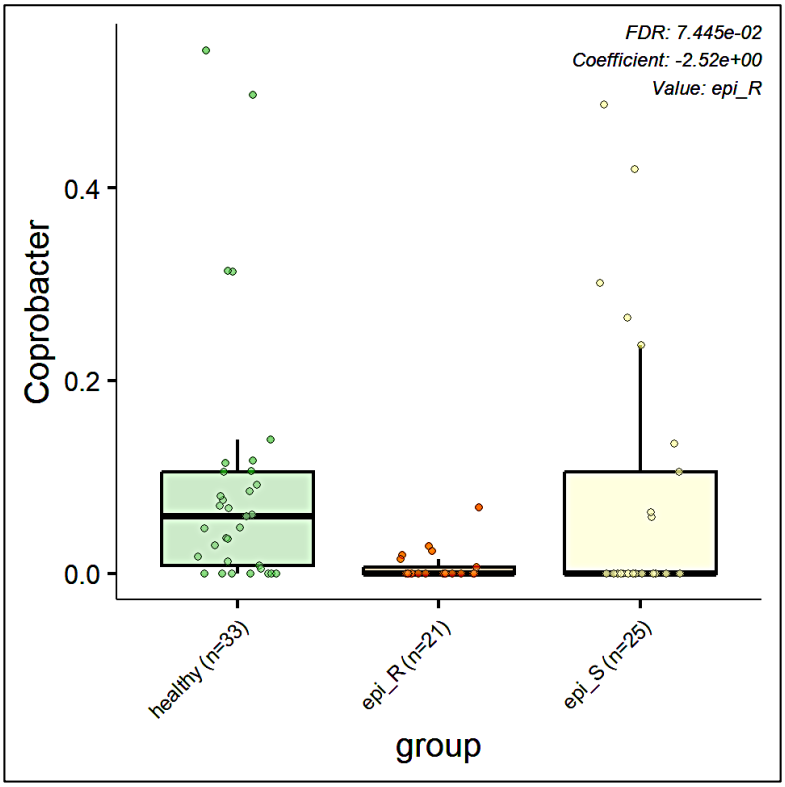



**Figure 33:** *Coprobacter* in the three subgroups

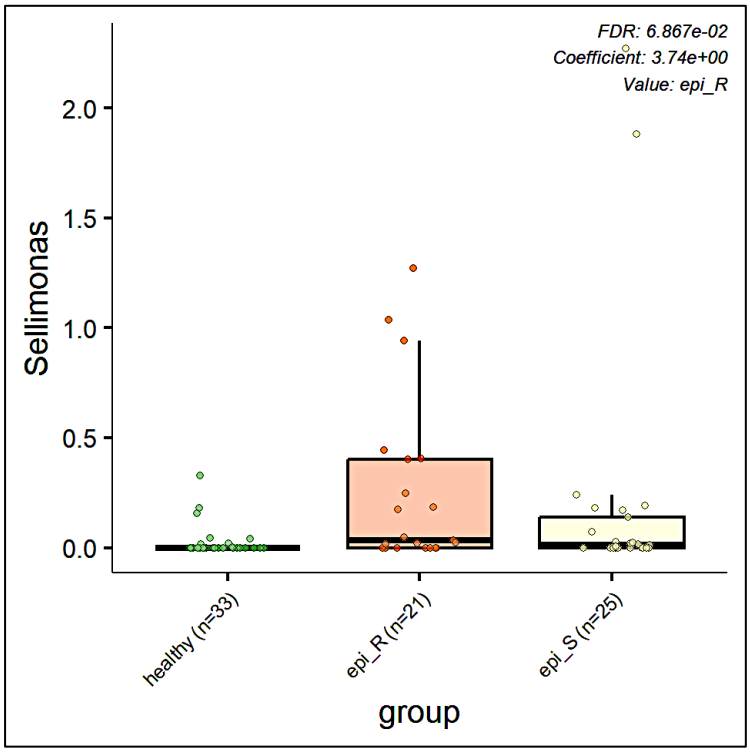



**Figure 34:** *Sellimonas* in the three subgroups

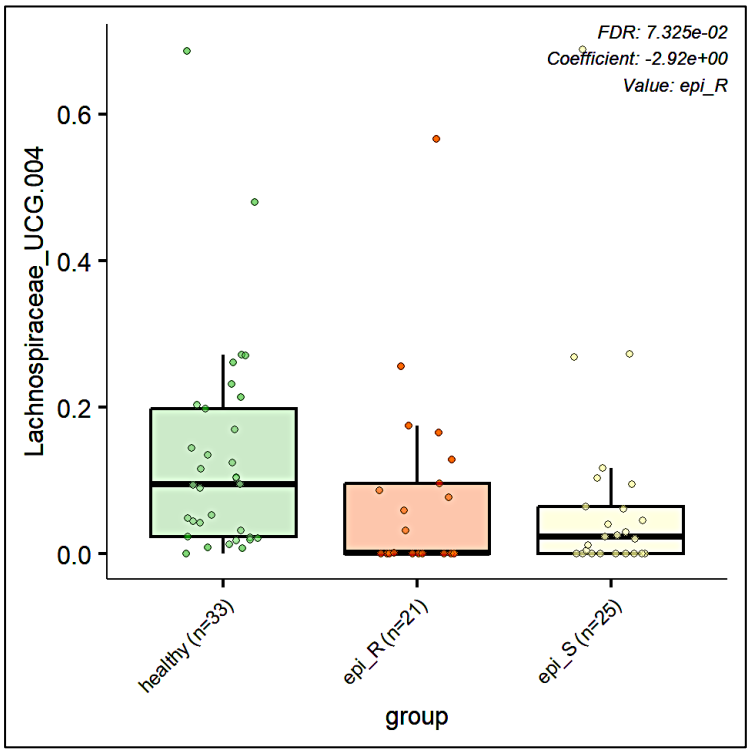



**Figure 35:** *Lachnospiraceae (UCG.004)* in the three subgroups

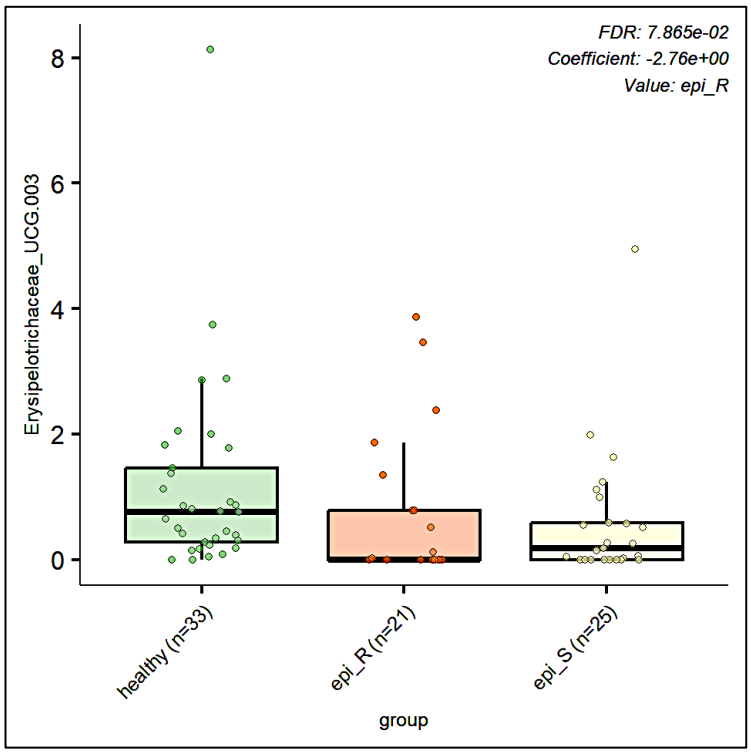



**Figure 36:** *Erysipelotrichaceae (UCG003)* in the three subgroups

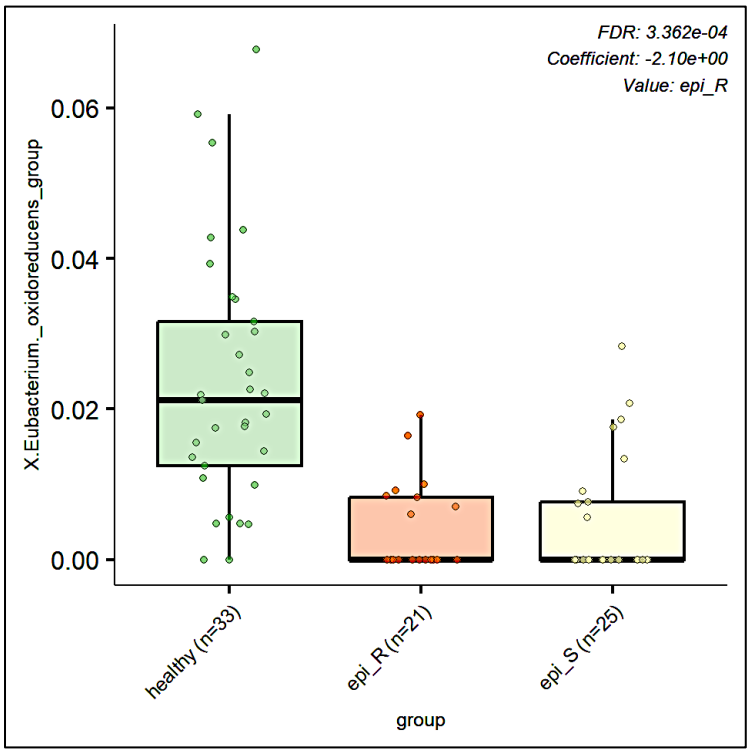



**Figure 37:** *X. Eubacterium (oxidoreducens group)* in the three groups

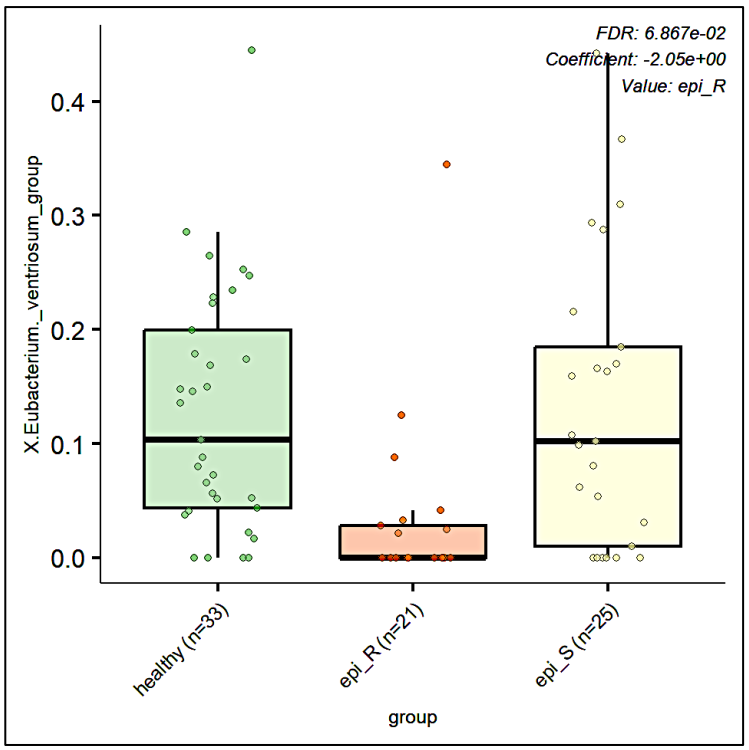



**Figure 38:** *X.Eubacterium (ventriosum group)* in the three subgroups

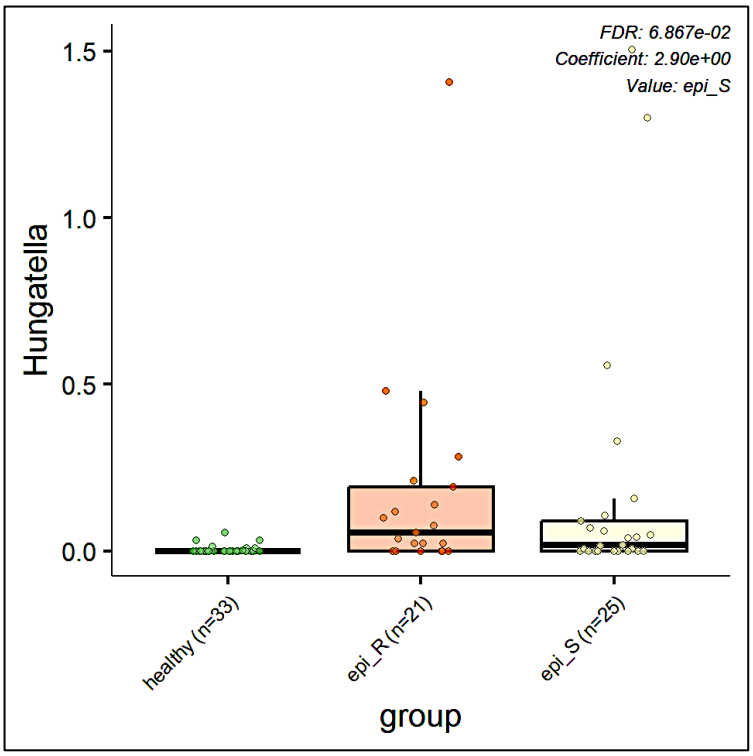



**Figure 39:** *Hungatella* genus in the three subgroups

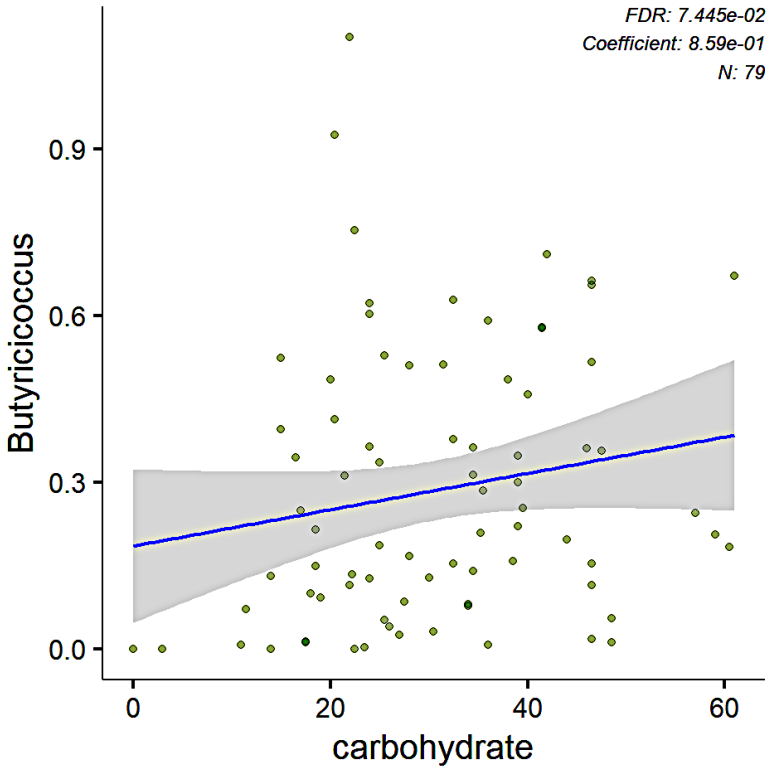



**Figure 40:** *Butyricicoccus* increases with carbohydrates

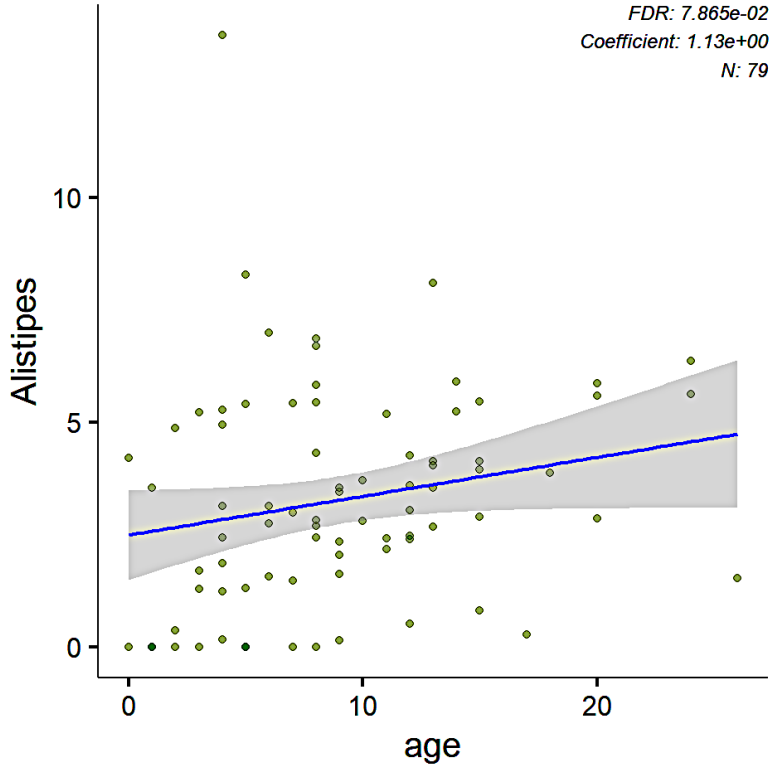



**Figure 41:** *Alistipes* increases with age

**Analysis n.2: comparison between DS and DR in patients with epilepsy**

The DS group was set as control.

The variables considered were: group, age, carbohydrate, and protein.

DR patients showed a significant association with these variables. Particularly, these parameters correlated with specific genera:

- **Age** showed a directly proportional relationship with *Clostridia vadin BB60 group* and *NK4A214 group;*
- **Carbohydrates** showed a directly proportional relationship with *Romboutsia;*
- **The parameter Group** presented a negative correlation with *X.Eubacterium (ventriosum group),* hence the bacterium was less represented in the DR group. (Figure 42)

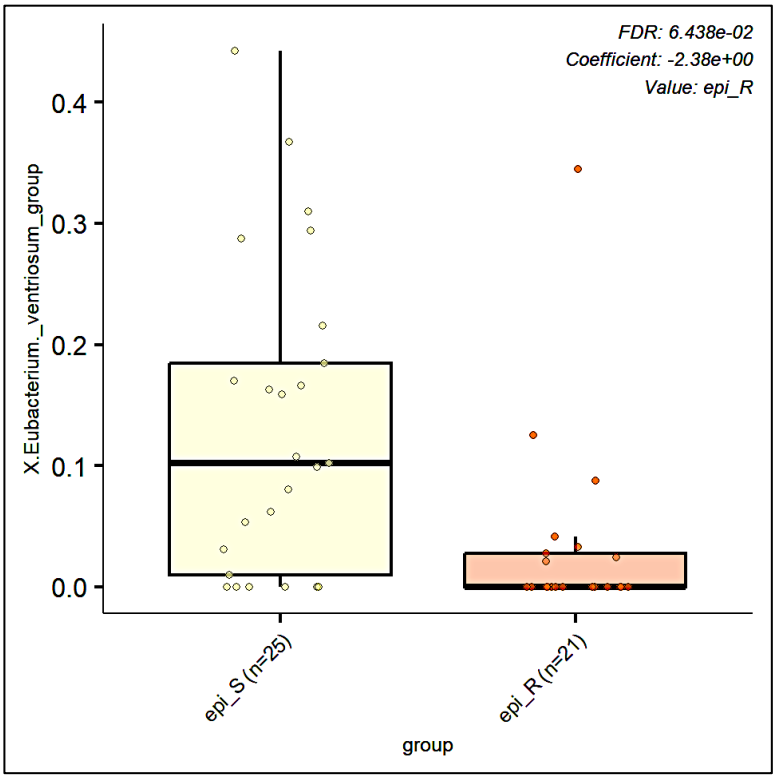



**Figure 42:** *X.Eubacterium (ventriosum group)* in DS and DR subgroup

## 5.5 Phylum

A phylum can be defined either as a group of organisms with a certain degree of morphological or developmental similarity or as a group of organisms with an evolutionary relationship. In classification for taxonomic rank, “Phylum” is placed below “Kingdom” and a few levels above “Genus”.

In the present study, the bacterial genera were grouped by the phylum to which they belonged.

The main phyla found were *Actinobacteriota, Desulfobacterota, Firmicutes, Proteobacteria* (significance for $p<0.05$; approaching significance for $0.05<p<0.1$).

All these phyla showed a significant relationship with age *(p*= 0.022; *p*= 0.022; *p*= 0.022; p=0.096 respectively). Specifically, *Actinobacteriota and Proteobacteria* were seen decreasing with age, whereas *Desulfobacterota* and *Firmicutes* showed an increase with age *(*Figures 43-46).

*Firmicutes* also showed a significant relationship with proteins (p=0.043), decreasing when proteins became higher, whereas *Proteobacteria* decreased with carbohydrates *(p*=0.022). (Figure 47,48)

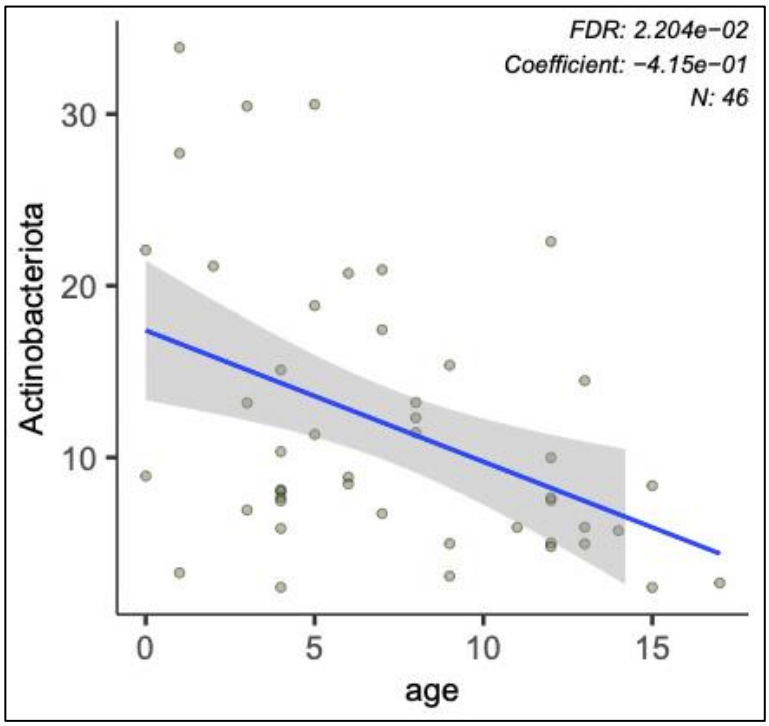



**Figure 43*:*** *Actinobacteriota* decreases with age

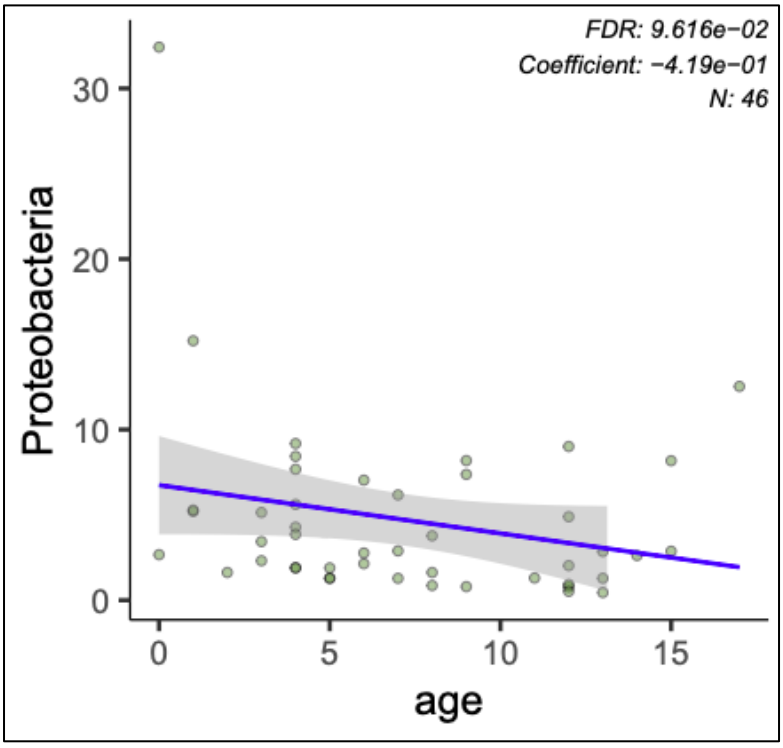



**Figure 44*:*** *Proteobacteria* decreases with age

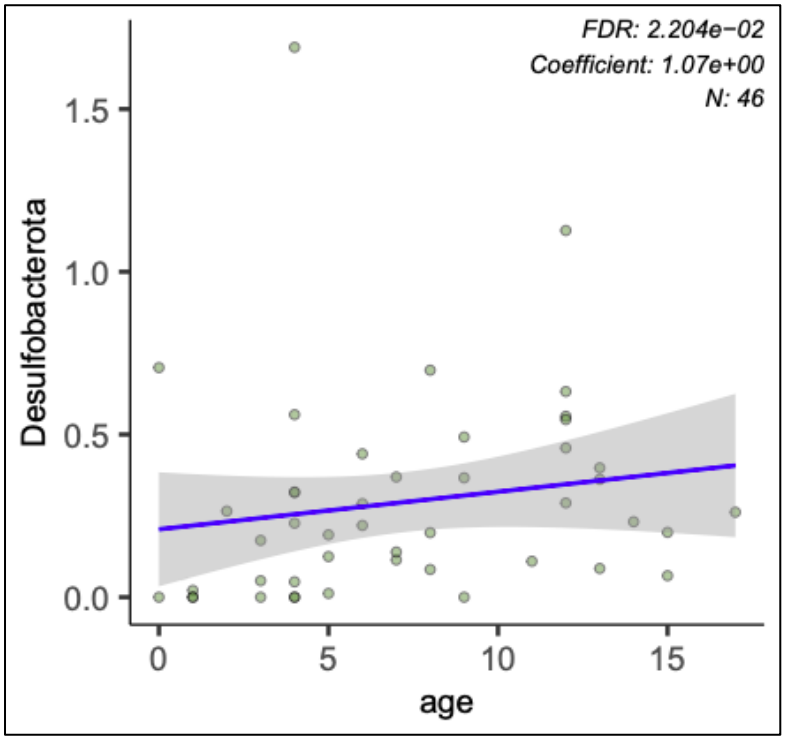



**Figure 45*:*** *Desulfobacterota* increases with age

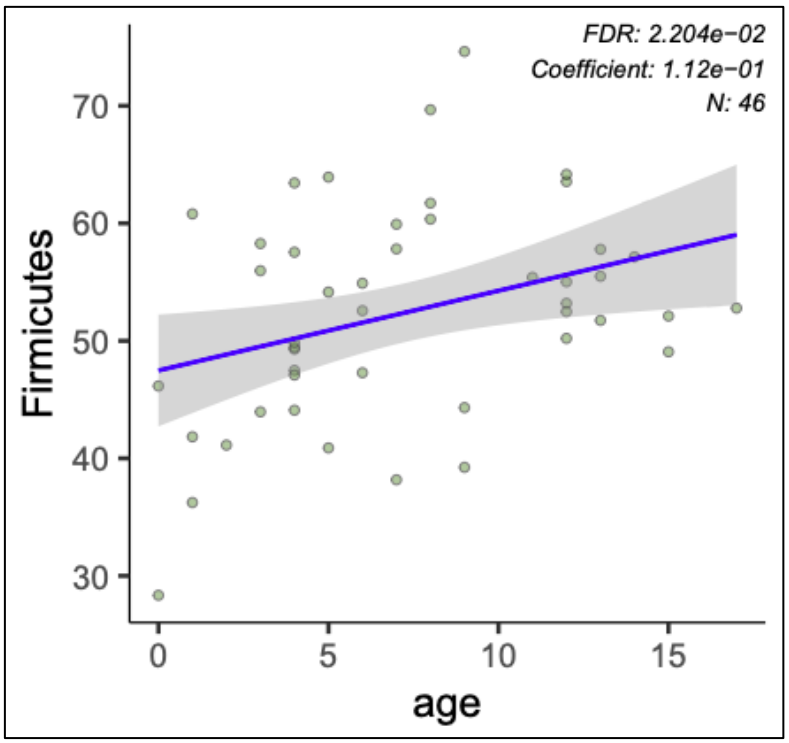



**Figure 46*:*** *Firmicutes* increases with age

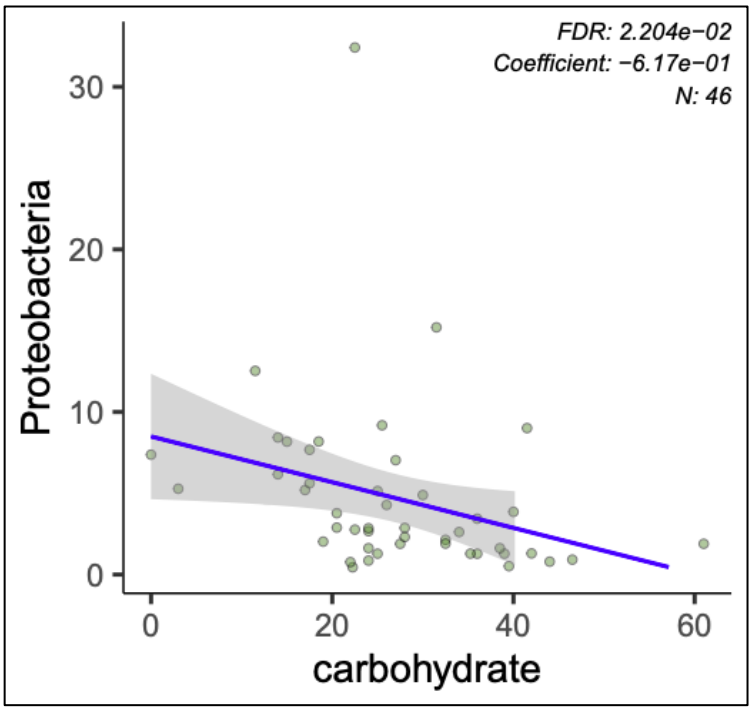



**Figure 47:** *Proteobacteria* phylum decreased with carbohydrate

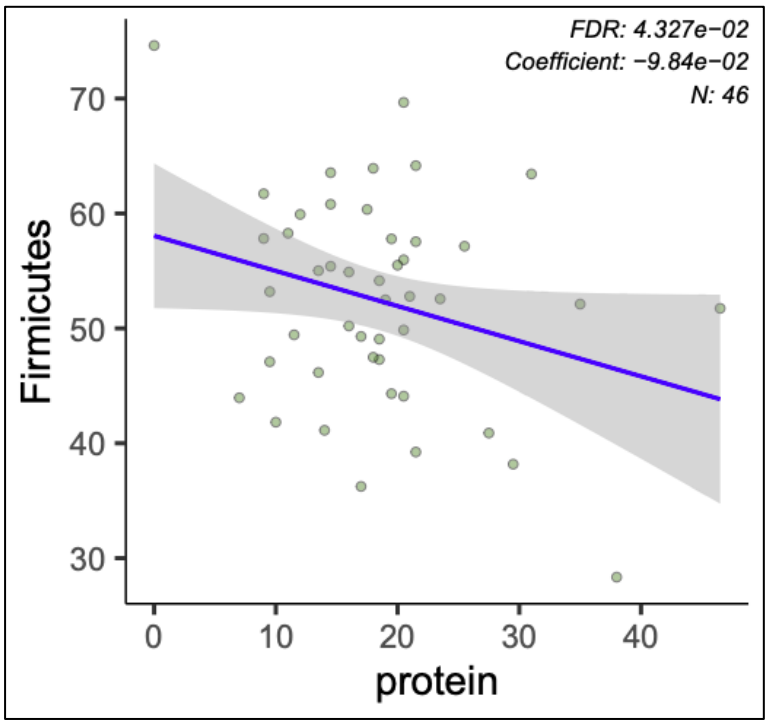



**Figure 48:** *Firmicutes* decreasing with proteins

# 6. Discussion

In recent years, the scientific community has given increasing attention to the role and impact that the composition of the gut microbiota can have on an individual's health and the occurrence of several diseases, also affecting CNS, such as epilepsy. Two of the main phyla in GM are *Firmicutes* and *Bacteroidetes*, whose ratio is related to a state of eubiosis.

The study aimed to analyze GM through modern gene sequencing techniques, along with symptoms and investigation of nutritional habits in a cohort of pediatric patients with epilepsy and neurotypical controls of the same age.

For what concerns alpha-diversity, in the present study it was significantly related to age and carbohydrate.

For what concerns beta-diversity, in the first analysis (three groups: healthy, DR, DS), using a higher quantity of parameters, the genus found were less, whereas when fewer parameters were used, the analysis produced more results for the genus.

Indeed, when four variables were considered (group, age, carbohydrate, protein), 23 genera with a significant association were found: *X. Eubacterium* (oxidoreducens group); *Hungatella; Negativibacillus; Family XIII (AD3011 group); Romboutsia; Sellimonas; X.Eubacterium (ventriosum group); X.Ruminococcus* (gauvreauii group); *Lachnospiraceae (UCG 004); Merdibacter; Butyricicoccus; Coprobacter; Odoribacter; Terrisporobacter; UCG.003; Roseburia; Alistipes; Christensenellaceae (R7 group); Erysipelotrichaceae (UCG003); Lachnospiraceae (NC2004 group); Subdoligranulum; X.Eubacterium (hallii group); Lachnospiraceae (ND3007 group).* Instead, when six variables were considered (group, age, carbohydrate, protein, fat, fiber), only 5 genera were found: *X. Eubacterium* (oxidoreducens group); *Hungatella; Negativibacillus; Sellimonas; X.Ruminococcus* (gauvreauii group).

In the second analysis, when the two groups of epileptic patients were compared (DS, DR), four types of genera were found: *Romboutsia; Clostridia vadin BB60 group; NK4A214 group; X.Eubacterium (ventriosum group).*

Thus, a consistent beta-diversity was found between the different groups.

It is interesting to notice that genus also differed within the epileptic patients whether it was considered the association with carbohydrates, proteins, or the group (DS, DR).

Indeed, if we consider the epileptic patients assuming carbohydrates, an increase of the following notable genus (all belonging to the *Firmicutes* phylum) was observed:

- ***Subdoligranulum****:* is known to be a promising probiotic candidate. *I*n early studies, it has been reported to be associated with depression, resulting depleted in individuals with generalized anxiety and depression. These studies were also supported by the fact that in the in omega-3 rich diet, beneficial for depression, *Subdoligranulum* resulted increased. Moreover, a negative correlation was observed between *Subdoligranulum* and fat mass, but its supplementation did not impact either the lipid metabolism, SCFAs or gut barrier.[132,133]
- ***Roseburia****:* they are obligate Gram-positive anaerobic bacteria that include five species: *Roseburia intestinalis*, *R. hominis*, *R. inulinivorans*, *R. faecis* and *R. cecicol. T*hey are part of commensal bacteria producing SCFAs, especially butyrate, affecting colonic motility, immunity maintenance, and anti-inflammatory properties. Diets that are high in fermentable carbohydrates increase the relative abundance of *Roseburia.* Modification in *Roseburia* spp. representation may affect various metabolic pathways and is associated with several diseases (including irritable bowel syndrome, obesity, Type 2 diabetes, nervous system conditions, and allergies). For instance, it was demonstrated that the abundance of *Roseburia* spp. is similar between healthy and diarrhea-predominant IBS children, whereas the population level of *Roseburia* is significantly lower in constipation-predominant patients compared with healthy subjects. This result could be of help for the study of epileptic patients with GI symptoms: there might be an association between the consumption of carbohydrates, the decrease of *Roseburia*, the constipation, and the frequency of the crisis. Supporting this hypothesis of a correlation between *Roseburia* and

constipation, several studies have shown that patients with Parkinson's disease present constipation and a reduction in *Roseburia* genus. At the same time, an increase in *Roseburia spp.* has been correlated with faster colonic transit. Finally, the abundance of *Roseburia*, a butyrate-producing genus, is positively related to colonic mucosal melatonin levels, whose antioxidant and anti-inflammatory functions are well known. Melatonin can also regulate gastrointestinal motility and moderate visceral hypersensitivity, alleviating the symptoms of GI disorders, such as irritable bowel syndrome (IBS) and ulcerative colitis (UC).[134,135]

- ***Butyricicoccus***: several studies showed a decreased abundance of the *Butyricicoccus* genus in stool samples of patients with IBD.[136]
- ***Romboutsia***: a reduction of this genus was observed in patients with depressive disorder (DD), anorexia nervosa (AN), and Dravet Syndrome.[137,138]

When compared to the healthy group, epileptic patients (both DR and DS) also showed:

- a lower abundance of ***Lachnospiraceae****:* DS group had a reduction of *Lachnospiraceae ND3007 group*, whereas the DR group had a less abundance of *Lachnospiraceae UCG004* and *Lachnospiraceae NC2004*. It is interesting to note that all these three genera, although found in two clinically different groups, have been found associated with constipation and decreased in patients with PD.[142]
- a higher abundance of ***Hungatella***: belonging to the family *Clostridiaceae* and phylum *Firmicutes*, it is known to produce the precursor molecule for trimethylamine-N-oxide (TMAO), and is implicated in cardiovascular and neurological diseases including depression.[133,139,]
- and a lower abundance of ***Eubacterium oxidoreducens group***: it is known to have quantitative alterations in relation to diet: studies on ruminants showed a reduction of it when fed with dietary protein

sources, such as cottonseed meal or rapeseed meal.[140] Studies on pigs, instead showed an increase of this genus in pigs assuming inulin.[141] The results of the present study, together with the scientific literature, may be of help for further research on the relationship between the consumption of fiber or proteins, the presence of *Eubacterium oxidoreducens group* and symptoms.

Along with these genera, the DR group was further characterized by an increased abundance of ***Sellimonas,*** and a lower abundance of ***Coprobacter, Eubacterium ventriosum, Erysipelotrichaceae****.* *Sellimonas* is known to increase as a result of dysbiosis and in IBD. *Coprobacter, Erysipelotrichaceae, and Lachnospiraceae* were seen to decrease in a study involving children with autistic spectrum disorder (ASD) with constipation.[144]

Finally, the ***Eubacterium ventriosum*** has been detected when comparing DR patients and the healthy group, and DS vs DR patients. This suggests a strong association of this genus with the DR group. *Eubacterium ventriosum* belongs to the family *Eubacteriaceae* and is significantly depleted after traumatic brain injury in mice and with an increase in depressive symptoms.[133, 143]

# 7. Conclusions

This study shows that the microbiota of healthy and epileptic patients is different, and differences can be detected within the epileptic population itself, between DS and DR.

The diversity of the GM within each group (healthy, DS, DR), that is *alpha diversity,* resulted associated with age and carbohydrates.

In the comparison of all three groups (*beta-diversity*), the GM diversity was influenced by age, the presence of epilepsy, and also carbohydrates. Comparing DS and DR patients, the factors that resulted in the greatest variation of the microbiota were age and carbohydrates.

The results of the genus that were found in the present study seem to corroborate the thesis of a correlation between epilepsy, diet, depression, and GI symptoms.

Indeed, although we didn't find a qualitative difference in terms of phylum in epileptic patients compared to the healthy group, a quantitative difference in terms of abundance and deficiency of certain phyla was detected. Particularly, the most represented phylum in epileptic patients was the one of *Firmicutes*.

The *Roseburia genus* was increased in epileptic patients eating carbohydrates and could thus be indicative of a correlation with GI symptoms in epileptic patients, such as constipation or diarrhea. Indeed, in scientific literature, the abundance of *Roseburia* was reported to faster colonic transit, and thus the assumption of carbohydrates may correlate with non-retentive fecal incontinence in epileptic patients. On the contrary, the lack of *Lachnospiraceae* in epileptic patients may relate to the functional constipation found in some of them. The decrease of *Eubacterium oxidoreducens group* in epileptic patients may be associated with a diet low in proteins.

The detection in this study of a lower abundance of *Eubacterium ventriosum* in DR patients is probably the most important result. It is less presented than in DS patients and could thus be a potential target of therapy in DR patients. Moreover, it is well known its association with depression, and it could be

interesting to evaluate whether DR patients with a lower abundance of *Eubacterium ventriosum* present more depressive symptoms.

In conclusion, our study highlights the importance of GMB-axis, demonstrating that Beta-diversity is influenced by diet and the sensitiveness or resistance to ASMs in epileptic patients. Finally, it claims an urgent need for causality studies, opening up glimpses for an always major detailed association between GM and epilepsy.

# 8. Appendix

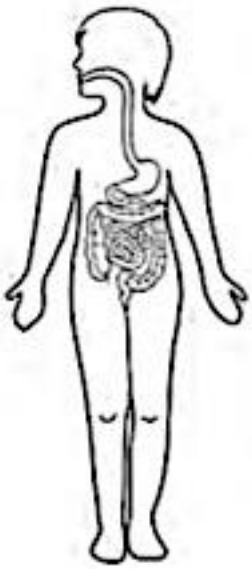
**Appendix 1:** Rome IV questionnaire sample for infants and toddlers (0-3 years)

**MODULO PER GENITORI DI NEONATI E BAMBINI PICCOLI**

**Questionario diagnostico Roma IV per il sistema gastrointestinale pediatrico**

**Disturbi per neonati e bambini piccoli (0-3 anni)**

(Questionario sui sintomi gastrointestinali pediatrici, versione bambino bambino, van Tilburg, Rouster, Argento, Pellegrini, Gao e Hyman, 2015)



**Istruzioni**

Questo questionario riguarda l'apparato digerente di tuo/a figlio/a (esofago, stomaco, intestino tenue e colon) e i problemi che può avere con esso. Alcuni problemi potrebbero applicarsi a tuo/a figlio e altri no.

*Prova a rispondere a tutte le domande nel miglior modo possibile.*

Fateci sapere se avete domande; saremo felici di aiutarti!

0.1. Quanti anni ha tuo/a figlio/a? ___Anni _____ Mesi

0.2. Tuo/a figlio/a sta guadagnando peso normalmente? ___Si _ No

## MODULO PARENT-REPORT PER NEONATI E BAMBINI PICCOLI

### Sezione A: Problemi gastrointestinali infantili

***Le seguenti domande sono applicabili solo ai bambini di età compresa tra 0 e 12 mesi.***

1. Tuo/a figlio/a ha rigurgitato o vomitato ogni giorno nelle ultime 3 settimane?
   *0.* No. ***Se no, vai alla domanda 5.***
   1. Sì

2. Quante volte al giorno, in media, tuo/a figlio/a rigurgita o vomita?
   0. 1 volta al giorno
   1. 2 volte al giorno
   2. 3-10 volte al giorno
   3. Più di 10 volte al giorno

3. Tuo/a figlio/a ha questi altri sintomi:

| | | |
|---|---|---|
| -Vomito misto a sangue | 0. ___No | 1___Si |
| -Respiro sibilante o problemi di respirazione | 0. ___No | 1___Si |
| -Conati di vomito o tentativi inutili di vomito | 0. ___No | 1___Si |
| -Testa e collo inclinati da un lato per lunghi periodi di tempo | 0. ___No | 1___Si |
| -Problemi di deglutizione | 0. ___No | 1___Si |
| -Problemi di allattamento/alimentazione | 0. ___No | 1___Si |

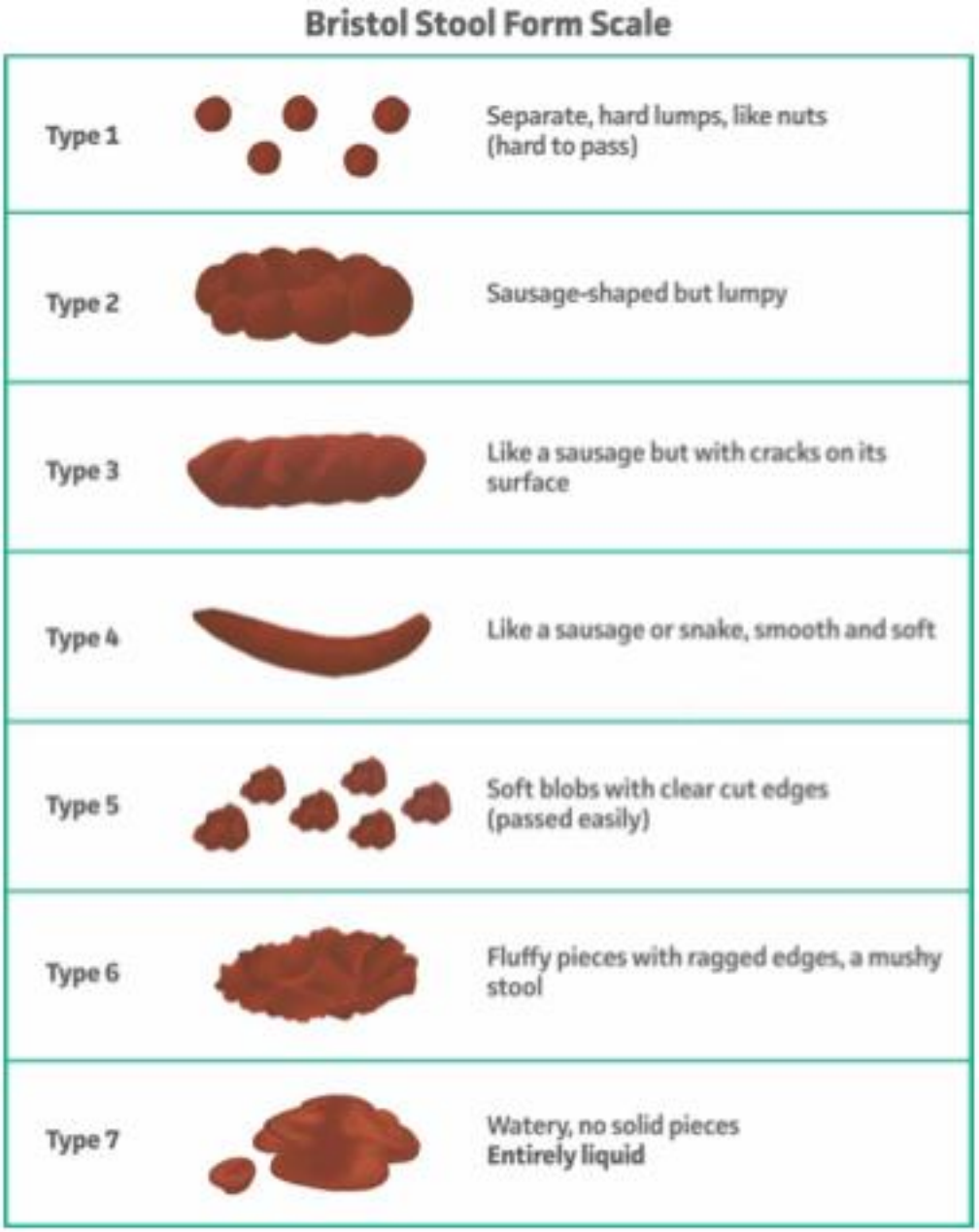
4. Tuo/a figlio/a è sano/a, eccetto il rigurgito e il vomito?
   0. ___No 1 ___Si
   Se no, si prega di spiegare _____

   _____

   _____

5. Nell'ultima settimana è stato/a irritabile, capriccioso/a (non pianto incessante ma non era nemmeno sereno/a), o ha pianto senza motivo?
   *0.* No, ***se no, vai alla domanda 9.***
   1. ___ Si

**Appendix 2:** Bristol Stool Form Scale



**Appendix 3:** Food frequency questionnaire

| CIBI e QUANTITA' | CONSUMO MEDIO NELLE ULTIME 12 SETTIMANE | | | | | | |
|---|---|---|---|---|---|---|---|
| (una porzione media) | Mai | 1-2 volte a settimana | 3-4 volte a settimana | 1 volta al giorno | 2-3 volte al giorno | 4-5 volte al giorno | 6+ volte al giorno |
| Carne rossa: arrosto; bistecca; stufato | | | | | | | |
| Pollo, coniglio o tacchino | | | | | | | |
| Insaccati: prosciutto o altri | | | | | | | |
| Pesce | | | | | | | |
| Pane | | | | | | | |
| Patate | | | | | | | |
| Pasta grano 00 | | | | | | | |
| Pasta grani antichi o farro | | | | | | | |
| Riso bianco | | | | | | | |
| Riso integrale, basmati o venere | | | | | | | |
| Pizza | | | | | | | |
| Yogurt | | | | | | | |
| Formaggio | | | | | | | |
| Uova | | | | | | | |
| Verdura cotta | | | | | | | |
| Verdura cruda | | | | | | | |
| Caffè | | | | | | | |
| Bevande zuccherate | | | | | | | |
| Frutta | | | | | | | |
| Legumi | | | | | | | |
| CONDIMENTI | Mai | < di 50 g a settimana | tra 50-100 g a settimana | tra 100-150g a settimana | > di 150 g a settimana | | |
| Burro | | | | | | | |
| Olio | | | | | | | |

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**Itaca** (1911 - Konstantinos Kavafis)

Quando ti metterai in viaggio per Itaca
devi augurarti che la strada sia lunga
fertile in avventure e in esperienze.
I Lestrigoni e i Ciclopi
o la furia di nettuno non temere,
non sarà questo il genere d'incontri
se il pensiero resta alto e un sentimento
fermo guida il tuo spirito e il tuo corpo.
I Ciclopi e i Lestrigoni, no certo
né nell'irato Nettuno incapperai
se non li porti dentro
se l'anima non te li mette contro.

Devi augurarti che la strada sia lunga.
Che i mattini d'estate siano tanti
quando nei porti – finalmente e con che gioia –
toccherai terra tu per la prima volta:
negli empori fenici indugia e acquista
madreperle coralli ebano e ambre
tutta merce fina, anche profumi
penetranti di ogni sorta, più profumi
inebrianti che puoi,
va in molte città egizie
impara una quantità di cose dai dotti.

Sempre devi avere in mente Itaca –
raggiungerla sia il pensiero costante.
Soprattutto, non affrettare il viaggio;
fa che duri a lungo, per anni, e che da vecchio
metta piedi sull'isola, tu, ricco
dei tesori accumulati per la strada
senza aspettarti le ricchezze da Itaca.
Itaca ti ha dato il bel viaggio,
senza di lei mai ti saresti messo
in viaggio: che cos'altro ti aspetti?

E se la trovi povera, non per questo Itaca ti avrà deluso.
Fatto ormai savio,con tutta la tua esperienza addosso
Già tu avrai capito ciò che Itaca vuole significare.