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ORIGINAL PAPERS

Methodological Aspects Regarding the Interactions Between Microflora and Neuropsychiatric/Metabolic Disorders

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Abstract. *Considering that intestinal microbiota a key factor in regulating the brain-intestinal axis and is also involved in the development and proper functioning of the hypothalamic-pituitary-adrenal axis, numerous studies have turned their attention to the composition of digestive microflora in most of the neuropschiatric disorders. Same goes for the metabolic deficits, which could be correlated with some microbiome dysfucntions, as well as with most of the existent neuropschiatric deficiences. In this context, considering also our group recent experience in this area of research, we are describing here some methodological aspects regarding the interactions between microflora and neuropsychiatric/metabolic disorders.*

Keywords: methodology, microflora, neuropsychiatric, metabolic.

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Introduction

Intestinal microflora has many important functions in the human body, also their populations of microorganisms have important roles on the pathophysiology of some diseases. The microflora of the digestive tract is the largest ecosystem present in the human body, and it maintains the homeostasis of the internal environment. Under physiological conditions of numerous processes, the disturbance of the balance of the microbiota occur in the initiation and development of some diseases (Chen et al., 2021).

Intestinal dysbiosis is most commonly associated with metabolic diseases such as irritable bol syndrome, type 2 diabetes and obesity (Jovel, et al., 2018). The neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease (Chen et al., 2021), and other neuropsychiatric conditions - autistic spectrum disease, schizophrenia, depression, anxiety (Rea et al, 2020) are connected with the intestinal dysbiosis as well.

Irritable bowel syndrome is defined as a series of diseases affecting the digestive bowl which presents discomfort, abdominal pain and abnormal defecation. It affects between 10 and 20% of adults and adolescents worldwide, and the main causes include: genetic factors, motor dysfunction of the gastrointestinal tract, visceral hypersensitivity, infections, inflammation. Variations in the composition of intestinal microflora are thought to be associated with the inflammatory process within this syndrome (Jovel et al., 2018).

Obesity and type 2 diabetes are also associated with intestinal dysbiosis. Recent studies in human subjects have revealed altered composition of intestinal microflora in obese individuals, in combination with that of individuals with normal body weight. Further investigation is needed to demonstrate the link between obesity and the different ratio of bacteria in the *Firmicutes* filum to those in *Bacteroidetes* (Jovel et al., 2018), but it is known that there is a significant decrease in *Bacteroidetes* species (Durack and Lynch, 2019).

Type 2 diabetes is a very complex disease, caused by both genetic and environmental factors, affecting public health worldwide. Recent studies indicate an association between this metabolic disease and the composition of the intestinal microbiota. For example, a decline in the number of beneficial bacteria that produce butyrate has been observed and an increase in the population of potentially pathogenic microorganisms (Jovel et al., 2018).

Parkinson's disease is a disease of the central nervous system that is characterized by degeneration and decrease of dopamine neurons in the black substance. Studies in this regard have highlighted a different composition of the

microbiota of patients compared to normal individuals. Thus, in patients with Parkinson's disease, there is a substantial increase in the bacteria of the genera *Bifidobacterium*, *Pasteurella* and *Enterococcus*, and a low number of species of *Brautella*, *Prevotella* and *Faecococcus* (Chen et al., 2021).

Alzheimer's disease is the most common neurodegenerative disease related to age and is represented by the decline in cognitive function. A multitude of clinical and experimental studies suggest the correlation between this disease and intestinal dysbiosis. Trough the axis linking the microbiota to the brain, the dysbiosis affects function and behavior. Among the mechanisms involved are the increase in β-amyloid deposits, phosphorylation of tau protein, neuroinflammatory processes, metabolic dysfunction and, oxidative stress (Wu et al., 2021).

These aspects could be also very relevant considering our group previous experience with the most important neurological disorders worldwide, judged by the number of affected patients, such as Alzheimer's disease and Parkinson's disease (Ciobica et al., 2009, Balmus et al., 2017), where the relevance of probiotics and phycobiotics is largely discussed lately (Sarkar et al., 2016).

Depression and anxiety are neuropsychiatric disorders that, together, affect about 10% of the global population annually. Numerous forms of psychotherapeutic and medicinal treatment have been generated in recent decades, but these still have a very high prevalence. In order to establish new treatment strategies, further studies on these diseases are needed in order to better understand the mechanisms related to their pathophysiology, especially in the case of comorbidities.

Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia, and it is mainly caused by abnormalities in the synthesis or use of insulin. These abnormalities inevitably affect the metabolism of carbohydrates, lipids and proteins, as insulin is a very important anabolic hormone (Kharroubi and Darwish, 2015). Diabetes-related hyperglycemia is also linked to numerous visceral dysfunction, so organs such as the eyes, kidneys, heart and blood vessels become affected in the long term (American Diabetes Association, 2010).

In this form of diabetes, the rate of destruction of pancreatic β cells varies among individuals, so it can be accelerated in children and slower in adults. Also, in some patients, especially children and adolescents, type 1 diabetes begins with ketoacidosis, while others have hyperglycemia that can take a severe form in the presence of an infection or stress factors. Some adults may have a reduced percentage of functional β cells that prevent the onset of ketoacidosis for several years, but over time they inevitably become insulin dependent and risk ketoacidosis (American Diabetes Association, 2010).

The idiopathic form of type 1 diabetes is a rare one, the origin of which is not yet known and which is less severe than the autoimmune variant. Patients diagnosed with this disease are found in Africa and Asia and have been observed to show variations in insulin deficiency and ketoacidosis (Kharroubi and Darwish, 2015).

Type 2 diabetes is the form of hyperglycemia and chronic hyperinsulinemia. Compared to type 1 diabetes, characterized by insulin deficiency, in this form, in most cases, the level of insulin in the blood is high - either constant or after the intake of glucose-rich foods. This is also known as "insulin resistance" (Westman, 2021).

According to the World Health Organization (WHO), in 2014, 8.5% of adults over the age of 18 were diagnosed with diabetes, and in 2019 this disease was the direct cause of 1.5 million deaths of these, 48% occurred in patients under the age of 70. In the case of well-developed countries, between 2000 and 2010, the rate of premature mortality from diabetes decreased and rose again between 2010 and 2016, while in middle and low-income countries, the rate of diabetes mortality increased in both time intervals. Type 2 diabetes is present in over 95% of patients diagnosed with diabetes (WHO, 2021).

As for determining the causes of type 2 diabetes, several factors are considered. The main risk factor is obesity, given that it is present in most patients diagnosed with this disease. The risk of developing type 2 diabetes is positively correlated with an increase in body mass index (BMI).For example, in Western countries it is estimated that about 50% of patients have BMI above 30. On the other hand, in Asian countries half of diabetic patients are not obese, and cases of undernourished patients have also been reported (Reed et al., 2021).

Other causes of diabetes are thought to be psychological stress and neuropsychiatric conditions. Thus, working under stressful circumstances or depression can be high-risk factors in the development of type 2 diabetes. The chances of disease can also increase with environmental changes. High levels of use of pesticides, drugs, and food additives in recent years can negatively influence the likelihood of diabetes onset. There are also correlations between some pathogens, such as herpes simplex virus type 1 and hepatitis C virus, and type 2 diabetes mellitus. Although the mechanism of this process is not yet fully elucidated, hepatitis C, for example, is associated with insulin resistance in the liver (Reed et al., 2021).

There are numerous symptoms that can indicate type 2 diabetes. They can be so mild that they are unnoticed. Symptoms include polyuria, polydipsia, numbness of the upper and lower limbs, fatigue, non-healing wounds, or recurrent

yeast infections. Dizziness, headaches and gradual increase in body mass may also be present (Dansinger, 2020).

Type 2 diabetes is a complex disease that progresses gradually, involves multiple metabolic abnormalities and affects multiple organs.

The main problems are the lack or reduction of insulin synthesis and its resistance in peripheral tissues (adipose, muscular tissues) and liver. The reason for the decrease in insulin synthesis is the structural and functional decline of pancreatic β cells (Pratley, 2013).

Insulin is a polypeptide hormone with important functions in terms of basically glucose homeostasis in the blood, but also cellular growth and metabolism. The role in regulating the body's energy is achieved by transporting glucose into the cells that are dependent on it – liver, muscle and adipose tissues. Although it has long been assumed that this hormone is synthesized strictly by pancreatic cells, studies have shown that, in low concentrations, it is also identified in some central nervous system neurons. Both biosynthesis and its secretion are controlled by blood glucose levels. For example, a blood glucose of 2-4 mm stimulates the synthesis of insulin, while 5 mm initiates its secretion. Food intake is the factor that triggers glucose metabolism, so there is an increase in the rate of insulin synthesis by pancreatic β cells and, implicitly, a decrease in the synthesis of glucagon, the hyperglycemic hormone produced by pancreatic α cells. Insulin once released from the cells is distributed into the systemic circulation and reaches the liver, the main storage location of glucose in the form of glycogen (Rahman et al., 2021).

There are both exogenous and endogenous factors in terms of influence on the increase in blood sugar. Among external factors, it is known that the main source of glucose is carbohydrates in the diet. They are digested and absorbed in the form of glucose, which the blood carries to the cells to maintain normal blood sugar. Endogenous factors include the main hyperglycemic hormones – glucagon, epinephrine and cortisol. The increase glucose levels are obtained by glycogenolysis and gluconeogenesis. These reactions would be able to generate a sufficient concentration of glucose for body without its dietary intake (Westman, 2021). At the level of all target cells, there is a receptor for insulin. They are made up of 4 subunits – 2 α and 2 β, connected by disulfide bonds. Normally, insulin attaches to the α subunit and activates the tyrosine-kinase enzyme in the β subunit. The second unit is self-phosphorylated, which involves the phosphorylation of 3 tyrosine residues – Tyr-1158, Tyr-1162, Tyr-1163. Most functions in regulating metabolism and decreasing insulin apoptosis are mediated by molecular signaling mechanisms that involve phosphorylation of proteins in insulin receptors (Saini, 2010).

In the case of insulin resistance of cells, there is a decrease in the rate of expression of insulin receptors on their surface and also reduced tyrosine kinase activity. It is assumed that in type 2 diabetes, insulin resistance is explained by post-receptor alterations of a protein, Insulin ligand – IRS-1 (insulin receptor substrates-1), which regulates phosphorylation and desphosphorylation. These alterations involve an imbalance between the phosphorylation of IRS-1 by tyrosine and serine. A low rate of phosphorylation by the amino acid tyrosine leads to decreased translocation of glucose transporter GLUT-4 (glucose transporter-4), a protein identified in the plasma membrane that helps in glucose influx. On the other hand, an increase in serine phosphorylation contributes to the activation of proteins with pro-inflammatory activity and which are involved in the degradation of insulin, which further leads to resistance against it (Blaslov et al., 2018).

As for the treatment of type 2 diabetes, a beneficial first step is given by lifestyle changes. Thus, the decrease in caloric intake and the increase in physical activity are factors with a positive impact on blood glucose control. At the same time, there are limitations in this aspect, as there may be difficulties in weight loss or the disease progressing naturally. In these circumstances, most patients require pharmacotherapy. Metformin is a commonly recommended drug for diabetic people because it has been shown to be effective in reducing blood sugar, even with a low risk of hypoglycemic effect. In the absence of the desired effect, treatment can be optimized by adding antihyperglycemic agents such as sulfonylurea, thiazolidindione, dipeptidyl peptidase-4 inhibitors or receptor agonists for GLP-1 peptide-1 (glucagon-like peptide-4). In the context in which, after taking 3 drugs, the desired values of glycated hemoglobin are not achieved, it is necessary to develop more complex treatment strategies, involving daily doses of insulin (Pratley, 2013).

Neuropsychiatric diseases are highly complex diseases whose biological mechanisms are not yet fully elucidated. Such diseases, commonly encountered, are schizophrenia, bipolar disorder, depression, and generalized anxiety disorder (Bray and O'Donovan, 2018).

Schizophrenia is a chronic neuropsychiatric disease characterized by symptoms such as hallucinations and disorganized behavior, and cognitive function is impaired. Studies show that the basis of the pathophysiology of this disease consists of abnormalities in the transmission of information at the neuronal level. Most theories that illustrate the mechanisms that cause schizophrenia symptoms have as a common denominator the existence of excess or deficiency of neurotransmitters such as dopamine, serotonin and glutamate (Patel et al., 2014).

Bipolar disorder is a severe neuropsychiatric disorder that leads to numerous cases of suicide and can be genetically inherited. Biologically, this disease is described as a disturbance in the balance between dopamine and serotonin in the brain caused by the pathology of some areas of the brain responsible for emotional regulation (Miklowitz and Johnson, 2006).

Depression is an increasingly common global mental illness that affects everyone, regardless of age, gender or race, and leads to major problems with physical health, interpersonal relationships, and cognitive function. From the symptomatology point of view, depression is characterized by sadness, fatigue, lack of interest, appetite, feelings of guilt, low self-esteem and very low concentration (Jesulola et al., 2018). The physiological dysfunction associated with depressed people differs from individual to individual. Thus, some individuals experience a disturbance in the function of the hypothalamic-pituitary-adrenal axis, some have hypercortisol and others hypocortisol. Some studies have demonstrated the presence of systemic inflammation in patients with depression, caused by the fact that changes in cortisol levels disrupt the body's homeostasis, the immune system, and promote the release of cytochines that further favor inflammatory processes (De Menezes et al., 2021).

Anxiety disorders are known as the most common diseases of a neuropsychiatric nature. Despite the fact that they involve less obvious symptoms than those specific to schizophrenia, depression or bipolar disorder, its effects affect the quality of life at least as much (Bystritsky et al., 2013). Anxiety disorders are of various types, among the most common being generalized anxiety disorder, panic disorder, social anxiety disorder, or other disorders involving phobias (National Institute of mental Health, 2022).

Generalized anxiety disorder is a common neuropsychiatric disease that affects approximately 20% of adults annually and is based on states of fear, worry and feeling overwhelmed, constant exhaustion. This condition is most commonly described by perpetual and unfounded concern about any aspect of daily life, for various reasons such as financial status, health or future issues (Munir, 2021).

This disorder can be caused primarily by the daily life stress. Various diseases, such as diabetes, may also be involved, or it can be inherited genetically from first-degree relatives At the same time, factors related to the environment, such as the existence of abuse in childhood and the consumption of substances can contribute to the development of this disorder (Munir, 2021).

As for the neurobiological explanation of anxiety, most symptoms are controlled by the autonomic nervous system, especially the sympathetic one. The underlying mediators are represented by the neurotransmitters norepinephrine,

dopamine and gamma-aminobutyric acid (GABA-gamma-aminobutyric acid). The amygdala also plays an important role in anxiety (Chand and Marwaha, 2022). This is a component of the brain, specifically the limbic system – a complex of structures known for its functions related to regulating emotions, behavior. The amygdala is located in the temporal lobe and consists of 13 nuclei (AbuHasan et al, 2021), grouped into 3 main categories according to position – central, basal and lateral nuclei. The central nuclei are responsible for regulating the fear response, implicitly regulating the release of cortisol through the paraventricular nucleus of the hypothalamus, and the basal and lateral nuclei are involved in the associated learning processes (Ressler, 2010). Studies to better explain anxiety neurobiology have shown that the amygdala has hyperactivity against fear-inducing stimuli, whether or not the subjects are aware of it (Forster et al., 2012).

Methods for treating anxiety disorder include, in principle, psychotherapy, medication administration or a combination of the two (Ströhle et al., 2018).

Cognitive-behavioral therapy is the first recommendation for controlling and treating anxiety and has been shown to have moderate to strong beneficial effects against any category of anxiety disorder (Ströhle et al., 2018). This type of therapy is based on the fact that thoughts, feelings and physical sensations are interconnected, and during meetings the patient discusses with the psychotherapist every disturbing aspect and potential changes that can be made to improve the quality of life (National Health Service, 2022).

In terms of pharmacotherapy, among the most effective anxiolytic drugs are selective serotonin and norepinephrine reuptake inhibitors which are useful in increasing the levels of these hormones in the blood (Ströhle et al., 2018).

The basic mechanism of this inhibitor effect is that neurotransmitters are, by obocele, reabsorbed by neurons after they have transmitted their information. Reuptake inhibitors basically block reabsorption and allow, by default, long-term retention of serotonin and norepinephrine outside nerve cells for the transmission of messages carried (National Health Service, 2022).

Methodological aspects

The useful technology for determining complex microbiomes in the human body has advanced greatly in recent years, which has also led to increased clinicians' interest in them and their importance (Allaband et al., 2019).

Microbiomics is the science that studies all aspects of the microbiome, such as its composition, diversity and functions. This has become a very important interdisciplinary field, which includes several classical fields such as

microbiology, chemistry, ecology, phylogenetics, metaprotheomics and metatranscriptomica (Bokulich et al., 2020).

The first step in analyzing the intestinal microbiome of individuals in a study is to establish a protocol aimed at obtaining samples from which to determine the intestinal microflora. The feces are considered to be the most accessible material, which allows its daily analysis compared to samples obtained by biopsy. However, in fecal matter not all microorganisms are identified in the gut – for example, those that are mucus-bound or those in the small intestine, especially in the ileum. This type of sample is also located far from the gastrointestinal area, meaning that pathologies at this level cannot be investigated. Despite these limitations, feces are accessible samples commonly used in the study of the digestive microbiome. The sample protocol is given by:

-sample collection;

- homogenizing immediate (with the help of a tissue homogenizer);

-cryopreservation in liquid nitrogen/dry ice (Allaband et al., 2019).

There are many molecular biology data that can be obtained by analyzing the intestinal microflora. Traditional methods of analysis focus their attention on the identification of the species and the presence of synthesized toxins in the case of pathogenic species. The new methods of analysis detect and describe all communities of microorganisms present in the samples (Allaband et al., 2019).

In the past, the analysis of the gut microbiome was dependent on the isolation of microganisms and crop achievement, but this method was of little efficiency as the anaerobic bacteria, abundant in the gut, were difficult to grow and this affected the accuracy of the analyzes. Thus, due to the development of new methods of genetic analysis in recent years, currently the most used technique is NGS sequencing (next generation sequencing) which allows the analysis of the microbial composition of a sample with high accuracy without the need for crop production (Tang et al., 2020).

The phylogenetic analysis of the composition of the intestinal microflora can be done through 2 main methods – crop dependent and crop independent.

The method of making crops has allowed over time the identification of the basic characteristics of intetsinal micororganisms: Morphology of colonies, bacterial growth, some biochemical aspects of them and, obviously, their analysis using microscopy. In 1980, many Gram-negative bacteria were identified from feces and later phylogenically classified according to their fermentative capacity. The method based on microbial crop production is the most advantageous in terms

of very low costs, but the major disadvantage is that about 30% of intestinal bacteria cannot be grown outside their natural habitat (Singh, 2021).

In order to counter the disadvantages of traditional analysis protocols – those based on crop production, microbiology specialists have developed methods independent of cultures, metagenomics being the field that develops in this way. They identified more than 80% of microorganisms that could not be determined by crop production. The techniques included in these methods are all based on gene identification for 16S ribosomal RNA – with major function in protein synthesis, a process well preserved in all bacterial cells. The metagenomic analysis protocol basically involves the extraction of nucleic acids from samples and amplification by the PCR-polymerase chain reaction of sequences of 1.500 base pairs, specific to the analyzed species, representing the genes for the 16S ribosomal RNA. After polymerization, DNA fragments are separated by gel electrophoresis (Singh, 2021).

NGS sequencing is similar in principle to the classical one – Sanger sequencing, based on capillary electrophoresis. The genomic cages are fragmented and the bases within each fragment are identified by issuing signals at the time of ligation of the respective fragments of the mold cages. In the case of the Sanger method, separate steps are required for sequencing, separation and detection of the resulting fragments, while NGS sequencing allows millions of reactions to take place in parallel. This indicates a high speed of results and low cost. The process includes 3 main steps:

a .Genomic library preparation – DNA is enzymatically or ultrasonic fragmented, the obtained fragments are ligated by adapters (fragments of double-stranded DNA that allow the attachment of complementary strands during sequencing);

b .PCR amplification – necessary for signals received from the sequencer to be strong enough to be accurately determined.

c.Sequencing itself – there are different methods developed by many companies (pyrosequencing, ion torrent sequencing, ligation sequencing) (ATDBio, 2005).

Once the information is obtained using NGS, it is analyzed using a software that includes a database of the genomes of the microorganisms that are intended to be identified. Based on the matches of the sequences obtained with the known ones, it can be achieved both the identification and quantification of the presence of microorganisms in the analyzed feces (Allaband et al., 2019).

Thus, considering that intestinal microbiota a key factor in regulating the brain-intestinal axis and is also involved in the development and proper functioning of the hypothalamic-pituitary-adrenal axis, numerous studies have turned their attention to the composition of digestive microflora in people diagnosed with anxiety or depression. The results in the literature are showing that the two conditions are associated with the abundance of pro-inflammatory microorganisms and a significant decrease in beneficial species producing short-chain fatty acids (Simpson et al., 2021).

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