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Targeting Hypothalamus: Diet impact, bacterial composition and memory impair

Universidade do Algarve – Faculdade de Ciências e Tecnologia

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Targeting Hypothalamus: Diet impact, bacterial composition and memory impair

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

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Abstract

Cholesterol metabolism is critical for neuronal functions such as, myelin formation, neurotransmitters release, membrane repair and also synaptogenesis, in the Central Nervous System (CNS), and its homeostasis is mainly maintained by CYPX. This enzyme catalyses the conversion of cholesterol into X-Hydroxycholesterol (X-HC) and may be a useful marker of neurodegenerative diseases. The X-HC is able to cross the blood-brain-barrier (BBB) into the circulation, having possible roles in the overall bile acid synthesis in the liver, therefore having an indirect impact on intestinal microbiota within human Gastrointestinal tract (GI). In the other hand, a diet mainly rich in fat, carbohydrates and protein, a typical Western diet, have been highly associated with changes in the GI environment, promoting alterations in intestinal microbiota composition, termed as dysbiosis, being associated with early metabolic physiopathological changes, such as increased intestinal barrier permeability and a state of low-grade intestinal inflammation. The main objective of this study was to determine the effects of either *Cyp* overexpression and silencing treatment and diet on intestinal bacterial groups, cognitive abilities and also on colon health status. C57BL/J6 mice were fed with either chow diet or an High Fat Diet (HFD), for 12 weeks. To determine the effects caused by both *Cyp* overexpression and silencing and also by the HFD and Control diet on the intestinal bacteriome, the quantification of several intestinal bacterial groups was performed using qPCR. The targeted bacterial groups included *Bacteroidetes*, *Firmicutes*, Sulphate-Reducing Bacteria (SRB), *Betaproteobacteria*, *Delta-and Gammaproteobacteria*, *Actinobacteria* and *Tenericutes*. The exposure of mice to an HFD resulted in variations in the loads of the intestinal bacteria. Interestingly, the *Cyp* silencing lead to an approximation of intestinal bacterial loads between mice exposed to the different diets. The *Cyp* silencing resulted in a decreased performance in Y-maze spontaneous alternation test, within subjects with a chow diet. In addition, both *Cyp* overexpression and silencing in mice exposed to an HFD showed increased signs of intestinal inflammation. Taken together, this study showed that intestinal microbiota is mainly influenced by dietary factors.

Key words: Hypothalamus; Cholesterol; Intestinal microbiota; HFD; Intestinal inflammation.

Resumo

Numa zona central do cérebro humano encontra-se o hipotálamo, sendo este constituído por quatros divisões principais, a área pré-ótica, hipotálamo anterior, hipotálamo tuberal e hipotálamo posterior. Sendo cada uma destas divisões constituída por diversos núcleos, desempenhando funções fundamentais para a manutenção da homeostasia do nosso organismo, tais como, termorregulação, controlo sobre o metabolismo energético, controlo sobre os estados de sono, respostas ao stress, crescimento, e também exerce o controlo sobre o nosso comportamento social, emocional, cognitivo e reprodutivo. Este pequeno órgão atua como uma central de comunicação entre o sistema nervoso central e a periferia, podendo regular vários órgãos periféricos como, a tiroide, o musculo, o osso e ainda o sistema gastrointestinal. A comunicação entre o sistema nervoso central e a periferia é essencialmente controlada pela barreira hematoencefálica, que para além de controlar a passagem de moléculas em ambos os sentidos, consoante as necessidades do organismo, também controla a entrada de toxinas e agentes patogénicos para o cérebro. Assim sendo, um bom funcionamento da barreira hematoencefálica é essencial tanto para o cérebro, como para os órgãos periféricos e, a sua disfunção, especialmente ao nível das "tight junctions", resulta num aumento de permeabilidade da barreira, aumento de infiltração de células inflamatórias e ainda uma desregulação dos processos de transporte de nutrientes para ambos os sistemas, sendo estes eventos cada vez mais associados ao desenvolvimento de doenças neurodegenerativas, como também doenças ao nível metabólico, como por exemplo, a doença de Alzheimer ou Diabetes.

O cérebro é o órgão mais rico em colesterol do corpo humano, contendo cerca de 23% do colesterol total existente no organismo, e ao contrário da zona periférica em que a homeostasia de colesterol é essencialmente dependente da dieta, a homeostasia de colesterol no cérebro parece ser maioritariamente realizada através de síntese *de novo* e reciclagem deste, a partir de acetilcoenzima A em astrócitos. Depois da sua síntese, o colesterol é conduzido até aos neurónios onde irá desempenhar funções a nível da formação de mielina, reparação da membrana, e ainda sinaptogênese. Diversos estudos têm sugerido que a acumulação de colesterol nos neurónios está associada a uma disfunção a nível cognitivo, sendo assim, este esterol tem sido associado a doenças neurodegenerativas, como a doença de Huntington ou a doença de Alzheimer. No entanto, apesar da sua importância, este composto não consegue atravessar a barreira hematoencefálica. Dentro da superfamília Citocromo P450, mais propriamente a proteína CYPx, está envolvida no metabolismo de colesterol no cérebro, e a sua ação reside na sua capacidade de converter colesterol a oxisterol,

ao introduzir um oxigénio na cadeia altamente hidrofóbica do colesterol, sendo fulcral para a sua translocação através da barreira hematoencefálica. Foram reportadas evidências em que cerca de 90% do oxisterol resultante da ação de CYPx (X-HC), atravessa a barreira hematoencefálica para a circulação, onde será conduzido essencialmente para o fígado, onde é utilizado para a síntese de ácidos biliares. Atualmente, são sugeridas duas vias principais para síntese de ácidos biliares no fígado, a via Clássica e a via Alternativa. Resumidamente, a via clássica envolve a hidroxilação do colesterol catalisado pela enzima CYP7A1, contribuindo com cerca de 75% da síntese total de ácidos biliares, enquanto que a via alternativa é catalisada pela ação das enzimas CYP27A1 e CYP7B1, sendo posteriormente conjugados com glicina ou taurina, e armazenados na vesicula biliar. A formação de estruturas micelares por parte dos ácidos biliares, deve-se às suas características anfipáticas, o que facilita a emulsificação, absorção e digestão de lípidos e vitaminas insolúveis. Porém, os ácidos biliares secundários, metabolitos resultantes da atividade microbiana podem ser altamente tóxicos para o organismo, e os seus efeitos têm sido associados a doenças como o cancro do colon e reto.

No intestino humano existe uma vasta e complexa comunidade de microrganismos, denominado de "Microbiota Intestinal". Mantendo uma relação de simbiose, a microbiota intestinal confere diversas vantagens ao hospedeiro, nomeadamente a nível de maturação do sistema imune, proteção contra agentes patogénicos, manutenção da permeabilidade da barreira gastrointestinal, fermentação e absorção de carboidratos e, portanto, manter uma microbiota equilibrada, em eubiose, assegura uma boa função não só digestiva, como também a nível cerebral. Existem várias evidências que mostram uma comunicação bidirecional entre a microbiota gastrointestinal e cérebro, denominado de "microbiota-gut-brain axis", assim sendo, a atividade da microbiota intestinal pode influenciar o nosso cérebro, e vice-versa. A via de comunicação entre estes sistemas ainda não está completamente compreendida, no entanto pensa-se que seja feita através do nervo Vago, Sistema Entérico nervoso e ainda através de metabolitos bacterianos. De entre todos os fatores que influenciam a microbiota intestinal, a dieta, é o fator que mais se destaca devido ao seu efeito notável. De facto, diversas publicações evidenciam um efeito negativo por parte de uma alimentação rica em gorduras, nomeadamente pela redução da diversidade microbiana no nosso intestino e ainda um aumento da permeabilidade da barreira gastrointestinal, possibilitando a passagem de endotoxinas e outros metabolitos bacterianos para a circulação, levando a um estado de inflamação intestinal de baixo grau. Para além disso, existem evidências que mostram que uma

alimentação rica em gorduras pode levar a uma disfunção a nível cognitivo, e ainda a uma desregulação do metabolismo de ácidos biliares, caracterizado pelo aumento dos níveis que ácidos biliares em circulação, podendo ter efeitos a nível da microbiota intestinal.

Este trabalho teve como principal objetivo analisar o efeito da sobre-expressão e silenciamento do gene *Cyp*, e de dois tipos de dieta, uma dieta controlo e uma dieta rica em gordura, na microbiota intestinal de ratinhos. Para além disso, os seus efeitos também foram analisados a nível cognitivo, bem como na inflamação intestinal.

Foram selecionados 70 ratinhos da espécie C57BL/J6 que foram divididos em dois grupos. O primeiro grupo foi destinado à sobre-expressão deste gene, enquanto que o segundo grupo foi destinado ao estudo do silenciamento deste. As duas populações de ratinhos foram alimentadas com uma dieta controlo e uma dieta rica em gordura por 12 semanas. A intervenção a nível do hipotálamo (*Cyp*) teve lugar após as primeiras quatro semanas. A análise da composição da microbiota intestinal foi realizada a partir de amostras fecais, através de qPCR utilizando *primers* direcionados para diferentes grupos bacterianos, nomeadamente *Bacteroidetes*, *Firmicutes*, Bactérias Sulfato-Redutoras (BSR), *Betaproteobacteria*, *Delta-e Gammaproteobacteria*, *Actinobacteria* e *Tenericutes*. Através dos resultados obtidos da análise da composição da microbiota intestinal, no ensaio da sobre- expressão foi possível distinguir a microbiota intestinal de indivíduos que foram sujeitos a uma dieta controlo, daqueles que foram sujeitos a uma dieta rica em gorduras, o que indica que as suas variações se deveram maioritariamente ao tipo dieta a que foram sujeitos. No entanto, o silenciamento deste gene também mostrou exercer influência, levando a uma aproximação em termos da microbiota intestinal, entre indivíduos com diferentes dietas.

A análise a nível cognitivo, utilizando o teste *Y-Maze spontaneous alternations*, indicou que contrariamente a estudos anteriores, uma alimentação rica em gorduras não afetou a memória a curto prazo dos ratinhos, no entanto, o silenciamento deste gene levou a uma diminuição da memória a curto prazo, em comparação com os ratinhos sujeitos à sobre-expressão.

Por último, através da análise de cortes histológicos do intestino, foi possível verificar que, uma desregulação do metabolismo de colesterol no cérebro, bem como uma alimentação rica em gorduras, levou a uma maior ocorrência de inflamação intestinal.

Palavras chave: Hipotálamo; Colesterol; Dieta; Microbiota intestinal; Inflamação intestinal; Memória.

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Abbreviations

ABC	ATP-biding cassette transporters
AD	Alzheimer disease
AgRP	Agouti-related peptide
ApoE	Apolipoprotein E
Arc	Arcuate nucleus
BACS	BA-CoA Synthase
BAT	BA-amino acid Transferase
BBB	Blood-Brain Barrier
BDNF	Brain-derived neurotrophic factor
CA	Cholic acid
CART	Amphetamine regulated transcript
CDCA	Chenodeoxycholic acid
CLA	Conjugated Linoleic acid
CLNA	Conjugated α-Linolenic acid
CNS	Central Nervous System
CYPx	Cholesterol X-hydrolase
CYP7A1	Cholesterol 7 alpha-hydroxylase
CYP27A1	Sterol 27-hydroxylase
CYP46A1	Cholesterol 24-hydroxylase
CYP7B1	25-hydroxycholesterol 7-alpha-hydroxylase
EC’s	Enterochromaffin cells
EEC’s	Enteroendocrine cells
EPS	Exopolysaccharides
FFA	Free Fatty Acid receptor
FMT	Fecal Microbiota Transplantation
GABA	Gamma-aminobutyric acid
GF	Germ free
GI	Gastrointestinal tract
HDAC	Histone Deacetylase

IBD	Irritable Bowel disease
IL	Interleukin
IκBα	Inhibitor kappa B alpha
LAB	Lactic-Acid Bacteria
LB	Luria Broth
LDLR	Low-density Lipoprotein receptor
LPS	Lipopolysaccharide
NFκB	Nuclear factor kappa B
NI	Non-Injected
NPY	Neuropeptide Y
PCR	Polymerase chain reaction
POMC	Proopiomelanocortin
qPCR	Quantitative real-time PCR
ROS	Reactive Oxygen Species
SCFAs	Short-Chain Fatty Acids
SOCS3	Suppressor of cytokine signalling 3
SRB	Sulphate-Reducing Bacteria
TLR4	Toll-like Receptor
TNF-α	Tumour Necrosis Factor
X-HC	X-Hydroxycholesterol

1. Introduction

1.1. Hypothalamus – A Master regulator; related metabolic disorders

The hypothalamus is one of the most important regions of the brain, lying just superior to the anterior pituitary gland, being part of the diencephalon (**Figure** 1.1 A) (Bertalan Dudás, 2013; Burbridge, Stewart and Placzek, 2016a). It acts as a master homeostatic regulator modulating crucial activities in our body, thereby being essential for species survival and propagation. In fact, it was found that hypothalamus contains cell-types that are highly conserved throughout evolution, from annelids to vertebrates (Tessmar-Raible *et al.*, 2007). The human hypothalamus can be divided into four main divisions, Preoptic area, Anterior hypothalamus, Tuberal hypothalamus and Posterior hypothalamus (**Figure** 1.1 B). Each one of this areas is constituted by several nuclei playing central roles in the maintenance of the organism, such as thermoregulation, control of energy metabolism and fluid balance, sleep-wake states, stress responses, growth, cognitive and reproductive behaviours and also emotional and social behaviours (Burbridge, Stewart and Placzek, 2016b; Kim and Choe, 2019).

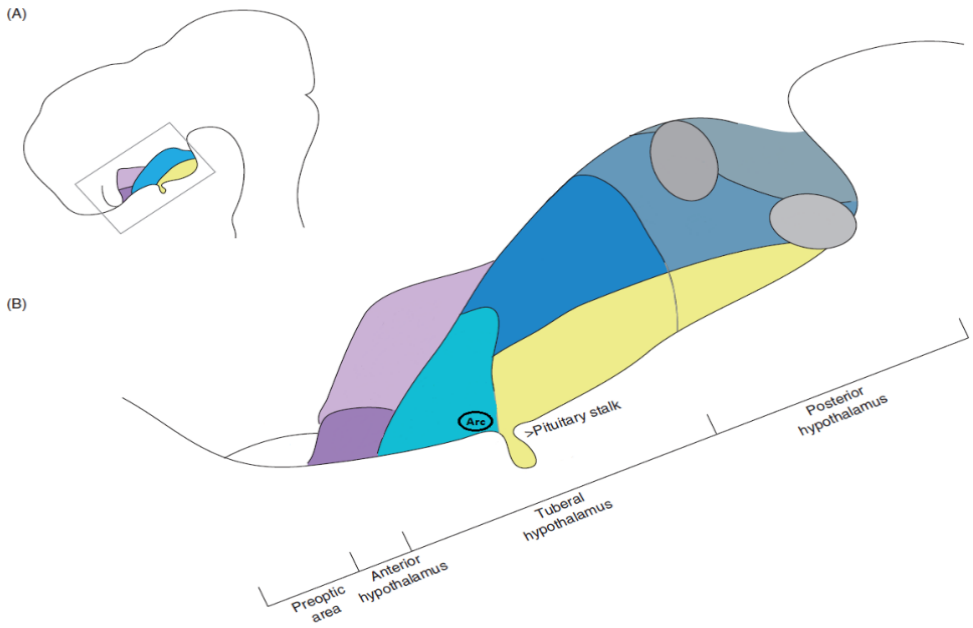


Figure 1.1. An overview on the architecture of the hypothalamus. (A) Represents the position of the hypothalamus within the brain. (B) Represents the main regions within the hypothalamus, each region is composed for several nucleus, and each one holds a specific function in our body regulation. Adapted from: Burbridge, S., Stewart, I. and Placzek, et al., 2016

This pivotal region of the brain, acts has the central of communication from the peripheral systems to the central nervous system (CNS) (Burbridge, Stewart and Placzek, 2016a), receiving numerous peripheral signals and sending efferent hormonal and neuronal signals, regulating diverse organs, such as thyroid, muscle, bone and gastrointestinal tract (Zaidi, 2007; Austin C. Stark, 2008). However, this bidirectional communication between systems is not an easy task. First, every molecule needs to cross the interface between the blood circulation and neural tissue termed as Blood-Brain Barrier (BBB), which is mainly formed by unique endothelial cells (Abbott, Rönnbäck and Hansson, 2006). By tightly controlling the passage of molecules and ions, this multicellular vascular structure can delivery almost instantly nutrients and oxygen depending on the organism needs. Thus, this highly impermeable barrier protects the brain from several toxins and pathogens (Obermeier, Daneman and Ransohoff, 2013). An intact BBB is essential for the proper function of the brain and also peripheral organs, and so, break-down of any of its components, especially tight junctions, results on increased permeability, impaired transport processes and increased immune cell infiltration (Palmela, Brites and Brito, 2012), leading to inflammatory processes which not only affects energy balance but also may contribute, for example, to obesity-associated insulin resistance (Guillemot-Legris and Muccioli, 2017). Since one of the primary functions of the hypothalamus is the control of energy homeostasis, by regulating feeding behaviour and energy expenditure to maintain body weight, these events are proposed to be involved in many metabolic disorders (Hotamisligil, 2006; Palmer, 2010; Gregor and Hotamisligil, 2011; Editor, Mullally and Hayes, 2019).

It has been established, that a state of hypothalamic inflammation is one of the primary causes for the development of obesity, with resistance to hormonal signals, such as insulin and leptin, therefore, as consequence, whole-body energy homeostasis might be affected (Manousopoulou *et al.*, 2016; Guillemot-Legris and Muccioli, 2017). Unhealthy lifestyles habits, such as reduced physical activity and increased consumption of highly-dense energy food are well-known factors contributing to obesity (Calder *et al.*, 2017). In fact, an high-fat diet causes neuronal mitochondria to be overloaded with fatty acids and glucose, resulting in elevated acetyl-CoA and NADH generation, with increased numbers of electrons that are able to occupy the intermembrane space, therefore increasing the production of reactive oxygen species (ROS), leading to the onset of oxidative stress and further inflammation (Cunarro *et al.*, 2018). Moreover, increased intake of saturated fatty acids induce an immune cell activation, which elicit an inflammatory response

(Guillemot-Legris and Muccioli, 2017). This immune response might be mediated through toll-like receptors that activate the transcription factors, nuclear factor kappa B (NFkB) and activator protein-1, which is turn, leads to the upregulation of pro-inflammatory cytokines, such as IL-1, IL-6, tumour necrosis factor-alpha and chemokines (Manousopoulou *et al.*, 2016; Guillemot-Legris and Muccioli, 2017). Indeed, 24 h exposure of a High Fat Diet (HFD) leads to increased hypothalamic expression of IL-6 and TNF-α in mice models (Mendes *et al.*, 2018), and in addition to this increased cytokines levels, endoplasmic reticulum stress, SOCS3 and the IKKb/NF-kB pathways were also upregulated within the hypothalamus (Zhang *et al.*, 2008)

Tanycytes, which are specialized glial cells, regulate not only BBB plasticity, but also allow the passage of molecules such as leptin and insulin (Langlet, 2014). Such molecules bind to specific neurons located in the Arcuate nucleus (Arc) (**Figure** 1.1), the melanocortin system, which consists of two antagonistic neuronal populations - orexigenic (AgRP/NPY neurons) and anorexigenic neurons (POMC/CART neurons), which exert opposite effects (Benite-Ribeiro *et al.*, 2016; Jais *et al.*, 2017). The first population express the orexigenic neuropeptides agouti-related peptide (AgRP) and the neuropeptide Y (NPY), whereas the second population expresses the anorexigenic peptides proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) (Jais *et al.*, 2017). Briefly, both circulating levels of insulin and leptin, inhibit AgRP neurons and activate POMC neurons, resulting in a decreased energy intake and an increased energy expenditure (Cowley *et al.*, 2001; Choudhury *et al.*, 2005), while under fasting conditions, AgRP neurons are activated to induce feeding, inhibit energy expenditure and also regulate glucose metabolism (Gropp *et al.*, 2005). In obesity-like state, there is an imbalance between those signalling pathways, which results in an overexpression of AgRP neuropeptides with a reduction of the expression of POMC/CART, leading to diminished satiety and hyperphagia (Bergen *et al.*, 1999). Additionally, POMC is expressed as a precursor peptide, which is processed in several peptides, including melanocortin receptor ligands that are released from synaptic endings of POMC neurons and interact with melanocortin receptors (MC3R/MC4R) expressed in secondary neurons to suppress food intake (Lee *et al.*, 2006; Vogt and Brüning, 2013)

1.1.1. Brain Cholesterol metabolism

The brain is the most cholesterol-rich organ in the human body, counting for almost 23% of total organism cholesterol (Ohyama *et al.*, 2006). In contrast to the periphery, where cholesterol

homeostasis is mainly diet-dependent, brain cholesterol homeostasis seems to be achieved by *de novo* synthesis and recycling (**Figure** 1.2) , being mainly synthetized by astrocytes from acetyl coenzyme A (**Figure** 1.2) (Gosselet, Saint-Pol and Fenart, 2014; Boussicault *et al.*, 2016; Loera-Valencia *et al.*, 2019). After synthesis, cholesterol is delivered to neurons by certain transporters, such as ATP-binding cassette transporters (ABC), specifically ABCA1 and ABCG1, and apolipoprotein E (ApoE), where is going to be used in synaptogenesis, myelin formation, neurotransmitters release and membrane repair (**Figure** 1.2) (Gosselet, Saint-Pol and Fenart, 2014; Zhang *et al.*, 2015; Loera-Valencia *et al.*, 2019). For this reason cholesterol metabolism is thought to play key roles in the physiopathology of several neurodegenerative diseases, such as Huntington disease or Alzheimer disease (AD), becoming a major focus on neuroscience research (Martins *et al.*, 2009; Boussicault *et al.*, 2016). As an example, silencing of *Cyp46a1* led to neuronal cholesterol accumulation, inducing apoptotic death of hippocampal neurons, followed by cognitive impairment and hippocampal atrophy (Djelti *et al.*, 2015). Another study showed that CYP46A1 has protective effects against Huntington's disease by improving cortico-striatal connectivity and synaptic transmission, vesicle and endosome trafficking, thereby showing an effect at regulating cholesterol homeostasis in zQ175 mice (Kacher *et al.*, 2019). Together with the aforementioned transporters, the enzyme HMG-CoA reductase, the low-density lipoprotein receptor (LDLR) and Cytochrome 450 enzymes super-family, are also involved in both cholesterol metabolism and excretion to the periphery (Benarroch, 2008). Cholesterol is not able to cross the BBB, due to physicochemical properties (Gosselet, Saint-Pol and Fenart, 2014; Zhang *et al.*, 2015; Ayciriex *et al.*, 2017; Björkhem, Leoni and Svenningsson, 2019), and in this situation CYP 450 enzymes superfamily comes in action, converting cholesterol into oxysterols, by introducing an oxygen into the highly hydrophobic cholesterol, being of critical importance for its translocation through the BBB (Meaney *et al.*, 2002).

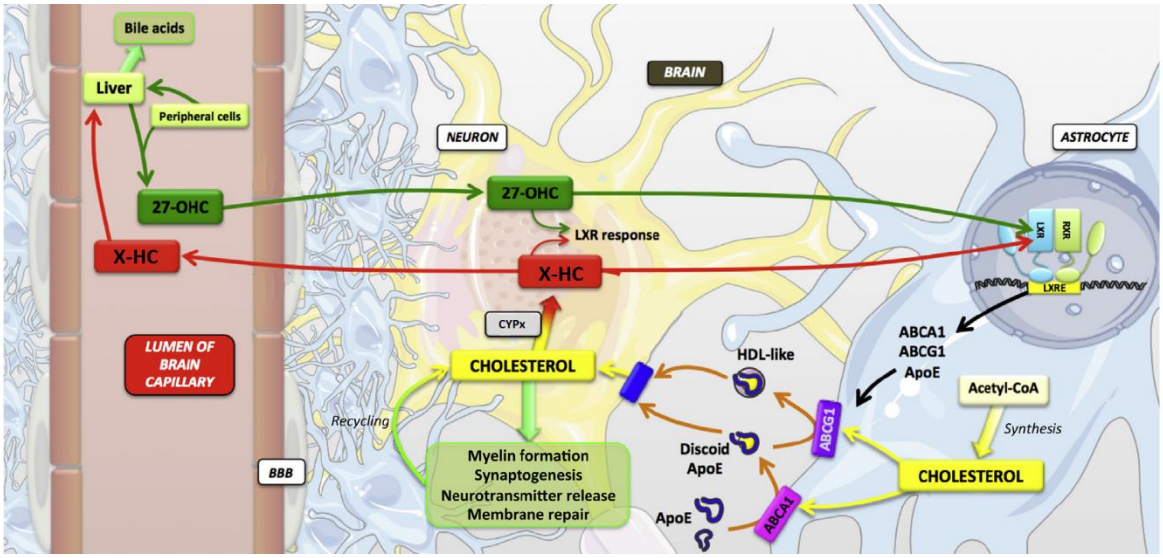


Figure 1.2. The main pathways of brain cholesterol homeostasis. Cholesterol homeostasis within our brain is thought to be mainly controlled by CYPx and CYP27A1.Source: Gosselet, F., Saint-Pol, J. and Fenart, L. *et al*., 2014.

The major excretion route of brain's cholesterol involves the enzyme cholesterol X-hydrolase (CYPx), which catalyses the conversion of cholesterol to X-hydroxycholesterol (X-HC). Indeed, 90% of X-HC produced in the brain, crosses the BBB to the circulation by diffusion (Björkhem, 2006), being conducted to several organs, specially to the liver where it will be used for bile acid synthesis, as final product (Reinicke *et al.*, 2018). In fact, it was found that bile acid synthesis is the major pathway of cholesterol catabolism (Hofmann and Hagey, 2008; Thomas *et al.*, 2008), and its levels in the circulation is a reflection of the balance between the production in the brain and the ability of the liver to metabolize it (Bretillon *et al.*, 2000). However, there are many other cholesterol hydrolases involved in the cholesterol metabolism, such as CYP27A1 or CYP7A1, which are mainly present in plasma (Loera-Valencia *et al.*, 2019). Two principal routes have been proposed for bile acid synthesis in the liver, named as the "classical" and "alternative" pathways (Chiang, 2004, 2009). Briefly, the classical pathway involves the hydroxylation of cholesterol catalysed by CYP7A1 contributing to at least 75% of total bile acid generation, while the alternative pathway is catalysed by CYP27A1, yielding 27-hydroxycholesterol, which is further hydroxylated by 7α-hydroxylase, CYP7B1(Enright *et al.*, 2018).

Furthermore, there are strong evidences showing a continuous flux from brain to circulation and vice-versa (**Figure** 1.2), and for example, HFD increased the flux of oxysterols from the periphery

into the brain (Rahman *et al.*, 2005; Mateos *et al.*, 2009; Park *et al.*, 2013). Moreover, memory impairment was reported in cholesterol-fed mice, however the same results were not observed in cholesterol-fed mutant mice lacking the enzyme CYP27A1, suggesting that diet can have detrimental effects at the brain level (Heverin *et al.*, 2015).

1.2. Human gastrointestinal tract (GI)

1.2.1. Intestinal Microbiota

Within human intestine resides a vast and complex community of microorganisms that is considered to be an essential component for host organism homeostasis, harbouring at least 100 trillion (10^{14}) bacteria per intestine (Grenham *et al.*, 2011; Tomasova, Konopelski and Ufnal, 2016). The coevolution of the intestinal microbiota with their host from the primordial beginnings of mankind resulted in a symbiotic relationship, contributing to crucial host functions, such as energy harvest and storage, maturation of the host immune system and promoting its functions, metabolism of xenobiotics, insulin sensitivity, production of inhibitory substances against pathogens, maintenance of intestinal barrier integrity and also fermentation and absorption of several carbohydrates, which cannot be degraded by host enzymes, in the large intestine (Clemente *et al.*, 2012; Hooper, Littman and Macpherson, 2012; Goubet *et al.*, 2018; Peng *et al.*, 2019; Viennois, Gewirtz and Chassaing, 2019). In addition, hosts can beneficiate from several metabolites of their metabolism, such as vitamin K, hormones, short chain fatty acids (SCFA), aldehydes, alcohols, and also as products of their metabolism are released certain gases, such as H2S, CO2 and NO (Sariaslani and Gadd, 2015; Wang and Wang, 2016; Kovatcheva-Datchary *et al.*, 2019). Human gut commensal microbiota is mainly composed of *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (Grenham *et al.*, 2011; Wang and Wang, 2016; Goubet *et al.*, 2018; Kovatcheva-Datchary *et al.*, 2019). However, this composition can suffer large variations due to numerous factors including maternal factors, mode of delivery at birth, breastfeeding or formula feeding, health status, lifestyle, antibiotic consumption, host genetics and essentially to the diet, which is distinct from each region (**Figure** 1.3) (Guo *et al.*, 2008; Holmes *et al.*, 2012; Sandhu *et al.*, 2016; Z. *et al.*, 2017; Dong and Gupta, 2019).

The impact of the diet on the microbial pattern can be exemplified with the study where the microbial profile of African children was compared with European children (De Filippo *et al.*,

2010). The African children consume 14.2 g fiber per day and display different intestinal microbiota profiles in comparison with European children which consume around 8.4 g of fiber per day (De Filippo *et al.*, 2010)

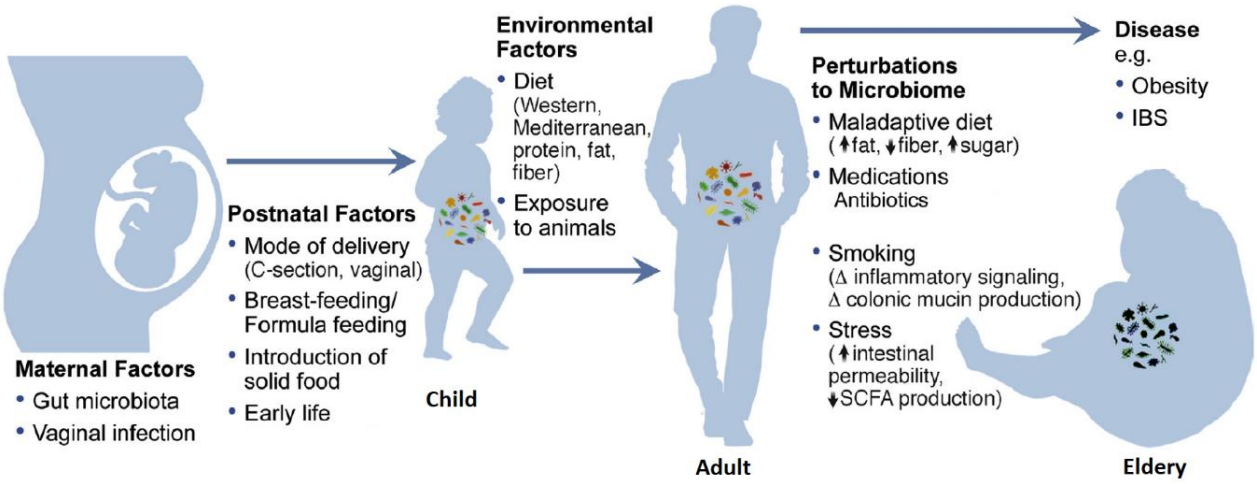


Figure 1.3. Maternal factors, Postnatal factors, environmental factors and perturbations to microbiota, such as unhealthy diet, smoking, stress and medications can change intestinal microbiota over time, leading to the development of metabolic diseases such as obesity or Irritable Bowel disease. **Source: Dong, T. S. and Gupta, *et al.*, 2019.**

In fact, diet and consequent nutritional quality have long been implicated to contribute to the most common human diseases, including cardiovascular, metabolic and neurodegenerative disorders (Chassaing *et al.*, 2015; Heverin *et al.*, 2015; Hooper *et al.*, 2015; Jeong, Jang and Kim, 2019). Over the past few years, many studies demonstrated that diet is the most important factor affecting intestinal microbiota (Gary D. Wu, Jun Chen, Christian Hoffmann, Kyle Bittinger, Ying-Yu Chen, Sue A. Keilbaugh, Meenakshi Bewtra, Dan Knights, William A. Walters, Rob Knight, Rohini Sinha, Erin Gilroy, Kernika Gupta, Robert Baldassano, Wu *et al.*, 2011; Llewellyn *et al.*, 2018; Shi *et al.*, 2019). For example, a 8-week HFD changed the intestinal microbiota causing an increase in the ratio of *Firmicutes* to *Bacteroidetes* in mouse models (Turnbaugh *et al.*, 2008). Another report, demonstrated a decrease in diversity and species richness after 12 weeks of a HFD in mouse models (He *et al.*, 2018). Furthermore, intestinal microbiota is thought to play an important role in the development of obesity, since germ-free (GF) mice does not become obese even if subjected to a HFD (Bäckhed *et al.*, 2004). HFD is associated to a low-grade systemic inflammation (Yang *et al.*,

2015) with increased circulating and fecal levels of lipopolysaccharide (LPS), a breakdown product of outer layer of Gram-negative bacteria (Erridge *et al.*, 2007), which in turn exerts an effect on the Toll-like receptor 4 (TLR4) (Yu *et al.*, 2015), activating inflammatory signalling pathways, such as inhibitor kappa B alpha (IκBα) and nuclear-factor-kappa B (NFκB) (Baker, Hayden and Ghosh, 2011). HFD also increases the expression of inflammatory cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 (Beck and Wallace, 1997; Ghanim *et al.*, 2009; Shi *et al.*, 2019). Furthermore, a low intake of fiber leads to a decreased intestinal microbial diversity and SCFA production, and in turn, the use of dietary fat increases bile acid production, possibly leading to increased levels of secondary bile acids inside the colon resulting in colonic inflammation (Makki *et al.*, 2018; Zou *et al.*, 2018). Indeed, epidemiological evidences suggests that increased dietary fibers consumption helps in ameliorating inflammatory-like states within human body. For example, the circulatory levels of pro-inflammatory mediators, such as TNFα, IL-6 and IL-8 were reduced after two weeks of diet supplemented with soluble fiber (Macfarlane *et al.*, 2013). Dietary fibers constitutes a spectrum of non-digestible carbohydrates such as non-starch polysaccharides, oligosaccharides, lignin and other polysaccharides with beneficial effects (Zeng *et al.*, 2019). The beneficial action of fibers, may be derived from microbial activity within large intestine, fermenting undigestible carbohydrates, that are not metabolized in the small intestine, producing SCFAs, such as acetate, propionate and butyrate (Li, van Esch, Wagenaar, *et al.*, 2018; Zeng *et al.*, 2019). Butyrate is essential for colonic health, by providing colonocytes with almost 70% of their energy (Roediger, 1980; Chen and Vitetta, 2018), whereas acetate and propionate are mainly transported to muscle and liver tissues, respectively (Hooper, Midtvedt and Gordon, 2002), however they are still metabolized in the epithelium providing energy and decreasing colonic pH (Rivière *et al.*, 2016). In addition, a low intake of dietary fibers, does not only results in a reduced microbial diversity and SCFA production, but also changes gut microbial metabolism through the utilization of less favourable substrates, such as host mucins, which may cause detrimental effects to the host (Zou *et al.*, 2018). One possible mechanism for the anti-inflammatory effects of SCFAs, is through activation of G-protein-coupled receptors, such as free fatty acid (FFA) receptors type 2 and 3 (FFA2 and FFA3 receptors) and G-protein coupled receptor 109A (GPR109A), which are differently expressed on cells and are the main receptors for SCFA's (Tan *et al.*, 2014; Li, van Esch, Wagenaar, *et al.*, 2018). The FFA2 receptor is mainly expressed on immune cells, such as neutrophils, eosinophils, dendritic cells and monocytes, thereby it is

proposed to play roles in immune responses and inflammatory processes, while FFA3 receptor is mainly expressed on pancreas, spleen and adipose tissue, being implicated in metabolic diseases, however this receptors are also expressed at lower levels on immune cells (Kim *et al.*, 2014; Li, van Esch, Wagenaar, *et al.*, 2018). As an example, SCFAs showed anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines IL-6 and IL-8 by endothelial activation, where FFA2 and FFA3 receptors are also expressed (Li, van Esch, Henricks, *et al.*, 2018), however there is contradictory results indicating a pro-inflammatory effect through this type of receptors (Kim *et al.*, 2013). In addition, SCFAs may act at epigenetic level. Acetylation of lysine residues within histones, results in gene activation, where the access of transcription factors to promoter regions is facilitated (MacDonald and Howe, 2009). SCFAs are known to inhibit histone deacetylases (HDAC) activity, thereby, the removal of acetyl groups from histones are not possible, altering gene expression in a wide variety of cells (Kim and Bae, 2011; Tan *et al.*, 2014; Li, van Esch, Wagenaar, *et al.*, 2018).

Recent reports have been suggesting a relation between an HFD and the levels of colonic bile acids and a possible dysmetabolism (Martínez-Augustin and de Medina, 2008; Murakami, Tanabe and Suzuki, 2016; La Frano *et al.*, 2017; Ghaffarzadegan *et al.*, 2018). For example, a HFD caused an increased colonic bile acids concentration, in comparison with Control diet subjects (Murakami, Tanabe and Suzuki, 2016). Moreover, bile acid dysmetabolism is being associated with fat malabsorption, diarrhoea, inflammation and liver injury (Pereira-Fantini *et al.*, 2014). The amphipathic structure of bile acids gives detergent properties, facilitating the emulsification, absorption and digestion of lipids and insoluble vitamins, therefore playing crucial roles on the maintenance of intestinal epithelial homeostasis (Hofmann and Hagey, 2008; Thomas *et al.*, 2008; Chiang, 2009). As mentioned on section 1.1.2, primary bile acids are synthesized on the liver, mainly by the action of cholesterol 7α-hydroxylase (CYP7A1) under normal conditions and by a an alternative pathway involving both CYP27A1 and CYP7B1 (Wahlström *et al.*, 2016), with further conjugation to both glycine or taurine through BA-CoA synthase (BACS) and BA-amino acid transferase (BAT) enzymes activities, and stored in the gallbladder (Chiang, 2009). In addition, a study involving murine models, demonstrated that gut microbiota was able to regulate the expression levels of CYP27A1, CYP7A1 and CYP7B1, among other enzymes involved in bile acid synthesis, in a FXR-dependent manner (Sayin *et al.*, 2013). The most abundant bile acids are the cholic acid (CA) and chenodeoxycholic acid (CDCA) (Thomas *et al.*, 2008). The majority of

these conjugated bile acids are reabsorbed in the large intestine, however 5-10% undergoes transformation to secondary bile acids, as a result of microbial metabolism (Ridlon, Kang and Hylemon, 2006; Chiang, 2009).

1.2.2. The Microbiota-Gut-brain axis

1.2.2.1. The microbial products and main routes of communication

The bidirectional communication between the gastrointestinal tract and the brain, is mainly regulated by both central and enteric nervous systems, hormones and immune system (Cryan and Dinan, 2012). Through the complex signalling network, the intestinal environment can have an influence on the brain functions, and inversely, the brain can influence intestinal functions such as motility, secretion and mucin production and also immune functions (Tracey, 2009; Foster, Rinaman and Cryan, 2017; Ge *et al.*, 2017). Recent reports have suggested a strong communication between the intestinal microbiota and the brain, designated by Microbiota-Gut-Brain axis (Oriach *et al.*, 2016). Using germ-free mice it was possible to show that intestinal microbiota helps the maintenance of the integrity of the blood-brain barrier, by positively regulating the expression of tight junctions proteins, occluding and claudin-5 (Braniste *et al.*, 2014). Another report demonstrated that germ-free mice showed larger and branched morphology in terms of microglial cells, in comparison with specific pathogen free mice (Erny *et al.*, 2015). In addition, a recent study demonstrated that diet-induced obese mice showed impaired recognition and spatial working memory, with decreased expression of the brain-derived neurotrophic factor (BDNF) (Zhang *et al.*, 2019). Numerous routes of communication have been proposed to explain how intestinal microbiota might exert an influence on brain functions, including the vagus nerve (which originates in the medulla oblongata of the CNS and extends into the periphery supplying the gut, heart and lungs), enteric nervous system, immune system and also bacterial metabolites, as shown on **Figure** 1.4 (Oriach *et al.*, 2016; Sandhu *et al.*, 2016; Cussotto *et al.*, 2018; Spielman, Gibson and Klegeris, 2018). Intestinal microbiota has been shown to produce a wide range of neurotransmitters, such as dopamine, norepinephrine, serotonin or gamma-aminobutyric acid (GABA), which might influence brain's behaviour (Cussotto *et al.*, 2018; Strandwitz, 2018). Serotonin synthesis is mainly dependent on the tryptophan availability, which is an essential amino acid (Le Floc'h, Otten and Merlot, 2011). Serotonin is widely used throughout the body, however 90-95% of serotonin content

resides within the gastrointestinal tract, mostly in epithelial cells (Gershon and Tack, 2007). Although several microbial strains have been reported to produce serotonin, such abilities were not identified on intestinal microbiota, instead, intestinal microbiota seems to influence serotonin levels by secreting SCFAs or secondary bile acids, which in turn activates tryptophan hydrolase expression within specialized endocrine cells, called enterochromaffin cells (EC's) (Yano *et al.*, 2015). Furthermore, the intestinal bacteria *Lactobacillus* spp. and *Bifidobacteria* spp. are able to metabolize glutamate to produce GABA, which is known to be a major inhibitor neurotransmitter of the central nervous system, being associated to scenarios, such as depression and anxiety (Möhler, 2012; Strandwitz *et al.*, 2019). Additionally, treatment with *L. rhamnosus* induced alterations in GABA receptor expression levels in the brain and anxiety-like behaviours via vagus nerve signalling (Bravo *et al.*, 2011). The vagus nerve is considered to be the major route for mediating the effects of intestinal microbiota on different physiological states within our body (Sandhu *et al.*, 2016). For instance, vagotomised mice with depressive-like behaviours were not able to recover even after being treated with potential probiotic *Lactobacillus rhamnosus* (Bravo *et al.*, 2011), suggesting the importance of this pathway for the communication of this bacteria with the brain. The vagus nerve does not enter directly in the intestinal lumen, its activation relies upon the secretion of chemical signals, including hormones (peptide YY), glucagon-like peptide 1, and cholecystokinin by enteroendocrine cells (EECs) in intestinal tract (Romijn *et al.*, 2008), therefore this specialized cells are critical for the communication between gut and brain.

EECs are located along the gastrointestinal tract in direct contact with luminal content and also with the gut microbiota, containing receptors for the most of the nutrients available on the lumen, such as receptors for amino acids, peptones, SCFAs and long-chain fatty acids, controlling several functions during the initiation of neural and hormonal responses, during digestion, and the regulation of appetite and insulin secretion (Furness *et al.*, 2013; Latorre *et al.*, 2016). It is acknowledge that SCFAs can interact with G-protein coupled receptor 41 (GPR41) expressed by EECs, causing a reduction of the expression of the peptide YY, increasing intestinal transit, inhibiting gut motility and also reducing nutrient contact time with the epithelium, thereby affecting host adiposity (Samuel *et al.*, 2008). In another study, administration of propionate, to neuronal cells caused a temporary intracellular acidification, which is known to affect neuronal excitability and signalling potential (Shono *et al.*, 2010). Although intestinal microbiota displays important

roles in the maturation of the immune system, there are reports demonstrating a negative effect in brain's behaviour.

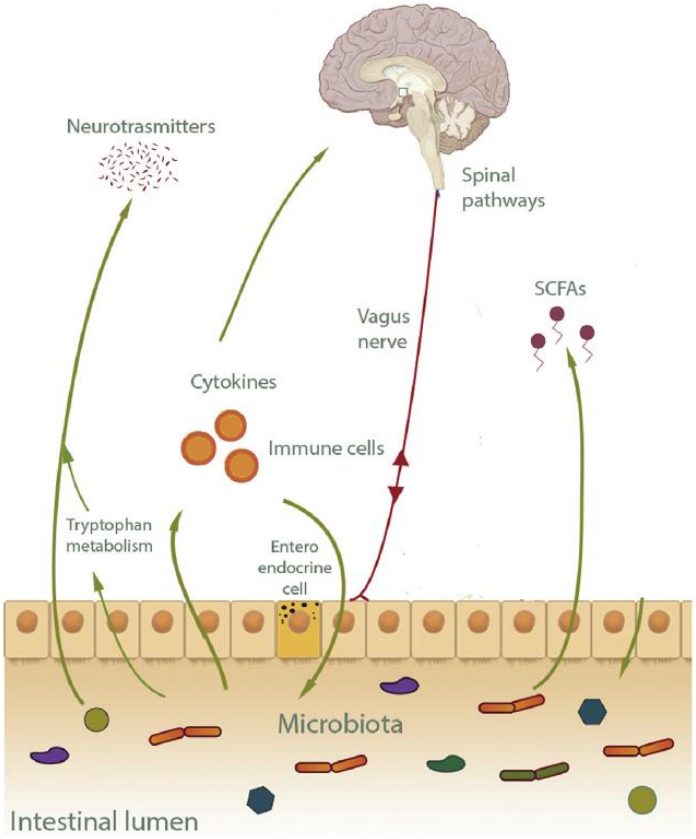


Figure 1.4. Principal pathways involved in the bidirectional communication between the gut microbiota and brain including the enteric nervous system, immune system, vagal nerve and bacterial metabolites. Source:Gadd, G. and Sariaslani, S *et al*, 2015

For example, peripheral administration of pro-inflammatory cytokines, induced depressive-like behaviours, such as reduced appetite, reduced exploratory behaviour and also sleep disturbances (Bilbo and Schwarz, 2012). Another report demonstrated that germ-free mice, exhibited diminished microglial activation with immature phenotype after LPS administration, which rises the hypothesis that the absence of microbiota attenuates immune responses at neuronal level (Erny *et al.*, 2015). Lastly, LPS is a strong promotor of the innate immune system and if the intestinal barrier is compromised, this bacterial component can be translocated from the lumen to the circulatory system leading to an immune response (Dantzer *et al.*, 2008).

1.2.3. Sulphate-Reducing Bacteria – an inflammation inducer

Being ubiquitous in mammalian colon, sulphate-reducing bacteria (SRB) are gram-negative bacteria, belonging to the phylum *Proteobacteria* (Ritz *et al.*, 2016; Tomasova, Konopelski and Ufnal, 2016). There are several microbial pathways involved in colonic sulphur metabolism, being widespread among several intestinal microbial groups, as shown on **Figure** 1.5. However, only this particular group is able to use inorganic sulphate, through the enzyme dissimilatory sulphate reductase in their metabolism (Carbonero *et al.*, 2012). The sulphur present within human organism is mainly acquired by the diet through protein ingestion, and it's either converted to sulphated compounds, absorbed by host cells or excreted (Stipanuk, 2004). The main food sources of inorganic sulphate include dried fruits, fermented beverages, commercial breads, red meat, eggs and also milk, whereas the last three contain elevated levels of cysteine, which is a sulphated amino acid (Carbonero *et al.*, 2012). Lastly, sulphate-reducing bacteria also plays roles in bile acid metabolism. For example, a HFD leads to an increase of hepatic taurine conjugated bile acids, providing sulphur-rich taurocholic acid in the gut, enhancing the growth of the bacterium *Bilophila wadsworthia*, being associated with the development of colitis (Devkota *et al.*, 2012; Z. *et al.*, 2017). The particular interest on this bacterial group, relies in the fact that they use sulphate, as a terminal electron acceptor, together with hydrogen (H_2) producing hydrogen sulphide (H_2S) (**Figure** 1.5 and **Figure** 1.6) (Carbonero *et al.*, 2012; Ritz *et al.*, 2016).

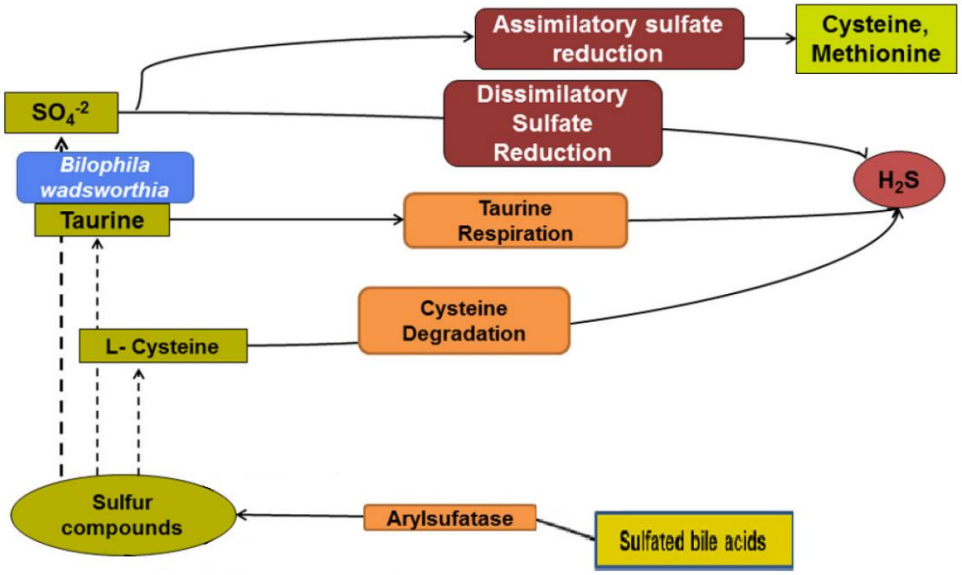


Figure 1.5. The main bacterial pathways involved in sulphur metabolism. The finals products of assimilatory sulphate reduction pathway, which is cysteine and methionine (sulphated amino acids) can be used by sulphate-reducing bacteria, producing hydrogen sulphite. Adapted from: Carbonero *et al*., 2012.

Hydrogen sulphite at physiological concentrations is essential regulating several systems including hypothalamic-pituitary-adrenal axis, long-term potentiation in the hypothalamus and also regulates smooth muscle functions (Diego, 1996; Dello Russo *et al.*, 2000; Gallego *et al.*, 2008). In addition, hydrogen sulphide have been proposed to play beneficial roles at intestinal level, such as providing energy for colonic epithelial cells, since it's oxidation in the gut results in ATP production, promotion of colonic mucus production, reverse dysbiosis caused by nonsteroidal anti-inflammatory drugs and can also show protective features at cardiovascular level (Goubern *et al.*, 2007; Blackler *et al.*, 2015; Motta *et al.*, 2015; Tomasova, Konopelski and Ufnal, 2016). However, this highly volatile gas is well-known to provoke detrimental effects at cognitive and gastrointestinal level. For example, administration of non-pathogenic H_2S-producers generated higher concentrations of H2S in the small intestine and cecum impairing cognitive function (Ritz *et al.*, 2016). Furthermore, production of H_2S (**Figure** 1.6) by sulphate-reducing bacteria affects colonic cell integrity, induced colonic mucosal hyperproliferation and can also be powerfully genotoxic, resulting in increased intestinal permeability and inflammation, for such have been associated with intestinal diseases, such as Irritable Bowel disease (IBD), colorectal cancer and even neurological diseases (Christl *et al.*, 1996; Attene-Ramos *et al.*, 2007; Nakamura *et al.*, 2009; Guidotti, 2010; Llewellyn *et al.*, 2018)

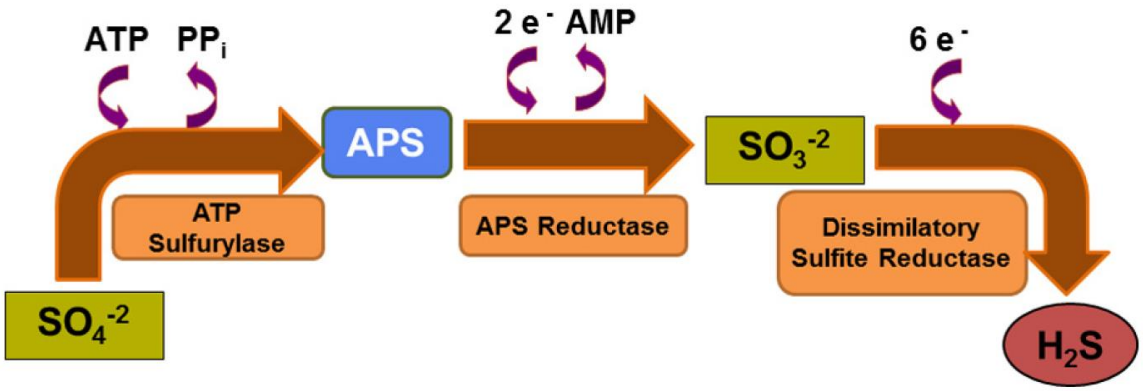


Figure 1.6. Cascade of enzymatic reactions involved in the production of hydrogen sulphite through the Dissimilatory sulphite reductase. Source: Carbonero *et al.*, 2012.

1.2.4. Bacterioterapy

Due to the astonishing development of advanced molecular and bioinformatics platforms in the last few years, scientists are getting closer to characterize the function and composition of microbiota within human intestine. Although intestinal microbiota is well-known to play several beneficial roles in maintaining body homeostasis, a situation of shifts in the gut microbiota, termed as dysbiosis is closely related to the development of intra-and extra-intestinal chronic inflammation, which is being associated with several diseases, such as metabolic syndromes, inflammatory bowel diseases, cardiovascular disorders, cancers and also neuropsychiatric diseases, as mentioned above (Lin *et al.*, 2014). To achieve the aim of amelioration of such diseases, the administration of probiotics is one of the best approaches widely accepted (Novik and Savich, 2019). Probiotic refers to "a preparation or a product containing viable microorganisms in adequate amounts, which alter the microflora conferring health benefits to the host" (Vrese, 2018), while a prebiotic can be defined by a " non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth or activity of a number of bacteria in the colon" (Gibson and Roberfroid, 1995). For example, Administration of *Lactobacillus* and *Bifidobacterium* spp. leads to increased levels of SCFAs, such as butyrate and propionate, which in turn possess antimicrobial activity and promote homeostasis by increasing the colonic integrity (Bindels *et al.*, 2015; Holscher, 2017), or even as stated in another report, where *L. rhamnusos* GG caused the reduction of blood glucose levels in genetic type 2 diabetic mouse model, by suppression of glucose absorption in the intestines (Honda *et al.*, 2012). Additionally, the administration of a probiotic mixture containing 8 different *Lactobacillus* and *Bifidobacterium* strains to mouse models, resulted in a significant reduction in depressive-like behaviours, thus the reduction was correlated with a diminished circulating levels of pro-inflammatory cytokines (Abildgaard *et al.*, 2017). Most of the probiotics used nowadays, are derived from the commensal microbiota found in the healthy human gut, and their propose is to mimic those of the homeostatic effects of a healthy microbiota (Quigley, 2019). Presently the most used probiotics are Lactic-acid bacteria (LAB) and *Bifidobacteria* which are able to produce numerous valuable substances, such as bacteriocins, vitamins and exopolysaccharides (EPS) (Novik and Savich, 2019). However, *Bifidobacterium* can be considered to be part of the LAB, since they use a specialized pathway termed as bifid shunt, which results in lactate and acetate production (Fushinobu, 2010).

Exopolysaccharides are long-chain polymers mainly produced by LAB consisting of branched, repeated units of sugars or sugar derivates, which have been shown positive effects by stimulating the immune system or exerting antitumoral activity in human and murine cell cultures (Harutoshi, 2013).

Bacteriocins are peptides that are largely produced by LAB and have shown to display microbial activity, mainly against Gram-positive bacteria (Novik and Savich, 2019). The bacteriocins can be classified into three different classes according to their spectrum of activity, molecular weight, biochemical properties and also by genetic origin (Mokoena, 2017). Among the bacteriocins, nisin is so far the best-studied, being widely used in the food industry, by the ability to extend the shelf life of food and food products by suppressing the growth of both degrading and pathogenic bacteria (Kuipers *et al.*, 1995; Novik and Savich, 2019).

Both LAB and *Bifidobacteria* have the ability to synthesize vitamins of the B group, such as riboflavin, folate and cobalamin, and also vitamin K, which humans are incapable of synthesizing (Novik and Savich, 2019). Furthermore, these species are able to produce low-calorie sweeteners, such as mannitol, sorbitol and tagatose, which can be used as sugars substitutes for patients with diabetes, and at the same time exhibit prebiotic and antioxidant effects (Patra, Tomar and Arora, 2009). Both LAB and Bifidobacterium also produce fatty acids, such as conjugated linoleic acid (CLA) and conjugated α-linolenic acid (CLNA), which are health-promoters, showing anti-obesity, anticarcinogenic, antioxidant, and anti-inflammatory properties both in humans and animal studies (Salsinha *et al.*, 2018).

Another recent therapeutic approach to ameliorate the gut microbiota and eliminate gut infection, such as the caused by *Clostridium difficile* is the fecal microbiota transplantation (FMT), which consists in the transplantation of fecal microbiota from a defined healthy individual into an unhealthy individual (Wang *et al.*, 2019). This process involves the full transplant of all microorganisms that compose an intact community of the gastrointestinal tract including bacteria, viruses, archaea, fungi, protozoa, together with residual amounts of colonocytes and metabolites (Bojanova and Bordenstein, 2016). In addition, this type of approaches can be cost-saving in comparison with the use of antibiotics (Zhang *et al.*, 2017). Over the last few years, this approach has been one of the main focus in gut microbiota research, being accepted and used with success in hospitals and clinics, mainly against *C. difficile* infections, nonresponsive to antimicrobial treatments (Hota *et al.*, 2018). The exact mechanisms of FMT efficacy is not yet clarified, however

it is mainly due to the restoration of the complex community of microorganisms (Liu *et al.*, 2017). The methods of administration of fecal material include upper gastrointestinal route, lower gastrointestinal route (colonoscopy) and oral capsule, where the colonoscopy seems to be the more effective, however it is the most invasive method, while oral capsule is the less invasive one (Wang *et al.*, 2019)

As mentioned before on section 1.2.4, it was demonstrated that intestinal microbiota can affect the central nervous system, playing roles in some neuronal pathophysiologic states. Therefore, bacteriotherapy, can also be used as alternative approach for the treatment of such diseases. For example, a recent study demonstrated that cerebral ischemic stroke triggered by intestinal microbiota dysbiosis and increased intestinal permeability was attenuated by a FMT rich in SCFAs (Runzhi Chen, Ying Xu, Peng Wu, Hao Zhou, Yi Lasanajak and Fang, Lan Tang, Ling Ye, Xing Li, Zheng Cai, 2019). In summary, fecal microbiota transplantation can be an excellent alternative method for the treatment or amelioration of several neurological and metabolic diseases, nevertheless further studies are required for its full understanding and serious guidelines to use FMT must be established since potential risk of serious infections caused by multidrug microorganisms were already identified (https://www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/important-safety-alert-regarding-use-fecal-microbiota-transplantation-and-risk-serious-adverse).

2. Objectives

The main goal of this study was to evaluate how the overexpression or silencing of a particular gene involved in the cholesterol metabolism (under corporate confidentiality) in the hypothalamus alters, in mice under different diets:

1) the intestinal microbiota of mice;
2) the cognitive abilities of mice;
3) The colon status of mice.

3. Material and Methods

3.1. Material

- Autoclave Uniclave 88 AJC (Lisbon, Portugal)
- Analytical balance AE 200, Mettler (USA)
- Analytical balance XS-410, Fisher Scientific (Portugal)
- Bio48 Laminar Flow Chamber, Faster (Italy)
- Mini-V / PCR camera, Telstar (Spain)
- Ultra-low temperature freezer -80ºC U725, Innova New Brunswick Scientific (USA)
- Electrophoresis Power Supply - EPS 301 (USA)
- *GNA 100* electrophoresis instrument, *Pharmacia Biotech (*Sweden)
- Binder Incubator, Keison (Germany)
- Kodak EDAS 290 (USA)
- Thermocycler T-Gradient (Biometra, Germany)
- Thermocycler T-personal (Biometra, Germany)
- Thermocycler T1 (Biometra, Germany)
- Vortex REAX 2000, Heidolph (Germany)
- Centrifuge Mikro 200, Hettich Zentrifugen (UK)
- Centrifuge Mikro 200R, Hettich Zentrifugen (UK)
- PCV-2400 Combi-spin combined centrifuge/vortex mixer, Grant-bio (UK)
- Multiplaces dry heating bath, Selecta (Spain)
- Real-Time PCR detection system CFX96, Bio-rad (USA)
- BTG shaking thermostatic bath, Bunsen (Spain)
- Axio Imager Z2, Zeiss (Norway)
- Heating Magnetic Stirrer, VELP Scientifica (Italy)
- NanoDrop 2000 Spectrophotometer, Fisher Scientific (Portugal)

3.1.1. Solutions

- X-Gal solution (50 mg / ml) (Sigma), dissolved in dimethyl sulfoxide (DMSO);
- Antibiotic Ampicillin solution (50 mg / ml) (Sigma), dissolved in sterile distilled water;
- Antibiotic Ampicillin solution (100 mg / ml) (Sigma), dissolved in sterile distilled water;
- Calcium chloride (50mM);
- Solution of potassium acetate (3M);
- Solution of 0.2 M NaOH / SDS 1% (w/v) solution;
- Ethylenediamine Tetra Acetic acid (EDTA) 0.5 M pH 8.0;
- Glucose-Tris-EDTA (GTE) buffer (50 mM glucose, 25 mM tris, 10mM EDTA) pH 8.0;
- Tris-Acetate-EDTA buffer (TAE) (40 mM Tris-acetate, 1 mM EDTA) pH 8.3;

3.1.2. Culture media

- Luria Broth base, Miller (LB) prepared according to the manufacturer's instructions.

When necessary, culture media was supplemented with Ampicillin (50 mg / mL) (Sigma) or Ampicillin (100 mg / mL) (Sigma), X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) (0.1%, w / v) (VWR) or agar (1.5% w / v) (Invitrogen).

3.1.3. Primers

The primers used in the current study were purchased from Sigma and are listed in the **Table** 3.1.

Table 3.1. List of DSR and 16S rRNA gene-targeted primers used in this study

Target Group	Primer	Sequence (5'–3')	Amplicon length (bp)	Optimal Annealing temperature(ºC)	Reference
Bacteroidetes	*BactesF*	CATGTGGTTTAATTCGATGAT	126	60	Weisburg et al., (1991)
	BactesR	AGCTGACGACAACCATGCAG	126		
Firmicutes	*Firm934F*	GGAGYATGTGGTTTAATTCGAAGCA	126	60	Yang, Y. W. et al., (2015)
	Firm1060R	AGCTGACGACAACCATGCAC	126		
Actinobacteria	*Act664F*	TGTAGCGGTGGAATGCGC	277	60	Yang, Y. W. et al., (2015)
	Act941R	AATTAAGCCACATGCTCCGCT	277		
Tenericutes	*Ten662F*	ATGTGTAGCGGTAAAATGCGTAA	200	60	Yang, Y. W. et al., (2015)
	Ten862R	CMTACTTGCGTACGTACTACT	200		
Betaproteobacteria	*Beta979F*	AACGCGAAAAACCTTACCTACC	174	59	Yang, Y. W. et al., (2015)
	Beta1130R	TGCCCTTTCGTAGCAACTAGTG	174		
Delta and Gammaproteobacteria	*Gamma877F*	GCTAACGCATTAAGTRYCCCG	189	60	Yang, Y. W. et al., (2015)
	Gamma1066R	GCCATGCRGCACCTGTCT	189		
16S rRNA gene	*27F*	AGAGTTTGATCCTGGCTCAG	aprox.1500	58	Yang, Y. W. et al., (2015)
	1525R	AAGGAGGTGWTCCARCC	aprox.1500		
Universal	*926F*	AAACTCAAAKGAATTGACGG	136	55	Yang, Y. W. et al., (2015)
	1062R	CTCACRRCACGAGCTGAC	136		
dsrB gene	*DSRp2060F*	CAACATCGTYCAYACCCAGGG	350	59	Kjeldsen et al., (2009)
	DSR4R	GTGTAGCAGTTACCGCA	350		

3.2. Methods

3.2.1. Animal experimentation

All the animal experiments were performed at the animal facility of the Centre of Biomedical Research (CBMR) of the University of Algarve in accordance with the Portuguese regulation, the Decree Law nº 113/2013, in accordance to the ethical issues for clinical research, European Directive for animal research (63/2010/EU) and the recommendations from the Federation of European Laboratory Animal Science Association (FELASA), regarding the use of animals for scientific purpose.

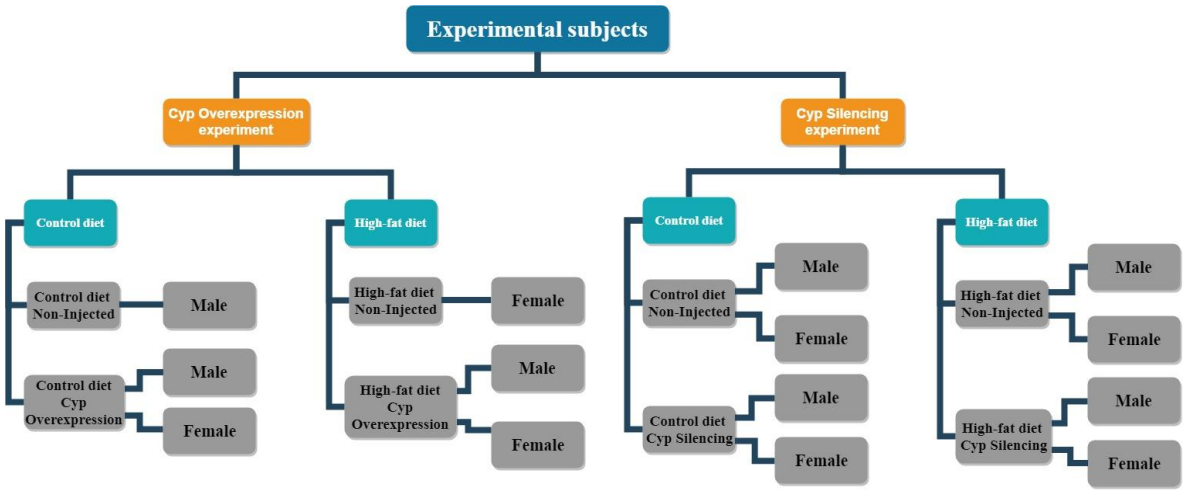


Figure 3.*1*. Schematic overview of the experimental approach for targeting *Cyp* Overexpression and Silencing. The animals were subjected to a Control diet and an HFD and included male and female individuals.

The experimental groups were divided in two cohorts, in a total of 69 C57BL/J6 mice: 29 of these mice were included in the first cohort, where the *Cyp* overexpression was performed (through lentiviral vectors), the remaining 40 mice were included in the second cohort for *Cyp* silencing (through a shRNA encoded by lentiviral vectors) and non-injected groups. In each experimental group both males and females were included. In each experimental group (CYP, shCYP and Non-injected controls) half of the animals were fed with a control diet (20% kcal protein, 10 % kcal fat, 70% kcal carbohydrate, Energy density 3.82 kcal/g, Research Diets, USA) and the other half fed with a High Fat Diet (HFD) (20 % kcal protein, 60% kcal fat, 20 % kcal carbohydrate, Energy density 5.21 kcal/g, Research Diets, USA), for 8 weeks after the stereotaxic injection to deliver the *Cyp* and the shRNA. Before the surgery the animals were already fed with the respective diets.

For each experimental group, fecal samples were collected once a month during the 3 months of the experiment. The samples were maintained at -80ºC until use for DNA extraction. In total the fecal samples were collected within four time points (T0-T3). However, in the first cohort, fecal collections from T1 were accidently lost, thereby bacterial quantifications for this time point are not shown.

3.2.2. DNA extraction from Fecal samples

For DNA extraction 100-240 mg of feces of mice were used. The DNA extraction was performed using the QIamp PowerFecal DNA Kit (Qiagen) according to the manufacturer's instructions with a slight modification on the lysis step in order to reduce the degradation of the extracted DNA. For this, instead of using beadbeater for fecal homogenization and bacterial lysis, temperature treatment was used as alternative by after the addition of C1 reagent the sample was homogenized by vortex for 5 seconds followed by water bath at 70ºC for 5 min, following a new vortex step for 5 seconds and a final water bath exposure at 70ºC for 5 min. The extracted DNA was eluted in 50 µl of C6 solution and maintained at -20ºC.

Each sample of DNA was quantified by Nanodrop (Fisher Scientific) and its integrity was analysed by agarose gel electrophoresis 1% (w / v) with the addition of 6.5 µl of the dye Green Safe (diluted 1:10). In each well, 2 µl of DNA and 1 µl of Loading buffer 6x concentrate (VWR) were loaded. The DNA ladder "GeneRuler ™ DNA Ladder Brews 1 kb" was used. The agarose gel electrophoresis was performed in 1 x TAE buffer at 120 V for 30 min.

3.2.3. Amplification of 16s rRNA and *dsr* genes by PCR

The genes *16S rRNA* and *dsr* were amplified by polymerase chain reaction (PCR) from fecal samples and used in the cloning process. Prior to the amplification, the best annealing temperature for each primer pair was determined using a gradient thermocycler (T-gradient, Biometra).

The amplification reaction was prepared for a final volume of 25 µl, and included 1 x PCR Buffer (Bioron), 0.2 mM of each dNTP, 10 ρmoles/µL of each primer, 5 U /µl of DFS-Taq DNA Polymerase, $MgCl_2$ 50mM/ml and 1 µl of DNA (132.1 ng/µl). The PCR conditions were one initial denaturing step for 5 min at 95ºC, followed by 30 cycles of 95ºC for 45s min, each annealing temperature for 45 seconds and the extension step was performed at 72ºC for 1,30 min, and a final step with 1 cycle of 72ºC for 3 min.

For the primers pair Firm934F/Firm1060R, a final volume of 25 µl was used containing 5 x Colorless GoTaq Flexi Buffer, 0.2 mM of each dNTP, 10 ρmoles/µL of each primer, 5 U /µl of GoTaq Flexi DNA Polymerase, $MgCl_2$ 25 mM/ml and 1 µl of DNA with the concentration of 132.1 ng/µl.

Each amplified fragment was analysed by agarose gel electrophoresis (1%, w / v) (Generose), 6.5 µl of the dye Green Safe at a concentration of 1:10. The gel electrophoresis was performed in 1 x TAE buffer at 120V for 30 minutes. In each well, 2 µl of DNA and 1 µl of Loading buffer 6x concentrate (VWR) were loaded. The DNA ladder "GeneRuler ™ DNA Ladder Brews 1 kb" was used.

3.2.4. Insertion of each amplified fragment of interest into the cloning vector

In the present study, vectors carrying each target fragment were used as standard DNA for the identification of the specific bacterial targets and qPCR quantifications. Since less or excess amounts of insert can inhibit the ligation reaction, the necessary amount of each insert was calculated according to the following formula:

$$ng\ of\ insert = \frac{(ng\ of\ the\ vector) \times insert\ size\ (kb)}{\text{vector size (kb)}} \times molar\ ratio\ of\ \frac{insert}{vector}$$

All ligation reactions were performed using the vector pCR ™ 2.1-TOPO (Invitrogen). The composition of each target insert is indicated in **Table 3.2**.

Table 3.2. List of the components of each reaction using the vector pCR ™ 2.1-TOPO.

Reagents	**Volume (µl)**	
	Sample	**Control**
Fresh PCR product (*Bacteroidetes*)	0.5 µl	------
Salt solution (1.2M NaCl & 0.06M $MgCl_2$)	1µl	1µl
Sterile H_2O	3.5 µl	5 µl
pCR ™ 2.1-TOPO (10 ng/µl)	1µl	1µl

Reagents	Volume (µl)	
	Sample	**Control**
Fresh PCR product (*Firmicutes*)	0.5 µl	------
Salt solution (1.2M NaCl & 0.06M $MgCl_{2)}$	1µl	1µl
Sterile H_2O	3.5 µl	5 µl
pCR ™ 2.1-TOPO (10 ng/µl)	1µl	1µl

Reagents	Volume (µl)	
	Sample	**Control**
Fresh PCR product (SRB)	4µl	------
Salt solution (1.2M NaCl & 0.06M $MgCl_{2)}$	1µl	1µl
Sterile H_2O	------	5µl
pCR ™ 2.1-TOPO (10 ng/µl)	1µl	1µl

Reagents	Volume (µl)	
	Sample	**Control**
Fresh PCR product (*Beta Proteobacteria*)	0.6 µl	------
Salt solution (1.2M NaCl & 0.06M $MgCl_{2)}$	1µl	1µl
Sterile H_2O	3.4 µl	5 µl
pCR ™ 2.1-TOPO (10 ng/µl)	1µl	1µl

Reagents	Volume (µl)	
	Sample	**Control**
Fresh PCR product (*Delta* and *Gamma Proteobacteria*)	0.6 µl	------
Salt solution (1.2M NaCl & 0.06M $MgCl_{2)}$	1µl	1µl
Sterile H_2O	3.4 µl	5 µl
pCR ™ 2.1-TOPO (10 ng/µl)	1µl	1µl

Reagents	Volume (µl)	
	Sample	**Control**
Fresh PCR product (*Tenericutes*)	0.5µl	------
Salt solution (1.2M NaCl & 0.06M $MgCl_{2)}$	1µl	1µl
Sterile H_2O	3.5µl	5 µl
pCR ™ 2.1-TOPO (10 ng/µl)	1µl	1µl

Reagents	Volume (µl)	
	Sample	**Control**
Fresh PCR product (*Actinobacteria*)	1 µl	------
Salt solution (1.2M NaCl & 0.06M $MgCl_{2)}$	1µl	1µl
Sterile H_2O	3 µl	5 µl
pCR ™ 2.1-TOPO (10 ng/µl)	1µl	1µl

3.2.5. Production of *Escherichia coli* MACH1 competent cells

Bacterial transformation is a natural process, where cells capture exogenous DNA from their environment, expressing the acquired genetic information, which is thought to be a source of genetic diversity or can also confer benefits to the host, such as antibiotic resistance. However, this process occurs at low frequency and not all bacterial species are able to take up foreign DNA from their environment. The ones which are able to undergo bacterial transformation are designed as competent cells. The standard chemical transformation protocol consists of using a suspension of bacteria in Log phase, in a cold calcium chloride solution increasing their permeability and then exposing them to a heat-shock treatment in the presence of the foreign DNA.

For generation of *Escherichia coli* MACH1 competent cells for further transformation with the vector pCR ™ 2.1-TOPO containing each fragment of interest, the *E. coli* MACH1 cells were firstly cultured in *Luria Bertani* (LB) agar. From this culture, 1 single colony was picked into 10 ml of *Luria Bertani* broth and grown overnight at 37 ºC with agitation at 120 rpm. From this overnight culture, 200 µl were transferred into 10 ml Luria Bertani broth and the incubation was

done at 37ºC with agitation at 120 rpm until an absorbance at 550 nm (A_{550nm}) of about 0.5-0.6 was reached. Afterwards the bacterial culture was centrifuged at 2790 x g for 5 min at 4ºC. The supernatant was discharged, and the bacterial pellet resuspended in 5 mL of 50 mM $CaCl_2$ previously cooled. The bacterial suspension was left on ice for 20 min, and then centrifuged at 2790 x g for 5 min at 4ºC, following the discharge of the supernatant. The bacterial pellet was resuspended in 1 mL of $CaCl_2$, previously cooled. The bacterial samples were maintained on ice for 1h 30 min. Afterwards, 1mL of LB with glycerol (50%, v/v) was added to the bacterial suspension. 150 µl of the bacterial cell suspension was distributed by cryotubes previously refrigerated and maintained at -80ºC until use.

3.2.6. Transformation of *Escherichia coli* MACH1 competent cells

After generating *E. coli* MACH1 competent cells, 2 µl of the ligation reaction were added into a vial of 150 µl containing MACH1 competent cells, mixed gently and maintained on ice for 20 min. After this time interval, a Heat-shock step (42ºC) for 30 s was performed and transferring the tube immediately to ice. This step is critical since the heat shock step strongly depolarizes the cell membrane of $CaCl_2$-treated cells decreasing the negative charge of the cell, which allows the movement of negatively charged DNA into the cell. The further cold shock raises again the membrane potential to its original value.

After heat-shock treatment, 250 µl of LB medium at room temperature was added, and the bacterial culture was allowed to grow at 37ºC for 1h with agitation (200 rpm). Afterwards, 50 µl from each transformation was spread on LB prewarmed plates (37ºC) supplemented with 50 mg / mL ampicillin and 40 µl of X-Gal (50 mg / ml). The inoculated plates were incubated at 37ºC overnight. After this incubation time, white colonies (the ones with successful transformation with the vector) were selected for further Plasmid DNA extraction and identification of the isolated clones.

3.2.7. Plasmid extraction and purification – Miniprep

As mentioned above, the transformed cells (white colonies) were selected for plasmid extraction and purification. For this purpose, each colony was grown overnight in 5 mL of LB medium

supplemented with 100 mg / mL ampicillin and grown in a water bath at 37 ºC with agitation (150-180 rpm.) After incubation, 2 mL of bacterial culture was transferred to an Eppendorf and centrifuged at 16 000 x g at 4ºC for 2 min. After the discharge of the supernatant, the pellet was resuspended in 200 µl of GTE buffer (which will provide a stable environment for the DNA), together with 20 µl of 10 mg / mL RNAse A, being briefly vortexed and left at room temperature for 10 min. After this time interval, 400 µl of a freshly prepared solution of NaOH 0.2 M / SDS 1% (w/v) solution was added, mixed by inversion and incubated on ice for 5 min (incubation should not take more than 5 min, since this solution may cause irreversible damage to the plasmid DNA). After alkaline lysis step, 300 µl of neutralizing solution 3M potassium acetate (cold) was added and mixed by inversion until a white precipitate was produced, following an incubation on ice for 30 min. After incubation, the mixture was centrifuged twice at 16000 x g at 4ºC for 15 min for complete protein elimination. The supernatant was transferred to a new Eppendorf (2 mL) and 1 mL of cold ethanol at 100% was added, and briefly vortexed, after which was maintained at room temperature for 2 min. Afterwards the precipitated DNA was centrifuged at 16 000 x g for 15 min (4ºC), after which the supernatant was discharged. A washing step was performed twice by adding 200 µl of ethanol (75 %) to the pellet and centrifuging at 16000 x g for 5 min (4ºC). The isolated plasmid DNA was air-dried near to a flame, accelerating the drying process and also avoiding any possible contamination, until all ethanol residues were evaporated. The final purified plasmid DNA was dissolved in nuclease-free water (Accugene, Lonza) and maintained on ice for 1-2 h for proper rehydration. Each extracted plasmid DNA sample was maintained at -20ºC until use. Furthermore, each clone was sequenced at the Molecular Biology Laboratory of the Centro de Ciências do Mar (CCMar) using Sanger sequencing. The reverse primer of each bacterial group was used for sequencing. The identification of each clone was performed using the Blast algorithm of the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.8. Quantitative Polymerase chain Reactions (qPCR)

In order to quantify each bacterial group of interest, quantitative real-time PCR (qPCR) was performed.

Prior to quantifications, standard curves were constructed (**Figure** 3.2) for each bacterial group by using dilutions of the plasmid DNA. Each PCR reaction for constructed standard curves consisted

in 5 µl of SsoFastTM kit Eva Green Supermix (BioRad), 3 µl of water, 0.5 µl (10 ρmoles/µL) of each primer and 1µl of the DNA sample.

The mass of each amplified fragment was calculated using the following formula:

$$Mass\ of\ each\ fragment\ (ng) = \text{size of the fragment (bp)} \times (1.096 \times 10^{-21})$$

Then, the mass of each fragment was used to calculate the Copy numbers per µl in each dilution using the following formula, and then converted to logarithmic scale.

$$Copy\ Numbers\ per\ \mu l = \frac{Concentration\ of\ each\ dilution\ (\frac{ng}{\mu l})}{\text{Mass of each amplified fragment (ng)}}$$

To perform the bacterial quantifications, SsoFastTM kit Eva Green Supermix (BioRad) was used and the PCR reaction was similar to the above PCR reaction but was used 1µl of each fecal DNA sample. The program consisted of 1 cycle of 95ºC for 10 min, followed by 40 cycles of 95ºC for 40 s, each target primer annealing temperature for 40 s and 72ºC for 30 s. Melting curve was performed by increment of 0.5ºC from temperature 72ºC to 95ºC at each 2 s during the extension step.

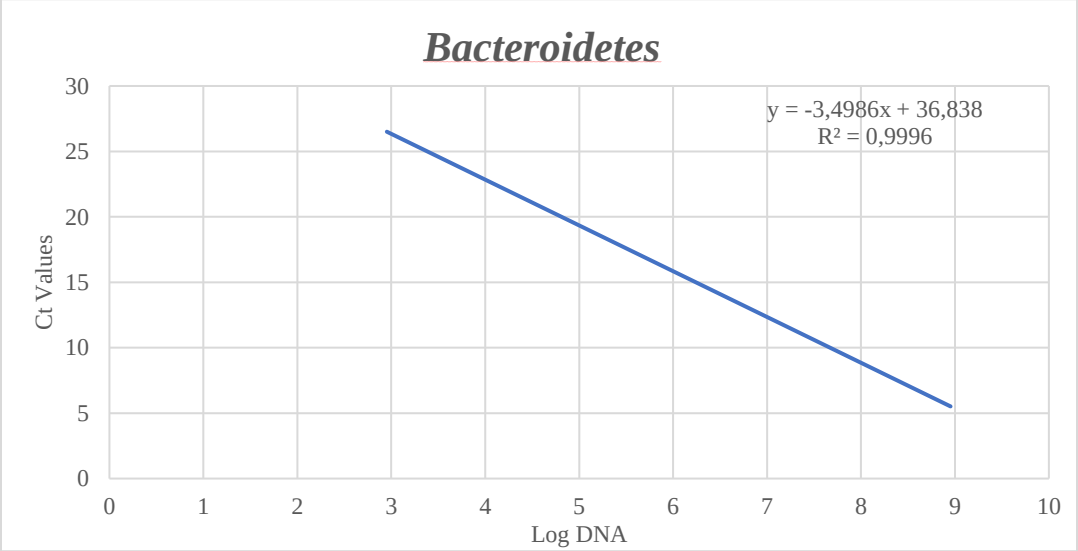
The copy numbers of each group present in each sample were inferred from each constructed standard curve.

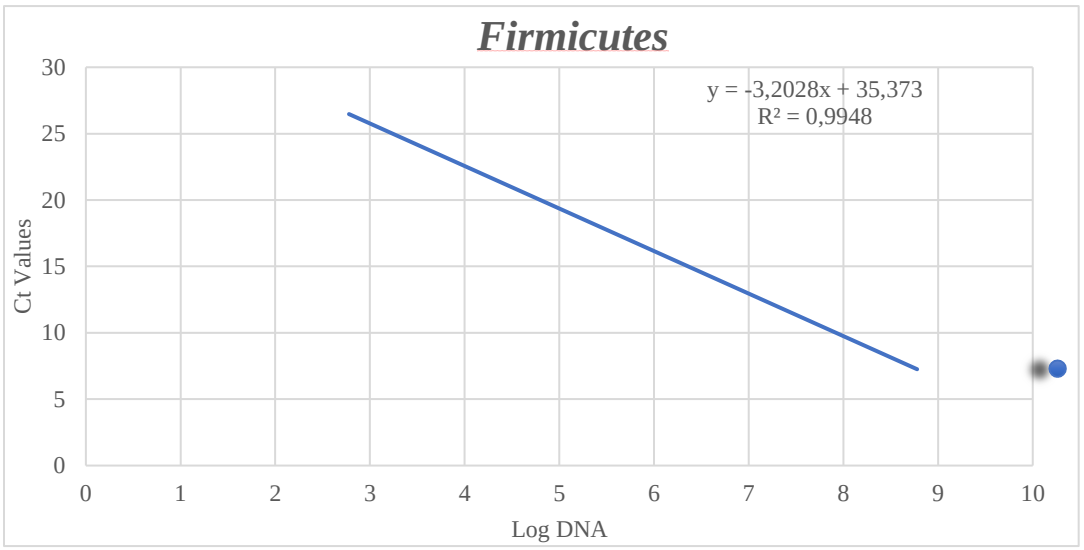
The amplification efficiency of each reaction was calculated using the formula below, and each efficiency value for the used primers is indicated in **Table** 3.3.

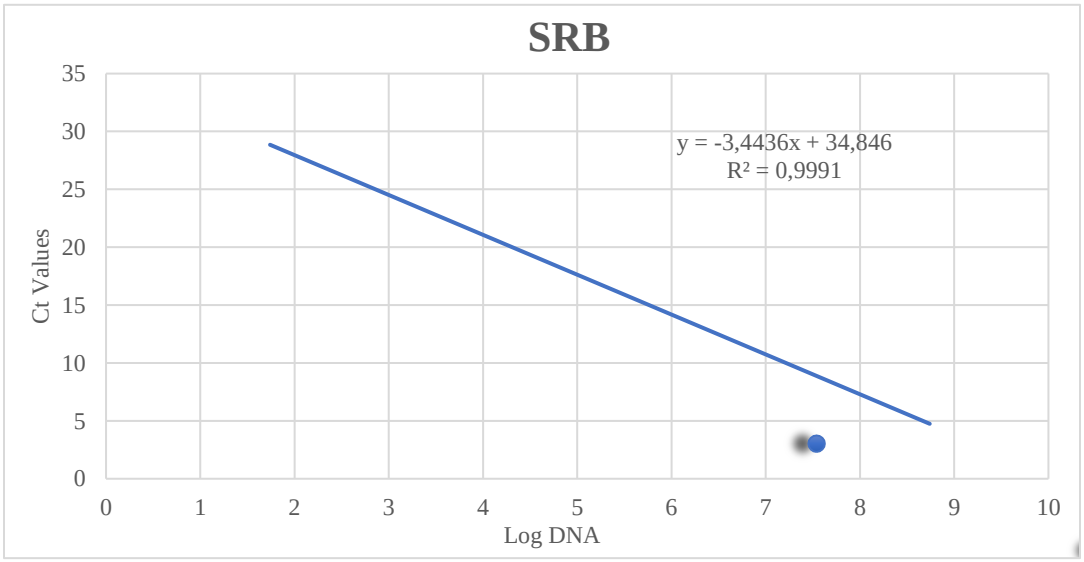
$$\text{Eficciency of each reaction} = 10^{(-\frac{1}{slope})} - 1$$

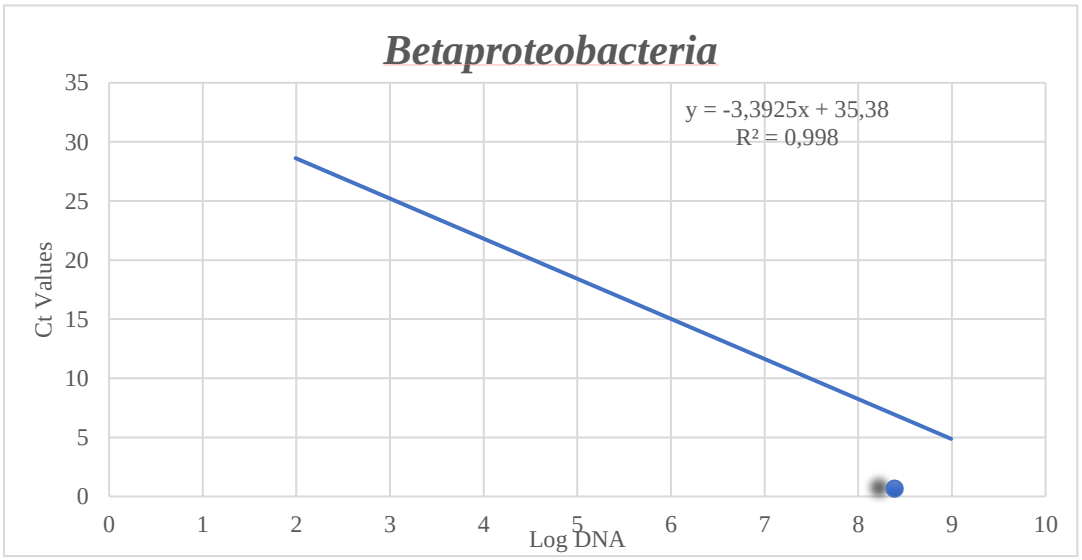
Table 3.3. Efficiencies of each pair primers calculated by qPCR.

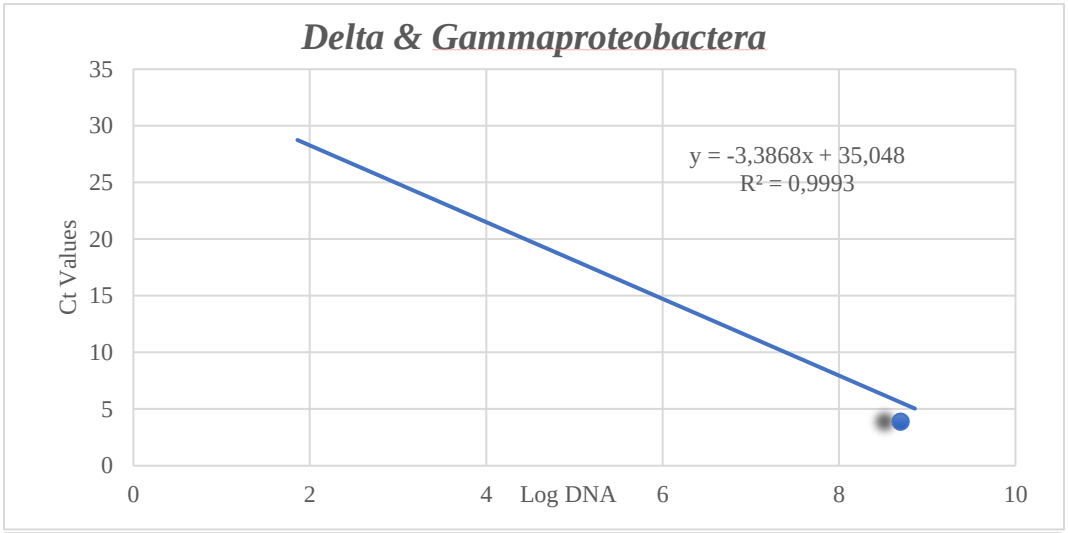
Primers	***Primer (%)*** ***Efficiency***
BactesF	93.10
BactesR	
Firm934F	105
Firm1060R	
Act664F	94.50
Act941R	
Ten662F	91.60
Ten862R	
Beta979F	97.10
Beta1130R	
Gamma877F	97.40
Gamma1066R	
DSRp2060F *DSR4R*	95.20

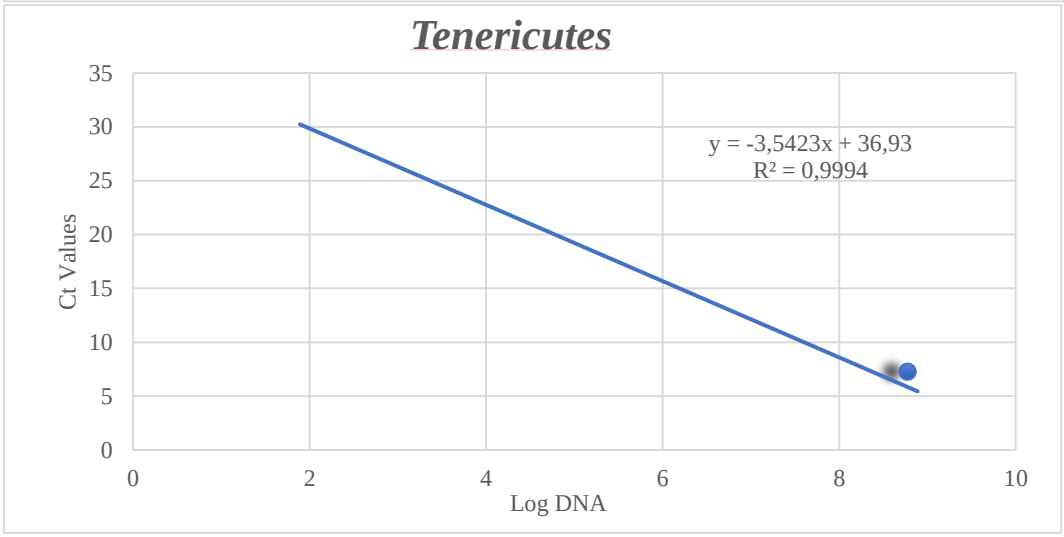



Firmicutes
y = -3,2028x + 35,373
R² = 0,9948
Ct Values
Log DNA
0
5
10
15
20
25
30
0
1
2
3
4
5
6
7
8
9
10


SRB
y = -3,4436x + 34,846
R² = 0,9991
Ct Values
Log DNA
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
7
8
9
10


Betaproteobacteria
y = -3,3925x + 35,38
R² = 0,998
Ct Values
Log DNA
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
7
8
9
10


Delta & Gammaproteobactera
y = -3,3868x + 35,048
R² = 0,9993
Ct Values
Log DNA
0
5
10
15
20
25
30
35
0
2
4
6
8
10


Tenericutes
y = -3,5423x + 36,93
R² = 0,9994
Ct Values
Log DNA
0
5
10
15
20
25
30
35
0
2
4
6
8
10

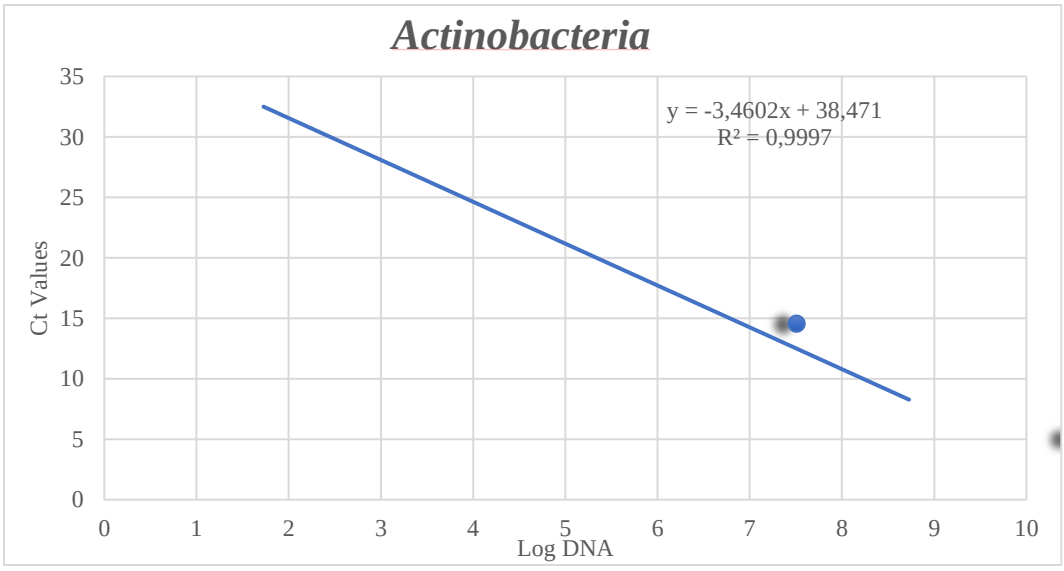

Actinobacteria
y = -3,4602x + 38,471
R² = 0,9997
Ct Values
Log DNA
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
7
8
9
10

Figure 3.2. Standard curves for qPCR of *Bacteroidetes*, *Firmicutes*, Sulphate-reducing bacteria (SRB), *Betaproteobacteria*, *Delta* -and *Gammaproteobacteria*, *Tenericutes and Actinobacteria*. Three replicates were used.

3.2.9. Y-maze spontaneous alternation test

The Y-maze spontaneous alternation test was conducted using a three-armed Y-shaped maze. The first measurement was performed just right before the Time point 1 (T1), where the individuals were previously subjected to the tested diets during 1 month (**Figure** 3.3). The next measurements were performed once a month, in a total of three measurements for the entire experiment. This type of test can be used to study several regions of the brain, such as hippocampus, septum, basal forebrain and prefrontal cortex (Fröhlich *et al.*, 2016). Every single mouse was placed at the beginning of the arm A and their movements were recorded for 5 minutes, for further analysis of total arm entries, total distance travelled and spontaneous alternations. Over the course of the experiment, each animal should show a tendency to visit new arms, instead of going back to the previously visited, in this way it was possible to evaluate their exploratory behaviour (Miedel *et al.*, 2017). Each recorded video was analysed in an automated way using ANY-maze software (http://www.anymaze.co.uk/). The percentage of spontaneous alternations was calculated according to the following formula and used for further analysis.

$$Spontaneous\ Alternations\ \% = \frac{\#Spontaneous\ Alternations}{\text{Total number of arm entries} - 2} \times 100$$

3.2.10. Intestinal histological analysis

After each animal sacrifice, intestines were placed in a tissue processing/embedding cassette. For dehydration, a series of graded ethanol baths were performed in the following way; treatment with 70% ethanol (v/v) for 1 h; 95% ethanol (v/v) for 45 min; 95% ethanol (v/v) for 40 min and a final treatment with 100% ethanol (v/v) for 1 h performed twice, followed by clearing with a treatment with xylene (Fisher chemical) performed twice and a last step of infiltration with two times treatment with paraffin wax (Luso Palex) in the incubator, at 56 ºC for 1 h. After this procedure, the intestinal samples were mounted in embedding molds (Tebu-bio), embedded with paraffin at

56ºC and cooled down at room temperature, obtaining paraffin blocks. Afterwards, the embedding molds were separated from the paraffin blocks.

After tissue treatment described above the intestinal samples were stained by Hematoxylin-eosin staining, according to the manufacturer's instructions (Merck Millipore).

Each intestinal sample images were acquired in the microscopy facility of the Centre of Biomedical Research at the Universidade do Algarve, using the Axio Imager Z2 microscope (Carl Zeiss), AXIOCAM-ICC3 camera and AxioVision software (Cal Zeiss), and finally imported to ImageJ – Fiji software for further analysis.

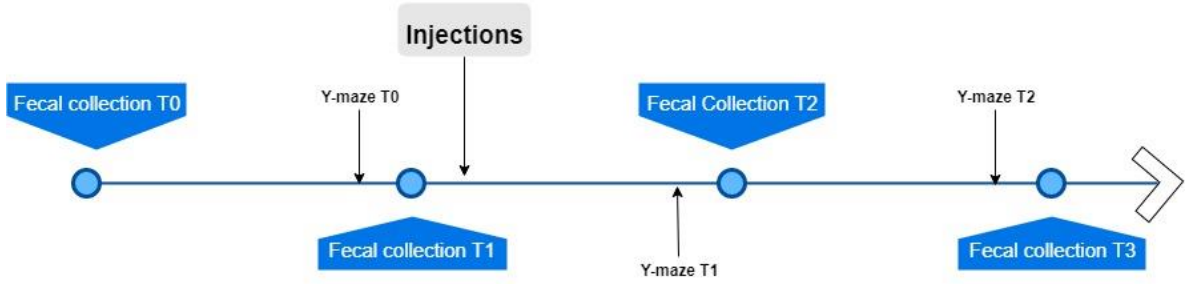


Figure 3.3. General overview of the experiment design. In total, the experiment lasted 3 months with four fecal collections performed once a month and three Y-maze spontaneous alternations tests performed once a month. Injections for *Cyp* overexpression and *Cyp* silencing were performed just right after the fecal collection on Time point 1 (T1). T0 - Start of the study; T1 - one month after intervention; T2 - two months after intervention; T3 - three months after intervention.

3.2.11. Statistical analysis

The statistical significance between samples was determined by one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test ($P<0.05$) using IBM SPSS Statistics 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

The linear regression method (Pearson's correlation) was used to analyse the correlation between body weight gained and the counts of both *Bacteroidetes* and *Firmicutes* groups, using IBM SPSS Statistics 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

For intestinal microbiota correlation analysis between diets and also *Cyp* intervention, correlation

analysis was conducted in R (R version 3.6.1, 2019). The Figures were obtained using the package ggplot2 (Wickham, 2009).

The statistical significance of spontaneous alternations, distance travelled and arm entries, between *Cyp* silencing and *Cyp* overexpression, a two-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com).

4. Results

4.1. Quantification of Bacterial Groups

The main goal of this work was to analyze the effects of the overexpression and silencing of a specific gene in the hypothalamus on the intestinal microbiota of mice. Therefore, the quantification of several intestinal bacterial groups was performed using qPCR. The quantified bacterial groups included *Bacteroidetes*, *Firmicutes*, Sulphate-Reducing Bacteria (SRB), *Betaproteobacteria*, *Delta-and Gammaproteobacteria*, *Actinobacteria* and *Tenericutes*. The results are summarized in the **Table** 4.1 and 4.2.

Regarding the results of the 1st Cohort where the overexpression of *Cyp* was analysed (CYPoverex), the quantifications of *Bacteroidetes* evidenced that all individuals at the start of the experiment (T0) showed similar numbers ($P>0.05$), except the male mice from the High Fat Diet (HFD) group and the female mice from the Control diet group that were subjected to *Cyp* overexpression, which showed the highest numbers of *Bacteroidetes* (8.27±0.04 and 8.60±0.00 Log_{10} DNA copies/g feces, respectively). However, overtime the numbers of *Bacteroidetes* decreased (**Table** 4.1). At the end of the experiment (T3) the HFD group, either subjected to *Cyp* overexpression or Non-injected (NI) showed similar numbers of *Bacteroidetes* ($P>0.05$), in contrast with the Control diet group, where the NI mice showed the lowest number ($P<0.05$) at this sampling time (6.55±0.04 Log_{10} DNA copies/g feces) and the female *Cyp* overexpression mice showed the highest numbers (8.44±0.02 Log_{10} DNA copies /g feces) (**Table** 4.1). The Pearson's correlation analysis revealed a negative correlation between the *Bacteroidetes* population and body weight ($r= -0.636$; $P < 0.01$), which denotes that with the increase on body weight, the number of *Bacteroidetes* decreases.

The quantification of *Firmicutes* at the start of the experiment (T0) showed that the mice in the HFD group are significantly different ($P<0.05$), namely the female NI, which showed the lowest number ($P<0.05$) of *Firmicutes* (6.60±0.01 Log_{10} DNA copies /g feces), whereas the female and male with *Cyp* overexpression showed similar higher numbers ($P>0.05$) (**Table** 4.1). The mice under the Control diet also showed significant differences ($P<0.05$), particularly the female with *Cyp* overexpression, which showed the lowest number of *Firmicutes* (7.55±0.03 Log_{10} DNA copies/g feces), whilst the male mice both NI and with *Cyp* overexpression showed higher similar

numbers (P>0.05), which were also similar to the HFD female with overexpression (P>0.05) (**Table** 4.1). As for the *Bacteroidetes* and for the *Firmicutes* phylum, the tendency to decrease the numbers over time was also observed. At the end of the experiment (T3), the Control diet NI male mice showed the lowest number of *Firmicutes* (6.63±0.14 Log_{10} copies /g feces), whereas all the other groups showed similar loads (P>0.05) (**Table** 4.1). Additionally, considering the ratio of *Firmicutes* to *Bacteroidetes*, no significant changes were observed on the tested groups, except the female *Cyp* overpexpression mice from the Control diet group and the female NI mice from the HFD group, showing both a slight increase overtime (**Table** 4.1).

Concerning Sulphate-Reducing Bacteria (SRB) at T0, the different groups showed significantly different numbers (P<0.05), either the mice under the Control diet or under the HFD (**Table** 4.1). The female NI mice in the HFD group showed the lowest (P<0.05) SRB numbers (3.21±0.05 Log_{10} DNA copies /g feces), in contrast with the male NI from the Control diet group that showed the highest (P<0.05) load (4.69±0.04 Log_{10} DNA copies /g feces). Over the experiment, it was observed an increase propensity in the SRB load in both diet groups. At T3 the HFD female *Cyp* overexpression mice showed the highest SRB numbers (5.08±0.04 Log_{10} DNA copies /g feces) followed by the male *Cyp* overexpression mice from the Control diet group (4.90±0.02 Log_{10} DNA copies /g feces) (**Table** 4.1).

Considering the results obtained for *Betaproteobacteria,* both diet groups showed similar numbers at T0 (P>0.05), except the male *Cyp* overexpression mice within control diet, which showed the lowest load (5.29±0.10 Log_{10} DNA copies /g feces) (**Table** 4.1). Through the sampling, a tendency towards a reduction of *Betaproteobacteria* was observed. The HFD mice together with the female with *Cyp* overexpression from the Control diet group achieved the highest load (P<0.05) of *Betaproteobacteria* **(Table** 4.1).

In terms of the Delta- and *Gammaproteobacteria* populations, it was observed that at T0 the members in the two diet groups showed significantly different (P<0.05) loads, the female with *Cyp* overexpression within control group mice displayed the highest content (5.34±0.02 Log_{10} DNA copies /g feces), whereas the male NI from the control diet group and the female NI mice from the HFD group showed the lowest load (4.68±0.08, 4.41±0.08 Log_{10} DNA copies /g feces, respectively) (P>0.05) (**Table** 4.1). In contrast with the populations of *Betaproteobacteria*, the populations of Delta- and *Gammaproteobacteria* have undergone an increase overtime. At the end of the experiment, the members of the HFD group showed similar numbers (P>0.05), which were

also similar to the male and female with *Cyp* overexpression mice from the Control diet group (P>0.05) (**Table** 4.1).

The numbers of *Tenericutes* at T0 were similar (P>0.05) for both diet groups, except for the female with *Cyp* overexpression mice from the Control diet group that showed the highest amount (6.20±0.00 Log_{10} DNA copies /g feces) (**Table** 4.1). Interesting for this bacterial group, the *Cyp* overexpression seems to affect differently the two genders in both diet groups, namely at time T3, as the male with *Cyp* overexpression mice from the Control diet group experienced a significant reduction (P<0.05), whereas the male with *Cyp* overexpression from the HFD group was able to recover the initial *Tenericutes* counts. In contrast, the female with *Cyp* overexpression from the HFD group, have undergone a more than 50% reduction on the *Tenericutes* levels. The female with *Cyp* overexpression from the Control diet group also experienced a reduction on the *Tenericutes* counts but not so prominent as observed in the female with *Cyp* overexpression mice from the HFD group (**Table** 4.1).

The results of the quantification of *Actinobacteria* group showed that at T0 the mice in both diet groups displayed significantly different amounts (P<0.05) (**Table** 4.1). The propensity towards an increase in *Actinobacteria* overtime was particularly evident for the HFD group, and in the female with *Cyp* overexpression from the Control diet group. The highest counts of *Actinobacteria* were observed in the female NI mice from the HFD group (7.28±0.01 Log_{10} DNA copies /g feces).

Table. 4.1. Quantification of the target intestinal bacterial groups in the 1st cohort (overexpression group [CYPoverex]). At time zero (T0) the new diets were introduced, after 8 weeks of exposure to the appropriate diet and also 4 weeks after injection(T2) and after 12 weeks of exposure to the same diet and also 8 weeks of injection (T3).

			Bacteroidetes (Log$_{10}$ DNA/g feces) *		
Target Group			T0	T2	T3
Control diet	Non-Injected	Male	7.92 ± 0.01^{cA}	7.16 ± 0.01^{bB}	6.55 ± 0.04^{aA}
	CYPoverex	Male	8.03 ± 0.03^{bA}	7.41 ± 0.03^{aC}	7.68 ± 0.14^{abB}
		Female	8.60 ± 0.00^{cC}	7.73 ± 0.00^{aD}	8.44 ± 0.02^{bC}

High Fat diet	Non-injected	Female	8.06±0.03^{bA}	6.97±0.10^{aB}	7.72±0.10^{bB}
	CYPoverex	Male	8.27±0.04^{cB}	6.30±0.07^{aA}	7.82±0.04^{bB}
		Female	8.06±0.09^{cA}	7.16±0.01^{aB}	7.76±0.04^{bB}

Firmicutes

Control diet	Non-Injected	Male	7.76±0.10^{bBC}	7.44±0.19^{bA}	6.63±0.14^{aA}
	CYPoverex	Male	7.90±0.06^{bC}	7.36±0.03^{aA}	7.64±0.16^{abB}
		Female	7.55±0.03^{bB}	7.22±0.04^{aA}	7.68±0.05^{bB}
High Fat diet	Non-injected	Female	6.60±0.01^{aA}	7.17±0.19^{abA}	7.22±0.15^{bB}
	CYPoverex	Male	7.81±0.09^{cC}	7.22±0.03^{bA}	7.24±0.03^{bB}
		Female	7.81±0.01^{bC}	7.31±0.01^{aA}	7.48±0.13^{aB}

Ratio of ***Firmicutes*/*Bacteroidetes***

			T0	T3
Control diet	Non-Injected	Male	0.98±0.01^{aCD}	1.01±0.02^{aC}
	CYPoverex	Male	0.98±0.01^{aD}	0.99±0.00^{aC}
		Female	0.88±0.01^{aB}	0.91±0.00^{bA}
High Fat diet	Non-injected	Female	0.82±0.00^{aA}	0.94±0.01^{bAB}
	CYPoverex	Male	0.94±0.02^{aC}	0.93±0.01^{aAB}
		Female	0.97±0.01^{aCD}	0.96±0.02^{aBC}

Sulphate-reducing bacteria

Control diet	Non-Injected	Male	4.69±0.04^{aE}	4.69±0.11^{aB}	4.42±0.03^{aA}
	CYPoverex	Male	3.80±0.02^{aB}	4.64±0.00^{bB}	4.90±0.02^{cC}

		Female	4.22 ± 0.06^{aCD}	4.38 ± 0.00^{aA}	4.69 ± 0.06^{bB}
High Fat diet	Non-injected	Female	3.21 ± 0.05^{aA}	4.78 ± 0.06^{cBC}	4.42 ± 0.02^{bA}
	CYPoverex	Male	4.20 ± 0.07^{aC}	4.67 ± 0.04^{bB}	4.30 ± 0.00^{aA}
		Female	4.41 ± 0.03^{aD}	4.96 ± 0.04^{bC}	5.08 ± 0.04^{bD}

Betaproteobacteria

Control diet	Non-Injected	Male	6.04 ± 0.02^{cB}	2.92 ± 0.00^{bB}	2.34 ± 0.02^{aB}
	CYPoverex	Male	5.29 ± 0.10^{cA}	4.09 ± 0.01^{bD}	1.89 ± 0.21^{aA}
		Female	6.20 ± 0.08^{bB}	3.73 ± 0.08^{aC}	3.79 ± 0.06^{aD}
High Fat diet	Non-injected	Female	6.10 ± 0.02^{cB}	2.82 ± 0.08^{aB}	3.75 ± 0.11^{bD}
	CYPoverex	Male	6.11 ± 0.01^{cB}	2.29 ± 0.03^{aA}	3.58 ± 0.01^{bCD}
		Female	6.05 ± 0.04^{cB}	3.89 ± 0.04^{bC}	3.32 ± 0.06^{aC}

Delta- and Gammaproteobacteria

Control diet	Non-Injected	Male	4.68 ± 0.08^{aAB}	6.01 ± 0.10^{cD}	5.48 ± 0.01^{bA}
	CYPoverex	Male	4.85 ± 0.03^{aBC}	5.59 ± 0.01^{bBC}	5.78 ± 0.11^{bAB}
		Female	5.34 ± 0.02^{aE}	5.95 ± 0.04^{bD}	5.95 ± 0.01^{bB}
High Fat diet	Non-injected	Female	4.41 ± 0.08^{aA}	5.80 ± 0.06^{bCD}	6.17 ± 0.21^{bB}
	CYPoverex	Male	5.19 ± 0.13^{aDE}	5.50 ± 0.07^{aAB}	6.01 ± 0.01^{bB}
		Female	5.03 ± 0.02^{aCD}	5.29 ± 0.03^{bA}	6.14 ± 0.04^{cB}

Tenericutes

Control diet	Non-Injected	Male	4.80 ± 0.13^{aA}	5.24 ± 0.09^{bE}	4.44 ± 0.00^{aB}
	CYPoverex	Male	5.16 ± 0.05^{bA}	5.37 ± 0.04^{bE}	2.67 ± 0.10^{aA}

		Female	6.20 ± 0.00^{cB}	4.85 ± 0.01^{aD}	5.63 ± 0.03^{bD}
High Fat diet	Non-injected	Female	4.63 ± 0.12^{bA}	3.05 ± 0.01^{aC}	4.26 ± 0.06^{abB}
	CYPoverex	Male	5.07 ± 0.05^{bA}	2.83 ± 0.04^{aB}	5.22 ± 0.04^{bC}
		Female	4.54 ± 0.10^{bA}	2.31 ± 0.04^{aA}	2.34 ± 0.20^{aA}

Actinobacteria

Control diet	Non-Injected	Male	6.78 ± 0.08^{abCD}	6.87 ± 0.07^{bCD}	6.51 ± 0.03^{aA}
	CYPoverex	Male	6.33 ± 0.03^{aA}	6.69 ± 0.06^{bC}	6.62 ± 0.06^{bA}
		Female	6.85 ± 0.03^{aD}	6.88 ± 0.03^{aD}	7.03 ± 0.05^{bB}
High Fat diet	Non-injected	Female	6.51 ± 0.01^{aB}	6.83 ± 0.02^{bCD}	7.28 ± 0.01^{cC}
	CYPoverex	Male	6.61 ± 0.05^{bBC}	6.36 ± 0.04^{aB}	7.03 ± 0.01^{cB}
		Female	6.88 ± 0.03^{bD}	5.73 ± 0.04^{aA}	7.09 ± 0.02^{cB}

* Data represent the mean ± standard deviation of two qPCR determinations replicates.

A principal component analysis (PCA) was performed to visualize the general differences among the 1st cohort samples, regarding the loads of the different bacterial groups. The PCA scatter plot showed that faecal samples from the start of the experiment (T0) were distinctly separated from the remaining samples (**Fig.** 4.1A), evidencing the consistency of the bacterial amounts at the start of the hypothalamus and diet intervention. The PCA scatter plot with the samples at the end of the experiment (**Fig.** 4.1B) evidences that the HFD mice are separated from the Control mice. However, is interesting to highlight that the female with *Cyp* overexpression from the Control group clustered with the female NI mice of the HFD group (**Fig.** 4.1B).

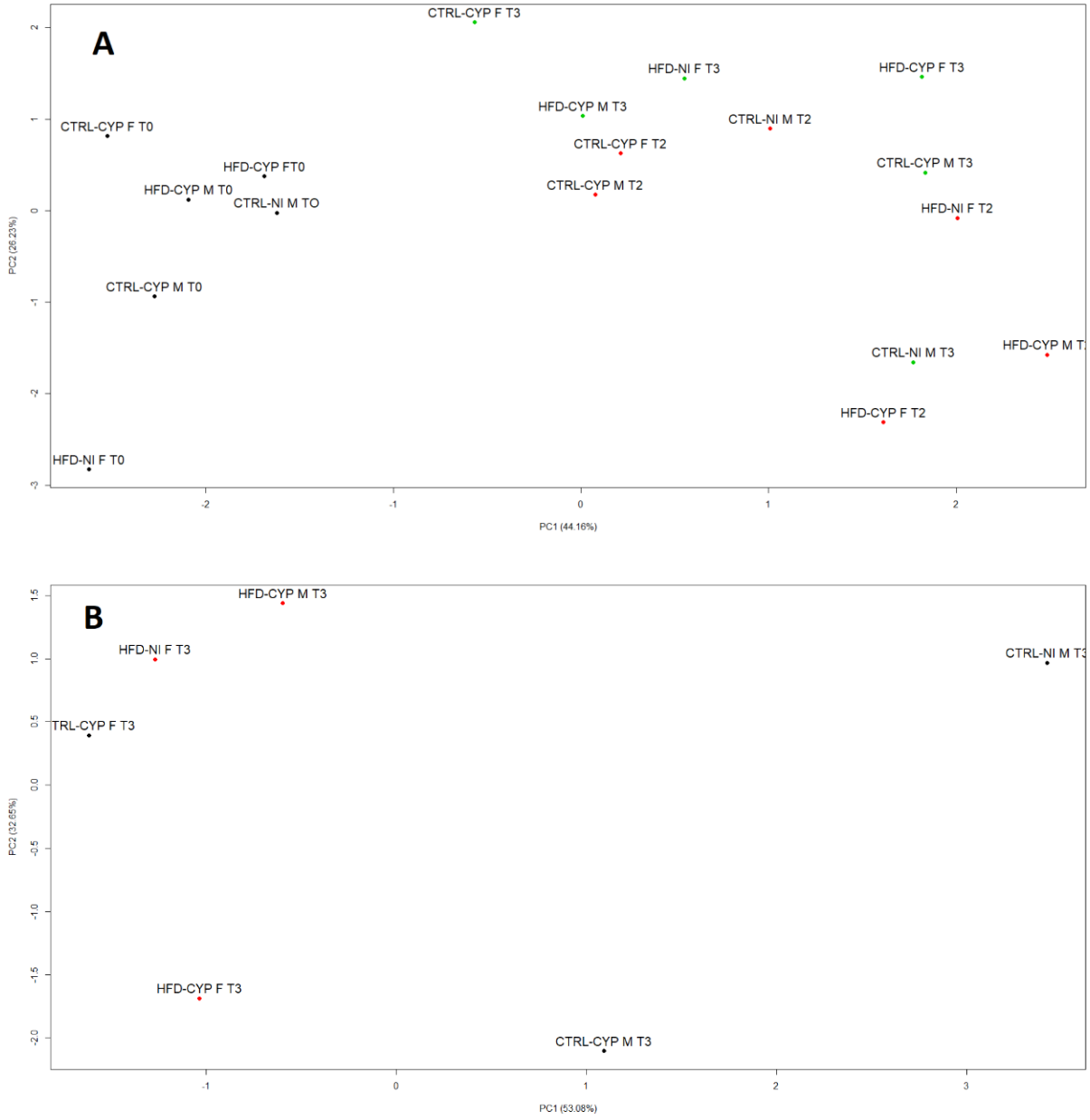


Figure 4.1. Intestinal bacteria showed significant diet-associated structure. **(A, B)** PCA scatter plot based on bacterial quantifications at different time points **(A)**, and at the end of the 1º cohort experiment (T3) **(B)**. Each circular point represents the fecal bacterial load of each-mice group, colored by time point **(A)**, and colored by the type of diet **(B)**.

The quantifications of *Bacteroidetes* on the *Cyp* silencing intervention (2nd cohort) showed that at the start of the experiment, the mice have distinct *Bacteroidetes* amounts, namely the female with the *Cyp* silenced from the HFD group, which showed the lowest content ($P<0.05$), whereas the mice from the Control diet group which were not injected (NI) (both male and female), the male

mice with the *Cyp* silenced from the Control diet group and the female NI from the HFD group, showed the highest *Bacteroidetes* counts (8.27±0.02, 8.33±0.02, 8.30±0.05 and 8.23±0.02 Log_{10} DNA copies /g feces, respectively) (**Table** 4.2). A propensity towards a slight decrease on *Bacteroidetes* counts overtime was observed. At the end of the experiment (T3) the most affected mice was the male NI from the Control group with 6.61±0.01 Log_{10} DNA copies /g feces (**Table** 4.2). In this cohort, also the *Bacteroidetes* loads were negatively correlated with the body weight gain, but in contrast with the 1st cohort, in this cohort the correlation value was not statistically significant (r=-0.337; P>0.05).

The quantifications of *Firmicutes* in this cohort showed that at the start of the experiment the mice also have distinct *Firmicutes* loads (**Table** 4.2). The female with *Cyp* silenced and the female NI from the HFD group showed the lowest (P<0.05) *Firmicutes* amounts. In contrast, the male with *Cyp* silenced mice from the control diet group, the male NI and the male with *Cyp* silenced from the HFD group, showed the highest *Firmicutes* counts (7.80±0.04; 7.62±0.06; 7.75±0.04 Log_{10} DNA copies /g feces, respectively). Overtime it was observed a decrease in the counts of *Firmicutes* in the male's mice, in contrast with the females that showed an increase in this phylum, except the female with *Cyp* silenced from the Control diet group, which remained unchanged at the end of the experiment. These finding seems to point out some gender effect on this phylum, for this cohort. At the end of the experiment (T3) large differences on *Firmicutes* counts were also observed, particularly in the male NI mice from the Control diet group which showed the lowest number of *Firmicutes* (P<0.05) (6.62±0.02 Log_{10} DNA copies /g feces), whereas the female NI from the Control diet group and from the HFD group showed the highest amounts (7.48±0.07 and 7.55±0.01 Log_{10} DNA copies /g feces, respectively) (**Table** 4.2). Furthermore, as for the 1º cohort, in the 2º cohort, the ratio *Firmicutes*/*Bacteroidetes* showed a tendency for a significant increase in all groups, except for the male NI mice from the HFD group that remained with the same ratio (**Table** 4.2).

The counts of SRB in the 2ºcohort also showed the propensity of this bacterial group to increase overtime as observed in the 1º cohort (**Table** 4.1 and 4.2). At T0 all the mice showed similar loads of SRB (P>0.05), however at T3, distinct statistical groups were observed, particularly the female mice either in the Control group or on the HFD seems to carry more SRB than males (**Table** 4.2).

The amounts of *Betaproteobacteria* at T0 were statistically different (P<0.05) in both diet groups, and as observed in the previous cohort, also in this cohort a decrease in the counts of

Betaproteobacteria was noticed. This decrease achieved about 3 logs (**Table** 4.2). In contrast to what was observed with the quantification of *Betaproteobacteria*, the counts of *Delta*-and *Gammaproteobacteria* at T0 were similar across all groups (P>0.05) and also as observed in the previous cohort, the counts of *Delta*-and *Gammaproteobacteria* were towards a slight increase (about 1 log) (**Table** 4.2). The female NI mice from the Control group showed the highest load (P<0.05) of *Delta*-and *Gammaproteobacteria* (5.73±0.02 Log_{10} DNA copies /g feces) (**Table** 4.2). The *Tenericutes* counts at T0 in the mice of this cohort were statistically different (P<0.05) (**Table** 4.2). At the end of the experiment, the HFD group was the most affected showing the lowest (P<0.05) *Tenericutes* counts in comparison with the Control group (**Table** 4.2) evidencing a loss of about 3 logs. The lowest counts were displayed by the female with *Cyp* silenced from the HFD group (1.47±0.04 Log_{10} DNA copies /g feces) (**Table** 4.2).

Regarding the quantification of *Actinobacteria* in this cohort, the results showed that at T0 the mice were divided in two distinct statistical groups, evidencing slightly differences in the counts of this bacterial group (**Table** 4.2). At T3, it was not possible to distinguish the groups of mice according to the counts of *Actinobacteria*. The female mice with *Cyp* silenced from the HFD group showed the lowest count (P<0.05) of *Actinobacteria* (5.34±0.01 Log_{10} DNA copies /g feces), whereas the female NI from the Control diet group showed the highest counts (P<0.05) (6.70±0.00 Log_{10} DNA copies /g feces) (**Table** 4.2).

Table 4.2. Quantification of the target intestinal bacterial groups in the 2nd cohort (silencing group) at time zero(T0, mice exposed to the new diets), after four weeks of exposure to the appropriate diet and also injection treatment (T1), after eight weeks of exposure to the diet(T2) and after 12 weeks of exposure to the same diet (T3).*

Target Group			***Bacteroidetes (Log$_{10}$ DNA/g Feces)***			
			T0	T1	T2	T3
Control Diet	Non-Injected	Male	8.27 ± 0.02^{dCD}	6.93 ± 0.09^{bA}	7.61 ± 0.07^{cBC}	6.61 ± 0.01^{aA}
		Female	8.33 ± 0.02^{cD}	7.45 ± 0.04^{bC}	7.09 ± 0.01^{aA}	7.37 ± 0.05^{bBC}
	Silenced	Male	8.30 ± 0.05^{cCD}	7.14 ± 0.01^{aB}	7.37 ± 0.07^{bAB}	7.27 ± 0.05^{abB}
		Female	8.08 ± 0.03^{cB}	7.83 ± 0.06^{bE}	7.61 ± 0.03^{abBC}	7.54 ± 0.12^{aC}
	Non-Injected	Male	8.01 ± 0.02^{cB}	$7.69\pm0.06b^{DE}$	7.90 ± 0.04^{cC}	7.38 ± 0.03^{aBC}
		Female	8.23 ± 0.02^{cCD}	7.42 ± 0.04^{aC}	7.57 ± 0.04^{bBC}	7.33 ± 0.01^{aBC}

High Fat Diet	Silenced	Male	8.22 ± 0.03^{bC}	ND	7.35 ± 0.11^{aAB}	7.40 ± 0.03^{aBC}
		Female	7.88 ± 0.02^{cA}	7.49 ± 0.00^{bCD}	7.08 ± 0.18^{aA}	7.17 ± 0.08^{abB}

Firmicutes

Control Diet	Non-Injected	Male	7.37 ± 0.11^{bCD}	6.64 ± 0.09^{aA}	7.64 ± 0.01^{bC}	6.62 ± 0.02^{aA}
		Female	7.16 ± 0.06^{aBC}	7.06 ± 0.03^{aBC}	6.99 ± 0.05^{aA}	7.48 ± 0.07^{bEF}
	Silenced	Male	7.80 ± 0.04^{cE}	6.88 ± 0.02^{aAB}	7.17 ± 0.02^{bAB}	7.18 ± 0.02^{bC}
		Female	7.38 ± 0.03^{aCD}	7.70 ± 0.03^{bD}	7.25 ± 0.06^{aB}	7.35 ± 0.03^{aDE}
High Fat Diet	Non-injected	Male	7.62 ± 0.06^{bDE}	7.73 ± 0.09^{bD}	7.66 ± 0.05^{bC}	6.96 ± 0.06^{aB}
		Female	7.09 ± 0.03^{aAB}	7.73 ± 0.12^{bD}	7.23 ± 0.03^{aB}	7.55 ± 0.01^{bF}
	silenced	Male	7.75 ± 0.04^{cE}	ND	7.30 ± 0.03^{bB}	7.15 ± 0.03^{aC}
		Female	6.89 ± 0.10^{aA}	7.37 ± 0.12^{bC}	6.96 ± 0.13^{abA}	7.33 ± 0.01^{bD}

Ratio of *Firmicutes/Bacteroidetes*

			T0	T3
Control Diet	Non-injected	Male	0.89 ± 0.01^{aAB}	1.00 ± 0.00^{bBCDE}
		Female	$0.86\pm0.0.1^{aA}$	1.01 ± 0.00^{cCDE}
	Silenced	Male	0.94 ± 0.00^{aC}	0.99 ± 0.01^{cABCD}
		Female	0.91 ± 0.00^{aBC}	0.97 ± 0.02^{bABC}
High Fat Diet	Non-Injected	Male	0.95 ± 0.01^{aC}	0.94 ± 0.00^{aA}
		Female	0.86 ± 0.01^{aAB}	1.03 ± 0.00^{cE}
	Silenced	Male	0.94 ± 0.00^{aC}	0.97 ± 0.01^{bAB}
		Female	0.87 ± 0.02^{aAB}	1.02 ± 0.01^{bDE}

Sulfate-Reducing Bacteria

Control Diet	Non-injected	Male	3.22 ± 0.02^{aA}	4.15 ± 0.04^{bA}	5.00 ± 0.02^{cE}	4.18 ± 0.16^{bA}
		Female	3.21 ± 0.05^{aA}	4.21 ± 0.03^{bA}	4.56 ± 0.03^{cCD}	4.86 ± 0.01^{dB}
	Silenced	Male	3.73 ± 0.26^{aA}	4.25 ± 0.01^{abA}	4.60 ± 0.04^{bD}	4.18 ± 0.25^{abA}
		Female	3.38 ± 0.20^{aA}	4.23 ± 0.13^{bA}	4.36 ± 0.01^{bBC}	4.42 ± 0.03^{bAB}

High Fat Diet	Non-Injected	Male	3.19±0.08^{aA}	4.56±0.03^{bAB}	4.76±0.10^{bD}	4.50±0.01^{bAB}
		Female	3.30±0.08^{aA}	4.92±0.01^{dB}	4.33±0.01^{bB}	4.73±0.06^{cB}
	Silenced	Male	3.70±0.05^{aA}	ND	4.24±0.02^{bAB}	4.15±0.20^{abA}
		Female	3.30±0.19^{aA}	4.73±0.23^{bB}	4.11±0.08^{bA}	4.61±0.10^{bAB}

Betaproteobacteria

Control Diet	Non-Injected	Male	5.93±0.02^{dCD}	4.56±0.03^{bD}	4.75±0.02^{cE}	3.50±0.01^{aC}
		Female	6.10±0.01^{cE}	3.50±0.04^{aC}	3.66±0.08^{aB}	4.69±0.05^{bD}
	Silenced	Male	5.92±0.06^{cCD}	4.79±0.02^{bD}	2.74±0.01^{aA}	4.69±0.09^{bD}
		Female	5.79±0.06^{cC}	4.48±0.01^{bD}	4.43±0.01^{bD}	3.53±0.05^{aC}
High Fat Diet	Non-Injected	Male	5.34±0.03^{cA}	3.72±0.03^{bC}	3.91±0.01^{bC}	2.74±0.10^{aAB}
		Female	6.00±0.06^{cDE}	3.03±0.22^{aB}	4.57±0.02^{bD}	2.82±0.01^{aAB}
	Silenced	Male	5.63±0.02^{cB}	ND	4.81±0.09^{bE}	2.66±0.06^{aA}
		Female	5.99±0.01^{dDE}	2.51±0.03^{aA}	4.54±0.03^{cD}	2.90±0.00^{bB}

Delta- and Gammaproteobacteria

Target Group			T0	T1	T2	T3
Control Diet	Non-Injected	Male	4.11±0.46^{aA}	4.65±0.17^{abAB}	5.26±0.07^{bB}	4.96±0.04^{abAB}
		Female	4.10±0.20^{aA}	4.70±0.28^{abAB}	5.26±0.00^{bcB}	5.73±0.02^{cE}
	Silenced	Male	4.45±0.24^{aA}	4.68±0.06^{abAB}	5.05±0.09^{bB}	5.38±0.01^{cD}
		Female	4.17±0.04^{aA}	5.55±0.04^{cC}	5.86±0.06^{dC}	5.26±0.09^{bBD}
High Fat Diet	Non-Injected	Male	4.21±0.03^{aA}	4.38±0.16^{aA}	4.12±0.25^{aA}	5.12±0.01^{bBCD}
		Female	4.02±0.22^{aA}	5.14±0.03^{bBC}	5.72±0.02^{cC}	5.00±0.01^{bABC}
	Silenced	Male	4.33±0.07^{aA}	ND	5.24±0.09^{bB}	5.31±0.07^{bD}
		Female	4.16±0.11^{aA}	4.71±0.03^{bAB}	4.98±0.03^{bB}	4.74±0.14^{bA}

Tenericutes

Control Diet	Non-Injected	Male	4.15±0.09^{bB}	3.75±0.03^{aC}	5.41±0.02^{dE}	4.61±0.09^{cE}
		Female	4.70±0.08^{aC}	5.37±0.03^{cE}	5.08±0.02^{bD}	5.04±0.08^{bF}
	Silenced	Male	3.82±0.02^{aA}	4.50±0.07^{bD}	4.57±0.10^{bC}	4.52±0.01^{bE}
		Female	5.03±0.01^{bD}	5.15±0.08^{bE}	5.13±0.00^{bD}	4.63±0.03^{aE}

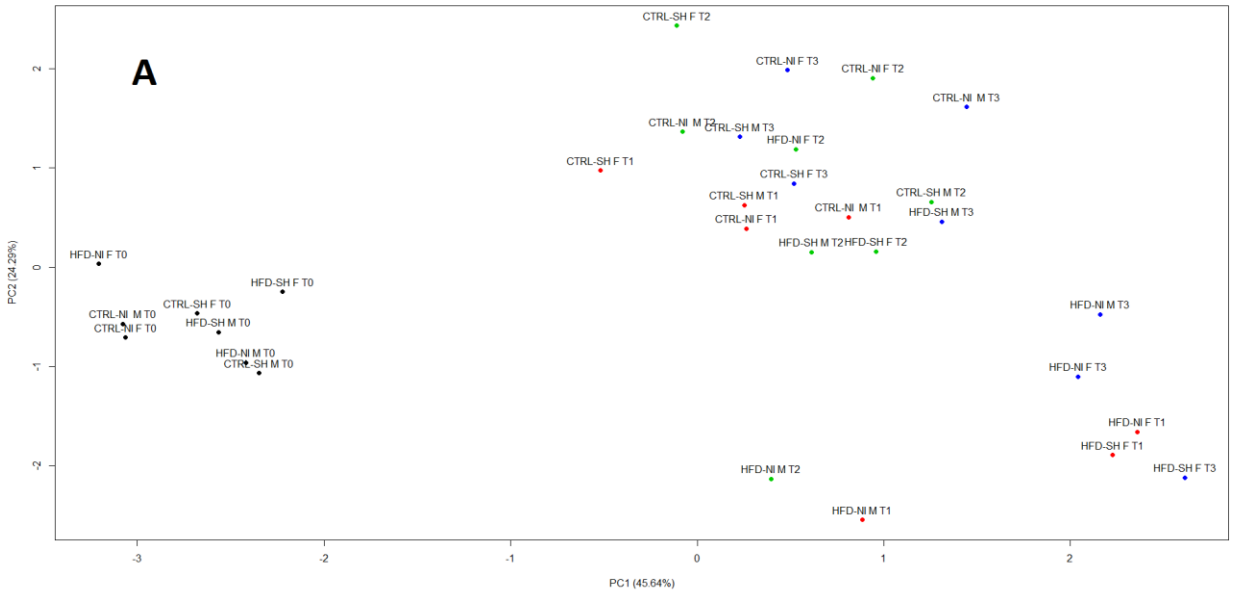
High Fat Diet	Non-injected	Male	4.23±0.01^{dB}	3.44±0.01^{cB}	2.36±0.14^{bA}	1.76±0.10^{aB}
		Female	5.04±0.02^{dD}	1.96±0.04^{aA}	2.68±0.05^{bB}	3.00±0.07^{cC}
	Silenced	Male	5.40±0.01^{cE}	ND	2.39±0.05^{aA}	3.33±0.01^{bD}
		Female	4.17±0.09^{cB}	2.18±0.14^{bA}	2.34±0.04^{bA}	1.47±0.04^{aA}

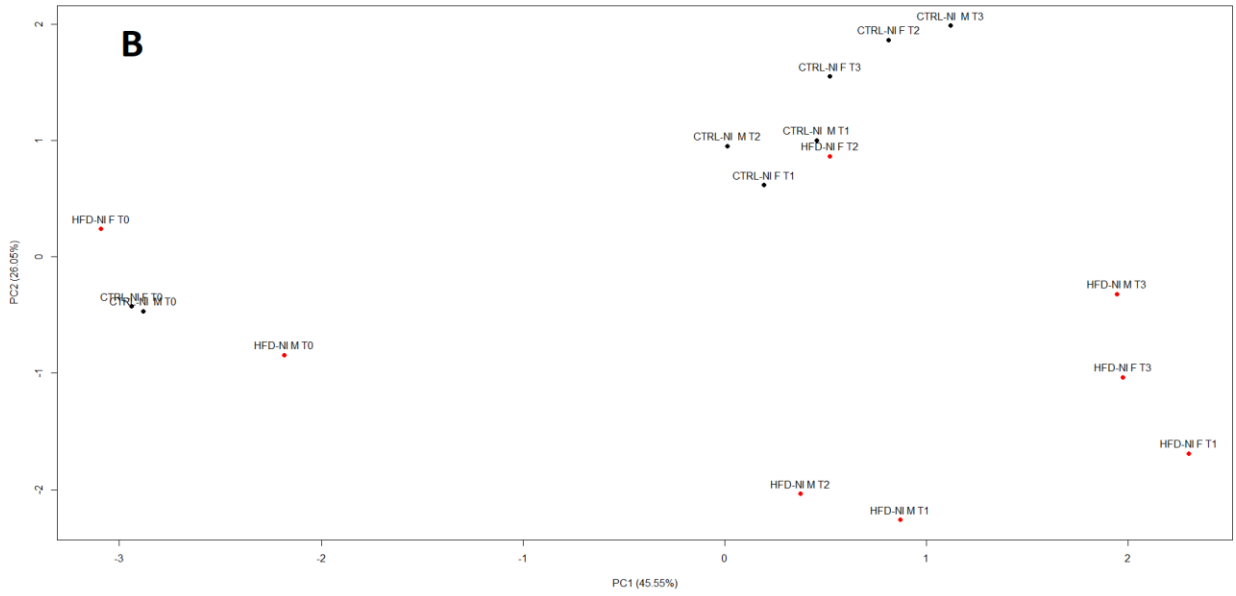
Actinobacteria

Control Diet	Non-Injected	Male	6.59±0.02^{cB}	5.99±0.02^{aC}	6.68±0.02^{dC}	6.23±0.00^{bD}
		Female	6.23±0.01^{aA}	6.03±0.12^{aC}	6.54±0.01^{bC}	6.70±0.00^{bF}
	Silenced	Male	6.40±0.04^{bAB}	6.10±0.02^{aC}	6.24±0.07^{abB}	6.39±0.01^{bE}
		Female	6.33±0.12^{aAB}	6.52±0.10^{abD}	6.89±0.10^{bD}	6.39±0.07^{aE}
High Fat Diet	Non-Injected	Male	6.34±0.10^{cAB}	5.25±0.09^{aA}	5.98±0.03^{bA}	5.99±0.06^{bC}
		Female	6.61±0.08^{bB}	5.63±0.00^{aB}	6.71±0.03^{bCD}	5.69±0.02^{aB}
	Silenced	Male	6.31±0.14^{aAB}	ND	6.34±0.01^{aB}	6.28±0.02^{aDE}
		Female	6.26±0.10^{bAB}	5.51±0.07^{aAB}	6.18±0.02^{bB}	5.34±0.01^{aA}

* Data represent the mean ± standard deviation of two replicates; ND not determined.

As for the 1st cohort also for the 2nd cohort, a PCA was performed to visualize the general differences among the cohort samples towards the loads of the different bacterial groups. The PCA scatter plot showed, as observed for the 1st cohort, that at T0 the faecal samples were distinctly separated from the remaining samples (**Fig.** 4.2 A), demonstrating also for this cohort a consistency on the bacterial amounts at the start of the hypothalamus and diet intervention. In this cohort the effect of the diet is highlighted in the PCA scatter for NI mice (**Fig.** 4.2 B) or *Cyp* silenced mice **(Fig.** 4.2 C). However, the *Cyp* silencing seems to bring some HFD mice closer to the Control diet mice (**Fig.** 4.2 C).


A
CTRL-SH F T2
CTRL-NI F T3
CTRL-NI F T2
CTRL-NI M T3
CTRL-NI M T2
CTRL-SH M T3
HFD-NI F T2
CTRL-SH F T1
CTRL-SH F T3
CTRL-SH M T1
CTRL-SH M T2
CTRL-NI M T1
HFD-SH M T3
CTRL-NI F T1
HFD-SH M T2
HFD-SH F T2
HFD-NI F T0
HFD-SH F T0
CTRL-SH F T0
CTRL-NI M T0
HFD-SH M T0
CTRL-NI F T0
HFD-NI M T0
CTRL-SH M T0
HFD-NI M T3
HFD-NI F T3
HFD-NI F T1
HFD-SH F T1
HFD-SH F T3
HFD-NI M T2
HFD-NI M T1
PC2 (24.29%)
PC1 (45.64%)


B
CTRL-NI M T3
CTRL-NI F T2
CTRL-NI F T3
CTRL-NI M T2
CTRL-NI M T1
HFD-NI F T2
CTRL-NI F T1
HFD-NI F T0
HFD-NI M T3
HFD-NI M T0
HFD-NI F T3
HFD-NI F T1
HFD-NI M T2
HFD-NI M T1
PC2 (26.05%)
PC1 (45.55%)

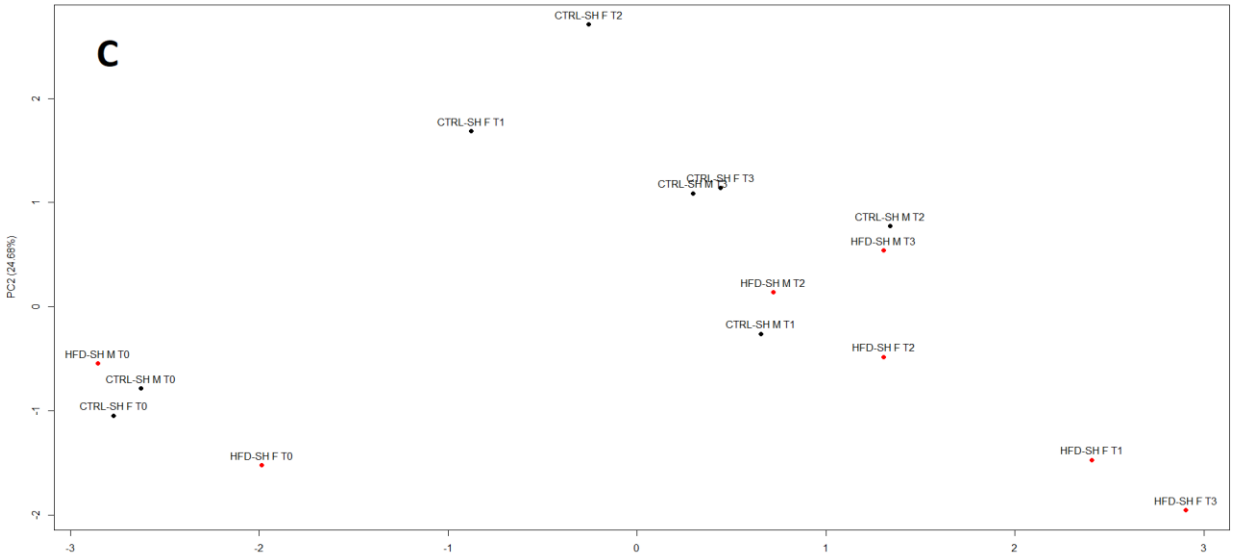


Figure 4.2. Distinction of the mice groups according to the impact of Diet- Injection- and gender on bacterial loads. **(A, B, C)** PCA scatter plot based on the tested bacterial groups of mice under Control and High Fat Diet (HFD) diet and *Cyp* silenced intervention. **(A),** PCA scatter plot based only on Non-injected mice overtime **(B)**, PCA scatter plot based only on *Cyp* silenced mice overtime **(C)**. Each circular point represents the bacterial load of mice of each group, colored by time point **(A)**, and colored by type of diet **(B, C)**.

4.2. Evaluation of mice cognitive abilities – Impact of the *Cyp* intervention

In order to examine the effect of the *Cyp* overexpression or silencing on the cognitive abilities, namely the Short-Term memory of mice, the Y-maze spontaneous alternation Test was performed. The diet did not show any impact on the short-term memory. The mice exposed to the HFD showed a similar (P>0.05) percentage of spontaneous alternations to the Control mice (**Fig.** 4.3 A).

The Y-Maze performances within the control diet mouse subjects, showed an effect of the *Cyp* intervention (*Cyp* overexpression versus Silencing). The Ctrl-*Cyp* overexpression mice displayed a more efficient short-term memory (P<0.05) in comparison with the Control silenced mice (Ctrl-SH). However, the Control mice without intervention (Ctrl-NI) showed similar abilities to the Control *Cyp* overexpression mice (**Fig.** 4.3 B). This effect was not observed on mice under the HFD; all mice showed similar Y-Maze performances (P>0.05) (**Fig.** 4.3 C)

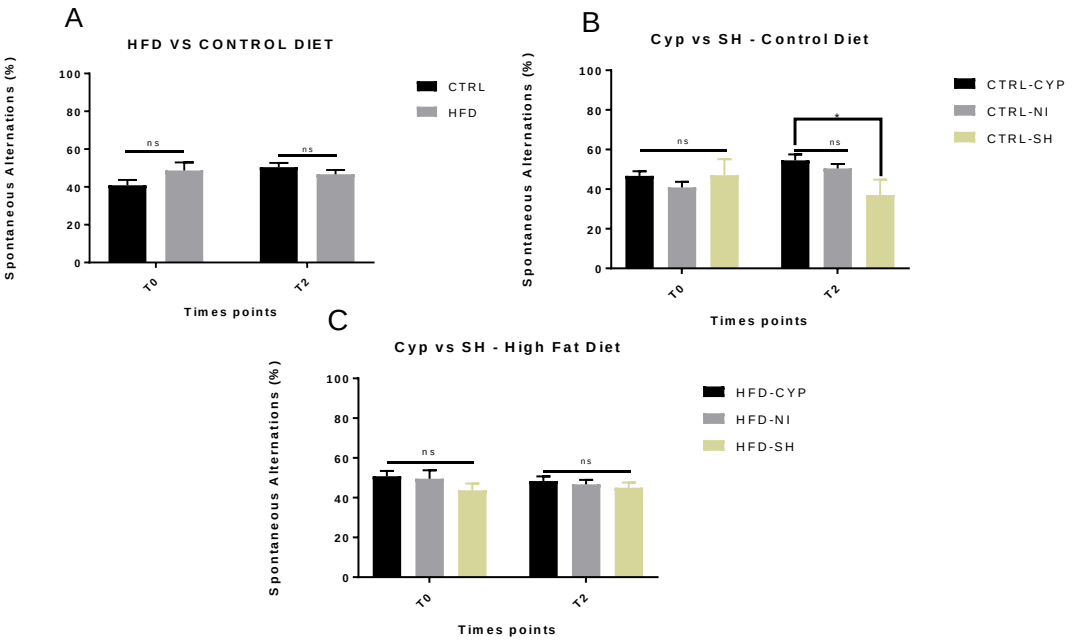


Figure 4.3. Effect of *Cyp* intervention and diet on memory impairment through variations on Spontaneous Alternations. Effect of diet, n (CTRL) = 7; n (HFD) = 6 (A); Spontaneous alternations between treatments within mice under Control diet, n (CTRL-CYP) = 6; n (CTRL-NI) = 7; n (CTRL-SH) = 4 (B); Spontaneous alternations between treatments within mice under HFD, n (HFD-CYP) = 5; n (HFD-SH) = 3; n (HFD-NI) = 6 (C). HFD - High-Fat Diet; CTRL - Control Diet; CYP - *Cyp* Overexpression; SH - *Cyp* Silencing; NI - Non-injected.

The total arm entries were also evaluated in order to examine the possible impact of the type of diet, and or the *Cyp* intervention on their movement abilities and distance travelled. The mice under the Control diet showed a better performance in comparison to the mice under the HFD, but the results were not statistically different (P>0.05) (**Fig.** 4.4 A). Furthermore, the Control mice with the *Cyp* overexpression or *Cyp* silencing (CTRL-CYP, CTRL-SH) showed a similar pattern to the CTRL-NI mice (**Fig.** 4.4 B). This same result was also observed within the HFD mice (**Fig.** 4.4 C). The results obtained for total arm entries also showed no statistical significance (P>0.05) between treatments (*Cyp* Silencing vs Overexpression) and between diets (Supplementary data).

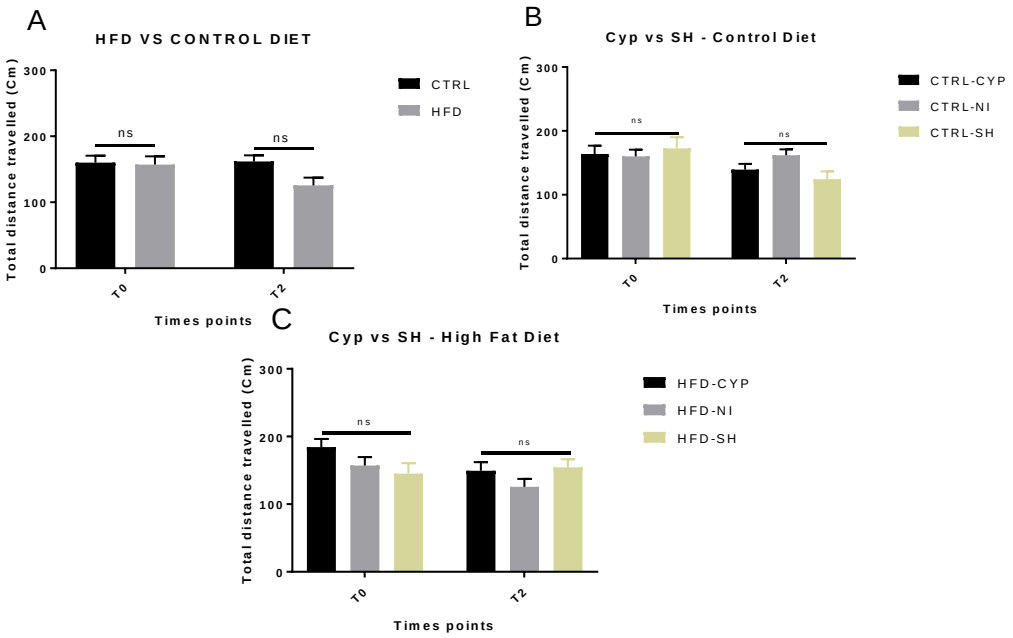


Figure 4.4. Effect of *Cyp* intervention and diet on movement abilities through variations on distance travelled. Effect of diet, n (CTRL) = 7; n (HFD) = 6 (A); Distance travelled between treatments within mice under Control diet, n (CTRL-CYP) = 6; n (CTRL-NI) = 7; n (CTRL-SH) = 4 (B); Distance travelled between treatments within mice under HFD, n (HFD-CYP) = 5; n (HFD-SH) = 3; n (HFD-NI) = 6 (C). HFD - High-Fat diet; CTRL - Control diet; CYP - *Cyp* Overexpression; SH - *Cyp* Silencing; NI - Non-injected.

4.3. The impact of the diet and *Cyp* intervention on the intestinal inflammation

The analysis of the histological samples of the colon of mice aiming to evaluate the impact of *Cyp* intervention (*Cyp* overexpression and silencing) and diet on intestinal inflammation was performed. Following the guide of histomorphological grade of intestinal inflammation in mouse models (U. *et al.*, 2014), the main alterations in the intestine of the different mice were registered. The observed disturbances are illustrated in **Fig.** 4.5. A-F.

The intestine of the mice under Control diet and not subjected to *Cyp* intervention (NI) (**Fig.** 4.5 A) show a pattern of goblet cells at the epithelial cells in contrast to the mice under the same Control diet but with *Cyp* overexpression, which showed a remarkable inflammatory cell aggregation (**Fig.**

4.5 B). The mice also exposed to Control diet but subjected to *Cyp* silencing showed alterations on the pattern of the goblet cells, increased inflammatory cell infiltration and the epithelial cells seemed damaged (**Fig.** 4.5. C).

The intestine of mice under the HFD but with no *Cyp* intervention showed a massive inflammatory cell infiltration (**Fig.** 4.5 D) whereas the intestine of mice also under the HFD but subjected to *Cyp* overexpression showed a reduced pattern of deformed goblet and epithelial cells (**Fig.** 4.5. E). The intestine of mice under HFD and subjected to *Cyp* silencing evidenced inflammatory cell infiltration (**Fig.** 4.5. E).

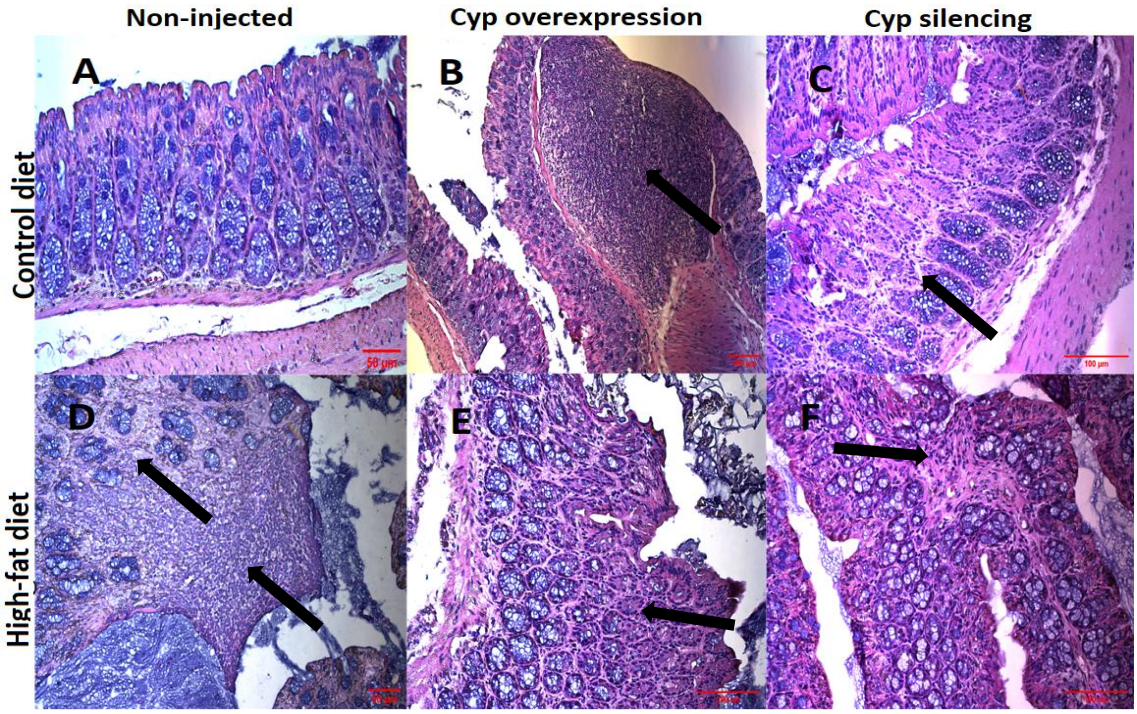


Figure 4.5. Alterations on the intestine of mice exposed to Control and HFD and subjected to *Cyp* intervention. (A) Intestine of mice under the Control diet and with no *Cyp* intervention (Control), (B) Intestine of mice under the Control diet and subjected to *Cyp* overexpression (black arrow evidences inflammatory cell aggregation); (C) Intestine of mice under the Control diet and subjected to *Cyp* silencing (black arrow indicates the damaged epithelial cells); (D) Intestine of mice under HFD with no *Cyp* intervention (black arrow indicates inflammatory cell aggregation); (E) Intestine of mice under HFD and subjected to *Cyp* overexpression (black arrow indicates reduced pattern of deformed goblet and epithelial cells); (F) Intestine of mice under HFD and subjected to *Cyp* silencing (back arrow evidences inflammatory cell infiltration).

5. Discussion

Numerous functions of our organism are regulated by hypothalamus, a small, but key region of the brain. These functions include among others, the control of energy metabolism and fluid balance, sleep-wake states, stress response, growth, cognitive and reproductive behaviors, thermoregulation, circadian rhythms and also emotional and social behaviors (Bertalan Dudás, 2013; Burbridge, Stewart and Placzek, 2016b).

Nowadays it is fully recognized that the network between the gut microbiota and the brain is crucial for the maintenance of human body homeostasis, and its disturbance may lead to a series of diseases, namely irritable bowel syndrome (IBD), obesity, diabetes, depression, anxiety and some cancer types (Ley RE, Turnbaugh PJ, Klein S, 2006; Fujimoto *et al.*, 2013; McCoy *et al.*, 2013; Forslund *et al.*, 2015; Fröhlich *et al.*, 2016; Wang and Wang, 2016; Halfvarson *et al.*, 2017; Malan-Muller *et al.*, 2018; Valles-Colomer *et al.*, 2019).

It is also clear, the influence of the diet on the composition and functionality of the gut microbiota (De Filippo *et al.*, 2010; David *et al.*, 2014; Olivier-Van Stichelen, Rother and Hanover, 2019; Wegorzewska *et al.*, 2019). A diet rich in high fat, high protein content, refined grains and low fiber levels (a typical Western diet) has been associated with the development of diseases such as obesity, diabetes, and cardiovascular, all increasing the death risk. This type of diet not only affects the immune cells, adipocytes and endocrine cells but also impairs the microbiome by markedly reducing the microbial diversity, thus promoting the dysbiosis (Turnbaugh *et al.*, 2006, 2009; Gupta, Paul and Dutta, 2017; Zinöcker and Lindseth, 2018).

In the current study, the mice subjected to HFD, either with *Cyp* overexpression or NI showed similar *Bacteroidetes* amounts at the end of the experiment. Also, for the *Cyp* silencing intervention within HFD subjects, either *Cyp* silencing or NI mice, showed similar *Bacteroidetes* amounts at the end of the experiment, suggesting the marked influence of the HFD in this bacterial group. The impact of the HFD on the reduction of *Bacteroidetes* was reported in previous studies (Bäckhed *et al.*, 2004; Ley *et al.*, 2005; Turnbaugh *et al.*, 2006; Guo *et al.*, 2008). However, this finding was not reported in the study conducted by (Onishi *et al.*, 2017) , in which Wistar rats were subjected to a HFD supplemented with 10 % of blueberry power. The mice whitin a Control diet, either *Cyp* overexpression, *Cyp* silencing or NI, also showed a slight reduction in *Bacteroidetes* amounts. It is possible that this decrease of *Bacteroidetes* amounts resulted from the sucrose and maltodextrin

supplementation in each diet (Control and HFD - 72.8 g sucrose and maltodextrin- 125 g). It is known that consumption of carbohydrates (including maltodextrin, a partially hydrolyzed form of corn starch) alters, among other factors, the composition of the gut microbiota (Parks *et al.*, 2013; Sorndech *et al.*, 2019). For instance, an earlier study conducted by Turnbaugh *et al.*, (2009), suggests the impact of a Western diet, mainly composed of fat and sucrose, on the reduction of *Bacteroidetes*, using C57BL/6J mouse models. Recently, Sorndech *et al.*, (2019) showed that the consumption of Resistant maltodextrin (RMD) and Resistant starch (RS) by humans caused a decrease in *Bacteroides* (a genus of *Bacteroidetes*) whereas bifidobacteria and lactobacilli increased.

In our results, *Bacteroidetes* populations were negatively correlated with body weight gain in both CYP experiments. The negative correlation between body weight and *Bacteroidetes* populations have been reported in previous studies (Ley RE, Turnbaugh PJ, Klein S, 2006).

The mice subjected to HFD, either *Cyp* overexpression or NI, showed similar amounts of *Firmicutes* populations at the end of the experiment. In the *Cyp* silencing experiment, within HFD group, either *Cyp* silenced female, NI female or NI male underwent a significant increase in *Firmicutes* counts after one month (T1) but at the end of the experiment every individual displayed distinct *Firmicutes* amounts. Furthermore, while their numbers decreased in both *Cyp* silencing and NI males, the opposite trend was observed in both *Cyp* silencing and NI females, suggesting a marked effect by diet and gender on this particular bacterial group. The positive correlation between an HFD and *Firmicutes* populations was reported in several studies (Hildebrandt *et al.*, 2009; Zhang *et al.*, 2012; Ecol, 2017; Heras *et al.*, 2019). Interestingly, individuals within a Control diet displayed a similar behavior in both CYP experiments. While HFD has been suggested to increase the *Firmicutes* population, it was reported that the addition of dietary fibers derived from potato starch with prebiotic properties into obese fecal samples collected from children (5-15 years old) under anaerobic conditions, inhibited *Firmicutes* growth (Barczynska *et al.*, 2015). However, this finding was not reported by Xie *et al.*, (2019) in which propionylated high-amylose maize starch promoted the relative abundance of *Firmicutes* in human fecal samples (3 healthy donors, age 20-25 years old). Furthermore, it was reported that women displayed higher proportions of *Firmicutes* regardless of the body mass index, in comparison with male subjects (Rangel-Zúñiga *et al.*, 2016), therefore ours and others findings suggest that gender may influence the *Firmicutes* populations. No correlation between *Firmicutes* populations and body weight were observed, a

finding that was reported in previous studies (Duncan *et al.*, 2008; Santos-Marcos, Perez-Jimenez and Camargo, 2019).

It has been assumed that the proportional ratio of the *Firmicutes* to *Bacteroidetes* may play key roles in the development of metabolic disorders, such as obesity or diabetes (Duncan *et al.*, 2007; Fleissner *et al.*, 2010; Larsen *et al.*, 2010). In this present study, within the *Cyp* silencing experiment, the individuals subjected to either HFD or Control diet, showed an increased *Firmicutes* to *Bacteroidetes* ratios, while in *Cyp* Overexpression experiment, this behavior could be observed only in the female *Cyp* overexpression mice from the Control diet group and in the Non-injected female mice from the HFD group. The increase in this ratio was reported in previous studies either with mice or human individuals, with high-carbohydrate-fat diets (Duncan *et al.*, 2008; Fleissner *et al.*, 2010). Furthermore, using an *in vitro* approach (Knudsen *et al.*, 2013), it was demonstrated that the fermentation of insoluble carbohydrates, such as waxy maize starch granules, pectin-rich potato fiber and potato lintner starch, increased the ratio of *Firmicutes* to *Bacteroidetes*, mainly by the suppression of *Bacteroidetes*.

Sulphate-reducing bacteria have long been suspected to play key roles in a variety of diseases, such as ulcerative colitis, but also in depression and impaired memory and cognitive levels through their use of sulphate in their metabolism, reducing and releasing it as H2S into the lumen, where it has been suggested to be cytotoxic to intestinal mucosa by inducing inflammatory responses and DNA damage (Carbonero *et al.*, 2012; Ritz *et al.*, 2016). In the current study, the mice subjected either to an HFD or Control diet, showed a significant increase in their sulphate-reducing bacteria numbers regardless of their diet type, in both experiments. Previous studies demonstrated that intestinal sulphate-reducing bacteria are mainly influenced by a protein-rich diet (Khalil *et al.*, 2014), therefore, this behavior might be due to the fact that both diets were supplemented with the same protein content (20%).

Several studies to date, endorse the concept that a bloom of *Proteobacteria* in the gut is a reflection of dysbiosis or an unhealthy gut microbial structure, therefore, are considered as a potential diagnostic signature for several diseases (Shin, Whon and Bae, 2015; Rizzatti *et al.*, 2017). In the current study, at *Betaproteobacteria* class level, the mice subjected either to an HFD and control diet, or even *Cyp* overexpression or *Cyp* silencing, showed a significant decrease in *Betaproteobacteria* amounts, whereas this reduction was even more pronounced in the HFD group within the *Cyp* silencing experiment (about 3 Log), suggesting a notable effect of the HFD on this

bacterial group. The same effect of the HFD on SRB group was reported in several studies, particularly, the consumption of a HFD feeding resulted in a decreased abundance of *Parasutterella*, a genus of *Betaproteobacteria* (Zhang *et al.*, 2012; Takiishi *et al.*, 2017; Ju *et al.*, 2019).

In the context of *Delta- & Gammaproteobacteria* class, *Cyp* overexpression resulted in an approximation between both *Cyp* overexpression male and female from Control group to the HFD mice group, while in the *Cyp* silencing experiment showed a highly variable results between treatments. However, it is important to underline that in both experiments, either mice under the Control diet or the HFD experienced in an increase in *Delta & Gammaproteobacteria* amounts. How Control diet lead to an increase on this bacterial group remains unclear, however, the impact of HFD was already reported in previous studies, particularly the increase of the members of the *Enterobacteriaceae* (included in the *Gammaproteobacteria* class) (Kim *et al.*, 2012; Heinritz *et al.*, 2016; Soderborg *et al.*, 2018).

In the present study, the mice subjected to HFD, either with the *Cyp* silencing or NI intervention, showed a remarkable decrease in *Tenericutes* counts at the end of the experiment, in comparison with Control diet group. In contrast, the results from the *Cyp* overexpression experiment are somehow inconsistent. The effect of a HFD on the reduction of *Tenericutes* was previously reported (Kang *et al.*, 2014; Jeong, Jang and Kim, 2019). However, there is some controversy since other study suggested an opposite finding, particularly the increase abundance of the class *Mollicutes* (in the phylum *Tenericutes*) when 6-week-old male Sprague Dawley rats were subjected to a HFD (Lecomte *et al.*, 2015).

The phylum *Actinobacteria* is a well-known component of the intestinal microbiota of healthy humans and includes the also well recognized genus *Bifidobacterium* (Eid *et al.*, 2017). The *Bifidobacterium* species are considered to be critical for human health, thereby, several *Bifidobacterium* species have been used as probiotics (microorganism that display beneficial impact on the health of the host) for the treatment of different gut diseases, such as Intestinal Bowel Syndrome (Eid *et al.*, 2017). In the present study, the mice subjected to the HFD, either in the *Cyp* overexpression or NI intervention, experienced an increase in *Actinobacteria* amounts suggesting that this increase was driven by the diet. However, conflict results were observed on the *Cyp* silencing intervention with HFD individuals, either *Cyp* silencing or NI showing a decrease suggesting that a not yet identified factor is affecting this bacterial group. The mice under the

Control diet showed unchanged or a slight increase in *Actinobacteria* counts, either in *Cyp* overexpression or silencing. Conflict results were also recently reported, namely Zhang *et al.* (2019) observed that *Actinobacteria* abundance was positively correlated with a HFD in Diet-induced-obese mice (Zhang *et al.*, 2019), in contrast with the reported by Meng *et al.*, 2019 that observed a negative effect of a HFD on *Actinobacteria* populations in comparison with mice feed with a standard diet.

In the present study, *Cyp* silencing changed the abundance of several intestinal bacterial groups in a way that mice under an HFD resemble that under the Control diet. It was reported that CYP barely contribute to the overall bile acid synthesis (Chiang, 2004) and a mixed strain of mice (C57Bl/6J;129S6/SvEv) lacking the *Cyp*, have normal bile acid synthesis (Lund *et al.*, 2003). So, the way that *Cyp* silencing affects the bacterial populations requires further investigation.

Unhealthy lifestyles habits, such as reduced physical activity and increased consumption of highly-dense energy food are well-known factors contributing the development of metabolic disorders (Calder *et al.*, 2017), such as obesity or IBD which, in turn have been strongly linked to cognitive impairment and depression-like behavior (Jais *et al.*, 2017)(Schachter *et al.*, 2018). In the current study, the mice exposed to an HFD did not show any significant differences in short-term memory, in comparison with mice under a Control diet, showing similar percentage of spontaneous alternations. The lack of correlation between diet and cognitive abilities was also reported by a recent study, using 7-month old male Sprague Dawley rats (Deshpande *et al.*, 2019). However, there are several reports evidencing the effect of HFD on hypothalamus functions (Guillemot-Legris and Muccioli, 2017; Cunarro *et al.*, 2018; Mendes *et al.*, 2018). In the study of André *et al.* (2014), it was reported an negative effect of HFD on hypothalamus functions, by impairing spatial working memory of C57BL/6 mice and increasing anxiety-like behavior. Furthermore, a one-day HFD feeding induced inflammation in the nodose ganglion and hypothalamus of mice, marked by increased neutrophils infiltration and macrophage recruitment, in male C57BL/6J mice (Waise *et al.*, 2015).

Brain's cholesterol metabolism is thought to be one of the major factors having functions in the development of neurogenerative disorders (Loera-Valencia *et al.*, 2019) (Boussicault *et al.*, 2016). In the present study, the mice exposed to the Control diet group and *Cyp* silencing experienced a lower performance in Y-maze spontaneous alternation test, in comparison with *Cyp* overexpression mice. However, both treatments did not show statistical significance when compared with NI mice.

On the other hand, HFD seems to attenuate the aforementioned effect, since no statistical significance (P>0.05) could be observed between treatments (*Cyp* silencing vs Overexpression vs Non-injected). In addition, the results obtained for total distance travelled and total arm entries, also showed no statistical significance (P>0.05) between treatments (*Cyp* Overexpression vs Silencing) and between diets (HFD vs Control diet). The correlation between CYP cholesterol metabolism and cognitive outcomes are strongly supported by previous studies (Zhang *et al.*, 2015; Kacher *et al.*, 2019). Particularly, an earlier report demonstrates that the silencing of *Cyp46a1* led to neuronal cholesterol accumulation, inducing apoptotic death of hippocampal neurons, followed by cognitive impairment and hippocampal atrophy, using both three-month-old female wild-type C57Bl/6 and 5-month-old APP23 transgenic mice (Djelti *et al.*, 2015).

Over-consumption of a HFD has been highly associated with increased incidence of many chronic inflammatory diseases of the gastrointestinal tract, which are generally accompanied by increased inflammatory cell infiltration, and changes at epithelial and mucosal level, resulting in a decrease in barrier function, impairment of specific transport mechanisms within the gut, allowing bacterial penetration and also the passage of bacterial derived endotoxins into the circulation (Erridge *et al.*, 2007; Murakami, Tanabe and Suzuki, 2016; Shi *et al.*, 2019). On the other hand, impaired bile acid metabolism has been suggested to play roles in the pathophysiology of such metabolic disorders (Tiratterra *et al.*, 2018). In the current study, within Control diet, the mice either subjected to *Cyp* overexpression or *Cyp* silencing lead to increased signs of intestinal inflammation in comparison with NI mice. Similarly, the mice exposed to an HFD, either subjected to *Cyp* overexpression or *Cyp* silencing, or even HFD NI, showed marked evidences of intestinal inflammation. Therefore, exposure to an HFD and the intervention at *Cyp* level, either silencing or overexpression, resulted in the development of a low-grade state of intestinal inflammation. The remarkable negative impact of an HFD on intestinal health has been strongly supported by previous studies (Ding *et al.*, 2010; Gulhane *et al.*, 2016; Murakami, Tanabe and Suzuki, 2016).

6. Conclusion and Future perspectives

The main objective of the current study was to evaluate the impact of *Cyp* intervention, in particular *Cyp* overexpression and silencing treatment in the hypothalamus in mice exposed to an HFD and

a Control diet on different intestinal bacterial groups and also on their cognitive abilities and colon health status.

In this study, it was possible to observe differences in the counts of the tested bacterial groups after 1 month of exposure to the HFD and Control diet, with a relative consistency in the following months. It was found that mice exposed to an HFD experienced shifts in the intestinal bacterial groups, mainly marked by decreased abundances of *Bacteroidetes*, *Betaproteobacteria* and *Tenericutes*, and increased abundances of *Firmicutes*, SRB, *Delta & Gammaproteobacteria* and *Actinobacteria*, in a HFD. In addition, it was observed that the *Cyp* silencing treatment lead to a resemblance in terms of intestinal bacterial loads, between mice exposed to the two types of diet.

After the analysis of intestinal microbiota of each mice group, their cognitive abilities were tested. It was found that an HFD does not influence mice cognitive abilities, arm entries and also their distance travelled in Y-Maze spontaneous alternations test. However, *Cyp* silencing treatment lead to a decreased short-term memory, in comparison with mice subjected to *Cyp* overexpression.

Finally, through histological analysis of the mice intestine, it was observed increased signs of intestinal inflammation in mice either subjected to *Cyp* overexpression, *Cyp* silencing or under and HFD, corroborating that a diet rich in fat content leads to a state of low-grade inflammation, and evidencing that a perturbation at brain's cholesterol metabolism can aggravate the state of inflammation.

In conclusion, these results show evidences that intestinal microbiota composition is highly influenced by dietary factors, and those changes can reflect a state of low-grade inflammation. It is noteworthy to mention, that this study also shows evidences that cholesterol metabolism at hypothalamus level, can display an impact on the intestinal bacterial loads. These results are promising for following new studies and to develop new approaches for amelioration or possibly treatment of neurological and metabolic disorders.

Although the current study adds a growing body of knowledge unravelling the impact of diet and brain's cholesterol metabolism on gut microbiota, there are questions that should be addressed in the future.

Since a limited number of studies have been performed about the effect of brain's cholesterol metabolism on intestinal microbiota, a study including the quantification of the expression of *Cyp7a1* and *Cyp27a1* genes (Classical and alternative pathways for bile acid synthesis), could

improve our understanding on the effect of *Cypx* overexpression or silencing on bile acid synthesis rate in the liver, and establish the link with the intestinal microbiota of mice, as a consequence. In addition, the bile acid quantification from liver and serum could be determined by ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS).

As previously mentioned, an HFD is associated with metabolic disorders such as, Irritable bowel disease or colitis, being accompanied by an increased intestinal permeability and a state low-grade inflammation. So, in order to clarify how the mucus barrier in the mice, exposed to the HFD and CYP, intervened the analysis of the intestinal MUC2 protein, would provide more information on the impact of CYPx treatment at hypothalamus level together with the diet affect the intestinal permeability. This approach can be performed for example, using anti-MUC2 antibodies.

Finally, since western diet is generally accompanied by a high content of fat, carbohydrates and protein (Myles, 2014; Hariharan, Vellanki and Kramer, 2015), a third diet type with different content of protein could be included. This approach would provide new perspectives on how protein content can influence sulphate-reducing bacteria and possible cognitive outcomes. To support this idea, analysis of intestinal H_2S could be determined by using gas chromatography, and correlate either with the *dsr* gene expression levels and with the cognitive outcomes.

7. References

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8. Attachments

8.1. **Mice weight measures over time**

Table 8.1. Weight measures over time of the overexpression experiment.

	T0	T2	T3
HFD-NI F	19.67±0.45[a]	24.77±2.47[a]	24.34±1.48[a]
CTRL-NI M	23.43±0.52[a]	25.99±0.54[b]	26.8±1.89[b]
CTRL-CYP M	24.6±0.59[a]	25.44±0.54[a]	27.21±0.63[b]
CTRL-CYP F	20.95±2.79[a]	20.8±1.52[a]	21.47±1.39[a]
HFD-CYP M	25.51±0.90[a]	30.09±4.02[a]	29.26±2.29[a]
HFD-CYP F	21.85±0.78[a]	24.47±0.66[a]	24.24±1.34[a]

Table 8.2. Weight measures over time of the silencing experiment.

	T0	T1	T2	T3
CTRL-NI M	26.15±2.01[a]	25.35±2.23[a]	28.59±2.15[a]	29.11±2.25[a]
CTRL-NI F	21.91±0.65[a]	21.98±0.12[a]	22.95±0.91[a]	23.89±1.45[a]
CTRL-SH M	27.32±0.91[a]	27.41±0.90[a]	35.69±2.28[b]	42.55±0.30[c]
CTRL-SH F	20.74±1.27[a]	21.68±0.11[a]	25.68±3.53[a]	32.97±7.05[a]
HFD-NI M	26.24±1.26[a]	33.51±0.40[a]	35.9±3.58[ab]	41.58±2.04[b]
HFD-NI F	19.91±0.37[a]	22.19±0.57[b]	24.29±1.63[c]	31.49±1.35[d]
HFD-SH M	27.72±1.16[a]	ND	37.37±2.00[b]	50.16±0.28[c]
HFD-SH F	20.01±0.60[a]	26.66±0.27[a]	29.77±5.31[a]	42.29±9.86[a]

8.2. Diets information

Table 8.3. Caloric information of each diet used in both experiments.

Caloric information	Control Diet	High Fat Diet
Protein	20% kcal	20% kcal
Fat	10% kcal	60% kcal
Carbohydrate	70% kcal	20% kcal
Energy density	3.82 kcal/g	5.21 kcal/g

8.3. ***Sequencing Results***

Table 8.4. NCBI BLAST search results and UniProt Taxonomy database classification of clones.

Clone Name	NCBI Sequence Match Description	Max Identity	Bacterial group	GenBank Acession Numbers	Primer	PCR Result
Clone 1 - *Actinobacteria*	Eggerthella sinensis JCM 14551 strain HKU14 16S ribosomal RNA, partial sequence	93.47%	*Actinobacteria*	NR_042840.1		+
	Slackia piriformis YIT 12062 strain JCM 16070 16S ribosomal RNA, partial sequence	97%	*Actinobacteria*	NR_113272.1	Act664F	+
	Slackia piriformis YIT 12062 16S ribosomal RNA, partial sequence	97%	*Actinobacteria*	NR_112898.1	Act941R	+
Clone 2 - *Actinobacteria*						-
					Act664F	-
					Act941R	-
Clone 1 - *Bacteroidetes*	Barnesiella viscericola strain C46 16S ribosomal RNA, complete sequence	100%	*Bacteroidetes*	NR_121773.2		+
	Zeaxanthinibacter aestuarii strain S2-22 16S ribosomal RNA, partial sequence	88.31%	*Bacteroidetes*	NR_151961.1	BactesF	+
	Muribaculum intestinale strain YL27 16S ribosomal RNA, partial sequence	100%	*Bacteroidetes*	NR_144616.1	BactesR	+
Clone 2 - *Bacteroidetes*	Porphyromonas loveana strain UQD444 16S ribosomal RNA, partial sequence	89.66%	*Bacteroidetes*	NR_152035.1		+
	Barnesiella intestinihominis strain JCM 15079 16S ribosomal RNA, partial sequence	91.89%	*Bacteroidetes*	NR_113073.1	BactesF	+
	Barnesiella intestinihominis YIT 11860 16S ribosomal RNA, partial sequence	91.89%	*Bacteroidetes*	NR_041668.1	BactesR	+
Clone 1- *Firmicutes*	[Clostridium] aldenense strain RMA 9741 16S ribosomal RNA, partial sequence	94.74%	*Firmicutes*	NR_043680.1		+
	[Clostridium] fimetarium strain Z-2189 16S ribosomal RNA, partial sequence	91.67%	*Firmicutes*	NR_024993.1	Firm934F	+
	Lachnoclostridium pacaense strain Marseille-P3100 16S ribosomal RNA, partial sequence	91.67%	*Firmicutes*	NR_147396.1	Firm1060R	+
	Butyrivibrio fibrisolvens strain ATCC 19171 16S ribosomal RNA, partial sequence	91.36%	*Firmicutes*	NR_025981.1		+
	Paenibacillus koleovorans strain NBRC 103111 16S ribosomal RNA, partial sequence	98.31%	*Firmicutes*	NR_114210.1	Firm934F	+

Clone 2 - *Firmicutes*	Paenibacillus koleovorans strain TB 16S ribosomal RNA, partial sequence	98.31%	*Firmicutes*	NR_024752.1	Firm1060R	+
Clone 1 - DSR	Bilophila wadsworthia dissimilatory sulfite reductase A and dissimilatory sulfite reductase B genes, complete cds	87.38%	*Proteobacteria*	AF269147.2	DSR2060F DSR4R	+
	Uncultured Desulfovibrionaceae bacterium partial dsrA and dsrB genes for dissimilatory sulfite reductase, clone ORIFRC-DSRMd-28	85.71%	*Proteobacteria*	HE856611.1		+
	Uncultured Desulfovibrionaceae bacterium partial dsrA and dsrB genes for dissimilatory sulfite reductase, clone ORIFRC-DSRMd-56	85.71%	*Proteobacteria*	HE856610.1		+
Clone 2 - DSR	Bilophila wadsworthia dissimilatory sulfite reductase A and dissimilatory sulfite reductase B genes, complete cd	87.86%	*Proteobacteria*	AF269147.2	DSR2060F DSR4R	+
	Uncultured Desulfovibrionaceae bacterium partial dsrA and dsrB genes for dissimilatory sulfite reductase, clone ORIFRC-DSRMd-28	86.01%	*Proteobacteria*	HE856611.1		+
	Uncultured Desulfovibrionaceae bacterium partial dsrA and dsrB genes for dissimilatory sulfite reductase, clone ORIFRC-DSRMd-56	86.01%	*Proteobacteria*	HE856610.1		+
Clone 1 - *Betaproteobacteria*	Parasutterella excrementihominis strain YIT 11859 16S ribosomal RNA, partial sequence	95.86%	*Betaproteobacteria*	NR_041667.1	Beta979F Beta1130R	+
	Massilia terrae strain J11 16S ribossomal RNA, partial sequence	91.10%	*Betaproteobacteria*	NR_157771.1		+
	Niveibacterium umoris strain MIC2059 16S ribosomal RNA, partial sequence	91.22%	*Betaproteobacteria*	NR_148584.1		+
Clone 2 - *Betaproteobacteria*	Turicimonas muris strain YL45 16S ribosomal RNA, partial sequence	97.14%	*Betaproteobacteria*	NR_144619.1	Beta979F Beta1130R	+
	Duganella zoogloeoides strain NBRC 102465 16S ribosomal RNA, partial sequence	92.31%	*Betaproteobacteria*	NR_114106.1		+
	Duganella zoogloeoides strain IAM 12670 16S ribosomal RNA, partial sequence	92.31%	*Betaproteobacteria*	NR_025833.1		+
Clone 1 - *Delta& Gammaproteobacteria*	Pseudomonas sp. BAKZL1118 gene for16S ribosomal RNA, partial sequence	99.60%	*GammaProteobacteria*	AB924632.1	Gamma877F Gamma1066R	+
	Pseudomonas sp. BAKZL1116 gene for 16S ribosomal RNA, partial sequence	99.60%	*GammaProteobacteria*	AB924630.1		+
	Pseudomonas sp. BAKZL1105 gene for 16S ribosomal RNA, partial sequence	99.60%	*GammaProteobacteria*	AB924632.1		+
Clone 2 - *Delta& Gammaproteobacteria*	Pseudomonas sp. BAKZL1118 gene for 16S ribosomal RNA, partial sequence	99.26%	*GammaProteobacteria*	AB924630.1	Gamma877F Gamma1060R	+
	Pseudomonas sp. BAKZL1116 gene for 16S ribosomal RNA, partial sequence	99.26%	*GammaProteobacteria*	AB924621.1		+
	Alteromonas sp. BAKZL1107 gene for 16S ribosomal RNA, partial sequence	99.26%	*GammaProteobacteria*	AB924621.1		+
	Anaeroplasma varium strain A2-T 16S ribosomal RNA, partial sequence	94.02%	*Tenericutes*	NR_044663.2		+

Clone 2 - *Tenericutes*	Anaeroplasma bactoclasticum strain JR 16S ribosomal RNA, partial sequence	94.02%	*Tenericutes*	NR_044675.2	Ten662F	+
	Anaeroplasma abactoclasticum strain 6-1 16S ribosomal RNA, partial sequence	93.01%	*Tenericutes*	NR_029167.1	Ten862R	+
Clone 3 - *Tenericutes*	Anaeroplasma varium strain A2-T 16S ribosomal RNA, partial sequence	94.51%	*Tenericutes*	NR_044663.2		+
	Anaeroplasma bactoclasticum strain JR 16S ribosomal RNA, partial sequence	94.51%	*Tenericutes*	NR_044675.2	Ten662F	+
	Anaeroplasma abactoclasticum strain 6-1 16S ribosomal RNA, partial sequence	93.48%	*Tenericutes*	NR_029167.1	Ten862R	+

+, positive; -, negative

8.4. Total arm entries results

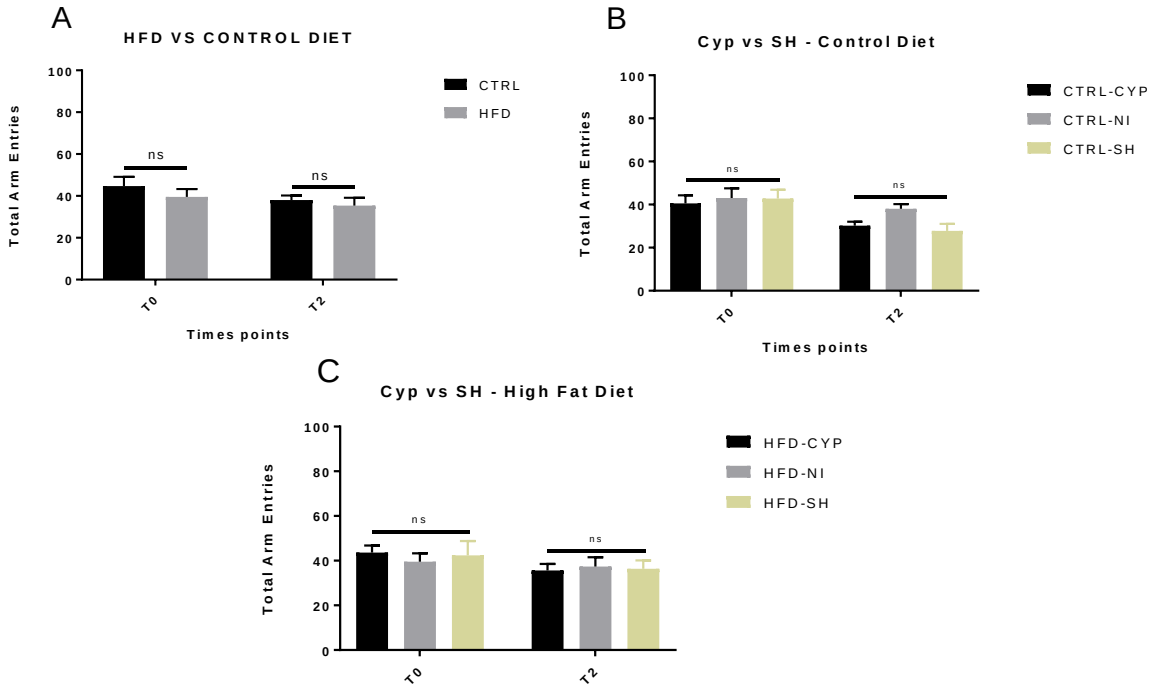

Figure 8.1. Effect of *Cyp* intervention and diet on total arm entries. Effect of diet, n (CTRL) = 7; n (HFD) = 6 (A); Arm entries between treatments within mice under Control diet, n (CTRL-CYP) = 6; n (CTRL-NI) = 7; n (CTRL-SH) = 4 (B); Arm entries between treatments within mice under HFD, n (HFD-CYP) = 5; n (HFD-SH) = 3; n (HFD-NI) = 6 (C). HFD - High-Fat diet; CTRL - Control diet; CYP - *Cyp* Overexpression; SH - *Cyp* Silencing; NI - Non-injected.