Instituto Tecnológico y de Estudios Superiores de Monterrey

Campus Monterrey

School of Engineering and Sciences


# Effect of a high protein diet from a vegetal and animal source on the Microbiota-Gut-Brain axis in a murine model

A thesis presented by

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Submitted to the
School of Engineering and Sciences
in partial fulfillment of the requirements for the degree of

Master of Science

In

Biotechnology

Monterrey Nuevo León, December 18th, 2020

## Dedication

This thesis is the result of two and a half years of constant effort, and fighting against unexpected circumstances. I want to thank you, for your support and encouragement:

- My parents: Emma and Rodolfo, my main drivers and motivators. Your actions and character have always been an example for me. You placed the rocks for the path I wanted to follow through.

- My sister: Miriam, for always reminding me of my strengths, despite how bad I felt on the inside at times. You always manage to put a smile on my face, and instantly make circumstances more bearable. I would not have come so far If it was not for your unconditional support and patience.

- My advisor: Dra. Arlette Santacruz, for guiding my research and offering support during difficult times. I am very thankful for the opportunity of working by your side, you are a very inspiring scientist in this community.

- My friend Laura: For being my lab partner during 2 years, and serving me company and support during despairing times. I think this project is just as successful as our joint effort. Thank you for reminding me of the meaning of hard work, diligence, and communication.

- My friend Karen: We met so much earlier, but during the thesis you caused the most impact. You were definitely a light in dark times. Thank you, because I always felt the confidence to share my thoughts and feelings with you, and you always gave back encouraging words.

- Gut Microbiota Team: Felipe, Blanca, Rebeca and Paulina, for sharing not only our work space, but for sharing dreams, intentions, and passions. You are such inspiring people, I have no doubt each one of you would go far.

"A scientist's work is determined by two things: his interests and those of his time. Everything has led to this."

## Acknowledgements

I would like to give special thanks to CONACyT for the support I received for living while working on my thesis.

To the Tecnológico de Monterrey for the support on tuition. I am very grateful for the opportunity of being accepted as a student in an institution with as much recognition.

To the Centro de Biotecnología FEMSA, for providing me the tools and equipment necessary for the experimental procedures in my thesis, and guiding my work along the way.

To the School of Medicine and Health Sciences, particularly to all the people working at the animal facility where all the behavioral tests were carried out. Thank you for your advice and constant support.

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## Abstract

The gut-brain axis is a complex bidirectional communication system that allows an intimate connection between both organs through different mechanisms. Recent studies regarding the gut microbiota now support its prominent role in modifying behavior and cognition. As consequence, there has been a growing interest in studying various dietary strategies to modulate behavior targeting the gut microbiota. High protein diets, both of plant and animal origin, have become popular as a dietary intervention due to their beneficial effects on metabolism; however, in high amounts, harmful effects on the intestinal microbiota can occur, so this type of diet is still a matter of debate. The present work aims to investigate whether diets high in protein from either plant or animal origin are capable of inducing changes in the gut-brain axis in a murine model. Thirty C57BL/6 mice were assigned to three treatment groups (n = 10 per group) and were fed a normal control diet (NC) or hyper-protein diets of plant (HPV) or animal (HPA) origin for 8 weeks, being monitored by weight, behavior and composition of the microbiota. The behavior analysis was carried out at the end of the dietary intervention using three behavioral tests: elevated plus maze test, 'Y' shaped maze, and forced swimming test. Different levels of activity with statistical significance ($p<0.05$) were present in all tests, except from the 'Y' shaped maze. Following dietary intervention, changes in the concentration of *Lactobacillus*, *Bifidobacterium* ($p<0.05$) and *Enterobacteriaceae* ($p<0.05$) were measured by quantitative PCR. Contrastingly, a significant reduction in the concentration of short chain fatty acids ($p<0.05$) was found on the luminal contents of mice, a measurement done via Gas Chromatography. The results of this research will allow to make more careful recommendations in the undertaking of dietary interventions with a greater contribution of protein in relation to other macronutrients.

**Keywords:** Gut microbiota, Gut-Brain-Axis, High protein diet, Animal behavior

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# Abbreviations

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine |
| BDNF | Brain Derived Neurotrophic Factor |
| CNS | Central Nervous System |
| CFU | Colony Forming Units |
| GABA | Gamma Amino Butyric Acid |
| GBA | Gut Brain Axis |
| GC | Gas Chromatography |
| GI | Gastro Intestinal |
| ECs | Enterochromaffin Cells |
| ENS | Enteric Nervous System |
| EPM | Elevated Plus Maze |
| FST | Forced Swim Test |
| HPA | Hypothalamic-Pituitary-Axis |
| IBS | Irritable Bowel Syndrome |
| qPCR | Quantitative PCR |
| SCFA | Short Chain Fatty Acids |

# Contents

# Chapter 1

## Introduction

The human body is host to 100 trillion microbes, outnumbering its own human cells in a ratio of 1.3:1 (Sender et al., 2016). Distributed across different systems in the body, the microbiota has a profound impact on host's health, and has just recently been receiving more attention from different fields of science, ranging from traditional medicine to more complex pharmacology. The microbiota which resides in the gastrointestinal (GI) tract, recognized as the gut microbiota, is probably one of the most studied, and which more information has been recollected about. At present, most of what is known about the gut microbiota is related to its function and composition. The scientific community has now agreed upon the fact that both diversity and stability are the main characteristics the gut microbiota should have to maintain a wholesome function of the digestive and immune system (Lozupone et al., 2012). However, the exact mechanisms by which it affects human physiology and how particular bacteria helps the host maintain a healthy state, still remains unclear.

Recently, a new paradigm shift in neuroscience has surged which now takes into consideration the influence of gut microbiota in the development of certain neurological conditions (Cryan et al, 2020). The microbiota-gut-brain axis, a complex bidirectional system that allows the bacteria in the gut, to communicate with the brain (Romijn et al, 2008), has emerged as a promising therapeutic target to treat certain psychiatric conditions. The connection between both organs is supported by specific mechanisms that involve the immune, nervous and endocrine system, as well as the synthesis of certain metabolites mediated by bacteria (Carabotti, 2015). Nonetheless, a challenge prevails to uncover the role of microbiota in coordinating these systems.

Moreover, a growing interest has been posed on the investigation of novel strategies to alter gut microbiota composition with the intention to modulate behavior

and cognition. Diet has the potential to modify the concentration of bacteria in the gut as well as products from its metabolism (Ojeda et al, 2016). A high-protein diet may prove capable of modifying colonic microbiota; however, its effect on the gut-brain-axis has not been explored yet. Furthermore, with the surge of plant based diets, the source of protein has also been given attention (Tomova et al, 2019).

Therefore, the main aim of this research is to test whether a high protein diet, depending on its source, vegetal or animal, could alter the concentration of specific bacteria in the gut, and its potential in modulating brain function (Tan & O'Toole, 2015). Therefore, the methodology in this project was carefully designed to measure the changes in concentration from the most common bacterial populations present in the gut from mice fed a high-protein diet. Behavioral tests were also used to assess changes in behavior and cognition.

Up to this day, psychiatric disorders are treated by pharmacological intervention, using drugs which could affect the diversity of the gut microbiota, and hence, alter its symbiotic relationship with the host. A dietary intervention, which supports the growth of probiotic bacteria could function as an adjunctive therapy to modulate behavior and cognition (Rea et al, 2020). This project could contribute to elucidate how changes in the ratio of intake of macronutrients could affect the microbiota-gut-brain axis.

# Chapter 2

# Literature Review

## 2.1 The Gut Microbiota

The Gastrointestinal tract (GI) is composed of a large number of microorganisms which have adapted to this environment, and together they are referred as gut microbiota (Zhan et al, 2018). It has been estimated that together these microbes can account for up to a trillion cells in the digestive tract (Guinane & Cotter, 2013), and therefore are considered and integral part of the body. Composition of gut microbiota is diverse, approximately 1,000 distinct bacterial species have been identified (Hooper & Macpherson, 2010), compromising variable effects for the host. Furthermore, the gut microbiota has major role in regulating multiple functions beneficial for the GI tract such as improved digestion (Tremaroli & Bäckhed, 2012), absorption of nutrients (Krajmalnik et al, 2012) and metabolism (Guinane & Cotter, 2013), and altogether it is considered essential for GI tract homeostasis (Sommer et al, 2013). In the process of digestion, bacteria is capable of fermenting carbohydrates which the body cannot digest; it also produces vitamins essential for the host, such as vitamin B and K synthesized by *E.coli*, and prevents infection by regulating the production of mucus and other molecules in the gut, aiding in the development of the immune system.

Different extrinsic factors have been recognized to alter the density of microbial communities in the GI tract, such as sleep, diet, exercise, and antibiotic use (Lobionda et al., 2019). Among these factors, diet has been shown to be one of the main factors altering not only microbiota composition, but its metabolism as well, regulating the production of substances like short chain fatty acids, neurotransmitters, and some toxic metabolites which could activate the immune system (Rowland et al, 2018). The use of supplements like probiotics has also been shown to influence microbial communities, and holds potential for the management of some behavioral disorders (Butel, 2014).

### 2.1.1 Probiotics

So far, one of the most common experimental strategies to modulate the microbiota-gut-brain axis has been the direct administration of probiotics, commonly defined as microorganisms capable of exerting a health benefit for the host. A correlation has been found between the ingestion of *Lactobacillus* species with a significant reduction of stress, anxiety- and depression- related behavior, by regulating the expression of certain GABA receptors (Bravo et al, 2011). Similar effects have been observed in Bifidobacterium species; a study conducted by Bercik et al, demonstrated that daily ingestion *B. longum* was able to provide an anxiolytic effect, as well as the normalization of the brain derived neurotrophic factor (BDNF) in mice with chronic colitis (Bercik et al, 2011). On the other hand, increased anxiety-like behaviors were observed to be induced by infection with *Campylobacter jejuni*; a phenomena that appeared to be mediated by the activation of specific brain regions that process sensory information coming from the intestines (Gaykema et al, 2004).

### 2.1.2 Short Chain Fatty Acids

Non-digestible carbohydrates are metabolized by gut bacteria, which ends up producing short-chain fatty acids (SCFAs), small molecules that serve as energy for cells in the colon. (MacFarlane, 2003) The main SCFAs, recognized by the benefits they hold over the gastrointestinal tract, are acetate, butyrate, and propionate. An adequate consumption of fiber has been shown to increase the concentration of these molecules, and to improve digestive function (Gill et al., 2018). Recently, it was demonstrated that they are capable of reaching the circulatory system, which increases the likelihood that the microbiota could signal the brain by means of this route (Haghikia et al., 2015). Moreover, the enteric nervous system (ENS) which uniformly covers the gut walls to promote motility, could be easily reached out by these type of molecules.

## 2.2 High Protein Diet

Diet is a major driver of microbiome diversity and one of the principal agents to produce quick shifts in gut microbiota. Regarding nutrition in humans, the nutrient requirements specified by the World's Health Organization include the macronutrients; protein, carbohydrates, lipids, and micronutrients; vitamins and minerals, as well as trace elements (WHO, 2018). The amount in which specific macronutrients are consumed in correspondence with the dietary habits of an individual can determine the impact on the gut microbiota (Scott et al, 2013). However, the recommended percentages in which these macronutrients should be consumed are also highly dependent on the subject's sex, age, weight, and activity levels.

Recently, a huge controversy regarding optimal protein intake (Cuenca-Sánchez et al, 2015) and its source has emerged. Evidence suggests that high protein-based meals may be beneficial to improve satiety levels in comparison with those carbohydrate-based (Hill & Blundell, 1986), and therefore, facilitate weight management. Nevertheless, other studies link a high consumption of this macronutrient with certain metabolic disturbances, particularly in calcium metabolism (Reddy et al, 2002) and may also induce important alterations in renal function (Friedman, 2004). What is more, microbes in the gut could be affected not only by the amount in which a specific macronutrient is consumed, but also from the origin of such. In the case of protein, there is an ongoing debate about whether protein coming from a vegetable or animal source is healthier for human consumption.

Protein coming from a vegetable source such as peas or soybeans, has been shown to modulate gut microbiota (Tomova et al, 2019), increasing the concentration of probiotic bacteria, such as *Lactobacilli* and *Bifidobacterium*. However, not as much research has been conducted on the impact of protein coming from other vegetable sources, like black beans and maize, which are consumed in large quantities Latin

American countries like Mexico (Monge et al, 2019). On the other hand, a high intake of animal protein often gets a bad reputation due to its association with an increased risk of cardiovascular disease and cancer (McAfee et al, 2010). Animal products generally contain higher levels of cholesterol which increases the likelihood of developing chronic illnesses. By the same token, the presence of certain carcinogens in processed meat (Zelver et al, 2018), or those generated during cooking methods (Sinha et al, 2005), constitute the reason why medical practitioners commonly advise against its consumption. However, some nutrients present in meat like iron and vitamin B-12 (McAfee et al, 2010), support important physiological functions and hence it is important to maintain adequate levels of such.

### 2.3 Influence of Gut Microbiota on Brain Axis Function

The interaction between the gut and the brain, was only until recently restricted to the scenario necessary for the transmission of hunger and satiety signals. Nonetheless, the gut also holds responsible for the transmission of other signals that regulate several other behaviors aside from those related to food intake, for instance, social behavior and the response to stress stimuli (Figure 1). New evidence supports a strong link between the communities residing in the gut and the function and development on the brain (Heijtz et al, 2011; Crumeyrolle et al, 2014). A study conducted by Dinan & Cryan, demonstrated that an altered response to stress was prevalent in animals raised in germ-free environment; what is more, they also found that these response was further normalized by the introduction of specific bacterial species (Dinan & Cryan, 2012). Similar studies have been conducted that draw homologous conclusions regarding anxiety-like (Bercik et al, 2011) and depressive behavior, for example, by regulating the biosynthesis of serotonin (Yano et al, 2015).

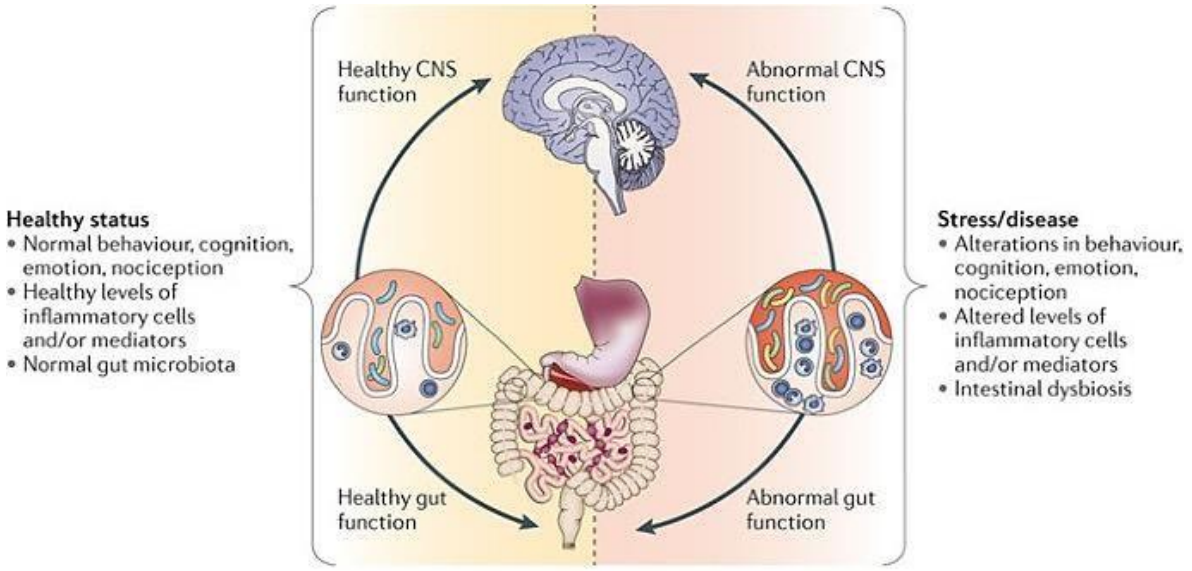


**Figure 1.** The impact of gut microbiota on the regulation of the CNS function. Differences in healthy status and stressed/disease conditions*. Taken from Cryan and Dinan, 2012.*

The biological interactions by which the gut is able to exert an effect on brain function, and modulate behavior under the influence of certain stimuli, compromises a communication system of high complexity, now recognized as the Gut-Brain-Axis (GBA) by the scientific community (Romijn et al, 2008). The gut microbiota has been identified as a major contributing factor to the regulation of mood, as well as the expression of emotion, and has been demonstrated to affect cognitive processes such as memory and learning. For instance, disturbances in gut microbial profile has shown significant associations with the development of neurological disorders such as autism, and condititions like Parkinson, and Alzheimer's Disease (de Theije et al, 2014; Mulak et al, 2015; Friedland, 2015). Conversely, preexisting conditions of psychological stress have been shown to contribute to inflammation, causing an imbalance in the microbial populations of the gut, also known as dysbiosis (Qin et al, 2014). Therefore, these interactions support the establishment of the gut-brain- axis as a system by which the brain is able to regulate intestinal function (Petra, 2015), and the microbial communities can modulate cognitive function and behavior

by stimulating the CNS (Collins & Bercik, 2009). Figure 2 shows a schematic representation of the Gut-Brain-Axis.

### 2.4 Signaling mechanisms implicated in the microbiota-gut-brain-axis

The mechanisms that underlie and support the interplay between the microbiota and the brain involve the action of specific mediators acting at three different levels: the immune, nervous and endocrine systems (Carabotti, 2015). As regards of the neural pathway, the vagus nerve has been acknowledged to be an important mediator for the signaling of the ingested nutrients to the brain (Tomokazu et al., 2009). Furthermore, neurotransmitters synthesized by the gut microbes have been shown to stimulate the axonal projections of the vagus nerve in the gut. Concerning the endocrine system, a recent model to describe Irritable Bowel Syndrome (IBS) has suggested an important role of stress hormones in the regulation of gut function. The authors proposed that psychological stress can induce the activation of the Hypothalamic-Pituitary-Axis (HPA) axis, and in turn, the secretion of cortisol, which increases the permeability in the gut favoring the entrance of molecules that increase the production of pro-inflammatory cytokines, and as a consequence, it impairs the metabolism of tryptophan needed for the synthesis of serotonin (Dinan & Cryan, 2017).

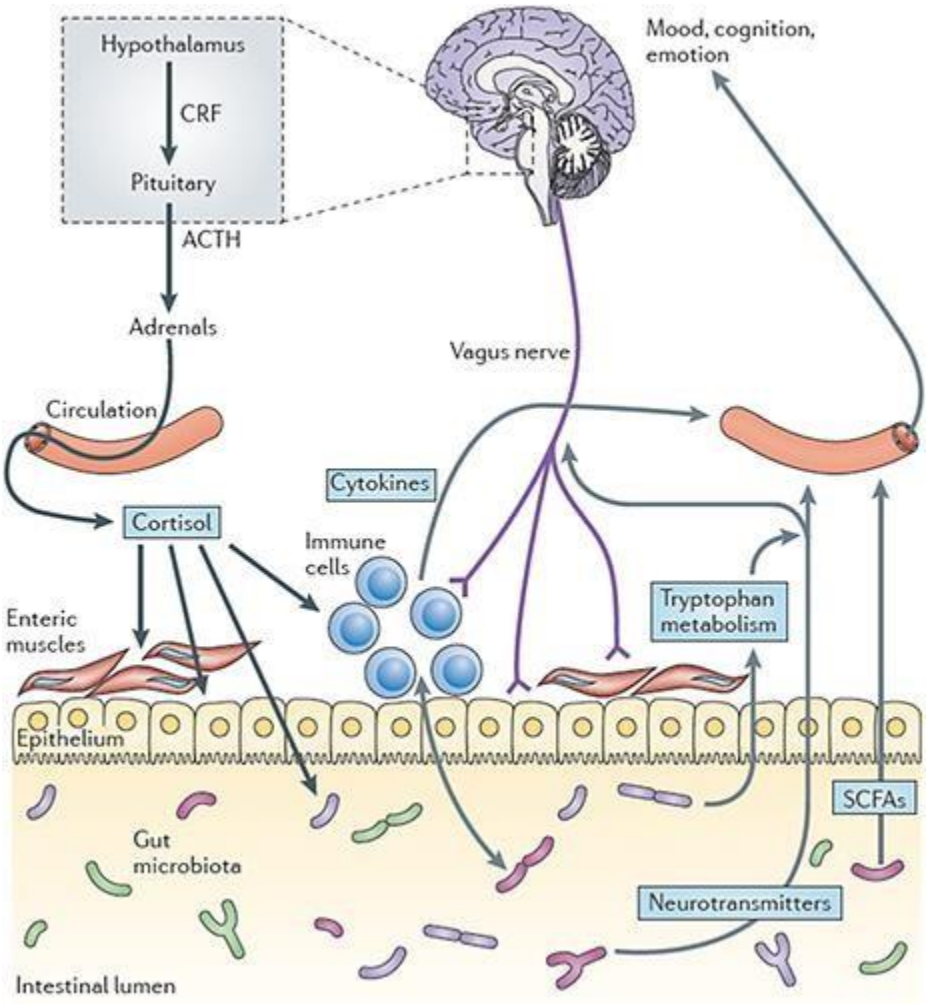


**Figure 2.** Pathways involved in bidirectional communication between the gut microbiota and the brain. *Taken from: Cryan and Dinan, 2012.*

Gut microbiota can also regulate the metabolism of tryptophan, and intervene in the signaling of serotonin or 5-hydroxytryptamine (5-HT), a monoamine neurotransmitter which modulates mood and cognition in the brain. It has been estimated that 90% of the body's serotonin is produced in the gut (Gershon & Tack, 2007). Bacteria in the gut also holds the capacity for the synthesis of others neurotransmitters such as: dopamine, which plays an important role in motivation and learning; noradrenaline, which mediates the physiological response to stress; GABA, which reduces neural activity and aids in the diminishing of anxiety, and acetylcholine, which acts on the nervous system to regulate motor activity.

Bacteria is a major driver for the synthesis of 5-HT mainly by stimulating the activity of the host colonic enterochromaffin cells (ECs) (Reigstad et al, 2015). In this way, gut microbiota regulates peripheral concentrations of 5-HT in the colon and serum (Yano et al., 2015). The ability to produce GABA, as well as the expression of *gad* genes, has been identified in a wide range of strains from *lactobacilli* and *bifidobacteria.* Nonetheless, *bifidobacteria* has been highlighted as the major producer of GABA from its precursor monosodium glutamate (Yunes et al., 2016).

In addition to the biosynthesis of neurotransmitters, the gut microbiota can communicate with the brain via the immune system (Pagliari et al, 2018). A mucous layer covers the gut epithelium, which provides a physical barrier and acts as a first line defense against pathogens. Nonetheless, in cases when the mucous layer proves to be insufficient to prevent microbial infection, the gut microbiota is also capable of inducing an immune response (D'Amelio & Sassy, 2018), promoting the synthesis and release of pro- and anti- inflammatory cytokines, which can communicate to the brain travelling through the circulatory system (Fung et al, 2017).

### 2.5 Behavioral tests

In neuroscience, a strategy that has been used successfully to study human behavior is through the adaptation of behavioral tests on animal models. Mice exhibit approximately 90% gene homology with humans, and the protein-coding regions of the mouse and human genomes are on average to 85% identical (Monaco et al., 2015), supporting their high validity to study the molecular and behavioral changes involved in neurological disorders. Behavioral testing in mice, depending on the set up, can be used to evaluate the changes in the levels of expression of stress, anxiety and depression.

### 2.5.1 Y-Maze Test

The Y-maze test is used to assess short term memory in mice (Kraeuter et al, 2019). Spontaneous alternation, a measure of spatial working memory, can be assessed by allowing mice to explore all three arms of the maze and is driven by an innate curiosity of rodents to explore previously unvisited areas. A mouse with intact working memory, and hence intact prefrontal cortical functions, will remember the arms previously visited and show a tendency to enter a less recently visited arm (Prieur et al, 2019).

### 2.5.2 Elevated Plus Maze (EPM) Test

The elevated plus maze test is one of the most widely used tests for measuring anxiety-like behavior (Komada et al, 2008). The test is based on the natural aversion of mice for open and elevated areas, as well as on their natural spontaneous exploratory behavior in novel environments. In this test, mice are given access to all of the arms and are allowed to move freely between them. The number of entries into the open arms and the time spent in the open arms are used as indices of open space-induced anxiety in mice (Faria et al, 2006).

### 2.5.3 Forced-Swim Test (FST)

The FST is based on the assumption that when placing an animal in a container filled with water (Can et al, 2012), it will first make efforts to escape but eventually will exhibit immobility that may be considered to reflect a measure of behavioral despair. Depression is often viewed as a lack of ability to handle with stress (Cryan et al, 2005).

# Chapter 3

## Hypothesis, General and Specific Objectives

### 3.1 Hypothesis

A high protein diet, depending on its source, could alter the density and concentration of certain microbial species in the gut and may also exert an effect on animal behavior.

### 3.2 General Objective

Evaluate the changes in the concentration of most representative probiotics groups in the gut microbiota as a result of the consumption of an animal (beef meat) and vegetal (*Phaseolus vulgaris L. and* Zein) high protein diet source, and study its effect on the different mechanisms of signaling between the gut and the brain, alongside the potential shifts in animal behavior.

### 3.3 Specific Objectives

I. Develop an animal model to verify the effect of a diet high in protein from either an animal (beef) or vegetable (beans and corn) source on the gut microbiota and behavior.
II. Assessment of anxiety and stress responses through behavioral tests post dietary intervention.
III. Quantify the alterations in the main gut microbiota populations (*Lactobacillus, Bifidobacterium,* and *Enterobacteriaceae*) by quantitative PCR, and changes in the concentration of short chain fatty acids produced from high protein diets.

# Chapter 4

## Materials and Methods

### 4.1 Protocol design for ethical animal use

In Mexico, the Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) is the regulatory agency which specifies the guidelines for the use of laboratory animals. It is required that the animals must be covered by an animal use protocol (AUP) approved by the Institutional Animal Care and Use Committee (IACUC) prior to acquisition and/or use of animals and throughout the entire period of animal use or maintenance. The Internal Committee that directs the animal house facility, at the School of Medicine and Health Sciences at the Tec de Monterrey Institute, required the researchers to design a protocol to justify the use of the animal model and to define the exact proceedings to be followed throughout the entire duration of the experimental phase. The procedures were verified to comply with all the guidelines expressed in the NOM-062-ZOO-1999 for the ethical animal use, particularly regarding the methods of euthanasia, and the folio number of 2019-013 was assigned to the protocol.

### 4.2 Protein Extraction

Black beans (*Phaseolus vulgaris*) donated from Centro de Biotecnología Monterrey, Nuevo León, were soaked (1:4 w/v) for 24 hours at 4°C to soften the hull and facilitate further removal. Later, the beans were dried in a convection oven set at a temperature of 60°C for 18 hrs. The flour was attained using a coffee grinder and was subsequently stored at -80°C until further use. The method of alkaline extraction and acid precipitation was used to obtain the vegetable protein extract. A batch of 200 grams of bean flour were mixed with 2 L of water (pH was adjusted to 9.0). The mix was maintained in constant agitation at a temperature of 52 °C for 1 h. The mixture was then centrifuged at 9000 rpm for 15 min at 21 °C (Thermo Scientific Sorvall Lynx 6000 Centrifuge). The precipitate was discarded, and supernatant was

preserved. The pH was adjusted to 4.5 with HCl 15% and centrifugation was repeated under the same conditions. This time, the precipitate with the protein extract was preserved and stored at –80 °C. The bean protein concentrate was later obtained by means of lyophilization.

**Figure 3.** Alkaline extraction of vegetable protein from bean flour and final protein isolate from black bean following the lyophilization process.

Beef meat was used as the source of protein in HPD from animal origin. Fresh *Pulpa Negra* was purchased from a local market in Monterrey, Nuevo León. Meat was cut into small cubes with a diameter of 0.5 mm and later was introduced to a convection oven set at a temperature of 50°C for 14 hours. Subsequently intramuscular fat was removed soaking the sample with hexane (1:10) at 20 °C for 6 hours and then placed in an extraction hood to remove the residual hexane. Dried meat extract was stored at -80°C until use. Detailed aminoacid composition from the vegetable and animal protein extract can be found on Table 1. The table also shows in highlight those aminoacids that act ad biosynthetic precursors of important neurotransmitters; such as glutamic acid for GABA, and tryptophan, tyrosine and phenylalanine for serotonin and dopamine.

**Table 1.** Aminoacid Profile from black bean, corn and red meat protein isolates.

| Aminoacid | Bean and Corn (9:1) Protein Isolate | Red Meat Protein Isolate |
|---|---|---|
| Taurine § | 0.03 | 0.07 |
| Hydroxyproline | 0.05 | 0.25 |
| Aspartic Acid | 9.68 | 7.04 |
| Threonine | 3.40 | 3.38 |
| Serine | 4.53 | 2.49 |
| Glutamic Acid | 13.78 | 11.47 |
| Proline | 4.05 | 2.91 |
| Lanthionine § | 0.21 | 0.28 |
| Glycine | 3.25 | 3.34 |
| Alanine | 4.10 | 4.44 |
| Cysteine | 0.62 | 0.82 |
| Valine | 5.18 | 4.05 |
| Methionine | 1.19 | 2.00 |
| Isoleucine | 4.62 | 3.97 |
| Leucine | 8.80 | 6.34 |
| Tyrosine | 3.59 | 3.44 |
| Phenylalanine | 5.64 | 3.24 |
| Hydroxylysine | 0.00 | 0.08 |
| Ornithine § | 0.04 | 0.08 |
| Lysine | 5.92 | 6.82 |
| Histidine | 2.57 | 3.01 |
| Arginine | 4.73 | 4.87 |
| Tryptophan | 1.06 | 1.09 |
| **Total** | 87.01 | 75.48 |

***§:*** *Non-proteionogenic aminoacid*

## 4.3 Diets

High protein diets, were prepared at the Centro de Biotecnología FEMSA using previously defined ingredients which have been reported to meet the caloric and nutritional requirements of mice from the C57BL/6 strain established by the American Institute of Nutrition (AIN) for laboratory rodents (Table 2.). Micronutrient requirements were fulfilled using both a mineral and a vitamin mix from MP Biomedicals ™. The PicoLab® Rodent Diet 20 was used for the control diet group (see Table 3 for macronutrient composition), and was supplied by the animal facility. High protein diets were designed with a higher ratio of protein in relation to carbohydrates and fats in comparison to the control group by 10%.

**Table 2.** Ingredients used to prepare animal feed pellets for the High Animal and Vegetal Protein treatments.

| Ingredient | High Animal Protein (HAP) (g/kg diet) | High Vegetal Protein (HVP) (g/kg diet) |
|---|---|---|
| Potato Starch | 265.6 | 265.6 |
| Beef Protein Isolate | 385 | - |
| Black Bean and Corn Protein Isolate (9:1) ratio | - | 385 |
| Maltodextrin | 155 | 155 |
| Sucrose | 55 | 55 |
| Soybean oil | 40 | 40 |
| Microcrystalline cellulose | 50 | 50 |
| Mineral mix (AIN-93G-MX) | 35 | 35 |
| Vitamin mix(AIN-93-VX) | 10 | 10 |
| L-Cysteine | 1.8 | 1.8 |
| Choline bitartrate (41.1% choline) | 2.5 | 2.5 |
| *tert*-Butilhidroquinone (TBHQ) *mg* | 8 | 8 |

The protein source in experimental diets was supplied from either a vegetable or animal protein extract. Table 3. shows the percentage composition of the three main macronutrients contained in the diet of the different treatments.

**Table 3.** Macronutrient composition of control diet and high protein diet treatments.

| Macronutrient | Control Diet (CD) PicoLab® Rodent Diet 20 (%) | High Animal Protein (HAP) (%) | High Vegetal Protein (HVP) (%) |
|---|---|---|---|
| Carbohydrate | 70 | 60 | 60 |
| Protein | 20 | 30 | 30 |
| Fat | 10 | 10 | 10 |
| Total | 100 | 100 | 100 |

The process of 'pelleting' was carried out manually at room temperature (20°C) to reduce the chemical and physical alterations that occur at higher values of temperature, such as the Maillard reaction and the degradation of essential vitamins for mouse development (Abdollahi et al., 2013). The ingredients specified in Table 1 were mixed with a small amount of water and cold pellets (1 cm in diameter and 2.5 cm longitude) were formed manually. Then, the pellets were dried at a low temperature in an oven (at a temperature of 60 ° C) for 1h. Later, they were stored at 4°C until administration to the animals. The safety is very important for a healthy diet, with the intention of determining the bacterial count present on manufactured diets, in addition to being a measure to assess the effectiveness of the selected decontamination method, the plate count method was employed with two different types of culture media: (1) Nutrient agar (Difco™, Becton Dickinson, Mexico) to evaluate the growth of a wide range of mesofilic aerobic total microorganisms and (2) Potato Dextrose Agar (Difco™, Becton Dickinson, Mexico) to discard any possible contamination from yeast and/or fungi.

**Figure 4.** Mouse feed pellets from animal source following drying in the oven.

### 4.4 Murine model and High protein diet

Thirty animal of 17-week-old male mice of the C57BL/ 6 strain were used for the investigational aims, and subjected to a dietary intervention with a total duration of 60 days (8 weeks). The age of the animal model was determined in a representative stage of the human equivalent corresponding to an adult (The Harrison Laboratory, 2019), in which the function of the diet is maintenance, thus the risk of interrupting the normal growth stage is decreased. Upon arrival at the animal house, the mice went through a one-week quarantine period so that they could get used to the cages, and to assess their initial health status prior to the experimental stage. After quarantine, mice were divided into three groups ensuring uniformity in their phenotype in each one of them (mice were kept in individual boxes with a density of 3-4 animals with the aim of reducing death due to aggressive and/or territorial behaviors). The number of animals was determined based on the survival percentage reported by the American College of Laboratory Animal Medicine, which estimates 50% survival for laboratory mice as part of the normal senescence process (Fox et al., 2007).

Mice were assigned to one of three groups with 10 animals each (n = 10); each group was fed a specific diet; first group was fed a control diet (NC) based on the nutritional requirements established by the American Institute of Nutrition (AIN) for laboratory rodents, and which are specified in the purified diet AIN-93 Maintenance (AIN-93M ); the second group was fed a high protein diet of animal origin (HAP) obtained from protein isolate of beef, and the third group with a hyper protein diet of vegetable origin (HVP) obtained from protein isolate of beans and corn ( ratio 9:1). Diets high in protein were reformulated with the same ingredients specified in the AIN-93M diet, altering their proportion, and raising the final protein percentage from 20% to 30%.

A percentage of protein (between 30-40%) has been reported in previous studies as sufficient to produce changes in the intestinal microbiota, without actually

causing other damage to mouse health (Walther et al., 2011; Blair et al. ., 2015). An intake equal to or greater than 50% can generate the accumulation of toxic metabolites and induce changes in the colon mucosa that are not the object of study of the project (Cui, 2018). Mice were provided with water and food ad libitum and monitored to detect common signs of health such as, normal levels of activity (by observation), coordination, appetite (by measuring deviation from the mean in food intake), softness of their hair (by observation and manipulation) (Burkholer et al., 2012). Mice were weighed every 7 days at the same time (11:30 AM) in order to keep an accurate record of the changes in their weight as a result of the administration of the different diets.

### 4.5 Behavioral Tests

Behavioral tests were used to measure changes in behavior resulting from the dietary intervention. Three different tests were carried out in order to obtain information on behaviors associated with stress, anxiety and depression: 1) Elevated plus maze test, 2) 'Y' maze test, and 3) Forced swim test. Although rodents are more active at night, all tests were performed in the light phase of the light-dark cycle, starting at 9:30 AM and ending at 12:00 PM, which was more convenient regarding the schedule of the vivarium. Previous studies have carried out similar tests in this phase without their results being affected (Li et al., 2009). The elevated plus maze test (EPM) is the most used to evaluate the characteristic behavior of anxiety in rodents, and assesses the level of exploration of the animal in aversive environments. The tests were carried out separately on different days, alternating the groups between tests.va

#### 4.5.1 'Y' Maze Test.

For the Y-maze test, a platform with three closed arms with the shape of a ‘Y’ was used (Figure 5). Previous to starting the test, the camera was placed in the appropriate position to record the activity and movement from the mice across the

three arms. Timer was set to 5 min. At the beginning of the test, each mouse was placed individually on the center of the Y-shape platform, and allowed to move freely through the maze for 5 minutes (Kraeuter et al, 2019). The number of entries and alternations between the arms was recorded. An alternation is considered only when the animal moves across and makes an entry to the three arms of the maze, without repeating a previously visited arm (Prieur et al, 2019).

**Figure 5.** Mice being positioned on the center of Y-Maze prior the recording of test.

### 4.5.2 Elevated Plus Maze (EPM) Test.

During the test, an elevated platform, with two open arms and two closed arms, forming a 'plus' was used to assess anxiety in mice (Figure 6). First, the mouse was placed in the center of such platform, and a camera was set to record the number of entries to the open and closed arms, as well as the distance travelled and time spent in both arms (Komada et al, 2008). Commonly, an entry is considered and registered when all four legs of the animal are placed on each arm. Conclusions would be drawn based on the level of exploration of each arm (num. of entries, time and distance) (Faria et al, 2006). The total duration of the test was set to 5 min.

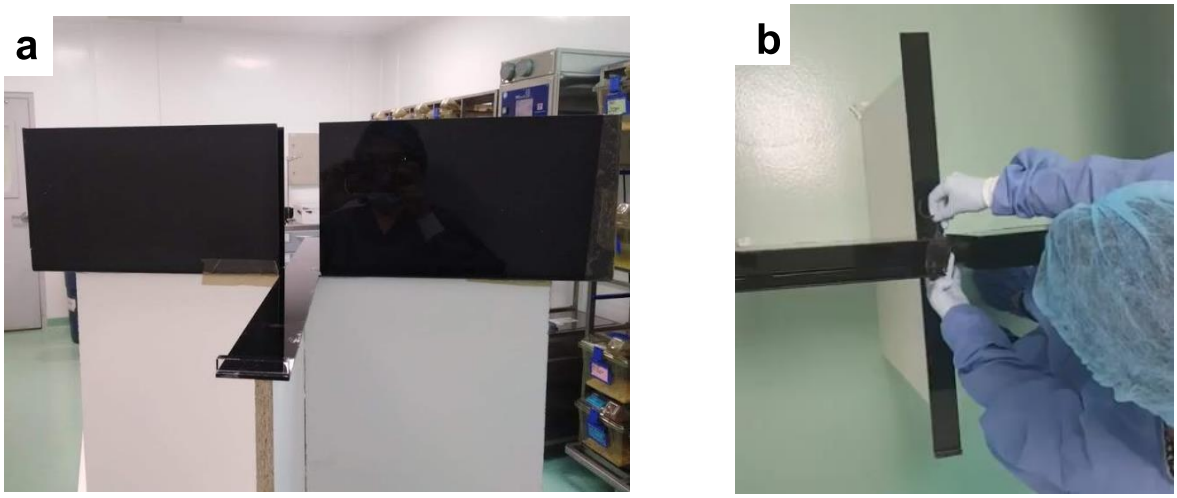


**Figure 6.** The Elevated Plus Maze test. Mice commonly feel secure on the closed arms and feel aversion towards the open arms. a) Side view of the platform, b) Mice being positioned on the center of the Elevated Plus Maze test platform.

### 4.5.3. Forced swim test.

The forced swim required a period of acclimatization to the water; in which the mouse was trained daily for 3 days in a row in a container with a low volume of water to prevent swimming (Figure 7). Acclimatization was carried out to reduce the stress caused by the novelty of the stimulus when carrying out the ultimate test. During the formal test, a camera was placed in the correct position to monitor the mobility of the mouse throughout the container. A transparent plastic cage, was filled with water at a temperature of 32°C to reach a level of 15 cm (Can et al, 2012). Carefully, the mouse was held gently by the tail and transferred to the swimming container. Subsequently, a previously set timer at 6 minutes (360 s) is started. Recording in the camera was stop by the end of the timer. The animal was removed from the water, and dried gently with a towel before transferring it to its original cage. Three types of activity were observed and recorded on camera for further analysis. The struggling time, which occurs typically during the first minutes of the test, where the mouse is captured being exceptionally dynamic in its attempt to escape from the cylinder. The mobility time, where the mouse moves freely in the container, but its activity no

longer is hallmarked by the rapid movements observed in the beginning. And finally, the immobility time were hardly any movement is recorded on the animal and the limbs are used only to stay afloat (Cryan et al, 2005).

**Figure 7.** A lateral side view of the container used for the forced swim test showing mice being tested.

### 4.6 Tissue samples collection and Quantitative PCR analysis

At the end of the behavioral tests, the mouse was euthanized using the anesthetic agent Sevoflurane, with the objective of obtaining the following tissues and organs: visceral fat, brain, caecum and blood samples. Blood samples were used to measure glucose levels. Total bacterial Genomic DNA in cecum samples was extracted using the DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany). Manufacturer's instructions were followed carefully to ensure a successful isolation of the DNA from the previously prepared samples of the caecum of mice. Then, isolated genomic DNA was quantified using the Nanodrop® at 230 nm (ND-1000, Software 3.7, Thermo Scientific$^{TM}$). Subsequently, dilutions were prepared to reach a concentration of 5 ng/ µl in 50 µl of final volume (Jian et al., 2020).

The standard curves were prepared beforehand with known concentrations from *Lactobacillus casei* (ATCC® 393), *Bifidobacterium* (ATCC® 55818), and Escherichia coli (CECT® 45). For the quantitative PCR analysis, a Rotor Gene RG-3000 from Corbett Research and SYBR green ® (Qiagen, Hilden, Germany), were

used. The total volume of DNA sample used for PCR reaction was 3 µL (5 ng/ µl). Table 4 shows the reactants and corresponding volumes used for each PCR reaction. With the aim of characterizing the gut microbiota, the primers sequences described in Table 5 were selected for the identification of specific genera of bacteria with relevance for the project regarding its probiotic effect (Cano et al., 2012).

**Table 4.** PCR mix used for each PCR Reaction

| Reactant | Volume (µL) |
|---|---|
| $H_2O$ | 7 |
| Forward primer | 1.25 |
| Reverse primer | 1.25 |
| DNA sample | 3 |
| SYBR Green | 12.5 |
| **Total Reaction Volume** | 25 |

**Table 5.** Primer sequences and PCR conditions for each bacterial group

| Bacterial group | Primers sequences | Anneling temperature (ºC) | Fragment (bp) |
|---|---|---|---|
| Total Bacteria (From *E. coli*) | Fw 5'-TGGCTCAGGACGAACGCTGGCGGC-3'<br>Rv 5'-CCTACTGCTGCCTCCCGTAGGAGT-3' | 61 | 339/ 539 |
| *Lactobacillus* | Fw 5'-GGAAACAGRTGCTAATACCG-3'<br>Rv 5'-CACCGCTACACATGGAG-3' | 61 | 340 |
| *Bifidobacteria* | Fw 5'-CTCCTGGAAACGGGTGG-3'<br>Rv 5'-CACCGCTACACATGGAG-3' | 55 | 549/ 563 |
| *Enterobacterium* (From *E. coli*) | Fw 5'-CATTGACGTTACCCGCAGAAGAAGC-3'<br>Rv 5'-CTCTACGAGACTCAAGCTTGC-3' | 62 | 340 |

## 4.7 Analysis of SCFAs by GC-FID

The colons of mice were collected after euthanasia and immediately frozen at -80°C and stored until further analysis. After thawing, the luminal contents were

gently removed from the proximal part and an aliquot of 100 mg was collected and transferred to an Eppendorf tube containing 0.25 mL of sulfuric acid 50% w/v. Sample is well homogenized and subsequently shaken for 3 min by vortex. Extraction was performed by adding 1mL of ethyl ether and centrifuged for 1.5 min at 8000 x g. The organic phase was transferred into a 2 mL vial. The extraction was repeated three times, and at the end 1.5 mL of collected organic phase was used for the GC analysis. The analysis was achieved employing a gas chromatograph Agilent Technologies 6850 (Santa Clara, CA) equipped with a split injector and a FID (Figure 8) Separation of SCFAs was performed using a capillary column (HP-FFAP column 25 m, 0.320 mm ID, 0.25 µm) purchased from Agilent Technologies. The injection was performed using split mode (5:1) and a volume of injection of 2 µL. The oven temperature was initially set at 120 °C, programmed at a rate of 15 °C / min up to 240 ° C. Pressure was set at 6.1 psi and flow rate at 1mL/min. The flow rates of carrier gases were established for hydrogen (40 mL/min), air (400 mL/min), and helium (15 mL/min). The FDI temperature was 300 ° C and the run time per sample was 15 minutes. The identity of SCFAs in mouse samples was assessed by comparison of their retention times with those of the corresponding standards obtained from the Volatile Free Acid Mix Analytical standard purchased from Sigma Aldrich.

**Figure 8.** Gas Chromatograph used in SCFA analysis (Agilent Technologies 6850).

# Chapter 5.

# Results and Discussion

## 5.1 Protocol design for ethical animal use and protein extraction

The protocol designed, which justifies the use of an animal model for behavioral research was accepted by the Ethical Committee from the School of Medicine of Tec de Monterrey on July 2019. The protocol received the folio number '2019-013' and it includes a detailed description of the experimental procedures in compliance with the guidelines from the NOM-062-ZOO-1999.

The methodology for the extraction and characterization of protein from an animal (red meat – Pulpa negra) and a vegetable source (Bean/Corn) was developed and carried out with satisfactory results in regards of protein quality and stability. Protein recovery from the alkaline extraction and acid precipitation of flour obtained from black beans had a yield of 15%. As for red meat, a 20% yield was obtained from the drying method in the oven with convective air.

## 5.2 Murine model and high protein diet

Substantial efforts were directed towards the minimization of the environmental factors which could play a role in the physiologic alterations within mice (Figure 9). Considering the profound effect that microenvironment has on both the health and welfare of the animals as well as on the validity and reproducibility of scientific data, mice were monitored for changes in weight and appetite following the dietary intervention; however, no significant changes in weight were found during the experimental period.

**Figure 9.** Caged mouse were closely monitored to assure a safe environment, as well as to verify individual signals of health in each specimen.

Diets for laboratory animals are available in different types of physical forms, with pelleted diets being the most common. While this diet is typically manufactured by injecting steam into the mixture of ground ingredients; the protocol adopted in this research was based on a variation of this same process known as ‘cold pelleting’, in aims of reducing the loss of important nutrients and prevent changes in protein stability. At the end of the process, high protein diets were allowed to dry until completely firm. Prior to storage, mouse feed pellets were subjected to short periods of irradiation to decontaminate diets, and prevent the growth of microorganisms, which could infect mice and compromise its health. To evaluate the safety of diets, the microbial analysis was carried out by duplicate, the microorganisms only grew on positive controls. Since mouse feed pellets were produced in batches, the analysis was repeated for each one, with no deviation in the results. Therefore, this results indicated that the pellets were safe for mice consumption and did not contain microorganisms.

### 5.3 Changes in Glucose and Fat Metabolism

It is commonly acknowledged that microbial metabolites can influence a wide variety of biological processes in the host, mediating cholesterol transport and hence, impacting lipid metabolism (Ghazalpour et al., 2016). However, this effect is most commonly observed in dietary interventions such as the adoption of a western type of diet, high in fats and simple sugars. In such type of diets, significant increases

in obesogenic microbial communities are notorious (Cao et al., 2019). Consequently, an impairment in processes which are commonly regulated by healthy bacteria, such as the production of secondary bile acids is disrupted which could lead to an increase in the normal levels of molecules like cholesterol. Since the fat and carbohydrate content in the experimental diets were maintained at fairly low level, with the intention of allowing for a higher concentration of protein, no significant changes in visceral fat were observed in mice (Figure 10a).

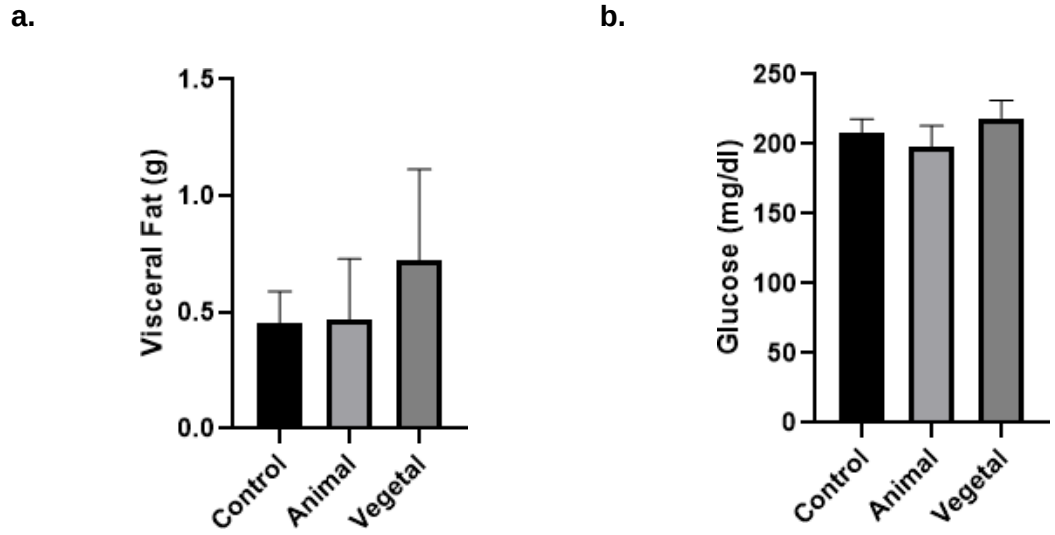


**Figure 10.** Graph bars showing the differences in measurement of (a) visceral fat in grams and (b) glucose levels (mg/dL) in mice after being fed with a control diet or a high protein diet from an animal or vegetable source.

A strong correlation has been established between excessive levels of visceral fat and conditions such as hypertriglyceridemia, low concentrations of high density lipoprotein (HDL) cholesterol, and insulin resistance (Bergman et al, 2006). However, as can be observed from Figure 10a, the groups that consumed a higher percentage of protein in their diets did not show a significant increase in visceral fat compared with the control, which shows healthy levels of adipose tissue (Cano et al., 2012). Similarly, no changes in glucose metabolism linked to insulin resistance or diabetes are presumed from the dietary intervention since blood samples from different groups show no significant differences (Figure 10b). A standard glucose tolerance test is frequently used to diagnose impairments in glucose metabolism; in

mice, a glucose level below 140 mg/dl should be reached within 2 hours of oral administration of glucose. A level between 140 and 200 mg/dl might be an indicator of an “impaired glucose tolerance”, and any level above 200 mg/dl is sufficient to diagnose diabetes (Zhang, 2011).

### 5.4 Behavioral Tests

Following treatment with high protein diets from either animal or vegetable source for 60 days (8 weeks), behavioral tests were used to measure changes in the expression of anxiety and depressive behavior in response to different stimuli. Three different tests were carried out for the analysis: 1) 'Y' maze test, 2) Forced swim test and 3) Elevated plus maze test.

#### 5.4.1 Y-Maze Test

The Y-maze test is used to assess short term memory in mice (Kraeuter et al, 2019). If working memory remains unaffected, the mouse would remember the arms previously visited and prefer to explore a less recently visited arm, therefore increasing the number of alternations in the maze. As can be noted from Figure. 11, there are not significant differences were observed on the activity among groups during the Y-Maze Test. A plausible explanation from this phenomena, could be that the spatial memory depends on the hippocampus, which is particularly vulnerable to aging, and since the age of mice was that corresponding a young adult, no change on the activity on this area of the brain could be detected during the experimental phase. However, is noteworthy that dietary protein restriction has been demonstrated to extend lifespan in a range of organisms, including rodents, and it has been found that cycles of dietary protein restriction hold the potential to ameliorate working memory deficits. (Parrella et al., 2013). Conversely, a study with rats concluded that a high-protein diet could worsen memory acquisition using a different test for working memory (Mendez et al., 2015).

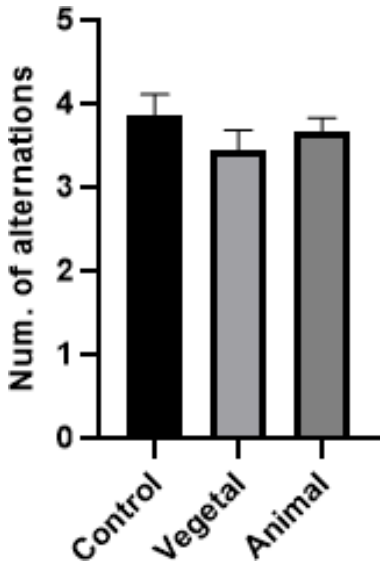


**Figure 11.** Number of alternations in the Y-Maze test for the different groups.

### 5.4.2 Forced Swim Test

In neuroscience, depression is often viewed as a lack of ability to handle changes in the environment, causing stress and hopelessness (Yang et al, 2015). The FST is based on the notion that when placing an animal in a container filled with water, it will first make efforts to escape but eventually will exhibit immobility, an indicator of behavioral despair (Yankelevitch, 2015). Significant differences were found in the levels of activity observed in this test. As observed in Figure. 12a, significant alterations from the behavior depicted by the control group in the immobile phase can be noted for both experimental groups in which a high protein diet was administered from either an animal or vegetable source. A significant reduction in the time spent in the immobile phase is clearly observed, an event that within the context of this test, is associated with a lower expression of depressive-like symptoms (Cryan et al, 2005).

It has been long recognized that carbohydrate fermentation tends to increase overall microbial fermentation and SCFA production, which could exert variable effects for the host (Harris et al, 2020). Alternatively, fermentation of protein can occur when large quantities of protein reach the gut, and bacteria is capable of

producing SCFAs, branched-chain FAs, sulfides, and phenolic and indolic compounds (Marco, 2020). Some of these metabolites are associated with gut diseases, as in the case of sulfides. Depending on the ratio of carbohydrate to protein fermentation by the gut microbiota, could alter the concentration of aforementioned metabolites. In pigs fed a 4 week western-type diet, added soluble fiber (wheat arabinoxylan) increased carbohydrate fermentation and reduced protein fermentation, therefore concluding that fiber may counteract the accumulation of harmful secondary metabolites from protein digestion (Gunness et al., 2016).

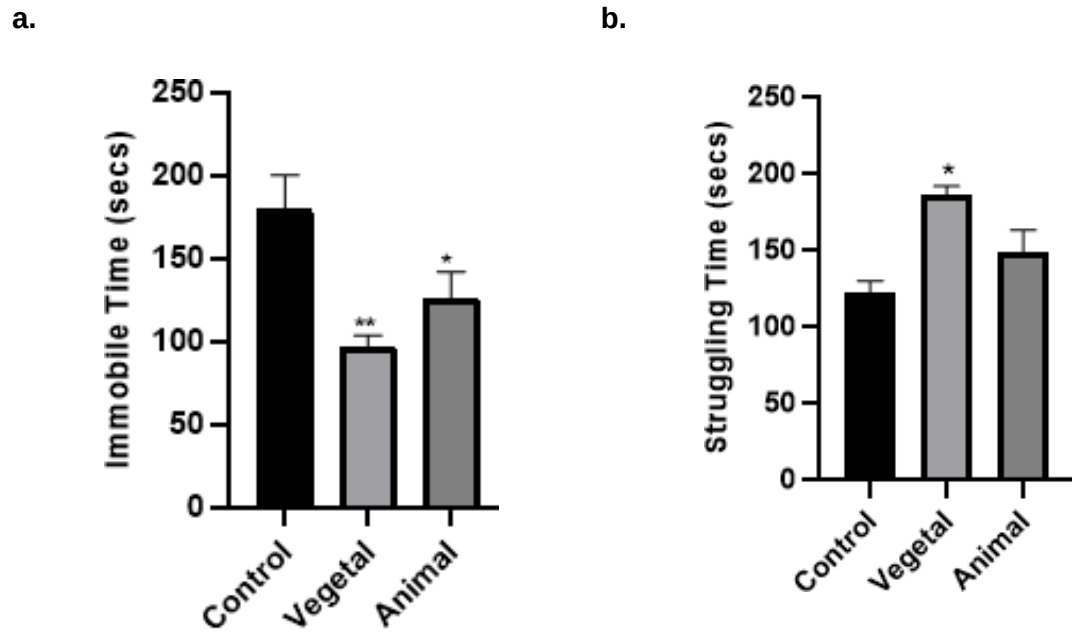


**Figure 12.** Graph bars showing the differences in time (secs) mice spent (a) immobile and (b) struggling during the Forced Swim Test for the different groups.

*Differences of statistical significance ANOVA ( p-Value <0.05) **( p-Value <0.001)

In Figure 12b, a visual difference can be observed in the struggling time among groups compared to the control. Time spent struggling is commonly associated with a higher drive for survival, which could also be an indicator of a lower prevalence of depressive moods. Long-term dietary patterns, such as increased consumption of protein or decreased consumption of fiber, can shift the composition of the microbiota, altering the fermentation pathways most frequently used by bacteria. A comparison between a diet rich in red meat or whole grains during a 10

week period showed that increased red meat consumption increased the genera *Clostridium* spp., which drives the synthesis of important neurotransmitters like GABA, which modulates the stress response (Foerster et al., 2014). Similarly, there is evidence that an increase in tryptophan availability in high protein diets, could be used as a precursor for certain bacterial metabolites that in turn improve gut barrier function and attenuate severity in certain neurological disorders such as multiple sclerosis (Diether & Willing, 2019; Gaetani et al., 2020).

### 5.4.3 Elevated Plus Maze Test

The elevated plus maze considers the natural aversion of mice for open and elevated areas (Komada et al, 2008). In this test, mice are allowed to access all of the arms and to move freely between them. The number of entries into and time spent in both arms is used to assess the anxiety response in mice. Significant differences were found for the number of entries to the open arms of the elevated plus maze apparatus for the vegetable group in comparison with the control (Figure 13). This observable fact suggests that mice fed a diet high in protein coming from a vegetable source could show less aversion to open spaces. This phenomena is commonly correlated with a lower expression of anxiety-like behaviors (Rosa et al, 2000).

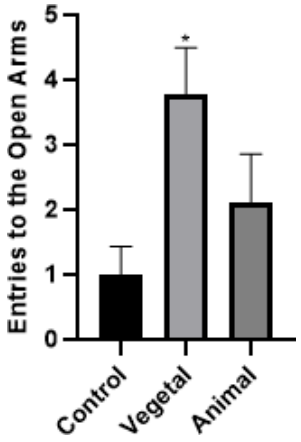


**Figure 13.** Number of entries to the open arms of the EPM test apparatus.
*Differences of statistical significance ANOVA ( *p*-Value <0.05)

The potential adverse effects which derive from a high protein diet are mainly associated with modifications in amino acid metabolism and alterations in the acid–

base balance caused by increased dietary acid load. In rats, a high protein diet rich in branched-chain amino acids (BCAAs) in combination with a high fat diet was able to induce insulin resistance causing oxidative stress (Newgard, 2012). However, it is likely that the combinatory effect of protein with a high fat diet might be held responsible for the oxidative stress, a phenomena that could impair downstream metabolic functions.

On the other hand, a high protein diet has been associated with an increase of particular molecules recognized as dietary polyamines, which are produced via a diverse set of pathways from decarboxylation of amino acids (Moinard et al, 2005). These polyamines play important roles in small intestine mucosal cell physiology and immune system development. Bacteria utilize polyamines in RNA synthesis, as structural components, and to protect against damage from reactive oxygen species or acidic environments (Shaw et al., 2012). Therefore, the production of these type of metabolites in high protein diets could offset the oxidative damage produced due to the increased acid load.

Regarding the role of neurochemicals, the neurotransmitters produced in the gut play a major role in the modulation of brain function. Gamma-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter of the vertebrate central nervous system (Obata et al 2013), mainly synthesized from glutamic acid which occurs naturally in high protein foods. As mentioned earlier, GABA is known for its analgesic effects and anti-anxiety activity (Liu et al, 2015). In a recent study the administration of vitamin B6 (180 mg/kg, i.p.) was capable of exerting an 'anxiolytic-like' effect in mice in the EPM apparatus. The authors also note that there was a significant increase in the levels of GABA whereas; the levels of glutamate and nitrite were decreased as compared to the control group (Walia et al., 2018).

Despite Figure 14 and 15 do not demonstrate differences of statistical significance for the travelled distance and time spent on the open arms, respectively, visually it can be detected that both the vegetable and animal group showed less

aversion towards the open arms in comparison to the control group, which is an indicator of lower levels of anxiety.

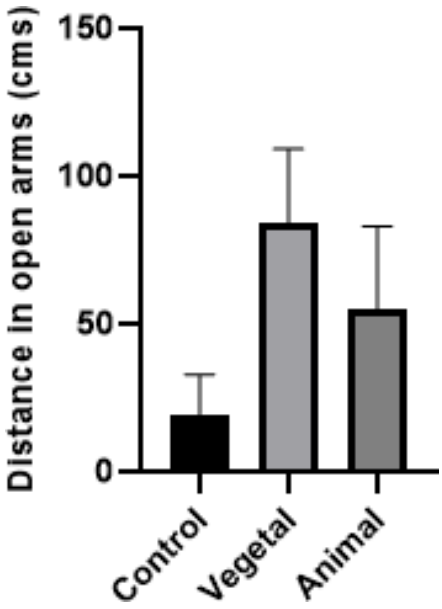


**Figure 14.** Travelled distance (cms) in the open arms of the EPM test apparatus for the different groups.

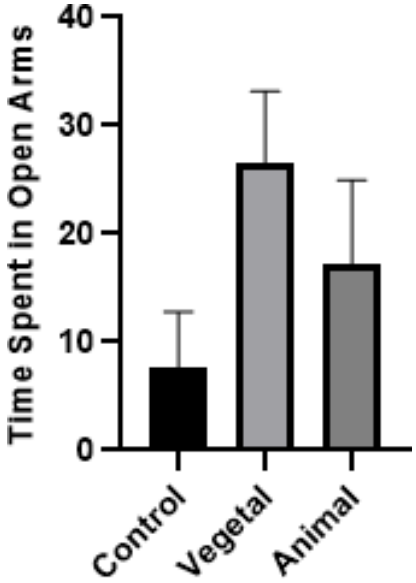


**Figure 15.** Time spent in open arms (secs) in the open arms of the EPM test apparatus for the different groups.

The levels of activity in this test could also be explained from the perspective of the endocrine system. As of late, paradoxical effects of glucocorticoids modulating the human physiological response to stress have been described. Traditionally, excessive levels of glucocorticoids have been implicated in the etiology of affective disorders in humans, and in a range of behavioral deficits in animals. However, new evidence suggests that that chronic, high levels of corticosterone are unlikely to cause anhedonia in rodents. (Barr et al., 2002). Corticosterone acting on the ventral hippocampal network has been observed to exert anxiolytic-like effects following fear exposure, highlighting its potential therapeutic value for anxiety disorders. (Albrecht et al, 2013).

### 5.5 Quantitative Polymerase Chain Reaction Analysis

The variation produced in the concentration of the genera of bacteria most abundant in the gut was evaluated by quantitative PCR analysis. A summary of the median concentrations found on samples from different groups can be found in Table 6. Bacterial species from the genera of *Lactobacilli* and *Bifidobacterium*, are also widely recognized for their probiotic effect.

**Table 6.** Mean concentration of the distinct bacterial groups found in caecum samples from mice post-dietary intervention.

| **Bacterial Group** | **Control Diet [log(UFC/g)]** | **High Animal Protein (HAP) [log(UFC/g)]** | **High Vegetal Protein (HVP) [log(UFC/g)]** | ***p*- Value** |
|---|---|---|---|---|
| Total Bacteria | 5.5 | 5.7 | 5.6 | 0.508 |
| *Lactobacillus* | 3.7 | 4.4 ***** | 3.3 | 0.005 |
| *Bifidobacteria* | 3.1 | 5.4 ***** | 4.1 | <0.005 |
| *Enterobacterium* | 1.9 | 3.6 ***** | 2.0 | <0.005 |

* Differences of statistical significance ANOVA ( *p*-Value <0.005)

Changes in the concentration of *Total Bacteria* from the different diet treatments did not show significant differences (Figure 16a). However, changes in the microbial community belonging to *Lactobacilli*, one of the most common studied bacteria and used as probiotic, actually did show differences of statistical significance across groups (Table 6). However, when means from the high protein diets are compared only against the mean of the control group, no statistical significance is observed (Figure 16b).

Nonetheless, despite the *p* value observed is not lower than alpha, visually from the graph in Figure 16b, a clear difference can be observed in the median concentration of *Lactobacilli* from the animal group to the rest, which is noticeable higher in comparison to the vegetable group. An augmentation in the concentration of *Lactobacilli* has been reported in other studies after increasing protein intake (Zhu et al., 2016). However, a recent publication demonstrated that such augmentation occurs only if and adequate amount of fiber is also present in the diet (Zhang et al., 2020), which could in turn explain why there is a slightly increase but not significant enough against the control. Nevertheless, relevant statistical changes in the concentration of *Bifidobacterium* were found for both animal and vegetable groups (Figure 16c). *Bifidobacterium* is one of the best characterized bacteria across the gut. Administration of this strain has long been advised to individuals looking forward to improve their mood and in the prevention of depression (Pinto et al., 2017). This changes in concentration could in turn, explain the behavior observed previously in both the Forced Swim Test, and the Elevated Plus Maze test.

A significant difference in the concentration of *Enterobacteriaceae* is also depicted in the graph from Figure 16d. Similar to previous scenario, the mice fed a high protein diet from an animal source, reported an evident increase in such microbial population compared to the control group. Despite the fact that bacteria from this strain often get a bad reputation for altering the healthy status of its host, certain microbial metabolites produced from Enterobacteriaceae actually aid in the maintenance of gastrointestinal homeostasis, as in the production of vitamins B and K.

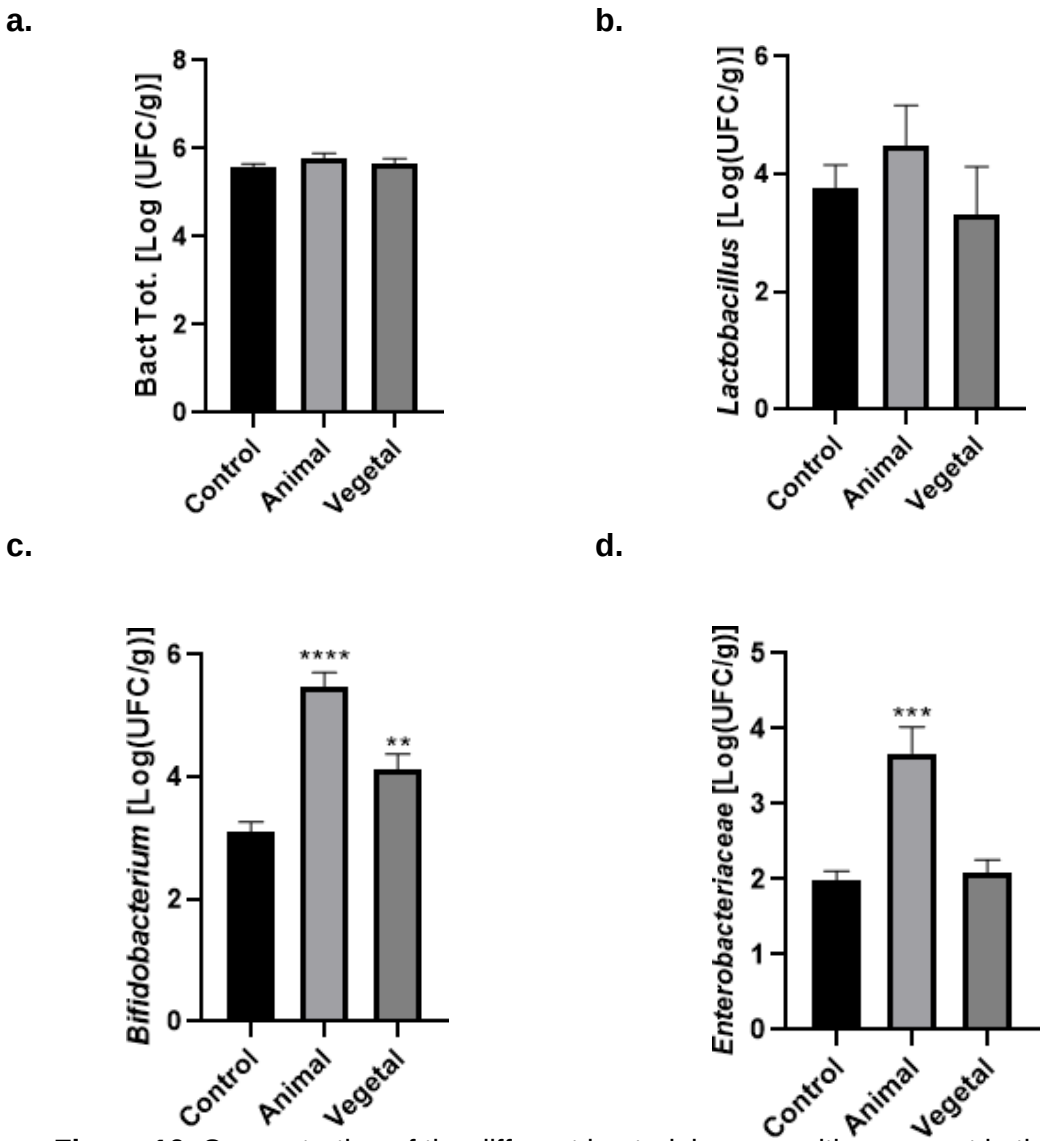


**Figure 16.** Concentration of the different bacterial communities present in the gut corresponding to: a. Total Bacteria, b. *Lactobacilli*, c. *Bifidobacterium*, and d. *Enterobacteriaceae* found in caecum samples from experimental groups.
** Differences of statistical significance ANOVA ( *p*-Value <0.01)
***( *p*-Value <0.001) ****( *p*-Value <0.0001)

## 5.6 Short Chain Fatty Acids Analysis

Short-chain fatty acids (SCFAs) are neuroactive bacterial metabolites of dietary fibers that also hold the potential to regulate brain function and behavior. Significant differences were found for the concentration of the main SCFAs: acetic,

propionic and butyric acid (Table 7). Acetate and propionate are mainly produced by gram-negative bacteria of the phylum of *Bacteroidetes*, meanwhile butyrate is synthesized mostly by members of gram-positive bacteria, from the phylum of *Firmicutes*.

**Table 7.** Dunnett´s multiple comparisons test of concentration of acetic, propionic and butyric acid.

| | Control vs Animal | | | Control vs Vegetal | | | |
|---|---|---|---|---|---|---|---|
| **SCFA** | Mean Control | Mean Animal | Mean Diff | Mean Control | Mean Vegetal | Mean Diff | Adjusted p-Value |
| Acetic Acid | 51.20 | 23.49 | 27.72 * | 51.20 | 17.60 | 33.60 * | <0.0001 |
| Propionic Acid | 7.42 | 2.15 | 5.27 * | 7.42 | 2.23 | 5.19 * | <0.0001 |
| Butiric Acid | 4.56 | 1.26 | 3.30 * | 4.56 | 0.81 | 3.75 * | <0.0001 |

Experimental groups are compared to the control with statistical significance.

*Differences of statistical significance (*p*-Value <0.0001)

The reduction in the concentration of SCFA's that prevails in both high protein diets, could be explained by a reduction in the availability of undigested carbohydrates like resistant starch (RS), which acts as the main substrate for its synthesis. Manually manufactured feed pellets show a reduction of 10% in its carbohydrate content, which could generate shifts in the most common metabolic pathways from gut bacteria. Aside from that, the source of fiber used in the experimental diets was supplied by microcrystalline cellulose that is more easily digested and metabolized by colon cells than other types of fibers, living little room for the production of SCFAs.

# Chapter 6.

# Conclusions

Following the period of dietary administration, different behavioral tests were employed effectively for the measurement of symptoms of depression, and for the assessment of stress and anxiety levels in mice, showing significant differences among groups.

Together with the behavioral tests, it was possible to identify a possible correlation between an increase in the concentration of *Bifidobacterium*, and a significant reduction in the depressive behavior in mice. There is also a potential effect of *Bifidobacterium* ameliorating the expression of anxiety and therefore, reducing the levels of stress of the animal in places where it naturally feels unsafe.

The quantification of short chain fatty acids from the luminal contents by Gas Chromatography, proved to an effective method to obtain data with little variability. Despite the results show an obvious reduction in the concentration of SCFAs in experimental treatments.

The behavioral tests conducted in mice showed important differences in the reduction of indicators of depression and anxiety, therefore providing support for an alternative mechanism of signaling between the gut and the brain, such as the production of other microbial metabolites, such as hormones, neurotransmitters and/or immune molecules.

# Chapter 7.

## Future Perspectives

The revealing of all the potential mechanisms underlying the microbiota-gut-brain axis still remains a challenge. Other analytical methods could be used to evaluate the presence of particular microbial metabolites; for instance, the use of liquid chromatography could be used as an efficient tool to measure biogenic amines produced in the gut.

Likewise, with the surge of more efficient and precise molecular techniques, such as next generation sequencing (NGS), it would be possible to attain a clearer picture of the actors involved in the signaling between the gut microbiota and the brain, which could have been used for example for a more precise description of the microbial profiles present in the mice following dietary intervention.

More and more, traditional fields of medicine like pharmacology, are recognizing the role of gut microbiota on the development of certain psychiatric disorders. The signaling in the microbiota-gut-brain axis is more than evident, and the targeting of gut bacteria holds great promise as an adjunctive therapy to traditional methods for the treatment of brain disorders.

# Chapter 8

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## Supplementary Material

Appendix 1. ANOVA Summary of the Visceral Fat Measurement among groups.

| | |
|---|---|
| **F** | 2.436 |
| **P value** | 0.1098 |
| **P value summary** | ns |
| **Significant diff. among means (P < 0.05)?** | No |
| **R square** | 0.1748 |

Appendix 2. ANOVA Summary of the Glucose Concentration among groups.

| | |
|---|---|
| **F** | 0.6430 |
| **P value** | 0.5349 |
| **P value summary** | ns |
| **Significant diff. among means (P < 0.05)?** | No |
| **R square** | 0.05295 |

Appendix 3. ANOVA Summary for the Y-Maze Test.

| | |
|---|---|
| **F** | 0.8246 |
| **P value** | 0.4515 |
| **Significant diff. among means (P < 0.05)?** | No |
| **R squared** | 0.06974 |

Appendix 4. Dunnetts's multiple comparisons test to assess the difference between treatments and control for the Y-Maze Test.

| **Dunnett's multiple comparisons test** | **Mean Diff.** | **95.00% CI of diff.** | **Significant?** | **Summary** | **Adjusted P Value** | **A-?** | |
|---|---|---|---|---|---|---|---|
| Control vs. Vegetal | 0.4127 | -0.3491 to 1.174 | No | ns | 0.3467 | B | Vegetal |
| Control vs. Animal | 0.1905 | -0.5713 to 0.9523 | No | ns | 0.7753 | C | Animal |

Appendix 5. ANOVA Summary for the Forced Swim Test in the immobile phase.

| | |
|---|---|
| **F** | 7.996 |
| **P value** | 0.0025 |
| **Significant diff. among means (P < 0.05)?** | Yes |
| **R squared** | 0.4209 |

Appendix 6. Dunnetts's multiple comparisons test to assess the difference between treatments and control for the Forced Swim Test in the immobile phase.

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Vegetal | 82.86 | 33.83 to 131.9 | Yes | ** | 0.0012 | B | Vegetal |
| Control vs. Animal | 53.86 | 4.833 to 102.9 | Yes | * | 0.0306 | C | Animal |

Appendix 7. ANOVA Summary for the Forced Swim Test in the struggling phase

| | |
|---|---|
| **F** | 8.516 |
| **P value** | 0.0018 |
| **Significant diff. among means (P < 0.05)?** | Yes |
| **R squared** | 0.4364 |

Appendix 8. Dunnetts's multiple comparisons test to assess the difference between treatments and control for the Forced Swim Test in the struggling phase

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Vegetal | -63.86 | -100.9 to -26.79 | Yes | ** | 0.0010 | B | Vegetal |
| Control vs. Animal | -25.86 | -62.92 to 11.21 | No | ns | 0.1934 | C | Animal |

Appendix 9. ANOVA Summary for the number of entries Elevated Plus Maze Test

| | |
|---|---|
| F | 4.015 |
| P value | 0.0326 |
| Significant diff. among means (P < 0.05)? | Yes |
| R squared | 0.2674 |

Appendix 10. Dunnetts's multiple comparisons test on the number of entries on the Elevated Plus Maze Test.

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Vegetal | -2.778 | -5.128 to -0.4272 | Yes | * | 0.0200 | B | Vegetal |
| Control vs. Animal | -1.111 | -3.462 to 1.239 | No | ns | 0.4356 | C | Animal |

Appendix 11. ANOVA Summary for the distance travelled on the open arm from the Elevated Plus Maze Test

| | |
|---|---|
| **F** | 1.616 |
| **P value** | 0.2214 |
| **Significant diff. among means (P < 0.05)?** | No |
| **R squared** | 0.1281 |

Appendix 12. Dunnetts's multiple comparisons test on the distance travelled on the Elevated Plus Maze Test.

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Vegetal | -64.43 | -148.8 to 19.91 | No | ns | 0.1470 | B | Vegetal |
| Control vs. Animal | -35.32 | -119.7 to 49.02 | No | ns | 0.5129 | C | Animal |

Appendix 13. ANOVA Summary for the time spent on the open arm from the Elevated Plus Maze Test

| | |
|---|---|
| **F** | 1.803 |
| **P value** | 0.1883 |
| **Significant diff. among means (P < 0.05)?** | No |
| **R squared** | 0.1408 |

Appendix 14. Dunnetts's multiple comparisons test on the time spent on the Elevated Plus Maze Test.

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Vegetal | -18.84 | -42.24 to 4.562 | No | ns | 0.1232 | B | Vegetal |
| Control vs. Animal | -9.508 | -32.91 to 13.90 | No | ns | 0.5316 | C | Animal |

Appendix 15. Dunnetts's multiple comparisons test on median concentration of Total Bacteria among groups.

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Animal | -0.1974 | -0.5818 to 0.1871 | No | ns | 0.3859 | B | Animal |
| Control vs. Vegetal | -0.1085 | -0.4929 to 0.2760 | No | ns | 0.7308 | C | Vegetal |

Appendix 16. Dunnetts's multiple comparisons test on median concentration of *Lactobacilli* among groups.

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant ? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Animal | -0.7175 | -1.494 to 0.05919 | No | ns | 0.0721 | B | Animal |
| Control vs. Vegetal | 0.4431 | -0.3117 to 1.198 | No | ns | 0.2984 | C | Vegetal |

Appendix 17. Dunnetts's multiple comparisons test on median concentration of *Bifidobacterium* among groups.

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant ? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Animal | -2.358 | -3.103 to -1.614 | Yes | **** | <0.0001 | B | Animal |
| Control vs. Vegetal | -1.028 | -1.773 to -0.2836 | Yes | ** | 0.0067 | C | Vegetal |

Appendix 18. Dunnetts's multiple comparisons test on median concentration of *Enterobacteriaceae* among groups.

| Dunnett's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant ? | Summary | Adjusted P Value | A-? | |
|---|---|---|---|---|---|---|---|
| Control vs. Animal | -1.692 | -2.516 to -0.8681 | Yes | *** | 0.0001 | B | Animal |
| Control vs. Vegetal | -0.1019 | -0.9258 to 0.7219 | No | ns | 0.9391 | C | Vegetal |